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Authors: Verslues, Paul E., and Sharma, Sandeep

Source: The Arabidopsis Book, 2010(8)

Published By: The American Society of Plant Biologists

URL: https://doi.org/10.1199/tab.0140

First published on November 3, 2010: e0140. 10.1199/tab.0140

Proline Metabolism and Its Implications for Plant-Environment Interaction

Paul E. Verslues[1] and Sandeep Sharma

Institute of Plant and Microbial Biology, Academia Sinica, No. 128 Sec. 2 Academia Rd, Nankang Dist., Taipei, 11529, Taiwan
[1]Address correspondence to paulv@gate.sinica.edu.tw

Proline has long been known to accumulate in plants experiencing water limitation and this has driven studies of proline as a beneficial solute allowing plants to increase cellular osmolarity during water limitation. Proline metabolism also has roles in redox buffering and energy transfer and is involved in plant pathogen interaction and programmed cell death. Some of these unique roles of proline depend on the properties of proline itself, whereas others depend on the "proline cycle" of coordinated proline synthesis in the chloroplast and cytoplasm with proline catabolism in the mitochondria. The regulatory mechanisms controlling proline metabolism, intercellular and intracellular transport and connections of proline to other metabolic pathways are all important to the *in vivo* functions of proline metabolism. Connections of proline metabolism to the oxidative pentose phosphate pathway and glutamate-glutamine metabolism are of particular interest. The N-acetyl glutamate pathway can also produce ornithine and, potentially, proline but its role and activity are unclear. Use of model systems such as *Arabidopsis thaliana* to better understand both these long studied and newly emerging functions of proline can help in the design of next-generation experiments testing whether proline metabolism is a promising metabolic engineering target for improving stress resistance of economically important plants.

I. INTRODUCTION

Proline, and its metabolism, is distinguished from other amino acids in several ways. The most fundamental is that proline is the only one of the proteogenic amino acids where the α-amino group is present as a secondary amine. While this may seem like a distinction more important to chemists than plant biologists, the unique properties of proline are highly relevant to understanding its role in plants. Another feature of proline, one that has been documented in studies too numerous to cite here, is that several types of plant stress cause proline to accumulate to high levels in many plant species (as well as bacteria and fungi). Figure 1A shows example data from *Arabidopsis thaliana* (hereafter Arabidopsis) seedlings where proline can accumulate more than 100-fold above the control level in seedlings exposed to low water potential. Upon stress relief, the process is fully reversible, with proline decreasing to a basal level over the course of a few days (Figure 1A). Lesser, but still significant, accumulation of proline occurs in salt-stressed plants (Figure 1B). As suggested by these data, proline accumulation primarily occurs in response to stresses that cause dehydration of the plant tissue such as drought (low water potential), salinity and freezing (Verslues et al., 2006), but can also occur at lower levels in response to heavy metal toxicity (Sharma and Dietz, 2009), plant pathogen interaction (Fabro et al., 2004) and other abiotic and biotic stimuli. Observations similar to Figure 1 have been the driver for most proline metabolism research in plant biology. However, recent data suggesting additional functions of proline are providing even more reasons to understand its metabolism.

The large accumulation of proline that occurs during drought may be explained in part by its basic chemical properties: proline is the most water soluble of the amino acids and exists much of the time in a zwitterionic state having both weak negative and positive charges at the carboxylic acid and nitrogen groups, respectively. Proline shares this property with other compounds collectively referred to as "compatible solutes" that are accumulated by a wide range of organisms to adjust cellular osmolarity (Yancey, 2005). Proline, however, clearly has more than one role in the plant and other aspects, such as cellular redox buffering, are also important in the overall function and regulation of proline metabolism. Many of these roles of proline have been discussed in recent reviews (Verbruggen and Hermans, 2008; Lehmann et al., 2010; Szabados and Savoure, 2010).

Thus, both the intrinsic properties of proline itself, as well as specific aspects of proline synthesis and catabolism, may explain why it is so prominent in plant stress biology. Also, mechanisms regulating proline differ substantially from other amino acids. Less and Galili (2008) analyzed publically available microarray data and found that, for many amino acids, abiotic stress altered transcription of the catabolism genes but had little effect on expression of genes encoding the biosynthetic enzymes. Notable exceptions to this were the connected pathways of proline and arginine metabolism, where abiotic stress caused extensive transcriptional regulation of the biosynthetic enzymes. In addition to specific regulation of its synthesis and catabolism pathways, the compartmentation of proline metabolism between cytoplasm, mitochondria and chloroplast is also of critical importance but incompletely understood. Proline transport, both intra- and inter-

cellular, tissue specific differences in proline metabolism, and the connections of proline metabolism to other metabolic pathways are all important both to understanding proline metabolism in a basic sense and knowing how it may be useful for plant improvement in an applied sense.

Proline metabolism is of interest to both those seeking to better understand plant stress physiology as well as those seeking to understand metabolic regulation. While in the past proline metabolism has been studied mostly by those interested in drought and other abiotic stresses, there is increasing evidence that proline is also relevant to plant pathogen interactions and is involved in programmed cell death and development. This summary of proline metabolism will bring in observations from all of these areas and consider proline metabolism in a larger metabolic context of connected pathways to highlight both what is known as well as some open questions about the unique metabolism and functions of proline.

II. THE CORE ENZYMES OF PROLINE METABOLISM AND THEIR REGULATION

The core of proline metabolism involves two enzymes catalyzing proline synthesis from glutamate in the cytoplasm or chloroplast, two enzymes catalyzing proline catabolism back to glutamate in the mitochondria, as well as an alternative pathway of proline synthesis via ornithine (Figure 2). The interconversion of proline and glutamate is sometimes referred to as the "proline cycle". The transcriptional upregulation of proline synthesis from glutamate and downregulation of proline catabolism during stress are thought to control proline levels, although exceptions to this pattern have been observed (Stines et al., 1999). Much data are consistent with this straightforward model of proline metabolism being mainly regulated by transcription of genes encoding the key enzymes. This is likely to not be the whole story, however, as post-translational regulation of these enzymes has been little explored and the role of ornithine as a proline precursor remains unclear. Likewise, the proline cycle may at first seem to be a futile cycle; however, understanding the coordinate regulation of this cycle and metabolic flux through it is key to overall understanding of proline metabolism and function. Our initial description of the core enzymes of proline metabolism will focus on their basic properties and regulation by abiotic stress, other functions and regulation by other factors are discussed in later sections of this review.

A. Δ^1 - Pyrroline – 5- Carboxylate Synthetase (P5CS)

P5CS (EC 2.7.2.11/1.2.1.41) is a bifunctional enzyme that converts glutamate into the intermediate glutamic semialdehyde, which spontaneously cyclizes into Δ^1- pyrroline-5-carboxlyate (P5C) (Figure 2). Like the *P5CS* gene that was first cloned from *Vigna aconitifolia* (mothbean) by complementation (Hu et al., 1992), the P5CS1 and P5CS2 enzymes of Arabidopsis encorporate both γ-glutamyl kinase as well as glutamic-γ-semialdehyde dehydrogenase activities (Hu et al., 1992; Delauney and Verma, 1993; Savoure et al., 1995). The two enzymatic domains of P5CS correspond to the ProB and ProA proteins of *Escherichia coli* and are also produced as separate polypeptides by *tomPRO1* in tomato (Fujita et al., 1998). *V. aconitifolia* P5CS was found to have leucine zipper sequences in each of the enzymatic domains (Hu et al., 1992), which may function intramolecularly to maintain the tertiary structure of the enzyme or intermolecularly in protein-protein interaction. Arabidopsis P5CS1 and P5CS2 also contain a

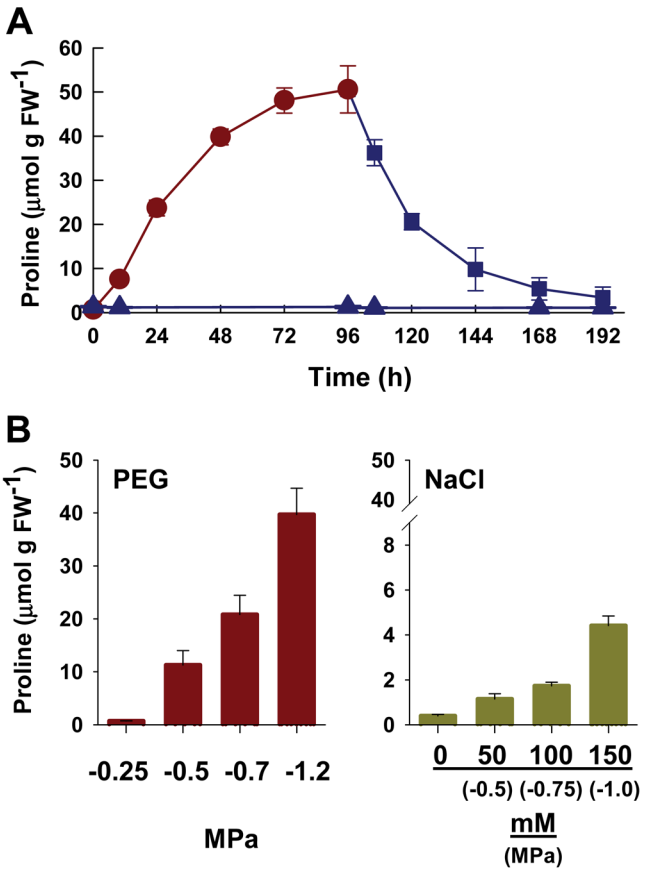


Figure 1. Examples of stress-induced proline accumulation in Arabidopsis.

(A) Proline content after transfer of 7-day-old seedlings to low water potential polyethylene glycol (PEG)-agar plates. Seedlings were held at -1.2 MPa for 4 days and then transferred back to high water potential media (-0.25 MPa). PEG-agar plates were used to mimic the decrease in water potential of a drying soil while allowing a constant and controlled severity of stress to be applied. Transfer to low water potential caused a nearly 100-fold increase in proline content over 4 days (circles). Proline levels declined back to control levels over a similar time period after return to high water potential (squares). Seedlings kept at -0.25 MPa for the entire experiment maintained proline content below 1 µmol g FW^{-1} (triangles) Figure is modified from Sharma and Verslues (2010). Data are means ± standard error (n = 10-12)

(B) Comparison of proline content after low water potential (PEG) or NaCl treatment. Seven-day-old seedlings were transferred to the indicated treatments and proline content measured 4 days later. Proline content increased with increasing severity of stress for low water potential and NaCl; however, low water potential imposed by PEG always elicited approximately ten-fold more proline accumulation than NaCl treatment of similar water potential. This is likely because proline is needed for osmotic adjustment in the low water potential treatment. In the salt treatment, ion uptake means there is less solute needed for osmotic adjustment and the proline that does accumulate may have other protective functions. Data are from Sharma and Verslues (2010) and are means ± standard error (n = 5-10).

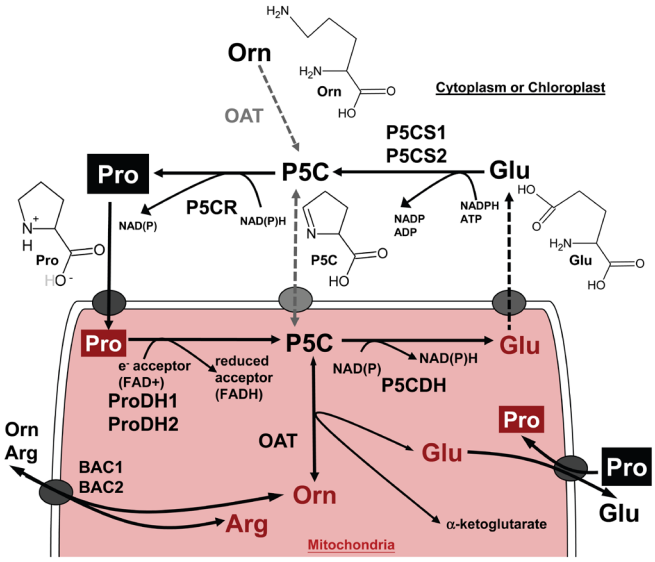


Figure 2. The core pathways of proline metabolism.

Proline synthesis from glutamate occurs in the cytoplasm and/or chloroplast via two enzymatic steps. Proline catabolism to glutamate occurs in the mitochondria also by two enzymatic steps. Proline synthesis and catabolism both use the common intermediate P5C (formed by spontaneous cyclization of glutamic-5-semialdehyde produced by P5CS or ProDH). Solid lines indicate known metabolic or transport steps. Dashed lines indicate proposed but not demonstrated steps. Both mitochondrial proline import carriers as well as a proline-glutamate exchanger have been demonstrated in transport studies of isolated mitochondria. Transport of P5C across the mitochondrial membrane to allow cyclic metabolism between ProDH and P5CR has been proposed but not demonstrated. The mitochondrial Basic Amino Acid Carrier1 (BAC1) and BAC2 may also influence proline metabolism by movement of arginine or ornithine. OAT had been thought to be a cytoplasmic enzyme that functioned as an alterantive route to proline but more recent evidence has placed it in the mitochondria. Arabidopsis gene identification numbers and all gene name abbreviations are given in Table 1.

leucine zipper region in each domain. However, these motifs are not present in an α helix and do not match the normal consensus of four heptad repeats (Savoure et al., 1995). These leucine zipper regions may still participate in protein-protein interaction (perhaps with reduced affinity) or be needed to maintain P5CS1 structure (Savoure et al., 1995).

P5CS1 is subjected to feedback inhibition by proline (Hu et al., 1992; Zhang et al., 1995). In bacteria, change of a conserved aspartate (at position 107) to asparagine, rendered the γ-glutamyl kinase much less sensitive to proline inhibition (Csonka et al., 1988; Dandekar and Uratsu, 1988) but in plants this conserved aspartate (at position 128) was not involved in feedback inhibition (Zhang et al., 1995). Instead, a F129A (phenylalanine129alanine) mutant of the *V. aconitifolia* P5CS enzyme had decreased feedback inhibition (Zhang et al., 1995). Tobacco plants ectopically expressing this feedback insensitive P5CS mutant had higher levels of proline than either untransformed plants or plants expressing the unmutated P5CS (Hong et al., 2000), suggesting that reduced feedback inhibition can lead to higher proline *in vivo*. Whether or not Arabidopsis P5CS1 exhibits a similar feedback regulation has not been reported. Also unclear is whether the feedback sensitiv-

Table 1. Arabidopsis genes mentioned in the main text. Includes both verified gene functions and gene annotations that have not been experimentally verified.

Description	AGI locus identifier
Δ^1- pyrroline-5-carboxlyate synthetase1 (P5CS1)	At2g39800
Δ^1- pyrroline-5-carboxlyate synthetase2 (P5CS2)	At3g55610
Δ^1 - Pyrroline – 5- carboxylate reductase (P5CR)	At5g14800
Proline dehydrogenase (ProDH)	At3g30775
Proline dehydrogenase2 (ProDH2)	At5g38710
SRO5	At5g62520
AtbZIP10	At4g02640
AtbZIP11	At4g34590
AtbZIP53	At3g62420
Δ^1 - Pyrroline – 5- carboxylate dehydrogenase (P5CDH)	At5g62530
Ornithine δ-aminotransferase (OAT)	At5g46180
Basic amino acid carrier1 (BAC1)	At2g33820
Basic amino acid carrier2 (BAC2)	At1g79900
Proline Transporter1 (ProT1)	At2g39890
Proline Transporter2 (ProT2)	At3g55740
Proline Transporter3 (ProT3)	At2g35590
Lysine histidine transporter1 (LHT1)	At5g40780
Amino acid permease5 (AAP5)	At1g44100
N-acetyl-L-glutamate synthase 1 (NAGS1)	At2g22910
N-acetyl-L-glutamate synthase 2 (NAGS2)	At4g37670
N-acetyl-L-glutamate kinase (NAGK)	At3g57560
N-acetyl-gamma-glutamyl-phosphate reductase	At2g19940
N^2-acetylornithine aminotransferase (NAOAT; OAT2)	At1g80600
N^2-acetylornithine glutamate acetyltransferase (NAOGAcT)	At2g37500
N^2-acetylornithine deacetylase (NAOD)	At4g17830
ornithine cyclodeaminase (OCD) (putative)	At5g52810
glucose-6-phosphate dehydrogenase1 (G6PDH1)	At5g35790
glucose-6-phosphate dehydrogenase2 (G6PDH2)	At5g13110
glucose-6-phosphate dehydrogenase3 (G6PDH3)	At1g24280
glucose-6-phosphate dehydrogenase4 (G6PDH4)	At1g09420
glucose-6-phosphate dehydrogenase5 (G6PDH5)	At3g27300
glucose-6-phosphate dehydrogenase6 (G6PDH6)	At5g40760
6-phosphogluconolactonase1 (PGL1)	At1g13700
6-phosphogluconolactonase2 (PGL2)	At3g49360
6-phosphogluconolactonase3 (PGL3)	At5g24400
6-phosphogluconolactonase4 (PGL4)	At5g24410
6-phosphogluconolactonase5 (PGL5)	At5g24420
6-phosphogluconate dehydrogenase	At1g64190
6-phosphogluconate dehydrogenase	At3g02360
6-phosphogluconate dehydrogenase	At5g41670
phosphoribulokinase (PRK)	At1g32060
glutamine synthetase1.1	At5g37600
glutamine synthetase1.2	At1g66200
glutamine synthetase1.3	At3g17820
glutamine synthetase1.4	At5g16570
glutamine synthetase1.5	At1g48470
glutamine synthetase2	At5g35630
glutamate synthase1 (Ferredoxin-dependent)	At5g04140
glutamate synthase1 (NADH-dependent)	At5g53460

continued

Table 1. (continued)

Description	AGI locus identifier
glutamate synthase2 (Ferredoxin-dependent)	At2g41220
glutamate dehydrogenase1	At5g18170
glutamate dehydrogenase2	At5g07440
glutamate dehydrogenase3	At3g03910
glutamate dehydrogenase (putative)	At1g51720
glutamate decarboxylase1 (GAD1)	At5g17330
glutamate decarboxylase2 (GAD2)	At1g65960
glutamate decarboxylase3 (GAD3)	At2g02000
glutamate decarboxylase4 (GAD4)	At2g02010
glutamate decarboxylase5 (GAD5)	At3g17760
arginase1	At4g08900
arginase2	At4g08870
arginine decarboxylase1	At2g16500
arginine decarboxylase2	At4g34710
(former) nitric oxide synthase1 (now shown to be effector of nitric oxide response)	At3g47450

ity of the enzyme may be modulated *in vivo* during stress to allow high levels of proline accumulation.

Many studies of proline metabolism have either explicitly or implicitly assumed a cytoplasmic localization of P5CS and proline synthesis. Recently, however, Szekely et al. (2008) showed that in stressed leaves P5CS1-GFP was found in punctuate structures either in, or closely associated with, chloroplasts. In the absence of stress, P5CS1 was more diffuse, raising the possibility of stress-induced relocalization of the enzyme. Additional studies are needed to build on this observation. However, a chloroplast localization would be a logical fit with some of the proposed metabolic roles of proline metabolism under stress and we will use this localization in discussing connections of proline to other metabolic pathways (see section IV.).

