Essential Role of Tissue-Specific Proline Synthesis and Catabolism in Growth and Redox Balance at Low Water Potential[1][W][OA]

Sandeep Sharma, Joji Grace Villamor[2], and Paul E. Verslues*

Institute of Plant and Microbial Biology, Academia Sinica, Taipei 11529, Taiwan

To better define the still unclear role of proline (Pro) metabolism in drought resistance, we analyzed Arabidopsis (*Arabidopsis thaliana*) Δ^1-*pyrroline-5-carboxylate synthetase1* (*p5cs1*) mutants deficient in stress-induced Pro synthesis as well as *proline dehydrogenase* (*pdh1*) mutants blocked in Pro catabolism and found that both Pro synthesis and catabolism were required for optimal growth at low water potential (ψ_w). The abscisic acid (ABA)-deficient mutant *aba2-1* had similar reduction in root elongation as *p5cs1* and *p5cs1/aba2-1* double mutants. However, the reduced growth of *aba2-1* but not *p5cs1/aba2-1* could be complemented by exogenous ABA, indicating that Pro metabolism was required for ABA-mediated growth protection at low ψ_w. *PDH1* maintained high expression in the root apex and shoot meristem at low ψ_w rather than being repressed, as in the bulk of the shoot tissue. This, plus a reduced oxygen consumption and buildup of Pro in the root apex of *pdh1-2*, indicated that active Pro catabolism was needed to sustain growth at low ψ_w. Conversely, *P5CS1* expression was most highly induced in shoot tissue. Both *p5cs1-4* and *pdh1-2* had a more reduced NADP/NADPH ratio than the wild type at low ψ_w. These results indicate a new model of Pro metabolism at low ψ_w whereby Pro synthesis in the photosynthetic tissue regenerates NADP while Pro catabolism in meristematic and expanding cells is needed to sustain growth. Tissue-specific differences in Pro metabolism and function in maintaining a favorable NADP/NADPH ratio are relevant to understanding metabolic adaptations to drought and efforts to enhance drought resistance.

Plant responses to low water potential (ψ_w) that occur during drought include changes in metabolite levels and the activity of specific metabolic pathways (Wilson et al., 2009). A continuing challenge is to identify the adaptive metabolic changes and determine how they contribute to drought resistance. In many plant species, including Arabidopsis (*Arabidopsis thaliana*), Pro accumulation is one of the main metabolic responses to abiotic stress. The highest levels of Pro accumulation are typically seen in response to low ψ_w with lower levels accumulating in response to salt or cold (Kaplan et al., 2007; Sharma and Verslues, 2010). Ecophysiological observations have also suggested a role of Pro in drought adaptation (Ben Hassine et al., 2008; Parida et al., 2008; Evers et al., 2010). However, even in Arabidopsis, the role of Pro in low-ψ_w stress is not clearly established and the specific effects of Pro on low-ψ_w resistance have been relatively less studied than salt or cold stress (Verslues and Sharma, 2010).

Early molecular studies of Pro in salt stress or under severe dehydration established a "standard model" whereby transcriptional up-regulation of Δ^1-*PYRROLINE-5-CARBOXYLATE SYNTHETASE1* (*P5CS1*), which catalyzes the first step of Pro synthesis (Hu et al., 1992; Yoshiba et al., 1995, 1999; Abrahám et al., 2003; Armengaud et al., 2004), and down-regulation of *PROLINE DEHYDROGENASE* (*PDH1*), which catalyzes the first step of Pro catabolism, were both required and sufficient for stress-induced Pro accumulation (Kiyosue et al., 1996; Peng et al., 1996; Yoshiba et al., 1997; Miller et al., 2005). Consistent with this standard model, *p5cs1* mutants have greatly reduced levels of Pro under both salt stress and low ψ_w (Székely et al., 2008; Sharma and Verslues, 2010). Székely et al. (2008) showed that *p5cs1* mutants had reduced growth and increased hydrogen peroxide (H_2O_2) content and reactive oxygen species (ROS) damage under salt stress, demonstrating the importance of Pro in salt resistance. Conversely, the standard model would suggest that suppression of *PDH1* would increase Pro content and enhance stress resistance. Some reports have been consistent with this model: antisense suppression of *PDH1* improved resistance to freezing and possibly salinity in one study (Nanjo et al., 1999a). However, another study of *PDH1* overexpression and antisense lines found no difference in growth or stress damage

[1] This work was supported by the National Science Council of Taiwan (grant no. NSC 97–2311–B–001–005 to P.E.V.), by a National Science Council postdoctoral stipend (to S.S.), and by an Academia Sinica Career Development Grant (to P.E.V.).

[2] Present address: Max Plank Institute for Plant Breeding Research, Cologne 50829, Germany.

* Corresponding author; e-mail paulv@gate.sinica.edu.tw.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Paul E. Verslues (paulv@gate.sinica.edu.tw).

[W] The online version of this article contains Web-only data.

[OA] Open Access articles can be viewed online without a subscription.

www.plantphysiol.org/cgi/doi/10.1104/pp.111.183210

in salt or high-osmolarity treatments (Mani et al., 2002). Many of these studies focused on salt or cold stress; there has been relatively little examination of *p5cs1* or *pdh1* mutants at low ψ_w indicative of drought.

Exceptions to the standard model of up-regulated Pro synthesis and down-regulated catabolism leading to Pro accumulation have been reported. For example, Kaplan et al. (2007) saw increased expression of *PDH1* in longer term (24–96 h) cold treatments even while Pro contents were high and increasing. Stines et al. (1999) found high levels of Pro despite low *P5CS1* expression in grape (*Vitis vinifera*) berries. Verslues and Sharp (1999) showed evidence that the high levels of Pro accumulated in the maize (*Zea mays*) root tip at low ψ_w were not due to de novo synthesis in the root tip but more likely came from Pro transport from other parts of the plant. Tissue-specific differences were also noted by Skirycz et al. (2010), who found that Pro accumulated in mature leaf tissue but not in expanding or meristematic tissue during long-term growth at moderate severity of osmotic stress, and Ueda et al. (2007), who found high utilization of Pro in the root apex during salt stress. Such observations suggest that stress-specific and tissue-specific differences in Pro metabolism may be more important than previously thought.

The function of drought-induced Pro accumulation in plants and how it enhances drought resistance is a long-standing question. Traditionally, Pro has been thought of mainly as a compatible solute that accumulates as part of osmotic adjustment; for example, there is strong evidence to support such a role in the root growth zone at low ψ_w (Voetberg and Sharp, 1991). In Arabidopsis seedlings at low ψ_w, osmotically significant levels of Pro (50–100 μmol g^{-1} fresh weight, roughly equivalent to 50–100 mM) are routinely observed in our laboratory (Sharma and Verslues, 2010). Such osmotic roles, as well as other potential roles in protecting cellular structure or ROS detoxification, depend on high bulk levels of Pro and have led to a "more-is-better" strategy of generating transgenic plants that constitutively overexpress *P5CS1* or suppress *PDH1* to increase Pro contents and, presumably, salt or drought resistance (Kishor et al., 1995; Zhu et al., 1998; Nanjo et al., 1999a, 1999b; Hong et al., 2000; Sawahel and Hassan, 2002; Parvanova et al., 2004; Vendruscolo et al., 2007).

Other proposed functions of Pro include redox buffering and storage or transfer of energy and reductant (Szabados and Savouré, 2010; Verslues and Sharma, 2010). These functions depend on spatial and temporal control of Pro synthesis and catabolism to either take up or release reductant and energy at the correct place and time to meet the plant's needs and do not lend themselves as well to the more-is-better theory of Pro accumulation. Of particular interest is the fact that both P5CS1 and Δ^1-PYRROLINE-5-CARBOXYLATE REDUCTASE (P5CR), which catalyzes the second step of Pro synthesis, prefer NADPH to NADH as an electron donor (Zhang et al., 1995; Murahama et al., 2001). It has been suggested that Pro synthesis could be a mechanism to regenerate $NADP^+$ in the chloroplast and thus prevent ROS production and photoinhibition caused by a lack of appropriate electron acceptors (Hare and Cress, 1997; Hare et al., 1998; Szabados and Savouré, 2010; Verslues and Sharma, 2010). However, there is no experimental evidence to test the role of Pro metabolism in controlling $NADP^+$ and NADPH levels during low-ψ_w stress.

These functions all require appropriate regulation of Pro metabolism and the coordination of Pro metabolism with many other stress-induced metabolic changes. Abscisic acid (ABA) is the main candidate for such a regulator and at the physiological level is well established to coordinate growth under low ψ_w by protecting root growth to maximize water uptake while restricting shoot growth to avoid water loss (Saab et al., 1990; Sharp et al., 1994). Pro accumulation is partially controlled by ABA, as ABA-deficient mutants such as *aba2-1* in Arabidopsis and *viviparous14* in maize had reduced Pro at low ψ_w (Ober and Sharp, 1994; Sharma and Verslues, 2010). However, whether Pro is an important factor in ABA regulation of growth at low ψ_w is unclear.

