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Overexpression of Δ^1-Pyrroline-5-Carboxylate Synthetase Increases Proline Production and Confers Osmotolerance in Transgenic Plants[1]

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Proline (Pro) accumulation has been correlated with tolerance to drought and salinity stresses in plants. Therefore, overproduction of Pro in plants may lead to increased tolerance against these abiotic stresses. To test this possibility, we overexpressed in tobacco the mothbean Δ^1-pyrroline-5-carboxylate synthetase, a bifunctional enzyme able to catalyze the conversion of glutamate to Δ^1-pyrroline-5-carboxylate, which is then reduced to Pro. The transgenic plants produced a high level of the enzyme and synthesized 10- to 18-fold more Pro than control plants. These results suggest that activity of the first enzyme of the pathway is the rate-limiting factor in Pro synthesis. Exogenous supply of nitrogen further enhanced Pro production. The osmotic potentials of leaf sap from transgenic plants were less decreased under water-stress conditions compared to those of control plants. Overproduction of Pro also enhanced root biomass and flower development in transgenic plants under drought-stress conditions. These data demonstrated that Pro acts as an osmoprotectant and that overproduction of Pro results in the increased tolerance to osmotic stress in plants.

Drought and salinity are two major osmotic stresses that dramatically limit plant growth and productivity (Boyer, 1982; LeRudulier et al., 1984; Skriver and Mundy, 1990). Many eubacteria, algae, and higher plants accumulate free Pro in response to osmotic stresses (Schobert, 1977; Csonka and Hanson, 1991; Delauney and Verma, 1993), a phenomenon first observed by Kemble and MacPherson (1954) in wilted rye grass. Accumulation of Pro is due primarily to de novo synthesis (Boggess et al., 1976; Stewart and Hanson, 1980; Rhodes et al., 1986; Voetberg and Sharp, 1991), although a reduced rate of catabolism has been observed (Stewart et al., 1977; Stewart and Hanson, 1980; Rhodes et al., 1986). In addition to acting as an osmoprotectant (Christian, 1955), Pro also serves as a sink for energy to regulate redox potentials (Blum and Ebercon, 1976; Saradhi and Saradhi, 1991), as a hydroxy radical scavenger (Smirnoff and Cumbes, 1989), as a solute that protects macromolecules against denaturation (Schobert and Tschesche, 1978), and as a means of reducing the acidity in the cell (Venekamp et al., 1989). Overexpression of a bacterial gene allowing mannitol synthesis has been demonstrated to confer salinity tolerance in transgenic plants (Tarczynski et al., 1993). Therefore, it is likely that overproduction of Pro, one of the natural osmolytes (Yancey et al., 1982) may enable crop plants to tolerate water stress.

The biosynthetic pathway of Pro in *Escherichia coli* has been well characterized. Glutamate is phosphorylated by γ-GK (encoded by the *proB* gene) to γ-glutamyl phosphate. This is then reduced to GSA by GSA dehydrogenase (encoded by the *proA* gene). GSA is spontaneously cyclized to P5C, which is reduced to Pro by P5CR (encoded by the *proC* gene). The *E. coli proBA* and *proC* loci have been cloned and sequenced (Deutch et al., 1982, 1984). In animals and higher plants, Pro is synthesized not only from glutamate but also from Orn (Bryan, 1990; Delauney and Verma, 1993).

We have recently isolated from soybean and mothbean (*Vigna aconitifolia*) genes involved in Pro biosynthesis. We have cloned cDNAs encoding P5CR and P5CS, a bifunctional enzyme that possesses both γ-GK and GSA dehydrogenase activities and catalyzes the first two steps in Pro biosynthesis (Delauney and Verma, 1990; Hu et al., 1992). Treatment of tobacco (*Nicotiana tabacum*) cell cultures with salt has been shown to increase the level of P5CR, but this step does not seem to be rate limiting for Pro synthesis (LaRosa et al., 1991). We independently reached the same conclusion by overexpressing soybean P5CR in transgenic tobacco (Szoke et al., 1992). We also demonstrated that the synthesis of Pro from Orn in plants proceeds via the δ-transamination of Orn to P5C and subsequent reduction to Pro, and we have isolated a cDNA clone encoding OAT. We further showed that Pro is made preferentially via Orn under normal conditions, whereas it is made directly from

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Abbreviations: CaMV, cauliflower mosaic virus; γ-GK, γ-glutamyl kinase; GSA, glutamic-γ-semialdehyde; OAT, ornithine aminotransferase; P5C, Δ^1-pyrroline-5-carboxylate; P5CR, Δ^1-pyrroline-5-carboxylate reductase; P5CS, P5C synthetase.

glutamate via P5CS under stress conditions (Delauney et al., 1993). This is consistent with the radiolabeled isotope studies that have revealed that glutamate rather than Orn is the principal precursor for Pro biosynthesis in osmotically stressed plants (Boggess and Stewart, 1976; Rhodes et al., 1986).

