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Environmental and developmental signals modulate proline homeostasis: Evidence for a negative transcriptional regulator

(amino acid catabolism/*Arabidopsis thaliana*/glutathione *S*-transferase/osmotic stress/proline oxidase)

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ABSTRACT **In many plants, osmotic stress induces a rapid accumulation of proline through *de novo* synthesis from glutamate. This response is thought to play a pivotal role in osmotic stress tolerance [Kishor, P. B. K., Hong, Z., Miao, G.-H., Hu, C.-A. A. and Verma, D. P. S. (1995) *Plant Physiol.* 108, 1387–1394]. During recovery from osmotic stress, accumulated proline is rapidly oxidized to glutamate and the first step of this process is catalyzed by proline oxidase. We have isolated a full-length cDNA from *Arabidopsis thaliana*, *At-POX*, which maps to a single locus on chromosome 3 and that encodes a predicted polypeptide of 499 amino acids showing significant similarity with proline oxidase sequences from *Drosophila* and *Saccharomyces cerevisiae* (55.5% and 45.1%, respectively). The predicted location of the encoded polypeptide is the inner mitochondrial membrane. RNA gel blot analysis revealed that *At-POX* mRNA levels declined rapidly upon osmotic stress and this decline preceded proline accumulation. On the other hand, *At-POX* mRNA levels rapidly increased during recovery. Free proline, exogenously added to plants, was found to be an effective inducer of *At-POX* expression; indeed, *At-POX* was highly expressed in flowers and mature seeds where the proline level is higher relative to other organs of *Arabidopsis*. Our results indicate that stress- and developmentally derived signals interact to determine proline homeostasis in *Arabidopsis*.**

As much as one-half of the irrigated areas of the world are affected by high salinity (1). Attention has therefore been focused on elucidation of the molecular details of such stress-induced gene expression, particularly the genes involved in proline biosynthesis, with the ultimate goal of engineering plant osmotic stress tolerance (2–6). Under conditions of osmotic stress, many plants rapidly accumulate proline through activation of *de novo* synthesis from glutamate. It is thought that this response is a key component of inducible mechanisms for drought and salt tolerance (6). However, it is also known that during recovery from stress, accumulated proline is rapidly converted to glutamate through two enzymic steps, the first of which is catalyzed by proline oxidase (EC 1.5.99.8) (7, 8).

Most studies of proline oxidase in plants have focused on its role in the regulation of cellular proline pools during and after osmotic stress. It is thought that biosynthesis of proline from glutamate during hyperosmotic stress occurs in the cytosol (2) and that oxidation during recovery occurs at the mitochondrial inner membrane (9). In plants that accumulate proline during osmotic stress, proline oxidase activity has been shown to decrease dramatically under such conditions in *Phaseolus aureus* (10), wheat (11), sweet pepper (12), and tomato (13). Clearly negative cross talk between the anabolic and catabolic pathways must act both in the effective establishment of osmoprotection and subsequently during recovery. During plant development, similar regulation must also occur to prevent a futile cycle.

During recovery, the accumulated proline is rapidly oxidized to glutamate (8). In addition to its role in osmoprotection, proline accumulation in plants may function in the storage of energy, amino nitrogen, and reducing power (8). Such an energy, reducing power, and amino nitrogen store would be of crucial importance in the maintenance of repair processes operative after osmotic stress and in the rapid restoration of cellular homeostasis. Indeed, proline is the primary energy source for sustaining the rapid growth observed during pollen tube elongation (14, 15). In other eukaryotes and in bacteria under nonstress conditions, proline is also used as a source of energy, carbon, or nitrogen. The oxidation of proline to glutamate in *Saccharomyces cerevisiae* permits growth when proline is the sole source of nitrogen (16). In bacteria, proline can be used as a sole carbon, nitrogen, and energy source (17). Proline is also accumulated and rapidly oxidized by the flight muscles of several insect species, where it is thought to be a readily accessible energy source (18). In strong support, the *slga* mutation in a *Drosophila* proline oxidase gene causes sluggish motor activity (19).

