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Proline Accumulation in Maize (*Zea mays* L.) Primary Roots at Low Water Potentials. II. Metabolic Source of Increased Proline Deposition in the Elongation Zone[1]

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The proline (Pro) concentration increases greatly in the growing region of maize (*Zea mays* L.) primary roots at low water potentials (ψ_w), largely as a result of an increased net rate of Pro deposition. Labeled glutamate (Glu), ornithine (Orn), or Pro was supplied specifically to the root tip of intact seedlings in solution culture at high and low ψ_w to assess the relative importance of Pro synthesis, catabolism, utilization, and transport in root-tip Pro deposition. Labeling with [^{3}H]Glu indicated that Pro synthesis from Glu did not increase substantially at low ψ_w and accounted for only a small fraction of the Pro deposition. Labeling with [^{14}C]Orn showed that Pro synthesis from Orn also could not be a substantial contributor to Pro deposition. Labeling with [^{3}H]Pro indicated that neither Pro catabolism nor utilization in the root tip was decreased at low ψ_w. Pro catabolism occurred at least as rapidly as Pro synthesis from Glu. There was, however, an increase in Pro uptake at low ψ_w, which suggests increased Pro transport. Taken together, the data indicate that increased transport of Pro to the root tip serves as the source of low-ψ_w-induced Pro accumulation. The possible significance of Pro catabolism in sustaining root growth at low ψ_w is also discussed.

Accumulation of Pro is a widespread plant response to environmental stresses, including low ψ_w (Yancey et al., 1982). Because of the high concentrations often observed, Pro has a clear role as an osmoticum. In particular, because of its zwitterionic, highly hydrophilic characteristics, Pro acts as a "compatible solute," i.e. one that can accumulate to high concentrations in the cell cytoplasm without interfering with cellular structure or metabolism (Yancey et al., 1982; Samaras et al., 1995). Other functions of Pro accumulation have also been proposed, including radical detoxification (Smirnoff and Cumbes, 1989) and regulation of cellular redox status by Pro metabolism (Hare and Cress, 1997).

The metabolic source of the Pro accumulated at low ψ_w is unclear. One potential mechanism is an increase in Pro synthesis. Two possible pathways of Pro synthesis, one using Glu and the other using Orn as a precursor, have been shown to exist in plants (Delauney and Verma, 1993). Studies of Pro metabolism have suggested that Pro synthesis from Glu can increase in response to low ψ_w (Boggess et al., 1976; Hanson and Tully, 1979b; Rhodes et al., 1986). More recent studies have focused on the expression of genes encoding Pro-synthesizing enzymes. Transcription of mRNA encoding P5CR and P5CS, which catalyze Pro synthesis from Glu, has been found to be induced by water deficits and salt stress (Delauney and Verma, 1990; Hu et al., 1992; Williamson and Slocum, 1992; Verbruggen et al., 1993; Yoshiba et al., 1995; Strizhov et al., 1997). Increased transcription was not found for Orn δ-aminotransferase, which catalyzes Pro synthesis from Orn (Delauney et al., 1993). The investigation of Pro synthesis from Glu has also been extended to include transgenic plants that overexpress P5CS (Kishor et al., 1995). Although these experiments did produce plants that had greater Pro accumulation, the effect of the enhanced Pro production on resistance to drought or salt stress is controversial (Blum et al., 1996; Sharp et al., 1996; Verma and Hong, 1996).

In addition to increased Pro synthesis, decreased Pro catabolism could also contribute to Pro accumulation at low ψ_w. Labeling studies such as those by Stewart et al. (1977) and Stewart and Boggess (1978) found a suppression of Pro oxidation, and other studies (Kiyosue et al., 1996; Verbruggen et al., 1996) found a decrease in Pro dehydrogenase mRNA accumulation at low ψ_w. Transport of Pro within the plant may also be important, as indicated by high Pro concentrations in the phloem sap of drought-stressed alfalfa (Girousse et al., 1996) and increased transcription of a Pro-specific amino acid transporter in response to water deficit or salt stress (Rentsch et al., 1996). To our knowledge, there have been no studies in which the effects of low ψ_w on the various possible contributors to Pro accumulation have been examined in the same organ under comparable conditions.

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Abbreviations: GABA, γ-aminobutyrate; P5CR, Δ^1-pyrroline-5-carboxylate reductase; P5CS, Δ^1-pyrroline-5-carboxylate synthetase; ψ_w, water potential(s).

Previous work in our laboratory has focused on mechanisms of growth maintenance in the maize primary root at low ψ_w. Although root growth is inhibited at low ψ_w, it is much less inhibited than shoot growth (Sharp et al., 1988). Maintenance of root elongation occurs preferentially toward the root apex (Sharp et al., 1988), in association with dramatic increases in Pro concentration to as much as 120 mmolal at a ψ_w of −1.6 MPa (Voetberg and Sharp, 1991). The accumulation of Pro in the apical region was shown to be largely attributable to an increased net rate of Pro deposition (Voetberg and Sharp, 1991). In contrast, increased deposition was not observed for K^+ and hexoses (Sharp et al., 1990); increases in the concentrations of these solutes occurred primarily in the more basal regions of the elongation zone and could be accounted for by decreased dilution resulting from growth inhibition.

The net Pro deposition rates reported by Voetberg and Sharp (1991) were calculated by combining spatial distributions of elongation rate and Pro content (see "Materials and Methods"). Although this analysis demonstrated unambiguously that more Pro was added to the solute pool in the root elongation zone at low ψ_w, it could not provide information concerning the metabolic processes responsible for the increased Pro deposition. This increase could be caused by increased synthesis of Pro in the elongation zone, increased Pro import from other parts of the seedling, decreased catabolism or utilization of Pro in the elongation zone, or a combination of these factors. In this study, we assessed the relative importance of these factors by applying labeled Pro or the Pro precursors Glu and Orn specifically to the apical region of intact roots growing at high or low ψ_w in solution culture. Rates of label incorporation into Pro and other amino acids were monitored and used to assess rates of Pro synthesis, catabolism, and utilization. The results show that none of these factors was responsible for Pro accumulation; however, Pro uptake did increase at low ψ_w. Taken together, our results indicate that increased transport of Pro to the root tip is the major source of Pro accumulated in the root elongation zone at low ψ_w.

MATERIALS AND METHODS

Seedling Culture and Labeling

Conditions for maize (*Zea mays* L. cv FR27 × FRMo17) seed germination, transplanting to solution culture, low ψ_w imposition, and seedling growth were as described by Verslues et al. (1998). Two ψ_w treatments were used: high ψ_w (−0.02 MPa, no PEG added) and low ψ_w (−1.6 MPa PEG). In both treatments, supplemental oxygenation was provided to increase the solution oxygen partial pressure from 20.4 (ambient) to 43 kPa, at which level tissue oxygen partial pressure within the root elongation zone was shown to be similar to that in roots growing in well-aerated vermiculite (Verslues et al., 1998). Clear plastic root guides were used to prevent damage from the vigorous aeration and to hold the roots in position for spatial growth analysis and labeling. In the low-ψ_w treatment, the solution ψ_w was reduced to −1.6 MPa over approximately 10 h by pumping PEG solution into the solution-culture box. The boxes, each containing 23 seedlings, were essentially the same as those described by Verslues et al. (1998).