Pro has been demonstrated to ameliorate dehydration-induced perturbation in proteins (Paleg et al., 1984), and exogenously supplied Pro confers some osmotic tolerance to the plants and cultured cells (Itai and Paleg, 1982). However, a cause-and-effect relationship between Pro levels and osmotic potential has not been established. Furthermore, the rate-limiting step in Pro biosynthesis has not been determined. We report here that introduction of *Vigna* P5CS cDNA into tobacco under the control of a constitutive promoter significantly increases production of Pro, which helps in maintenance of osmotic potential of transgenic plants during water stress.

MATERIALS AND METHODS

Transformation of Tobacco Plants

A plasmid (pBI-P5CS, Fig. 1) containing *Vigna aconitifolia* P5CS cDNA (Hu et al., 1992) under the control of the CaMV 35S promoter in vector pBI121 was introduced into *Agrobacterium tumefaciens*. Tobacco (*Nicotiana tabacum* cv Xanthi) leaf discs were transformed with *A. tumefaciens* containing pBI-P5CS or pBI121 (used as control). A total of 250 plants was regenerated on kanamycin and screened for GUS activity as well as for Pro production. Ten independent primary transformants (T_0) showing high levels of Pro, and two transgenic lines having similar levels of Pro, compared to the control, were allowed to flower and set seeds. Cross-pollination was prevented by placing a paper bag on flowers prior to opening. Seeds were collected from

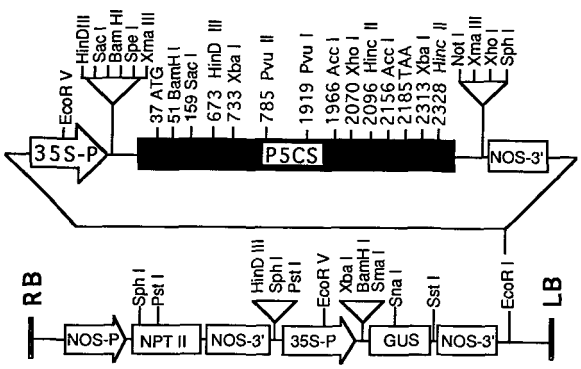

Figure 1. Restriction map of the plasmid pBI-P5CS used for producing transgenic plants. *Vigna* P5CS cDNA (Hu et al., 1992) was placed between the CaMV 35S promoter (35S-P) and NOS-3′ regions, and the resulting construct was inserted into the *Eco*RI site of vector pBI121. The vector also contains the *NPT II* and *uid A* (GUS) coding regions that were used for selection of transgenic plants on kanamycin and as reporter of transformation, respectively. The positions of restriction enzyme sites of the *Vigna* P5CS cDNA are indicated on the map. The coding region of the P5CS cDNA is marked by ATG at nucleotide position 37 and TAA at 2185, and other regions are depicted diagramatically.

transformants and germinated on medium containing kanamycin. Seedlings (T_1) resistant to kanamycin were transferred to Metromix (A.H. Hummert Seed Co., St. Louis, MO) and grown at 25 ± 2°C in a growth chamber (with 16-h light and 8-h dark cycles) or in a greenhouse. Plants were watered with half-strength Hoagland nutrient solution and fertilized with 20 mM NH_4NO_3 in the Hoagland solution once a week.

Salinity or Drought Treatments

Six-week-old seedlings (T_1) were subjected to a 10-d drought period by withholding water. Salinity stress was imposed by growing plants in Metromix or vermiculite saturated with 250 and 400 mM NaCl. For each experiment, appropriate controls (well-watered plants or plants without salt stress) were included. To minimize variability among the individuals, plants of similar height and age (before stress) were selected. Plants were harvested as indicated.

Southern, Northern, and Western Blot Analyses

Genomic DNA was isolated from young leaves of control and transgenic (T_0) plants (Dellaporta et al., 1983). DNA (10 μg) was digested with *Hin*dIII, resolved on a 0.6% agarose gel, and blotted on a GeneScreen nylon membrane (DuPont/NEN). The blot was probed with radiolabeled *Vigna* P5CS cDNA (Hu et al., 1992) at 62°C as described earlier (Delauney and Verma, 1990). Total RNA (15 μg) isolated from the transgenic and control plants was subjected to electrophoresis in a 1.2% agarose gel, transferred to the GeneScreen membrane, and hybridized to *Vigna* P5CS cDNA (Hu et al., 1992) labeled with [^{32}P]dATP. Hybridization was carried out at 60°C (Delauney and Verma, 1990), and filters were exposed to x-ray film at −70°C.