In plants, while the molecular characterization of the proline biosynthetic pathway is advanced, nothing is known about proline breakdown. Given the broad range of cellular activities in which proline oxidase functions, we initiated a molecular characterization of proline oxidase gene expression in the genetically amenable model plant species, *Arabidopsis thaliana*. A detailed understanding of the molecular nature of interactions that underlie changes in proline oxidase activity could be of critical importance in the design of strategies for the genetic engineering of osmotolerance. Novel insights into mechanisms that determine energy supply would also be forthcoming.

To these ends, we describe the isolation and characterization of the *At-POX*, a cDNA from *A. thaliana* that encodes proline oxidase. We present expression analysis of *At-POX* during or after osmotic stress, in the presence of high concentrations of exogenous proline, and in different plant organs.

MATERIALS AND METHODS

Plant Material and Growth Conditions. *A. thaliana* (L.) Heynh., ecotypes Columbia and Landsberg *erecta* (provided by M. Anderson, Nottingham Arabidopsis Stock Center, United Kingdom) were grown in the greenhouse (250 microeinsteins; 16 h light/8 h dark, 60% humidity, 22°C) or *in vitro* on K1 medium as described (3). Seeds were suspended in K1 medium containing 0.2% (wt/vol) agarose and pipetted onto nylon

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Data deposition: The sequence reported in this paper has been deposited in the GenBank data base (accession number X97075).

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filters placed on K1 medium solidified with 0.6% (wt/vol) agarose. Incubations were performed by transferring filters to Petri dishes containing liquid K1 medium supplemented with 20% (wt/vol) PEG 6000, or 5 or 10 mM of proline. All manipulations were done under sterile conditions.

cDNA Cloning and Sequence Analysis. An *At-POX* cDNA was isolated using a PCR-based approach. Degenerated primers were designed corresponding to the amino acid sequence motifs LVRGAY and YLLRR conserved between the proline oxidase from *Drosophila melanogaster* (19) and *S. cerevisiae* (20). A 300-bp fragment was amplified and similarity with known proline oxidase genes was confirmed by sequencing. Two cDNA libraries were screened; one from *A. thaliana* ecotype Landsberg *erecta* plants in λgt11 vector (Clontech) and the other from well-watered roots in λzap (21). Nine positive clones from 200,000 λgt11 clones screened with the 300-bp PCR fragment were obtained and analyzed. They showed identical sequences; only partial cDNAs were found. From 200,000 λzap clones screened with a 300-bp fragment isolated from the 5′ end of the longest λgt11-positive clone, only one clone was obtained and sequenced. One hundred percent identity with the λgt11 clones was found.

Primer Extension. The position of the 5′ end of the *At-POX* transcript was mapped by the primer extension method as described (22) using two different 36-bp primers, 119 and 141 bp downstream from the transcription start site.

DNA and Protein Sequence Analysis. Double-stranded plasmid DNA was sequenced on both strands by the dideoxy chain termination method on an automated DNA sequencer using dye primers (Applied Biosystems). Sequence comparison with the databases was performed using the Blast Enhanced Alignment Utility (BEAUTY). Alignment of the deduced At-POX protein sequence with other deduced proline oxidase sequences was done with CLUSTAL W (version 1.5). Analysis of protein motifs and signal peptides was performed using the PSORT program available on the Internet (http://psort.nibb.ac.jp/helpwww.htm1). Analysis of transmembrane domains was done using the TMPRED program on the Internet (http://ulrec3.unil.ch/tmbase/TMPRED_doc.htm1) and the SOAP program of the PC/Gene package (GCG).

