Regulation of Levels of Proline as an Osmolyte in Plants under Water Stress

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Compatible osmolytes are potent osmoregulators that play a role in counteracting the effects of osmotic stress. Proline (Pro) is one of the most common compatible osmolytes in water-stressed plants. The accumulation of Pro in dehydrated plants is caused both by activation of the biosynthesis of Pro and by inactivation of the degradation of Pro. In plants, L-Pro is synthesized from L-glutamic acid (L-Glu) via \( \Delta^1 \)-pyrroline-5-carboxylate (P5C) by two enzymes, P5C synthetase (P5CS) and P5C reductase (P5CR). L-Pro is metabolized to L-Glu via P5C by two enzymes, proline dehydrogenase (oxidase) (ProDH; EC 1.5.99.8) and P5C dehydrogenase (P5CDH; EC 1.5.1.12). Such metabolism of Pro is inhibited when Pro accumulates during dehydration and it is activated when rehydration occurs. Under dehydration conditions, when expression of the gene for P5CS is strongly induced, expression of the gene for ProDH is inhibited. By contrast, under rehydration conditions, when expression of the gene for ProDH is strongly induced, expression of the gene for P5CS is inhibited. Thus, P5CS, which acts during the biosynthesis of Pro, and ProDH, which acts during the metabolism of Pro, appear to be the rate-limiting factors under water stress. Therefore, it is suggested that levels of Pro are regulated at the level of transcriptional the genes of these two enzymes during dehydration and rehydration. Moreover, it has been demonstrated that Pro acts as an osmoregulator and that overproduction of Pro results in increased tolerance to osmotic stress of transgenic tobacco plants. Genetically engineered crop plants that overproduce Pro might, thus, acquire osmotolerance, namely, the ability to tolerate environmental stresses such as drought and high salinity.

Key words: Abscisic acid — Proline — Proline dehydrogenase (oxidase) (EC 1.5.99.8) — \( \Delta^1 \)-pyrroline-5-carboxylate synthetase — Transcriptional regulation — Water stress.

Plants are exposed to many types of environmental stress. Among these stresses, osmotic stress, in particular that due to drought and salinity, is the most serious problem that limits plant growth and crop productivity in agriculture (Boyer 1982).

Many plants, including halophytes, accumulate compatible osmolytes, such as proline (Pro), glycine betaine and sugar alcohols, when they are exposed to drought or salinity stress (Hellebust 1976, Yancey et al. 1982, Csonka 1989, Deauney and Verma 1993). It has been suggested that compatible osmolytes do not interfere with normal biochemical reactions and act as osmoregulators during osmotic stress. Among known compatible solutes, Pro is probably the most widely distributed osmolyte. The accumulation of Pro has been observed not only in plants but also in eubacteria, marine invertebrates, protozoa, and algae (McCue and Hanson 1990, Deauney and Verma 1993).

Genes for enzymes involved in the biosynthesis and metabolism of Pro have been isolated from various plants, and their expression and the functions of their gene products have been characterized. Results of investigations of the relationship between the expression of these genes and the accumulation of Pro under water stress indicate that the level of Pro in plants is mainly regulated at transcriptional level during water stress. Moreover, the overproduction of Pro results in the increased tolerance of transgenic tobacco plants to osmotic stress. In this review, we shall discuss the transcriptional regulation of the level of Pro during dehydration and rehydration, as well as the possibility of breeding of transgenic plants that can tolerate water stress.