After transfer to solution culture, the seedlings were allowed to grow for approximately 32 h in the high-ψ_w treatment and for 52 h in the low-ψ_w treatment. At these times, root elongation rates (measured by monitoring the position of the root apex on the side of the box) and root-tip amino acid concentrations were steady (see "Results"). Average primary root lengths were approximately 150 mm at high ψ_w and 115 mm at low ψ_w; seedlings with primary roots that were substantially shorter or longer than this were removed from the box. Sixteen seedlings were left in the box and were height adjusted so that their primary root apices were all the same distance (±1 mm) from the bottom of the box. Height adjustments were made by gently lifting the caryopsis of each seedling and supporting it using a toothpick inserted into a Plexiglas pegboard. The solution in the box was then drained until only the apical 12 to 15 mm of each primary root remained submerged. The aeration rate was reduced to 550 mL min^{-1} to avoid excessive splashing of the solution onto more basal parts of the roots, and the seedlings were allowed to acclimate to these conditions for approximately 15 min.

To label the root apical region, 20 μCi of ^{3}H-labeled Glu or Pro or 9 μCi of ^{14}C-labeled Orn was added to a 25-mL aliquot of solution removed from the root box (L-[2,3,4-^{3}H]Glu, 60 Ci $mmol^{-1}$; L-[3,4-^{3}H]Pro, 40 Ci $mmol^{-1}$; and L-[1-^{14}C]Orn, 50 mCi $mmol^{-1}$; American Radiolabeled Chemicals, St. Louis, MO; a specific activity of 40 Ci $mmol^{-1}$ is equivalent to 8.8×10^7 dpm $nmol^{-1}$). This resulted in the following amino acid concentrations in the labeling solution: Glu, 1.3×10^{-5} mM; Pro, 2.0×10^{-5} mM; and Orn, 7.2×10^{-3} mM. The solution containing labeled amino acid was put into a second root box (the "labeling box"), and the Plexiglas holder containing the height-adjusted seedlings was then transferred to the labeling box, which was configured so that the 25 mL of labeling solution covered the apical 12 to 15 mm of each primary root. Total aeration rate in the labeling box was kept at 550 mL min^{-1}.

Sections of four primary roots were collected 10, 30, 60, and 120 min after transfer of seedlings to the labeling box. At each sampling time, four seedlings were removed from the box and their root tips submerged for 3 min in an ice-cold aliquot of growth medium of the same ψ_w as that in the box. This allowed efflux of labeled amino acids that had entered the root apoplast but had not yet been taken up by root cells. Three minutes was chosen because preliminary experiments showed that there was a rapid efflux of radioactivity from the root for 3 min, which was followed by a slower, steady efflux, presumably from the symplast (data not shown). The seedlings were then removed and blotted dry. After excision of the apical 0.5 mm to remove the majority of the root cap, root sections were collected using a razor-blade holder with the razor blades adjusted to correspond to the positions shown in Figure 1. The sections were placed in preweighed microcentrifuge vials and immediately frozen in liquid nitrogen. Samples were then weighed, freeze dried, and reweighed to obtain the weight of water by difference.

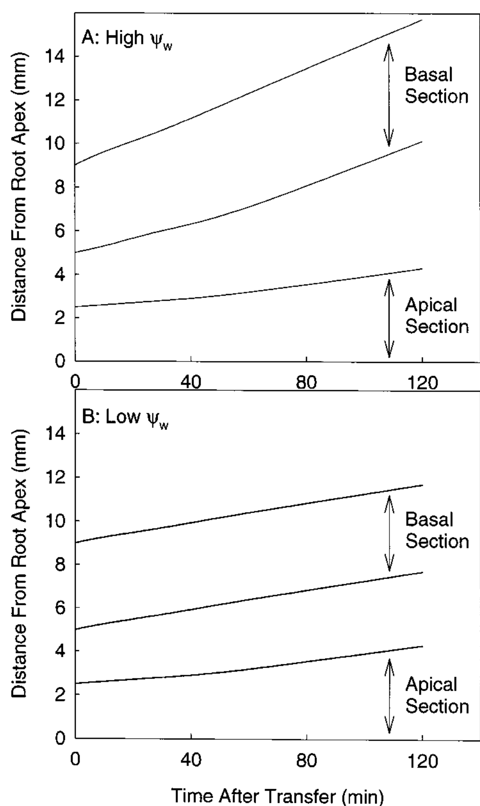

Figure 1. Time courses of location and dimensions of sections harvested in root-tip labeling experiments. Displacement velocities and strain rates (see Fig. 4A) for roots at high (−0.02 MPa) (A) or low (−1.6 MPa) (B) ψ_w were used to calculate the section of the root tip that encompassed the same tissue at each sampling time. At all time points, the apical section was entirely within the region of the root that had high Pro deposition rates at low ψ_w (Fig. 4B) and the basal section was in the region that had negative or zero Pro deposition rates at low ψ_w (see Fig. 4B). Harvesting times were 10, 30, 60, and 120 min after the start of labeling. Displacement immediately behind the root cap (a distance of 0 mm from the root apex in this figure) was negligible during the time frame of these experiments.

Determining a time course of labeling of root sections for these experiments required that expansion and displacement of tissue within the root tip be taken into account. The effect of displacement on labeling experiments was discussed by Silk et al. (1984), who showed that local variation in labeling rates is lost when tissue displacement is not considered. The approach that we used to determine a true time course of tissue labeling was to track the movement of tissue through the root tip. Therefore, we performed labeling only over relatively short periods (2 h) so that tissue displacement would not be so great that the labeling could not be assigned to a specific region. Sections of the apical 4 mm of the primary root, where Pro deposition was high at low ψ_w, were compared with tissue basal to 5 mm, where Pro deposition was negative or zero. The expansion and displacement of these sections was taken into account by sampling different sections of the root tip over time (Fig. 1); the boundaries of the sections were calculated from displacement velocity and longitudinal strain rate profiles (see below). The initial sizes of the apical and basal sections were chosen to provide adequate tissue for analysis while ensuring that the apical section was not displaced into the region of negative Pro deposition during the experiment. This approach allowed Pro metabolism to be compared in regions of high and low Pro deposition. Pro uptake from the labeling solution was measured by removing small aliquots at the same times that root tip samples were removed. ^{3}H activity in these aliquots was determined by scintillation counting and used to calculate the rate of ^{3}H uptake per root tip.

The results of the Glu-, Orn-, and Pro-labeling experiments are presented as means of two replicates except as noted otherwise. Calculations in "Results" are based on these means, but in all cases the same calculations for the two experiments individually led to the same conclusions, demonstrating the reproducibility of the results.

Before actual labeling experiments were conducted, it was necessary to ensure that the necessary manipulations did not inhibit the root elongation rate. Seedlings were transferred to the labeling box as described above, and root elongation was measured at 30-min intervals using a razor blade to mark the position of the root apex (viewed with a magnifying lens) on a clear plastic sheet mounted on the side of the box. The distance between the marks was then measured using the eyepiece reticle of a stereomicroscope. This procedure allowed more accurate quantification of small root length increases than marking the side of the box as was routinely used.

Spatial Growth Analysis and Calculation of Pro Deposition Rate

Spatial growth analysis was performed as described by Silk et al. (1984) and Sharp et al. (1988), with some modifications. Seedlings at high or low ψ_w were grown for 32 or 52 h, respectively, as described above. At these times, most of the primary roots had reached the ends of the root guides through which they were growing; seedlings with shorter roots were removed from the box. The Plexiglas holder and attached root guides containing the seedlings were then removed from the root box so that the tips of the primary roots could be reached through slots cut in the ends of the guides. The apical 15 mm of each root was gently blotted dry and marked at approximately 1-mm intervals with waterproof ink (no. 17 black, Pelikan, Hannover, Germany) using a small paintbrush. The seedlings were then returned to the solution and allowed to recover for 15 min. A series of five photographs of the whole box was then taken at 15- or 30-min intervals. The photographs were scanned, and for each root the mark displacement was measured using image-analysis software (SigmaScan, Jandel Scientific, San Rafael, CA).