For western blot analysis, fresh leaves (1 g) from transgenic or control plants were homogenized in a buffer (5 mL) containing 10 mM β-mercaptoethanol and 1 mM PMSF in 50 mM Tris (pH 7.8). Following centrifugation at 80,000*g* for 30 min, total soluble proteins were resolved on SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted using antibodies raised against the purified *Vigna* P5CS protein (C. Zhang and D.P.S. Verma, unpublished data).

P5CS Enzyme Assay of Transgenic Plants

Leaves from transgenic plants were homogenized in an extraction buffer (100 mM Tris-Cl, pH 7.5, 10 mM β-mercaptoethanol, 10 mM $MgCl_2$, and 1 mM PMSF). The extracts were centrifuged at 10,000*g* for 15 min. Ammonium sulfate (15–35% saturation) precipitation was carried out, and the resulting pellets were collected and dissolved in the extraction buffer. This fraction was dialyzed against 10 mM Tris-Cl (pH 7.5) containing 5 mM $MgCl_2$ for 4 h with three changes of the buffer. P5CS enzyme assay was performed in the presence of [^{14}C]glutamate (Z. Hong and D.P.S. Verma, unpublished results). This assay was carried out in a mixture containing 50 mM Tris, 20 mM $MgCl_2$, 10 mM ATP, 5 mM NADPH, 0.1 μCi [^{14}C]glutamate (DuPont/

NEN), and leaf extract (10 μg of protein) in a final volume of 15 μL and final pH of 7.0 to 7.5. A blank reaction (−ATP) was performed in a mixture containing leaf extract of the transgenic line 22 and other components except ATP. P5CS enzyme was partially purified from *Escherichia coli* by 35% ammonium sulfate precipitation and DEAE-cellulose chromatography (C. Zhang and D.P.S. Verma, unpublished data). The reaction containing 0.1 μg of the partially purified P5CS served as a positive control. After incubation at 35°C for 15 min, the mixture was chilled on ice. The products (2 μL of the mixture) were resolved by TLC on a silica gel (Analtech, Inc., Newark, DE). P5C was prepared as described earlier (Szoke et al., 1992). Glu, Gln, Pro, and P5C (1 μg of each) were used as reference markers. [^{14}C]Glutamate and [^{14}C]Pro (DuPont/NEN) were also used as standards. TLC gel was developed with a mobile solution (phenol:water:acetic acid, 75:25:5 [w/v/v]) containing 0.3% (w/v) ninhydrin in a saturated chamber. After development, the gel was dried at 65°C for 15 min or until the amino acid spots appeared. The gel was wrapped with Saran Wrap and analyzed on a PhosphorImage (Molecular Dynamics, Sunnyvale, CA) or exposed to x-ray film.

Amino Acid Analysis

Leaves of transgenic plants (T_1) were used for amino acid analysis. Because the Pro contents vary from leaf to leaf and also with the age of the plant, precaution was taken to select leaves of similar age and size. One gram of leaf tissue collected from turgid and stressed leaves was quickly frozen and ground in liquid nitrogen. The tissue was extracted with sulfosalicylic acid (10%) and, after centrifugation, the supernatant was used for total amino acid analyses and for determination of Pro content as described by Bates et al. (1973).

Osmotic Potential Measurements

The osmotic potentials of expressed leaf sap were measured with a vapor pressure osmometer (Wescor, Inc., Logan, UT, model 5100 C) using potassium chloride solutions as standards at 25°C. The chamber was equilibrated for different time periods (1–3 min) before taking measurements, and the stability of the instrument during the measurement period was tested in each experiment.

RESULTS AND DISCUSSION

Expression of *Vigna* P5CS cDNA in Transgenic Tobacco Plants

A mothbean P5CS cDNA was fused to the CaMV 35S promoter (pBI-P5CS, Fig. 1) and was introduced into tobacco. Genomic Southern blot analysis of five transgenic plants showed that two to three copies of the P5CS cDNA were inserted into the genome of these transgenic lines (Fig. 2). A common band of 0.7 kb was detected in all transgenic lines, because *Hin*dIII enzyme releases a 5′ terminal fragment from the *Vigna* P5CS cDNA (for restriction map, see Fig. 1). In addition to this common band, two to three bands at different sizes were detected in different transgenic lines, suggesting that these lines were produced from different transformation events and carry multiple insertions.