Pathways for the biosynthesis and metabolism of proline in plants—The pathway for the biosynthesis of Pro in plants was elucidated by reference to the pathway in *Escherichia coli* (Leisinger 1987). Figure 1 shows this pathway in...
bacteria and the pathways for the biosynthesis and metabolism of pro in plants. The pathway in bacteria begins with the ATP-dependent phosphorylation of the γ-carboxy group of L-glutamic acid (L-Glu) by γ-glutamyl kinase (γ-GK). The product of γ-GK is reduced to glutamic-γ-semialdehyde (GSA) by GSA dehydrogenase (GSADH), with which γ-glutamyl kinase forms an obligatory enzyme complex. GSA cyclizes spontaneously to form Δ1-pyrroline-5-carboxylate (P5C), which is finally reduced to pro by P5C reductase (P5CR). It has been suggested that, in plants, pro is synthesized either from Glu or from ornithine and that the pathway from Glu is the primary route for the synthesis of pro under conditions of osmotic stress and nitrogen limitation, while the pathway from ornithine predominates at high levels of available nitrogen (Delauney et al. 1993). The biosynthetic pathway to pro from Glu is thought to involve conversion of Glu to pro via the intermediates γ-glutamyl phosphate, GSA and P5C, as is the case in E. coli, because a cDNA clone for P5C synthetase (P5CS) was isolated from mothbean (Vigna aconitifolia) by complementation of a mutant of E. coli, and recombinant P5CS protein, expressed in E. coli, had both γ-GK and GSA dehydrogenase activities (Hu et al. 1992). cDNAs for plant P5CS have been isolated from mothbean (Hu et al. 1992), Arabidopsis thaliana (Yoshiba et al. 1995, Savoure et al. 1995), and rice (Igarashi et al. 1997). A cDNA for a plant P5CR was also isolated by complementation of an E. coli mutant from soybean (Delauney and Verma 1990), and homolog of this cDNA were isolated from pea (Williamson and Slocomb 1992), and Arabidopsis (Verbruggen et al. 1993). The second important factor that controls levels of pro in plants is the degradation or metabolism of pro. 1-Pro is oxidized to p5C in plant mitochondria by proline dehydrogenase (oxidase) (ProDH; EC 1.5.99.8), and P5C is converted to t-Glu by P5C dehydrogenase (P5CDH; Bogess et al. 1977, Elthon and Stewart 1981). Such oxidation of pro is inhibited during the accumulation of pro under water stress and is activated in rehydrated plants (Stewart et al. 1977, Rayapati and Stewart 1991). ProDH and P5CDH catalyze reactions that are the reverse of those catalyzed by P5CS and P5CR, respectively, in the biosynthesis of pro. cDNA for plant ProDH was isolated from Arabidopsis (Kiyosue et al. 1996, Verbruggen et al. 1996, Peng et al. 1996). However, no gene for P5CDH has yet been isolated. Recently, the P5CDH (EC 1.5.1.12) protein was puri-
fied from cultured cells of potato (Forlani et al. 1997), and we anticipate that the corresponding gene will be cloned in the near future.

**Water stress and the expression of genes for enzymes involved in the biosynthesis of proline**—In plants, L-Pro is produced from L-Glu via PSC, in reactions catalyzed by two enzymes, P5CS and P5CR. Many plants, when they are exposed to water stress, drought or salinity stress, accumulate Pro. The expression of genes for P5CS and P5CR has been analyzed under dehydration conditions in mothbean and *Arabidopsis* (Hu et al. 1992, Verbruggen et al. 1993, Yoshida et al. 1995, Savouré et al. 1995). In *Arabidopsis*, mRNA for P5CS appeared within 2 h of the start of dehydration, and its level increased for up to 10 h (Fig. 2).

When plants were dehydrated for 10 h and then rehydrated by transfer to water, the level of the transcript decreased within 5 h, returning eventually to the level in untreated plants. Limited accumulation of mRNA for P5CS in response to cold stress was also observed 24 h after the initiation of low-temperature treatment. Western blotting analysis, demonstrated that the level of P5CS protein increased after the initiation of dehydration treatment in direct proportion to the level of accumulated mRNA for P5CS (Yoshida et al. unpublished results).

The expression of the gene for P5CR appears not to be enhanced to any significant extent by dehydration, high salinity, or treatment with ABA (Yoshida et al. 1995). Verbruggen et al. (1993) reported that, in *Arabidopsis* under salt stress, levels of mRNA for P5CR were 5-fold higher in leaves and 2-fold higher in roots than in non-stressed plants. However, the level of expression of the gene for P5CR was very low compared with that of the gene for P5CS in *Arabidopsis*. These results suggest that the gene for P5CR might play a more important role than the gene for P5CR in the accumulation of Pro under osmotic stress.