DNA Blot Analysis. Genomic DNA was isolated from young leaves of *Arabidopsis* ecotypes Columbia and Landsberg *erecta* as described (4) and DNA blot analysis was carried out using a ^{32}P-labeled *Sph*I–*Eco*RI 1.5-kb fragment of the *At-POX* cDNA clone at 55°C as described (3). Membranes were washed at medium [55°C, 2× SSC (1× SSC = 150 mM NaCl/15 mM sodium citrate, pH 7.0)/0.1% SDS] or high stringency (65°C, 0.1× SSC/0.1% SDS).

RNA Gel Blot Analysis. Total RNA was isolated from 10-day-old seedlings and from the different organs of mature *Arabidopsis* plants as described (3). RNA blot analysis was performed as described (3). Quantification of signal was done with a PhosphorImager 445SI (Molecular Dynamics). Filters were also exposed for 4 days to x-ray films (Kodak). Quantities of RNA loaded were evaluated by hybridization with a 25S RNA probe.

Proline Determination. Free proline content was measured according to the Bates method (23) using proline as standard.

RESULTS

Isolation and Predicted Subcellular Location of *At-POX* cDNA. A 1.86-kb full-length *At-POX* cDNA was isolated from a cDNA library prepared from roots of well-watered *Arabidopsis* seedlings (21) by PCR (see *Materials and Methods*). The 1.86-kb-long *At-POX* cDNA was full length because the 5′ end matched with the transcription start determined by primer extension, 121 bp upstream from the start codon (data not shown), and the 3′ end contained a poly(A) tail. The deduced amino acid sequence of the single open reading frame encoded a putative protein of 499 amino acids with a molecular mass of 55 kDa (Fig. 1). We identified *At-POX* cDNA as encoding proline oxidase on the basis of its deduced amino acid sequence showing similarity with the PUT1 proline oxidase from the yeast *S. cerevisiae* (20), the SLGA proline oxidase of *D. melanogaster* (19), and with the NH_2 terminal part of the PutA proline dehydrogenase from *Escherichia coli* (25) (Table 1). Relatively low identity (32–20%) was found over the length of the four proteins (Table 1). The closest similarity among the four sequences was located at the COOH-terminal part of the deduced proteins in two regions, residues 282–362 and 400–474, where At-POX shared 65% or 62% similarity with SLGA, 52% or 55% with PUT1, 53% or 55% with PutA in the first or second most conserved region, respectively (Fig. 2).

We predicted (see *Discussion*) that the At-POX protein was located in the mitochondrial inner membrane because the NH_2 terminus contained a 17-amino acid mitochondrial signal peptide, with a consensus Gavel (24) cleavage motif YRLPAF (Fig. 1) and a motif for translocation to the mitochondrial inner membrane (predicted with 76% certainty by the PSORT software). A hydropathy plot of the At-POX-deduced amino acid sequence did not show characteristically alternating blocks of hydrophobic and hydrophilic residues, but analysis by the TMPRED, PSORT, and SOAP programs suggested the presence of one 17-residue transmembrane helix at position 190–206, which was long enough to span the membrane (26). Putative potential myristyl anchor sites were predicted at positions 62, 115, 119, 158, 358, 392, 396, 433, 441, and 477.

DNA Gel Blot Analysis. Genomic DNA of Columbia or Landsberg *erecta* plants digested with *Bgl*II, *Dde*I, *Dra*I, *Eco*RI, *Hin*dIII, and *Xba*I was probed with the coding sequence of the *At-POX* cDNA (Fig. 3). Polymorphism was observed between the DNA of the Columbia and Landsberg ecotypes digested with *Bgl*II and *Xba*I (Fig. 3). Hybridization performed under medium stringency suggested the presence of at least one gene related to *At-POX* (Fig. 3*A*). Sixty-three recombinant inbred lines were used to map *At-POX* using the *Bgl*II polymorphism and high-stringency conditions. The *At-POX* gene mapped on