The levels of P5CS transcripts in transgenic plants were determined by northern blot analysis (Fig. 3A). Hybridization at high stringency with *Vigna* P5CS cDNA showed only the mRNA from the P5CS transgene and no detectable cross-reaction with the tobacco native P5CS mRNA (Fig. 3A, lanes 1 and 2). Very high levels of expression of P5CS mRNA were detected in transgenic lines 22, 88, 136, and 162, compared to that in the control plants (Fig. 3A, lanes 1 and 2) or that in *Vigna* leaves (data not shown). No detectable expression was observed in the transgenic line 128 (Fig. 3A, lane 5), although it showed positive GUS activity and contained at least two copies of the *Vigna* P5CS cDNA insert in the genome, as revealed by genomic Southern blot analysis (Fig. 2, lane 4). Transgenic plants expressing high levels of P5CS mRNA (lines 22, 88, 136, and 162) also accumulated high levels of P5CS protein, as detected by western blotting using P5CS antibodies (Fig. 3B). A very weak protein band of the expected size of P5CS (72 kD) was detected in the control plants (Fig. 3B, lanes 1 and 2), indicating a cross-reaction of the *Vigna* P5CS antibodies with the tobacco native P5CS protein. These data demonstrated that *Vigna* P5CS cDNA is expressed at high levels and produces stable proteins.

Increase in P5CS Activity in Transgenic Plants

P5CS activity was assayed based on the conversion of [^{14}C]glutamate to P5C that is reduced to Pro by endogenous P5C reductase. When leaf extracts of the transgenic plants were incubated with [^{14}C]glutamate in the presence of Mg^{2+}, ATP, and NADPH, a significant amount of Pro was produced (Fig. 4, lanes 6, 7, 9, and 10). This activity

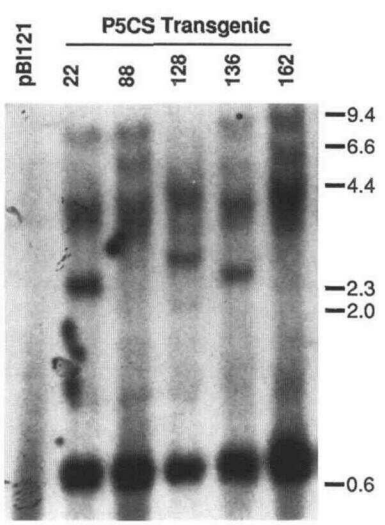

Figure 2. Genomic Southern blot of five lines of tobacco plants transformed with pBI-P5CS. The plant transformed with vector pBI121 served as control. Genomic DNA was digested by *Hin*dIII and resolved on 0.6% agarose gel. The blot was probed by ^{32}P-labeled *Vigna* P5CS cDNA. Molecular size (kb) of *Hin*dIII-digested λ

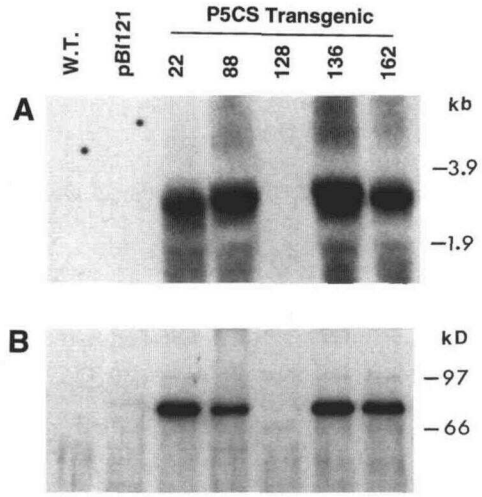

Figure 3. Expression of *Vigna* P5CS in transgenic tobacco plants. A, Northern blot of total RNA from wild-type (W.T.) and pBI121 plants (lanes 1 and 2) and the P5CS transgenic lines 22, 88, 128, 136, and 162 (lanes 3–7). The membrane was probed with ^{32}P-labeled *Vigna* P5CS cDNA. Molecular sizes (kb) of 28S and 18S rRNA are indicated. B, Western blot of total soluble proteins from leaves of the same plants used for RNA blotting. The membrane was reacted with antibodies to purified *Vigna* P5CS protein. Molecular sizes (kD) of protein markers (Bio-Rad) are indicated.

was dependent on ATP, because exclusion of ATP from the reaction mixture diminished the conversion of [^{14}C]glutamate to Pro (Fig. 4, lane 3). This activity was also correlated with expression levels of P5CS mRNA and proteins in the transgenic plants, because line 128, which produced low levels of P5CS mRNA and proteins (Fig. 3, A and B, lane 5), did not show the elevated activity required to convert [^{14}C]glutamate to Pro (Fig. 4, lane 8). That the reaction did not result in accumulation of P5C, instead of Pro, can be explained by the observation that P5C reductase is in excess and is not a rate-limiting factor in Pro biosynthesis in plants (LaRosa et al., 1991; Szoke et al., 1992). The partially purified P5CS from *E.coli* expressing *Vigna* P5CS cDNA catalyzed the production of P5C (Fig. 4, lane 11), although a portion of the radioactivity was also seen in the Pro spot, possibly due to the presence of *E. coli* P5C reductase contaminating this preparation. The leaf extracts also appeared to contain certain inhibitory elements to P5CS, because when the partially purified P5CS was mixed with the leaf extract of transgenic line 22 in the reaction mixture, no additional P5CS activity was shown (Fig. 4, lane 12). The unidentified inhibitory component(s) could not be removed by dialysis, suggesting that they are nondialyzable macromolecules. This inhibition could be one of the reasons why it is difficult to detect P5CS activity in wild-type plants.