The levels of Pro in *Arabidopsis* under dehydration and rehydration stress were compared with the levels of mRNAs for P5CS and P5CR. The level of Pro increased during dehydration and decreased during rehydration in proportion to the level of the mRNA for P5CS. It was reported that transgenic tobacco plants that expressed soybean P5CR had an elevated level of P5CR activity but did not accumulate Pro to any significant extent (Szoke et al. 1992). By contrast, transgenic tobacco plants expressing mothbean P5CS produced a large amount of the enzyme and subsequently accumulated 10-fold more Pro than did control plants (Kavi Kishor et al. 1995). Therefore, our results and these reports suggest that P5CS is the principal enzyme involved in the biosynthesis of Pro in plants under water stress.

It was reported recently that the expression of the gene for P5CS is independent of ABA upon exposure to cold and osmotic stress, even though expression of this gene can be triggered by treatment with exogenous ABA (Savouré et al. 1997). The expression of the gene for P5CS was induced by osmotic stress and exogenous application of ABA both in wild-type and in ABA-deficient (aba1) and ABA-insensitive (abi1 and abi2) mutants of *Arabidopsis*. These observations suggest that the expression of the gene for P5CS can be induced by two different pathways, an ABA-independent and an ABA-dependent pathway, under dehydration conditions (Fig. 3).

**Water stress and the expression of genes for enzymes involved in the metabolism of proline**—1-Proline is metabolized to Glu by two enzymes, ProDH and P5CDH, which catalyze reactions that are the reverse of those catalyzed by P5CR and P5CS, respectively, in the biosynthesis of Pro (Fig. 1). It was proposed initially that the metabolism of

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**Fig. 2** Time course of the accumulation and the degradation of proline (Pro), the mRNA for Δ1-pyrroline-5-carboxylate synthetase (P5CS), and the mRNA for proline dehydrogenase (ProDH) in *Arabidopsis* during dehydration (A) and rehydration after 10-h dehydration (B).
Pro might be inhibited during the accumulation of Pro under water stress and activated by rehydration (Stewart et al. 1977, Rayapati and Stewart 1991). It was difficult in early experiments to purify ProDH in substantial amounts because of the localization of the enzyme on the matrix side of the inner membrane of mitochondria. ProDH seems to donate electrons directly to the respiratory electron transport system (Elthon and Stewart 1981, 1982). However, cDNA sequences encoding ProDH were recently isolated from Arabidopsis by three groups (Kiyosue et al. 1996, Verbruggen et al. 1996, Peng et al. 1996). Kiyosue et al. isolated a cDNA clone (ERD5) for ProDH from a cDNA library of one-hour-dehydrated plants by differential screening. Verbruggen et al. and Peng et al. found a conserved sequence of ProDH in the Arabidopsis EST database by using the amino acid sequences of ProDH from Saccharomyces cerevisiae and from Drosophila melanogaster, and then they isolated cDNA clones. Comparison of the deduced amino acid sequence with other sequences in sequence databases revealed that the ProDH protein of Arabidopsis is 34.5% and 23.6% homologous to those of Drosophila (Hayward et al. 1993) and S. cerevisiae (Wang and Brandriss 1987), respectively. The amino acid sequence of ProDH protein from Arabidopsis contains a putative signal for mitochondrial localization that is characteristic of proteins that are imported into mitochondria. The ProDH protein appears to be targeted to mitochondria and the gene product was detected immunologically in a mitochondrial fraction from Arabidopsis cells (Kiyosue et al. 1996).