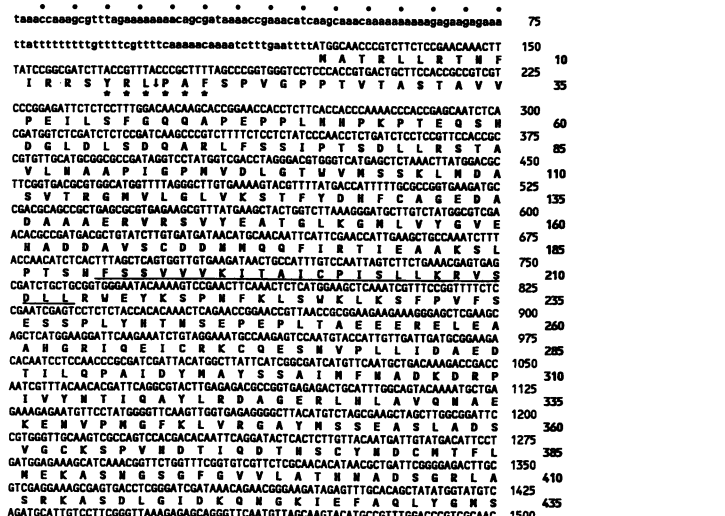

FIG. 1. Nucleotide sequence of *At-POX* cDNA and deduced amino acid sequence with positions indicated at the right and far right, respectively. The consensus Gavel cleavage motif (24) is marked with stars. The putative cleavage site of the mitochondrial transit peptide is indicated by an arrow. The putative transmembrane domain is underlined.

Table 1. Similarity/identity (percent) of deduced amino acid sequence of At-POX with other proline oxidase-deduced sequences

	Dm-SLGA	Sc-PUT1	Ec-PutA*
At-POX	55.7/32.9	45.1/22.4	45.2/23.5
Dm-SLGA		46.4/24.0	44.7/21.1
Sc-PUT1			40.8/20.0

At, *A. thaliana*; Dm, *D. melanogaster*; Ec, *E. coli*; Sc, *S. cerevisiae*. *Only the first 700 amino acids containing the proline oxidase activity (25) were used in the comparison.

chromosome 3 at ≈69.4 centimorgans between the mi413 and mi358 phage clones.

Expression of *At-POX* in Plant Tissues. To study expression of *At-POX in planta*, roots, leaves, flowers, green siliques, and mature seeds were analyzed for *At-POX* steady-state transcript level by RNA gel blot analysis (Fig. 4*A*). A single band corresponding to an mRNA of 1.8-kb was detected. After normalization of the signal using 25S RNA hybridization as control, *At-POX* expression was ≈2.5-fold less than in stems, ≈5.4-fold less than in green siliques, ≈6-fold less than in roots, ≈12-fold less than in mature seeds, and ≈19-fold less than in flowers (Fig. 4*A*). High levels of *At-POX* expression correlated with high free proline levels determined in these organs (Fig. 5*A*).

Expression of *At-POX* During and After Osmotic Stress. Regulation of proline oxidase gene expression during and after osmotic stress was determined by RNA gel blot analysis (Fig. 4*B*). Ten-day-old seedlings were transferred with media supplied with 20% (wt/vol) PEG for 12 h and then transferred to control media for the next 12 h. *At-POX* transcript levels declined sharply, and only 2 h after the addition of PEG were barely detectable. This corresponded to the onset of free proline accumulation. After transferring plants from PEG-containing medium to control medium, *At-POX* was rapidly induced up to 15-fold after 2 h, correlating with a rapid decline in free proline (Fig. 5*B*).

Expression of *At-POX* in Plants Exposed to Exogenous Proline. To study the possible role of proline as a signaling molecule in the activation of proline oxidase gene expression, 10-day-old plants were incubated for 2, 6, 12, and 24 h in the presence of proline (5 or 10 mM). Under these conditions, proline rapidly accumulated (Fig. 5*C*). RNA gel blot analysis was performed with total RNA extracted from these plants (Fig. 4*C*) and it was shown that exogenously added proline induced *At-POX* expression. The level of *At-POX* transcripts was increased up to 15-fold above the control level by 10 mM proline.

