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"Toward Increasing Tolerance of Forage Legumes to Salinity and Drought"

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L. Szabados
Biological Reseach Center, Szeged
Hungary

Y. Kapulnik
Volcani Center
Beit-Dagan
Israel

A. Zilberstein
Dept. of Botany
Tel-Aviv Univ.
Israel

Part A - submitted by Laszlo Szabados, Hungary
Part B - by Aviah Zilberstein and Yoram Kapulnik, Israel.

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General Objectives:

Development of a proline over-production system in plants as a mean to increase stress withstanding.
Understanding of proline biosynthesis and degradation cycle in plants.
Elucidation of salinity effects on proline cycle.

Summary

In the course of this study, E. coli proline biosynthesis genes linked to constitutive plant promoters were introduced into plants and the gene products were targeted either to the cell cytosol or chloroplasts. The expression of the genes did not lead to a significant increase in proline content until the native proB was substituted by a proline indifferent mutant proB74. The combination of proA and proB74 encoding proteins that are imported to the chloroplast yielded about six-fold increase in proline levels in the transgenic tobacco and alfalfa plants.

P5CS (delta-pyrroline 5-carboxylate synthase) genes and cDNAs from alfalfa and Arabidopsis were isolated, characterized and their expression under salt stress (alfalfa and Arabidopsis) or different hormonal conditions (Arabidopsis) was evaluated.

The Arabidopsis cDNA linked to constitutive or root specific promoters was introduced into tobacco and led to increased proline accumulation.

A partial cDNA clone of proline dehydrogenase (PDh) was isolated from alfalfa and introduced, linked to the CaMV promoter, with or without proA and proB74 into tobacco and alfalfa. Transgenic plants are currently being analysed molecularly and physiologically.

The effect of salinity on transcript levels of P5CS and PDh was evaluated in young alfalfa plants. It was shown that the increase of proline content under stress is mainly due to the decrease in PDh transcription which is rapidly increased during recovery from stress.

In the physiological tests (salinity and drought) it was demonstrated that the transgenic tobacco plant (P9) accumulates higher level of proline under optimal growth conditions. A significant higher proline level was detected in wild type plants upon exposure to saline conditions, indicating that the regulation of proline accumulation processes were not affected by the introduced transgenes – not at the precursor level nor at the product feedback inhibition level. Although, as yet, a significant enhancement in plant development has not been recorded in the described preliminary physiological studies, a tendency of root growth improvement was noticed. Further experiments are now carried out under different growth conditions to unravel more effects of proline over-production.

Part A: BRC, Szeged, Hungary

1. Introduction.

The increase of salinity and drought tolerance of forage legumes is of principal importance in many countries. Our research project was initiated to evaluate the possibility to engineer alfalfa plants by increasing the internal concentrations of proline, which can function as osmoprotectant molecule in stressed cells (Delaunay and Verma, 1993). To achieve this goal, we try to increase the proline synthesis of the engineered plants by decreasing the feed-back inhibition of the first step in the proline biosynthetic pathway. In bacteria this step is metabolized by the g-glutamyl-kinase (GK), coded by the proB gene (Mahan and Csonka, 1983). In plants, a bifunctional enzyme, the Δ^1-pyrrolin-5-carboxylate synthetase (P5CS), is responsible for the first and secod step of proline synthesis (Hu et al., 1992). Both enzymes are subjected to feed-back inhibition by high concentrations of proline. The feed-back inhibition of the GK was eliminated in some bacterial mutants, resulting in the overproduction of proline and an increased tolerance to osmotic stress (Maha and Csonka, 1983, Csonka et al., 1988). Based on these data, we decided to evaluate, whether similarly engineered proline overproduction can result in increased osmotolerance in plants.

When out grant proposal was submitted, no information was available about plant, homologs of the bacterial proA or proB genes. The first publication about molecular cloning of the first plant proAB gene, coding for the Δ^1-pyrrolin-5-carboxylate synthetase from *Vigna acutifolia* , appeared later (Hu et al., 1992). Therefore, in the original proposal we suggested the introduction of a proline insensitive bacterial proB mutant gene into alfalfa. However, the possibility of using a plant gene for similar purposes is perhaps more attractive, because the codon usage of a bacterial proB gene might lead to inefficient translation in plant cells. At the start of the research, we agreed to evaluate both the bacterial proB and plant proAB genes in order to find an efficient system for engineering of the target plant. *Arabidopsis thaliana* , the model species of plant molecular biology (Redei et al., 1988), was choosen for cloning of the plant P5CS gene, because cDNA and genomic DNA phage libraries were available in the laboratory of Dr. Csaba Koncz, Max-Planck-Institut fur Zuchtungsforschung, Cologne, Germany.

2. Cloning of the *Arabidopsis* P5CS genes.

In order to clone cDNA homologous to the published *Vigna* proAB sequence, two cDNA libraries were screened with a synthetic oligonucleotide, whose sequence is

homologous to the conserved region of *E. coli* proB and *Vigna* proAB genes. Arabidopsis cDNA and genomic DNA phage libraries, prepared in lZapII and EMBL4 vectors respectively, were provided by Dr. Csaba Koncz, MPI, Cologne. Part of the cloning work and DNA sequencing was done in collaboration with Dr. Nikolai Strichov and Dr. C. Koncz, MPI, Cologne. The molecular methods were used according to Sambrook et al., (1989). Several clones were obtained and purified from both cDNA libraries. The inserts were subcloned into pBluescript plasmid and sequenced (Figure 1,2). Determination of the nucleotide sequence of the isolated cDNS and genomic clones was done using Pharmacia sequencing kit or with automated sequencing. Nucleotide sequence analysis of 6 clones (EMBL/Genbank X86777) from the seedling cDNA library revealed complete sequence identity with the P5CS cDNA from Savoure et al. (1995), and some mismatches in comparison to a similar sequence reported by Yoshiba et al. (1995). In contrast, 6 clones derived from the cell suspension cDNA library carried segments of a novel P5CS cDNA, which was converted to a full-length cDNA clone by 5'-RACE PCR amplification (Frohman et al., 1988, EMBL/Genbank Y09355). In comparison with the known AtP5CS1 cDNA, encoding the P5CS1 enzyme of 717 amino acids (77.7 kDa), the open reading frame of new AtP5CS2 cDNA consisted of 726 codons coding for the P5CS2 isoenzyme of 78.8 kDa. The two AtP5CS cDNAs shared an overall homology of 82%, yielding an amino acid similarity of 93% between the two isoenzymes (Figure 3, table 1). Close phylogenetic relationship of the plant-derived P5CS genes suggested conserved function of the encoded enzymes (Figure 4.).

Table 1. Sequence similarity of the AtproAB peptide sequences

Peptide	AtP5CS1	AtP5CS2
A. thaliana proAB1	100,0%	93,0%
A. thaliana proAB2	93,0%	100,0%
A. thaliana P5CS	99,6%	92,6%
V. aconitifolia P5CS	74,4%	86,3%
S. marcescens GSA dehydr.	62,4%	61,9%
S. marcescens g-GK	56,0%	57,3%
E. coli GSA dehydr.	62,2%	62,2%
E. coli g-GK	56,0%	56,8%
S. cerevisiae g-GK	49,9%	51,2%

In order to perform a functional test, the cloned *Arabidopsis* P5CS1 cDNA was introduced into a proline autotroph bacterial mutants, *E. coli* JM83 and CSH26. These

mutant are unable to grow on M9 minimal medium, in the absence of proline. Complementation of the E. coli proAB mutant was obtained when the 5' end of *Arabidopsis* AtP5CS1 cDNA was inserted next to the lac promoter in the pBluescript plasmid (Figure 1), but not in the inverted orientation (Table 2). These results showed, that the cloned AtP5CS1 cDNA codes for a functional P5CS enzyme which is active in *E. coli.*

Table 2. Complementation of the pro- mutant of *Escherichia coli* with the P5CS1 cDNA of *Arabidopsis thaliana*.. M9 medium was supplemented by 1 mM IPTG. Proline and ampicilline were used at the concentration of 20mM and 50 mg/l, respectively. Plasmids of pPC8-1 and pPC8-2 (Figure 1) were used for the complementation experiments.

Bacterium	M9	M9 +proline	LB	LB + Amp
JM83	-	+	+	-
JM83/PC8-1	-	+	+	+
JM83/PC8-2	+	+	+	+

3. Analysis of the *Arabidopsis* P5CS genes.

Sequence analysis of the *Arabidopsis* P5CS cDNA clones suggested, that two P5CS genes exist in the genome of *Arabidopsis thaliana* . Southern analysis confirmed the existence of two AtP5CS genes. Hybridization of EcoRI, XbaI and KpnI digested genomic DNA of *A. thaliana* (landraces Columbia wt. and Landsberg erecta) with gene specific cDNA probes resulted in clearly different hybridization patterns (Figure 5). Using the cDNA probes, a restriction fragment length polymorphism (RFLP) was found with KpnI for AtP5CS2, and a previously reported EcoRI RFLP for AtP5CS1 was confirmed (Savoure et al., 1995). The AtP5CS1 probe hybridized to overlapping YACs CIC10A6, CIC10A12 and CIC9E4, but not to the neighboring clones CIC10F12, CICG9 and CIC11C8. This allowed us to refine the previous mapping data (Savoure et al., 1995) indicating that AtP5CS1 is located at position 78.5 cM of chromosome 2, in close proximity of the RFLP marker 05841 between the left end of CIC2G9 and the right end of CIC11C8 YAC clones. The position of the AtP5CS2 gene and corresponding YAC-contig was mapped by linkage analysis, using recombinant inbreds (Lister and Dean, 1993) and the KpnI RFLP. The AtP5CS2 gene was located close to position 101.3 cM of chromosome 3 marked by the RFLP marker m457 (Figure 6).

Characterization of 4 genomic clones isolated with the AtP5CS2 probe resulted in a physical map clearly different from that of the AtP5CS1 locus (Figure 6; Savoure et al., 1995). Nucleotide sequencing showed that the transcribed region of the AtP5CS2 gene (EMBL/Genbank X86778) is 4.9 kb, including 20 exons (Figure 6). Comparison of the exon-intron structure of both AtP5CS genes indicated that the lengths of their exons were identical, except for exon 20. The homology between exon sequences of the AtP5CS genes ranged from 80% to 94%, whereas the intron sequences showed less conservation (from 39% to 65% identity) and a variation in length. The conserved 5'-splicing sites corresponded to AG/GT at 15 AtP5CS1 and 16 AtP5CS2 exon/intron junctions, whereas TG/GT occured at 4 splicing sites in AtP5CS1 and 3 sites in AtP5CS2. Results have been published in a scientific paper (Strizhov et al., 1997).

4. Expression of the Arabidopsis P5CS genes.

In order to characterize the transcription regulation of the two AtP5CS genes, Northern analysis was performed. In order to get gene specific hybridization probes , the divergent 3´ regions of the cDNA clones were amplified by PCR using a cDNA specific primer and the T7 sequencing primer of the pBluescript plasmid. The amplified and gel-purified DNA fragments were used to generate radiolabelled hybridization probes.

Total RNA was isolated from different organs (leaf, roots, stem, flowers, calli) of *A. thaliana* Col. wt. plants. RNA was isolated also from salt or drought-stressed or hormone-treated plants. 4 weeks-old in vitro-grown plants or cell suspension cultures were subjected to salt stress by transferring them to culture medium containing 200mM NaCl. Dessication treatment was performed by opening the lid of the culture wessels and subjecting the plants to continuous ventillation. Samples were collected by freezing the plants in liquid nitrogen at different timepoins after the treatment. RNA was isolated according to Pawlowski et al., (1994). Northern analysis was performed using the gene specific hybridization probes. Expression of the two AtP5CS genes was clearly different in both stressed and non-stressed plants. The AtP5CS-1 gene strongly expressed in leaves, stem and flower tissues, while AtP5CS-2 expression was higher in calli, and roots (Figure 7). Expression of both genes could be induced by salt and dessication treatment, although the increase in the accumulation of the AtP5CS-1 transcript was stronger in plant tissues (Figure 8). Accumulation of the AtP5CS-1 transcript could be detected at relatively low, non-lethal salt concentrations also, visible effect of salt on the plants could not be observed (Figure 8).

5. Construction of expression vectors and introduction of the AtP5CS gene constructs into tobacco.

The AtP5CS-1 cDNA was used to construct plant expression vectors. Expression cassettes were made in various binary plant transformation vectors, carrying different plant promoter constructs. The expression of the AtP5CS-1 cDNA was controlled by the constitutive pCaMV35S (pROK2), the light-induced rbcS (pROK8) the root-enhanced Srglb-35S (pLBP2) and Srglb-nos promoters (pLBP4) (Szabados et al., 1990; Figure 9). Agrobacterium-mediated transformation was used to introduce the constructed genes into tobacco (SR1). Kanamycin resistant transgenic plants were rooted and transferred to greenhouse. Plants were allowed to flower and set seeds.

Expression of the introduced AtP5CS gene in the transgenic tobacco plants was investigated by Northern RNA hybridization. Leaf material of the transgenic plants was used for RNA isolation, as described by Pawlowski et al., (1994). 20 μg of total RNA was electrophoresed in denaturing agarose gels, and transferred onto Hybond nylon membranes. Radiolabeled AtP5CS1 cDNA fragment was used to detect the expression of the introduced gene constructs (Figure 10). According to Northern hybridization data most of the regenerated transgenic tobacco plants expressed the introduced Arabidopsis cDNA, although notable differences were detected among the individually regenerated plants. Highest transcription rates were detected in the plant with the pROK2 plasmid, where the pCaMV35S promoter controls the expression of the AtP5CS1 gene (Figure 11).

Internal prolin concentration was investigated by testing the proline content in leaf, stem and root tissues of in vitro propagated or greenhouse-grown tobacco plants, as described (Bates, 1973). Several transgenic plants, expressing the AtP5CS1 gene in the sense orientation, accumulated higher proline concentrations than the non-transformed control (figure 12). None of the transgenic plants with the antisense AtP5CS1 gene construct had higher proline content than the control. These results suggest, that the constructed expression vectors can be used for proline overproduction.

Progenies of transgenic tobacco plants were tested in order to determine the ability of the foreign gene constructs to produce higher internal proline accumulation. Proline concentrations were repeatedly tested in stressed and non-stressed plants. Although great variability was found between the individual lines, in some plants increased proline concentrations could be detected. Proline accumulation in these non-stressed plants was 2-3 fold higher than in the control SR1 plants. During stress treatment sharp increase in proline accumulation could be detected in both transgenic and non-transgenic control

plants (Figure 13). Shoot and root growth was affected by NaCl treatment of both transgenic plants and the control SR1 plants. 0.2M NaCl had completely blocked shoot and root elongation of the SR1 plants, while some of the transgenic plants could maintain a reduced rate of shoot and/or root elongation (Figure 13). These results suggest the positive effect of proline accumulation in maintaining root and shoot viability in NaCl stress conditions.

6. Genetic transformation of alfalfa.

The proA and proB genes of Escherichia coli have been cloned to create pPCV-type plant expression vectors (Koncz and Schell, 1986), where the *pro* A or proB genes were put under the control of the constitutive CaMV35S promoter, and had plant polyA termination region. Some of the constructs were made with the rbcS transit peptide, facilitating the targeting of synthesized proteines into chloroplasts. The pPCV-proA vectors have the pnos-nptII-pAg4 selectable marker gene, conferring kanamycin resistance. In order to improve the efficiency of the gene constructs the proB74 mutation in the proB gene has been reconstructed and used for plant transformation. The pGif-proB vectors carry the pnos-hph-pAg4 gene construct, conferring hygromycin resistance to the transformed cells. The list of the expression vectors is given below:

Table 3. Binary vectors used in alfalfa transformation experiments.

pPCV-proA	(KmR)
pPCV-proATP	(KmR)
pGif-proB	(HygR)
pGif-proBTP	(HygR)
pGif-proB74	(HygR)
pGif-proB74TP	(HygR)

Culture and handling of the *Agrobacterium* strains was done according to Koncz et al., (1994). *Agrobacterium*-mediated genetic transformation of alfalfa (*Medicago sativa* RA3) was performed according to Deák et al., (1986), with some modifications. Culturing of the *Agrobacterium* strains was done according to Koncz et al., (1994). *Agrobacterium* cultures were prepared by inoculating liquid YEB culture medium, supplemented by apropriate antibiotics and cultured with continuous agitation at 28°C overnight, or until the culture reached OD 0,6. Bacteria were pelleted by centrifugation, and resuspended in MS liquid culture medium. Leaves and stem segments of in vitro propagated alfalfa plants were cut into 1cm pieces, and immersed into the bacterial

solution for 3 hrs. The explants were gently blotted on dry filter paper, and transferred onto agar-solidified callus induction medium, and incubated at 24°C for 2 days. After the *Agrobacterium* coincubation the explants were transferred onto callus induction medium, supplemented by 500 mg/l claforan, and incubated for 1 week. Explants were than transferred to callus induction medium, supplemented by 300 mg/l claforan and 100 mg/l kanamycin or 20 mg/l hygromycin, depending on the selection marker used. Explants were transferred to fresh culture medium at every 2-3 weeks, until calli developed on them. Callus cells were subjected to auxin shock, by incubating them on high 2,4-D containing (10 mg/l) medium for 4 days. Calli were transferred to hormone free regeneration medium, containing 100 mg/l claforan and kanamycin or hygromycin as described above, and subcultured at 2-3 weeks interwals. Developing embryos were separated from the calli and transferred to fresh culture medium, where they formed shoots and roots. Regenerated plantlets were propagated in vitro on hormonfree medium, and rooted shoots are transferred to greenhouse.

Several transformation experiments were initiated using the vector constructs. Numerous kanamycin or hygromycin resistant calli were obtained with each vector. Plants could be regenerated from the selected calli via somatic embryogenesis. Regenerated plantlets were propagated in vitro on hormon free medium, and rooted shoots were transferred to greenhouse.