Transcriptional regulation and study of knockout mutants indicates that the two Arabidopsis *P5CS* genes have clearly distinct functions. *P5CS1* is expressed most highly in shoot tissue but not in dividing cells (Strizhov et al., 1997; Yoshiba et al., 1999) and transcription of *P5CS1* is strongly induced under high salinity, dehydration and cold (Delauney and Verma, 1990; Hu et al., 1992; Yoshiba et al., 1995; Yoshiba et al., 1997; Yoshiba et al., 1999; Hong et al., 2000). Thus, the transcriptional up-regulation of *AtP5CS1* is well correlated with proline accumulation in short-term dehydration experiments (Yoshiba et al., 1995) although it is unclear how tight this correlation is under longer-term stress or other types of stress (Kaplan et al., 2007). Consistent with the upregulation of *AtP5CS1*, *p5cs1* knockout mutants have greatly reduced proline levels during salt or low water potential stress (Szekely et al., 2008; Sharma and Verslues, 2010). *p5cs1* mutants were also reported to have reduced growth and altered reactive oxygen levels, suggesting that they are hypersensitive to salt, osmotic stress and low water potential (Nanjo et al., 1999b; Szekely et al., 2008).

Studies of abscisic acid (ABA)-deficient mutants and exogenous ABA treatment have indicated that proline accumulation under stress is partially regulated by ABA (Ober and Sharp, 1994; Savoure et al., 1997; Verslues and Bray, 2006; Sharma and Verslues, 2010) but ABA applied in the absence of stress is insufficient to induce high levels of proline (Ober and Sharp, 1994; Verslues and Bray, 2006; Sharma and Verslues, 2010). ABA is well known to be required for the up regulation of many stress-induced genes (Shinozaki and Yamaguchi-Shinozaki, 2007). However, conflicting data have indicated either ABA-dependent (Yoshiba et al., 1995; Strizhov et al., 1997) or ABA-independent regulation of P5CS1 (Savoure et al., 1997). Recently, quantitative comparison of *P5CS1* expression to known ABA-induced genes found largely ABA-independent regulation of *P5CS1*, implying that the underlying mechanisms controlling *P5CS1* expression are distinct from that of many commonly studied stress marker genes (Sharma and Verslues, 2010). *P5CS1* expression has also been found to be stimulated by light (Hayashi et al., 2000) and nitric oxide (Zhao et al., 2009). In contrast, *P5CS1* expression was decreased by brassinosteroid application (Abraham et al., 2003).

Other than effects of plant hormones, the signal transduction controlling *P5CS1* is not well known. Calcium signaling is likely to be involved: by using a *Glycine max* (soybean) salt-inducible calmodulin, Yoo et al. (2005) described a mechanism whereby calmodulin activated a MYB transcription factor which then activated a number of downstream genes including *P5CS1*. Another calcium signaling component, phospholipase C, upregulated expression of *P5CS1* in Arabidopsis under salt but not osmotic stress (Parre et al., 2007).

In contrast to the *P5CS1*, gene expression of *P5CS2* has little or no transcriptional up-regulation under stress (Strizhov et al., 1997; Zhang et al., 1997; Szekely et al., 2008) and all indications are that it has only a minor role in proline accumulation induced by abiotic stress (Strizhov et al., 1997; Abraham et al., 2003; Szekely et al., 2008). *P5CS2* can be induced by pathogen response (Fabro et al., 2004) and may be more actively involved in plant-pathogen interaction. Also, *P5CS2* is expressed more abundantly in actively dividing callus and cell suspension culture (Strizhov et al., 1997). *p5cs2* mutants are embryo lethal despite the presence of functional P5CS1 (Szekely et al., 2008), suggesting a specialized developmental role of P5CS2. While exogenous proline can rescue some *p5cs2* mutants, the mutant plants were sterile (Szekely et al., 2008). This is likely to be more complex than simple proline auxotrophy as ectopic expression of *P5CS1* can also lead to developmental abnormalities (Mattioli et al., 2008) and the *rolD* gene of *Agrobacterium rhizogenens* also alters both proline metabolism and development (Mauro et al., 1996; see section V.-D. below for further discussion). P5CS2 was observed to have a more predominantly cytoplasmic localization than P5CS1 (Szekely et al., 2008). How P5CS expression affects development and whether the different functions of P5CS1 and P5CS2 may also involve different post-translational regulation and/or compartmentation of the two remains to be established. The enzymatic properties of Arabidopsis P5CS2 have been relatively little studied.

Post translational regulation is also unknown for either P5CS1 or P5CS2. Phosphorylation of P5CS1 has been found in large scale proteomics studies (Benschop et al., 2007; Reiland et al., 2009) but its significance in regulating P5CS1 activity is not known. Also, there have been reports that a protein component of plant extracts inhibits P5CS1 activity *in vitro* (Kavi Kishor et al., 1995; Zhang et al., 1995) but no further experiments to identify the inhibitor have been reported.

B. Δ^1 - Pyrroline – 5- Carboxylate Reductase (P5CR)

P5CR (EC 1.5.1.2) converts the intermediate P5C to proline and is encoded by a single gene in Arabidopsis. It is commonly thought of as not being a rate-limiting factor in proline synthesis based mainly on its lack of transcriptional regulation by stress (Szoke et al., 1992; Delauney and Verma, 1993; Verbruggen et al., 1993; Hua et al., 1997; Sharma and Verslues, 2010) and a report that ectopic expression of the gene did not change proline content (Szoke et al., 1992). There may, however, be species and stress specific differences as *P5CR* overexpression was reported to affect proline levels in soybean (de Ronde et al., 2004a; de Ronde et al., 2004b). In Arabidopsis, there was transcriptional induction of *P5CR* by heat and salt stress but not dehydration (Hua et al., 1997). However, the increased transcript levels did not lead to increases in P5CR protein level or enzyme activity. Instead, it was found that a 92 bp fragment of the *P5CR* 5'UTR acted as a regulator to control mRNA stability and inhibit protein translation of P5CR under heat or salt stress thus controlling protein level independently of transcript level (Hua et al., 2001). In addition to this post-transcriptional regulation; transcript levels of *P5CR* are developmentally regulated with highest expression in root tips, shoot meristem, guard cells, hydathodes, pollen grains, ovule and developing seeds (Szoke et al., 1992; Hua et al., 1997).

Similar to P5CS, the Arabidopsis P5CR is actually less studied at the protein level than the enzyme from other plants. Two isoenzymes of *Spinacea oleracea* (spinach) leaf P5CR, P5CR-1 and P5CR-2, were found to be differentially distributed with P5CR-2, but not P5CR-1 isolated from intact chloroplasts (Murahama et al., 2001). P5CR-1 and P5CR-2 had similar K_m values for NADPH (9 and 19 μM, respectively) and P5C (0.122 and 0.162 mM). Both isoenzymes had strong preference for NADPH rather than NADH as the electron donor and also showed inhibition by ATP and Mg^{2+} (Murahama et al., 2001). The preference for NADPH is likely to be significant in linking proline metabolism to the pentose phosphate pathway (see section IV.-A. below).

C. Proline Dehydrogenase (ProDH)

ProDH (EC 1.5.99.8, also referred to as proline oxidase), the first enzyme of the proline catabolism pathway (Figure 2), catalyzes oxidation of proline to P5C in the mitochondria. Arabidopsis *ProDH* was identified by sequence analysis of an Arabidopsis cDNA clone, *ERD5* (Early Response to Dehydration5). ERD5 encodes a protein highly similar to yeast *PUT1* (Proline Utilization1 (PUT1) (23.6% identical over 364 amio acids) and *Drosophila melanogaster* sluggish-A gene (34.5% identical over 255 amino acids), both of which encode proline dehydrogenases. Furthermore, the C terminus of ERD5 contains a highly conserved region also found in PUTA, the proline dehydrogenase of *E. coli* (Kiyosue et al., 1996). There is surprisingly little known about the enzymology of plant ProDH. Reductant from ProDH contributes to electron transport and ATP generation in the mitochondria. The initial acceptor to which ProDH passes electrons is not conclusively known in plants but, by analogy to the bacterial enzyme (Lee et al., 2003), is thought to be an FAD moiety connected to the mitochondrial electron transport chain. The enzyme kinetics of plant ProDH and posttranslational regulation also remain unexplored.

ProDH gene expression is downregulated by low water potential, dehydration and salinity (Kiyosue et al., 1996; Verbruggen et al., 1996; Verslues et al., 2007; Sharma and Verslues, 2010), but upregulated by stress release and exogenous proline (Kiyosue et al., 1996; Peng et al., 1996; Verbruggen et al., 1996; Yoshiba et al., 1997; Satoh et al., 2002; Sharma and Verslues, 2010). Downregulation of *ProDH* expression during stress is widely accepted as one control point that can promote proline accumulation under stress. However, upregulation of ProDH during stress when high levels of proline were accumulating has been reported (Kaplan et al., 2007). Also calling into question whether downregulation of *ProDH* is needed for proline accumulation are the observations of Mani et al (2002), who found that increased or decreased *ProDH* expression in transgenic Arabidopsis had relatively little effect on proline accumulation. Antisense suppression of *ProDH* was suggested to lead to increased proline and stress tolerance in one study (Nanjo et al., 1999b) although whether or not this conclusion holds for longer term low water potential treatments is uncertain. Antisense suppression of *ProDH* in tobacco cell cultures led to altered growth and cell morphology (Tateishi et al., 2005). Tissue-specific differences in the stress regulation of *ProDH* may also exist but have been little investigated.

More recently, a second *ProDH* (*ProDH2*) was identified in Arabidopsis by sequence homology and its function confirmed both by complementation of a yeast mutant defective in ProDH activity and characterization of the Arabidopsis knockout mutant (Funck et al., 2010). *ProDH2* is expressed at a lower level and in samples of whole seedlings was less affected by stress than *ProDH* (Sharma and Verslues, 2010). *ProDH2* can also be induced by exogenous proline and is downregulated by exogenous sugar (Hanson et al., 2008; Funck et al., 2010; Sharma and Verslues, 2010). Similar to ProDH, there is essentially no protein level information or enzymatic properties known for ProDH2.

ProDH, and possibly *ProDH2* as well, have a unique pattern of transcriptional regulation. Promoter analysis of *ProDH* and its induction by exogenous proline led to the identification of a cis-acting element (ACTCAT) which was named the proline-response element (PRE) (Satoh et al., 2002). Further work has shown that induction of *ProDH* by high proline and the PRE involves *bZIP* transcription factors of the ATB2 subgroup, particularly AtbZIP11 and AtbZIP53 (Satoh et al., 2004). AtbZIP11 also regulates *ProDH2* in response to sugar signaling. Ectopic expression of bZIP11 in Arabidopsis induced both *ProDH2* and *ProDH* and led to reduced proline levels in unstressed plants (Hanson et al., 2008). This suggests that bZIP11 may function in coordinating sucrose and proline metabolism. Another study used chromatin immunoprecipation (ChIP) assay to show that *ProDH* is a direct target of the group S bZIP transcription factor AtbZIP53 and group C AtbZIP10. These transcription factors form heterodimers on the ACTCAT element (Weltmeier et al., 2006). Presumably these bZIPs lie downstream of an unknown mechanism that senses proline levels either directly or indirectly via metabolic effects of exogenous proline.

Given this, it is even more striking that *ProDH* is downregulated (Figure 3) by drought or salt stress when high levels of proline are present. Thus, stress can override the proline induction of *ProDH* (Miller et al., 2005). This raises the question of whether the increase of *ProDH* upon stress release is the result simply of removing the stress inhibition of *ProDH,* thus allowing it to be

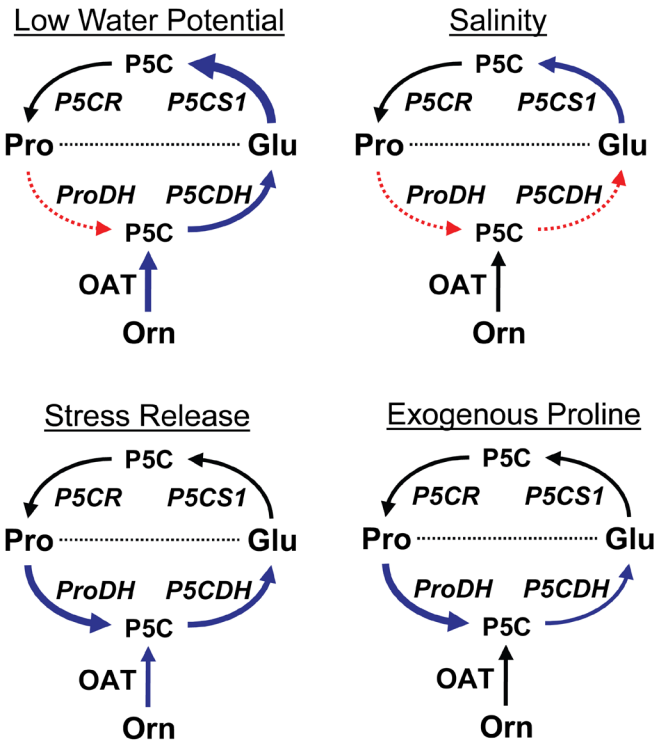


Figure 3. Transcriptional regulation of core genes of proline metabolism by low water potential, salt stress or stress recovery.

Consensus transcriptional regulation of proline metabolism from literature, our own work, and publically available microarray data (Genevestigator). Black lines indicate no change from basal level expression in unstressed conditions. Blue lines indicate up-regulation with the thickness of line approximately corresponding to the extent of up-regulation. Dashed red lines indicate down regulation. Stress release refers to recovery immediately after either low water potential or salt stress.

induced by the high level of proline which has built up in the plant. This, however, seems not to be the case as *p5cs1* mutants, which had greatly reduced levels of proline, still had wild type levels of *ProDH* induction by stress release (Sharma and Verslues, 2010). The same study used ABA-deficient mutants to show that the downregulation of *ProDH* by low water potential is ABA-independent. Thus, both the stress down regulation and stress release up-regulation of *ProDH* are controlled by novel signaling mechanisms which have yet to be elucidated.

D. Δ^1- Pyrroline – 5- Carboxylate Dehydrogenase (P5CDH)

P5CDH (EC 1.5.1.12) catalyzes the second step of proline oxidation and converts the intermediate P5C to glutamate. Arabidopsis P5CDH was identified by functional complementation of a yeast P5CDH (*ScPUT2)* deletion strain unable to utilize proline as a sole nitrogen source (Deuschle et al., 2001). *P5CDH* is a single copy gene in Arabidopsis and the encoded protein is mitochondria localized (Deuschle et al., 2001). P5CDH purified from potato appeared to be a tetramer with 60kD subunits. It has K_m of 0.11 and 0.46 mM for NAD^+ and P5C, respectively but could use either NAD^+ or $NADP^+$ as an electron acceptor (Forlani et al., 1997). It is interesting to note that plant ProDH and P5CDH differ from their bacterial counterparts in that the bacterial PutA protein incorporates both ProDH and P5CDH activities and produces proline without releasing P5C (Srivastava et al., 2010). That the two activities are separate in plants is one of several lines of evidence suggesting a role for P5C, possibly in programmed cell death (see sections V.-B. and VI.-B. below for more discussion of this topic).

Data on the physiological role of P5CDH have come mainly from the study of *p5cdh* mutants in Arabidopsis. *p5cdh* mutants had only slightly higher than wild type or unchanged proline content under salt stress (Deuschle et al., 2004; Borsani et al., 2005). However, *p5cdh* mutants did maintain higher proline content after stress release or removal of exogenous proline (Deuschle et al., 2004), consistent with a requirement for P5CDH during rapid proline catabolism. When grown in presence of proline, arginine or ornithine, which can all be metabolized to produce P5C, *p5cdh* mutants had tissue damage likely related to ROS accumulation (Deuschle et al., 2004). It is thought that this damage was primarily caused by increased P5C, which may elicit ROS production in the mitochondria. Such a role of P5C is difficult to prove directly as it is maintained at low levels under most conditions and has been difficult to assay accurately. In contrast, another study found no change in P5C content of ProDH overexpressing plants and *p5cdh* mutants (Miller et al., 2009). They proposed that P5C levels were controlled by transport to the cytoplasm or chloroplast where it could be used as a substrate for P5CR (Figure 2). Such a cycle would maintain P5C at a constant level but could still lead to ROS production in the mitochondria by activity of ProDH. While such a cyclic metabolism of P5C within the larger proline cycle is an intriguing idea, more evidence is needed before such a pathway can be conclusively said to exist in plants. In particular, it is not yet known whether or how P5C can move between mitochondria and cytosol or chloroplast compartments.

P5CDH is transcriptionally regulated by both environmental signals and developmental factors. Developmentally, *P5CDH* is expressed at a basal level in all tissues except flowers, where it has higher expression (Deuschle et al., 2001). Stress regulation of *P5CDH* is more varied. *P5CDH* was upregulated by low water potential (Sharma and Verslues, 2010), an observation consistent with publically available microarray data (Genevestigator; www.genevestigator.com), and was also found to be upregulated in response to exogenous proline (Deuschle et al., 2004). However, under salt stress, *P5CDH* expression was downregulated by siRNAs generated from the overlapping transcripts of *P5CDH* and *SRO5*, a mitochondrial protein potentially important in ROS regulation (Borsani et al., 2005). Production of the siRNA targeting P5CDH was specific to salt stress and not seen under osmotic stress, dehydration or ABA treatments (Borsani et al., 2005). This mechanism is of considerable interest because it demonstrated a new class of siRNAs generated by overlapping gene transcripts. The involvement of *SRO5* also suggested a link between proline and reactive oxygen. The specificity of the mechanism to salt stress and the different regulation of *P5CDH* between low water potential and salt stress (Figure 3) suggest different roles for proline metabolism under these two stresses. Salt-induced changes in proline may be closely linked to reactive oxygen while the low

water potential response may be more related to production of proline for osmotic adjustment, redox buffering and other metabolic roles (Sharma and Verslues, 2010).