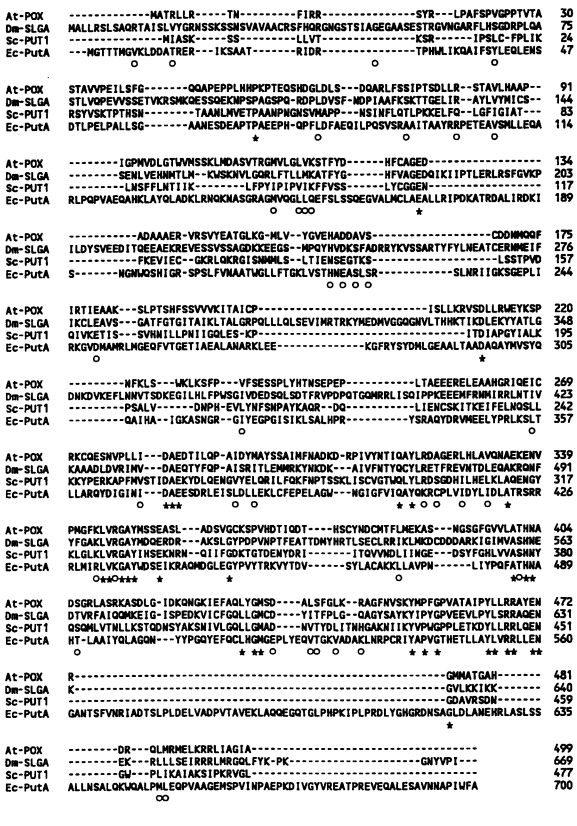

FIG. 2. Comparison of the deduced proline oxidase amino acid sequence of At-POX with that of *D. melanogaster* (Dm), *S. cerevisiae* (Sc), and with the NH_2 terminus of the bifunctional proline dehydrogenase of *E. coli* (Ec). Identical and structurally similar amino acids are indicated with stars and open circles, respectively.

DISCUSSION

We have isolated a full-length cDNA from *Arabidopsis* mapping to a single locus on chromosome 3, designated *At-POX*. The cDNA contained a single open reading frame encoding a predicted polypeptide of 499 amino acids. The At-POX protein shared significant homology only with the known proline oxidase-deduced sequences from *Drosophila* (19), yeast (20), and the NH_2 terminus of the proline dehydrogenase from *E. coli* (25) (Table 1, Fig. 2). Functional analysis of the full-length *At-POX* cDNA through complementation of yeast mutants lacking proline oxidase activity failed, despite the clear identity of this cDNA with proline oxidase sequences (data not shown).

Examination of the NH_2-terminal region of the predicted At-POX protein revealed characteristics of a mitochondrial import sequence (24). The predicted mitochondrial inner membrane location is in agreement with the location of rat liver proline oxidase (27), as well as the proposed location in plants (9). The rat and yeast enzymes appear to be tightly linked to the mitochondrial respiratory chain. Indeed, in the latter, active electron transport is a strict requirement for proline oxidase activity (16). The hydropathy plot of At-POX indicated that it is not an integral membrane protein, although it has a short putative transmembrane helix at position 190–206. Several potential myristyl anchor sites that would bind At-POX to the inner membrane were predicted toward the C terminus.