7. Analysis of the transgenic alfalfa plants.

Transgenic alfalfa plants were subjected to RNA analysis, in order to determine the expression of the foreign pro genes. RNA isolation and Northern hybridization was performed as described befoire. Northern analysis resulted in the identification of transgenic alfalfa plants that expressed the introduced genes (Figure 14.). Variation in the expression level have been detected with each vector construct.

Alfalfa plants, transformed by the proA or proB gene constructs were used in physiological experiments, in order to determine their internal proline content. Stem cuttings of greenhouse grown alfalfa plants were rooted in hydroponic conditions, and proline content of the leaves was determined in three weeks old plantlets. Experiments were repeated in stressed conditions, when salt treatment was applied in the hydroponic cultures before proline determination. Proline determination was made according to Bates (Bates, 1973). Results of a typical experiment are shown in table 4. In our culture conditions, the internal proline content in the transgenic alfalfa plants was not significantly higher, when compared to the non-transformed controls. Only few plants had somewhat

higher proline contents. Upon salt treatment proline concentrations increased 8-12 times in leaves, and 2-4 times in roots.

Table 4. Determination of proline concentrations in transgenic alfalfa plants.
A2 or RA3 lines were transformed by proA or proB gene constructs. Stem cuttings were rooted in hydroponic conditions and proline content in leaf and root tissues was determined after 3 days of salt treatment (200mM in nutrient solution).
Legends: L/c: leaf, control, L/Na: leaf, NaCl treatment, R/c: root, control, R/Na: root, NaCl treatment

Medicago sativa RA3

Plant	L/c	L/Na	R/c	R/Na
R/proA2/3	0.90	11.42		
R/proB3	1.35	14.12	2.43	2.90
R/proB9	2.98	6.01	3.75	7.34
R/proBTP11	2.48	12.64	12.92	15.12
R/proBTP16	2.60	15.16		
R/proBTP2	0.89	10.63	19.39	28.93
R/proBTP23	0.67	12.40	0.71	3.70
R/proBTP4	0.42	6.79	0.70	0.64
R/proBTP7	0.62	17.15	1.49	0.93
RA3/c	1.24	2.33	12.20	14.90

Medicago varia A2

Plant	L/c	L/Na	R/c	R/Na
A/proATP1	1.93	6.44	3.08	7.58
A/proATP2	2.18	4.37	4.89	14.06
A/proATP3	3.05	8.29		
A/proATP5	1.24	6.14	0.50	10.31
A/proB1	2.11	4.77	5.30	8.50
A/proB10	0.05	8.69	0.21	0.51
A/proB13	3.21	8.81		
A/proB14	0.58	10.85	5.18	10.03
A/proB15	1.48	13.28	1.53	1.40
A/proB17	1.09	1.87	12.30	16.35
A/proB19	0.75	11.56	8.38	12.92
A/proB20	0.49	11.26	2.36	1.63
A/proB3	1.48	14.03		
A/proB30	0.09	11.30	0.38	3.50
A/proB5	0.46	8.78	0.05	1.06
A/proB6	0.75	13.78	5.65	7.85
A/proB7	4.02	13.93		
A/proB8	1.05	10.51	2.58	10.93
A/proBTP2	0.30	10.56	0.73	3.19
A/proBTP3	1.48	6.26	1.90	10.07
A/proBTP4	0.59	23.89	2.06	4.54
A2/c	1.50	3.15	3.8	4.43

As plants have a bifunctional P5CS enzyme, possessing both γ-Glutamyl kinase and P5C dehydrogenase activity, it is possible, that increase in the γ-GK activity by the bacterial proB gene constructs does not necessarily result in significant change in proline synthesis. In order to combine the proB and proA genes in the same transgenic plant, second transformation experiments were initiated, using transgenic alfalfa plants, where the expression of the introduced proA gene could be detected by RNA hybridization (e.g. A2/proA-1, A2/proATP5). The new transformation experiments were initiated using a modified proB74 construct, where the feed-back inhibition site of the γ-glutamyl kinase protein is removed. Plant transformation and regeneration was made as described above. Expression of the introduced genes in the transgenic plants was analysed by Northern hybridization. To our surprise, expression of the previously introduced proA gene could not allways been detected (Figure 15.). Several plants were identified, that expressed both proA and proB genes (Figure 16.). In order to compare the efficiency of the bacterial proA/proB and plant-derived P5CS genes, transgenic alfalfa plants were generated using the pLBP4 vector as well.

Proline content of in in-vitro propagated transgenic plants was determined as described above. 2-4 times increase in proline accumulation was detected in several double transformed transgenic alfalfa lines (Table 5.).

The data of table 5 shows, that double transformation resulted in a 2-3 times increase in proline content of some of the tested transgenic alfalfa lines when compared to untransformed controls. Increase of the internal proline content of RA3 plants was somewhat higher, when the feedback insensitive proB74 gene was combined with proA. Transgenic alfalfa plants have been and are being transferred to greenhouse. Proline content of plants expressing the introduced proA and proB genes was measured in a few greenhouse-grown plants as well. When compared to non-transformed control, or proA-transformed plants, increased proline concentrations were detected in some plants, expressing the proB74 gene with the chloroplast targeting signal (Figure 17). These plant are being propagated in order to evaluate their stress tolerance.

In order to evaluate the possibility to use the Arabidopsis AtP5CS1 gene for engineering the proline concentrations of alfalfa, transformation experiments were recently initiated with the pLBP-P5CS1 constructs. Several kanamycin resistant shoots has been obtained in this experiments. Proline concentration has been determined in these in-vitro propagated plantlets. Increase in the internal proline concentration has been Increase in the internal proline concentration has been detected in at least one transformed shoot (Table 6.). Further evaluation of the regenerated plants will have to confirm the expression of the introduced gene, and the effect on osmotic stress tolerance.

Table 5. Proline content of in-vitro propagated transgenic alfalfa lines posessing proA and proB genes. A2 and RA3 untransformed control plants are indicated by bold letters. Transgenic plants carrying the proA gene, that were used for re-transformation are indicated by italics. Plants with * carries mutant proB74 gene, and T indicates constructs with transit peptides.

Medicago varia line A2

Lines	proline (mM)
A2	**37,5**
A2/A1	*44,8*
A2/AB11	21,6
A2/AB5	40,3
A2/AB2	50,3
A2/AB*20	40,0
A2/AB*21	28,7
A2/AT3	*36.5*
A2/ABT3	60,0
A2/ABT31	48,8
A2/ABT9	50,2
A2/ABT36	30,2
A2/ABT15	41,8
A2/ABT27	73,8
A2/ABT38	83,2

Medicago sativa line RA3.

Lines	proline (mM)
RA3	**38,8**
R/A2	*44,2*
R/AB5	60,6
R/AB6	43,6
R/AB7	53,7
R/AB14	69,7
R/AB17	59,6
R/AB19	50,7
R/AB22	55,0
R/AB24	56,6
R/AB*9	68,4
R/AB*29	59,6
R/AB*11	70,2
R/AB*18	54,7
R/AB*15	97,6
R/AB*66	72,2
R/AB*73	42,3
R/AB*25	29,3
R/AB*71	59,7

Table 6. Proline content of transgenic Medicago varia A2 lines carrying the pLBP constructs.

Medicago varia line A2

Lines	proline (mM)
A2	37,5
A2/L4-1	56,5
A2/L4-2	131,4
A2/L4-3	49,6
A2/L2-B	49,7

8. References.

Bates, L.S. (1973) Rapid determination of free proline content for water-stress studies. Plant Soil. 39:205-207

Lister, C. and Dean, C. (1993) Recombinant inbred lines for mapping RFLP and phenotypic markers in Arabidopsis thaliana. Plan J. 4, 745-750.

Mahan, M.J., and Csonka, L.N. (1983) Genetic analysis of the proBA genes of *Salmonella typhimurinum*: physical and genetic analysis of the cloned proB+ A+ genes of *Escherichia coli* and of a mutant allele that confers proline overproduction and enhanced osmotolerance. J. Bacteriol. 156:1249-1262.

Pawlowski, K., Kunze, R., de Vries,S. and Bisseling, T. (1994) Isolation of total poly(A) and polysomal RNA from plant tissues. In Plant Molecular Biology Manual, Volume D5, (Gelvin, S.G. and Schilperoort, R.A., eds.). Dordrecht: Kluwer Academic Publ., pp. 1-13.

Rédei, G.P., Koncz, C. and Schell, J. (1988) Transgenic Arabidopsis. In: Chromosome Structure and Function. (Eds.: J.P.Gustafson, R.Appels), Plenum Press, New York, pp. 175-200.

Sambrook, J., Fritsek, E.F., Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, CSHL Press, Cold Spring Harbour.

Savoure, A., Jaoua, S., Hua, X.-J., Ardiles, W. and Van Montagu, M. (1995) Isolation, characterization and chromosomal location of a gene encoding the D1-pyrroline-5-carboxylate synthase in *Arabidopsis thaliana*. FEBS Lett. 372, 13-19.

Strichov, N., Ábrahám, E., Ökrész, L., Blickling, S., Zilberstein, A., Schell, J., Koncz, C., Szabados, L., (1997) Differential expression of two P5CS genes controlling proline accumulation during salt-stress is regulated by *ABA1, ABI1 and AXR2* in *Arabidopsis*. Plant J. 12:557-569.

Szabados, L., Ratet, P., Grunenberg, B., de Bruijn, F. (1990) Functional analysis of the Sesbania rostrata leghemoglobin glb3 gene 5'-upstream region in transgenic Lotus corniculatus plants. Plant Cell 2:973-986.

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

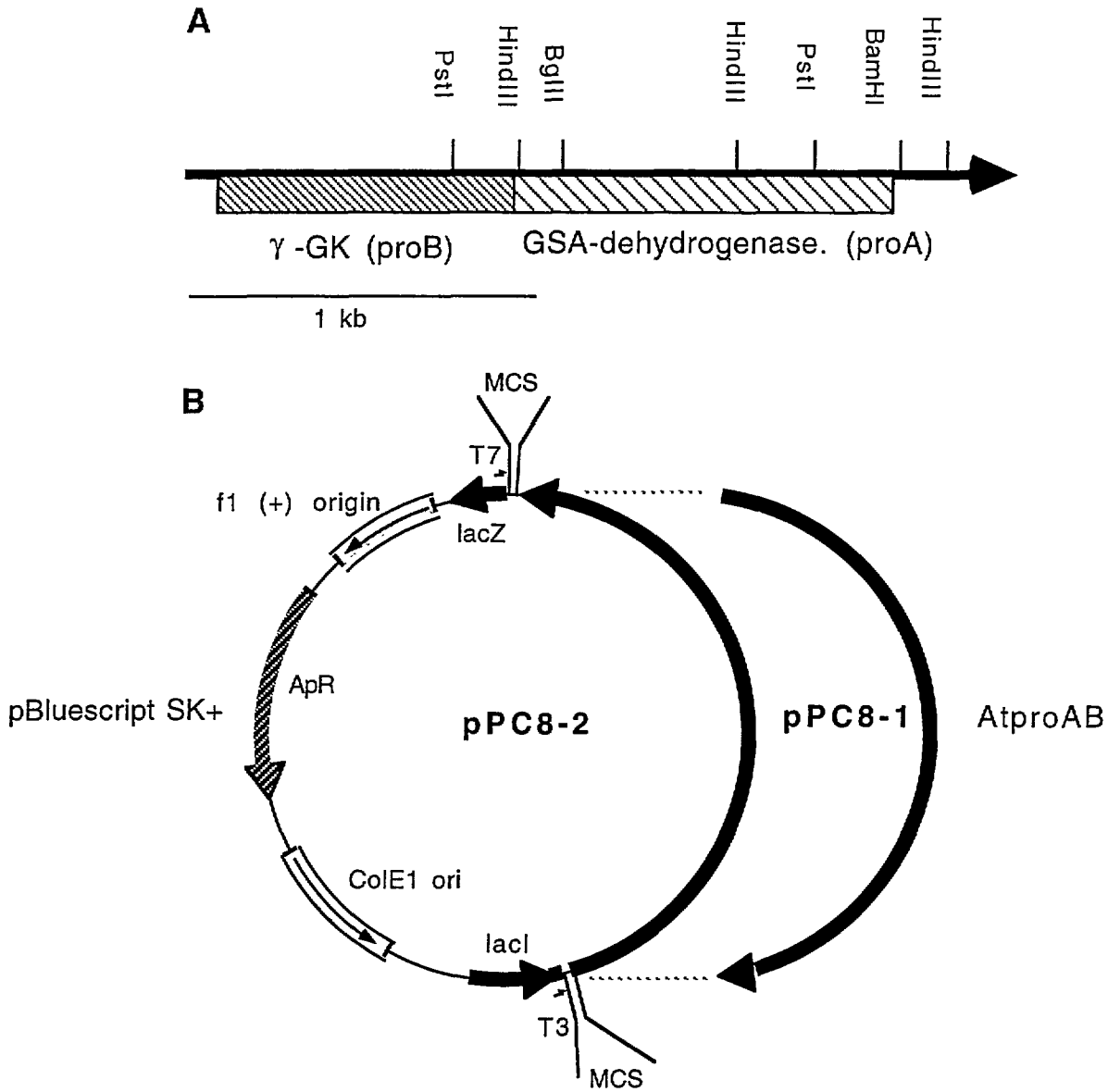

Figure 1. Map of the longest AtP5CS1 (AtproAB) cDNA clone isolated from *Arabidopsis thaliana*.. A. Restriction map showing the regions coding for the g-glutamyl-kinase (homologous to proB) and GSA dehydrogenase (homologous to proA). B. Map of the plasmids pPC8-1 and pPC8-2 having the same cDNA clone in inverted orientation.

```
   1  ATTCCCGGGC AGGTGCTTCA CTGAGTCCGA CTCAGTTAAC TCGTTCCTCT
  51  CTCTGTGTGT GGTTTTGGTA GACGACGACG ACGATAATGG AGGAGCTAGA
 101  TCGTTCACGT GCTTTTGCCA GAGACGTCAA ACGTATCGTC GTTAAGGTTG
 151  GGACAGCAGT TGTTACTGGA AAAGGTGGAA GATTGGCTCT TGGTCGTTTA
 201  GGAGCACTGT GTGAACAGCT TGCGGAATTA AACTCGGATG GATTTGAGGT
 251  GATATTGGTG TCATCTGGTG CGGTTGGTCT TGGCAGGCAA AGGCTTCGTT
 301  ATCGACAATT AGTCAATAGC AGCTTTGCGG ATCTTCAGAA GCCTCAGACT
 351  GAACTTGATG GGAAGGCTTG TGCTGGTGTT GGACAAAGCA GTCTTATGGC
 401  TTACTATGAG ACTATGTTTG ACCAGCTTGA TGTGACGGCA GCTCAACTTC
 451  TGGTGAATGA CAGTAGTTTT AGAGACAAGG ATTTCAGGAA GCAACTTAAT
 501  GAAACTGTCA AGTCTATGCT TGATTTGAGG GTTATTCCAA TTTTCAATGA
 551  GAATGATGCT ATTAGCACCC GAAGAGCCCC ATATCAGGAT TCTTCTGGTA
 601  TTTTCTGGGA TAACGATAGC TTAGCTGCTC TACTGGCGTT GGAACTGAAA
 651  GCTGATCTTC TGATTCTTCT GAGCGATGTT GAAGGTCTTT ACACAGGCCC
 701  TCCAAGTGAT CCTAACTCAA AGTTGATCCA CACTTTTGTT AAAGAAAAAC
 751  ATCAAGATGA GATTACATTC GGCGACAAAT CAAGATTAGG GAGAGGGGGT
 801  ATGACTGCAA AAGTCAAAGC TGCAGTCAAT GCAGCTTATG CTGGGATTCC
 851  TGTCATCATA ACCAGTGGGT ATTCAGCTGA GAACATAGAT AAAGTCCTCA
 901  GAGGACTACG TGTTGGAACC TTGTTTCATC AAGATGCTCG TTTATGGGCT
 951  CCGATCACAG ATTCTAATGC TCGTGACATG GCAGTTGCTG CGAGGGAAAG
1001  TTCCAGAAAG CTTCAGGCCT TATCTTCGGA AGACAGGAAA AAAATTCTGC
1051  TTGATATTGC CGATGCCCTT GAAGCAAATG TTACTACAAT CAAAGCTGAG
1101  AATGAGTTAG ATGTAGCTTC TGCACAAGAG GCTGGGTTGG AAGAGTCAAT
1151  GGTGGCTCGC TTAGTTATGA CACCTGGAAA GATCTCGAGC CTTGCAGCTT
1201  CAGTTCGTAA GCTAGCTGAT ATGGAAGATC CAATCGGCCG TGTTTTAAAG
1251  AAAACAGAGG TGGCAGATGG TCTTGTCTTA GAGAAGACCT CATCACCATT
1301  AGGCGTACTT CTGATTGTTT TTGAATCCCG ACCTGATGCA CTTGTACAGA
1351  TAGCTTCACT TGCCATCCGT AGTGGAAATG GTCTTCTGCT GAAGGGTGGA
1401  AAGGAGGCCC GGCGATCAAA TGCTATCTTA CACAAGGTGA TCACTGATGC
1451  AATTCCAGAG ACTGTTGGGG GTAAACTCAT TGGACTTGTG ACTTCAAGAG
1501  AAGAGATTCC TGATTTGCTT AAGCTTGATG ACGTTATCGA TCTTGTGATC
1551  CCAAGAGGAA GCAACAAGCT TGTTACTCAG ATAAAAAATA CTACAAAAAT
1601  CCCTGTGCTA GGTCATGCTG ATGGAATCTG TCATGTATAT GTCGACAAGG
1651  CTTGTGATAC GGATATGGCA AAGCGCATAG TTTCTGATGC AAAGTTGGAC
1701  TATCCAGCAG CCTGTAATGC GATGGAAACC CTTCTTGTGC ATAAGGATCT
1751  AGAGCAGAAT GCTGTGCTTA ATGAGCTTAT TTTTGCTCTG CAGAGCAATG
1801  GAGTCACTTT GTATGGTGGA CCAAGGGCAA GTAAGATACT GAACATACCA
1851  GAAGCACGGT CATTCAACCA TGAGTACTGT GCCAAGGCTT GCACTGTTGA
1901  AGTTGTAGAA GACGTTTATG GTGCTATAGA TCACATTCAC CGACATGGGA
1951  GTGCACACAC AGACTGCATT GTGACAGAGG ATCACGAAGT TGCAGAGCTA
2001  TTCCTTCGCC AAGTGGATAG CGCTGCTGTG TTCCACAACG CCAGCACAAG
2051  ATTCTCAGAT GGTTTCCGAT TTGGACTTGG TGCAGAGGTG GGGGTAAGCA
2101  CGGGCAGGAT CCATGCTCGT GGTCCAGTCG GGGTCGAAGG ATTACTTACA
2151  ACGAGATGGA TAATGAGAGG AAAAGGACAA GTTGTCGACG GAGACAATGG
2201  AATTGTTTAC ACCCATCAGG ACATTCCCAT CCAAGCTTAA ACAAGACTTC
2251  CGAGTGTGTG TTTGTGTATT TGGTTGAGAC TTGAGGAGAG ACACAGAGGA
2301  GGATGGGCTT TTTTGTTTCC TCTCTGCTTA GTACTCACCT GCCCGGG
```

Figure 2. Sequence of the AtP5CS1 cDNA from *Arabidopsis thaliana*. Translation initiation and termination sites are underlined.