Roots that elongated at a rate near (within 0.4 mm h^{-1}) the premarking mean during photography and that had retained a sufficient quantity of ink were selected for growth analysis. Mark position over time was used to calculate displacement velocities; these values were then interpolated to 0.5-mm intervals using cubic splines and differentiated to yield the spatial distribution of the longi-

tudinal strain rate (Sharp et al., 1988). In similar experiments, the apical 12 mm of 15 to 20 roots was harvested into 1-mm sections (collected by position) and used to determine the profile of Pro content via a ninhydrin-based assay (Bates et al., 1973). The spatial growth and Pro-content data were combined to calculate profiles of net Pro deposition rate using the continuity equation, as described previously (Silk et al., 1984; Sharp et al., 1990).

The boundaries of the root sections harvested in labeling experiments were determined by fitting a polynomial equation to the high- and low-ψ_w displacement velocity profiles. These equations were then put into a computer program that calculated the displacement of a specific point on the root surface over time. From these data, the positions of the apical and basal boundaries of the section at each sampling time were determined.

HPLC Analysis and Quantification of Amino Acid Labeling

HPLC separation of amino acids was performed as described previously (Verslues et al., 1998). Derivatized amino acids were separated by reversed-phase HPLC (modified from Yang and Sepulveda, 1985; Ebert, 1986). HPLC results were corrected for recovery (always within the range of 60%–80%) of the internal standard α-aminoadipic acid. The amino acids Pro, Glu, Asp, Asn, Ser, Gln, Arg, GABA, Thr, and Ala were routinely quantified using this method. However, the method was unable to quantify Orn because of its low concentration in the root apical region (Voetberg and Sharp, 1991) and coelution with other, unidentified peaks. In labeling experiments, individual amino acid peaks were collected in 7-mL scintillation vials by a computer-controlled fraction collector (Cygnet, Isco, Lincoln, NE). The ^{3}H or ^{14}C activity of each collected peak was determined by scintillation counting for 10 min (LS-6000-IC, Beckman) using Opti-Fluor scintillation fluid (Packard Instruments, Meriden, CT); all samples were well above the limit of detection. Counting results were corrected for background ^{3}H or ^{14}C activity.

For samples in which labeled Pro was applied to the root apical region, incorporation of Pro into the aqueous insoluble fraction was measured by drying the residual organic phase and hydrolyzing the residue in 6 M HCl at 110°C for 12 to 15 h. HPLC fractions were collected and ^{3}H activity was quantified as described above.

RESULTS

Root Growth and Amino Acid Concentrations

After the seedlings were transferred to solution culture at high ψ_w, the root elongation rate accelerated and reached a steady value at approximately 30 h (Fig. 2). In the low-ψ_w (−1.6 MPa PEG) treatment, the root elongation rate first decreased and then recovered, reaching a near-steady value at approximately 35 h. These results are similar to those obtained in similar experiments by Verslues et al. (1998). Thirty-two hours (high ψ_w) and 52 h (low ψ_w) were chosen as the appropriate times for analysis of Pro deposition and for labeling experiments because of the steady

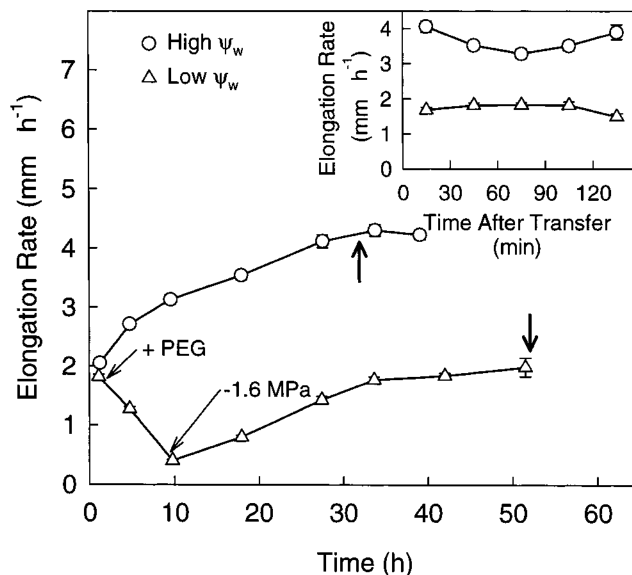

Figure 2. Time course of root elongation rate at high (−0.02 MPa) or low (−1.6 MPa) ψ_w. Arrows show the time at which the addition of PEG was started and the time at which solution ψ_w reached −1.6 MPa in the low-ψ_w treatment; solution ψ_w was constant at later times. Boldface arrows indicate the times after transfer when ^{3}H or ^{14}C labeling and quantification of the spatial patterns of Pro concentration and growth within the root tip were performed. Root elongation rates are means ± SE (n = 50–100) from three to five experiments. Inset, Root elongation rate measured in 30-min intervals after seedlings were transferred to the labeling box. Data are means ± SE (n = 25–55) from four experiments.

root elongation rates and root-tip amino acid concentrations (see below). Because root-tip labeling involved transfer of the seedlings to another box and was done over a short time interval, it was also necessary to examine the root elongation rate immediately after transfer to the labeling box to ensure that substantial fluctuations did not occur. Figure 2, inset, shows that root elongation was stable in the 2 h after transfer of seedlings at −1.6 MPa. In the high-ψ_w treatment, there was a small decrease in the root elongation rate during the initial 90 min after transfer, followed by recovery to the normal rate. These root elongation rates were judged to be sufficiently stable for the labeling experiments.

It was also necessary to measure time courses of root-tip amino acid concentrations in the high- and low-ψ_w treatments before and after transfer to the labeling box to ensure that interpretation of labeling was not complicated by increases or decreases in concentration over time. Time courses of root-tip (apical 10 mm) concentrations of Pro, Glu, Gln, Arg, GABA, Asp, Asn, Ala, Ser, Thr, and Gly were measured. The results for Glu, Arg, and Pro are shown in Figure 3 because these amino acids were the focus of much of the labeling studies and because the response of Glu and Arg concentrations to low ψ_w was similar to that of the other amino acids measured.

With the exception of Pro, all of the amino acids examined exhibited a rapid increase in concentration after the addition of PEG, which was followed by a decline either to