We used Arabidopsis Pro metabolism mutants subjected to constant, precisely controlled low-ψ_w stress to demonstrate the essential role of both Pro synthesis and Pro catabolism in promoting growth and maintaining a more oxidized NADP/NADPH ratio at low ψ_w. Analysis of *p5cs1*, *p5cs1/aba2-1*, and *pdh1* mutants, tissue-specific expression of Pro metabolism genes, and the effects of root- and shoot-applied Pro all suggest that Pro supply from the shoot and its catabolism in the root are essential for continued growth at low ψ_w. The *p5cs1/aba2-1* mutants demonstrated that these roles of Pro metabolism are a key part of ABA-mediated growth responses to low ψ_w. Both Pro synthesis and catabolism were needed to maintain a more oxidized NADP/NADPH ratio in the shoot. These results provide a new model that emphasizes dynamic transport and turnover of Pro with tissue-specific synthesis and catabolism, rather than static cell-autonomous Pro accumulation, as fundamental to the protective role of Pro during drought.

RESULTS

Pro Is Required for ABA Protection of Root Elongation and Stimulates Growth at Low ψ_w

We generated *p5cs1-4/aba2-1* and *p5cs1-1/aba2-1* double mutants in which Pro accumulation is only about 15% of the wild-type level (Sharma and Verslues, 2010) and ABA content is only a few percent of the wild-type level (Schwartz et al., 1997; Verslues and Bray, 2006). These *p5cs* alleles have been shown to be null (*p5cs1-4*) or to have very low expression (*p5cs1-1*; Székely et al., 2008). To test the low-ψ_w response of these mutants,

5-d-old seedlings were transferred from normal medium to polyethylene glycol (PEG)-infused agar plates (Verslues et al., 2006) to impose a precisely controlled low-ψ_w stress. As transpiration was low in this system, dehydration caused by impaired stomatal regulation in *aba2-1* was minimized, allowing the growth response to low ψ_w to be accurately compared among genotypes. Root elongation, fresh weight, and dry weight were all measured as indicators of growth at low ψ_w. All media were prepared without added sugar to better mimic the environment of soil-grown plants.

At −0.25 MPa (unstressed control), all genotypes grew similarly to the ecotype Columbia (Col-0) wild type over 7 d after transfer except *aba2-1* and the *p5cs1/aba2-1* mutants, which had an approximately 25% reduction in root elongation (Fig. 1). When seedlings were exposed to a low-ψ_w stress (−1.2 MPa), root elongation of all genotypes was reduced; however, the root elongation of *aba2-1*, *p5cs1-1*, *p5cs1-4*, and the *p5cs1/aba2-1* double mutant was further reduced to half that of the wild type, and overall seedling growth indicated by dry weight and fresh weight was also reduced (Fig. 1).

Root elongation and dry weight of *aba2-1* were restored to the wild-type level by the addition of 2 μM ABA to the media (Fig. 1). However, ABA treatment did not increase the root growth or dry weight of *p5cs1-1*, *p5cs1-4*, or the *p5cs1/aba2-1* double mutant. This indicated that Pro accumulation mediated by *P5CS1* was required for the protective effect of ABA on root elongation and growth at low ψ_w. In contrast, application of 10 mM Pro could restore the root elongation and dry weight of all genotypes (Fig. 1). Pro application also had a stimulatory effect on dry weight, fresh weight (Fig. 1A), and shoot growth (Fig. 1B) of ABA-deficient seedlings. Because ABA normally acts to restrict shoot growth under low ψ_w (Saab et al., 1990; Verslues et al., 2006), seedlings that did not accumulate ABA (*aba2-1* and *p5cs1/aba2-1* mutants) were able to respond more to the stimulation of growth by Pro. Adding Pro to seedlings under control conditions (−0.25 MPa) had no effect on root growth or fresh weight (Supplemental Fig. S1).

Measurement of root elongation at −1.2 MPa in the presence of a range of Pro concentrations from 0 to 10 mM indicated that as little as 0.2 mM exogenous Pro was sufficient to stimulate root elongation of both *aba2-1* and *p5cs1-4* as well as the double mutant (Fig. 2). We also tested whether other amino acids could duplicate the effects of Pro. The branched-chain amino acids (Leu and Ile) have also been observed to accumulate in response to dehydration (Nambara et al.,

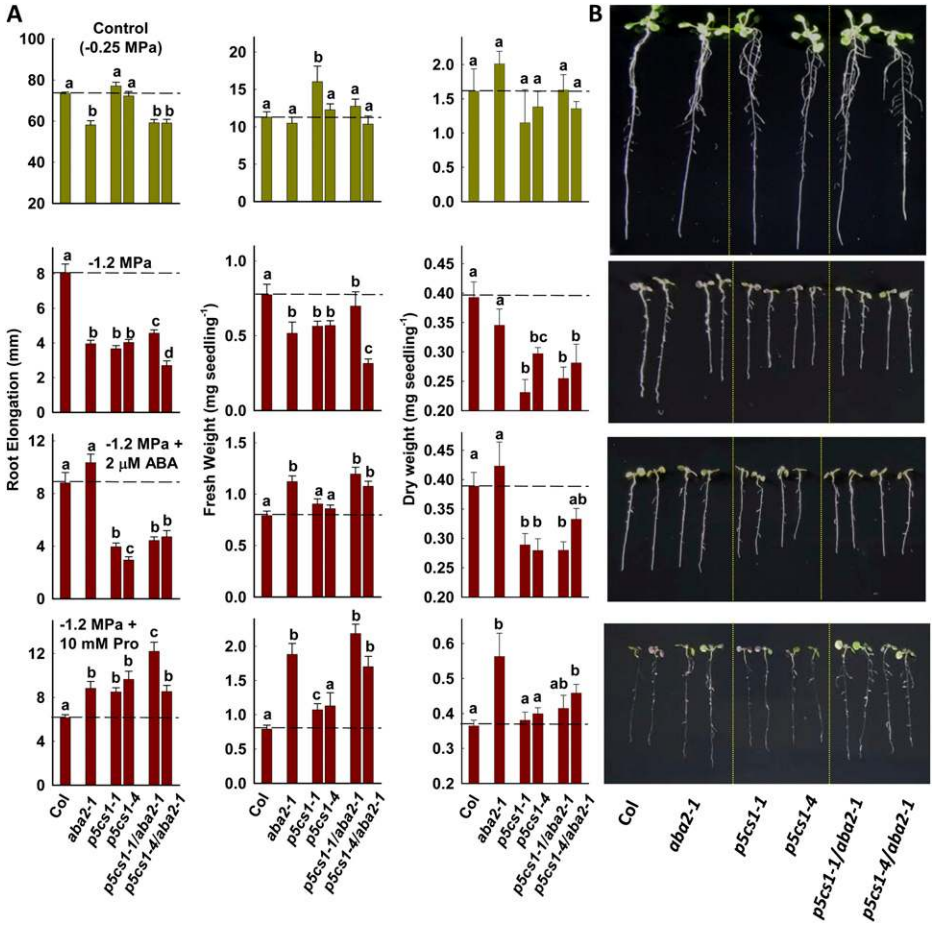

Figure 1. Root elongation and fresh weight of the Col-0 wild type, *aba2-1*, *p5cs1*, and *p5cs1/aba2-1* mutants. Five-day-old seedlings were transferred to either control medium (−0.25 MPa) or PEG-infused agar plates for low ψ_w (−1.2 MPa) or low ψ_w with added ABA (2 μM) or Pro (10 mM). Root elongation was measured over the next 7 d for control seedlings and 10 d for low-ψ_w-treated seedlings. Fresh weight and dry weight were quantified at the end of an experiment. A, Quantification of root elongation, fresh weight, and dry weight. Within each panel, significant differences found by two-factor ANOVA are indicated by different letters. Data shown are means ± SE combined from two to four independent experiments, with each experiment including more than 30 measurements for root elongation, 12 measurements (each including five to six seedlings) for fresh weight, or three to six measurements (each including 25–35 seedlings) for dry weight. Dashed lines in each panel mark the wild-type (Col-0) value. B, Photographs of representative seedlings from each treatment taken at the end of an experiment. One seedling of each genotype is shown for the control, and two seedlings of each genotype are shown for low-ψ_w treatments.

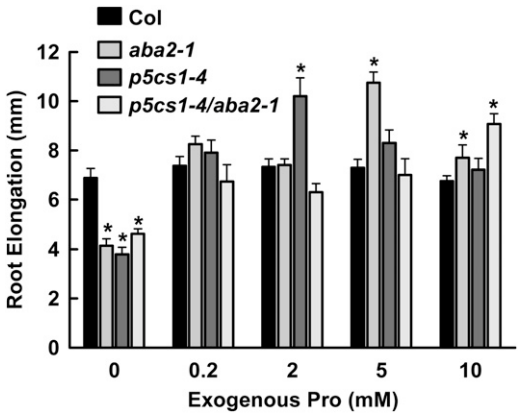

Figure 2. Response of root elongation at −1.2 MPa to various concentrations of exogenous Pro for the Col-0 wild type, *aba2-1*, *p5cs1-4*, and *p5cs1-4/aba2-1* mutants. Five-day-old seedlings were transferred to PEG-infused agar plates (−1.2 MPa) containing Pro (0–10 mM). Root elongation was measured over the next 10 d. Data shown are means ± SE combined from two independent experiments (n = 20–30). Asterisks indicate significant differences compared with the Col-0 wild type ($P > 0.05$).