A.

```
ATPAB2    MTE-IDRSRAFAKDVKRIVVKVGTAVVTGKGGRLALGRLGAICEQ-VAEL
ATPAB1    MEE-LDRSRAFARDVKRIVVKVGTAVVTGKGGRLALGRLGALCEQ-LAEL
ATP5CS    MEE-LDRSRAFARDVKRIVVKVGTAVVTGKGGRLALGRLGALFEQ-LAEL
VAPAB     MESAVDPSRGFMKDVKRVIIKVGTAVVTREEGRLAVGRLGALCEQ-IKQL
YPB       MKDANE-SKSYT-----IVIKLGSSSLVDEKTKEPKLAIMSLIVETVVKL
ECPB      MSDSQ------T-----LVVKLGTSVLTGGSRRLNRAHIVELVRQCA-QL
SMPB      MNGSQ------T-----LVVKLGTSVLTGGSLRLNRAHIVELVRQCA-QQ
          *                ...*.*.. .     .     .  .  .

ATPAB2    NSDGFEVILVSSGAVGLGRQRLRYRQLVNSRFADLQKPQMELDGKACAGV
ATPAB1    NSDGFEVILVSSGAVGLGRQRLRYRQLVNSSFADLQKPQTELDGKACAGV
ATP5CS    NSDGFKVILVSSGAVGLGRQRLRYRQLVNSSFADLQKPQTELDGKACAGV
VAPAB     NSLGYDIILVSSGPVGIGRQRLRFRKLINSSFADLQKPQLELDGKACAAV
YPB       RRMGHKVIIVSSGGIAVGLRTMRMNKR----------PKHLAEVQAIAAI
ECPB      HAAGHRIVIVTSGAIAAGREHLGYPEL----------PATIASKQLLAAV
SMPB      HAAGHRIVIVTSGAIAAGREHLGYPEL----------PATIASKQLLAAV
             *  ...*.** .. *   .               *      .  *..

ATPAB2    GQSSLMAYYETMFDQVDVTVAQ-MLV-TDSSFRDKDFRKQLSETVKAMLRMR
ATPAB1    GQSSLMAYYETMFDQLDVTAAQ-LLV-NDSSFRDKDFRKQLNETVKSMLDLR
ATP5CS    GQSSLMAYYETMFDQLDVTAAQ-LLV-NDSSFRDKDFRKQLNETVKSMLDLR
VAPAB     GQNSLMALYDTLFTQLDVTSAQ-LLV-TDNDFRDKDFRKQLTETVKSLLALK
YPB       GQGRLIGRWDLLFSQFDQRIAQILLTRNDI--LDWTQYKNAQNTINELLNMG
ECPB      GQSRLIQLWEQLFSIYGIHVGQMLLTRADM--EDRERFLNARDTLRALLDNN
SMPB      GQSRLIQLWEQLFSIYGIHVGQMLLTRADL--EDRERFLNVRDTMTALLDNR
          **  *.   . .*   .   .* .*.  *    *     .  .*.  .*

ATPAB2    VIPVFNENDAISTRRAPYKDSTGIFWDNDSLAALLSLELKADLLILLSDV
ATPAB1    VIPIFNENDAISTRRAPYQDSSGIFWDNDSLAALLALELKADLLILLSDV
ATP5CS    VIPIFNENDAISTRRAPYQDSSGIFWDNDSLAALLALELKADLLILLSDV
VAPAB     VIPVFNENDAVSTRKAPYEDSSGIFWDNDSLSALLALELKADLLVLLSDV
YPB       VIPIVNENDTLSVREIKFG-------DNDTLSAITSALIHADYLFLLTDV
ECPB      IVPVINENDAVATAAIKVG-------DNDNLSALAAILAGADKLLLLTDQ
SMPB      IVPVINENDAVATAEIKVG-------DNDNLSALAAILAGADKLLLLTDQ
          ..*. ****...              *** *.*. .    ** * **.*

ATPAB2    EGLYTG-PPSDSTSKLI---HTFIKEKHQDEITFGEKSKLGRGGMTAKVK
ATPAB1    EGLYTG-PPSDPNSKLI---HTFVKEKHQDEITFGDKSRLGRGGMTAKVK
ATP5CS    EGLYTG-PPSDPNSKLI---HTFVKEKHQDEITFGDKSRLGRGGMTAKVK
VAPAB     EGLYSG-PPSDPHSKLI---YTYNKEKHQNEITFGDKSRVGRGGMTAKVK
YPB       DCLYTDNPRTNPDAMPILVVPDLSKGLPGVNTAGGSGSDVGTGGMETKLV
ECPB      KGLYTADPRSNPQAELIKDVYGIDDALRAI--AGDSVSGLGTGGMSTKLQ
SMPB      QGLYTADPRNNPQAELIREVHGIDDALRAI--AGDSVSGLGTGGMGTKLQ
            **.  *  .. .  *                . .  * .* *** .*.

ATPAB2    AAVNAAYGGVPVIITRGYAAENISKVLRGLRVGTLFHQDAHLWAPVVDTTSR
ATPAB1    AAVNAAYAGIPVIITSGYSAENIDKVLRGLRVGTLFHQDARLWAPITDSNAR
ATP5CS    AAVNAAYAGIPVIITSGYSAENIDKVLRGLRVGTLFHQDARLWAPITDSNAR
VAPAB     AAVHAAEAGIPVVITSGFAPENIINVLQGQRIGTLFHKDAHEWAQVKEVDAR
YPB       AADLATNAGVHTLIMKSDTPANIGRIVEYMQTLELDDENKVKQAYNGDLTDL
ECPB      AADVACRAGIDTIIAAGSKPGVIGDVMEGISVGTLFHAQATPLENRKRWIFG
SMPB      AADVACRAGIDVVIAAGSKPGVVADVIEGKPVGTRFHALETPLENRKRWIFG
          **  *  .*.  .*  .  .  .  .. .   .....

YPB       QKREFEKLKALNVPLHTKFIANDNKHHLKNREFWILHGLVSKGAVVI--D
ECPB      ----------------------------------------APPAGEITVD
SMPB      ----------------------------------------APPAGEITVD

YPB       QGAYRALTRKNKAGLLPAGVIDVQGTFHELECVDIKVGKKLPDGTLDPDF
ECPB      EGATAAILERG-SSLLPKGIKSVTGNFSRGEVIRI------------CNL
SMPB      DGAVEAMMARG-SSLLPKGIREVKGDFSRGEVIRI------------RNL
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B.
ATPAB2      DMAVAA---RESSRKLQALSSEDRKQILHDIANALEVNEKTIKAENDLDV
ATPAB1      DMAVAA---RESSRKLQALSSEDRKKILLDIADALEANVTTIKAENELDV
ATP5CS      DMAVAA---RESSRKLQALSSEDRKKILLDIADALEANVTTIKAENELDV
VAPAB       EMAVAAGNVREGSRRYLQRKGN---KILLKIADALEANEKIIRIENEADV
ECPA        MLEQMGIAAKQASYKLAQLSSREKNRVLEKIADELEAQSEIILNANAQDV
SMPA        MLEQMGKAAKQASWQLAVLSTAKKNQVLSVMADRLEANSEAILLANEQDM
             .   .   ...* .          ..*  .*. ** .   *   *  *.

ATPAB2      AAAQEAGYEESLVARLVMKPGKISSLAASVRQLAEMEDPIGRVLKKTQVA
ATPAB1      ASAQEAGLEESMVARLVMTPGKISSLAASVRKLADMEDPIGRVLKKTEVA
ATP5CS      ASAQEAGLEESMVARLVMTPGKISSLAASVRKLADMEEPIGRVLKKTEVA
VAPAB       TAAQEAGYEKSLVARLALKPGKIASLANNMRIIANMEDPIGRVLKRTELS
ECPA        ADARANGLSEAMLDRLALTPARLKGIADDVRQVCNLADPVGQVIDGGVLD
SMPA        AQARATGMSEALLDRLLLTPARLAAIANDVRQVCRLNDPVGHVLDGNLLD
            . *.  *   ... ** . *... ..*  .* .  . .*.*.*.    .

ATPAB2      DDLILEKTSSPIGVLLIVFEFRPDALVQIASLAIRSGNGLLLKGGKEARR
ATPAB1      DGLVLEKTSSPLGVLLIVFESRPDALVQIASLAIRSGNGLLLKGGKEARR
ATP5CS      DGLVLEKTSSPLGVLLIVFESRPDALVQIASLAIRSGNGLLLKGGKEARR
VAPAB       DGLILEKTSSPLGVLLIVFESRPDALVQIASLAIRSGNGLLLKGGKEAKR
ECPA        SGLRLERRRVPLGVIGVIYEARPNVTVDVASLCLKTGNAVILRGGKETCR
SMPA        SGLKLERRRVPLGVIGVIYEARPNVTIDVASLCLKTGNAVILRGGKETHN
             .* **.   *.**. ...* **.  ...*** ...**...*.****.

ATPAB2      SNAILHKVITDAIPE-TVGGKLIGLVTS--REEIPDLLKLDDVIDLVIPR
ATPAB1      SNAILHKVITDAIPE-TVGGKLIGLVTS--REEIPDLLKLDDVIDLVIPR
ATP5CS      SNAILHKVITDAIPE-TVGGKLIGFVTS--REEIPDLLKLDDVIDLVIPR
VAPAB       SNAILHKVIIEAIPD-NVGGKLIGLVTS--REEIPELLKLDDVIDLVIPR
ECPA        TNAATVAVIQDALKSCGLPAGAVQAIDNPDRALVSEMLRMDKYIDMLIPR
SMPA        TNQATVKVIQQALEQCGLPAAAVQAIDSPDRALVNELLRLDRYVDMLIPR
            .*     ** .*.    . .  .  . .  *  . ..*..*  .*..***

ATPAB2      GSNKLVSQIKNSTKIPVLGHADGICHVYVDKSGKLDMAKRIVSDAKLDYP
ATPAB1      GSNKLVTQIKNTTKIPVLGHADGICHVYVDKACDTDMAKRIVSDAKLDYP
ATP5CS      GSNKLVTQIKNTTKIPVLGHADGICHVYVDKACDTDMAKRIVSDAKLDYP
VAPAB       GSNKLVSQIKSSTKIPVLGHADGICHVYVDKSANVEMAKRIVLDAKVDYP
ECPA        GGAGLHKLCREQSTIPVITGGIGVCHIYVDESVEIAEALKVIVNAKTQRP
SMPA        GGAGLHKLCREQSTIPVITGGIGVCHTYVDADVDFDKALTVIENAKIQRP
            *.  *    .  . ***.  . *.** ***       *  .. .** . *

ATPAB2      AACNAMETLLVHKDLEQNGFLDDLIYVLQTKGVTLYGGPRASAKLNIPET
ATPAB1      AACNAMETLLVHKDLEQNAVLNELIFALQSNGVTLYGGPRASKILNIPEA
ATP5CS      AACNAMETLLVHKDLEQNAVLNELIFALQSNGVTLYGGPRASKILNIPEA
VAPAB       AACNAMETLLIHKDLIEKGWLKEIILDLRTEGVILYGGPVASSLLNIPQA
ECPA        STCNTVETLLVNKNIADS-FLPALSKQMAESGVTLHADAAALAQLQAGPA
SMPA        SACNSLETLLVNRSIAAE-FLPALSAKMAAAGVTLHAAENALPLLQGGPA
            ..**..****... .     *  .   .   ** * .   *   *.   .

ATPAB2      K-------SFHHEYSSKACTVEIVEDVYGAIDHIHQHGSAHTDCIVTEDS
ATPAB1      R-------SFNHEYCAKACTVEVVEDVYGAIDHIHRHGSAHTDCIVTEDH
ATP5CS      R-------SFNHEYCAKACTVEVVEDVYGAIDHIHRHGSAHTDCIVTEDH
VAPAB       H-------SFHHEYSSLACTAEIVDDVYAAIDHINLYGSAHTDSIVAEDN
ECPA        KVVAVKAEEYDDEFLSLDLNVKIVSDLDDAIAHIREHGTQHSDAILTRDM
SMPA        TVVPVNAEDYDDEWLSLDLNVLLVDDIDQAIDHIRTHGTNHSDAILTRSL
                      ...*  .      .* *.  ** **   *. *.* *..

ATPAB2      EVAEIFLRQVDSAAVFHNASTRFSDGFRFGLGAEVGISTSRIHARGPVGV
ATPAB1      EVAELFLRQVDSAAVFHNASTRFSDGFRFGLGAEVGVSTGRIHARGPVGV
ATP5CS      EVAELFLRQVDSAAVFHNASTRFSDGFRFGLGAEVGVSTGRIYARGPVGV
VAPAB       EVANVFLRQVDSAAVFHNASTRFSDGARFE-----------TRRRG----
ECPA        RNAQRFV----------NASTRFTDGGQFGLGAEVAVSTQKLHARGPMGL
SMPA        SSAEHFVRAVDSSAVYVNASTRFTDGGQFGLGAEVAVSTQKLHARGPMGL
              *. *.          ******.** .*                **
```

Figure 3. Comparison of the deduced amino acid sequences of the P5CS, g-GK (A), and GSA-dehydrogenase (B). ATPAB1, ATPAB2: new*A. thaliana* proAB clones; ATP5CS: P5CS of *A. thaliana* , VAPAB: P5CS of *V. aconitifolia*, YPB: g-GK of yeast; ECPB: g-

GK of *E.coli* ; SMPB: g-GK of *S.marcescens* . ECPA: GSA dehydrogenase of *E.coli* ; SMPA: GSA dehydrogenase of *S.marcescens* . Identical amino acids are indicated by *.

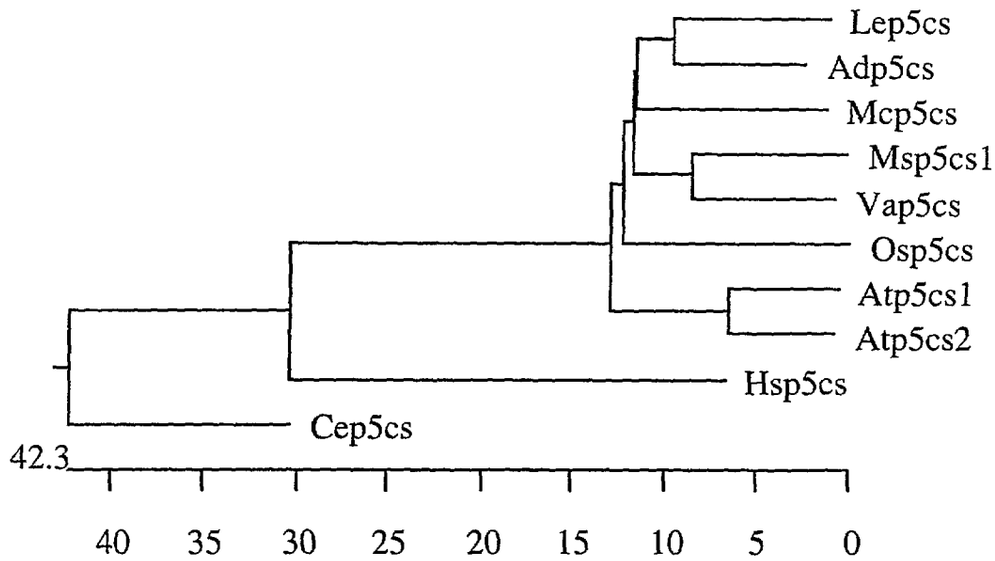


Figure 4. Phylogenetic relationship between reported P5CS sequences.
Legend: Lep5cs: *Lycopersicum esculentum* (Garcia-Rios et al., 1997); Adp5cs: *Actinidia deliciosa* (Walton et al., 1998), Mcp5cs: *Mesembryanthemum crystallinum* (Michalowski, unpublished), Msp5cs: *Medicago sativa* (Ginzberg et al., 1998); Vap5cs: *Vigna aconitifolia* (Hu et al., 1992); Osp5cs: *Oryza sativa* (Igarashi et al., 1997); Atp5cs: *Arabidopsis thaliana* (Yoshiba et al., 1995; Savoure et al., 1995; Strichov et al., 1997); Hsp5cs: *Homo sapiens* (Aral et al., 1996); Cep5cs: *Caenorhabditis elegans* (McMurray, unpublished).