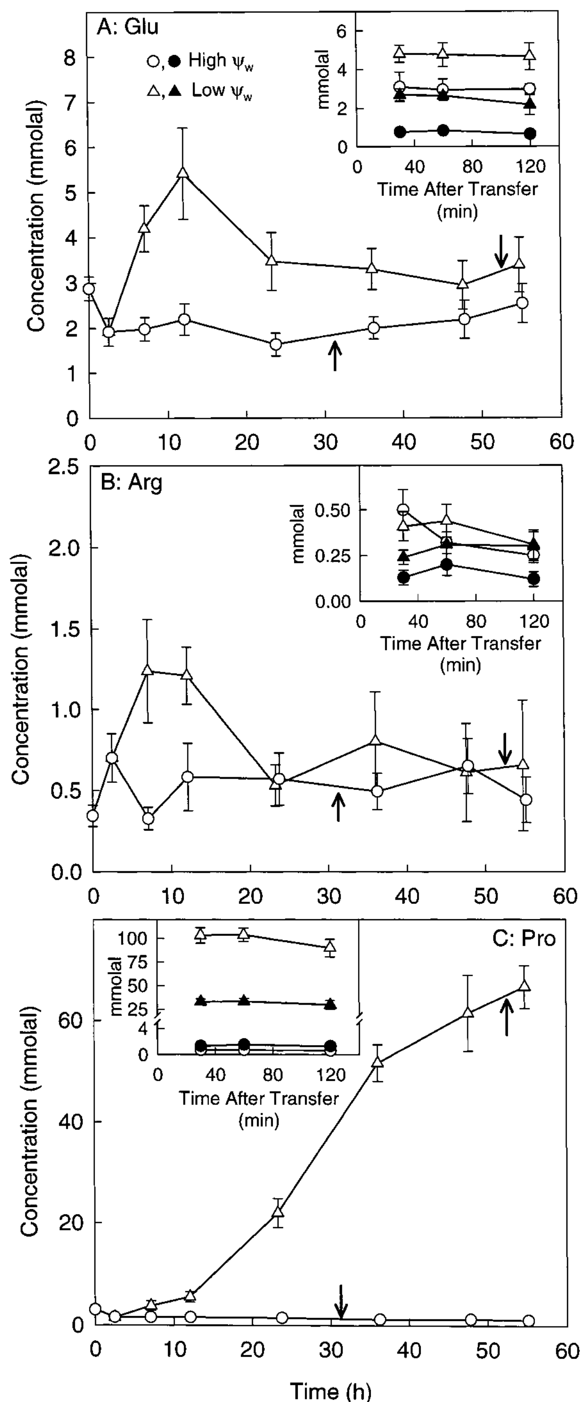

Figure 3. Time courses of Glu (A), Arg (B), and Pro (C) concentrations in the root tip. Data are means ± SE of three to eight samples from different experiments. Each sample contained the apical 10 mm of two roots. The inset in each panel shows the time course of amino acid concentration after transfer to the labeling box. In the insets, open symbols represent the apical section and closed symbols represent the basal section (harvested as described for Fig. 1). Data in the insets are means ± SE of six samples, each from a separate experiment. Time courses of the amino acids Asp, Asn, Ser, Gln, Arg, GABA, Thr, and Ala were also analyzed and exhibited responses to low ψ_w similar to those of Glu and Arg.

the prestress value (Arg [Fig. 3B]) or to a concentration greater than the prestress level (Glu [Fig. 3A]). It is likely that the initial increase in concentration was attributable at least in part to continued amino acid deposition while growth (and hence water deposition) was reduced (Fig. 2), causing the solutes to "pile up" in the elongation zone. The insets in Figure 3, A and B, show that in both the apical and basal sections Arg and Glu concentrations (as well as those of the other amino acids measured) were stable after height adjustment, solution draining, and transfer to the labeling box. Transfer was performed at the times indicated by the arrows in the main figure.

The behavior of Pro in response to decreasing solution ψ_w differed strikingly from that of the other amino acids. Pro concentration increased immediately after low-ψ_w imposition (note the different scales in Fig. 3, A–C), but unlike the other amino acids, the most rapid rate of Pro accumulation did not occur until 15 to 35 h after the start of PEG addition (Fig. 3C). During this time, solution ψ_w was steady at −1.6 MPa (Fig. 2) and concentrations of the other amino acids had either stabilized or begun to decline. The time course of the Pro response was very similar to that reported for seedlings transplanted to vermiculite at −1.6 MPa (Ober and Sharp, 1994). The late increase in Pro concentration showed that Pro did not accumulate merely because root growth slowed; indeed, the root elongation rate was increasing during the period when Pro concentration increased most rapidly (compare Figs. 2 and 3C). The lag before the most rapid phase of Pro accumulation suggests either that induction of metabolic or transport components is necessary or that Pro is catabolized rapidly during the period of initial adaptation to low ψ_w. Figure 3C, inset, shows that the Pro concentration was stable after transfer to the labeling box.

Pro Deposition

Vermiculite was the medium used in previous studies in which Pro deposition in the root elongation zone increased at low ψ_w (Voetberg and Sharp, 1991; Ober and Sharp, 1994). Therefore, for the present study it was first necessary to show that similar results could be obtained in solution culture. To calculate Pro deposition rates, the spatial distributions of the longitudinal strain rate (relative elongation rate) and Pro content were measured at the same times after transfer to solution culture as the labeling experiments. Figure 4A shows that the length of the elongation zone was reduced at low ψ_w to approximately 6 mm compared with 11 mm at high ψ_w. The maximum strain rate was also reduced at low compared with high ψ_w; however, the strain rate in the apical 3 mm was not affected. These results are very similar to those obtained with vermiculite-grown roots at the same ψ_w (Sharp et al., 1988). Profiles of Pro content (not shown) and concentration (Fig. 4B, inset) were also similar to those obtained in vermiculite-grown roots (Voetberg and Sharp, 1991), except that in the −1.6 MPa treatment Pro concentrations were even higher in the apical few millimeters but lower in the 8- to 12-mm region.

The strain rate and Pro profiles were used to compute the pattern of the net Pro deposition rate (Fig. 4B). At high ψ_w, a low positive rate of Pro deposition occurred over the

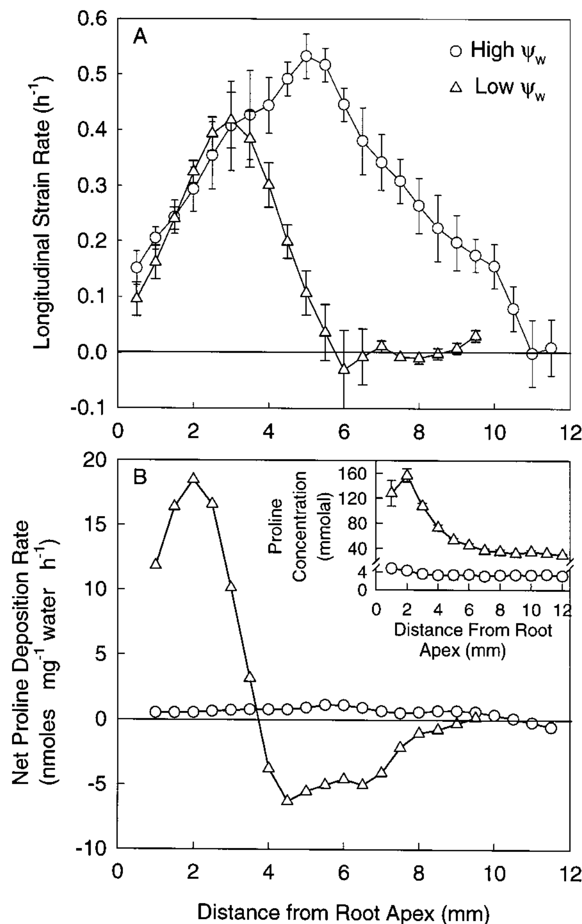

Figure 4. A, Longitudinal strain rate profiles in the apical 12 mm of roots at high (−0.02 MPa) or low (−1.6 MPa) ψ_w. Data are means ± SE (n = 6 or 7) from two experiments. B, Spatial distribution of net Pro deposition rate in the apical 12 mm of roots at high or low ψ_w. Inset, Spatial distribution of Pro concentration used in calculating Pro deposition rates. Data are means ± SE (n = 3–4) from three or four experiments.

entire elongation zone. At low ψ_w, Pro deposition was greatly increased in the apical 3 mm and then declined steeply, such that deposition rates were negative from 4 to 9 mm. A negative deposition rate indicates a net loss of Pro from that section of the root by catabolism, by utilization in the synthesis of protein or other compounds, or by export. These results are similar to those obtained in vermiculite-grown roots (Voetberg and Sharp, 1991), except that in the latter, deposition rates in the basal region were close to zero rather than negative.