Pro Accumulation in Transgenic Tobacco Plants Expressing Elevated Levels of P5CS

Free Pro contents of transgenic lines that produced high levels of *Vigna* P5CS mRNA and proteins were analyzed, and the effect of this accumulation [illegible] water stress was studied. Plants expressing high levels of P5CS accumulated high levels of Pro (see below). Ten independent primary transformants (T_0) that produced high levels of Pro and two lines that produced normal levels of Pro were selected for further analysis. Transgenic line 128 (Fig. 2, lane 4), which did not produce *Vigna* P5CS mRNA (Fig. 3A, lane 5) or P5CS protein (Fig. 3B, lane 5), was found to have levels of Pro as low as the control plants (data not shown). Lack of expression of *Vigna* P5CS in this line might be a consequence of a positional effect of the transgene in the tobacco genome. Pro levels in leaves of 10 transgenic lines ranged from 830 to 1590 μg/g fresh weight of leaves (average 1100 μg/g), compared to 80 to 89 μg/g in control plants (Fig. 5A). Corresponding to the level of expression of P5CS, Pro contents were increased in transgenic lines 22, 88, 136, and 162. Because overproduction of P5CR does not increase Pro content in transgenic plants (Szoke et al., 1992), a direct correlation between P5CS expression level and Pro accumulation in the transgenic plants clearly suggests that P5CS, catalyzing the first two steps in the pathway, is rate limiting in Pro biosynthesis.

Pro Accumulation Facilitates Maintenance of Osmotic Potential during Water Stress

Pro levels were increased in both control and transgenic plants after drought treatment (Fig. 5A). These values increased from about 80 μg Pro/g fresh leaf (before stress) to

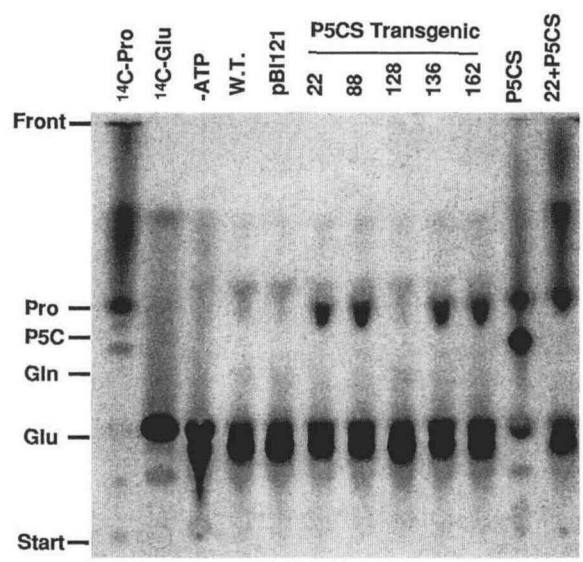

Figure 4. P5CS enzyme assay of transgenic plants. Protein extracts from transgenic and control plants (see "Materials and Methods") were incubated with [^{14}C]glutamate in the presence of ATP and NADPH and the reaction products were resolved by TLC as described in "Materials and Methods." Standard amino acid spots were recorded after the gel was dried at 65°C for 15 min, and the ^{14}C-labeled spots were analyzed on a PhosphorImager (Molecular Dynamics). The blank reaction (−ATP) contained the extract of line 22 and other reaction components except ATP. A partially purified P5CS enzyme from *E. coli* expressing pVAB2 (Hu et al., 1992) was used as a positive control. The positions of standard amino acids and the TLC [illegible] are shown on the left side.

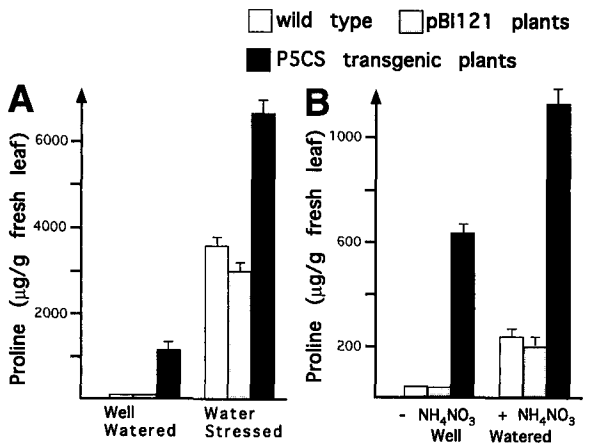

Figure 5. Pro content in leaves of the wild-type (open box), pBI121 (stippled box), and P5CS transgenic plants (solid box) before and after a 10-d drought treatment (A) or before and after growth with 20 mM NH_4NO_3 (B). Ten independent transgenic lines (T_1) with six plants each were used for this analysis, and only an average value is shown.