In many plants, during hyperosmotic stress, proline levels increase markedly (Fig. 5*B*). It has previously been shown that during this adaptive response in *Arabidopsis*, transcriptional activation of the proline biosynthetic pathway is a key factor in determining proline accumulation (3–5). Here, we provide evidence that in addition, under the same conditions, there is a concomitant down-regulation of proline oxidase transcript levels (Fig. 4*B*). Upon release from hyperosmotic stress conditions, the accumulated proline is rapidly broken down by oxidation to glutamate. We have demonstrated that proline catabolism is triggered by elevation of proline oxidase transcript levels. Proline accumulation during hyperosmotic stress is an essential adaptive response to these conditions in *Arabidopsis*. Rapid oxidation of proline is an equally important process in recycling the accumulated free proline and providing reducing power, amino nitrogen, and energy in the restoration of cellular homeostasis during recovery from osmotic stress.

Our results indicate the presence of a negative repressor that we propose is rapidly activated by signals arising at the onset of osmotic stress and acts to lower the steady-state levels of *At-POX* transcripts (Fig. 4*B*). Upon release from the stress, the repressor is inactivated and *At-POX* transcript levels rise. The results presented here highlight significant cross talk between the pathways for proline anabolism and catabolism in the osmotic stress response. It has previously been shown that transcriptional up-regulation of proline synthesis genes occurs at the onset of osmotic stress and is down-regulated on recovery (3–5). Studies of the transcriptional regulation of proline metabolism in yeast reveal interesting parallels with