By integrating the Pro deposition rate over distance from the apex, it could be calculated that the total Pro deposition rate for the apical 9 mm of the root was 19 nmol h^{-1} at low ψ_w, compared with 5.1 nmol h^{-1} at high ψ_w. In the apical 4 mm, the Pro deposition rate was 36.4 nmol h^{-1} at low ψ_w, compared with 1.9 nmol h^{-1} at high ψ_w. For the 5- to 9-mm region, the Pro deposition rate was −14.3 nmol h^{-1} at low ψ_w, compared with 2.8 nmol h^{-1} at high ψ_w. These deposition rates represent the minimum rates (because Pro-consuming processes in the root tip, such as Pro catabolism and utilization in protein synthesis, are not accounted for in the calculation) of Pro synthesis or import needed to maintain the root-tip Pro concentration over time. Thus, these deposition rates are useful in interpreting the results of the root-tip-labeling experiments (see below).

Pro Synthesis from Glu

Pro synthesis from Glu has been proposed to be the major source of Pro accumulated under drought or salinity stress (Delauney and Verma, 1993). We examined this possibility in our system by applying [^{3}H]Glu to the apical region of roots growing at high or low ψ_w. Figure 5 shows Glu and Pro content per root section (A and D), labeling of Glu and Pro (B and E), and specific activity of Glu and Pro (C and F). Amino acid content per root section changed over time because of the expansion of the root section and displacement of the tissue through the gradient of amino acid concentration in the elongation zone. These changes in amino acid content make it essential to analyze specific activity when interpreting the labeling data.

Glu specific activity was greater at low ψ_w than at high ψ_w in both the apical and basal regions of the elongation zone (Fig. 5C). Despite this, the specific activity of Pro at low ψ_w was less than that at high ψ_w (Fig. 5F). Based on the simplifying assumptions that in the apical root section at low ψ_w the only fate of Pro is accumulation as free Pro (i.e. Pro does not turn over and is not catabolized) and synthesis from Glu is the source of all of the accumulated Pro (i.e. the rate of Pro synthesis from Glu equals the Pro deposition rate), the predicted specific activity of Pro can be conservatively estimated. This was done by taking the Pro deposition rate (36.4 nmol h^{-1}, the total deposition rate for the apical 4 mm, as calculated above) and multiplying it by the Glu specific activity (130 dpm nmol^{-1}, the specific activity observed in the apical section at the 30-min time point; Fig. 5C) to calculate the amount of label predicted to be converted to Pro. This result was then divided by the Pro content of the root section (111 nmol [Fig. 5D]) to obtain the predicted Pro specific activity. The predicted Pro specific activity at the 2-h time point for the apical section at low ψ_w would be approximately 67 dpm nmol^{-1} using this method. This is already much higher than the observed Pro specific activity of 3.9 dpm nmol^{-1} after 2 h of labeling (Fig. 5F). Pro turnover and catabolism (which, based on labeling with [^{3}H]Pro, is known to occur at both high and low ψ_w; see below) could further increase the predicted specific activity by requiring the use of a Pro synthesis rate higher than the net Pro deposition rate. Thus, synthesis from Glu could not have been the major source of Pro accumulated in the apical section at low ψ_w.

In addition, if Pro synthesis from Glu in the apical section was responsible for the high Pro deposition at low ψ_w, it would be expected that Pro specific activity would be much less in the basal section, where the Pro deposition rate was negative or zero (thus having an expected labeling of zero using our simplifying assumptions). This was not the case, and in fact, Pro specific activity was slightly higher in the basal section, reaching 7.7 dpm nmol^{-1} after 2 h of labeling (Fig. 5F). Labeling of Pro in the basal section,

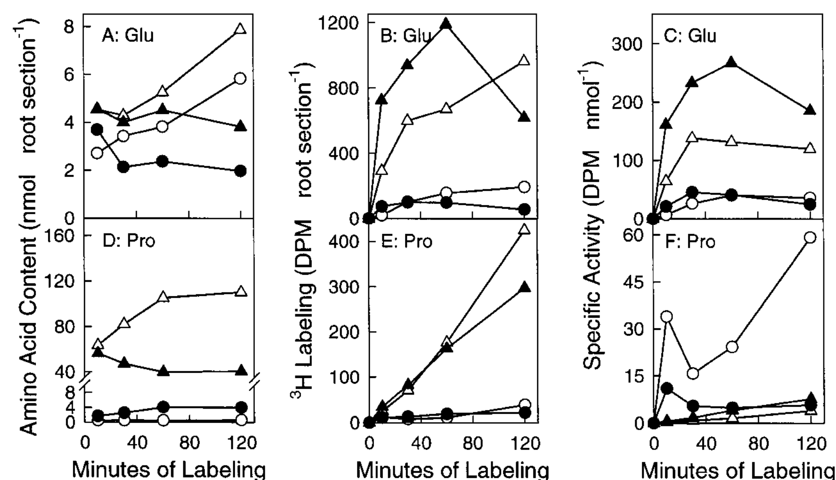

Figure 5. Labeling of Glu (upper panels) and Pro (lower panels) by ^{3}H supplied as Glu in apical and basal sections of the root tip at high (−0.02 MPa) or low (−1.6 MPa) ψ_w. Peaks corresponding to individual amino acids were collected after HPLC separation, and ^{3}H activity was quantified. A and D show Glu and Pro contents; B and E show total ^{3}H activity; and C and F show specific activity. Each data point is the mean of two samples from two separate experiments, and each sample contained four sections. Apical and basal sections were harvested as shown in Figure 1. ○, High-ψ_w apical section; ●, high-ψ_w basal section; △, low-ψ_w apical section; and ▲, low-ψ_w basal section.

despite negative Pro deposition, could be explained by Pro turnover or by the synthesis and export of Pro. However, the negative rate of Pro deposition in the basal section (−14.3 nmol h^{-1}) was insufficient to account for the increased Pro deposition in the apical section (36.4 nmol h^{-1}) without additional Pro synthesis. If significant quantities of Pro had been synthesized in the basal section and then transported to the apical section, this would have resulted in high Pro specific activities in both the apical and basal sections. The similarity of labeling in the two regions suggests that there is a basal rate of Pro synthesis that is unrelated to treatment differences in Pro deposition.

At high ψ_w, the same calculation of predicted Pro labeling in the apical section yields a somewhat different result. Using the Pro deposition rate in the apical 4 mm of 1.9 nmol h^{-1}, as calculated above, and the Glu specific activity at 30 min of 40 dpm nmol^{-1} (Fig. 5C), the predicted Pro specific activity at the 2-h time point would be approximately 115 dpm nmol^{-1}. Compared with the actual Pro specific activity after 2 h of labeling (approximately 60 dpm nmol^{-1}), this calculation indicates that Pro synthesis from Glu can account for a substantial fraction (as much as half) of the Pro deposited. For the basal (5- to 9-mm) section, Pro deposition was 2.8 nmol h^{-1} and the predicted Pro specific activity would be 47 dpm nmol^{-1} at the 2-h time point. However, the actual Pro specific activity remained low (5.7 dpm nmol^{-1} at the end of the experiment), indicating that synthesis from Glu accounts for a smaller fraction of Pro deposition in the basal section than in the apical section. As was the case at low ψ_w, the predicted Pro specific activities are probably underestimates because Pro turnover and catabolism are not accounted for.

It should be noted that the much higher ^{3}H labeling in Pro in the low-ψ_w treatment (Fig. 5E) was not indicative of increased Pro synthesis, because the specific activity of Pro was much lower than at high ψ_w (Fig. 5F). The higher ^{3}H activity can be accounted for by labeled Pro being "trapped": The presence of a large pool of Pro makes it unlikely that labeled Pro that enters this pool will be catabolized. Thus, total ^{3}H activity can accumulate faster in the low-ψ_w treatment with little or no increase in the rate of Pro synthesis.