E. Ornithine δ-aminotransferase (OAT)

OAT (EC 2.6.1.13) catalyzes the reversible transamination of Δ^1-pyroline-5-carboxylate (P5C), the common intermediate in both proline synthesis and catabolism (Figure 2). The first plant *OAT* cDNA was isolated from *V. aconitifolia* by *trans*-complementation of proline auxotrophic mutant of *E. coli* (Delauney et al., 1993) and encodes a polypeptide of 48.1kD. The native enzyme expressed *in E. coli* had a K_m of 2 mM for ornithine and 0.75mM for α-ketoglutarate (Delauney et al., 1993). An *OAT* cDNA encoding a similar protein was later isolated from Arabidopsis (Roosens et al., 1998). Analysis of OAT enzymatic activity in extracts of salt stressed Arabidopsis showed that OAT activity doubled in the first 24 h of salt stress and continued to increase up to 72 h after the start of salt treatment (the maximal activity was 82 μmol P5C mg^{-1} protein h^{-1}). However, the OAT K_m did not show any difference between control and salt stressed plants (91 and 87 mM for ornithine in control and stressed plants, respectively). The same study also found that OAT activity and transcript varied with plant age being 10-fold higher in 12-day-old plants than in 4-week-old plants (Roosens et al., 1998). It is not known why the apparent K_m for the bacterially expressed OAT was different from that observed in Arabidopsis extracts or whether this represents different properties of the *V. aconitifolia* versus Arabidopsis enzymes. To our knowledge, there are no other studies examining the enzymatic properties or posttranslational regulation of OAT.

OAT has often been thought of as an alternative route of proline production in the cytoplasm (Figure 2). Studies showing decreased proline accumulation in the presence of the OAT inhibitor gabaculine (Hervieu et al., 1995; Yang and Kao, 1999) seemed to support the idea of OAT having a significant role in proline synthesis, although it is still clearly secondary to proline synthesis from glutamate by P5CS1. In addition, overexpression of *OAT* resulted in enhanced proline accumulation (Roosens et al., 2002; Wu et al., 2003). Although the difference in proline content between wild type and transgenics was not large, this still seemed to support OAT as being involved in proline synthesis in the cytosol. However, Funck et al. (2008) showed localization of GFP-tagged OAT in the mitochondria, consistent with earlier reports of mitochondrial localization sequences in OAT (Delauney et al., 1993; Roosens et al., 1998). Funck et al. (2008) also proposed that OAT was not involved in salt stress-induced proline accumulation based on analysis of *oat* Arabidopsis mutants. The phenotype of such mutants under low water potential, where proline accumulates to higher levels, is an interesting question for further experiments. It is also unclear whether *OAT* is transcriptionally upregulated in response to stress: Delauney et al. (1993) did not observe an upregulation of *OAT*; however, more recent microarray data available through Genevestigator do show a 3-fold upregulation of *OAT* expression under osmotic stress, and upregulation of *OAT* has also been observed in other studies (Armengaud et al., 2004; Sharma and Verslues, 2010); illustrated in Figure 3). Another study observed *OAT* induction by salt stress in seedlings but not older plants (Roosens et al., 1998).

These conflicting hypotheses about the role of OAT in proline metabolism have yet to be resolved. If mitochondrial OAT does contribute to proline synthesis, as indicated by its up-regulation under low water potential, this presumably occurs by production of P5C in the mitochondria. Then there is a question of whether mitochondrial P5C moves to the cytoplasm or chloroplast for proline synthesis (as proposed above in studies of *p5cdh* mutants) or whether glutamate formed in the mitochondria is transported out for proline synthesis elsewhere (Figure 2). More indirect connections between OAT activity in the mitochondria and proline synthesis are also possible.

III. ADDITIONAL PROTEINS POTENTIALLY INVOLVED IN PROLINE METABOLISM OR TRANSPORT

Having discussed the genes and enzymes that are well-established to be the core of proline metabolism, we can now consider other proteins required for proline metabolism as a whole. We can also begin to place proline metabolism in a larger cellular context. Both the core of proline metabolism (Figure 2) as well as an expanded view (Figure 4) suggest that there are both proteins that are clearly needed for proline metabolism but yet to be identified, as well as known proteins whose role in proline metabolism is unclear.

A. Intracellular Transporters

The complex compartmentation of proline metabolism, with proline synthesis in the chloroplast and cytosol but proline catabolism in the mitochondria, raises a clear need for intracellular movement of proline and related metabolites (Figure 2, Figure 4). The high levels of proline accumulated under stress, as well as possible movement of proline among different parts of the plant and cyclic proline synthesis and degradation, suggest that the flux of proline into and out of specific compartments is likely to be substantial. Transport assays using isolated wheat mitochondria have shown the existence of both a mitochondrial proline importer as well as a proline-glutamate exchanger (Di Martino et al., 2006; illustrated in Figure 2). However, the genes encoding these activities have not been identified in any plant. Less is known about proline transport on the chloroplast envelope, although the recent data supporting proline synthesis in the chloroplast of stressed plants (Szekely et al., 2008; Szabados and Savoure, 2010) make it more likely that such transporters exist and move large fluxes of proline in stressed plants. Most probably these transporters are among the many Arabidopsis genes annotated as mitochondrial-type transporters but with no data on their substrate specificity or subcellular localization.

Part of the intracellular exchange of metabolites related to proline metabolism may involve the transport of ornithine and/or arginine between the cytoplasm and the mitochondria. Basic Amino Acid Carrier1 (BAC1) and BAC2 have been shown to be mitochondrial transporters that move ornithine and arginine out of the mitochondria (Figure 2). Although BAC1 and BAC2 are similar, their pH optimum, substrate specificities and gene expression differ, with BAC2 being less specific for basic amino acids (Hoyos et al., 2003; Palmieri et al., 2006). Genevestigator data and our own experiments also show that *BAC2* is induced 10-15 fold by ABA or

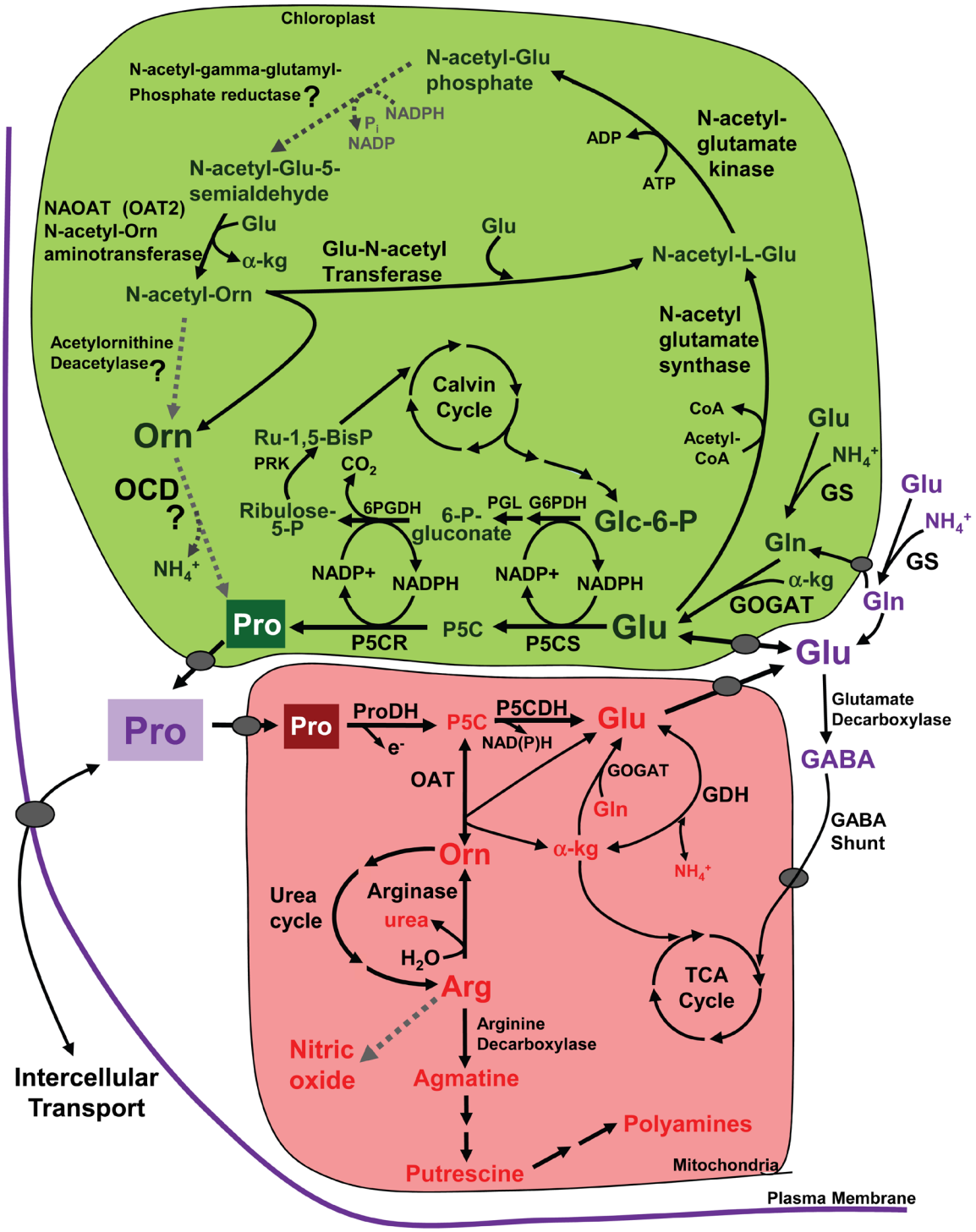


Figure 4. Connections of proline metabolism to other pathways.

Connections of proline synthesis to nitrogen assimilation by GS and GOGAT; the N-acetyl-glutamate pathway (chloroplast); the oxidative pentose phosphate pathway (chloroplast); GABA metabolism (cytoplasm and mitochondria); and, ornithine-arginine metabolism (mitochondria). Note that in this illustration, proline synthesis is depicted solely in the chloroplast; however, cytoplasmic proline synthesis is also possible. Dashed lines indicate reactions that have not been experimentally observed in Arabidopsis. Transport steps are drawn merely as the most direct route as transporters for these steps remain mostly unidentified. Note that many of these reactions are catalyzed by multiple isozymes with different compartmentation; not all of the possible isozymes/localizations are shown. Arabidopsis gene identification numbers and gene name abbreviations are given in Table 1.

osmotic stress treatments (S. Sharma and P.E. Verslues, unpublished observations). *bac2* mutants accumulate slightly more proline than wild type after osmotic stress (Toka et al., 2010). Thus, BAC2 may be involved in transport related to proline metabolism although its exact role is still unclear.

B. Intercellular Transporters

Several lines of evidence support intercellular movement of proline, including the observation of high levels of proline in the phloem of drought-stress plants (Girousse et al., 1996; Lee et al., 2009) and experiments showing that glutamine synthetase activity in phloem companion cells influences proline levels (Brugiere et al., 1999; see section IV.-B. for additional discussion of the role of glutamine synthetase). Such observations suggest the need for plasma membrane transporters that move proline. Arabidopsis Proline Transporter1 (ProT1), ProT2 and ProT3 were identified based on a combination of yeast complementation and sequence comparison (Rentsch et al., 1996; Grallath et al., 2005) and shown to be plasma membrane localized. The ProTs transport not only proline but also GABA and glycine betaine, two additional compatible solutes, but with varying affinities (Rentsch et al., 1996; Grallath et al., 2005). The three *ProT*s also have differing patterns of tissue-specific expression, with *ProT2* being highest in roots and *ProT1* being higher in stems and flowers (Grallath et al., 2005). It might be expected that a proline transporter would be induced by stress when proline levels are also increasing. However, Genevestigator data show little or no increase in *ProT* expression by osmotic stress, salt stress or ABA. Only *ProT2* has some induction by salt stress (approximately 3-fold). Such data, however, do not address whether there may be localized induction of these transporters important for redistribution of proline within the plant.

Interestingly, a proline-specific transporter induced by salt stress specifically in barley root tip has been identified (Ueda et al., 2001). Tissue-specific overexpression of this transporter in the Arabidopsis root tip led to increased proline content of the root apex (0-5 mm) and increased growth under control conditions (Ueda et al., 2008), although the effects of such targeted overexpression on growth under stress conditions were not tested. Whether or not this function in Arabidopsis is performed by one of the ProTs, other transporters known to have proline uptake activity in roots such as LHT1 or AAP5 (Hirner et al., 2006; Rentsch et al., 2007; Svennerstam et al., 2008), or other yet uncharacterized transporters will be an interesting question for further experiments. Long distance proline transport may be important in taking proline produced in photosynthetic tissue as a way to buffer cellular redox status and moving it to non-photosynthetic tissue such as roots, where it can contribute to osmotic adjustment or be catabolized to support growth (Figure 5A; see sections IV.-A. and V.-A. for further discussion). This idea is supported by observations that the elongation zone of maize roots accumulates high levels of proline at low water potential but little of that proline is synthesized in the root tip (Verslues and Sharp, 1999). Ueda et al. (2007) noted that both proline content and use of proline in protein synthesis increased in salt-stressed barley roots whereas increase in P5CS1 activity was minimal. This, along with the increased proline transporter expression in the root tip (Ueda et al., 2001), strongly suggests import of proline under stress.

C. The N-Acetyl-Glutamate Pathway, Acetyl-Ornithine Amino Transferase and Ornithine Cyclodeaminase

The non-protein amino acid ornithine is a precursor of proline and an interesting metabolite in its own right because it is an intermediate in several key pathways. The best known is the urea cycle in the mitochondria. Less well studied is the acetyl-glutamate pathway which resides in the chloroplast (Figure 4). The importance of this pathway was recently indicated by Kalamaki et al. (2009), who cloned (from tomato) the first plant N-acetyl-glutamate synthase (EC 2.3.1.1) and overexpressed it in Arabidopsis. The transgenic plants had elevated levels of ornithine, were more able to germinate in the presence of salt, and soil grown plants were more resistant to salt or soil drying (Kalamaki et al., 2009). Whether or not these plants also had higher amounts of proline was not reported so it is not known whether proline accumulation contributed to the observed stress phenotypes.

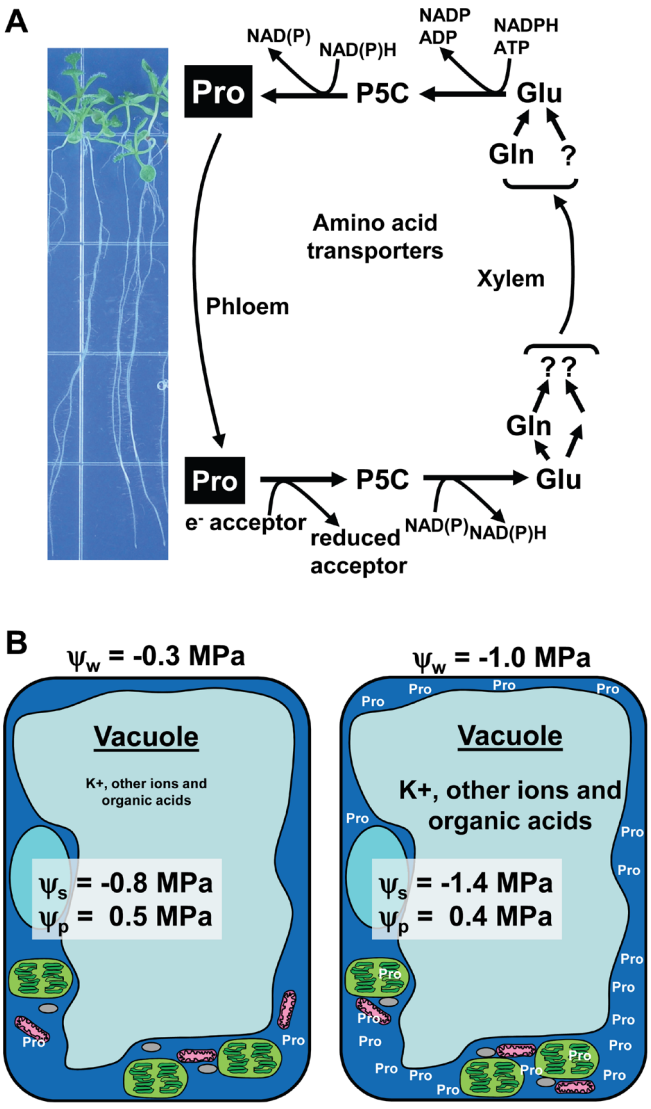
The genes and enzymes of the acetyl-glutamate pathway are all relatively uncharacterized in Arabidopsis and other plants. Two Arabidopsis genes have been annotated as N-acetyl-L-glutamate synthases based on homology to the tomato enzyme but have not been characterized in their own right. Arabidopsis has one gene encoding an N-acetyl-L-glutamate kinase, which is regulated by direct interaction with the metabolic regulatory protein PII (Chen et al., 2006) and one gene encoding an N-acetyl-gamma-glutamyl-phosphate reductase (Figure 4).

Three remaining enzymes in the pathway are key to connecting the acetyl-ornithine pathway to proline synthesis in the chloroplast and have been little studied. The first, N^2-acetyl-ornithine aminotransferase (NAOAT: EC 2.6.1.11; sometimes referred to as OAT2) is a single gene in Arabidopsis and has 26% sequence similarity with δ-ornithine aminotransfease (OAT), particularly at the argD domain (Marchler-Bauer et al., 2003; Slocum, 2005). NAOAT catalyzes the conversion of N-acetyl-glutamate-5-semialdehyde to N-acetyl-ornithine (Figure 4). Proteomic analysis has found NAOAT in the chloroplast (Kleffmann et al., 2004). Interestingly, NAOAT is perhaps better known as WIN1, a protein which interacts with the HOPW1 effector protein of *Pseudomonas syringae* and is involved in defense against bacterial pathogens (Lee et al., 2008). Thus, while *NAOAT* seems not to be induced by abiotic stress (Genevestigator data), it may instead be part of the response of proline to plant pathogen interaction (see section V.-B.).

To convert N-acetyl-ornithine directly to ornithine requires activity of an acetyl-ornithine deacetylase (EC 3.5.1.16). So far this activity has not been identified in plants and ornithine may instead be produced by Glu-N-acetyl-transferase (EC 2.3.1.35) (Figure 4). One Arabidopsis gene has been listed as an acetyl-ornithine deacetylase in previous report (Ferrario-Mery et al., 2006) but biochemical and physiological verification of this function are lacking. A functional glutamate-N-acetyl-transferase has been purified from *Citrullus lanatus* (watermelon; Takahara et al., 2005) and one Arabidopsis gene is also proposed to have this activity (Ferrario-Mery et al., 2006). In addition, there are 22 Arabidopsis genes annotated as “GCN5-related N-acetyltransferase family proteins” (the two N-acetyl-glutamate synthases are also GCN5-related), which are of unknown function but could act as amino acid acyl-transferases. Experiments such as examining whether proline is elevated in N-acetyl-glutamate synthase overexpressing plants (Kalamaki et al., 2009) and whether ornithine

and proline levels are affected in knockout mutants of the various candidate acyl-transferases and deacetylases mentioned above could more firmly identify the genes of the N-acetyl-glutamate pathway and their physiological roles.