1998; Joshi and Jander, 2009), but neither Leu, Ile, Val, nor Ala could restore the root elongation of *aba2-1*, *p5cs1-4*, or *p5cs1-4/aba2-1* to the wild-type level at low ψ_w (Supplemental Fig. S2). In fact, these amino acids had a general inhibitory effect on root elongation. γ-Aminobutyrate (GABA) also could not restore root elongation (see below), and the Pro-related amino acids Glu, Gln, Arg, and Orn were strongly inhibitory to root elongation. Inhibition of plant growth by exogenous amino acids has been observed in a number of studies (Bonner and Jensen, 1997; Chen et al., 2010), and this is likely related to feedback inhibition or disturbance of metabolic equilibrium caused by applied amino acids or disruption of metabolite transport. Such observations also illustrate the uniqueness of Pro in promoting growth at low ψ_w.

Tissue-Specific Gene Expression Suggests Spatial Separation of Pro Synthesis and Catabolism as Well as Continued Pro Catabolism at Low ψ_w

To understand the basis of the unique growth-promoting effect of Pro, we quantified Pro metabolism gene expression and found dramatic differences between shoot and root. Shoot tissue had the well-established pattern of *P5CS1* induction and *PDH1* repression after 96 h at −1.2 MPa (Fig. 3A). In contrast, *PDH1* expression was induced by low ψ_w rather than repressed in the 0- to 10-mm root section containing the root meristem and expanding cells (Fig. 3A). Thus, *PDH1* went from being expressed at a relatively even level across root and shoot in unstressed seedlings to being expressed 12-fold higher in the 0- to 10-mm root section than in the bulk of the shoot at low ψ_w. Arabidopsis also contains a second Pro dehydrogenase (*PDH2*), which has only more recently been studied (Funck et al., 2010). *PDH2* had a similar pattern as *PDH1*, although its level of expression was much lower.

A contrasting pattern was observed for *P5CS1*, which was expressed at a much lower level in root than in shoot (Fig. 3A). *P5CR*, which encodes the other enzyme of Pro synthesis, was also significantly induced in shoot but not in root. *Δ^1-PYRROLINE-5-CARBOXYLATE DEHYDROGENASE* (*P5CDH*) and *ORNITHINE AMINOTRANSFERASE* had increased root expression, especially in the 0- to 10-mm section. Overall, the gene expression pattern of the root apex during low ψ_w was similar to that observed in whole seedlings during stress release when rapid Pro catabolism was occurring (Sharma and Verslues, 2010). However, this pattern was different from the combination of high *PDH1* expression but low *P5CDH* expression that was associated with ROS production during pathogen infection (Cecchini et al., 2011).

To further define the tissue-specific expression of *PDH1*, we generated $PDH1_{pro}$:*GUS* plants. After 96 h at −1.2 MPa, there was high *PDH1* promoter activity in the apical few millimeters of the root containing the root meristem and expanding cells, consistent with the quantitative PCR results, as well as high GUS activity in the shoot meristematic region and young expanding leaves (Fig. 3B). The combined gene expression results suggested that expanding and dividing cells in both the root and shoot meristem had substantial Pro catabolism at low ψ_w, while the bulk of the photosynthetic tissue in the shoot had high Pro synthesis but repressed Pro catabolism.

PDH Expression Is Required to Sustain Growth at Low ψ_w

To test the importance of continued Pro catabolism at low ψ_w, two homozygous T-DNA mutant lines were isolated for *PDH1*. The *PDH1* mutants were null or had extremely low expression based both on a previous report (Funck et al., 2010) and our own data (Supplemental Fig. S3A). The *pdh1* mutants grew similarly to the Col-0 wild type in the control (−0.25 MPa; Fig. 4A). At −1.2 MPa, however, root growth, fresh weight, and dry weight of *pdh1* were reduced, indicating the importance of Pro catabolism for both root and shoot growth. Exogenous Pro could not restore the growth of *pdh1* mutants (Fig. 4, A and B). Tests of a range of exogenous Pro concentrations from 0.2 to 10 mM found that none could restore the root elongation of *pdh1-2* (Fig. 4C). Higher exogenous Pro levels (10 mM) inhibited the root growth of *pdh1-2* at both low ψ_w (Fig. 4, A and C) and high ψ_w (Supplemental Fig. S3B). This inhibition could be caused by high levels of Pro inhibiting the transport of other metabolites to the root tip, which may be more critical to sustaining root growth when Pro catabolism is blocked. ABA application also could not restore the root elongation of *pdh1-2* (Supplemental Fig. S3C).

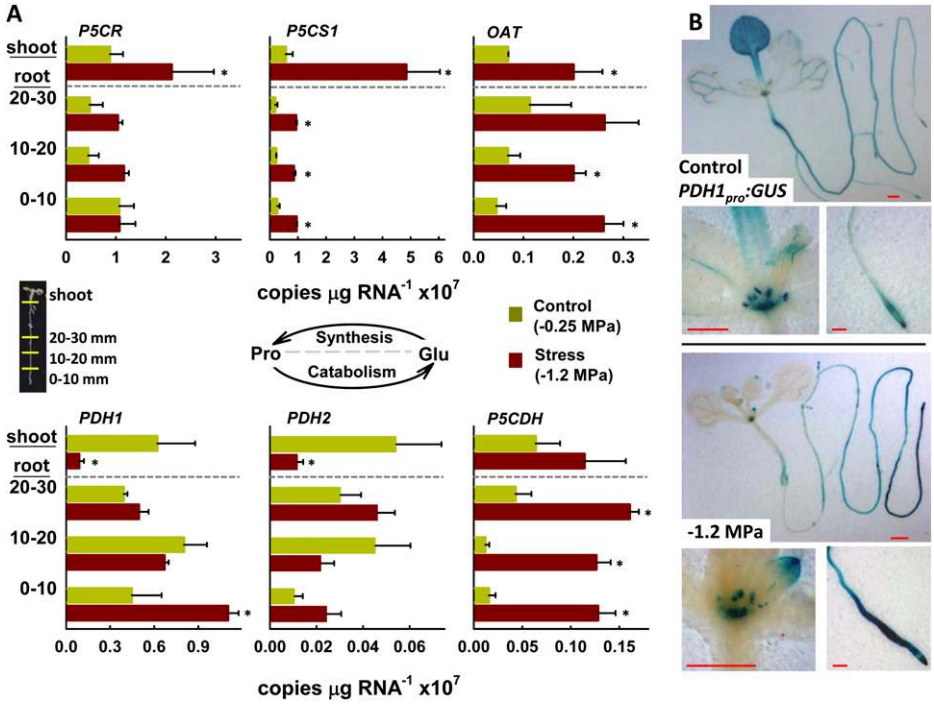

Figure 3. Tissue-specific expression of Pro metabolism genes in wild-type seedlings. A, Quantitative PCR analysis of gene expression. Seven-day-old seedlings were transferred to either control (−0.25 MPa) or low-ψ_w (−1.2 MPa) medium for 96 h, and samples of shoot tissue as well as three sequential sections of the root were collected (the sampling scheme is shown in the diagram in the middle of the panel). Asterisks indicate significant differences between control and low-ψ_w treatments based on results of an unpaired *t* test ($P < 0.05$). Data are shown as means ± SE ($n = 3$–4) from one representative experiment out of two independent experiments. B, Histochemical GUS staining of T3 homozygous plants expressing $PDH1_{pro}$:*GUS* containing a 1.6-kb fragment of the *PDH1* promoter. Control and low-ψ_w treatments were performed as described in A. Data are for one representative transgenic line out of three independent lines analyzed. Bars = 1 mm.

As a further test of the importance of Pro metabolism for growth at low ψ_w, we quantified root elongation of both *p5cs1* and *pdh1* mutants at the moderate stress level of −0.7 MPa, where growth continues at a more rapid rate than at −1.2 MPa. Root elongation was reduced in *p5cs1-4* and to an even greater extent in *pdh1-2* (Supplemental Fig. S4A), demonstrating that both Pro synthesis and catabolism were required for maximal growth across a range of low-ψ_w severities. It was also seen that Pro application at −0.7 MPa inhibited the root growth of *pdh1-2* seedlings even more than low ψ_w alone (Supplemental Fig. S4B), suggesting that when Pro catabolism was blocked, simply adding additional Pro was not sufficient to promote growth and may in fact interfere with the transport or metabolism of other substrates.

Lack of *PDH1* Expression Decreases Root Respiration Despite Increased Pro Content

One possible explanation for the reduced growth of *pdh* or *p5cs1* mutants is that inhibited Pro catabolism or Pro supply caused a decrease in respiration needed to sustain growth. Consistent with this hypothesis, we found reduced root tip oxygen consumption of *pdh1-2* at low ψ_w while the Col-0 wild type and *p5cs1-4* were unaffected (Fig. 5A). Adding 10 mM Pro to seedlings at −1.2 MPa could not restore oxygen consumption of *pdh1-2* and in fact further decreased oxygen consumption, consistent with the effects on root elongation. The impaired oxygen consumption of *pdh1-2* but not *p5cs1-4* suggests that although both *p5cs1* and *pdh1* mutants have similar effects on root elongation, the main factor limiting growth may be different.