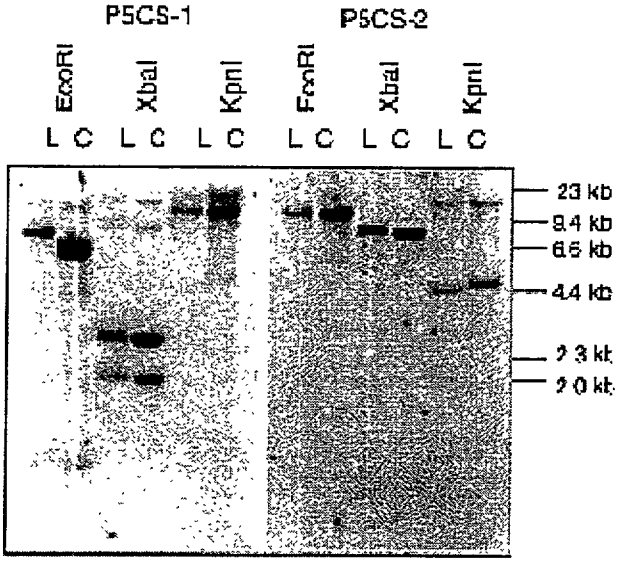


Figure 5. Genomic Southern blot analysis of the AtP5CS-1 and AtP5CS-2 genes in *Arabidopsis thaliana*. Genomic DNA of landraces Landsberg erecta (L) and Columbia wt. (C) was digested with the restriction enzymes EcoRI, XbaI and KpnI, blotted and probed with radiolabelled AtP5CS-1 and AtP5CS-2 cDNA. Note the differences in the hybridization patterns. EcoRI fragment length polymorphism was detected by the AtP5CS-1 probe, and the KpnI fragment length polymorphism was detected by the AtP5CS-2 probe.

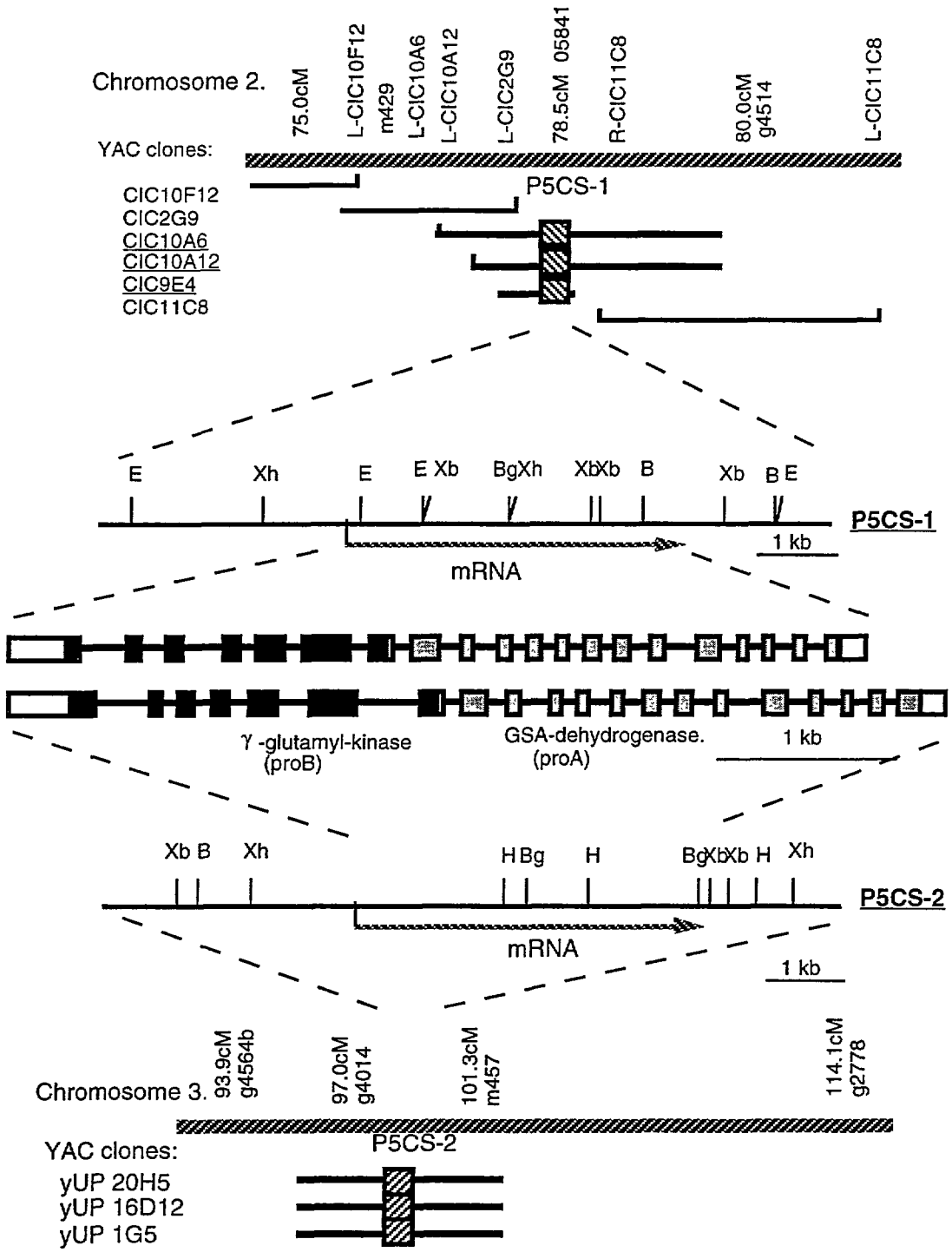


Figure 6. Gene structure and chromosomal localization of the AtP5CS1 and AtP5CS2 genes.

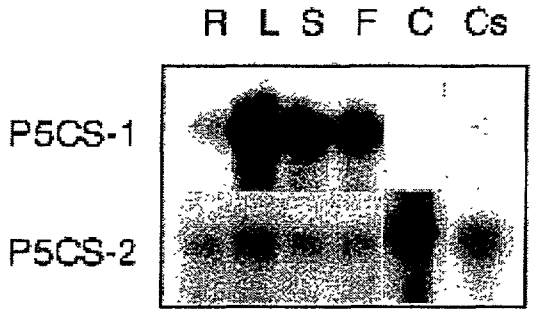


Figure 7. Nothern blot analysis of the AtP5CS-1 and AtP5CS-2 transcripts in different plant organs. 20 mg of total RNA isolated from roots (R), leaves (L), stems (S), flowers (F) callus (C) and cell suspension (CS) was hybridized with the radiolabelled gene specific 3´ fragments of the AtP5CS-1 and AtP5CS-2 cDNA.

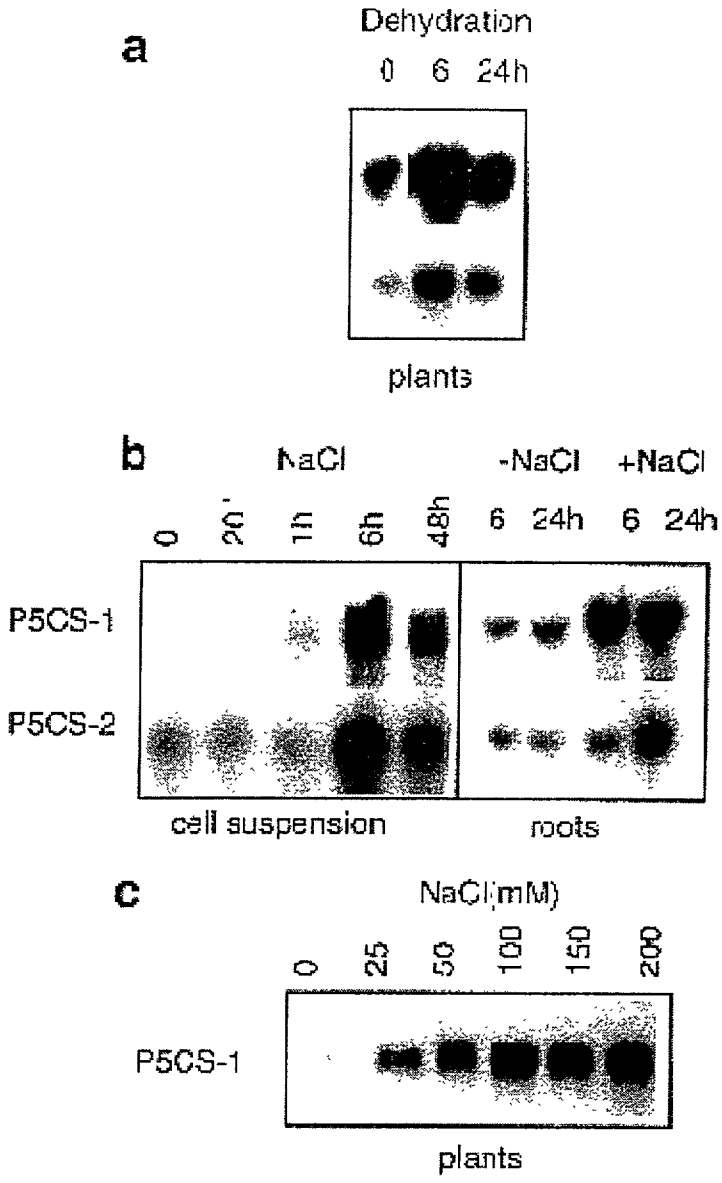


Figure 8. Effect of osmotic stress on the expression of the AtP5CS genes. A) Influence of dehydration stress on the accumulation of AtP5CS transcripts in *Arabidopsis* plants. 4 weeks old, in vitro grown plants were dehydrated for 6 or 24 hours. B). Effect of salt stress on the accumulation of the AtP5CS transcripts in cell suspensions and root cultures. NaCl was added to the medium of cell suspension culture of *A. thaliana* at the final concentration of 200mM and RNA was extracted from samples taken at various timepoints: 0 min, 20 min, 1hr., 6 hrs., 48 hrs. Three weeks old root cultures of *A. thaliana* were stressed for 6 and 24 hours, by 200mM NaCl. C) Accumulation of the AtP5CS-1 transcript in *Arabidopsis* plants cultured on agar solidified SG medium, containing various concentrations of NaCl, for 8 days.

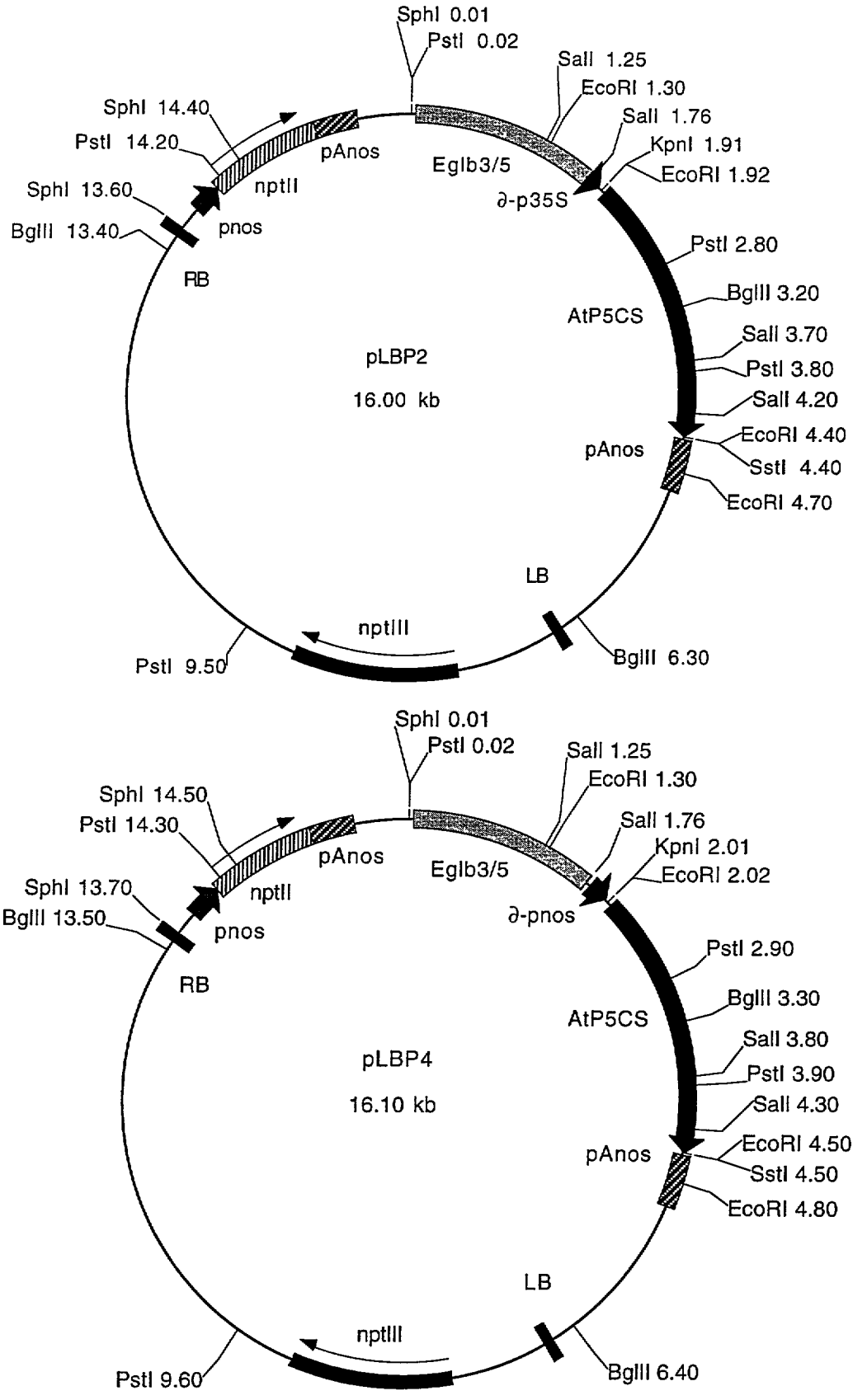


Figure 9. Map of the pLBP2 and pLBP4 binary expression vectors carrying the AtP5CS cDNA under the control of the chimaeric Srglb3-pnos or Srglb3-p35S promoters. pLBP3 is the antisense version of pLBP2.

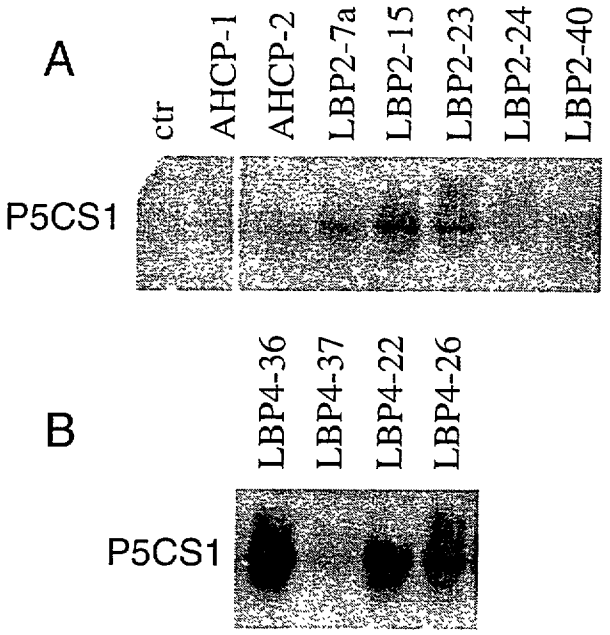


Figure 10. Expression of the AtP5CS1 gene in transgenic tobacco plants. Plants were transformed by the pLBP2 (A) and pLBP4 (B) vectors. Expression of the inserted gene constructs was detected by northern hybridization of total RNA samples. Transgenic plants carrying the pLBP4 construct usually showed higher transcript accumulation (B).

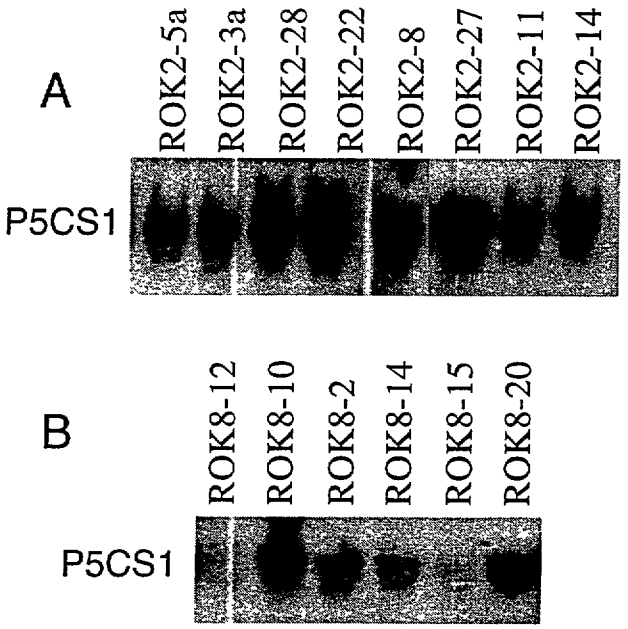


Figure 11 Expression of the the AtP5CS1 gene in transgenic tobacco, carrying the pROK2 (A) and pROK8 (B) vectors.