about 3000 μg/g (after stress) in control (wild-type and pBI121) plants, and from 1000 μg/g to an average of 6500 μg/g in transgenic lines. Although Pro content was approximately 14-fold greater in transgenic lines than in control plants before stress, it was only about 2-fold greater after stress. Control plants started wilting 5 to 6 d after drought treatment, whereas wilting was delayed by at least 2 to 3 d in transgenic plants, and the wilting was more severe in controls than in transgenic plants. The water contents of the root-supporting media (vermiculite or Metromix), as determined by drying for 24 h, were identical in all plants. High constitutive levels of Pro in P5CS transgenic lines, rather than the induced level of Pro, may be responsible for the observed effect on wilting.

We tested the effect of Pro accumulation on osmotic potential in leaf cells. Osmotic potentials of the leaf sap were very similar between controls and transgenic lines before water stress but were different after stress (Table I). The osmotic potentials declined from about −0.78 MPa (before stress) to −1.06 MPa in the top leaves of control plants after stress, whereas smaller changes were observed for transgenic plants (from about −0.70 to −0.73 MPa). More drastic changes in osmotic potentials were observed in the lower leaves of control plants after stress. Osmotic potentials of leaf sap dropped from −0.74 to less than −1.27 MPa in the lower leaves of control plants, but declined only to −0.73 MPa in comparable leaves from P5CS transgenic plants (Table I). These results suggest that Pro accumulation in transgenic plants helped the cells to maintain osmotic potential and thus enhanced the ability of the plants to tolerate water stress. This effect was more pronounced in the lower (the seventh to ninth) leaves of the plants, which are more sensitive to water stress and start wilting first during dehydration of the plant.

Data on amino acid analysis showed that accumulation of Pro occurs at the expense of glutamate, a precursor for P5C (Table II). In addition, Gly contents declined. This indicates that the availability of glutamate may act as a factor for Pro overproduction under water or salt stress. This conclusion is consistent with our observation that transgenic plants expressing high levels of P5CS and supplied with 20 mM NH_4NO_3 produced nearly 5 times more Pro than did the pBI121 controls. The total amount of Pro in control plants was 200 to 230 μg/g fresh weight of leaves, as opposed to 1120 μg/g fresh weight in transgenic plants (Fig. 5B). These results further confirm that P5CS activity is the rate-limiting factor in Pro synthesis, provided that nitrogen is available. The levels of Pro also regulate P5CS activity. Our data using purified P5CS enzyme (C. Zhang and D.P.S. Verma, unpublished data) indicate that *Vigna* P5CS is feedback inhibited to 50% by 5 mM Pro in vitro (Hu et al., 1992). Thus, P5CS activity is controlled by substrate as well as the end product of the pathway. We have recently demonstrated that Pro synthesis is also regulated by the flux of nitrogen (i.e. from glutamate versus Orn) by controlling the level of OAT and P5CS under stress conditions (Delauney et al., 1993).

Overproduction of Pro Enhances Biomass Production and Flower Development under Salt-Stress Conditions

To determine the effect of Pro accumulation on plant growth and development, we measured root length, root dry weight, capsule number, and seed number in both control and transgenic plants under normal and salt-stressed conditions. Control and transgenic plants did not differ statistically in these four parameters when grown under greenhouse conditions (Fig. 6, A–D, unstressed).

Table I. *Osmotic potential of leaf sap from wild-type and P5CS-transgenic plants*

The data represent the mean (±SE) of 4 replicates from wild-type plants and the mean of 12 replicates from P5CS-transgenic plants. Leaf sap was squeezed through a syringe from freshly picked leaves. Data shown in boldface are values obtained from transgenic plants after water stress.

Plant Type	Osmotic Potential					
	Before stress			After stress		
	Top leaf	Middle leaf	Lower leaf	Top leaf	Middle leaf	Lower leaf
	MPa					
Wild type	−0.78 (±0.04)	−0.70 (±0.06)	−0.74 (±0.07)	−1.06 (±0.06)	−1.11 (±0.06)	−1.27 (±0.05)
P5CS	−0.77 (±0.07)	−0.77 (±0.10)	−0.70 (±0.12)	**−0.70** (±0.11)	**−0.73** (±0.15)	**−0.73** (±0.12)

Table II. *Composition of free amino acids in leaves of tobacco plants transformed with pBI121 (control) or P5CS cDNA*

Data shown in boldface are Glu and Pro contents that changed significantly after water stress. nd, Not detected.