The expression of the gene for ProDH was strongly induced by rehydration after dehydration for 10 h, but not by heat or cold stress (Kiyosue et al. 1996). A low level of mRNA for ProDH was detected transiently for 1 h after the start of dehydration and then the level decreased (Fig. 2). When plants were dehydrated for 10 h and then rehydrated, accumulation of the mRNA for ProDH began to increase within 2 h, and large amounts of the mRNA were detectable after rehydration for up to 48 h. The level for Pro decreased rapidly for 10 h from the beginning of rehydration but then decreased gradually for the next 38 h. Figure 2 shows the relationship between the accumulation of Pro and the level of expression of the gene for ProDH during dehydration and rehydration. Furthermore, the expression of the gene for ProDH was strongly induced by exogenous L-Pro and D-Pro. Thus, the expression of the gene for ProDH in plants was induced by Pro but repressed by
osmotic stress (Kiyosue et al. 1996). Dehydration of plants caused osmotic stress and led subsequently to elevated levels of Pro in plant cells. The Pro formed several hours after the onset of osmotic stress as a result of biosynthesis de novo could not induce the expression of the gene for ProDH because of repression by osmotic stress. When plants were rehydrated, the expression of this gene became inducible by Pro because of the removal of osmotic stress and absence of repression (Fig. 3).

In dehydrated plants, the accumulation of Pro occurs as the result of both the activation of its biosynthesis and the inactivation of its degradation, whereas a decrease in the accumulation of Pro occurs as a result of both the inactivation of biosynthesis and the activation of degradation in rehydrated plants. The decrease in ProDH activity in water-stressed plants and the increase in this activity in rehydrated plants were observed by Rayapati and Stewart (1991). They postulated that the decrease in activity of ProDH might have been caused by a specific change in the inner membrane of mitochondria. Our results demonstrate that both the inactivation of ProDH in dehydrated plants and its activation in rehydrated plants are regulated at the level of the accumulation of the mRNA (Fig. 3). Therefore, the activity of ProDH is regulated not only by the level of the enzyme but also by the level of gene expression in plants.

Transcriptional regulation of the level of proline under water stress—The transcriptional regulation of the level of Pro under dehydration and rehydration conditions in Arabidopsis is shown schematically in Figure 3. The gene for P5CS is induced by dehydration but is repressed by rehydration. Expression of the gene for P5CR is slightly upregulated by dehydration. These results indicate that the induction of the gene for P5CS plays a major role in the biosynthesis of Pro. The plant hormone ABA accumulates under environmental stresses such as drought, high salinity, and low temperature and is involved in responses and tolerance to dehydration (Giraudat et al. 1994). Many genes that respond to water stress are also induced by the exogenous application of ABA (Bohnert et al. 1995, Ingram and Bartels 1996, Shinozaki and Yamaguchi-Shinozaki 1996, Bray 1997). It appears that water stress triggers the production of ABA which, in turn, induces various genes. Because the gene for P5CS is also induced by the exogenous application of ABA, it seems likely that this gene is also one of many ABA-inducible genes. However, evidence for the ABA-independent expression of the gene for P5CS under dehydration conditions was also suggested by studies of an ABA-deficient mutant (Yoshida et al. unpublished results). These observations indicate, therefore, that the expression of the gene for P5CS is induced by two different pathways, an ABA-independent and an ABA-dependent pathway, under dehydration conditions (Fig. 3).

Four independent signal-transduction pathways that control dehydration-induced genes have been proposed. Two pathways are ABA-dependent and two are ABA-independent (Shinozaki and Yamaguchi-Shinozaki 1996). Two different cis-acting elements appear to function in ABA-dependent and ABA-independent gene expression under water stress. The ABRE (ABA-responsive element; PyACGTGTC) functions as a cis-acting element that is involved in ABA-responsive transcription. DNA-binding proteins that contain the bZIP motif have been shown to bind to the ABRE sequence (Chandler and Robertson 1994, Giraudat et al. 1994). A coupling element is also required in conjunction with the ABRE to generate the ABA-responsive complex (Shen and Ho 1995). By contrast, the DRE (dehydration-responsive element; TACCGACAT) has been identified as a cis-acting element involved in ABA-independent gene expression under dehydration, high-salinity, and low-temperature conditions (Yamaguchi-Shinozaki and Shinozaki 1994). The CCGAC core motif has been found in the promoter regions of many cold- and drought-inducible genes (Nordin et al. 1993, Baker et al. 1994, White et al. 1994, Wang et al. 1995). Analysis of the promoter of the P5CS gene should provide further information about the control of the expression of this gene under dehydration conditions.