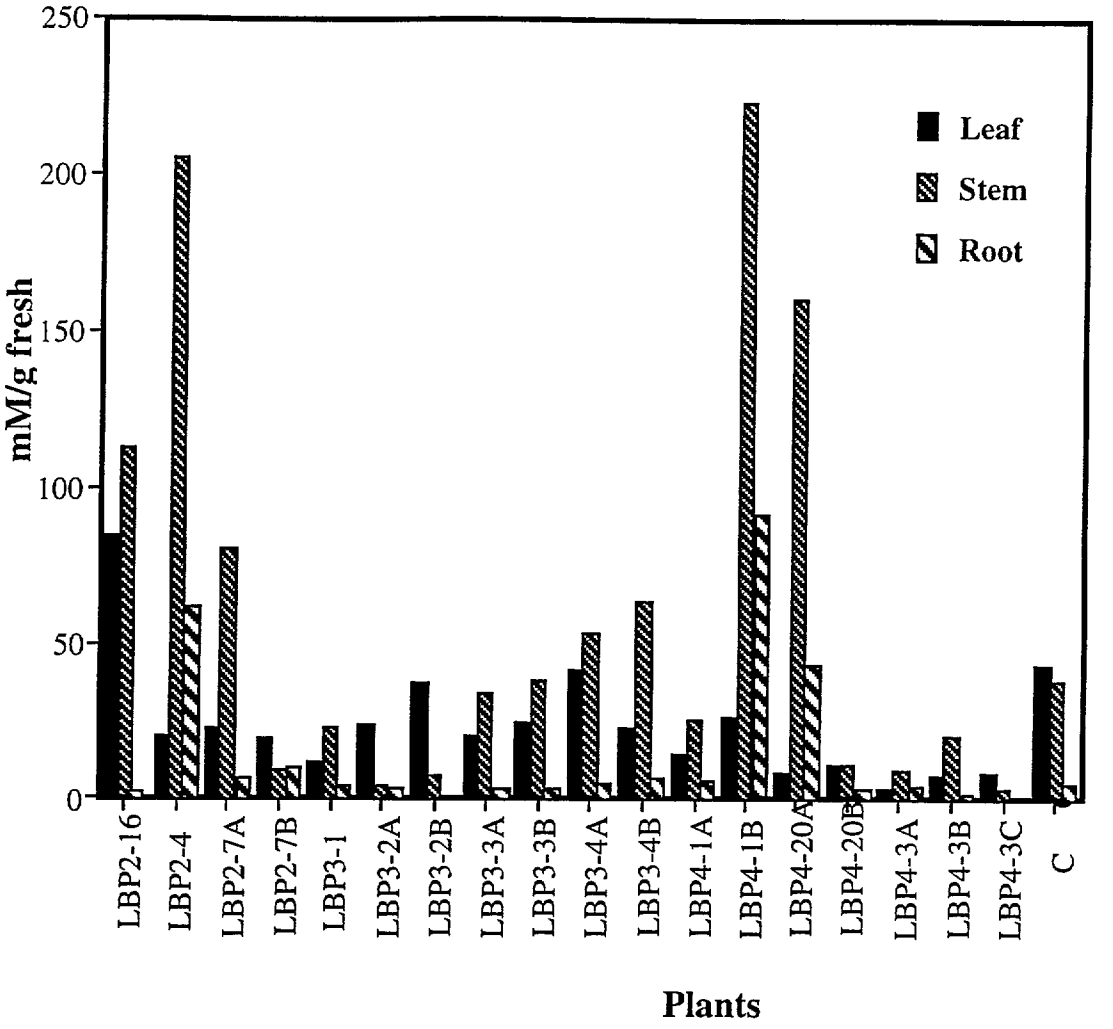


Figure 12. Proline determination in transgenic tobacco plants. Free proline was measured in leaf, stem and root tissues of in-vitro propagated plants. Diagram show the proline concentrations in independent clones. Several plants carrying the sense constructs (LBP2 and LBP4), have higher proline content than the SR1 control, while none of the antisense construct have increased proline content.

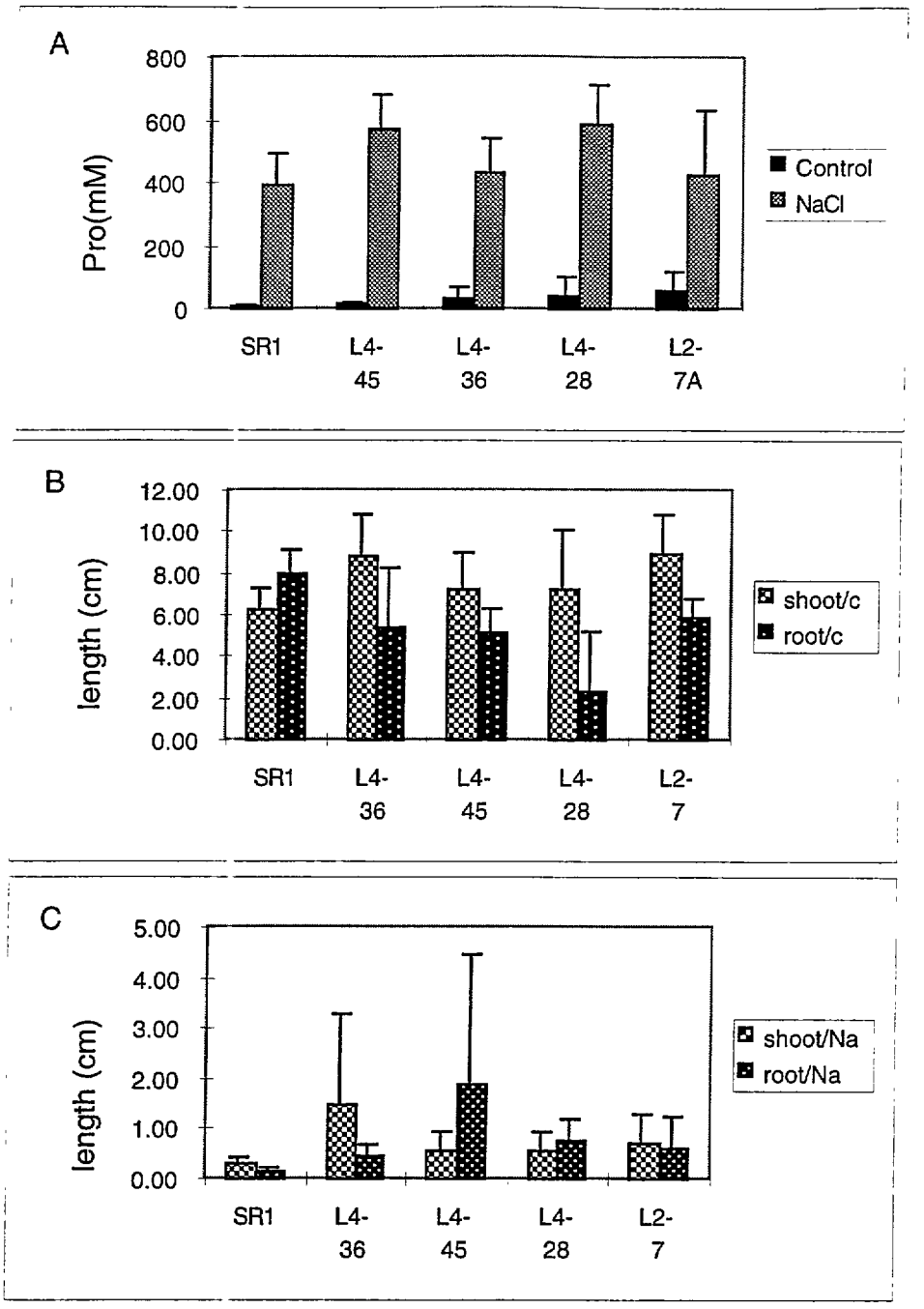


Figure 13. Proline concentration, shoot and root growth of transgenic tobacco plants carrying the AtP5CS1 gene constructs. Progenies of transgenic plants were grown in hydroponic culture conditions. Culture medium (Hoagland solution) of four weeks old plants was replaced by fresh solution, some of them containing 0.2M NaCl. Proline accumulation, growth of shoot and root was measured seven days after the initiation of stress treatment. A) Accumulation of proline in stressed and non-stressed plants. B) Growth of shoot and root of non-stressed plants in seven days (in cm). C) Growth of shoot and root of NaCl-stressed plants in seven days (in cm).

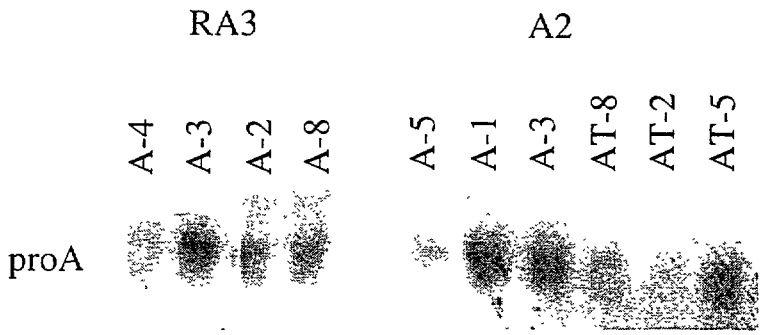


Figure 14. Expression of te bacterial proA gene in transgenic A2 and RA3 alfalfa plants.

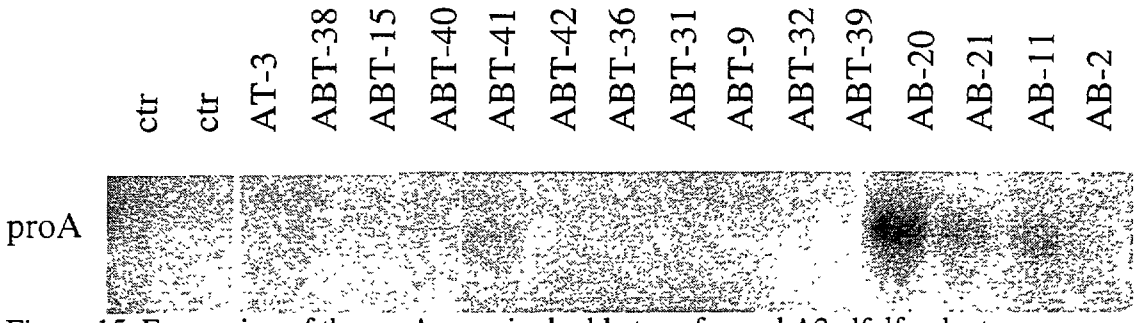


Figure 15. Expression of the proA gene in double transformed A2 alfalfa plants.

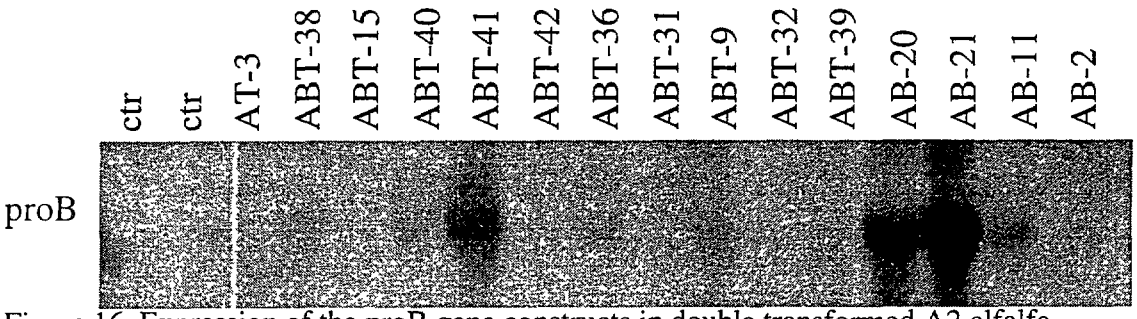


Figure 16. Expression of the proB gene constructs in double transformed A2 alfalfa plants.

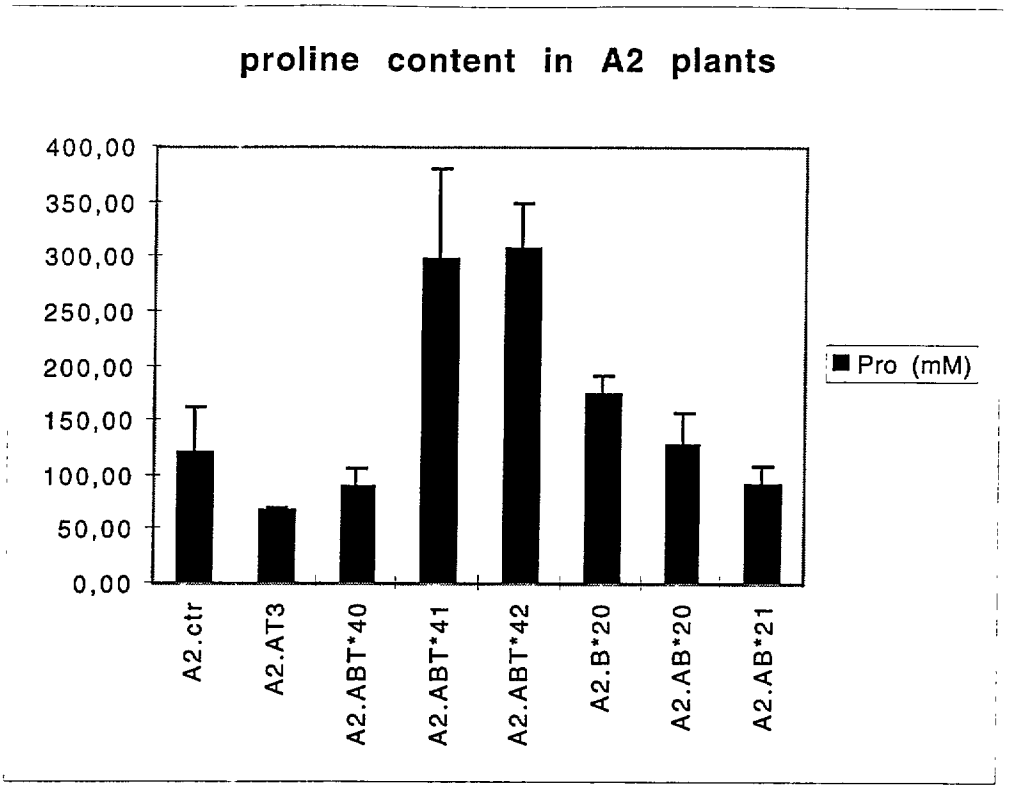


Figure 17. Proline concentration of transgenic alfalfa plants (A2, Medicago varia) expressing the proA and proB74 genes. Two of the tested lines had increased proline concentrations.

PART B – Tel Aviv University and Volcani Center, Israel

I.Introduction

I.1. Main aims of the study

Common environmental stresses such as salinity, drought and low temperature strongly affect the growth of most plants and limit their distribution and agricultural potential worldwide. Although the end result of each of these stresses is lack of available water molecules, different stress tolerance mechanisms, which are sometimes species-specific, may be involved in counteracting their diverse negative effects. Stress tolerance is a multigenic character, encompassing various metabolic pathways that bacteria, fungi, algae and plants have developed throughout the evolutionary struggle for survival. All these interlinked pathways help the plant to achieve osmotic adjustment, facilitate the retention or acquisition of water, maintain ion homeostasis, ameliorate shifts in redox potential, detoxify radicals and protect chloroplast functions. Some of these anti-stress mechanisms operate during exposure to biotic and abiotic stresses.

This study has been focused on the anti-stress potential of the simple cycle of proline biosynthesis and degradation in plants. This cycle mediates cellular proline levels, accumulation of proline as an osmolyte during the imposition of a diverse array of stresses, and intensive proline degradation as an energy, carbon and nitrogen source during recovery from stress. Intensive operation of the proline cycle may also maintain high NADP+/ NADPH ratios needed for normal functioning of the oxidative pentose phosphate pathway and avoidance of photoinhibition damage.

I.2. Proline accumulation in different organisms during stresses- current opinion

Proline appears to be the preferred organic osmolyte in many organisms. Genetic studies in prokaryotes demonstrated that proline is an essential compatible solute capable of conferring osmo-protection (Csonka, 1989). An array of diverse stresses induces proline accumulation in many plants. It includes salinization, water deprivation, high and low temperature, toxicity of heavy metals, pathogen infections, anaerobiosis, nutrient deficiencies, atmospheric pollution and UV irradiation. In many cases it has been shown

that during the imposition of a stress, the elevation of proline levels *in planta* is linked to the amelioration of negative physiological effects (Hare and Cress, 1997). Analysis of experimental evidence collected from many studies suggests that proline accumulation may fortuitously serve more than one function.

Compelling evidence based on studying *E. coli* and *Salmonella* mutants showed the direct role of proline accumulation in counteracting stress effects (Csonka, 1989). Experimental evidence for extreme proline accumulation linked to stress tolerance have also been reported in plants. In maize primary roots subjected to water deprivation, proline accumulation accounted for about 45% of the total osmotic adjustment (Voetberg and Sharp, 1991). However, this stress induced proline accumulation in plants is not an immediate phenomenon. During the first hours of stress imposition, due to the concomitant activity of proline synthase and dehydrogenase, a certain balance between proline synthesis and degradation exists. Proline starts to be significantly accumulated when the transcription and activity of proline dehydrogenase and P5C dehydrogenase (Fig. 1) are gradually abolished (Yoshiba *et al.*, 1997).

Thus, a genetic manipulation that would increase the accumulation of proline independent of stress imposition may ameliorate immediate and long-lasting stress effects. It should also have a profound economical benefit, provided that the increase would be devoid of negative physiological effects.

I.3. Proline biosynthesis - summary of current knowledge

In *E. coli*, the first two steps in the main pathway of proline synthesis from glutamate (Fig. 1) is catalyzed by gamma-glutamyl kinase (GK), encoded by the *pro*B gene and gamma-glutamylphosphate reductase (GPR or GSD), encoded by *pro*A , which together form a stable enzymatic complex. The delta-pyrroline-5-carboxylate (P5C) intermediate formed by the complex, or by the side pathway of arginine biosynthesis (Fig. 1), is reduced to proline in a reaction catalyzed by delta-pyrroline-5-carboxylate reductase (P5CR), encoded by *pro*C.

The first enzyme, GK, is feed-back regulated by proline and therefore proline accumulation does not occur in wild-type *E. coli* cells. The *pro*B74 mutant encodes a

modified GK enzyme, which is insensitive to high proline concentrations, up to 100mM, and therefore can drive proline synthesis in the presence of high proline concentration in the cells. The proB74 gene has a change from wild-type aspartic acid to asparagin at position 107 of ProB amino acid sequence (Csonka et al., 1988).

In young plants proline is produced from glutamate or ornithine, while in mature plants or during imposition of stress the glutamate pathway usually dominates (Roosens et al., 1998). A bifunctional enzyme, delta-pyrroline-5-carboxylate synthase (P5CS, Fig. 1) catalyzes the first two steps of P5C formation and shows a certain amino acid homology to the bacterial *pro*B and *pro*A (Fujita *et al.*, 1998; Ginzberg *et al*, 1998; Hu *et al*, 1992; Strizhov *et al.*, 1997). P5CS is feed-back regulated. A concentration of 10mM proline completely blocks its activity (Hu *et al.*, 1992), while under stress the cellular levels of proline may exceed 100mM. Ectopic expression of the cDNA encoding this wild-type enzyme in tobacco rendered the recipient plants relatively tolerant to salt stress (Kishor et al., 1995) despite of the enzyme sensitivity to low proline concentrations and the measurements related to osmotic adjustment that were not accepted by some plant physiologists (Blum *et al.*, 1996). A mutated P5CS, showing a relative proline insensitivity was obtained by site directed mutagenesis (Zhang et al., 1995).