Amino Acid	Before Stress		After Stress	
	pBI121	P5CS	pBI121	P5CS
	%	%	%	%
Asp	28.52	14.00	5.67	1.98
Glu	**35.31**	**16.80**	**4.68**	**9.90**
Hyp	nd	nd	nd	nd
Ser	3.25	1.89	1.30	0.85
Gly	15.01	8.50	6.23	1.71
His	0.00	0.90	0.00	0.22
Arg	1.84	2.17	1.46	1.26
Thr	3.27	2.00	1.48	1.29
Ala	3.27	2.39	1.47	1.18
Pro	**3.60**	**48.18**	**73.06**	**78.95**
Tyr	2.60	1.15	0.94	0.31
Val	1.13	0.56	1.72	0.43
Met	0.32	0.07	0.11	0.13
Cys	nd	nd	nd	nd
Ile	0.33	0.34	0.14	0.29
Leu	0.66	0.38	0.17	0.44
Phe	0.51	0.42	0.90	0.57
Lys	0.37	0.30	0.67	0.51
Trp	nd	nd	nd	nd

However, we observed significant differences between control and P5CS-transgenic plants when grown under saline-stressed conditions (Fig. 7A). Compared to control plants, roots on transgenic plants were 40% longer and had 2-fold greater biomass (Fig. 6, A and B, Fig. 7B). More than twice the number of capsules and the number of seeds per capsule developed in transgenic plants (Fig. 6, C–D). Root growth is very sensitive to water conditions, and accordingly, a significant difference in root length was noticed between control and P5CS transgenic plants (Fig. 7B). These results suggest that Pro accumulation in plants enhances biomass production and facilitates flower development under stress conditions.

It is known that salt and water stresses can severely affect developmental traits such as flowering, fertilization, and seed setting. The age of the plant appears to be critical for the adaptation to salinity stress (Tarczynski et al., 1993). Plants that initiated flower primordia (7 weeks old) before being subjected to stress proceeded almost normally with flower blooming and capsule formation in both transgenic plants and controls. However, imposing the same stress earlier (before the initiation of flowering) resulted in significant reduction (average of 12 flowers as opposed to 80 in nonstressed plants) of flowering in transgenic plants, whereas flowering was totally suppressed in the control plants. This indicates that there is a time-limited capacity for the plant to adjust to a changing environment. This finding resembles the phenomena that occur in many vertebrates (Gurdon, 1987) and invertebrates (Bidwell et al., 1990), in which environmental factors trigger developmental processes such as morphogenic timing during the early stages of embryogenesis. Tissues sensitive to dehydration have been found to have a poor capacity to bind water tightly (Vertucci and Leopold, 1987). It was also found that the accumulation of Cl^-, K^+, and Pro was concurrent with an increase in the "binding strength" of water in wheat leaves (Rascio et al., 1994). The primary function of the accumulation of these solutes may be the regulation of intracellular water activity (LaRosa et al., 1991). Both Pro and Gly betaine under water-stressed conditions may be able to induce the formation of strong H-bonded water around the protein, preserving the native state of the cell biopolymers (Rascio et al., 1994). These osmolytes have been suggested to directly influence protein solvation by protecting from dehydration-induced thermodynamic perturbations in proteins (Paleg et al., 1984). Externally added Pro has been demonstrated to protect the structural and functional integrity of enzymes in vitro (Deutch et al., 1984).

Plants well adapted to a saline environment manifest a variety of changes for sustained growth. Accumulation of Pro is only one of the factors that facilitate this adjustment, whereas other processes must allow the plant to overcome osmotic stress and sustain growth. Expression of the *E. coli mtlD* gene in plants has been demonstrated to accumulate mannitol, which also helped sustain growth, albeit at a reduced rate (Tarczynski et al., 1993). Other osmolytes such as Gly betaine may be equally effective, provided that the basal metabolism of the plant can sustain a high rate of