The gene for ProDH is induced by rehydration but is repressed by dehydration. Moreover, its expression is induced by Pro and repressed by osmotic stress. The expression of the gene for ProDH might be inducible by an elevated level of intracellular Pro during dehydration, but it might be repressed by osmotic stress under dehydration conditions (Fig. 3). Analysis of the promoter of the gene for ProDH is in progress in an attempt to identify cis-acting elements that are involved in the Pro-inducible expression and repression of this gene by osmotic stress.

Localization and transportation of proline under water stress—The maintenance of an appropriate water potential during a water deficit can be achieved by osmotic adjustment. A reduction in the cellular water potential to below the external water potential, resulting from a decrease in osmotic potential, allows water to move into the cell. The osmotic potential inside the cell is lowered by the accumulation in the cytosol of compatible solutes, such as Pro. Accumulation of compatible solutes occurs preferentially in the cytosol under water stress (Hall et al. 1978, Leigh et al. 1981, Pahlisch et al. 1983, Matoh et al. 1987). Fricke and Pahlisch (1990) demonstrated that 34% of the total intracellular Pro was accumulated in vacuoles in non-water-stressed cultured cells of potato. They also observed that the total amount of Pro in cells increased and the amount of Pro in vacuoles decreased when cultured cells were exposed to water stress. Thus, it seems that the plant vacuole plays an important role in the accumulation and transportation of Pro during water stress.

Recently, two genes encoding a proline transporter
(ProT) were isolated from Arabidopsis by use of a yeast targeting mutant (Frommer et al. 1993, Rentsch et al. 1996). A yeast mutant lacking SHR3, a protein that is specifically required for the correct targeting of plasma membrane-localized amino acid permeases, was used to isolate novel SHR3-independent transporters of amino acids. The SHR3 gene was isolated by complementation of the shr mutant with a cDNA library from Arabidopsis and it was shown to encode a membrane protein that is located in the endoplasmic reticulum and seems to serve as a cargo specifier for plasma membrane amino acid permeases in vesicles of the endoplasmic reticulum (Ljungdahl et al. 1992). Genes encoding eight different transporters of amino acids, including two genes for specific proline transporters (ProT1 and ProT2) were isolated by Rentsch et al. (1996). ProT1 mRNA was found in all organs and, in particular in roots, stems, and flowers. ProT2 mRNA was found ubiquitously in almost all tissues, but its level was strongly enhanced under water or salt stress. Thus, it is suggested that, under water stress, ProT2 might play an important role in the distribution of Pro. Under water-stressed conditions, massive changes in the partitioning of nitrogen and carbon take place, for example, depression of the delivery of nitrate to shoots (Shaner and Boyer 1976) and a reduction in phloem transport (Tully et al. 1979).

The relationship between the accumulation of proline and tolerance to water stress—The role of Pro as an osmoprotectant was demonstrated in Salmonella oranienburg. Christian (1955a, 1955b) reported that exogenous Pro could alleviate the inhibition of growth of S. oranienburg that was due to osmotic stress. It was reported subsequently that a wide variety of osmotically stressed bacteria accumulate Pro (Measures 1975). Moreover, a mutation (proB74) in E. coli that resulted in the overproduction of Pro also endowed a resistance to osmotic stress (Csonka et al. 1988). These observations indicated that Pro can act as an osmoprotectant, in other words, it can protect bacteria from osmotic stress.

Eubacteria, protozoa, marine invertebrates, and many plants including algae (e.g., halophytes, tobacco, spinach, potato, tomato, Arabidopsis, alfalfa, field bean, soybean, wheat, barley and rice) can all accumulate Pro (McCue and Hanson 1990, Delauney and Verma 1993). Therefore, among compatible organic solutes, it is probable that Pro is the most widely distributed osmolyte. Tomato cells cultured under water stress rapidly accumulated about 300 times more Pro than non-water-stressed cells, and they adapted to osmotic stress (Handa et al. 1983, 1986, Rhodes et al. 1986). These observations indicate that many plants have the ability to adapt to water stress at the cellular level and that Pro is involved in tolerance to osmotic stress, acting as a compatible osmolyte.