It is also informative to look at the labeling of other amino acids metabolically related to Glu (Table I). Both Gln and GABA are synthesized from Glu by a single enzymatic reaction that does not remove any of the ^{3}H label. Arg is synthesized from Glu by a series of steps that also leave the ^{3}H-labeled carbon backbone of Glu intact. All three of these amino acids were labeled to a much higher specific activity than Pro at low ψ_w. This indicates that the relatively low Pro labeling at low ψ_w was not caused by the labeled Glu being unable to participate in biosynthetic reactions. Also,

Table I. *Labeling of amino acids by ^{3}H supplied as Glu in apical and basal sections of the root tip at high (−0.02 MPa) or low (−1.6 MPa) ψ_w.*

Apical and basal sections were harvested after 120 min of labeling, as shown in Figure 1. Data are means of two samples from two experiments. RS, Root section.

Amino Acid	High ψ_w				Low ψ_w			
	Apical		Basal		Apical		Basal	
	dpm RS^{-1}	*dpm nmol^{-1}*	*dpm RS^{-1}*	*dpm nmol^{-1}*	*dpm RS^{-1}*	*dpm nmol^{-1}*	*dpm RS^{-1}*	*dpm nmol^{-1}*
Asp	47	16	33	32	118	30	90	46
Asn	19	12	13	3	23	4	22	2
Ser	8	7	13	6	25	10	17	5
Gln	174	12	87	4	1830	70	847	48
Arg	27	57	30	116	162	274	104	398
GABA	72	118	53	113	250	58	44	108
Thr	16	17	24	10	56	29	30	10
Ala	8	2	9	1	29	4	9	1

if the total ^{3}H activity in Gln, Arg, and GABA is summed, it is greater than the total ^{3}H activity in Pro at either high or low ψ_w and in either the apical or basal section. Although the labeling of Gln, Arg, and GABA is also influenced by catabolism and compartmentation that we cannot account for, the relatively heavy labeling of these amino acids suggests that, in all of our samples, synthesis of Pro is not the major metabolic fate of Glu.

Pro Synthesis from Orn

Orn can also serve as a precursor of Pro via the action of Orn δ-aminotransferase (Delauney and Verma, 1993). Some studies have suggested that the Orn pathway of Pro synthesis is of minor importance in water-stressed plants (Delauney et al., 1993), although studies of cotyledons have indicated that Orn can be a major precursor of Pro in certain tissues (Hervieu et al., 1995). When we applied [^{14}C]Orn to the root apical region, the pattern of Pro labeling (Fig. 6, D–F) was qualitatively similar to that obtained by labeling with Glu (Fig. 5, D–F) in both the apical and basal sections. Total ^{14}C labeling in Pro was higher at low than at high ψ_w (Fig. 6E), and yet the specific activity remained low in the low-ψ_w treatment (Fig. 6F). Because the HPLC analysis used here was unable to quantify Orn, it was not possible to measure the specific activity of Orn directly. Instead, the labeling of Arg is presented (Fig. 6, A–C). Arg is synthesized from Orn through the urea cycle. ^{14}C incorporation into Arg was greater than that into Pro (Fig. 6, B and E), despite the much lower Arg content of the root sections (Fig. 6, A and D). This observation provides an initial indication that conversion to Pro may not be a major metabolic fate of Orn in the maize primary root tip at either high or low ψ_w.

If we make the assumption that the specific activity of Arg is equivalent to that of Orn, the predicted specific activity of Pro can be calculated in the same manner as described above for Glu labeling. In the apical section at low ψ_w, the predicted specific activity of Pro after 2 h of labeling would be approximately 318 dpm nmol^{-1}. The actual specific activity was 12.4 dpm nmol^{-1}. In the apical section at high ψ_w, the predicted specific activity of Pro would be 1544 dpm nmol^{-1}, whereas the actual specific activity was 347 dpm nmol^{-1}. Thus, synthesis from Orn cannot account for more than a small fraction of Pro deposition in the apical section. Similar results were obtained for the basal sections. It should be emphasized that the assumption that the specific activity of Orn is similar to that of Arg is highly conservative. In reality, the specific activity of Orn should be considerably higher than that of Arg because of the small pool size of Orn (Voetberg and Sharp, 1991) and the time needed to synthesize Arg from Orn. Thus, the predicted specific activities of Pro were almost certainly lower than they would have been if they had been calculated directly from the specific activity of Orn. As was the case for Glu labeling, if Pro turnover were taken into account, the predicted specific activity of Pro would be even higher. It should be noted that at low ψ_w Pro labeling was similar in the apical and basal sections. As detailed above for Glu labeling, this is not what would be expected if synthesis from Orn were a major source of the increase in Pro deposition in the apical section.

Because of the different specific activities of the [^{3}H]Glu and [^{14}C]Orn and the different pool sizes of Glu and Orn in the root tip, it is not valid to directly compare the labeling or specific activity in Figure 5, E and F, and Figure 6, E and F, and draw any conclusions about whether Pro was synthesized more rapidly from Glu or Orn. Nonetheless, the relatively low rate of Pro synthesis from either Glu or Orn shows that Pro synthesis could not have accounted for more than a small fraction of the Pro deposition in the root elongation zone at low ψ_w. This leaves decreased Pro catabolism and/or utilization and increased Pro transport to the root tip as the possible sources of Pro accumulation.

Pro Catabolism, Utilization, and Uptake

To assess Pro catabolism, [^{3}H]Pro was applied to root tips and the appearance of ^{3}H in Glu and other amino acids was monitored. After 30 min of labeling, the specific activity of Pro in the apical section was 11-fold higher at high ψ_w than at low ψ_w (Fig. 7F). However, the specific activity of

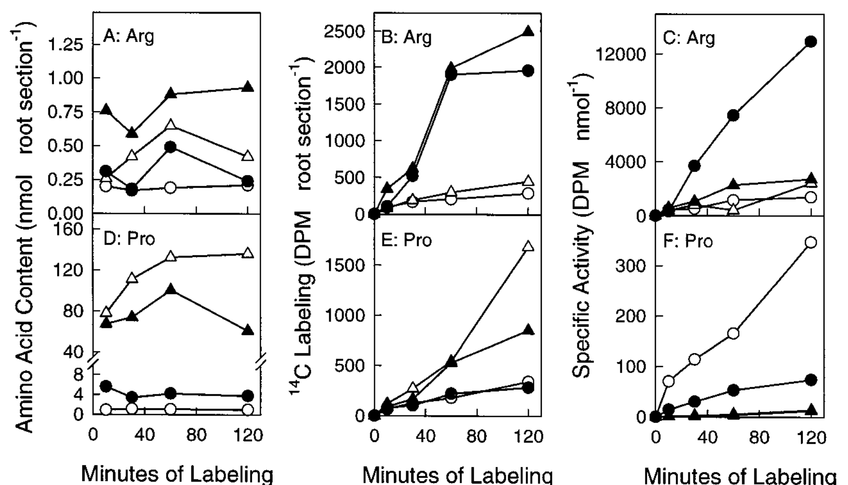

Figure 6. Labeling of Arg (upper panels) and Pro (lower panels) by ^{14}C supplied as Orn in apical and basal sections of the root tip at high (−0.02 MPa) or low (−1.6 MPa) ψ_w. Sampling and data presentation are as described for Figure 5. In F, the plot of the low-ψ_w apical section is obscured by the low-ψ_w basal section data. ○, High-ψ_w apical section; ●, high-ψ_w basal section; △, low-ψ_w apical section; ▲, low-ψ_w basal section.