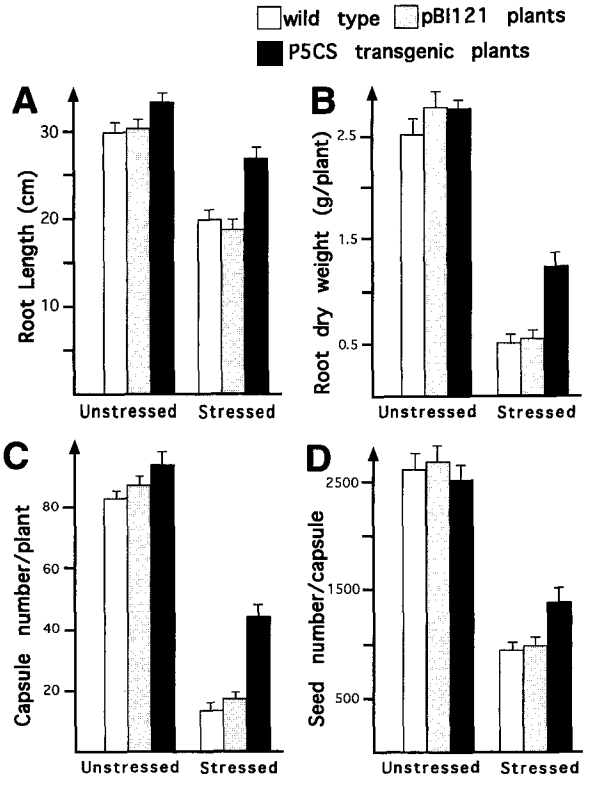

Figure 6. Comparison of the wild-type (open box), pBI121 (stippled box), and P5CS transgenic plants (solid box) in root length (A), root dry weight (B), pod number (C), and seed number (D). The plants were grown to maturity in Metromix, supplied with 0.5 M NaCl. Ten independent transgenic lines (T_1) with six plants each were used for this analysis, and only an average value is shown.

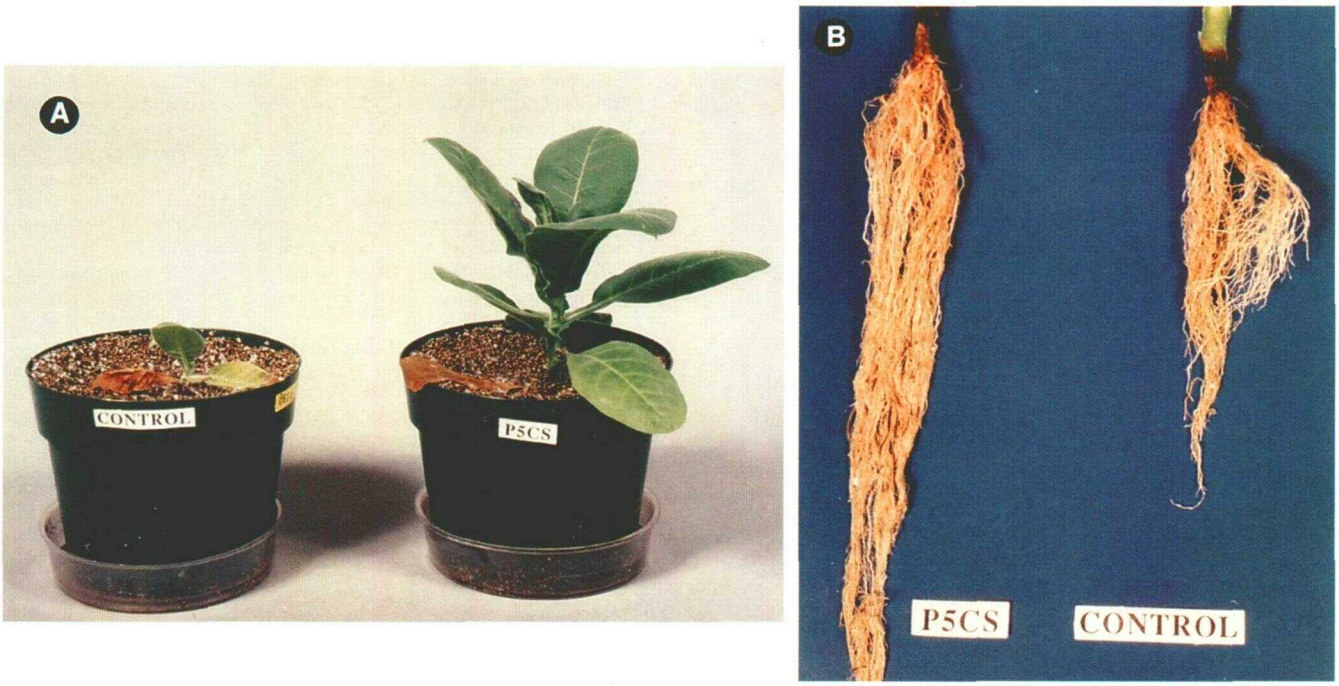

Figure 7 A, Phenotype of control and P5CS transgenic plants treated with salinity stress. Plants of wild type and transgenic line 22 (T_1) were grown in vermiculite, and at the four-leaf stage, the pots were transferred to trays containing 0.4 M NaCl and allowed to stand in the solution for 3 weeks. B, Root phenotype of wild type and transgenic line 22 (T_1) treated with drought stress. The plants were potted in Metromix, and 6-week-old plants were subjected to drought conditions until flowering. The roots at the time of flowering were washed and photographed.

synthesis of these compounds. Thus, enhancing accumulation of putative osmolytes does facilitate osmotic adjustment, opening the possibility of genetic engineering of crops for tolerance to water stress. Understanding of the regulatory mechanism that allows sustained growth under stress is essential for improving crops for osmotolerance.

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