To further determine how root oxygen consumption and growth were related to Pro, the same shoot and root section sampling scheme used for gene expression measurements was used to measure Pro content of seedlings at −1.2 MPa. *pdh1-2* had Pro content more than double that of the wild type in the root apex (0- to 10-mm section; Fig. 5B), while Pro contents of the shoot, 20- to 30-mm root, and 10- to 20-mm root sections were only slightly increased or unchanged. This again suggested an active Pro catabolism in the root apex at low ψ_w, such that in *pdh1-2*, where this catabolism was blocked, Pro accumulated to high levels while root respiration was inhibited.

In contrast, Pro content of *p5cs1-4* was much lower than that of the wild type in all tissues measured (Fig. 5B). Interestingly, *p5cs1-4* maintained a relatively

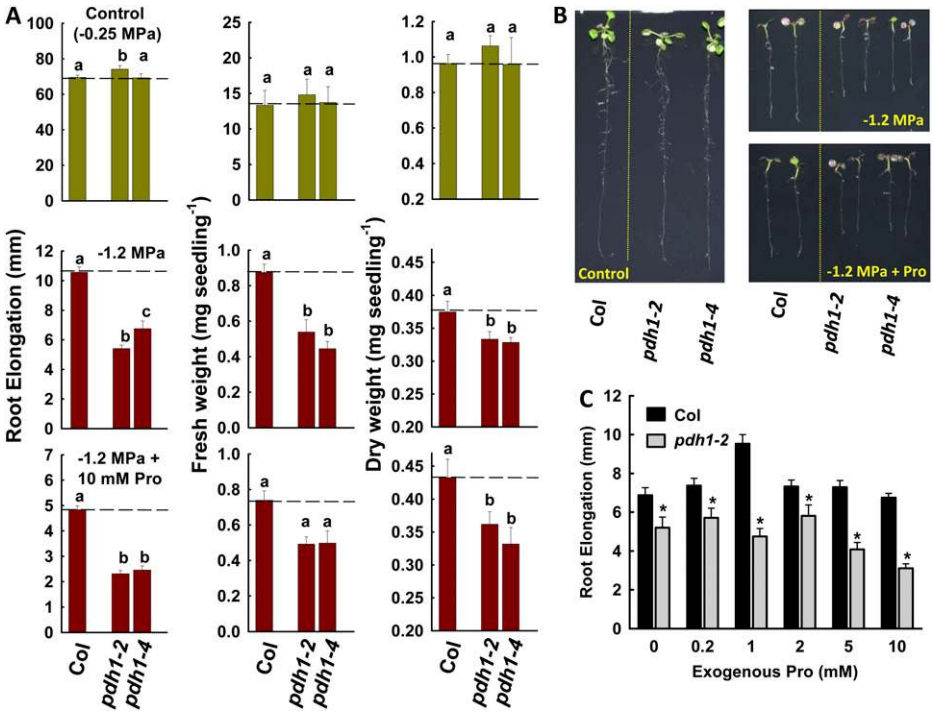

Figure 4. *pdh1* growth response to low ψ_w and Pro. Five-day-old seedlings were transferred to control (−0.25MPa), low-ψ_w (−1.2 MPa), or low-ψ_w plus Pro (10 mM) medium. A, Root elongation, fresh weight, and dry weight data were collected as described for Figure 1A. Within each panel, statistically significant differences detected by two-factor ANOVA and/or *t* test are indicated by different letters ($P > 0.05$). Data are shown as means ± SE combined from two to four independent experiment, with each experiment including more than 15 measurements for root elongation, eight or more measurements for fresh weight, or three or more measurements for dry weight. B, Photographs of representative seedlings of the Col-0 wild type and *pdh1* mutants taken at the end of an experiment. One seedling of each genotype is shown for the control, and two seedlings of each genotype are shown for the low-ψ_w treatments. C, Response of root elongation to a range of Pro concentrations from 0 to 10 mM. Data shown are means ± SE combined from two independent experiments (n = 20–30). Asterisks indicate significant differences compared with the Col-0 wild type ($P > 0.05$).

higher Pro content in the 0- to 10-mm root section (22% of the wild type) than in the shoot (9% of the wild type), despite higher *P5CS1* expression in the shoot (Fig. 3A). One possible explanation for this would be shoot-to-root movement of Pro. *aba2-1* was also analyzed to see if its reduced growth at low ψ_w may be caused by altered Pro distribution. Pro content was reduced to half the wild-type level in both the shoot and 0- to 10-mm root sections, although Pro content was not different from the wild type in the other root sections (Fig. 5B). Thus, *aba2-1* may be affected by Pro shortage in the root growing region at low ψ_w as well as by reduced Pro synthesis in the shoot tissue.

Stimulation of Root Growth by Shoot-Applied Pro Suggests Active Shoot-to-Root Transport

The gene expression pattern, oxygen consumption, and Pro contents all suggested that Pro synthesized in the shoot may be transported to the root to support growth at low ψ_w. Consistent with this idea, we found that Pro applied specifically to the shoot could stimulate root elongation of the wild type, *p5cs1-1*, and *p5cs1-4* at low ψ_w. Split agar plates were constructed by removing the regular agar medium from half of a PEG-infused agar plate (−1.2 MPa) and replacing it with the same low-ψ_w medium supplemented with Pro (Supplemental Fig. S5). Seedlings were then transferred to these plates such that only the root or shoot was in contact with the Pro-containing medium. Pro diffusion across the boundary separating the two plate halves was not detected (Supplemental Fig. S5).

When split plates were constructed with both halves lacking Pro, both root elongation and Pro content of the 0- to 10-mm root section (Fig. 6A) were similar to those seen in other experiments (compare Fig. 6A with Figs. 1A and 5B). Addition of Pro specifically to the root stimulated root elongation, and this was associated with a large increase in root tip Pro content (Fig. 6A). Interestingly, however, addition of Pro specifically to the shoot stimulated root elongation to a greater extent than either Pro applied to whole seedlings or specifically to the root (compare Fig. 6A with Fig. 1A). Shoot-applied Pro only increased root Pro content back to the wild-type level or even slightly below the wild-type level, showing that it was not a large buildup of Pro in

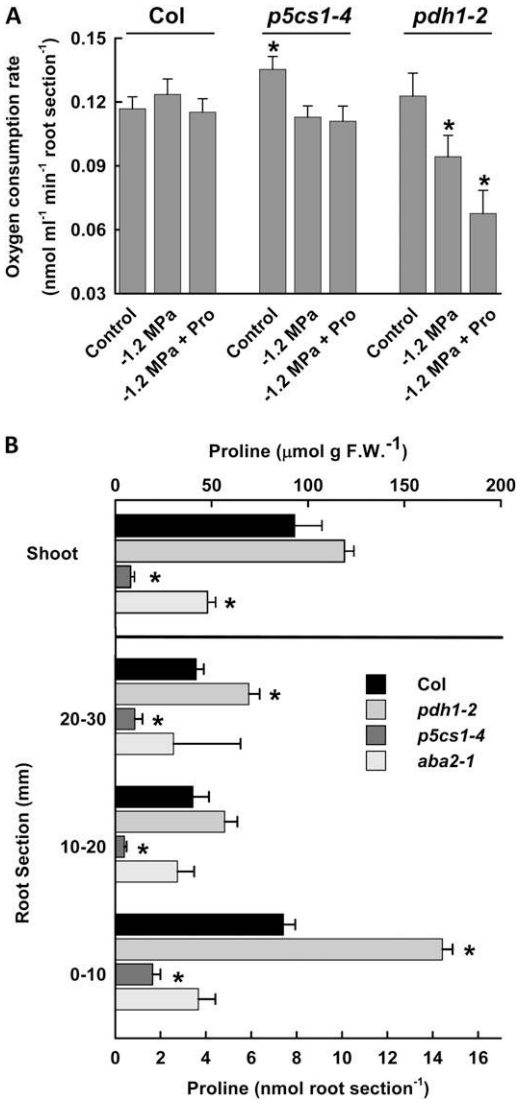

Figure 5. Root tip oxygen consumption rates and tissue-specific Pro contents of the Col-0 wild type and Pro metabolism mutants. A, Five-day-old seedlings were transferred to control (−0.25 MPa), low-ψ_w (−1.2 MPa), or low-ψ_w plus Pro (10 mM) medium for 96 h. Root sections (0–10 mm) were excised and immediately used for oxygen consumption rate measurement in a Clark-type electrode. Data are means ± SE (n = 7–8) from two independent experiments. Asterisks indicate significant differences compared with the wild type in the same treatment detected by unpaired t test ($P < 0.05$). B, Free Pro content measured in shoot and 10-mm root sections after 96 h at low ψ_w (−1.2 MPa). Note that shoot data are expressed as μmol g^{-1} fresh weight (F.W.), while root data are expressed as nmol root $section^{-1}$. Data are means ± SE (n = 6–8) combined from two independent experiments. Asterisks mark significant differences from the wild type detected by unpaired t test ($P < 0.05$).

the root that was most needed to enhance growth. The observation that shoot-applied Pro could stimulate root elongation suggests shoot-to-root movement of Pro; however, we cannot rule out the possibility that shoot-applied Pro stimulated the production of other metabolites that were transported to the root.