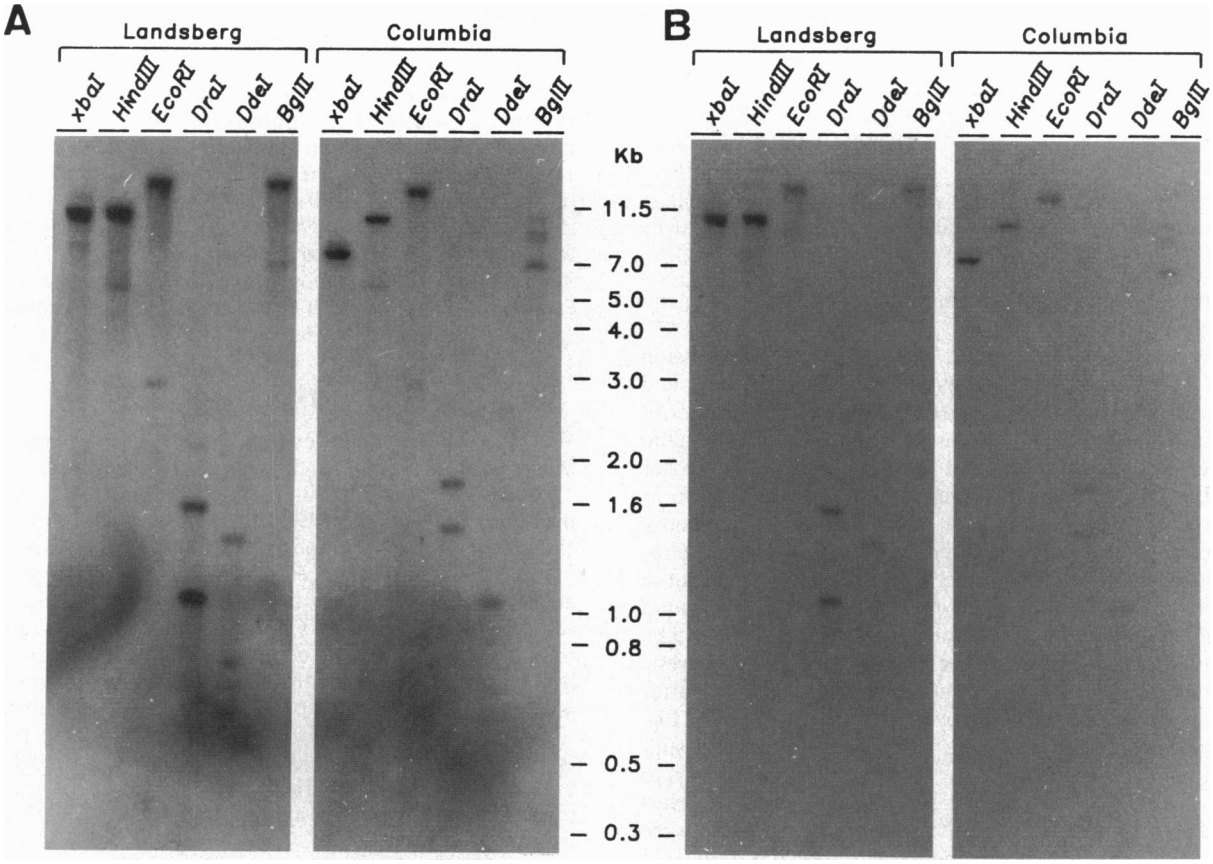

FIG. 3. Genomic DNA gel blot analysis of *A. thaliana*. Hybridization was done at 55°C overnight with the translated coding region. (*A*) Wash performed in 2× SSC/0.1% SDS at 55°C. (*B*) Wash performed in 0.1× SSC/0.1% SDS at 65°C.

what we have now discovered in the model plant *Arabidopsis*. As in *Arabidopsis* (see below), proline oxidase gene expression is regulated by the level of the free proline pool (16) (Figs. 4*C* and 5*C*). When proline catabolism is not needed as an alternative nitrogen source, transcription of *PUT1* (proline oxidase) and *PUT2* (pyrroline-5-carboxylate reductase dehydrogenase) is repressed by the *URE2* negative regulator (28). It is possible that proline oxidase gene expression in yeast and *Arabidopsis* share common regulatory factors. It is intriguing that *URE2* has significant sequence homology with glutathione *S*-transferases (29), multifunctional enzymes that are associated with responses to a wide variety of stress conditions (30, 31).

Developmental signals also determine the steady-state level of *At-POX* transcripts. Our data indicate that one of these signals is free proline itself because exogenous proline application elevated *At-POX* transcript levels in *Arabidopsis* seedlings (Figs. 4*C* and 5*C*). The results in Figs. 4*C* and 5*C* show that when free proline is the only inducer, *At-POX* transcript levels and free proline can accumulate. In this respect, it is interesting to note that the synthesis and catabolism of other amino acids such as lysine, are also coordinately regulated during seed development (32). Under conditions of hyperosmotic stress, however, *At-POX* expression is clearly unresponsive to the free proline that accumulates (Figs. 4*B* and 5*B*). This lends further support to the proposition that a stress-activated repressor exists and whose activity overrides the stimulatory effect of free proline on *At-POX* transcript levels. A further indication that proline acts in the regulation of *At-POX* transcript levels is high levels of *At-POX* transcripts in flowers and mature seeds (Figs. 4*A* and 5*A*). Here, the proline level and expression of the proline synthesis genes are high (4). It is interesting to note that the water potential of florets and