Once ornithine is produced from the N-acetyl-glutamate pathway or other sources, it was generally thought that in plants OAT was the only route to convert ornithine to proline. There is, however, another possibility: bacteria are known to possess ornithine cyclodeaminase (OCD; also known as L-ornithine ammonia lyase or ornithine cyclase; EC 4.3.1.12). OCD can both deaminate ornithine releasing ammonium and cyclize it to produce proline without passing through the intermediate P5C (Figure 4). A suggestion that such an enzyme can function in plants comes from the discovery that the *rolD* gene present in the T-DNA of *A. rhizogenes* encodes an OCD (Trovato et al., 2001). One Arabidopsis gene has recently been annotated as an *OCD* based on sequence homology. However, its enzymatic activity and physiological role remain to be tested.



IV. CONNECTIONS OF PROLINE METABOLISM TO OTHER KEY PATHWAYS

Proline occupies a central place in metabolism and is connected to other pathways through both ornithine and glutamate. The large increases in proline that occur in response to abiotic stress make it worthwhile to consider how proline metabolism may influence and be influenced by other pathways, particularly those that are likely to have their own functions in plant stress response. We discuss some direct metabolic connections here; many more indirect connections mediated by stress and metabolic signaling factors are also likely to exist and influence proline metabolism.

A. Oxidative Pentose Phosphate Pathway

Proline synthesis consumes reductant, principally in the form of NADPH. In the chloroplast, this consumption of NADPH may be linked to the oxidative pentose phosphate pathway as a way of moving reductant out and buffering the redox status of the chloroplast (Hare and Cress, 1997; Hare et al., 1998). In the pentose phosphate pathway (Figure 4), glucose-6-phosphate (from sucrose metabolism) is oxidized to glucono-d-lactone by glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49) and to 6-phospho-gluconate by 6-phosphogluconolactonase (EC 3.1.1.31; PGL). 6-phospho-gluconate is then oxidized again to ribulose-5-phosphate (with loss of CO_2) by 6-phospho-gluconate dehydro-

Figure 5. Functions of proline and proline transport in growth and osmotic adjustment.

(A) Scheme of possible long distance proline transport in plants experiencing water limitation. In this scenario, proline synthesized in the photosynthetic tissue is transported to non-photosynthetic tissues (such as root) or other sinks and used to maintain metabolism and growth. A product of proline catabolism could then be transported back to the source tissue to sustain the cycle. Such a scenario would be consistent with high levels of proline observed in the phloem of drought stressed plants and may be one role of proline transporters that have been identified but whose physiological function is unclear.

(B) Osmotic adjustment and proline compartmentation. Cell on the left is unstressed (high water potential, -0.3 MPa) and has relatively low levels of proline and other solutes. Cell on the right is exposed to a moderate low water potential stress (-1.0 MPa). The cell on the right has decreased osmotic potential (ψ_s) to maintain water potential (ψ_w) equilibrium with its surroundings. The solute accumulation reflected in the decreased ψ_s has allowed cell volume and turgor (ψ_p) to be maintained (as ψ_s and ψ_p are the dominant components of water potential in this example the cellular water status can be expressed as $\psi_w = \psi_s + \psi_p$). Note that the -0.6 MPa change of ψ_s in the cell at -1.0 MPa corresponds to an increased solute concentration of approximately 240 mM. Accumulation of proline (and other compatible solutes) in the relatively small volume of cytoplasm is matched by accumulation of potassium and other solutes in the larger vacuole volume. Thus, an increase in proline that may not seem significant when expressed on a bulk tissue basis can cause larger changes in osmotic potential by eliciting matching accumulation of other solutes in the larger vacuole. Note that the figure also depicts the close association of chloroplasts (green), mitochondria (red) and peroxisomes (gray) which is likely to be beneficial in stressed plants (Rivero et al., 2009).

genase (6PGDH; EC 1.1.1.43). The ribulose-5-phosphate can re-enter the Calvin cycle through phosphoribulokinase (EC 2.7.1.19) or be further metabolized to glyceraldehyde-3-phosphate through a series of reactions included in the non-oxidative portion of the pentose phosphate pathway (Kruger and von Schaewen, 2003).

The production of ribulose-5-phosphate generates two molecules of NADPH and one CO_2. The NADPH can be used in the synthesis of proline and the CO_2 reassimilated by carbon fixation with the net result of reductant generated by photosynthesis being stored in proline. This may be beneficial under stress conditions where CO_2 in the chloroplast can be limited because of stomatal closure. The regeneration of CO_2 and ribulose-1,5-bis phosphate can allow continued carbon reduction and prevent photoinhibition and excess ROS production in the chloroplast. The resulting proline is a useful osmoticum for coping with water loss, can be transported into the mitochondria to pass reductant directly into the electron transport chain, or can be transported to non-photosynthetic tissue (such as roots) as a substrate for continued growth or osmotic adjustment. In this scenario, proline is a "buffer" for cellular redox status under stress and a transfer and storage molecule for that reductant in addition to its traditional roles as a compatible solute.

Whether or not such a coupling of proline and the oxidative pentose pathway exists is not well established. All three enzymes of the oxidative pentose phosphate pathway have both cytosolic and plastid-localized isozymes (Kruger and von Schaewen, 2003; Wakao and Benning, 2005; Xiong et al., 2009) and there is exchange of metabolic intermediates across the chloroplast envelope at several points (Kruger and von Schaewen, 2003). Thus, several variations on the scheme shown in Figure 4 are possible, with different partitioning of reactions between cytosol and plastid. Some of the enzymes of the oxidative pentose phosphate pathway may be stress-induced including PGL (Hou et al., 2007), although differences between gene expression and enzyme activity for other genes (Wakao and Benning, 2005) make their activity under stress difficult to predict. Replacement of cytosolic G6PDH with a plastid isozyme less sensitive to feedback inhibition by NADPH increased resistance to *Phytophthora nicotinianae* infection in tobacco and may also have increased drought resistance (Scharte et al., 2009). Also, knockout of the plastid PGL (*pgl3*) led to decreased growth and constitutive activation of defense responses (Xiong et al., 2009). These phenotypes were not seen in knockouts of the cytosolic PGL isozymes. Thus, there is evidence that the oxidative pentose phosphate pathway is important for plant stress response, and whether this is connected to proline metabolism is worthy of further investigation.

Such scenarios would fit with observations that proline accumulation induced by salt is further stimulated by light (Hayashi et al., 2000). It is also possible that regeneration of $NADP^+$ in chloroplasts by proline synthesis is beneficial even without coupling to the oxidative pentose phosphate pathway. In either case, increased photosynthetic capacity may necessitate higher proline production as a stronger buffer of cellular redox status. If such proline is then transported to non-photosynthetic tissues such as roots (Figure 5A), this would explain the high proline levels in phloem of stressed plants. In addition, some of the proline may be catabolized in the mitochondria with the reducing potential dissipated by alternative oxidase activity. The alternative oxidases, which allow electron flow that bypasses complex III and complex IV of mitochondrial electron transport, are often thought of as a "safety valve" to dissipate excess reductant and a number of studies have suggested that the alternative oxidases contribute to stress tolerance by limiting ROS generation (Umbach et al., 2005; Giraud et al., 2008; Zsigmond et al., 2008; Smith et al., 2009; Van Aken et al., 2009; Skirycz et al., 2010).

Other amino acids (such as leucine and isoleucine) that accumulate under stress and whose synthesis in the plastid consumes NADPH and catabolism in the mitochondria releases reductant could also be part of such a mechanism. These other amino acids would seem to be lesser contributors though because their biosynthetic pathways are not transcriptionally upregulated by stress in the same way that proline biosynthesis is (Less and Galili, 2008). It would seem that proline is the main compound involved because it combines the high accumulation and protective properties of a compatible solute with function as a metabolic shuttle. Also important is that ProDH donates electrons directly to the mitochondrial electron transport chain instead of reducing NADH or NADPH. Further experiments are needed to test such hypotheses.

This view that energy dissipation and redox buffering are important functions of drought-induced metabolic changes is supported by recent characterization of drought-resistant, cytokinin-overproducing plants (Rivero et al., 2007). Drought resistance of these plants may in part derive from increased photorespiration as a way to dissipate excess energy and regenerate ribulose-bisphosphate for operation of the Calvin cycle (Rivero et al., 2009). These concepts are in turn part of an emerging view that mitochondria and energy dissipating reactions in the mitochondria are important for drought tolerance (Atkin and Macherel, 2009).

B. Nitrogen Assimilation and Glutamate/Glutamine Availability

Both the dramatically reduced proline in *p5cs1* mutants and studies of amino acid labeling during stress suggest glutamate as a main precursor of proline (Rhodes et al., 1986; Szekely et al., 2008; Sharma and Verslues, 2010). Glutamate availability is ultimately controlled by nitrogen assimilation, primarily through the action of glutamine synthetase (GS; EC 6.3.1.2), which uses glutamate and ammonium to produce gluatamine. Glutamate is then regenerated by the action of glutamate synthase (GOGAT; EC 1.4.1.13; Figure 4). Another possible route of generating glutamate in the mitochondria is glutamate dehydrogenase (GDH; EC 1.4.1.3), which can either directly produce glutamate from ammonium and α-ketoglutarate or deaminate glutamate to produce α-ketoglutarate (Forde and Lea, 2007)(Figure 4).

Although the role of GDH is not clear, there is evidence that it can be important in supplying glutamate for proline synthesis. Tobacco GDH protein level and activity were induced by salt stress and ^{15}N-labeling experiments performed in the presence of inhibitors to block the GS-GOGAT pathway of glutamate production suggested that a significant amount of glutamate and proline were produced by GDH rather than the GS-GOGAT pathway (Skopelitis et al., 2006). Genetic experiments to further test the importance of GDH under other stresses such as drought will be of future interest. Such experiments may resolve the apparent conflict between these observations of GDH as important for proline production with other observations that the deaminase activ-

ity of GDH rather than glutamate synthesis is predominant under standard growth conditions (Skopelitis et al., 2007; Labboun et al., 2009).

Because glutamate is involved in many reactions and its levels are tightly controlled (Forde and Lea, 2007), glutamine can serve as a reservoir of nitrogen through transamination with glutamate. The importance of this glutamine reservoir for proline metabolism was shown by Brugiere et al (1999) who specifically inhibited expression of *Glutamine Synthetase1* (*GS1*) in phloem companion cells of tobacco. This resulted in decreased levels of glutamine and proline (but unchanged glutamate) in both unstressed and salt stressed plants as well as increased salt sensitivity of the transgenics. The results showed that glutamine was metabolized to proline via a pool of glutamate that remained unchanged in amount but turned over rapidly. In addition, Díaz et al. (2010) found that a *Lotus japonicus* mutant (*Ljgln2-2*) deficient in plastid-localized glutamine synthetase also had reduced proline accumulation during drought and altered expression of proline metabolism genes. Transcript profiling found more extensive upregulation of stress-induced genes in *Ljgln2-2,* suggesting that the mutant was more stress-damaged than wild type, presumably because of the reduced glutamine and proline synthesis.

The results of these studies show that the source of glutamate for proline synthesis may vary with nitrogen source and stress condition and can be a limiting factor for proline synthesis. Although the metabolism of glutamate is complex (GS, GOGAT, and GDH are all encoded by multiple genes and the enzymes can both be present in multiple compartments; Figure 4), consideration of the metabolic source of glutamate is likely to be important in attempts to engineer plants that overproduce proline as an osmoprotectant.

C. The GABA Shunt

One glutamate product that stands out from a stress perspective is GABA. GABA shares many of proline's characteristics as a compatible osmolyte and also accumulates in response to various stresses (Shelp et al., 1999), although in Arabidopsis at low water potential the levels of GABA are much lower than that of proline (P.E. Verslues, unpublished). There are many of the same questions about the role of GABA under stress as there are about proline and thus they would seem to be complimentary compounds, although they may in fact be antagonistic in function. GABA is produced by glutamate decarboxylase (EC 4.1.1.15) in the cytoplasm and elevated glutamate levels can stimulate GABA production (Shelp et al., 1999). Thus GABA and proline synthesis are potentially in competition for the same substrate (although their compartmentation may be different). GABA can be catabolized in the mitochondria by transaminases to produce either glutamate or alanine and succinic semialdhyde which is reduced to succinate that can enter the TCA cycle. The observations that the GABA-to-proline ratio influences quorum sensing and virulence in *Agrobacterium tumefacians* (Haudecoeur et al., 2009) and that GABA may deter insect feeding (MacGregor et al., 2003) also make the interaction of GABA and proline metabolism a question of further interest (see section V.-B. below for further discussion).

D. Arginine, Nitric Oxide and Polyamines

Arginine can be both a precursor and product of ornithine through the action of the urea cycle with arginase as the key enzyme converting arginine to ornithine (Figure 4). Arginine, via the action of arginine decarboxylase (EC 4.1.1.19), is also the precursor of the polyamines putrescine, spermidine and spermine (direct decarboxylation of ornithine by ornithine decarboxylase is also possible but this enzyme has not been found in Arabidopsis; see Alcazar et al., 2010). A number of studies have described polyamines as protective compounds for plants exposed to abiotic stress or perhaps regulators of stress responses (see for example Panicot et al., 2002; Perez-Amador et al., 2002; Cuevas et al., 2008; Alcazar et al., 2010). The levels of polyamines, particularly spermine and spermidine, are typically low compared with proline and most other amino acids. Polyamine and proline metabolism may interact through ornithine as a common intermediate.

Arginine may also be a source of nitric oxide via nitric oxide synthase (EC 1.14.13.39) which converts arginine to citrulline and nitric oxide. Whether or not nitric oxide synthase exists in plants is controversial (Crawford et al., 2006; Zemojtel et al., 2006). However, it has been observed that arginase mutants, which are predicted to have higher mitochondrial arginine levels, also produced more nitric oxide (Flores et al., 2008). Thus, even though the gene has not been identified, nitric oxide synthase may still be present and may be important for stress resistance (Zhao et al., 2007). As polyamines can also be metabolized to produce nitric oxide (Alcazar et al., 2010) and the oxidative pentose phosphate pathway may also be a source of NADPH for nitric oxide synthesis (Scharte et al., 2009) proline metabolism could influence nitric oxide production at several points. This is of interest because nitric oxide may also be one factor regulating the expression of *P5CS1* and *ProDH* (Zhao et al., 2009). Such interaction between proline and ornithine-arginine-nitric oxide may also have role in the newly emerging connection of proline metabolism and pathogen response (Section V.-B).

V. FUNCTIONS OF PROLINE METABOLISM IN PLANT ENVIRONMENT INTERACTION AND DEVELOPMENT

A. Abiotic Stress

Whether or not proline accumulation is an adaptive response to abiotic stress has been debated since Kemble and McPherson (Kemble and Macpherson, 1954) first observed proline accumulation in wilted ryegrass. Apparent correlations between high proline and greater stress induced injury led some to conclude that proline was a symptom of injury or merely a result of decreased growth and metabolism rather than an adaptive response (Stewart and Hanson, 1980). Indeed, it can seem contradictory that plants make large amounts of proline while at the same time restriction of carbohydrate production by photosynthesis is a limitation on growth and reproductive performance during drought (Boyer, 2010). However, controlled experiments show that proline accumulation occurs even in plant tissues where growth continues and injury is minimal (Voetberg and Sharp, 1991; Ober and Sharp, 1994) and also occurs to significant extent under mild and moderate stress treatments (Sharma and Verslues, 2010). Pro-

line also accumulates to extremely high levels in pollen and this is likely related to pollen desiccation tolerance (Schwacke et al., 1999). Moreover, a number of groups have noted higher proline in more drought adapted varieties of wild or cultivated plants (Ben Hassine et al., 2008; Parida et al., 2008; Evers et al., 2010). Also, the salt-tolerant Arabidopsis relative *Thellungiella halophila* accumulates more proline during salt stress than the standard Columbia-0 ecotype of Arabidopsis (Kant et al., 2006). Proline may also serve as a precursor for proline- or hydroxyproline-betaines as even more effective osmoprotectants for plants in chronically dry environments (Hanson et al., 1994).

The advent of molecular data has shown that proline metabolism is actively regulated by stress signals and the use of reverse genetics (such as *p5cs1* mutants; Szekely et al., 2008) has allowed more direct tests of the requirement of proline accumulation for stress resistance. Such data have shown extensive regulation of proline metabolism by abiotic stress, especially at the transcriptional level, and have firmly established that stress-induced proline accumulation is an adaptive response. The challenge remains to answer questions of how proline contributes to plant stress resistance, the metabolic regulation that allows high levels of proline to accumulate, and whether modification of proline metabolism is useful for biotechnological improvement of plants.

The many studies testing response to stresses that change plant water status (drought, salinity, freezing) have found several likely functions of proline in stress resistance:

1. *Osmotic adjustment:* Accumulation of proline (and other solutes) decreases cellular osmotic potential to balance decreases in soil water potential while allowing turgor and water cotent to be maintained (Figure 5B), Evidence such as high levels of proline in unvacuolated cells of the root tip (Voetberg and Sharp, 1991; Verslues and Sharp, 1999) and fractionation studies (Bussis and Heineke, 1998) indicate that proline is accumulated in the cytoplasm and chloroplast stroma while other solutes (sugars, organic acids, potassium) are accumulated in the vacuole. Because the cytoplasm is a small fraction of cellular volume, local concentrations of proline in the cytoplasm can be much higher than the bulk tissue level. Relatively small (on a bulk tissue basis) changes in proline may have larger effects on total osmotic adjustment by causing additional accumulation of potassium and other solutes in the vacuole (Figure 5B). Specific measurements of total cellular solute content (osmotic potential) and water content are needed to quantify the effect of changing proline content on osmotic adjustment (especially important for studies of transgenic plants).
2. *Protection of cellular structure during dehydration:* Proline has properties (highly soluble, zwitterionic) observed in compatible solutes that accumulate in organisms as diverse as bacteria and deep sea animals (Yancey et al., 1982; Yancey, 2005). These properties allow proline to accumulate to high levels without disrupting cellular structure (hence its preferential accumulation in cytoplasm). When cellular water content decreases, proline, other compatible solutes and some intrinsically unstructured proteins (such as certain classes of Late Embryogenesis Abundant proteins) can act as "water substitutes" to stabilize cellular structure through hydrophilic interactions and hydrogen bonding.
3. *Redox buffering:* Synthesis of proline uses NADPH in the chloroplast or cytosol, whereas catabolism of proline releases reductant in the mitochondria. Adjusting the balance of proline synthesis and degradation can alleviate imbalances in cellular redox (as described above for the connection of proline metabolism to the oxidative pentose phosphate pathway; Section IV.-A).
4. *Storage and transfer of reductant (and nitrogen):* This function may be part of redox buffering at the whole plant level. Synthesis and catabolism of proline can be separated in either time (proline synthesis during stress; proline catabolism after stress release) or space (for example synthesis of proline in photosynthetic tissue, catabolism in other tissues such as root, Figure 5A). Observations of high levels of proline in phloem of drought-stressed plants (Girousse et al., 1996; Lee et al., 2009) and lack of *ProDH* downregulation in Arabidopsis root tip or shoot meristem during low water potential stress (S. Sharma, J.G.C. Villamor and P.E. Verslues, unpublished) suggest the importance of proline movement within the plant and separation of synthesis and catabolism between different tissues. Rapid decreases in proline levels after stress release may be one factor in resumption of growth after stress which is also an important determinant of overall stress tolerance (Hayano-Kanashiro et al., 2009).
5. *A signaling molecule:* Transport of proline between different parts of the plant may serve as a metabolic signal (Hare and Cress, 1997; Hare et al., 1998, 1999). As noted above, proline can induce expression of *ProDH* and perhaps other genes via the PRE. Regulation of gene expression by P5C application has been noted (Iyer and Caplan, 1998) and P5C is proposed to enhance mitochondrial reactive oxygen production and apoptosis (see section V.-B.). Regulation of cell death has obvious importance for incompatible plant-pathogen interactions, but may also be relevant to drought as plants with delayed senescence were reported to have dramatically improved drought resistance (Rivero et al., 2007).
6. *Reactive oxygen scavenging:* In this case proline itself reacts with and detoxifies ROS (Smirnoff and Cumbes, 1989). Although often mentioned in the literature, the products of such reactions and the importance of proline relative to other ROS scavenging systems are unclear. This mechanism may be most important in cases of extreme dehydration of the plant tissue.