It is assumed that P5CS activity is confined to the cytosol, since all the full size cDNAs isolated thus far are devoid of a defined plastid targeting transit peptide sequence. Zhang et al., 1995, also reported about the presence of P5CS inhibitor that inactivates its enzymatic activity in roots.

The plant P5CR is also considered to be a cytosolic enzyme although cell fractionation studies detected P5CR activity in both cytoplasm and chloroplast (Szoke et al., 1992). Overexpression of P5CR in transgenic plants did not affect proline levels and therefore the native enzyme is not considered as a very promising candidate for ectopic expression in transgenic crops (Szoke et al., 1992).

Here we have summarized the four years study on proline biosynthesis and degradation in plants and the development of an alien system, based on *E. coli* genes, that enables over-production of proline in the cytosol or chloroplasts of transgenic plants.

II. Results

II.1. Introduction of E. coli proline biosynthesis genes into plants.

Construction of plant transformation vectors carrying the *E. coli pro*B^m or proB74 and *pro*A genes driven by a constitutive promoter and generation of transgenic tobacco and alfalfa plants.

Performed mainly by Tel-Aviv University. Alfalfa transformation was carried out at the Volcani Institute).

In the course of this study, three rounds of transgenic tobacco plants have been generated, each based on the assessment of the results of the previous round. Prior to the construction of plant transformation vectors, an attempt to obtain mutated *pro*B gene versions encoding proline indifferent GKs was carried out, using selection for AZT (L-azetidine carboxylate) indifferent GK in *E. coli.* A mutated proB^m was identified, characterized and inserted in the first version of proB^m-plant transformation vectors

Two sets of plant vectors were then constructed designed to target the transgenic mutated GK and GPR either to the cytoplasm or to the chloroplasts of the recipient plants. To target the proteins into the chloroplast, the *pro*B^m mutated gene, defined by its capability to confer resistance to L-azetidine carboxylate (AZT) (described in the 1st Semi Annual Report), was translationally fused downstream to the pea *rbc*S 5' sequence encoding the transit peptide (as detailed in the 1st Semi-Annual Report). Similarly, the *pro*A gene was also fused to the same sequence. Both genes, with or without the sequence of the transit peptide, were linked downstream to the CaMV 35S promoter and the TMV omega sequence (Gallie et al., 1987).

To be able to assay the function of the translationl fusions in *E. coli* JM83 (lacking *pro*BA), *pro*B alone or its translational fusion with the transit peptide were also cloned in pACYC184. Co-introduction of the two sets of plasmids; pACYC*pro*B^m and pCE*pro*ATP or pACYC*pro*B^{m}TP and pCEproA into *E. coli* JM83 allowed the survival of the transformed colonies on a minimal medium lacking proline. This complementation

assays indicated that the translational fusions of *pro*B and *pro*A encode functional GK and GPR, respectively, and the addition of the *rbc*S transit peptide which is not cleaved out in E. coli cells does not interfere with their enzymatic activity.

The *pro*$\mathbf{B^m}$ cassettes with or without the *rbc*S transit peptide sequence, were inserted into the **pGIF** *Agrobacterium*-plant shuttle vector vector (Fig. 2). This vector carries the *hph* gene linked to the *nos* promoter as a selectable marker that confers resistance to hygromycin B. It also contains the *pat* gene driven by the p2 promoter of mannopine synthase from octopine type T-DNA. This gene confers resistance *in planta* to the non-selective herbicide "Basta".

The *pro*A cassettes were inserted into the *Agrobacterium*-plant shuttle vector **pPCV702** (Koncz et al., 1989). This vector carries the *npt*II gene linked to the *nos* promoter and confers resistance to kanamycin in plants (Fig. 2).

Two combinations of plasmids were used to co-transform tobacco (*Nicotiana tabacum* NN): The plasmids pPCV*pro*A and pGIF*pro*B^m were co-introduced to produce GK and GPR in the cytoplasm of the double transgenic plants. In parallel, the plasmids pPCV*pro*ATP and pGIF*pro*B^mTP were also co-transformed to develop double transgenic plants with GK and GPR activity in the chloroplasts. In both cases, *Agrobacterium* mediated transformations were carried out using kanamycin and hygromycin B containing medium for selection of double transgenic plants.

Each of the four constructs was also introduced alone into tobacco or alfalfa.

Six types of transgenic plants were obtained and analyzed for proline content. Molecular analysis of transgenic tobacco plants that carry both genes showed that although the proteins were well expressed and processed during chloroplast uptake, no elevated accumulation of proline was recorded. Similar results were obtained by the Hungarian group (see the Hungarian part of the report) in transgenic alfalfa.

Coupled GK – GPR enzymatic assays, based on the isolation of the over-expressed proteins from *E. coli,* showed that *pro*B74 gene product (Csonka et al., 1988) is capable of maintaining its enzymatic activity in the presence of high proline levels. Therefore, a second set of plant transformation vectors with the proB74 gene (kindly supplied by L.N.

Csonka), was constructed and the *pro*B74, with or without *rbc*S transit peptide was introduced into plants that were already analyzed and found to express high levels of *pro*A. Transgenic tobacco and alfalfa plants were obtained and molecularly analyzed. Western analyses showed that high levels of both GK and GPR are expressed and the proteins with the SSU leader (RUBISCO small subunit leader) are correctly processed while being taken into the chloroplasts (Fig. 4). An endogenous increase in proline levels, up to six folds, was observed in tobacco. The increase was more prominent when alien proteins were targeted to the chloroplasts (Fig. 5).

Transgenic alfalfa plants expressing GK encoded by proB74 and GPR have been generated. The levels of proline in these transgenic plants is currently being evaluated.

II.2. **Characterization of cDNAs encoding the first enzyme of proline biosynthesis pathway in alfalfa.**

(Tel-Aviv University and Volcani Center)

In order to understand the endogenous modulation of the proline cycle during stress imposition and recovery, we have isolated two different cDNA clones, representing two genes that code for delta-pyrroline-5-carboxylate synthase (P5CS), the key enzyme of proline biosynthesis (Ginzberg et al., 1998). The clones were isolated from a Lambda-ZAP-cDNA library that we constructed from salt stressed alfalfa roots. The MsP5CS-1 cDNA (2.6kb) clone, has an open reading frame of 717 amino acids as well as a non-spliced intron at a position corresponding to the evolutionary fusion point of the bacterial *pro*B and *pro*A genes. The second cDNA clone, MsP5CS-2, is a partial clone, sharing 65% nucleotide sequence identity and 74% amino acid homology with MsP5CS-1 (Fig. 6).

II.3. Changes in transcription of alfalfa P5CS during stress imposition and recovery.

(Tel-Aviv University and Volcani Center)

The effect of salinity on transcription of MsP5CS-1 and MsP5CS-2 genes was studied using northern blot analysis and RT-PCR approach. By using this RT-PCR highly sensitive approach, a rapid increase in transcript level of P5CS genes in roots was observed following the exposure of roots to 90mM NaCl. A higher response (up to six folds) was observed for MsP5CS-2 (Fig. 7). However, Northern blot analyses showed that the general transcript levels of P5CS in both roots and shoots before, during and after exposition to salt stress are high and the immediate increase, observed by RT-PCR, probably plays a minor role in the overall elevated proline accumulation during salinity stress in alfalfa (Fig. 8). Thus we assumed that the high increase in proline content in roots and shoots during stress (Fig. 8) might be due to less proline degradation.

II.4. Proline dehydrogenase cDNA and its transcription during salt stress

(Tel-Aviv University)

A partial cDNA clone of proline dehydrogenase was isolated from the alfalfa cDNA library by using degenarate primers corresponding to the amino acids highly conserved region present in Drosophila as well as in yeast proline dehydrogenase (PDh). This cDNA clone was used as a probe to evaluate the effect of salt stress on PDh transcript levels. Northern analyses showed that following stress imposition a great decrease in PDh transcript level is observed both in roots and in shoots (Fig. 9). This decrease correlates well with a concomitant increase in proline accumulation. Thus increased proline accumulation during stress in alfalfa is mainly due to a decline in proline breakdown and not to an increased synthesis.

II.5. Anti-sensing PDh transcription in tobacco (Tel-Aviv University)

The partial PDh cDNA clone was inserted downstream to the CaMV 35S promoter and cloned in a plant transformation vector with or without proA and proB74 genes, each linked to the same promoter. The different vectors were separately introduced into tobacco and alfalfa plants by Agrobacterium mediated transformation. Transgenic plants are currently being generated. These plants will be evaluated for proline content and response to salinity stress.

II.6. Salinity experiments (Volcani center)

The objective of the following experiments was to evaluate the response of transgenic tobacco plants to saline and drought growth conditions relative to the wild type plants. Tobacco plants were germinated and allowed to grow for 4 weeks in vermiculate under non-stressed growth conditions in controlled environment (27/24 °C day/night). When plants reached an age of 4 weeks, both transgenic (line P9) and non-transgenic plants, were transferred to aerated hydrophonic system provided with nutrient solution (1/4 strength of Hoglland) for one week. Plants were subjected gradually to salinity by initially exposing the roots to half (of the final salt level) strength for 3 days and only then to the final concentration. All salt treatments were applied to the standard nutrient solution. In recovery experiments plants were exposed to the final salt level for two weeks before being transferred to non-saline conditions. Samples were taken before, during and after salt application and proline and reduced sugars were determined in the first fully exposed leaf. Leaf development was evaluated by following elongation rates of first and second leaves of the tested plant.

Proline accumulation

At optimal growth conditions (0 mM of NaCl), a significant higher proline concentration was found in transgenic P9 plants relative to concentrations obtained in non-trasgenic

controls. Plants exposure to 45 or 90 mM NaCl resulted in a farther increase in proline level in both, transgenic and wild type plants (Fig. 1). However, the relative increase in proline concentration of transgenic P9 plants at the highest salt level tested in a second experiment (90mM of NaCl) was lower relative to the elevation obtained in the non-transgenic wild type plants (Fig. 2). The increase in proline concentration of the trasgenic line (P9) and the wild type plants was not necessarily linear.

II.6.1. Plant growth

Leaf growth rates (leaf 1 and 2) were followed through out the exposure to saline growth conditions. In non-saline conditions (0 mM NaCl) the transgenic line exhibited higher elongation rates (relative to elongation detected before the salt exposure) then the wild type plants (Fig. 3). In both lines (P9 and wt), elevation of salinity in the rooting media reduced leaf elongation rates by 45% relative to the elongation obtained in nonsaline conditions. However, no significant differences between the two lines was obtained at both salt levels tested (Fig. 4). At the end of the experiment, no significant differences in plant biomass accumulation was obtained between the two lines.

Recovery experiments were carried in a similar experimental design. In these experiments plants were grown under saline growth conditions for 10 days and then were exposed to non-saline conditions. All the parameters tested above were also detected in the recovery experiments and no significant differences in the performances of the transgenic plant relative to the wild type were found.

Concentrations of reduced sugars in both, transgenic and wild type plants (detected by the Antron) declined by 40% as salinity increases in the rooting media. No significant differences was obtained between the two tobacco lines tested (Fig, 5).

Table 1: The effect of salinity growth conditions (45 and 90 mM NaCl) on roots and shoots dry weight of transgenic (P9) and wild type (NN) plants.

Salt level (mM)	Plant genotype	Root DW (g/plant)	Shoot DW (g/plant)
0	Wild type	.55±.04	3.18±.25
	P9	.59+.07	3.71+.71
45	Wild type	.50+.10	3.72+.9
	P9	.68+.09	3.73+.55
90	Wild type	.14+.03	1.59+.20
	P9	.24+.07	1.97+.62

II.6.2. Drought experiments

The effect of drought growth conditions on the performance of the high proline transgenic P9 line was tested in two different experimental systems – PEG and pots experiments.

PEG experiment

Plants were grown in vermiculite for 5 weeks and then were transferred stepwise to PEG 8,000 solution to obtain a final water potential of –9.2 bar. Plants were allowed to grow for 2 more weeks in controlled environment and analyzed for relative water content and osmotic adjustment. No significant genotype effect (P=0.05) was found in both parameters (Table 2).

II.6.3 Pot experiment

Plants were grown in vermiculite for 5 weeks and then were planted in 15 litter pots with sand as a rooting media. Plants were grown under 25% shade in net-house during June of 1999. Plants were allowed to grow for 20 days and then irrigation stopped. 22 days later (42 days after transferred to the pots) plants were tested for RWC, OA and shoot

biomass. No significant differences were found between the tested transgenic plant to the wild type (Table 3).

Table 2: The effect of stress conditions on relative water content (RWC) and osmotic adjustment (OA) of transgenic tobacco plant contains high level of proline (P9) and wild type (NN).

Tobacco line	Treatment	RCW (%)	OA (bar)
N N	Stressed	85.5	3.17
NN	Non-stressed	91.5	
P9	Stressed	86.7	2.23
P9	Non-stressed	91.9	

Table 3: The effect of stress conditions on relative water content (RWC), osmotic adjustment (OA) and biomass production of transgenic tobacco plant (P9) and wild type (NN). Experiment was conducted in 15 L sand pots and data was collected 6 weeks after transplanting.

Tobacco line	Treatment	RCW (%)	AO (bar)	Shoot biomass (g/plant)	% of the control
N N	Stressed	73.0	1.9	10.0	95.5
NN	Non-stressed	90.7		10.4	
P9	Stressed	75.7	0.38	8.0	94.4
P9	Non-stressed	91.4		8.5	

References

Blum, A., R. Munns, J. B. Passioura, and N. C. Turner. 1996 Genetically Engineered Plants Resistant to Soil Drying and Salt Stress - How to Interpret Osmotic Relations. Plant Physiol. 110:1051-1051.

Csonka LN, Gelvin SB, Goodner BW, Orser CS, Siemieniak D, Slightom JL 1988 Nucleotide sequence of a mutation in the proB gene of Escherichia coli that confers proline overproduction and enhanced tolerance to osmotic stress. Gene 64: 199-205.

Csonka LN 1989 Physiological and genetic responses of bacteria to osmotic stress. Microbiol. Reviews 53: 121-147.

Fujita T, Maggio A, Garcia-Rios M, Bressan RA, Csonka LN 1998 Comparative analysis of the regulation of expression and structures of two evolutionarily divergent genes for delta1-pyrroline-5-carboxylate synthetase from tomato. Plant Physiol. 118: 661-674.

Ginzberg I, Stein H, Kapulnik Y, Szabados L, Strizhov N, Schell J, Koncz C, Zilberstein-Aviah1998 Isolation and characterization of two different cDNAs of DELTA1-pyrroline-5-carboxylate synthase in alfalfa, transcriptionally induced upon salt stress. Plant Molecular Biology 38: 755-764.

Hare PD, Cress WA 1997 Metabolic implications of stress-induced proline accumulation in plants. Plant Growth Regulation 21: 79-102.

Hu CA, Delauney AJ, Verma DP 1992 A bifunctional enzyme (delta1-pyrroline-5-carboxylate synthetase) catalyzes the first two steps in proline biosynthesis in plants. Proc. Natl. Acad. Sci. USA 89: 9354-9358.

Kishor PBK, Hong Z, Miao G-H, Hu C-AA, Verma DPS 1995 Overexpression of delta1-pyrroline-5-carboxylate synthetase increases proline production and confers osmotolerance in transgenic plants. Plant Physiol. 108: 1387-1394.

Koncz, C., Martini, M., Mayerhofer, R., Koncz-Kalman, Z., Korber, H., Redei, G. P., Schell, J. 1989 High-frequency T-DNA-mediated gene tagging in plants. Proc. Natl. Acad. Sci. USA 86:8467-8471.

Roosens NHCJ, Thu TT, Iskander HM, Jacobs M 1998 Isolation of the ornithine-delta-aminotransferase cDNA and effect of salt stress on its expression in Arabidopsis thaliana. Plant Physiol. 117: 263-271.

Strizhov N, Abraham E, Okresz L, Blickling S, Zilberstein A, Schell J, Koncz C, Szabados L 1997 Differential expression of two P5CS genes controlling proline accumulation during salt-stress requires ABA and is regulated by ABA1, ABI1 and AXR2 in Arabidopsis. Plant J. 12: 557-569.

Szoke A, Miao GH, Hong Z, Verma DPS 1992 Subcellular location of delta1-pyrroline-5-carboxylate reductase in root/nodule and leaf of soybean. Plant Physiol. 99: 1642-1649.

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

Yoshiba Y, Kiyosue T, Nakashima K, Yamaguchi-Shinozaki K, Shinozaki K 1997 Regulation of levels of proline as an osmolyte in plants under water stress. Plant Cell Physiol. 38: 1095-1102.

Zhang CS, Lu Q, Verma DP 1995 Removal of feedback inhibition of delta1-pyrroline-5-carboxylate synthetase, a bifunctional enzyme catalyzing the first two steps of proline biosynthesis in plants. J. Biol. Chem. 270: 20491-20496.

Publications derived from this study

Ginzberg I, Stein H, Kapulnik Y, Szabados L, Strizhov N, Schell J, Koncz C, Zilberstein-A. 1998 Isolation and characterization of two different cDNAs of DELTA1-pyrroline-5-carboxylate synthase in alfalfa, transcriptionally induced upon salt stress. Plant Molecular Biology 38: 755-764.

Strizhov N, Abraham E, Okresz L, Blickling S, Zilberstein A, Schell J, Koncz C, Szabados L 1997 Differential expression of two P5CS genes controlling proline accumulation during salt-stress requires ABA and is regulated by ABA1, ABI1 and AXR2 in Arabidopsis. Plant J. 12: 557-569.

In the course of this year (1999-2000) additional manuscripts will be submitted. The information will be delivered to the AID Office upon acceptance for publication.