Glu was only 3.4-fold higher at high ψ_w (Fig. 7C). If Pro catabolism to Glu had been inhibited at low ψ_w, it would be expected that the difference in Glu specific activity between high and low ψ_w would be greater than the difference in Pro specific activity. This would occur because the lower flux from Pro to Glu at low ψ_w would limit the amount of ^{3}H being converted from Pro to Glu. Thus, the results indicate that Pro catabolism was not decreased in the apical section at low ψ_w and may even have been increased. Conversely, in the basal section, Pro specific activity at 30 min was 2-fold higher at high than at low ψ_w but Glu specific activity was 14.2-fold higher at high ψ_w, indicating that a suppression of Pro catabolism occurred at low ψ_w. The labeling of Pro at high ψ_w (Fig. 7E) decreased slightly at later times during the experiments, complicating interpretation. Because of the low Pro content (Fig. 7D) in this treatment, the decrease in labeling had a relatively large effect on Pro specific activity (Fig. 7F). Caution must be used in interpreting these results quantitatively because of the high turnover rate of Glu, indicated by extensive labeling of other amino acids (see below). This high turnover rate of Glu and the fact that we do not know the Glu deposition rate made it impractical to perform the calculations of predicted specific activity that were described for Glu and Orn labeling.

The labeling of amino acids other than Glu and Pro lends additional support to a relatively high rate of Pro catabolism in the root apex. In both the high- and low-ψ_w treatments, all of the other amino acids analyzed, with the exception of Gln, were labeled more heavily in the experiments in which ^{3}H was supplied as Pro (Table II) than when ^{3}H was supplied directly as Glu (Table I), despite a lower Glu specific activity in the Pro-labeling experiments (compare Figs. 5C and 7C). These other amino acids also had greater labeling than was found in Pro after labeling with Glu (compare Table II and Fig. 5E). This shows that the rate of flux in the direction of Pro to Glu exceeded the rate from Glu to Pro in both the apical and basal sections, suggesting that, instead of being a relatively inert solute or storage form of carbon and reductant, Pro is actively catabolized at both high and low ψ_w.

The differential labeling between ^{3}H applied as Glu and Pro may also show the effect of metabolic compartmentation. Several studies have shown that Pro catabolism occurs in the mitochondria (Stewart and Lai, 1974; Elthon et al., 1984), whereas Pro synthesis is cytoplasmic (Szoke et al., 1992). Therefore, the Glu produced from Pro catabolism is produced inside the mitochondria, where it can be rapidly deaminated for entry into the TCA cycle. This would explain the heavier labeling of amino acids such as Ala and Asp when ^{3}H is supplied as Pro instead of Glu, and it may also explain the high specific activity of some amino acids such as Arg (Tables I and II). It is also consistent with Pro catabolism serving as a source of energy and reductant in the root tip (see "Discussion").

Hydrolysis of the water-insoluble portion of the root-section extracts showed that decreased Pro utilization in protein synthesis also did not contribute to increased deposition of free Pro at low ψ_w. Pro released by hydrolysis contained more ^{3}H activity at low ψ_w than at high ψ_w in both the apical and basal sections after 2 h of labeling (823 and 1636 dpm per root section in the apical section at high and low ψ_w, respectively, and 303 and 656 dpm per root section in the basal section at high and low ψ_w, respectively).

Increased Pro transport to the root tip is left as the most likely source of the Pro accumulated at low ψ_w. In support of this conclusion, we observed that the rate of loss of ^{3}H activity from the Pro-labeling solution was greater at low than at high ψ_w, indicating that Pro uptake by the root tips increased at low ψ_w. Additional uptake experiments were performed to verify this observation, and the results are shown in Figure 8. The root tips at low ψ_w took up Pro at nearly twice the rate as those at high ψ_w. The high rate of ^{3}H uptake in the first 10 min after [^{3}H]Pro was applied is consistent with an influx of Pro into the root apoplast. The later, sustained higher rate of ^{3}H uptake at low ψ_w suggests an increased rate of Pro import into the root cells. It should be noted that the amount of root tissue exposed to the labeling solution at high and low ψ_w was roughly the same at the beginning of the experiments, but the roots at high ψ_w elongated at a higher rate than the roots at low ψ_w (Fig. 2). Thus, by the end of the experiments, it is likely that

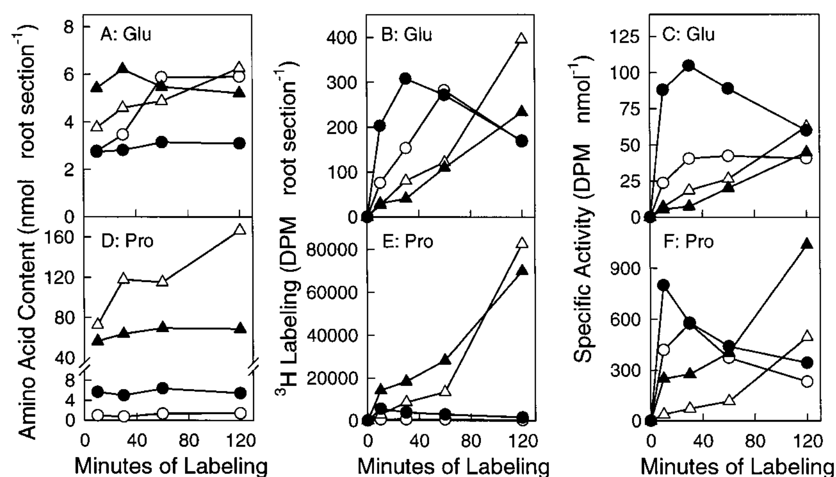

Figure 7. Labeling of Glu (upper panels) and Pro (lower panels) by ^{3}H supplied as Pro in apical and basal sections of the root tip at high (−0.02 MPa) or low (−1.6 MPa) ψ_w. Sampling and data presentation are as described for Figure 5. ○, High-ψ_w apical section; ●, high-ψ_w basal section; △, low-ψ_w apical section; ▲, low-ψ_w basal section.

Table II. *Labeling of amino acids by ^{3}H supplied as Pro in apical and basal sections of the root tip at high (−0.02 MPa) or low (−1.6 MPa) ψ_w.*

Apical and basal sections were harvested after 120 min of labeling, as shown in Figure 1. Data are means of two samples from two experiments. RS, Root section.

Amino Acid	High ψ_w				Low ψ_w			
	Apical		Basal		Apical		Basal	
	dpm RS^{-1}	*dpm nmol^{-1}*	*dpm RS^{-1}*	*dpm nmol^{-1}*	*dpm RS^{-1}*	*dpm nmol^{-1}*	*dpm RS^{-1}*	*dpm nmol^{-1}*
Asp	130	72	136	165	193	79	129	48
Asn	107	157	325	60	260	76	276	16
Ser	43	64	86	68	107	46	94	13
Gln	278	16	397	14	595	22	213	6
Arg	101	412	312	745	1324	5247	819	1942
GABA	320	313	1972	1258	696	138	1314	993
Thr	106	184	571	144	257	144	380	71
Ala	823	302	550	35	1486	174	1420	49

more root tissue was taking up Pro at high than at low ψ_w. Accordingly, the difference between high and low ψ_w in the rate of Pro uptake per unit of root tissue was probably greater than that indicated in Figure 8. Typically, less than 20% of the ^{3}H in solution was taken up by the end of the experiment. Measurements of label disappearance from the solutions in the Glu- and Orn-labeling experiments did not indicate increased uptake of Glu or Orn at low ψ_w (data not shown), suggesting that the increase in Pro uptake was not caused by a general increase in amino acid uptake. However, the increased Pro uptake must be interpreted with caution because it is not known how accurately the uptake of exogenous Pro from the apoplast reflects delivery of endogenous Pro to the root tip.