These functions are not mutually exclusive and likely the reason that plants accumulate proline is that it fulfills several of these roles at once. Another key point is that the function of proline likely varies for different stresses, as suggested by the differential accumulation of proline under low water potential and salt stress, for example (Figure 1B and C) and the differential regulation of some proline metabolism enzymes by salt and low water potential (Figure 3). Some of these functions, such as osmotic adjustment and acting as a compatible solute, depend on the properties of proline itself and the amount accumulated during stress. Other functions, such as redox buffering, depend on the metabolism of proline and flux through proline synthesis or catabolism.

One can also ask how unique proline is in these functions. Many metabolites exhibit at least a low level of accumulation (3-fold or less) in drought-stressed plants. In some cases this can be explained by dehydration of the plant tissue that decreases the

volume of water in the plant tissue or by reduced growth, which alters the balance between metabolite synthesis and dilution by water uptake and growth. Note that such reasons have been shown not to explain low water potential-induced proline accumulation (see for example Voetberg and Sharp, 1994). In addition to proline, other amino acids accumulate upon drought stress including leucine, isoleucine, valine, lysine, methionine and phenylalanine (Charlton et al., 2008; Joshi and Jander, 2009). Several of these amino acids, particularly the branched chain amino acids (leucine, isoleucine and valine) and threonine, are present at higher levels than other amino acids and have synthesis pathways that consume NADPH in the plastids and catabolism pathways that release reductant in the mitochondria (pathway information is available from AraCyc). Thus they could participate in similar redox buffering and energy transfer mechanisms (proline functions number 3, 4 and 5 above) as proline metabolism. In most cases, proline accumulates to the highest levels and is likely to be the most important component of redox buffering under drought stress. It is possible that there are exceptions to this in certain tissues or Arabidopsis ecotypes that accumulate only low levels of proline. It should also be acknowledged that because there is a specific colorimetric assay for proline (Bates et al., 1973; Verslues, 2010) many studies have only measured proline and not compared its level to that of other metabolites.

The protective functions of proline may be shared with specialized compatible solutes that accumulate during stress, such as glycine betaine and sugar alcohols. A detailed discussion of compatible solutes can be found in Yancey (2005). These compounds are typically not catabolized rapidly and thus unlikely to function in redox buffering but may be important in osmotic adjustment and protection of cellular structure (proline functions number 1 and 2 above). Complex carbohydrates such as raffinose are also likely to have stress protective functions. Metabolomic studies have shown that even in Arabidopsis there are many additional complex carbohydrates that accumulate under stress but remain to be identified (Wilson et al., 2009). Proline is often present at the highest concentration and makes the most substantial contribution of osmotic potential, especially in growing tissue (Voetberg and Sharp, 1991). However, the sum of these other compounds is also significant in osmotic adjustment and it is likely that the mix of compatible solutes accumulated is more protective of cellular structure than any one of them alone. Our focus on proline here should not be seen as neglecting the role of these other metabolic adaptations. Nonetheless, observations that many plants accumulate large amounts of proline as part of their stress response strategy indicate an advantage over other possible strategies. More clearly defining what such advantages are is a key question for future proline research.

B. Biotic Interactions (pathogen and nodulation) and Programmed Cell Death

Pathogen infection can also affect proline content and gene expression of proline metabolism. Fabro et al. (2004) found that inoculation of Arabidopsis with avirulent *P. syringae* that produced a hypersensitive response led to increased proline and the induction of *P5CS2* and *ProDH* expression around the inoculation site. Conversely, inoculation with a virulent strain of the same pathogen that did not induce a hypersensitive response also failed to induce *P5CS2*, *ProDH* or proline accumulation. The authors suggested that induction of both *P5CS2* and *ProDH* led to increased levels of P5C, which activated programmed cell death during the hypersensitive response (Fabro et al., 2004). This was consistent with reports of cell death in plants with mis-regulated *ProDH* expression (Hellmann et al., 2000) or findings that *p5cdh* mutants were prone to cell death and showed altered pathogen response (Deuschle et al., 2004). Cell death (apoptosis) activated by proline metabolism and P5C has also been described in mammalian systems. Another report showed induction of *P5CDH* by compatible fungal infection (Ayliffe et al., 2002), possibly a mechanism used by the pathogen to keep P5C levels low and inhibit cell death and the hypersensitive response. Although the possible role of P5C as an inducer of programmed cell death has been mentioned in a number of places, there is no information on the underlying mechanism except that the salicylic acid pathway may be involved (Deuschle et al., 2004).

The ratio of proline to GABA plays a role in *A. tumefacians* virulence (Haudecoeur et al., 2009). Plant-produced GABA attenuates *A. tumefacians* virulence by enhancing the degradation of a quorum sensing signal needed to activate T-DNA transfer. Proline blocks the effect of GABA by competing with it for uptake into the *A. tumefacians* cell. Thus, high GABA concentrations decrease tumor initiation, whereas high proline promotes tumor initiation. Increased GABA production may be part of the plant's defense against infection. Conversely, the pathogen may promote proline synthesis to overcome the GABA defense. High levels of proline are seen in *A. tumefacians*-induced tumors (Wachter et al., 2003; Deeken et al., 2006; Efetova et al., 2007), although GABA was also high in at least one case (Deeken et al., 2006). At least part of the proline production may be mediated by the bacteria themselves: the *A. tumefacians* Ti plasmid encodes an OCD to produce proline from ornithine (Farrand and Dessaux, 1986; Sans et al., 1988) and in *A. rhizogenes* the OCD is part of the T-DNA and enhances virulence (Mauro et al., 1996). Using OCD for proline synthesis in tumors may avoid the production of P5C which could promote apoptosis and limit tumor growth. It has also been suggested that high levels of proline (and ABA) are present in plant tumors because tumors have high rates of water loss and are prone to dehydration (Efetova et al., 2007). In this case, determining the metabolic source of proline (synthesis from ornithine or glutamate) would also tell much about the reason for its accumulation.

Proline also figures prominently in nodulation; another type of plant microbe interaction. *Rhizobium meliloti* lacking expression of OAT nodulate less effectively than wild type *R. meliloti* strains (Soto et al., 1994) and soybean nodulated with *R. meliloti* deficient in ProDH activity had reduced nitrogen fixation (Curtis et al., 2004). Conversely, supply of exogenous proline increases nitrogen fixation (Zhu et al., 1992). Ability of the bacteriods to catabolize proline was even more important under drought stress (Kohl et al., 1991; Straub et al., 1995; Straub et al., 1997). Proline metabolism in nodules may be coupled to the pentose phosphate pathway, similar to the mechanism described above for leaf tissue (Section IV.-A). In bacteroids, proline synthesis could produce $NADP^+$ which would allow continued operation of the pentose phosphate pathway to generate precursors for ureide synthesis (Kohl et al., 1988; Kohl et al., 1990).

C. Plant Insect Interactions-Proline as an Attractant and Fuel

Plant nectars have high levels of proline, up to 2 mM (Carter et al., 2006), and this high level of proline is thought to be an attractant, as several species of insects prefer high-proline nectars (Carter et al., 2006; Bertazzini et al., 2010). It was also reported that application of proline to plants stimulated insect feeding (Haglund, 1980), whereas plant-produced GABA was an inhibitor of insect feeding (MacGregor et al., 2003). Proline was also found to be the most highly increased (more than 10-fold) amino acid in response to compatible infections of wheat with *Mayetiola destructor* (Hessian fly), a plant parasite (Zhu et al., 2008). No increase in proline was seen in incompatible *M. destructor* interactions. The increase in proline involved upregulation of *P5CS1* expression, but the reason for proline to accumulate in this case is not known. It is interesting that several studies have shown the proline is an important metabolic fuel used during insect flight (Micheu et al., 2000; Scaraffia and Wells, 2003), perhaps explaining the apparent affinity of insects for proline.

D. Development

Proline can also influence plant development via unknown mechanisms. Proline content is high in reproductive tissues (Mutters et al., 1989; Chiang and Dandekar, 1995; Schwacke et al., 1999; Matsui et al., 2008; Mattioli et al., 2009). *P5CS1* and *P5CR* were also found to be highly expressed in reproductive tissues (Verbruggen et al., 1993; Savoure et al., 1995). Consistent with this, decreased *P5CS1* expression in antisense Arabidopsis plants caused impaired bolting and delayed flowering as well as decreased proline and hydroxyproline in the cell wall protein fraction (Nanjo et al., 1999b). Similar reduced proline and late flowering phenotypes were also observed in *p5cs1* T-DNA mutants, and an even more severe effect was seen in *p5cs1*$^{-/-}$/*p5cs2*$^{+/-}$ mutant plants (Nanjo et al., 1999b; Mattioli et al., 2008; Mattioli et al., 2009). Conversely, constitutive expression of *P5CS1* caused early flowering in transgenic plants under normal (Mattioli et al., 2008) or salt-stress conditions (Kavi Kishor et al., 1995). A portion of Arabidopsis plants ectopically expressing *P5CS1* were shorter in size and produced many coflourescences (clusters of flowers), had an overall "bushy" appearance because of reduced elongation of the main inflourescence stem, and had an extended lifespan compared to wild type (Mattioli et al., 2008). Overexpression of *A. rhizogenes rolD*, which encodes an OCD, also induced early flowering in transgenic tobacco (Mauro et al., 1996) and tomato (Bettini et al., 2003). This would suggest that increased proline can cause early flowering no matter which pathway of proline synthesis is used.

Interestingly, *P5CS2* was identified as a target of CONSTANS, a transcriptional activator involved in floral transition of Arabidopsis (Samach et al., 2000), suggesting that effects of proline on flowering are part of normal development and not an artifact caused by ectopic expression of proline synthesis or extreme restriction of proline supply in *p5cs* mutants. The combined observations are consistent with proline, and induction of proline synthesis at a specific time/developmental stage, acting as a signal to promote flowering. How such a proline signal may be perceived and affect floral transition is not known.

VI. OBSERVATIONS FROM NON-PLANT SYSYTEMS

Observations from other systems are informative about the roles and regulation of proline metabolism in plants. Bacteria and fungi also accumulate proline in response to increased external osmolarity and loss of water (Jennings and Burke, 1990; Madkour et al., 1990; Poolman and Glaasker, 1998). Proline metabolism also comes up in some interesting, and perhaps unexpected, places in mammalian biology.

A. Microbial Systems-Proline, Osmoregulation and Oxidative Stress

Bacteria accumulate proline in response to hypo-osmolarity, either by synthesizing it or by taking it up from the surrounding media, and the role of proline as a compatible, osmoregulatory solute is well established in the microbiology literature (Grothe et al., 1986). The direct link of proline to osmoregulation in bacteria is illustrated by studies of ProP, an *E. coli* transporter that takes up external proline. ProP is a direct osmosensor: transport is activated by osmotic shifts and is intrinsic to the ProP protein as ProP alone reconstituted in lipid bilayers is able to open and close normally in response to osmotic shifts (Culham et al., 2008). Thus, in bacteria, proline accumulation is directly linked to osmoregulation. Plant osmosensing and osmoregulation are not understood but may also be linked to proline metabolism as proline is an important osmoregulatory solute. Another unique feature of bacterial proline metabolism is the bacterial PutA protein that functions as both a proline catabolic enzyme and a DNA-binding transcriptional repressor of the Put (Proline Utilization) operon (Zhou et al., 2008).

A yeast gene, MPR1, was found to encode a novel P5C-acetyl transferase (Nomura and Takagi, 2004). Acylation of P5C by MPR1 prevents its function in apoptosis (or "detoxifies" it depending on one's perspective). Such removal of P5C enhances tolerance to oxidative stress. Thus in yeast, as in other systems, there is evidence that P5C is a potent inducer of cell death. Such studies also make clear the need to distinguish the functions of proline metabolism from the properties of proline itself: in contrast to the effect of proline metabolism leading to P5C production, exogenous proline applied to a fungal pathogen inhibited ROS-induced cell death and was proposed to be a ROS scavenger (Chen and Dickman, 2005).

B. Mammalian Biology-Connection of Proline Metabolism to Apoptosis and Cancer.

Further evidence of a link between proline metabolism and apoptosis comes from cancer biology where *ProDH* is known as a pro-apoptosis gene and is regulated by the tumor-suppressor p53 (Maxwell and Davis, 2000; Donald et al., 2001). Expression of *ProDH* can inhibit growth of tumorigenic cell lines by activating apoptosis (Maxwell and Davis, 2000; Donald et al., 2001). This occurs via proline-stimulated reactive oxygen production in the mitochondria and activation of multiple pro-apoptosis pathways (Liu et al., 2005a; Liu et al., 2006; Liu et al., 2008). The P5C-induced reactive oxygen signaling appears to involve super oxide

as co-expression of super oxide dismutase can block the pro-apoptotic effect of ProDH (Liu et al., 2005b). One study has also reported p53 regulation of ALDH4, a human P5CDH (Yoon et al., 2004), perhaps providing another point of p53 control over P5C levels and apoptosis. The regulation of *ProDH* by p53 and its role in apoptosis are consistent with the expression of plant *ProDH* in incompatible plant-pathogen interactions, where cell death also occurs (Fabro et al., 2004), and observations of cell death phenotypes in *p5cdh* mutants (Deuschle et al., 2004).

Defects in *ProDH* cause nervous system disorder in the *Sluggish* mutant of Drosophila (Hayward et al., 1993) and mouse mutants (Gogos et al., 1999). In humans, variants of *ProDH* are associated with schizophrenia (Kempf et al., 2008; Willis et al., 2008) and proline is thought to be a modulator of synaptic transmission (Gogos et al., 1999). Certainly, it is not only plant biologists interested in special properties and metabolism of proline.

VII. BIOTECH MANIPULATION OF PROLINE PLANTS

Efforts to engineer increased proline accumulation and enhance plant stress tolerance began almost immediately after the first enzymes had been cloned but have produced somewhat ambiguous results. Kavi Kishor et al. (1995) produced transgenic tobacco ectopically expressing *V. aconitifolia* P5CS. The plants accumulated more proline than wild type, but further interpretation of the phenotype was hampered by concerns about water relations measurements of the transgenic plants (Blum et al., 1996). The same group also produced transgenic plants ectopically expressing P5CS with reduced feedback inhibition (Hong et al., 2000). These plants produced even more proline than plants expressing wild type P5CS and were more resistant to salt stress.

A number of other laboratories have followed this work by ectopically expressing P5CS in several of plant species and reporting increased resistance to soil drying/dehydration, salinity or cold (see for example: Zhu et al., 1998; Hong et al., 2000; Sawahel and Hassan, 2002; Su and Wu, 2004; Vendruscolo et al., 2007; Parvanova et al., 2004; Gleeson et al., 2005). In aggregate, these studies support a role for proline in stress resistance. Individually, the strength of the data supporting increased stress tolerance varies greatly between studies. This is especially a concern for drought stress, where appropriate experimental design and water relations measurements are needed to properly interpret differences between transgenic and control plants (Verslues et al., 2006). In most studies, the increase in proline has been relatively small compared to the already substantial accumulation of proline in the wild type background. This often has been interpreted as an indication that non-osmotic roles of proline such as reactive oxygen scavenging were most important to the proposed increase in stress tolerance. However, this must be taken with some caution, as few studies have directly measured the effect of manipulating proline content on osmotic adjustment. As illustrated in Figure 5B, an increased proline content of the relatively small volume of the cytoplasm could elicit a matching increase of other solutes in the much larger volume of the vacuole to maintain osmotic balance between cytoplasm and vacuole.

A more limited number of papers have sought to engineer proline accumulation via overexpression of *OAT* or further repression of *ProDH*. *OAT* over-expression lines of Arabidopsis (Roosens et al., 2002) and rice (Wu et al., 2003) had increased proline content and higher biomass or germination in osmotic stress. Increased or decreased *ProDH* level in sense and anti-sense transgenic plants led to 50% decreased or 25% increased proline level, respectively. However, the change in proline content did not show any correlation with osmotolerance (Mani et al., 2002). On the other hand, Nanjo et al. (1999a) proposed a positive correlation between increased proline accumulation and freezing and salt tolerance in *ProDH* anti-sense transgenic plants. The apparent contradiction between these last two studies highlights the importance of detailed phenotypic measurements to determine how stress response of the transgenic differs from that of wild type. Also of concern, but not always well documented, is how the manipulation of proline metabolism affected plant performance in the absence of stress.

VIII. FUTURE PROSPECTS

In this review, we have brought up a number of questions for future research. These include metabolic pathways where the relevant genes have yet to be identified and experimentally verified (such as the acetyl-glutamate pathway), the identity of intracellular transporters to move proline and related metabolites among cellular compartments, long-distance transport of proline and its functional significance, post-translational regulation of proline metabolism and the sensing and signal transduction controlling proline metabolism. Some of these questions, such as the role of long-distance transport of proline in the phloem, will require a “whole plant” perspective, whereas other questions will require detailed biochemical studies of the core enzymes and transporters. The level of proline can also serve as a “readout” of stress sensing and signaling and thus can be an important phenotype to identify signaling mutants. As proline accumulation and expression of proline metabolism genes are regulated in a largely ABA-independent manner, use of proline as a marker is likely to identify different genes than approaches focused on ABA signaling. Proline has traditionally been studied in the context of abiotic stresses such as drought and salinity. While this will continue, we think there is ample evidence to stimulate studies of proline metabolism in plant pathogen interaction and programmed cell death.

The need to establish such basic knowledge as a prerequisite to applied studies means that Arabidopsis and other model species can contribute relatively more to the study of proline metabolism than has been the case in the past. In addition to forward and reverse genetic approaches, the genetic diversity among Arabidopsis accessions is a resource to be developed in understanding both the role of proline under stress and metabolic regulation more generally. Our laboratory is already finding substantial variation in the proline accumulation among Arabidopsis accessions (unpublished data).