Figures:

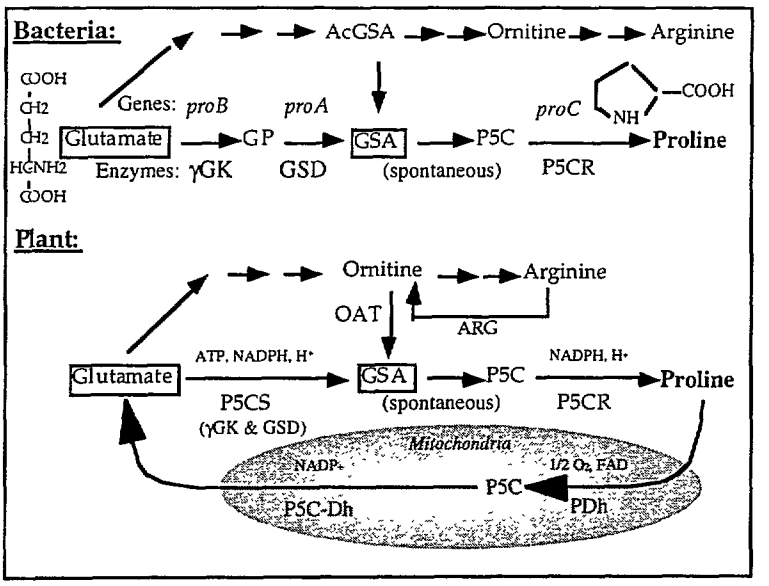
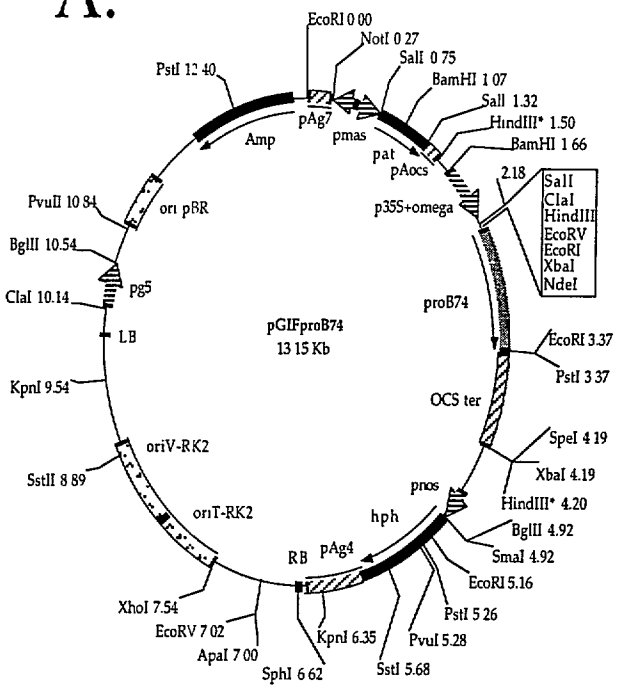


Figure 1: Proline biosynthesis pathways in bacteria and plants and related genes and enzymes.

AcG, N-actyl glutamate; GSA, glutamic γ-semialdehyde; GP, glutamyl phosphate; P5C, Δ1-pyrroline-5-carboxylate. Enzymes: ARG, araginase; γGK, γ-Glutamyl Kinase; GSD, Glutamic γ-Semialdehyde Dehydrogenase (also known as GPR); OAT, ornitine amino transferase; P5CS, P5C synthetase; P5CR, P5C reductase; PDh, Proline dehydrogenase; P5C-Dh, P5C dehydrogenase.

A.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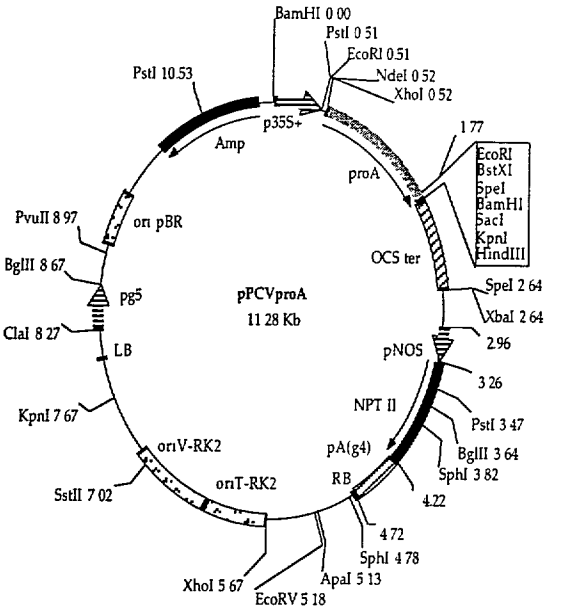
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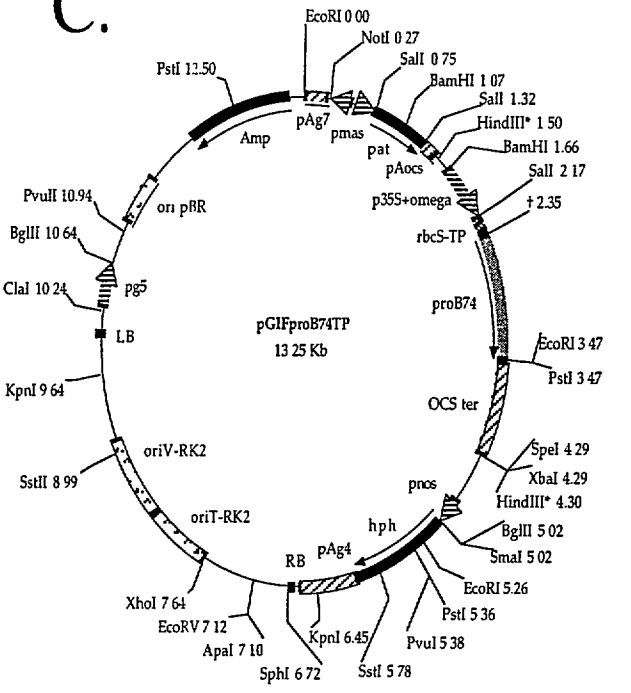
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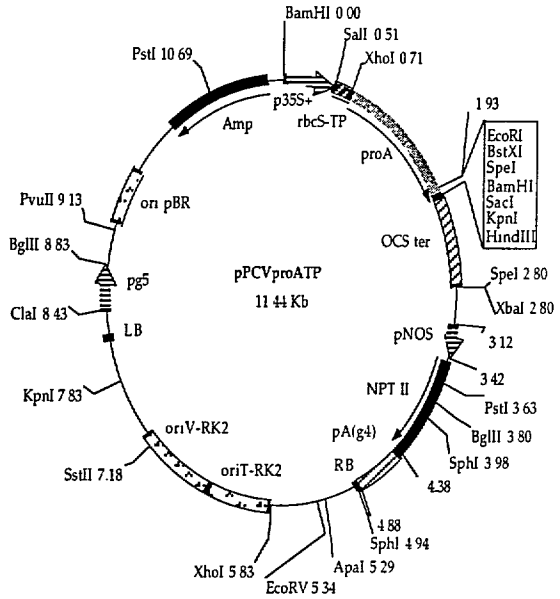
SstI 5 78

D.


BamHI 0 00
SalI 0 51
XhoI 0 71
PstI 10 69
p35S+
rbcS-TP
Amp
1 93
EcoRI
BstXI
SpeI
BamHI
SacI
KpnI
HindIII
proA
PvuII 9 13
ori pBR
OCS ter
BglII 8 83
pg5
pPCVproATP
11 44 Kb
SpeI 2 80
XbaI 2 80
ClaI 8 43
LB
pNOS
3 12
3 42
KpnI 7 83
NPT II
PstI 3 63
oriV-RK2
pA(g4)
BglII 3 80
SphI 3 98
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RB
4.38
4 88
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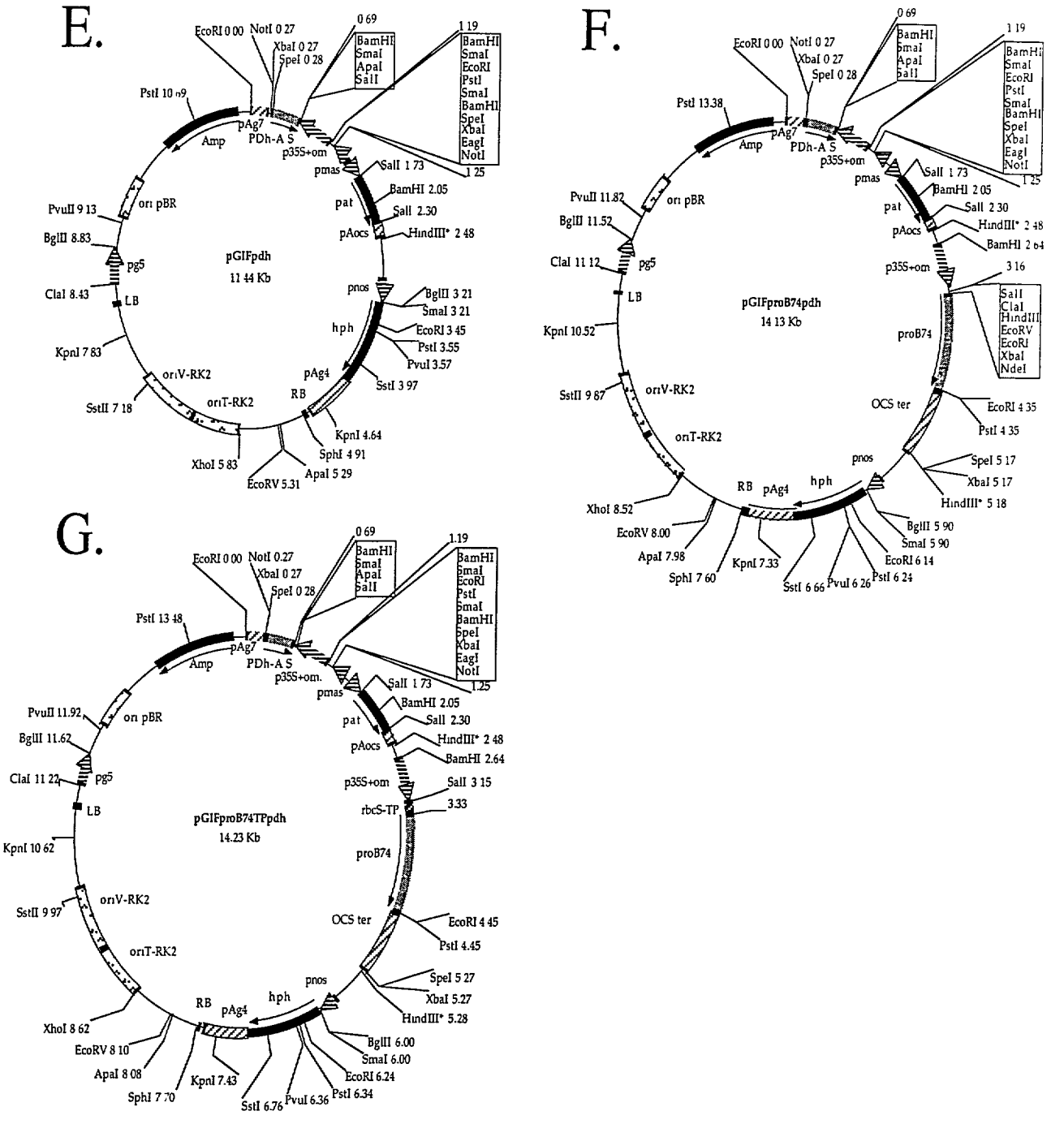


Figure 2. Physical maps of plant transformation vectors carrying the *pro*B74 gene and *pro*A with (A, B) or without (C, D) *rbc*S transit peptide sequence, driven by the constitutive CaMV 35S promoter. In E, F, G the PDh cDNA fragment is inserted in an anti-sense orientation to down-regulate endogenous PDh expression in tobacco and alfalfa.

All vectors belong to a similar binary vector system designed for *Agrobacterium* mediated transformation.

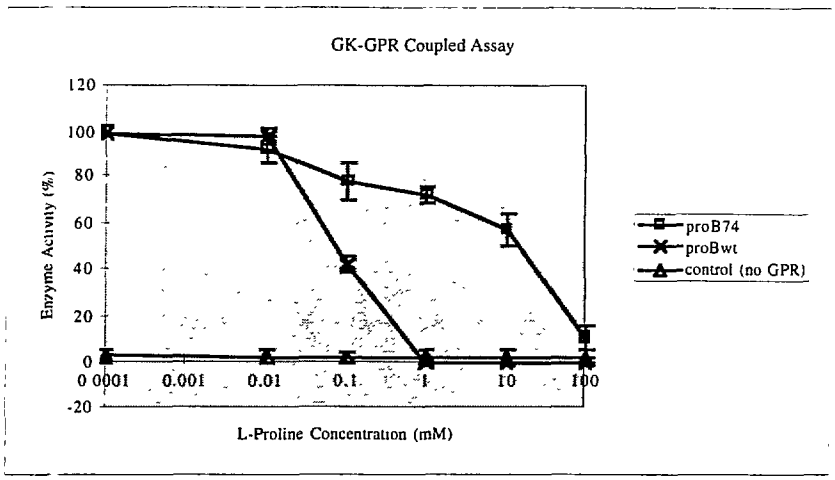


Figure 3. L-proline effect on GK and GPR activity measured by a coupled assay. The enzymes were over expressed in *E. coli* and then assayed *in vitro*. Bars represent a standard error of four replicates.

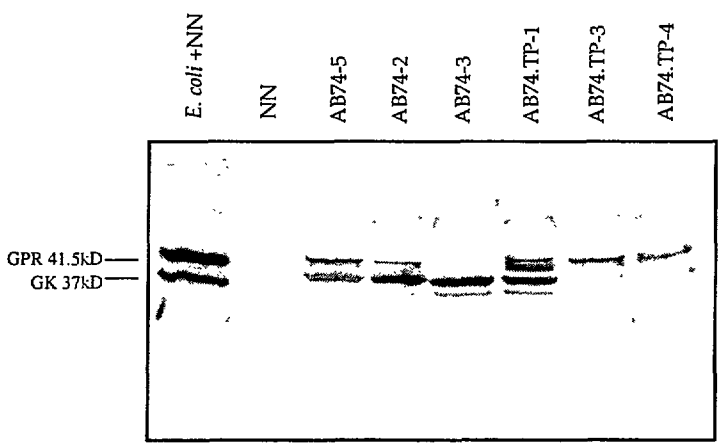


Figure 4. Expression of GK and GSD in the cytosol (AB74) and chloroplasts (AB74.TP) of transgenic tobacco plants.
Western blot analysis of total leaf proteins extracted from control (NN) and transgenic plants. A mixture of total proteins extracted from *E. coli* that overexpressed GK and GSD (200ng) and leaves of a wild-type plant (60mg) is separated on lane 1.
The bacterial enzymes constituted approximately 0.1% of the total protein content in the leaf. In the AB74.TP transgenic plants, two forms of GSD are observed; the 42kDa band representing the unprocessed form of with the transit peptide and a 41.5kD band representing the processed form following the removal of the transit peptide during the uptake into the chloroplast. Additionally, a 38.5kD band representing a truncated form of GSD is also observed, probably resulting from proteolytic activity.

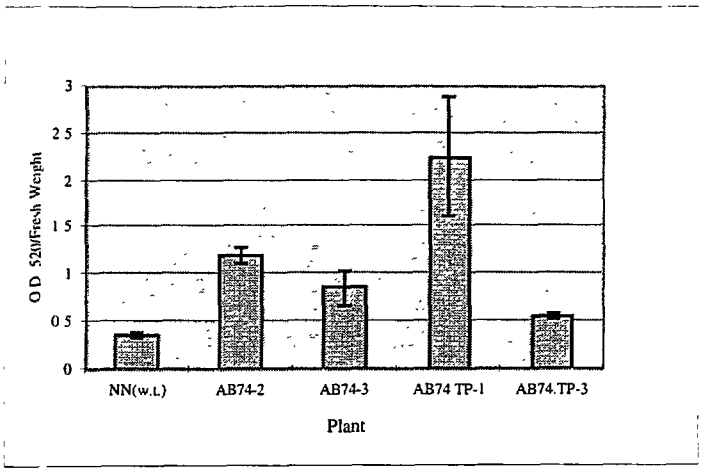


Figure 5. Proline level in transgenic tobacco plants grown under normal conditions. .Proline content in leaves of the wild-type tobacco (NN) and GK+GSD transgenic lines (AB74 and AB74.TP) was determined as described by Bates *et. al.* (1973). Each point represents the mean value of four replicates.