GABA can stimulate root elongation in unstressed seedlings (Mirabella et al., 2008), is often thought to have similar stress-protective roles as Pro, and is also catabolized in the mitochondria. However, GABA application to either root or shoot could not duplicate the effect of Pro in restoring root elongation of *p5cs1* mutants (Fig. 6B), again demonstrating a specific effect of Pro on growth at low ψ_w.

Pro Metabolism Is Required to Maintain an Oxidized NADP/NADPH Ratio

These data still left open the question of why Pro synthesis would be greater in the shoot than in the root. Our data, plus recent observations that *P5CS1* may be in, or closely associated with, the chloroplast (Székely et al., 2008; Reiland et al., 2009) led us to test the possibility that Pro synthesis serves to regenerate $NADP^+$ and maintain an appropriate NADP/NADPH ratio (Hare and Cress, 1997; Szabados and Savouré, 2010; Verslues and Sharma, 2010). In the Col-0 wild type, low ψ_w (−1.2 MPa for 96 h) increased NADP content by approximately 4-fold while NADPH content increased by nearly 6-fold (Fig. 7A). Thus, the NADP/NADPH ratio was decreased by half from 4.9 at high ψ_w to 2.4 at low ψ_w. This trend to a more reduced ratio at low ψ_w was exacerbated significantly in both *p5cs1-4* and *pdh1-2*, where the NADP/NADPH ratio at −1.2 MPa was 1.5 and 1.6 for *p5cs1-4* and *pdh1-2*, respectively. This change in ratio resulted from higher NADPH and lower NADP contents, such that the total pyridine nucleotide content was little changed in the mutants compared with the wild type. It was particularly interesting that we found similar NADP/NADPH in both *p5cs1-4* and *pdh1-2* at −1.2 MPa, as *PDH1* expression in most of the shoot tissue was reduced to a low level in this treatment (Fig. 3). Also interesting was the observation that *pdh1-2* had a significantly more reduced NADP/NADPH ratio at high ψ_w (2.9 versus 4.9 for the wild type). This demonstrated a role for Pro metabolism in maintaining an oxidized NADP/NADPH ratio even at high ψ_w, when Pro content was low. A specific effect of Pro metabolism on NADP/NADPH was further indicated by the lack of any significant difference between the wild type and *p5cs1-4* or *pdh1-2* in either NAD or NADH content or NAD/NADH ratio at high or low ψ_w (Fig. 7B).

We also assayed H_2O_2 to see if any of the Pro metabolism mutants had increased overall ROS content. Consistent with previous reports (Verslues et al., 2007; Fujii et al., 2011), low ψ_w caused an increase in H_2O_2 content of the wild type (Supplemental Fig. S6). This increase also occurred in *aba2-1*, *p5cs1*, and *prodh* mutants; however, none of the mutants had H_2O_2 levels above that of the wild type at either high or low ψ_w. This lack of H_2O_2 accumulation suggested that general ROS accumulation or damage was unlikely to cause the altered growth or other phenotypes observed for *p5cs1* or *pdh1*.

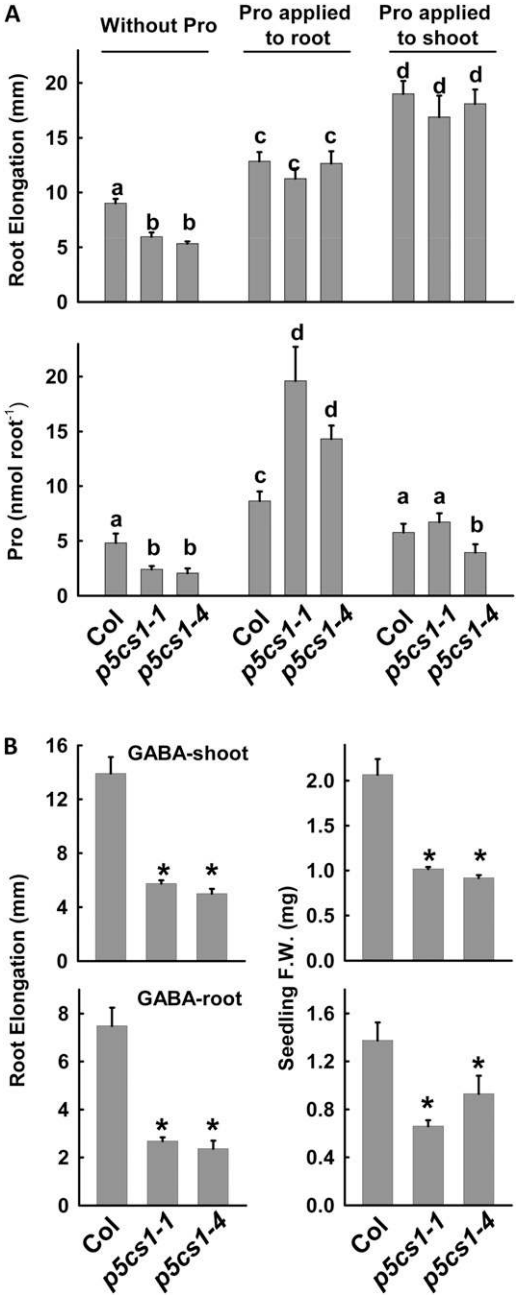

Figure 6. Effect of Pro or GABA applied specifically to shoot or root using a split-plate assay system. A, Five-day-old seedling were transferred to low ψ_w (−1.2MPa) with the addition of 10 mM Pro to either root or shoot using split plates (the split-plate system is shown in Supplemental Fig. S5). Root elongation was then monitored over the next 10 d. For root elongation, data are means ± SE ($n > 40$) combined from three independent experiments. Pro contents were determined in the 0- to 10-mm root section at 10 d after transfer and are means ± SE ($n = 3–6$) combined from three independent experiments. Within each panel, different letters indicate significant differences detected by two-factor ANOVA ($P < 0.05$). B, Effect of 10 mM GABA applied to root or shoot on root elongation and seedling fresh weight of the wild type or *p5cs1* mutants. The experimental design is the same as in A. Data are means ± SE ($n = 20–25$). Asterisks indicate significant differences compared with the wild type detected by unpaired *t* test ($P < 0.05$).

DISCUSSION

Several novel observations presented here shed new light on the role of Pro metabolism in low-ψ_w resistance. First, the *p5cs1/aba2-1* double mutants demonstrated that Pro was essential for ABA protection of root elongation and overall growth at low ψ_w. Second, the similar phenotypes of *p5cs1* and *pdh1* mutants demonstrated that both Pro synthesis and catabolism were required to maintain growth at low ψ_w. Third, the localized expression of *PDH1* and *P5CS1*, the ability of shoot-applied Pro to greatly enhance root elongation, and the large buildup of Pro in the root apex of *pdh1-2* all suggest the transport of Pro and/or related metabolites from shoot to root and catabolism in the root apex as essential to continued growth at low ψ_w. Fourth, the NADP/NADPH ratio, but no change in NAD/NADH, in both *p5cs1-4* and *pdh1-2* suggest a specific role of Pro turnover in maintaining a more oxidized NADP/NADPH ratio.

Together, these data lead to a new model of Pro metabolism (Fig. 8). At low ψ_w, Pro synthesis is focused in "Pro source" tissue (primarily the photosynthetic tissue of the shoot), where it regenerates NADP to help maintain an appropriately oxidized NADP/NADPH ratio. Some of this Pro accumulates in the leaf as a compatible solute. However, a portion of the Pro is transported to "Pro sink" tissue in the root and shoot meristem, where it can be catabolized both to support continued growth at low ψ_w and to maintain a sink for continued Pro export from the source tissue. Thus, the effectiveness of Pro metabolism in buffering energy and redox imbalances within the plant, in addition to Pro function as a protective compatible solute for osmotic adjustment, is key to its role in low-ψ_w resistance.

Pro Metabolism Maintains a More Oxidized NADP/NADPH Ratio

Low ψ_w caused a shift in NADP/NADPH to a more reduced state, and this was exacerbated in both *p5cs1-4* and *pdh1-2*. The NADP/NADPH ratio we observed at high ψ_w was consistent with previous reports (Wang and Pichersky, 2007). However, we could find no previous data on NADP/NADPH ratio at low ψ_w. Consistent with our hypotheses, De Ronde et al. (2004) found increased levels of NADP in drought-stressed soybean (*Glycine max*) plants overexpressing *P5CR* and decreased NADP in *P5CR* antisense plants; however, they did not measure NADPH levels or calculate NADP/NADPH ratio, the key measure of redox status. The specific effect of Pro metabolism on NADP/NADPH, but not NAD/NADH, further suggests a link of Pro metabolism to redox buffering in the chloroplast, which is prone to overreduction under changing environmental conditions (Dietz and Pfannschmidt, 2011). The NADP/NADPH ratio is critical not only for photosynthetic electron transport but also for ROS generation and signaling by NADPH