For these resources to be used most effectively and the results utilized by the crop science community, Arabidopsis researchers must be realistic about the strengths and limitations of their experimental system. For example, crop researchers have noted that limitation of photosynthetic carbohydrate supply is a major problem in drought stress, particularly in reproductive development (Boyer, 2010). Many Arabidopsis experiments, however, are done in the presence of high levels of sugar in the media. Thus,

the carbon limitation encountered by stressed plants in the field is not incorporated in many Arabidopsis genetic experiments. Many Arabidopsis experiments look only at the short term (a few hours or less) response, whereas other responses (such as proline accumulation, Figure 1A) can take longer to occur. One can certainly come up with other examples and it is not possible (or even desirable) for Arabidopsis genetic experiments to reflect all aspects of field stress conditions. An honest assessment of the pros and cons of each experimental system will be essential to apply knowledge gained in Arabidopsis to other plants.

From an applied science perspective, proline is still of interest as a strategy for increasing plant stress tolerance, but increasing basic knowledge should lead to more refined approaches of engineering proline metabolism. Overall, the first generation biotechnological manipulation of proline metabolism used relatively unregulated ectopic expression of proline metabolism genes, typically using standard tools such as the 35S promoter. Stress-inducible (Su and Wu, 2004) and/or targeted expression of the relevant enzymes and transporters will be needed to achieve a desired metabolic effect rather than just a general overproduction of proline. For example, targeted overexpression of proline synthesis in the shoot, perhaps coupled with expression of transporters to move proline to destination tissue such as the root could be beneficial in some stress situations. Detailed measurements of plant phenotype, and water relations measurements in the case of drought experiments, will continue to be of importance. Side effects of manipulating proline metabolism on other phenotypes, such as pathogen response and insect feeding, will need to be kept in mind if such transgenics are promising enough for the ultimate test of field trails. Overall, a better understanding of the many questions about proline metabolism left unanswered in this review will allow a next generation of experiments testing the value of proline metabolism in plant improvement.

ACKNOWLEDGEMENTS

This work was supported by an Academia Sinica Career Development Award and funding from the National Science Council of Taiwan (to P.E.V). S.S. was supported by a postdoctoral stipend from the National Science Council of Taiwan.

We would like to acknowledge the many older studies of proline metabolism and related topics that we were unable to cite here but nonetheless contributed to present understanding. We also thank Dr. Wendy Hwang-Verslues (Genomics Research Center, Academia Sinica) for critical reading of the manuscript.

REFERENCES

Abraham, E., Rigo, G., Szekely, G., Nagy, R., Koncz, C., and Szabados, L. (2003). Light-dependent induction of proline biosynthesis by abscisic acid and salt stress is inhibited by brassinosteroid in Arabidopsis. Plant Molecular Biology **51,** 363-372.

Alcazar, R., Altabella, T., Marco, F., Bortolotti, C., Reymond, M., Koncz, C., Carrasco, P., and Tiburcio, A.F. (2010). Polyamines: molecules with regulatory functions in plant abiotic stress tolerance. Planta **231,** 1237-1249.

Armengaud, P., Thiery, L., Buhot, N., Grenier-de March, G., and Savoure, A. (2004). Transcriptional regulation of proline biosynthesis in *Medicago truncatula* reveals developmental and environmental specific features. Physiologia Plantarum **120,** 442-450.

Atkin, O.K., and Macherel, D. (2009). The crucial role of plant mitochondria in orchestrating drought tolerance. Annals of Botany **103,** 581-597.

Ayliffe, M.A., Roberts, J.K., Mitchell, H.J., Zhang, R., Lawrence, G.J., Ellis, J.G., and Pryor, T.J. (2002). A plant gene up-regulated at rust infection sites. Plant Physiology **129,** 169-180.

Bates, L.S., Waldren, R.P., and Teare, I.D. (1973). Rapid determination of free proline for water-stress studies. Plant and Soil **39,** 205-207.

Ben Hassine, A., Ghanem, M.E., Bouzid, S., and Lutts, S. (2008). An inland and a coastal population of the Mediterranean xero-halophyte species *Atriplex halimus* L. differ in their ability to accumulate proline and glycinebetaine in response to salinity and water stress. Journal of Experimental Botany **59,** 1315-1326.

Benschop, J.J., Mohammed, S., O'Flaherty, M., Heck, A.J.R., Slijper, M., and Menke, F.L.H. (2007). Quantitative phosphoproteomics of early elicitor signaling in Arabidopsis. Mol Cell Proteomics **6,** 1198-1214.

Bertazzini, M., Medrzycki, P., Bortolotti, L., Maistrello, L., and Forlani, G. (2010). Amino acid content and nectar choice by forager honeybees (*Apis mellifera* L.). Amino Acids **39,** 315-318.

Bettini, P., Michelotti, S., Bindi, D., Giannini, R., Capuana, M., and Buiatti, M. (2003). Pleiotropic effect of the insertion of the *Agrobacterium rhizogenes* rolD gene in tomato (*Lycopersicon esculentum* Mill.). Theoretical and Applied Genetics **107,** 831-836.

Blum, A., Munns, R., Passioura, J.B., Turner, N.C., Sharp, R.E., Boyer, J.S., Nguyen, H.T., Hsiao, T.C., Verma, D.P.S., and Hong, Z. (1996). Genetically engineered plants resistant to soil drying and salt stress: How to interpret osmotic relations? Plant Physiology **110,** 1051-1053.

Borsani, O., Zhu, J., Verslues, P.E., Sunkar, R., and Zhu, J.-K. (2005). Endogenous siRNAs derived from a pair of natural cis-antisense transcripts regulate salt tolerance in Arabidopsis. Cell **123,** 1279-1291.

Boyer, J.S. (2010). Drought decision-making. Journal of Experimental Botany **61,** 3493-3497.

Brugiere, N., Dubois, F., Limami, A.M., Lelandais, M., Roux, Y., Sangwan, R.S., and Hirel, B. (1999). Glutamine synthetase in the phloem plays a major role in controlling proline production. Plant Cell **11,** 1995-2012.

Bussis, D., and Heineke, D. (1998). Acclimation of potato plants to polyethylene glycol-induced water deficit - II. Contents and subcellular distribution of organic solutes. Journal of Experimental Botany **49,** 1361-1370.

Carter, C., Shafir, S., Yehonatan, L., Palmer, R.G., and Thornburg, R. (2006). A novel role for proline in plant floral nectars. Naturwissenschaften **93,** 72-79.

Charlton, A.J., Donarski, J.A., Harrison, M., Jones, S.A., Godward, J., Oehlschlager, S., Arques, J.L., Ambrose, M., Chinoy, C., Mullineaux, P.M., and Domoney, C. (2008). Responses of the pea (*Pisum sativum* L.) leaf metabolome to drought stress assessed by nuclear magnetic resonance spectroscopy. Metabolomics **4,** 312-327.

Chen, C.B., and Dickman, M.B. (2005). Proline suppresses apoptosis in the fungal pathogen *Colletotrichum trifolii*. Proceedings of the National Academy of Sciences of the United States of America **102,** 3459-3464.

Chen, Y.M., Ferrar, T.S., Lohmeir-Vogel, E., Morrice, N., Mizuno, Y., Berenger, B., Ng, K.K.S., Muench, D.G., and Moorhead, G.B.G. (2006). The PII signal transduction protein of *Arabidopsis thaliana* forms an arginine-regulated complex with plastid N-acetyl glutamate kinase. Journal Of Biological Chemistry **281,** 5726-5733.

Chiang, H.H., and Dandekar, A.M. (1995). Regulation of proline accumulation in *Arabidopsis thaliana* (L) Heynh during development and in response to desiccation. Plant Cell and Environment **18,** 1280-1290.

Crawford, N.M., Galli, M., Tischner, R., Heimer, Y.M., Okamoto, M., and

Mack, A. (2006). Response to Zemojtel et al: Plant nitric oxide synthase: back to square one. Trends in Plant Science **11,** 526-527.

Csonka, L.N., Gelvin, S.B., Goodner, B.W., Orser, C.S., Siemieniak, D., and Slightom, J.L. (1988). Nucleotide-sequence of a mutation in the PROB gene of *Escherichia coli* that confers proline overproduction and enhanced tolerance to osmotic stress. Gene **64,** 199-205.

Cuevas, J.C., Lopez-Cobollo, R., Alcazar, R., Zarza, X., Koncz, C., Altabella, T., Salinas, J., Tiburcio, A.F., and Ferrando, A. (2008). Putrescine is involved in Arabidopsis freezing tolerance and cold acclimation by regulating abscisic acid levels in response to low temperature. Plant Physiology **148,** 1094-1105.

Culham, D.E., Vernikovska, Y., Tschowri, N., Keates, R.A.B., Wood, J.M., and Boggs, J.M. (2008). Periplasmic loops of osmosensory transporter ProP in *Escherichia coli* are sensitive to osmolality. Biochemistry **47,** 13584-13593.

Curtis, J., Shearer, G., and Kohl, D.H. (2004). Bacteroid proline catabolism affects N_2 fixation rate of drought-stressed soybeans. Plant Physiology **136,** 3313-3318.

Díaz, P., Betti, M., Sánchez, D.H., Udvardi, M.K., Monza, J., and Márquez, A.J. (2010). Deficiency in plastidic glutamine synthetase alters proline metabolism and transcriptomic response in *Lotus japonicus* under drought stress. New Phytologist **DOI 10.1111/j.1469-8137.2010.03440.x**.

Dandekar, A.M., and Uratsu, S.L. (1988). A single base pair change in proline biosynthesis genes causes osmotic stress tolerance. Journal of Bacteriology **170,** 5943-5945.

de Ronde, J.A., Cress, W.A., Kruger, G.H.J., Strasser, R.J., and Van Staden, J. (2004a). Photosynthetic response of transgenic soybean plants, containing an Arabidopsis *P5CR* gene, during heat and drought stress. Journal of Plant Physiology **161,** 1211-1224.

de Ronde, J.A., Laurie, R.N., Caetano, T., Greyling, M.M., and Kerepesi, I. (2004b). Comparative study between transgenic and non-transgenic soybean lines proved transgenic lines to be more drought tolerant. Euphytica **138,** 123-132.

Deeken, R., Engelmann, J.C., Efetova, M., Czirjak, T., Muller, T., Kaiser, W.M., Tietz, O., Krischke, M., Mueller, M.J., Palme, K., Dandekar, T., and Hedrich, R. (2006). An integrated view of gene expression and solute profiles of Arabidopsis tumors: A genome-wide approach. Plant Cell **18,** 3617-3634.

Delauney, A.J., and Verma, D.P.S. (1990). A soybean gene encoding pyrroline-5-carboxylate reductase was isolated by functional complementation in *Escherichia coli* and is found to be osmoregulated. Molecular General Genetics **221,** 299-305.

Delauney, A.J., and Verma, D.P.S. (1993). Proline biosynthesis and osmoregulation in plants. Plant Journal **4,** 215-223.

Delauney, A.J., Hu, C.A., Kishor, B.P., and Verma, D.P. (1993). Cloning of ornithine delta-aminotransferase cDNA from *Vigna aconitifolia* by transcomplementation in *Escherichia coli* and regulation of proline biosynthesis. Journal of Biological Chemistry **268,** 18673 - 18678.

Deuschle, K., Funck, D., Hellmann, H., Daschner, K., Binder, S., and Frommer, W.B. (2001). A nuclear gene encoding mitochondrial Δ^1-pyrroline-5-carboxylate dehydrogenase and its potential role in protection from proline toxicity. Plant Journal **27,** 345-355.

Deuschle, K., Funck, D., Forlani, G., Stransky, H., Biehl, A., Leister, D., van der Graaff, E., Kunzee, R., and Frommer, W.B. (2004). The role of Δ^1-pyrroline-5-carboxylate dehydrogenase in proline degradation. Plant Cell **16,** 3413-3425.

Di Martino, C., Pizzuto, R., Pallotta, M.L., De Santis, A., and 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.

Donald, S.P., Sun, X.Y., Hu, C.A.A., Yu, J., Mei, J.M., Valle, D., and Phang, J.M. (2001). Proline oxidase, encoded by p53-induced gene-6, catalyzes the generation of proline-dependent reactive oxygen species. Cancer Research **61,** 1810-1815.

Efetova, M., Zeier, J., Riederer, M., Lee, C.-W., Stingl, N., Mueller, M., Hartung, W., Hedrich, R., and Deeken, R. (2007). A central role of abscisic acid in drought stress protection of Agrobacterium-induced tumors on Arabidopsis. Plant Physiology **145,** 853-862.

Evers, D., Lefevre, I., Legay, S., Lamoureux, D., Hausman, J.-F., Gutierrez Rosales, R.O., Tincopa Marca, L.R., Hoffmann, L., Bonierbale, M., and Schafleitner, R. (2010). Identification of drought-responsive compounds in potato through a combined transcriptomic and targeted metabolite approach. Journal of Experimental Botany **61,** 2327-2343.

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

Farrand, S.K., and Dessaux, Y. (1986). Proline biosynthesis encoded by the NOC and OCC loci of *Agrobacterium* Ti plasmids. Journal of Bacteriology **167,** 732-734.

Ferrario-Mery, S., Besin, E., Pichon, O., Meyer, C., and Hodges, M. (2006). The regulatory PII protein controls arginine biosynthesis in Arabidopsis. FEBS Letters **580,** 2015-2020.

Flores, T., Todd, C.D., Tovar-Mendez, A., Dhanoa, P.K., Correa-Aragunde, N., Hoyos, M.E., Brownfield, D.M., Mullen, R.T., Lamattina, L., and Polacco, J.C. (2008). Arginase-negative mutants of Arabidopsis exhibit increased nitric oxide signaling in root development. Plant Physiology **147,** 1936-1946.

Forde, B.G., and Lea, P.J. (2007). Glutamate in plants: metabolism, regulation, and signalling. Journal of Experimental Botany **58,** 2339-2358.

Forlani, G., Scainelli, D., and Nielsen, E. (1997). Δ^1-pyrroline-5-carboxylate dehydrogenase from cultured cells of potato - Purification and properties. Plant Physiology **113,** 1413-1418.

Fujita, T., Maggio, A., Garcia-Rios, M., Bressan, R.A., and Csonka, L.N. (1998). Comparative analysis of the regulation of expression and structures of two evolutionarily divergent genes for Δ^1-pyrroline-5-carboxylate synthetase from tomato. Plant Physiology **118,** 661-674.

Funck, D., Stadelhofer, B., and Koch, W. (2008). Ornithine-delta-aminotransferase is essential for arginine catabolism but not for proline biosynthesis. BMC Plant Biology **8**, 40

Funck, D., Eckard, S., and Muller, G. (2010). Non-redundant functions of two proline dehydrogenase isoforms in Arabidopsis. BMC Plant Biology **10,** 70.

Giraud, E., Ho, L.H.M., Clifton, R., Carroll, A., Estavillo, G., Tan, Y.-F., Howell, K.A., Ivanova, A., Pogson, B.J., Millar, A.H., and Whelan, J. (2008). The absence of ALTERNATIVE OXIDASE1a in Arabidopsis results in acute sensitivity to combined light and drought stress. Plant Physiology **147,** 595-610.

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

Gleeson, D., Lelu-Walter, M.A., and Parkinson, M. (2005). Overproduction of proline in transgenic hybrid larch (*Larix x leptoeuropaea* (Dengler)) cultures renders them tolerant to cold, salt and frost. Molecular Breeding **15,** 21-29.

Gogos, J.A., Santha, M., Takacs, Z., Beck, K.D., Luine, V., Lucas, L.R., Nadler, J.V., and Karayiorgou, M. (1999). The gene encoding proline dehydrogenase modulates sensorimotor gating in mice. Nature Genetics **21,** 434-439.

Grallath, S., Weimar, T., Meyer, A., Gumy, C., Suter-Grotemeyer, M., Neuhaus, J.-M., and Rentsch, D. (2005). The AtProT family. Compat-

ible solute transporters with similar substrate specificity but differential expression patterns. Plant Physiology **137,** 117-126.

Grothe, S., Krogsrud, R.L., McClellan, D.J., Milner, J.L., and Wood, J.M. (1986). Proline transport and osmotic stress response in *Escherichia coli* K-12. . Journal of Bacteriology **166,** 253-259.

Haglund, B.M. (1980). Proline and valine-cues which stimulate grasshopper herbivory during drought stress. Nature **288,** 697-698.

Hanson, A.D., Rathinasabapathi, B., Rivoal, J., Burnet, M., Dillon, M.O., and Gage, D.A. (1994). Osmoprotective compounds in the *Plumbaginaceae*: a natural experiment in metabolic engineering of stress tolerance. Proceedings of the National Academy of Sciences of the United States of America **91,** 306-310.

Hanson, J., Hanssen, M., Wiese, A., Hendriks, M., and Smeekens, S. (2008). The sucrose regulated transcription factor bZIP11 affects amino acid metabolism by regulating the expression of *ASPARAGINE SYNTHETASE1* and *PROLINE DEHYDROGENASE2*. Plant Journal **53,** 935-949.

Hare, P.D., and Cress, W.A. (1997). Metabolic implications of stress-induced proline accumulation in plants. Plant Growth Regulation **21,** 79-102.

Hare, P.D., Cress, W.A., and Van Staden, J. (1998). Dissecting the roles of osmolyte accumulation during stress. Plant Cell and Environment **21,** 535-553.

Hare, P.D., Cress, W.A., and van Staden, J. (1999). Proline synthesis and degradation: a model system for elucidating stress-related signal transduction. Journal of Experimental Botany **50,** 413-434.

Haudecoeur, E., Planamente, S., Cirou, A., Tannieres, M., Shelp, B.J., Morera, S., and Faure, D. (2009). Proline antagonizes GABA-induced quenching of quorum-sensing in *Agrobacterium tumefaciens*. Proceedings of the National Academy of Sciences of the United States of America **106,** 14587-14592.

Hayano-Kanashiro, C., Calderon-Vazquez, C., Ibarra-Laclette, E., Herrera-Estrella, L., and Simpson, J. (2009). Analysis of gene expression and physiological responses in three Mexican maize landraces under drought stress and recovery irrigation. PLoS ONE **4,** e7531.

Hayashi, F., Ichino, T., Osanai, R., and 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 and Cell Physiology **41,** 1096-1101.

Hayward, D.C., Delaney, S.J., Campbell, H.D., Ghysen, A., Benzer, S., Kasprzak, A.B., Cotsell, J.N., Young, I.G., and Miklos, G.L.G. (1993). The sluggish-A gene of *Drosophila melanogaster* is expressed in the nervous system and encodes proline oxidase, a mitocondrial enzyme involved in glutamte biosynthesis. Proceedings of the National Academy of Sciences of the United States of America **90,** 2979-2983.

Hellmann, H., Funck, D., Rentsch, D., and Frommer, W.B. (2000). Hypersensitivity of an Arabidopsis sugar signaling mutant toward exogenous proline application. Plant Physiology **122,** 357-367.