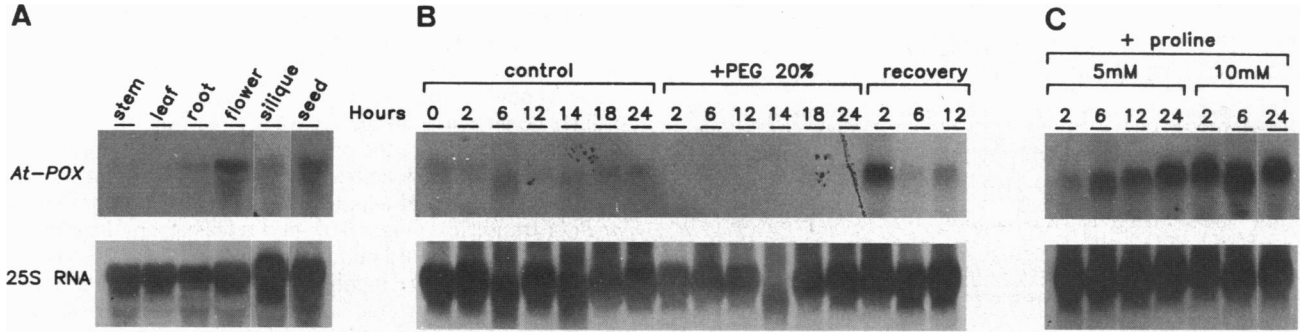

FIG. 4. RNA gel blot analysis of total RNA from different organs of *Arabidopsis* (*A*), during osmotic stress and upon recovery (*B*), and during incubation in the presence of exogenous proline (*C*). Total RNA was isolated from 10-day-old seedlings after treatment (2, 6, 12, 14, 18, and 24 h) with PEG 20% or upon recovery (2, 6, 12 h) in control medium after 12 h PEG treatment and after treatment (2, 6, 12, and 24 h) with proline (5 mM or 10 mM).

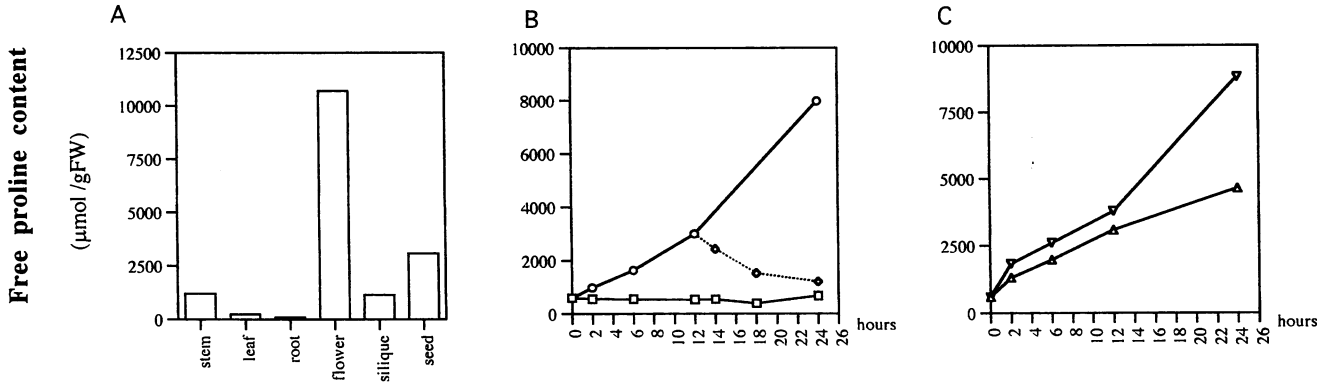

FIG. 5. Levels of free proline from the same organs of *Arabidopsis* (*A*) and under the treatments (*B* and *C*) as described in Fig. 4. (*B*) During osmotic stress with 20% PEG (○) or upon recovery (2, 6, and 12 h) in control medium after 12 h of PEG treatment (◇) and in controls (□). (*C*) After treatment with 5 mM proline (△) or 10 mM proline (▽).

mature seeds is low compared with other organs. A direct linear correlation between the water content of different *Arabidopsis* organs and free proline level has been shown (33). It is possible that in these organs cells are adapted to low-water content and that osmotic stress signals do not arise. It could be envisaged that under conditions of developmentally controlled desiccation, the postulated negative repressor of *At-POX* transcript levels is not activated and transcripts accumulate. In tissues with high H_2O content exposed to rapid and severe osmotic stress, the negative repressor would be activated and, as we have demonstrated, *At-POX* transcript levels decline. In flowers and seeds, proline oxidase activity may serve a useful function in providing energy and/or carbon and nitrogen sources through initiating turnover of the free proline pool.

In plants, the precise roles of proline oxidation are not known. However, our results indicate that proline oxidation may carry out a regulatory role in a wide variety of adaptive and developmental processes. The cloning of a cDNA encoding proline oxidase now opens new routes to study the mechanisms involved in the regulation of proline metabolism and the physiological significance of proline oxidation in higher plants.

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