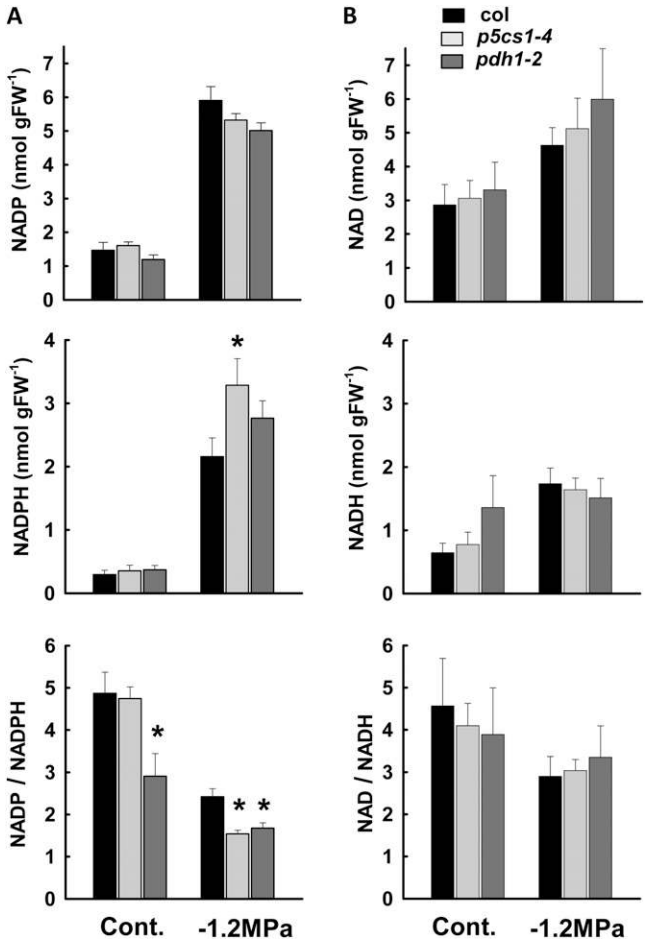

Figure 7. Pyridine nucleotide contents and NADP/NADPH and NAD/NADH ratios in the wild type (Col-0), *p5cs1-4*, and *pdh1-2* after control or low-ψ_w treatment. Seven-day-old seedlings were transferred to control (−0.25 MPa) or low-ψ_w (−1.2 MPa) medium for 96 h, and samples of shoot tissue were collected for pyridine nucleotide assay. A, Assay of NADP and NADPH and calculation of NADP/NADPH ratio. NADP and NADPH contents are representative data from two independent experiments (means ± SE; $n = 8–12$). One ratio was calculated for each independent experiment, and the NADP/NADPH ratio shown is the mean of ratios from four independent experiments. Asterisks indicate significant differences relative to the wild type detected by unpaired *t* test ($P < 0.05$). B, Assay of NAD and NADH and calculation of NAD/NADH ratio. Data and ratio calculation are as described for A. No significant differences were detected between mutants and the wild type for NAD, NADH, or NAD/NADH. FW, Fresh weight.

oxidases, activity of many NADPH-dependent enzymes, and nitric oxide production (Zhang et al., 2009; Takahara et al., 2010; Dietz and Pfannschmidt, 2011). Therefore, control of NADP/NADPH ratio by Pro metabolism could impact other metabolic and signaling pathways. It will be of particular interest to investigate the possible coupling of Pro synthesis and the oxidative pentose phosphate pathway, which is also known to have a role in controlling the NADP/NADPH ratio (Verslues and Sharma, 2010; Dietz and Pfannschmidt, 2011).

The surprising observation that *pdh1-2* had a substantial role in maintaining the NADP/NADPH ratio even in unstressed seedlings suggests that the cycle of Pro synthesis and catabolism was important at both high and low ψ_w. Thus, restricted Pro catabolism in *pdh1-2* may have caused a reduction in NADPH consumption by Pro synthesis by mechanisms such as reduced regeneration of Glu for Pro synthesis. However, other more indirect effects of *PDH1* on NADP/NADPH, such as a restricted supply of Glu generated by Pro catabolism causing altered glutathione production, cannot be ruled out.

Growth Maintenance at Low ψ_w Depends on Both Pro Accumulation and Pro Metabolism

The reduced dry weight and root elongation of *p5cs1* and *pdh1* mutants demonstrated the essential role of Pro metabolism in growth at low ψ_w. The role of Pro was particularly prominent in root elongation, and the ability of Pro, but not ABA, to rescue *p5cs1* and *p5cs1/aba1-2* root elongation demonstrated that Pro metabolism was part of the well-established function of ABA in promoting root elongation at low ψ_w. The observation that both *p5cs1* and *pdh1* mutants had decreased root elongation (as well as reduced dry weight) suggests that two mechanisms are likely to be involved in Pro promotion of growth. In the case of *p5cs1-4*, root oxygen consumption remained high but root Pro content was low (Fig. 5). Pro could be catabolized for respiration, but then insufficient Pro was left for other essential functions such as osmotic adjustment. For *pdh1-2*, oxygen consumption was reduced but there

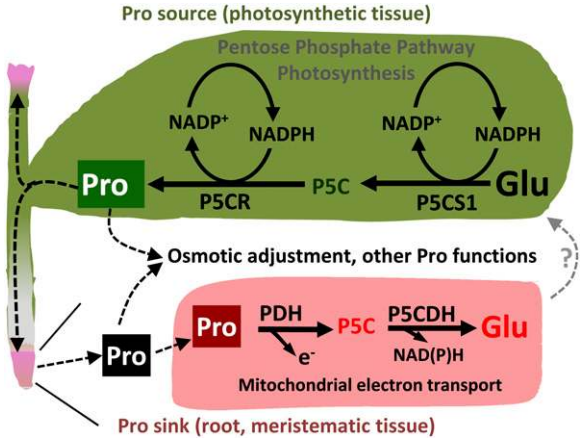

Figure 8. Model of tissue-specific Pro synthesis and catabolism at low ψ_w. At low ψ_w, Pro synthesis increases in the photosynthetic tissue of the shoot (Pro source), as indicated by increased expression of both *P5CS1* and *P5CR*. This synthesis generates NADP to maintain a more oxidized NADP/NADPH ratio. A portion of the Pro is transported to Pro sinks in the growing regions of root and shoot. In Pro sink tissues, Pro is catabolized in the mitochondria to support growth. Whether a product of Pro catabolism moves back to the shoot to continue the cycle is not known. In both the Pro source and Pro sink regions, Pro accumulation is also necessary for other functions such as osmotic adjustment.

was nearly twice as much Pro in the root as in the wild type. In this case, the catabolism of Pro as a respiratory substrate was the limiting factor.

In both of these cases, the data suggest that delivery of Pro to the root growing region was critical for continued growth at low ψ_w. This is consistent with previous work describing a stress-induced, root-specific Pro transporter from barley (*Hordeum vulgare*; Ueda et al., 2001) and increased root growth (at high ψ_w) of plants with root tip-specific overexpression of this transporter (Ueda et al., 2008). Also consistent with our model of Pro movement are previous suggestions that the root growth zone has limited Pro synthesis but high Pro utilization (Verslues and Sharp, 1999; Ueda et al., 2007) and reports of high levels of Pro in the phloem of drought-stressed plants (Girousse et al., 1996; Lee et al., 2009). Identifying the transport mechanisms that move Pro from leaf to root at low ψ_w will be of interest for future experiments.

While we focused more on the maintenance of root growth, our data are also consistent with those of Skirycz et al. (2010), who performed a detailed analysis of gene expression and metabolite profiles in shoots of Arabidopsis plants grown under long-term mild osmotic stress. They found high expression of *PDH1* in leaf primordial tissue, which was not down-regulated in plants adapted to osmotic stress, all consistent with our $PDH1_{pro}$:*GUS* staining pattern. Also, Pro content was higher in mature leaf than in primordial or expanding tissue. In agreement with our model, they postulated a role for Pro in protecting photosynthesis in mature leaves and a role of mitochondrial metabolism in stress adaptation of the primordial leaf tissue.

Pro Metabolism Is a Means to Buffer Energy and Redox Imbalances at Low ψ_w

The combined data point to Pro metabolism and transport as part of a strategy to deal with a fundamental imbalance caused by low ψ_w: photosynthetic tissue has an overabundance of reductant and a decreased ability to use it effectively because of reduced CO_2 uptake and restricted shoot growth. Conversely, the root is still growing and has an increased need for energy to support processes such as solute accumulation and cell wall metabolism. A need to correct energy imbalances within the plant at low ψ_w has also been indicated by the observation that alternative oxidase overexpression lines had less reduction in leaf growth during soil drying (Skirycz et al., 2010).

The movement of Pro from shoot to root can be one way for the plant to correct such energy and redox imbalances. An obvious question is why Pro transport, instead of other sugars or amino acids, would be beneficial under low ψ_w. Part of the answer seems to be the benefit of continued Pro synthesis in the shoot to generate $NADP^+$. On the catabolism side, the unique catabolic pathway of Pro (PDH1 donates electrons directly to the mitochondrial electron transport chain) and the high energy content of Pro (30 ATP equivalents per Pro molecule; Mattioli et al., 2009), as well as the possibility that Suc supply from the shoot may be diminished (Boyer, 2010), make Pro a good substrate for continued respiration at low ψ_w. Function of Pro as a specialized respiratory substrate has been demonstrated in other biological systems (Verslues and Sharma, 2010). In addition, *PDH1* is known to be up-regulated during darkness-induced energy starvation (Dietrich et al., 2011), where Pro may also substitute for other respiratory substrates. Our observations place Pro metabolism within the emerging view that energy and redox management, including important roles for chloroplast and mitochondrial metabolism, are key to drought adaptation (Atkin and Macherel, 2009; Skirycz et al., 2010; Dietz and Pfannschmidt, 2011). We believe that this role of Pro metabolism is complementary to the role of Pro as a compatible solute for osmotic adjustment.