Hervieu, F., Ledily, F., Huault, C., and Billard, J.P. (1995). Contribution of ornithine aminotransferase to proline accumulation In NaCl-treated radish cotyledons. Plant Cell And Environment **18,** 205-210.

Hirner, A., Ladwig, F., Stransky, H., Okumoto, S., Keinath, M., Harms, A., Frommer, W.B., and Koch, W. (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.

Hong, Z.L., Lakkineni, K., Zhang, Z.M., and Verma, D.P.S. (2000). Removal of feedback inhibition of Δ^1-pyrroline-5-carboxylate synthetase results in increased proline accumulation and protection of plants from osmotic stress. Plant Physiology **122,** 1129-1136.

Hou, F.Y., Huang, J., Yu, S.L., and Zhang, H.S. (2007). The 6-phosphogluconate dehydrogenase genes are responsive to abiotic stresses in rice. Journal of Integrative Plant Biology **49,** 655-663.

Hoyos, M.E., Palmieri, L., Wertin, T., Arrigoni, R., Polacco, J.C., and Palmieri, F. (2003). Identification of a mitochondrial transporter for basic amino acids in *Arabidopsis thaliana* by functional reconstitution into liposomes and complementation in yeast. Plant Journal **33,** 1027-1035.

Hu, C.A., Delauney, A.J., and Verma, D.P.S. (1992). A bifunctional enzyme (Δ^1-pyrroline-5-carboxylate synthase) catalyzes the first two steps in proline biosynthesis in plants. Proceedings of the National Academy of Sciences of the United States of America **89,** 9354 - 9358.

Hua, X.J., vandeCotte, B., VanMontagu, M., and Verbruggen, N. (1997). Developmental regulation of pyrroline-5-carboxylate reductase gene expression in Arabidopsis. Plant Physiology **114,** 1215-1224.

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

Iyer, S., and Caplan, A. (1998). Products of proline catabolism can induce osmotically regulated genes in rice. Plant Physiology **116,** 203-211.

Jennings, D.H., and Burke, R.M. (1990). Compatible solutes– The mycological dimension and their role as physiological buffering agents. New Phytologist **116,** 277-283.

Joshi, V., and Jander, G. (2009). Arabidopsis methionine γ-lyase is regulated according to isoleucine biosynthesis needs but plays a subordinate role to threonine deaminase. Plant Physiology **151,** 367-378.

Kalamaki, M.S., Alexandrou, D., Lazari, D., Merkouropoulos, G., Fotopoulos, V., Pateraki, I., Aggelis, A., Carrillo-Lopez, A., Rubio-Cabetas, M.J., and Kanellis, A.K. (2009). Over-expression of a tomato N-acetyl-L-glutamate synthase gene (SlNAGS1) in *Arabidopsis thaliana* results in high ornithine levels and increased tolerance in salt and drought stresses. Journal of Experimental Botany **60,** 1859-1871.

Kant, S., Kant, P., Raveh, E., and Barak, S. (2006). Evidence that differential gene expression between the halophyte, *Thellungiella halophila*, and *Arabidopsis thaliana* is responsible for higher levels of the compatible osmolyte proline and tight control of Na^+ uptake in *T. halophila*. Plant Cell And Environment **29,** 1220-1234.

Kaplan, F., Kopka, J., Sung, D.Y., Zhao, W., Popp, M., Porat, R., and Guy, C.L. (2007). Transcript and metabolite profiling during cold acclimation of Arabidopsis reveals an intricate relationship of cold-regulated gene expression with modifications in metabolite content. Plant Journal **50,** 967-981.

Kavi Kishor, P.B., Hong, Z., Miao, G.H., Hu, C.A.A., and Verma, D.P.S. (1995). Overexpression of Δ^1-pyrroline-5-carboxylate synthase increases proline production and confers osmotolerance in transgenic plants. Plant Physiology **108,** 1387 - 1394.

Kemble, A.R., and Macpherson, H.T. (1954). Liberation of amino acids in perennial rye grass during wilting. Biochemistry **58,** 46-49.

Kempf, L., Nicodemus, K.K., Kolachana, B., Vakkalanka, R., Verchinski, B.A., Egan, M.F., Straub, R.E., Mattay, V.A., Callicott, J.H., Weinberger, D.R., and Meyer-Lindenberg, A. (2008). Functional polymorphisms in PRODH are associated with risk and protection for schizophrenia and fronto-striatal structure and function. PloS Genetics **4** : e1000252.

Kiyosue, T., Yoshiba, Y., YamaguchiShinozaki, K., and 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.

Kleffmann, T., Russenberger, D., von Zychlinski, A., Christopher, W., Sjolander, K., Gruissem, W., and Baginsky, S. (2004). The *Arabidopsis thaliana* chloroplast proteome reveals pathway abundance and novel protein functions. Current Biology **14,** 354-362.

Kohl, D.H., Lin, J.-J., Shearer, G., and Schubert, K.R. (1990). Activities of the pentose phosphate pathway and enzymes of proline metabolism

in legume root nodules. Plant Physiology **94,** 1258-1264.

Kohl, D.H., Schubert, K.R., Carter, M.B., Hagedorn, C.H., and Shearer, G. (1988). Proline metabolism in N_2-fixing root nodules. Energy transfer and regulation of purine synthesis. Proceedings of the National Academy of Sciences of the United States of America **85,** 2036-2040.

Kohl, D.H., Kennelly, E.J., Zhu, Y.X., Schubert, K.R., and Shearer, G. (1991). Proline accumulation, nitrogenase (C_2H_2 reducing) activity and activities of enzymes related to proline metabolism in drought-stressed soybean nodules. Journal of Experimental Botany **42,** 831-837.

Kruger, N.J., and von Schaewen, A. (2003). The oxidative pentose phosphate pathway: structure and organisation. Current Opinion in Plant Biology **6,** 236-246.

Labboun, S., Terce-Laforgue, T., Roscher, A., Bedu, M., Restivo, F.M., Velanis, C.N., Skopelitis, D.S., Moshou, P.N., Roubelakis-Angelakis, K.A., Suzuki, A., and Hirel, B. (2009). Resolving the role of plant glutamate dehydrogenase. I. *in vivo* real time nuclear magnetic resonance spectroscopy experiments. Plant and Cell Physiology **50,** 1761-1773.

Lee, B.R., Jin, Y.L., Avice, J.C., Cliquet, J.B., Ourry, A., and Kim, T.H. (2009). Increased proline loading to phloem and its effects on nitrogen uptake and assimilation in water-stressed white clover (*Trifolium repens*). New Phytologist **182,** 654-663.

Lee, M.W., Jelenska, J., and Greenberg, J.T. (2008). Arabidopsis proteins important for modulating defense responses to *Pseudomonas syringae* that secrete HopW1-1. Plant Journal **54,** 452-465.

Lee, Y.H., Nadaraia, S., Gu, D., Becker, D.F., and Tanner, J.J. (2003). Structure of the proline dehydrogenase domain of the multifunctional PutA flavoprotein. Nature Structural Biology **10,** 109-114.

Lehmann, S., Funck, D., Szabados, L., and Rentsch, D. (2010). Proline metabolism and transport in plant development. Amino Acids **39,** 949-962

Less, H., and Galili, G. (2008). Principal transcriptional programs regulating plant amino acid metabolism in response to abiotic stresses. Plant Physiology **147,** 316-330.

Liu, Y., Borchert, G.L., Surazynski, A., and Phang, J.M. (2008). Proline oxidase, a p53-induced gene, targets COX-2/PGE(2) signaling to induce apoptosis and inhibit tumor growth in colorectal cancers. Oncogene **27,** 6729-6737.

Liu, Y., Borchert, G.L., Surazynski, A., Hu, C.A., and Phang, J.M. (2006). Proline oxidase activates both intrinsic and extrinsic pathways for apoptosis: the role of ROS/superoxides, NFAT and MEK/ERK signaling. Oncogene **25,** 5640-5647.

Liu, Y.M., Borchert, G., Surazynski, A., Hu, C.A.A., and Phang, J.M. (2005a). Proline oxidase, a p53-induced gene, metabolically activates both intrinsic and extrinsic pathways for apoptosis: The role of superoxide radicals, NFAT and MEK/ERK signaling. Clinical Cancer Research **11,** 9038S-9038S.

Liu, Y.M., Borchert, G.L., Donald, S.P., Surazynski, A., Hu, C.A., Weydert, C.J., Oberley, L.W., and Phang, J.M. (2005b). MnSOD inhibits proline oxidase-induced apoptosis in colorectal cancer cells. Carcinogenesis **26,** 1335-1342.

MacGregor, K.B., Shelp, B.J., Peiris, S., and Bown, A.W. (2003). Overexpression of glutamate decarboxylase in transgenic tobacco plants deters feeding by phytophagous insect larvae. Journal of Chemical Ecology **29,** 2177-2182.

Madkour, M.A., Smith, L.T., and Smith, G.M. (1990). Preferential osmolyte accumulation: a mechanism of osmotic stress adaptation in diazotrophic bacteria. Appl Environ Microbiol **56,** 2876-2881.

Mani, S., Van de Cotte, B., Van Montagu, M., and Verbruggen, N. (2002). Altered levels of proline dehydrogenase cause hypersensitivity to proline and its analogs in Arabidopsis. Plant Physiology **128,** 73-83.

Marchler-Bauer, A., Anderson, J.B., DeWeese-Scott, C., Fedorova, N.D., Geer, L.Y., He, S.Q., Hurwitz, D.I., Jackson, J.D., Jacobs, A.R., Lanczycki, C.J., Liebert, C.A., Liu, C.L., Madej, T., Marchler, G.H., Mazumder, R., Nikolskaya, A.N., Panchenko, A.R., Rao, B.S., Shoemaker, B.A., Simonyan, V., Song, J.S., Thiessen, P.A., Vasudevan, S., Wang, Y.L., Yamashita, R.A., Yin, J.J., and Bryant, S.H. (2003). CDD: a curated Entrez database of conserved domain alignments. Nucleic Acids Research **31,** 383-387.

Matsui, A., Ishida, J., Morosawa, T., Mochizuki, Y., Kaminuma, E., Endo, T.A., Okamoto, M., Nambara, E., Nakajima, M., Kawashima, M., Satou, M., Kim, J.M., Kobayashi, N., Toyoda, T., Shinozaki, K., and Seki, M. (2008). Arabidopsis transcriptome analysis under drought, cold, high-salinity and ABA treatment conditions using a tiling array. Plant and Cell Physiology **49,** 1135 - 1149.

Mattioli, R., Marchese, D., D'Angeli, S., Altamura, M.M., Costantino, P., and Trovato, M. (2008). Modulation of intracellular proline levels affects flowering time and inflorescence architecture in Arabidopsis. Plant Molecular Biology **66,** 277-288.

Mattioli, R., Falasca, G., Sabatini, S., Altamura, M.M., Costantino, P., and Trovato, M. (2009). The proline biosynthetic genes *P5CS1* and *P5CS2* play overlapping roles in Arabidopsis flower transition but not in embryo development. Physiologia Plantarum **137,** 72-85.

Mauro, L.M., Trovato, M., De Paolis, A., Gallelli, A., Constantino, P., and Altamura, M.M. (1996). The plant oncogene rolD stimulates flowering in transgenic tobacco plants. Developmental Biology **180,** 693-700.

Maxwell, S.A., and Davis, G.E. (2000). Differential gene expression in p53-mediated apoptosis-resistant vs. apoptosis-sensitive tumor cell lines. Proceedings of the National Academy of Sciences of the United States of America **97,** 13009-13014.

Micheu, S., Crailsheim, K., and Leonhard, B. (2000). Importance of proline and other amino acids during honeybee flight (*Apis mellifera* carnica POLLMANN). Amino Acids **18,** 157-175.

Miller, G., Stein, H., Honig, A., Kapulnik, Y., and Zilberstein, A. (2005). Responsive modes of *Medicago sativa* proline dehydrogenase genes during salt stress and recovery dictate free proline accumulation. Planta **222,** 70-79.

Miller, G., Honig, A., Stein, H., Suzuki, N., Mittler, R., and Zilberstein, A. (2009). Unraveling Δ^1-pyrroline-5-carboxylate-proline cycle in plants by uncoupled expression of proline oxidation enzymes. Journal of Biological Chemistry **284,** 26482-26492.

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

Mutters, R.G., Ferreira, L.G.R., and Hall, A.E. (1989). Proline content of the anthers and pollen of heat-tolerant and heat-sensitive cowpea subjected to different temperatures. Crop Science **29,** 1497-1500.

Nanjo, T., Kobayashi, M., Yoshiba, Y., Kakubari, Y., Yamaguchi-Shinozaki, K., and Shinozaki, K. (1999a). Antisense suppression of proline degradation improves tolerance to freezing and salinity in *Arabidopsis thaliana*. Febs Letters **461,** 205-210.

Nanjo, T., Kobayashi, M., Yoshiba, Y., Sanada, Y., Wada, K., Tsukaya, H., Kakubari, Y., Yamaguchi-Shinozaki, K., and Shinozaki, K. (1999b). Biological functions of proline in morphogenesis and osmotolerance revealed in antisense transgenic *Arabidopsis thaliana*. Plant Journal **18,** 185-193.

Nomura, M., and Takagi, H. (2004). Role of the yeast acetyltransferase Mpr1 in oxidative stress: Regulation of oxygen reactive species caused by a toxic proline catabolism intermediate. Proceedings of the National Academy of Sciences of the United States of America **101,** 12616-12621.

Ober, E.S., and Sharp, R.E. (1994). Proline accumulation in maize (*ZEA-MAYS* L) primary roots at low water potentials. 1. Requirement for increased levels of abscisic acid. Plant Physiology **105,** 981-987.

Palmieri, L., Todd, C.D., Arrigoni, R., Hoyos, M.E., Santoro, A., Polacco, J.C., and Palmieri, F. (2006). Arabidopsis mitochondria have two basic amino acid transporters with partially overlapping specificities and differential expression in seedling development. Biochimica Et Biophysica Acta-Bioenergetics **1757,** 1277-1283.

Panicot, M., Minguet, E.G., Ferrando, A., Alcazar, R., Blazquez, M.A., Carbonell, J., Altabella, T., Koncz, C., and Tiburcio, A.F. (2002). A polyamine metabolon involving aminopropyl transferase complexes in Arabidopsis. Plant Cell **14,** 2539-2551.

Parida, A.K., Dagaonkar, V.S., Phalak, M.S., and Aurangabadkar, L.P. (2008). Differential responses of the enzymes involved in proline biosynthesis and degradation in drought tolerant and sensitive cotton genotypes during drought stress and recovery. Acta Physiologiae Plantarum **30,** 619-627.

Parre, E., Ghars, M.A., Leprince, A.S., Thiery, L., Lefebvre, D., Bordenave, M., Richard, L., Mazars, C., Abdelly, C., and Savoure, A. (2007). Calcium signaling via phospholipase C is essential for proline accumulation upon ionic but not nonionic hyperosmotic stresses in Arabidopsis. Plant Physiology **144,** 503-512.

Parvanova, D., Ivanov, S., Konstantinova, T., Karanov, E., Atanassov, A., Tsvetkov, T., Alexieva, V., and Djilianov, D. (2004). Transgenic tobacco plants accumulating osmolytes show reduced oxidative damage under freezing stress. Plant Physiology and Biochemistry **42,** 57-63.

Peng, Z., Lu, Q., and Verma, D.P.S. (1996). Reciprocal regulation of Δ^1-pyrroline-5-carboxylate synthetase and proline dehydrogenase genes controls proline levels during and after osmotic stress in plants. Molecular and General Genetics **253,** 334-341.

Perez-Amador, M.A., Leon, J., Green, P.J., and Carbonell, J. (2002). Induction of the arginine decarboxylase ADC2 gene provides evidence for the involvement of polyamines in the wound response in Arabidopsis. Plant Physiology **130,** 1454-1463.

Poolman, B., and Glaasker, E. (1998). Regulation of compatible solute accumulation in bacteria. Molecular Microbiology **29,** 397-407.

Reiland, S., Messerli, G., Baerenfaller, K., Gerrits, B., Endler, A., Grossmann, J., Gruissem, W., and Baginsky, S. (2009). Large-scale Arabidopsis phosphoproteome profiling reveals novel chloroplast kinase substrates and phosphorylation networks. Plant Physiology **150,** 889-903.

Rentsch, D., Schmidt, S., and Tegeder, M. (2007). Transporters for uptake and allocation of organic nitrogen compounds in plants. FEBS Letters **581,** 2281-2289.

Rentsch, D., Hirner, B., Schmelzer, E., and Frommer, W.B. (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.

Rhodes, D., Handa, S., and Bressan, R.A. (1986). Metabolic changes associated with adaptation of plant cells to water stress. Plant Physiology **82,** 890-903.

Rivero, R.M., Shulaev, V., and Blumwald, E. (2009). Cytokinin-dependent photorespiration and the protection of photosynthesis during water deficit. Plant Physiology **150,** 1530-1540.

Rivero, R.M., Kojima, M., Gepstein, A., Sakakibara, H., Mittler, R., Gepstein, S., and Blumwald, E. (2007). Delayed leaf senescence induces extreme drought tolerance in a flowering plant. Proceedings of the National Academy of Sciences of the United States of America **104,** 19631-19636.

Roosens, N., Thu, T.T., Iskandar, H.M., and Jacobs, M. (1998). Isolation of the ornithine-delta-aminotransferase cDNA and effect of salt stress on its expression in *Arabidopsis thaliana*. Plant Physiology **117,** 263-271.

Roosens, N.H., Al Bitar, F., Loenders, K., Angenon, G., and Jacobs, M. (2002). Overexpression of ornithine-delta-aminotransferase increases proline biosynthesis and confers osmotolerance in transgenic plants. Molecular Breeding **9,** 73-80.

Samach, A., Onouchi, H., Gold, S.E., Ditta, G.S., Schwarz-Sommer, Z., Yanofsky, M.F., and Coupland, G. (2000). Distinct roles of CONSTANS target genes in reproductive development of Arabidopsis. Science **288,** 1613-1616.

Sans, N., Schindler, U., and Schroder, J. (1988). Ornithine cyclodeaminase from Ti plasmid C58-DNA sequence, enzyme properties and regulation of activity by arginine. European Journal of Biochemistry **173,** 123-130.

Satoh, R., Nakashima, K., Seki, M., Shinozaki, K., and 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 Physiology **130,** 709-719.

Satoh, R., Fujita, Y., Nakashima, K., Shinozaki, K., and Yamaguchi-Shinozaki, K.Y. (2004). A novel subgroup of bZIP proteins functions as transcriptional activators in hypoosmolarity-responsive expression of the *ProDH* gene in Arabidopsis. Plant and Cell Physiology **45,** 309-317.