DISCUSSION

The combined results of this study argue strongly that Pro transported to the root tip is the main source of the dramatic increases in Pro deposition rate and Pro concentration that occur in the maize primary root elongation zone at low ψ_w. The results showed no evidence for a substantial increase in Pro synthesis at low ψ_w. In the apical section, where the highest Pro deposition rates were found, there was also no evidence for a decrease in Pro catabolism or utilization. Preliminary evidence in support of increased Pro transport to the root tip at low ψ_w was seen in the form of increased Pro uptake from solution.

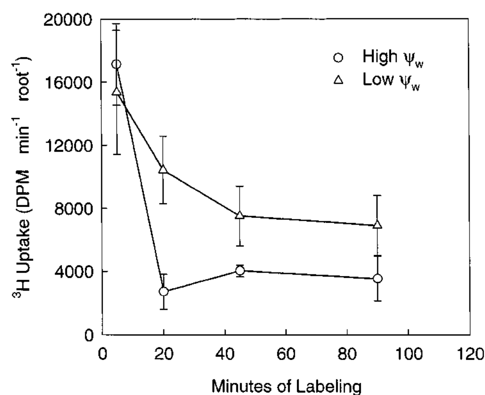

Figure 8. Uptake of [^{3}H]Pro by the root tips at high or low ψ_w measured as the rate of loss of ^{3}H activity from the labeling solution in which the roots were growing. Data are means ± SE of four experiments.

Metabolism and Function of Root-Tip Pro

Several studies of Pro metabolism have shown that Pro synthesis from Glu is increased at low ψ_w in leaves (Boggess et al., 1976; Hanson and Tully, 1979b) and in cell cultures (Rhodes et al., 1986). In addition, expression of both P5CS and P5CR mRNA has been shown to increase in response to salinity or dehydration (Delauney and Verma, 1990; Hu et al., 1992; Williamson and Slocum, 1992; Verbruggen et al., 1993; Yoshiba et al., 1995). A possible explanation for the difference between the conclusion reached in those studies and that reached here is our examination of a very specific region, the primary root elongation zone. Oaks (1966) examined Pro synthesis in excised maize primary root tips at high ψ_w. Consistent with the results presented here, it was found that ^{14}C applied as acetate was incorporated into Pro slowly. It was suggested that Pro synthesis in the root tip was insufficient to meet the requirements for Pro and, therefore, that Pro must be transported to the root tip to make up for this deficit. The increase in Pro deposition at low ψ_w would compound this deficit in Pro synthesis unless a large shift occurred in the metabolic fate of Glu or Orn. Our experiments showed no evidence of such a shift. In support of our conclusion, preliminary experiments in which seedlings at high and low ψ_w were grown in the presence of ^2H_2O and ^{2}H incorporation into root-tip amino acids was analyzed showed that Pro labeling in the root apex was approximately one-half the amount expected if all of the Pro had been synthesized since germination. The unlabeled Pro must have been released from seed storage and transported to the root tip (D. Rhodes, P.E. Verslues, and R.E. Sharp, unpublished data).

Pro catabolism has often been proposed to be suppressed under water or salt stress. Labeling studies such as those by Stewart et al. (1977) and Stewart and Boggess (1978) found a suppression of Pro oxidation, and in other studies (Kiyosue et al., 1996; Verbruggen et al., 1996) a decrease in Pro dehydrogenase mRNA accumulation was reported. Again,

it is important to consider the tissues involved. Stewart et al. (1977) and Stewart and Boggess (1978) used mature leaves, and Kiyosue et al. (1996) and Verbruggen et al. (1996) analyzed either whole Arabidopsis plants or whole root systems. The results presented here show that in the apical root section Pro catabolism was not decreased at low ψ_w, and at both high and low ψ_w, the flux from Pro to Glu exceeded the flux from Glu to Pro. These observations agree with those of Barnard and Oaks (1970), who showed that applied Pro is rapidly catabolized in excised 5-mm-long maize root tips at high ψ_w. They suggested that Pro is required by the root tip as an energy and nitrogen source. It should be noted that a lack of suppression of Pro catabolism relative to high ψ_w does not imply a lack of regulation during water stress. It seems unlikely that Pro could accumulate to such high levels if Pro catabolism were allowed to increase in proportion to Pro concentration. Also, Pro catabolism may be suppressed at low relative to high ψ_w in other tissues that serve as a source of the Pro transported to the root tip.

Our observations also fit well with some of the ideas of Hare and Cress (1997) and Kohl et al. (1988). Hare and Cress (1997) suggested that under water stress Pro from "effector cells" is transported to "target cells," which have a high energy requirement. The effector cells, which export Pro, are proposed to be cells that use the synthesis of Pro from Glu to regenerate $NADP^+$ for processes such as the synthesis of purine nucleotides. The apical growing region of the primary root is likely to be a tissue with a high energy requirement and so would be a good candidate for such a "target tissue." A similar system has been described in soybean nodules by Kohl et al. (1988). Soybean nodules export nitrogen in the form of purine derivatives. The authors proposed that Pro synthesis in the cytoplasm of bacteroid-infected plant cells generates $NADP^+$ used in the synthesis of purines. The Pro in the plant cytoplasm is then transported into the bacteroid, where it is catabolized as an energy source to fuel nitrogen fixation. It is interesting to note that Pro dehydrogenase activity in bacteroids is increased at low ψ_w (Kohl et al., 1991), whereas in other tissues Pro dehydrogenase activity or mRNA expression is suppressed (Sudhakar et al., 1993; Kiyosue et al., 1996; Verbruggen et al., 1996). It has also been demonstrated that bacteroid Pro catabolism is significant in terms of plant performance: Soybean plants inoculated with a *Bradyrhizobium japonicum* strain unable to catabolize Pro had a greater reduction in yield after moderate water stress than plants inoculated with wild-type *B. japonicum* (Straub et al., 1997). This suggests that it would be of interest to specifically modify Pro metabolism in the root tip and measure the resulting effects on root growth and plant performance at low ψ_w.

Pro Transport

Pro transport at low ψ_w has been much less studied than Pro metabolism. Despite the smaller number of studies, there are still seemingly contradictory reports. A series of studies using barley leaves (Hanson and Tully, 1979a; Tully and Hanson, 1979; Tully et al., 1979) found that the Pro content of phloem sap increased only slightly in response to water stress and that Pro could account for only a small fraction of the nitrogen exported from the stressed leaves. In contrast, Girousse et al. (1996) found an increase of up to 60-fold in Pro concentration of phloem sap collected from stems of water-stressed alfalfa plants. Rentsch et al. (1996), working with Arabidopsis, found that Pro transporter mRNA expression could be induced by dehydration, whereas expression of a general amino acid permease was suppressed. In addition, expression of P5CR associated with the vascular tissue in Arabidopsis was shown to increase under water stress (Hare and Cress, 1996; Hua et al., 1997). To our knowledge, no studies of Pro transport have been performed using a seedling system comparable to the system used here. Our characterization of the metabolic mechanisms responsible for Pro deposition in the maize primary root tip makes this an attractive system for further studies of the regulation of Pro transport and deposition. Previous work demonstrated that ABA accumulation is required for the increased Pro deposition at low ψ_w (Ober and Sharp, 1994), suggesting that ABA may play a role in regulating Pro transport to the root tip.

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