A number of studies have used transgenic strategies to modify Pro metabolism and, presumably, enhance drought resistance. Such studies have generally been designed on the premise that Pro accumulation is a cell autonomous process and that any combination of increased synthesis and decreased catabolism that leads to higher bulk Pro levels would increase drought resistance. Our model suggests that tissue-specific modifications of Pro metabolism, such as enhanced Pro synthesis in the shoot coupled with increased transport to the root, could be a more effective strategy.

MATERIALS AND METHODS

Plant Material, Growth, and Stress Treatments

T-DNA insertion lines of Arabidopsis (*Arabidopsis thaliana*) were obtained from the Arabidopsis Biological Resource Center, and homozygous plants were identified by PCR screening as described (http://signal.salk.edu/tdnaprimers.2.html). Double mutants of *p5cs1/aba2-1* were isolated as described previously (Sharma and Verslues, 2010). A summary of the T-DNA lines used is presented in Supplemental Table S1.

Seedling growth and stress treatments were performed in a manner similar to previous experiments (Verslues et al., 2006; Sharma and Verslues, 2010). Briefly, sterilized Arabidopsis seeds were plated on half-strength Murashige and Skoog (MS) medium (pH 5.7) without the addition of sugar. After 48 h of cold treatment at 4°C, the plates were placed in a growth chamber (25°C, continuous light at 80–100 μmol photons m^{-2} s^{-1}) oriented vertically so that seedlings grew along the surface of the agar. Five- or 7-d-old seedlings were transferred to PEG-infused agar plates to impose low-ψ_w stress (Verslues et al., 2006). Where indicated, *S*(+)ABA or Pro was added to the medium after sterilization. For root elongation and fresh weight measurements, seedlings were transferred to the indicated medium at 5 d of growth and root elongation was monitored over the subsequent 10 d for stress treatments and 7 d for the control.

To apply Pro or other metabolites specifically to root or shoot, split agar plates were prepared by removing half of the agar from PEG-infused plates of −1.2 MPa and replacing that half of the agar with PEG-infused agar containing Pro or other amino acid. Five-day-old seedlings were transferred to these split plates such that only the root or shoot was in contact with the Pro-containing medium, and root growth was monitored for the subsequent 10 d. The split agar plate experiment is diagrammed and further explained in Supplemental Figure S5.

Quantitative Real-Time PCR

Gene expression analysis was carried out with shoot tissue and three different root sections each of 10 mm from the root tips. Three or four biological samples were collected, and total RNA was isolated using the

RNeasy Plant Mini Kit (Qiagen) with DNase treatment. RNA was quantified using a Nanodrop spectrophotometer, and 0.5 to 1.0 μg of RNA was reverse transcribed using the SuperScript III (Invitrogen) cDNA synthesis kit. cDNAs were diluted appropriately to fall within the range of standard curves generated using known copy numbers of cloned cDNAs, and real-time PCR was performed using FAM fluorophore/BHQ quencher-labeled TaqMan probes as described previously (Sharma and Verslues, 2010).

Oxygen Consumption

Oxygen uptake rate in 10-mm root tips was measured by Clark-type electrode (Hansatech) in a stirred 1-mL reaction volume at 25°C. The incubation mixture contained half-strength MS (pH 5.7) for control samples or half-strength MS with mannitol added to match the ψ_w of the PEG-infused agar plates. Each sample consisted of 40 to 50 (control) or 30 to 35 (low ψ_w) root tips. Three or four samples were assayed in each independent experiment.

Assay of Nicotinamide Coenzyme

Oxidized and reduced nicotinamide coenzyme contents were measured using enzymatic assays as described previously by Hayashi et al. (2005) with some modifications. Samples of 100 to 200 mg (control) or 30 to 50 mg (low ψ_w) were ground in liquid N_2 and homogenized with 0.1 M HCl (for NAD and NADP assay) or 0.1 M NaOH (for NADH and NADPH assay). Samples were heated at 95°C for 2 min, cooled in an ice bath, and pH adjusted using NaOH (for NAD and NADP assay) or HCl (for NADH and NADPH assay). Samples were centrifuged for 10 min at 4°C, and the supernatants were used for coenzyme assay. For NADP and NADPH measurement, sample aliquots were added to a reaction mixture containing 50 mM glyglycine buffer (pH 7.4), 5 mM Glc-6-P, 0.25 mM phenazine methosulfate, 0.5 mM thiazolyl blue, 20 mM nicotinamide, and Glc-6-P dehydrogenase at a final concentration of 0.5 μg mL^{-1}. For NAD and NADH measurement, the reaction mixture consisted of 50 mM glyglycine buffer (pH 8.8), 8% ethanol, 0.25 mM phenazine methosulfate, 0.5 mM thiazolyl blue, 20 mM nicotinamide, and alcohol dehydrogenase at a final concentration of 2.5 μg mL^{-1}. Assays were performed using a reaction volume of 200 μL on 96-well plates, and absorbance was measured at 565 nm from 0 to 30 min after the start of the reaction. The concentration of NAD(P)(H) was determined by comparing sample values with standard curves.

Construction of the *PDH1*$_{pro}$:*GUS* Transgenic Plants and Histochemical GUS Assay

The *PDH1* promoter and 5′ untranslated region from −1,500 to +115 was amplified (forward primer, 5′-AAAAAGCAGGCTTGAAGGAACTTCTCAAAA-3′; reverse primer, 5′-AGAAAGCTGGGTAAAATTCAAAGATTTTGT-3′; these primers include Gateway cloning sequences, and a second nested PCR was performed to add the remaining portion of the Gateway recognition sequences) from Col-0 genomic DNA, cloned into entry vector pDONR207 (Invitrogen) by BP reaction, and subsequently transferred by LR reaction into the pGWB433 binary vector (Nakagawa et al., 2007) to generate a promoter:GUS fusion. The construct was transferred into *Agrobacterium tumefaciens* strain GV3101 and used for transformation of the Arabidopsis Col-0 wild type. Transgenic plants were selected on half-strength MS medium containing 50 μg mL^{-1} kanamycin. Lines exhibiting segregation ratios consistent with a single locus insertion were selected, and three independent homozygous T3 lines were used for GUS staining. Similar staining patterns were obtained with all lines, and representative results are shown. GUS staining was performed following standard protocols (Weigel and Glazebrook, 2002).

Pro and H_2O_2 Measurement

Free Pro was assayed using the ninhydrin assay (Bates et al., 1973) adapted to 96-well format (Verslues, 2010). For H_2O_2 assay, 50 to 100 mg of plant material was collected and extracted as described previously (Shin and Schachtman, 2004), and H_2O_2 was quantified using an Amplex Red H_2O_2 assay kit (Invitrogen) following the manufacturer's protocol.

Statistical Analysis

Data were analyzed by two-factor ANOVA with the Holm-Sidak posttest as implemented in Sigma Plot 11 (Systat Software). Other comparisons were performed by two-tailed *t* test as indicated in the text and figure legends.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Root elongation and fresh weight of Pro-treated *aba2-1*, *p5cs1*, and *p5cs1/aba2-1* mutant seedlings at high ψ_w.

Supplemental Figure S2. Amino acids other than Pro did not restore root elongation of the *aba2-1*, *p5cs1-4*, and *p5cs1-4/aba2-1* mutants at low ψ_w.

Supplemental Figure S3. Characterization of *pdh1* mutants.

Supplemental Figure S4. Root elongation of the wild type and Pro metabolism mutants at −0.7 MPa.

Supplemental Figure S5. The split agar plate system for root- or shoot-specific application of Pro and other metabolites.

Supplemental Figure S6. H_2O_2 content of the wild type, *aba2-1*, and Pro metabolism mutants.

Supplemental Table S1. T-DNA lines used in this study.

ACKNOWLEDGMENTS

We thank Na Lin for assistance in the laboratory and Mei-Jane Fang for assistance with photography of the GUS-stained plants.

Received July 11, 2011; accepted July 25, 2011; published July 26, 2011.