Kavi Kishor et al. (1995) reported that transgenic tobacco plants that expressed a cDNA for mothbean P5CS under the control of the 35S promoter of cauliflower mosaic virus produced a high level of the enzyme and subsequently accumulated 10- to 18-fold more Pro than control plants. They also reported that overproduction of Pro enhanced root biomass and the development of flower in transgenic plants exposed to drought. Thus, overproduction of Pro resulted in the increased tolerance of plants to osmotic stress and it seems likely that Pro-overproducing crop plants obtained by genetic engineering, might acquire osmotolerance, namely, the ability to tolerate environmental stresses such as high salinity.

Igarashi et al. (1997) recently isolated a cDNA for P5CS from rice, and they compared the level of the mRNA for P5CS and the level of accumulation of Pro in a salt-sensitive rice, IR28, and a salt-tolerant rice, Dee-gee-woo-gen (DGWG), under high-salinity conditions. The transcript of the rice gene for P5CS appeared within 10 h after the start of salt treatment, and the level of the transcript increased for up to 48 h in both kinds of rice, but the level of the transcript in salt-tolerant DGWG was higher than that in salt-sensitive IR28. The level of Pro that accumulated in DGWG was also higher than that in IR28. These observations suggest that expression of the gene for P5CS and the accumulation of Pro might be correlated with salt tolerance in rice.

Future perspectives—Plants respond to various types of water stress, such as drought, high salinity, and low temperature, by a number of physiological and developmental changes. During water stress, plant cells can undergo changes in concentrations of solutes, in cell volume and in the shape of cell membranes, as well as disruption of gradients in water potential, loss of turgor, disruption of membrane integrity and the denaturation of proteins. Three genes for enzymes involved in the biosynthesis and metabolism of Pro have been cloned to date, and their expression has been analyzed. A gene for P5CDH, which catalyzes the conversion of P5C to Glu, has not been cloned from plants, but the P5CDH protein has recently been purified. In the near future, all the genes involved in the synthesis and degradation of Pro should be cloned. From an analysis of these genes, the functions of their products and the regulation of their gene expression, we should develop a better understanding of the role of Pro in stress responses and development at the molecular level. Regulation of the expression of genes for P5CS, P5CR, and ProDH is now being analyzed at the transcriptional level by use of transgenic plants. Both cis- and trans-acting elements that are involved in the regulation of expression of these genes should also be identified in the near future. An understanding of the transcriptional regulation of Pro-related genes should give us better insight into the roles and regulation of level of Pro in the stress responses of plant cells, where Pro acts as an osmolyte and as a source of energy and nitrogen. Moreover, details of the tissue-specific expression of Pro-re-
lated genes should provide more information about the role of Pro in plant development.

The synthesis of Pro represents one response of plant cells to a water deficit. Other known compatible solutes are sugar alcohols (e.g., pinitol), other sugars (e.g., fructans) and quaternary ammonium compounds (e.g., glycine betaine), and they can accumulate at high levels without disruption of protein functions. It was reported that when Synechococcus was transformed with genes for enzymes related to the synthesis of glycine betaine, the cells exhibited enhanced tolerance to salt stress (Nomura et al. 1995, Deshnium et al. 1995). In the case of higher plants, transgenic tobacco harboring the gene from E. coli for mannitol-1-phosphate dehydrogenase accumulated mannitol in the cytosol and this accumulation increased the tolerance of plants to high salinity (Tranczynski et al. 1993). The accumulation of fructan or trehalose has also been shown to promote stress tolerance in transgenic tobacco (Pilon-Smits et al. 1995, Holmström et al. 1996). In all such transformants, the demonstrated tolerance to water stress did not appear to be due to osmotic adjustment because the amount of osmolyte that accumulated was insufficient to account for an alteration in gradients in water potential at the cellular level. However, the cited reports do suggest that the application of gene-engineering techniques to many crops might improve their tolerance to water stress. In the future, application of genetic engineering to the breeding of environmental stress-tolerant transgenic plants should lead to improvements in crop production in unfavorable environments, such as those with high salinity or insufficient water.

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