Savoure, A., Hua, X.J., Bertauche, N., VanMontagu, M., and Verbruggen, N. (1997). Abscisic acid-independent and abscisic acid-dependent regulation of proline biosynthesis following cold and osmotic stresses in *Arabidopsis thaliana*. Molecular and General Genetics **254,** 104-109.

Savoure, A., Jaoua, S., Hua, X.J., Ardiles, W., Vanmontagu, M., and Verbruggen, N. (1995). Isolation, characterization and chromosomal location of a gene encoding the Δ^1-pyrroline-5-carboxylate synthetase in *Arabidopsis thaliana*. Febs Letters **372,** 13-19.

Sawahel, W.A., and Hassan, A.H. (2002). Generation of transgenic wheat plants producing high levels of the osmoprotectant proline. Biotechnology Letters **24,** 721-725.

Scaraffia, P.Y., and Wells, M.A. (2003). Proline can be utilized as an energy substrate during flight of *Aedes aegypti* females. Journal of Insect Physiology **49,** 591-601.

Scharte, J., Schon, H., Tjaden, Z., Weis, E., and von Schaewen, A. (2009). Isoenzyme replacement of glucose-6-phosphate dehydrogenase in the cytosol improves stress tolerance in plants. Proceedings of the National Academy of Sciences of the United States of America **106**, 8061-8066.

Schwacke, R., Grallath, S., Breitkreuz, K.E., Stransky, E., Stransky, H., Frommer, W.B., and Rentsch, D. (1999). LeProT1, a transporter for proline, glycine betaine, and γ-amino butyric acid in tomato pollen. Plant Cell **11,** 377-392.

Sharma, S., and Verslues, P.E. (2010). Mechanisms independent of ABA or proline feedback have a predominant role in transcriptional regulation of proline metabolism during low water potential and stress recovery. Plant Cell and Environment **33,** 1838-1851.

Sharma, S.S., and Dietz, K.J. (2009). The relationship between metal toxicity and cellular redox imbalance. Trends in Plant Science **14,** 43-50.

Shelp, B.J., Bown, A.W., and McLean, M.D. (1999). Metabolism and functions of γ-aminobutyric acid. Trends in Plant Science **4,** 446-452.

Shinozaki, K., and Yamaguchi-Shinozaki, K. (2007). Gene networks involved in drought stress response and tolerance. Journal of Experimental Botany **58,** 221-227.

Skirycz, A., De Bodt, S., Obata, T., De Clercq, I., Claeys, H., De Rycke, R., Andriankaja, M., Van Aken, O., Van Breusegem, F., Fernie, A.R., and Inze, D. (2010). Developmental stage specificity and the role of mitochondrial metabolism in the response of Arabidopsis leaves to prolonged mild osmotic stress. Plant Physiology **152,** 226-244.

Skopelitis, D.S., Paranychianakis, N.V., Kouvarakis, A., Spyros, A., Stephanou, E.G., and Roubelakis-Angelakis, K.A. (2007). The iso-

enzyme 7 of tobacco NAD(H)-dependent glutamate dehydrogenase exhibits high deaminating and low aminating activities *in vivo*. Plant Physiology **145,** 1726-1734.

Skopelitis, D.S., Paranychianakis, N.V., Paschalidis, K.A., Pliakonis, E.D., Delis, I.D., Yakoumakis, D.I., Kouvarakis, A., Papadakis, A.K., Stephanou, E.G., and Roubelakis-Angelakis, K.A. (2006). Abiotic stress generates ROS that signal expression of anionic glutamate dehydrogenases to form glutamate for proline synthesis in tobacco and grapevine. Plant Cell **18,** 2767-2781.

Slocum, R.D. (2005). Genes, enzymes and regulation of arginine biosynthesis in plants. Plant Physiology and Biochemistry **43,** 729-745.

Smirnoff, N., and Cumbes, Q.J. (1989). Hydroxyl radical scavening activity of compatible solutes. Phytochemistry **28,** 1057-1060.

Smith, C.A., Melino, V.J., Sweetman, C., and Soole, K.L. (2009). Manipulation of alternative oxidase can influence salt tolerance in *Arabidopsis thaliana*. Physiologia Plantarum **137,** 459-472.

Soto, M.J., Zorzano, A., Garciarodriguez, F.M., Mercadoblanco, J., Lopezlara, I.M., Olivares, J., and Toro, N. (1994). Identification of a novel *Rhizobium meliloti* nodulation efficiency NFE gene homolog of Agrobacterium ornithine cyclodeaminase. Molecular Plant-Microbe Interaction **7,** 703-707.

Srivastava, D., Schuermann, J.P., White, T.A., Krishnan, N., Sanyal, N., Hura, G.L., Tan, A.M., Henzl, M.T., Becker, D.F., and Tanner, J.J. (2010). Crystal structure of the bifunctional proline utilization A flavoenzyme from *Bradyrhizobium japonicum*. Proceedings of the National Academy of Sciences of the United States of America **107,** 2878-2883.

Stewart, C.R., and Hanson, A.D. (1980). Proline accumulation as a metabolic response to water stress. *In* Adaptation of Plants to Water and High Temperature Stress, N.C. Turner and P.J. Kramer, eds (New York: John Wiley and Sons). pp173-189

Stines, A.P., Naylor, D.J., Hoj, P.B., and van Heeswijck, R. (1999). Proline accumulation in developing grapevine fruit occurs independently of changes in the levels of Δ^1-pyrroline-5-carboxylate synthetase mRNA or protein. Plant Physiology **120,** 923-931.

Straub, P.F., Shearer, G., and Kohl, D.H. (1995). Effect of the ability of bacteroids to catabolize proline on seed yields of water stressed soybeans. Plant Physiology **108,** 108-108.

Straub, P.F., Shearer, G., Reynolds, P.H.S., Sawyer, S.A., and Kohl, D. (1997). Effect of disabling bacteroid proline catabolism on the response of soybeans to repeated drought stress. Journal of Experimental Botany **48,** 1299-1307.

Strizhov, N., Abraham, E., Okresz, L., Blickling, S., Zilberstein, A., Schell, J., Koncz, C., and Szabados, L. (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 Journal **12,** 557-569.

Su, J., and Wu, R. (2004). Stress-inducible synthesis of proline in transgenic rice confers faster growth under stress conditions than that with constitutive synthesis. Plant Science **166,** 941-948.

Svennerstam, H., Ganeteg, U., and Nasholm, T. (2008). Root uptake of cationic amino acids by Arabidopsis depends on functional expression of amino acid permease. New Phytologist **180,** 620-630.

Szabados, L., and Savoure, A. (2010). Proline: a multifunctional amino acid. Trends in Plant Science **15,** 89-97.

Szekely, G., Abraham, E., Cselo, A., Rigo, G., Zsigmond, L., Csiszar, J., Ayaydin, F., Strizhov, N., Jasik, J., Schmelzer, E., Koncz, C., and Szabados, L. (2008). Duplicated *P5CS* genes of Arabidopsis play distinct roles in stress regulation and developmental control of proline biosynthesis. Plant Journal **53,** 11-28.

Szoke, A., Miao, G.H., Hong, Z.L., and Verma, D.P.S. (1992). Subcellular location of Δ^1-pyrroline-5-carboxylate reductase in root nodule and leaf of soybean. Plant Physiology **99,** 1642-1649.

Takahara, K., Akashi, K., and Yokota, A. (2005). Purification and characterization of glutamate N-acetyltransferase involved in citrulline accumulation in wild watermelon. Febs Journal **272,** 5353-5364.

Tateishi, Y., Nakagawa, T., and Esaka, M. (2005). Osmotolerance and growth stimulation of transgenic tobacco cells accumulating free proline by silencing proline dehydrogenase expression with double-stranded RNA interference technique. Physiologia Plantarum **125,** 224-234.

Toka, I., Planchais, S., Cabassa, C., Justin, A.-M., De Vos, D., Richard, L., Savoure, A., and Carol, P. (2010). Mutations in the hyperosmotic stress-responsive mitochondrial BASIC AMINO ACID CARRIER2 enhance proline accumulation in Arabidopsis. Plant Physiology **152,** 1851-1862.

Trovato, M., Maras, B., Linhares, F., and Costantino, P. (2001). The plant oncogene rolD encodes a functional ornithine cyclodeaminase. Proceedings of the National Academy of Sciences of the United States of America **98,** 13449 - 13453.

Ueda, A., Yamamoto-Yamane, Y., and Takabe, T. (2007). Salt stress enhances proline utilization in the apical region of barley roots. Biochemical and Biophysical Research Communications **355,** 61-66.

Ueda, A., Shi, W., Sanmiya, K., Shono, M., and Takabe, T. (2001). Functional analysis of salt-inducible proline transporter of barley roots. Plant and Cell Physiology **42,** 1282-1289.

Ueda, A., Shi, W., Shimada, T., Miyake, H., and Takabe, T. (2008). Altered expression of barley proline transporter causes different growth responses in Arabidopsis. Planta **227,** 277-286.

Umbach, A.L., Fiorani, F., and Siedow, J.N. (2005). Characterization of transformed Arabidopsis with altered alternative axidase levels and analysis of effects on reactive oxygen species in tissue. Plant Physiology **139,** 1806-1820.

Van Aken, O., Giraud, E., Clifton, R., and Whelan, J. (2009). Alternative oxidase: a target and regulator of stress responses. Physiologia Plantarum **137,** 354-361.

Vendruscolo, E.C.G., Schuster, I., Pileggi, M., Scapim, C.A., Correa Molinari, H.B., Marur, C.J., and Esteves Vieira, L.G. (2007). Stress-induced synthesis of proline confers tolerance to water deficit in transgenic wheat. Journal of Plant Physiology **164,** 1367-1376.

Verbruggen, N., and Hermans, C. (2008). Proline accumulation in plants: a review. Amino Acids **35,** 753-759.

Verbruggen, N., Villarroel, R., and Vanmontagu, M. (1993). Osmoregulation of a pyrroline-5-carboxylate reductase gene in *Arabidopsis thaliana*. Plant Physiology **103,** 771-781.

Verbruggen, N., Hua, X.J., May, M., and VanMontagu, M. (1996). Environmental and developmental signals modulate proline homeostasis: Evidence for a negative transcriptional regulator. Proceedings of the National Academy of Sciences of the United States of America **93,** 8787-8791.

Verslues, P.E. (2010). Quantification of water stress-induced osmotic adjustment and proline accumulation for *Arabidopsis thaliana* molecular genetic studies. *In* Plant Stress Tolerance. Methods and Protocols, R. Sunkar, ed (New York: Humana Press), pp. 301-316.

Verslues, P.E., and Sharp, R.E. (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 Physiology **119,** 1349-1360.

Verslues, P.E., and Bray, E.A. (2006). Role of abscisic acid (ABA) and *Arabidopsis thaliana* ABA-insensitive loci in low water potential-induced ABA and proline accumulation. Journal of Experimental Botany **57,** 201-212.

Verslues, P.E., Kim, Y.S., and Zhu, J.K. (2007). Altered ABA, proline and hydrogen peroxide in an Arabidopsis glutamate: glyoxylate aminotransferase mutant. Plant Molecular Biology **64,** 205-217.

Verslues, P.E., Agarwal, M., Katiyar-Agarwal, S., Zhu, J.H., and Zhu, J.K. (2006). Methods and concepts in quantifying resistance to drought, salt and freezing, abiotic stresses that affect plant water status. Plant Journal **45,** 523-539.

Voetberg, G.S., and Sharp, R.E. (1991). Growth of the maize primary root at low water potentials. 3. Role of increased proline deposition in osmotic adjustment. Plant Physiology **96,** 1125-1130.

Wachter, R., Langhans, M., Aloni, R., Gotz, S., Weilmunster, A., Koops, A., Temguia, L., Mistrik, I., Pavlovkin, J., Rascher, U., Schwalm, K., Koch, K.E., and Ullrich, C.I. (2003). Vascularization, high-volume solution flow, and localized roles for enzymes of sucrose metabolism during tumorigenesis by *Agrobacterium tumefaciens*. Plant Physiology **133,** 1024-1037.

Wakao, S., and Benning, C. (2005). Genome-wide analysis of glucose-6-phosphate dehydrogenases in Arabidopsis. Plant Journal **41,** 243-256.

Weltmeier, F., Ehlert, A., Mayer, C.S., Dietrich, K., Wang, X., Schutze, K., Alonso, R., Harter, K., Vicente-Carbajosa, J., and Droge-Laser, W. (2006). Combinatorial control of Arabidopsis proline dehydrogenase transcription by specific heterodimerisation of bZIP transcription factors. EMBO Journal **25,** 3133-3143.

Willis, A., Bender, H.U., Steel, G., and Valle, D. (2008). PRODH variants and risk for schizophrenia. Amino Acids **35,** 673-679.

Wilson, P.B., Estavillo, G.M., Field, K.J., Pornsiriwong, W., Carroll, A.J., Howell, K.A., Woo, N.S., Lake, J.A., Smith, S.M., Millar, A.H., von Caemmerer, S., and Pogson, B.J. (2009). The nucleotidase/phosphatase SAL1 is a negative regulator of drought tolerance in Arabidopsis. Plant Journal **58,** 299-317.

Wu, L.Q., Fan, Z.M., Guo, L., Li, Y.Q., Zhang, W.J., Qu, L.J., and Chen, Z.L. (2003). Over-expression of an Arabidopsis delta-OAT gene enhances salt and drought tolerance in transgenic rice. Chinese Science Bulletin **48,** 2594-2600.

Xiong, Y.Q., DeFraia, C., Williams, D., Zhang, X.D., and Mou, Z.L. (2009). Characterization of Arabidopsis 6-phosphogluconolactonase T-DNA insertion mutants reveals an essential role for the oxidative section of the plastidic pentose phosphate pathway in plant growth and development. Plant and Cell Physiology **50,** 1277-1291.

Yancey, P.H. (2005). Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses. Journal of Experimental Biology **208,** 2819-2830.

Yancey, P.H., Clark, M.E., Hand, S.C., Bowlus, R.D., and Somero, G.N. (1982). Living with water stress: evolution of osmolyte systems. Science **217,** 1214-1222.

Yang, C.W., and Kao, C.H. (1999). Importance of ornithine-delta-aminotransferase to proline accumulation caused by water stress in detached rice leaves. Plant Growth Regulation **27,** 189-192.

Yoo, J.H., Park, C.Y., Kim, J.C., Heo, W.D., Cheong, M.S., Park, H.C., Kim, M.C., Moon, B.C., Choi, M.S., Kang, Y.H., Lee, J.H., Kim, H.S., Lee, S.M., Yoon, H.W., Lim, C.O., Yun, D.J., Lee, S.Y., Chung, W.S., and Cho, M.J. (2005). Direct interaction of a divergent CaM isoform and the transcription factor, MYB2, enhances salt tolerance in Arabidopsis. Journal of Biological Chemistry **280,** 3697-3706.

Yoon, K.A., Nakamura, Y., and Arakawa, H. (2004). Identification of *ALDH4* as a p53-inducible gene and its protective role in cellular stresses. Journal of Human Genetics **49,** 134-140.

Yoshiba, Y., Kiyosue, T., Nakashima, K., Yamaguchi-Shinozaki, K., and Shinozaki, K. (1997). Regulation of levels of proline as an osmolyte in plants under water stress. Plant Cell Physiol **38,** 1095-1102.

Yoshiba, Y., Nanjo, T., Miura, S., Yamaguchi-Shinozaki, K., and Shinozaki, K. (1999). Stress-responsive and developmental regulation of Δ^1-pyrroline-5-carboxylate synthetase 1 (*P5CS1*) gene expression in *Arabidopsis thaliana*. Biochemical and Biophysical Research Communications **261,** 766-772.

Yoshiba, Y., Kiyosue, T., Katagiri, T., Ueda, H., Mizoguchi, T., Yamaguchi-Shinozaki, K., Wada, K., Harada, Y., and Shinozaki, K. (1995). Correlation between the induction of a gene for Δ^1-pyrroline-5-carboxylate synthetase and the accumulation of proline in *Arabidopsis thaliana* under osmotic stress. Plant Journal **7,** 751 - 760.

Zemojtel, T., Frohlich, A., Palmieri, M.C., Kolanczyk, M., Mikula, I., Wyrwicz, L.S., Wanker, E.E., Mundlos, S., Vingron, M., Martasek, P., and Durner, J. (2006). Plant nitric oxide synthase: a never-ending story? Trends in Plant Science **11,** 524-525.

Zhang, C.S., Lu, Q., and Verma, D.P.S. (1995). Removal of feedback inhibition of Δ^1-pyrroline-5-carboxylate synthetase, a bifunctional enzyme catalyzing the first two steps of proline biosynthesis in plants. Journal of Biological Chemistry **270,** 20491-20496.

Zhang, C.S., Lu, Q., and Verma, D.P.S. (1997). Characterization of Δ^1-pyrroline-5-carboxylate synthetase gene promoter in transgenic *Arabidopsis thaliana* subjected to water stress. Plant Science **129,** 81-89.

Zhao, M.G., Tian, Q.T., and Zhang, W.H. (2007). Nitric oxide synthase-dependent nitric oxide production is associated with salt tolerance in Arabidopsis. Plant Physiology **144,** 206-217.

Zhao, M.G., Chen, L., Zhang, L.L., and Zhang, W.H. (2009). Nitric reductase-dependent nitric oxide production is involved in cold acclimation and freezing tolerance in Arabidopsis. Plant Physiology **151,** 755-767.

Zhou, Y.Z., Larson, J.D., Bottoms, C.A., Arturo, E.C., Henzl, M.T., Jenkins, J.L., Nix, J.C., Becker, D.F., and Tanner, J.J. (2008). Structural basis of the transcriptional regulation of the proline utilization regulon by multifunctional PutA. Journal of Molecular Biology **381,** 174-188.

Zhu, B.C., Su, J., Chan, M.C., Verma, D.P.S., Fan, Y.L., and Wu, R. (1998). Overexpression of a Δ^1-pyrroline-5-carboxylate synthetase gene and analysis of tolerance to water- and salt-stress in transgenic rice. Plant Science **139,** 41-48.

Zhu, L., Liu, X.M., Liu, X., Jeannotte, R., Reese, J.C., Harris, M., Stuart, J.J., and Chen, M.S. (2008). Hessian fly (*Mayetiola destructor*) attack causes a dramatic shift in carbon and nitrogen metabolism in wheat. Molecular Plant-Microbe Interaction **21,** 70-78.

Zhu, Y.X., Shearer, G., and Kohl, D.H. (1992). Proline fed to intact soybean plants influences acetylene reducing activity and content and metabolism of proline in bacteroids. Plant Physiology **98,** 1020-1028.

Zsigmond, L., Rigo, G., Szarka, A., Szekely, G., Otvos, K., Darula, Z., Medzihradszky, K.F., Koncz, C., Koncz, Z., and Szabados, L. (2008). Arabidopsis PPR40 connects abiotic stress responses to mitochondrial electron transport. Plant Physiology **146,** 1721-1737.