LITERATURE CITED

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

Armengaud P, Thiery L, Buhot N, Grenier-De March G, Savouré A (2004) Transcriptional regulation of proline biosynthesis in *Medicago truncatula* reveals developmental and environmental specific features. Physiol Plant **120:** 442–450

Atkin OK, Macherel D (2009) The crucial role of plant mitochondria in orchestrating drought tolerance. Ann Bot (Lond) **103:** 581–597

Bates LS, Waldren RP, Teare ID (1973) Rapid determination of free proline for water-stress studies. Plant Soil **39:** 205–207

Ben Hassine A, Ghanem ME, Bouzid S, 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. J Exp Bot **59:** 1315–1326

Bonner CA, Jensen RA (1997) Recognition of specific patterns of amino acid inhibition of growth in higher plants, uncomplicated by glutamine-reversible 'general amino acid inhibition.' Plant Sci **130:** 133–143

Boyer JS (2010) Drought decision-making. J Exp Bot **61:** 3493–3497

Cecchini NM, Monteoliva MI, Alvarez ME (2011) Proline dehydrogenase contributes to pathogen defense in Arabidopsis. Plant Physiol **155:** 1947–1959

Chen H, Saksa K, Zhao F, Qiu J, Xiong L (2010) Genetic analysis of pathway regulation for enhancing branched-chain amino acid biosynthesis in plants. Plant J **63:** 573–583

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

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

Dietz K-J, Pfannschmidt T (2011) Novel regulators in photosynthetic redox control of plant metabolism and gene expression. Plant Physiol **155:** 1477–1485

Evers D, Lefèvre I, Legay S, Lamoureux D, Hausman J-F, Rosales RO, Marca LR, Hoffmann L, Bonierbale M, Schafleitner R (2010) Identification of drought-responsive compounds in potato through a combined

transcriptomic and targeted metabolite approach. J Exp Bot **61:** 2327–2343

Fujii H, Verslues PE, Zhu J-K (2011) Arabidopsis decuple mutant reveals the importance of SnRK2 kinases in osmotic stress responses in vivo. Proc Natl Acad Sci USA **108:** 1717–1722

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

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

Hare PD, Cress WA (1997) Metabolic implications of stress-induced proline accumulation in plants. Plant Growth Regul **21:** 79–102

Hare PD, Cress WA, Van Staden J (1998) Dissecting the roles of osmolyte accumulation during stress. Plant Cell Environ **21:** 535–553

Hayashi M, Takahashi H, Tamura K, Huang JR, Yu LH, Kawai-Yamada M, Tezuka T, Uchimiya H (2005) Enhanced dihydroflavonol-4-reductase activity and NAD homeostasis leading to cell death tolerance in transgenic rice. Proc Natl Acad Sci USA **102:** 7020–7025

Hong ZL, Lakkineni K, Zhang ZM, Verma DPS (2000) Removal of feedback inhibition of delta(1)-pyrroline-5-carboxylate synthetase results in increased proline accumulation and protection of plants from osmotic stress. Plant Physiol **122:** 1129–1136

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

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

Kaplan F, Kopka J, Sung DY, Zhao W, Popp M, Porat R, Guy CL (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 J **50:** 967–981

Kishor P, Hong Z, Miao GH, Hu CAA, Verma DPS (1995) Overexpression of Δ^1-pyrroline-5-carboxylate synthase increases proline production and confers osmotolerance in transgenic plants. Plant Physiol **108:** 1387–1394

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

Lee BR, Jin YL, Avice JC, Cliquet JB, Ourry A, Kim TH (2009) Increased proline loading to phloem and its effects on nitrogen uptake and assimilation in water-stressed white clover (Trifolium repens). New Phytol **182:** 654–663

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

Mattioli RC, Costantino P, Trovato M (2009) Proline accumulation in plants: not only stress. Plant Signal Behav **4:** 1016–1018

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

Mirabella R, Rauwerda H, Struys EA, Jakobs C, Triantaphylidès C, Haring MA, Schuurink RC (2008) The Arabidopsis *her1* mutant implicates GABA in E-2-hexenal responsiveness. Plant J **53:** 197–213

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

Nakagawa T, Suzuki T, Murata S, Nakamura S, Hino T, Maeo K, Tabata R, Kawai T, Tanaka K, Niwa Y, et al (2007) Improved Gateway binary vectors: high-performance vectors for creation of fusion constructs in transgenic analysis of plants. Biosci Biotechnol Biochem **71:** 2095–2100

Nambara E, Kawaide H, Kamiya Y, Naito S (1998) Characterization of an *Arabidopsis thaliana* mutant that has a defect in ABA accumulation: ABA-dependent and ABA-independent accumulation of free amino acids during dehydration. Plant Cell Physiol **39:** 853–858

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

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

Ober ES, Sharp RE (1994) Proline accumulation in maize (*Zea mays* L) primary roots at low water potentials. I. Requirement for increased levels of abscisic acid. Plant Physiol **105:** 981–987

Parida AK, Dagaonkar VS, Phalak MS, Aurangabadkar LP (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 Physiol Plant **30:** 619–627

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

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

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

Saab IN, Sharp RE, Pritchard J, Voetberg GS (1990) Increased endogenous abscisic acid maintains primary root growth and inhibits shoot growth of maize seedlings at low water potentials. Plant Physiol **93:** 1329–1336

Sawahel WA, Hassan AH (2002) Generation of transgenic wheat plants producing high levels of the osmoprotectant proline. Biotechnol Lett **24:** 721–725

Schwartz SH, Léon-Kloosterziel KM, Koornneef M, Zeevaart JA (1997) Biochemical characterization of the *aba2* and *aba3* mutants in *Arabidopsis thaliana*. Plant Physiol **114:** 161–166

Sharma S, Verslues PE (2010) Mechanisms independent of abscisic acid (ABA) or proline feedback have a predominant role in transcriptional regulation of proline metabolism during low water potential and stress recovery. Plant Cell Environ **33:** 1838–1851

Sharp RE, Wu YJ, Voetberg GS, Saab IN, Lenoble ME (1994) Confirmation that abscisic acid accumulation is required for maize primary root elongation at low water potentials. J Exp Bot **45:** 1743–1751

Shin R, Schachtman DP (2004) Hydrogen peroxide mediates plant root cell response to nutrient deprivation. Proc Natl Acad Sci USA **101:** 8827–8832

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

Stines AP, Naylor DJ, Høj PB, van Heeswijck R (1999) Proline accumulation in developing grapevine fruit occurs independently of changes in the levels of Δ1-pyrroline-5-carboxylate synthetase mRNA or protein. Plant Physiol **120:** 923–931

Szabados L, Savouré A (2010) Proline: a multifunctional amino acid. Trends Plant Sci **15:** 89–97

Székely G, Abrahám E, Cséplo A, Rigó G, Zsigmond L, Csiszár J, Ayaydin F, Strizhov N, Jásik J, Schmelzer E, et al (2008) Duplicated *P5CS* genes of Arabidopsis play distinct roles in stress regulation and developmental control of proline biosynthesis. Plant J **53:** 11–28

Takahara K, Kasajima I, Takahashi H, Hashida SN, Itami T, Onodera H, Toki S, Yanagisawa S, Kawai-Yamada M, Uchimiya H (2010) Metabolome and photochemical analysis of rice plants overexpressing Arabidopsis NAD kinase gene. Plant Physiol **152:** 1863–1873

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

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

Ueda A, Yamamoto-Yamane Y, Takabe T (2007) Salt stress enhances proline utilization in the apical region of barley roots. Biochem Biophys Res Commun **355:** 61–66

Vendruscolo ECG, Schuster I, Pileggi M, Scapim CA, Molinari HB, Marur CJ, Vieira LG (2007) Stress-induced synthesis of proline confers tolerance to water deficit in transgenic wheat. J Plant Physiol **164:** 1367–1376

Verslues PE (2010) Quantification of water stress-induced osmotic adjustment and proline accumulation for *Arabidopsis thaliana* molecular ge-

netic studies. *In* R Sunkar, ed, Plant Stress Tolerance: Methods and Protocols. Methods in Molecular Biology, Vol 639. Humana Press, New York, pp 301–316

Verslues PE, Agarwal M, Katiyar-Agarwal S, Zhu JH, Zhu JK (2006) Methods and concepts in quantifying resistance to drought, salt and freezing, abiotic stresses that affect plant water status. Plant J **45:** 523–539

Verslues PE, Bray EA (2006) Role of abscisic acid (ABA) and *Arabidopsis thaliana* ABA-insensitive loci in low water potential-induced ABA and proline accumulation. J Exp Bot **57:** 201–212

Verslues PE, Kim YS, Zhu JK (2007) Altered ABA, proline and hydrogen peroxide in an Arabidopsis glutamate:glyoxylate aminotransferase mutant. Plant Mol Biol **64:** 205–217

Verslues PE, Sharma S (2010) Proline metabolism and its implications for plant-environment interaction. The Arabidopsis Book **8:** e0140, doi/10.1199/tab.0140

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

Voetberg GS, Sharp RE (1991) Growth of the maize primary root at low water potentials. III. Role of increased proline deposition in osmotic adjustment. Plant Physiol **96:** 1125–1130

Wang GD, Pichersky E (2007) Nicotinamidase participates in the salvage pathway of NAD biosynthesis in Arabidopsis. Plant J **49:** 1020–1029

Weigel D, Glazebrook J (2002) Arabidopsis: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Wilson PB, Estavillo GM, Field KJ, Pornsiriwong W, Carroll AJ, Howell KA, Woo NS, Lake JA, Smith SM, Harvey Millar A, et al (2009) The nucleotidase/phosphatase SAL1 is a negative regulator of drought tolerance in Arabidopsis. Plant J **58:** 299–317

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

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

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

Zhang YY, Zhu HY, Zhang Q, Li MY, Yan M, Wang R, Wang LL, Welti R, Zhang WH, Wang XM (2009) Phospholipase Dα1 and phosphatidic acid regulate NADPH oxidase activity and production of reactive oxygen species in ABA-mediated stomatal closure in *Arabidopsis*. Plant Cell **21:** 2357–2377

Zhu BC, Su J, Chan MC, Verma DPS, Fan YL, 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 Sci **139:** 41–48