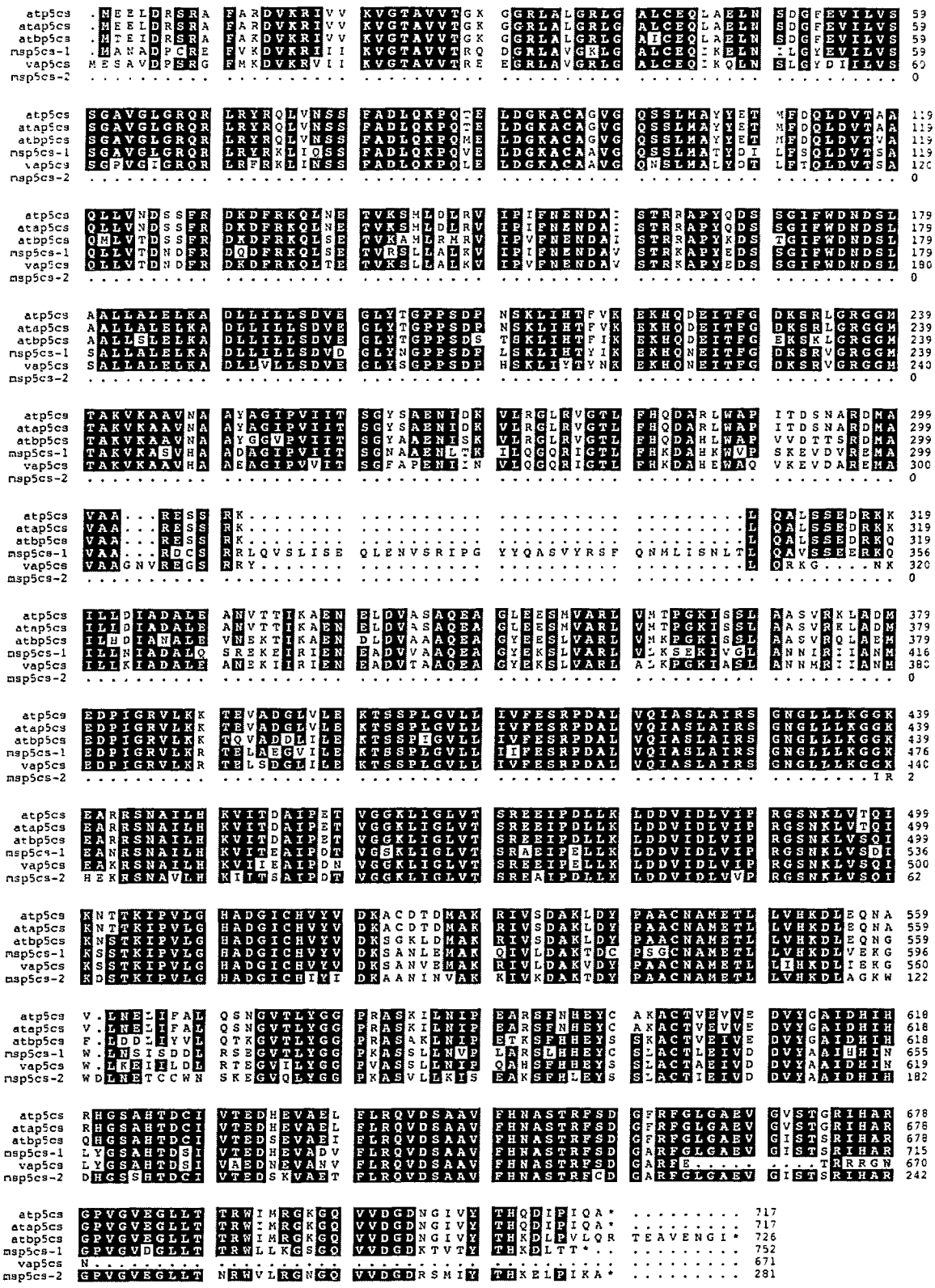
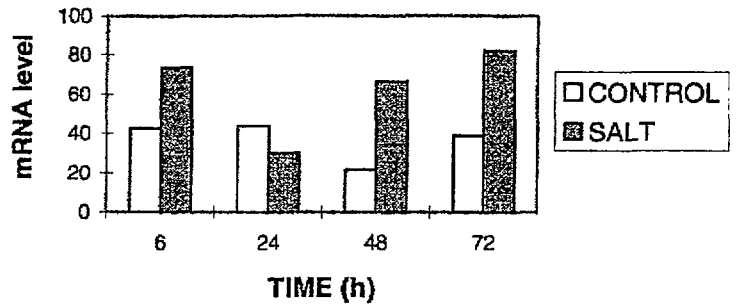


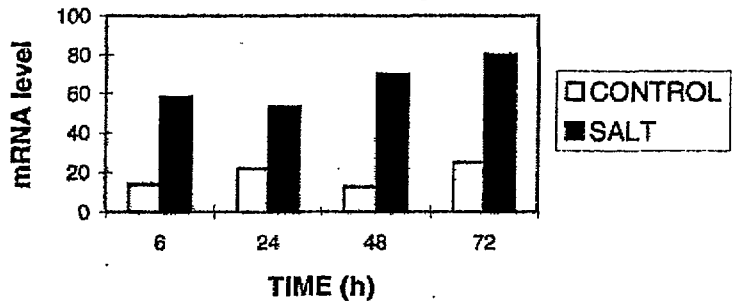
Figure 6. Alignment of the predicted amino acid sequences of the P5CS polypeptides from the following plants: *A. thaliana* (EMBL accession no.: atp5cs X87330, atap5cs X86777, atbp5cs X86778), *M. sativa* (msp5cs-1 and msp5cs-2), and *V. aconitifolia* (vap5cs). A putative ATP binding site and a NAD(P)H binding domain are located

between amino acids 62-69 and 469-504 of the *MsP5CS-1*, respectively. Amino acid sequence from position 310 to 345 in *MsP5CS-1* although being in frame, belongs to an intron which is homologous to intron 7 of the corresponding *Arabidopsis* genes.

a. *MsP5CS-1* mRNA steady-state level.



b. *MsP5CS-2* mRNA steady-state level.



c. Induction level of *MsP5CS* genes.

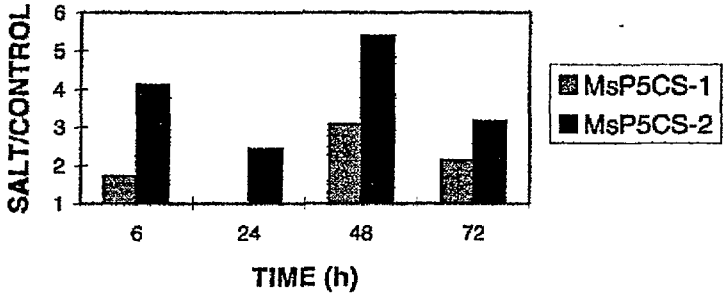


Figure 7. Induction of alfalfa *P5CS-1* and *P5CS-2* transcription by salt stress. Total RNA extracted from seedling roots exposed to 90 mM NaCl (gray and black boxes) and from control plants (white boxes) was subjected to RT-PCR analysis using *MsP5CS-1* or *MsP5CS-2* gene-specific primers. Southern blots of the PCR products were hybridized with their corresponding ^{32}P-cDNA clones and the radioactivity values were analyzed by Phosphor-imager program. Values obtained were normalized per equal rRNA content in each sample. Panels (a) and (b) display mRNA steady-state levels of *MsP5CS-1* and *MsP5CS-2*, respectively. The relative induction of each gene is shown in (c). The RNA samples were within the range of template dependent linearity.

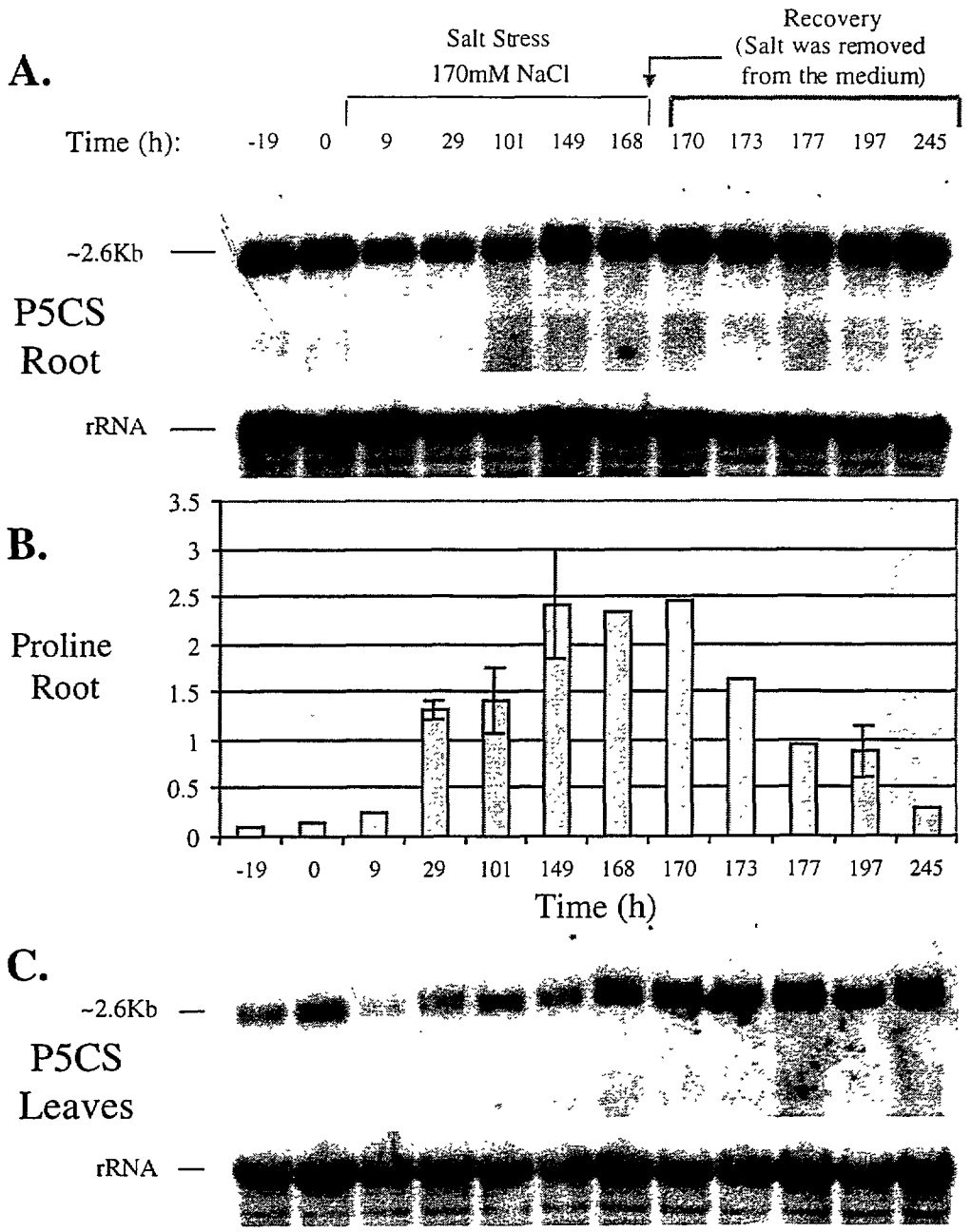


Figure 8. P5CS RNA and free Pro levels in roots and leaves of alfalfa seedlings during salt stress and recovery. A. Northern blot analysis of P5CS mRNA levels in roots of alfalfa plants during salt stress (170mM NaCl) and recovery. Alfalfa P5CS cDNA and ribosomal DNA probe (18S) were used as probes. B. Proline content in roots of alfalfa plants during salt stress and recovery. Bars represent standard errors of three replicates. C. Northern blot analysis of P5CS mRNA levels in leaves of alfalfa plants during salt stress (170mM NaCl) and recovery. Alfalfa PDh cDNA and ribosomal DNA probe (18S) were used as probes. Identical daily timing of sampling was kept to avoid circadian rhythm effects

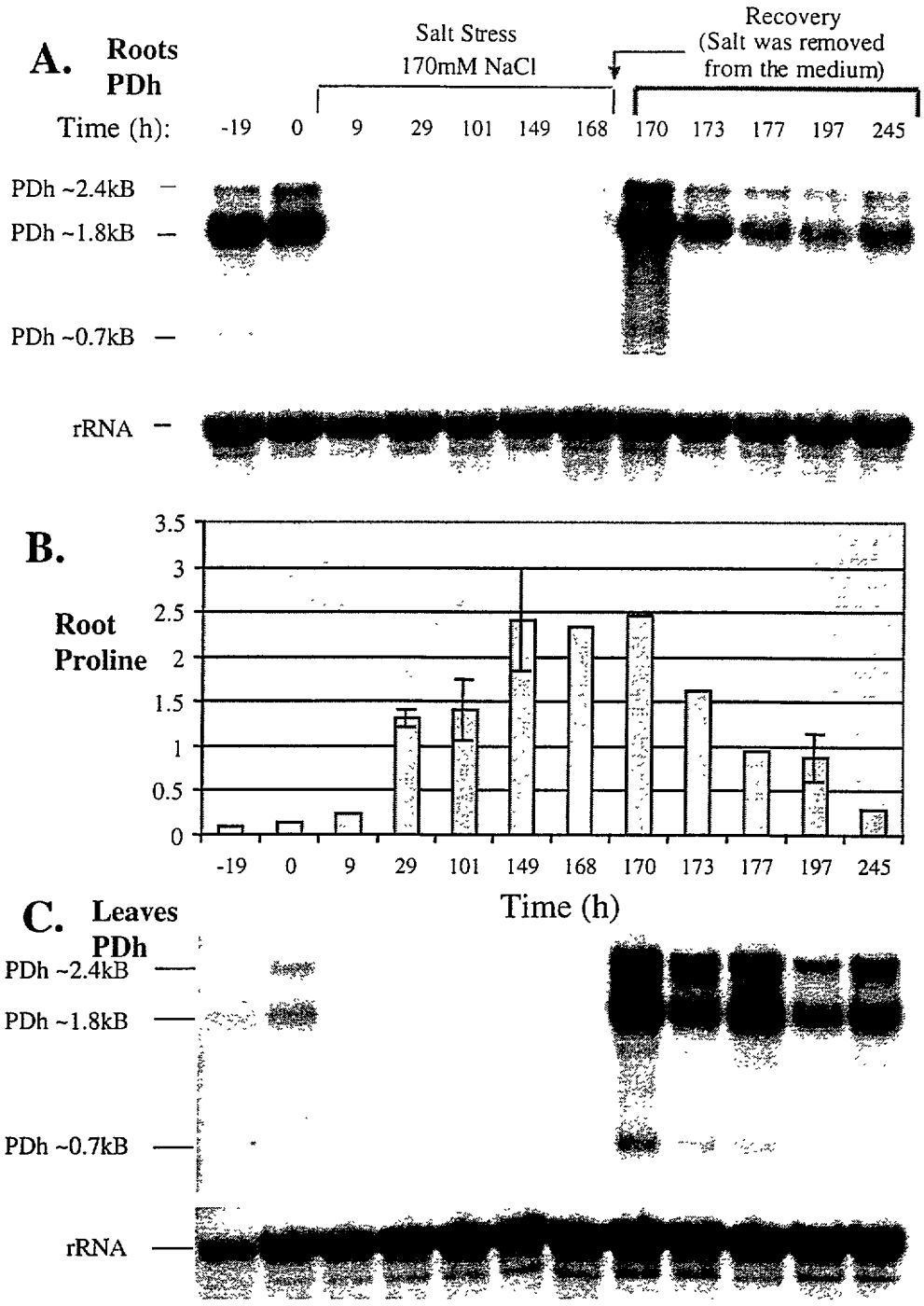


Figure 9. Evaluation of PDh mRNA and free Pro levels in roots and leaves of alfalfa seedlings during salt stress (170mM NaCl) imposition and recovery. A. Northern blot analysis of PDh mRNA levels in roots. Alfalfa PDh cDNA and ribosomal DNA (18S) fragment were used as probes.
B. Proline content in roots. Bars represent standard errors of three replicates.
C. Northern blot analysis of PDh mRNA levels in leaves.
Identical daily timing of sampling was kept to eliminate differences due to circadian rhythm.

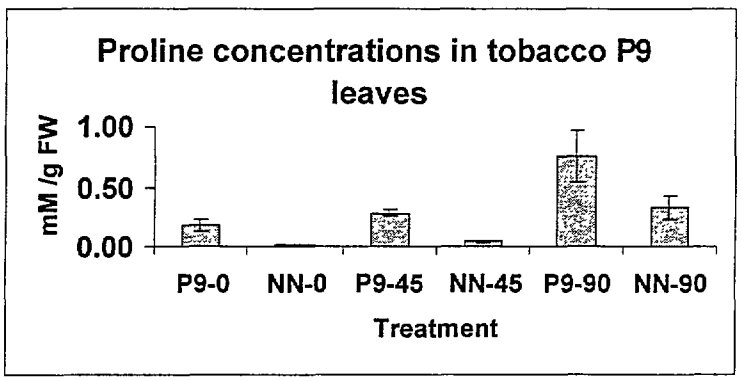


Figure 10: Proline concentration in Transgenic (P9) and wild type (NN) tobacco plants exposed to 45 and 90 mM NaCl. Plants were grown hydroponically and proline was detected from the first fully exposed leaf of each plant.

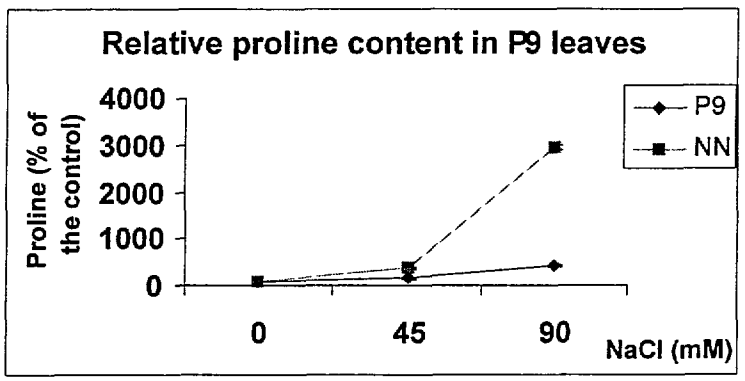


Figure 11: Relative elevation of proline levels in the Transgenic (P9) and wild type (NN) tobacco plants exposed to 45 and 90 mM NaCl. Plants were grown hydroponically and proline was detected from the first fully exposed leaf of each plant.

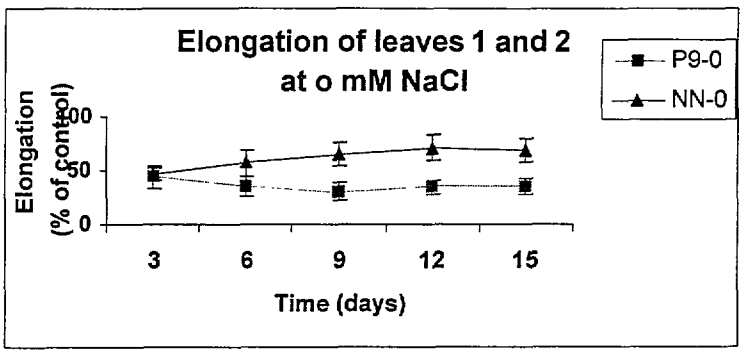


Figure 12: Elongation of leaves 1 and 2 of transgenic (P9) and wild type (NN) plants at non-saline growth conditions during the two weeks experiment.

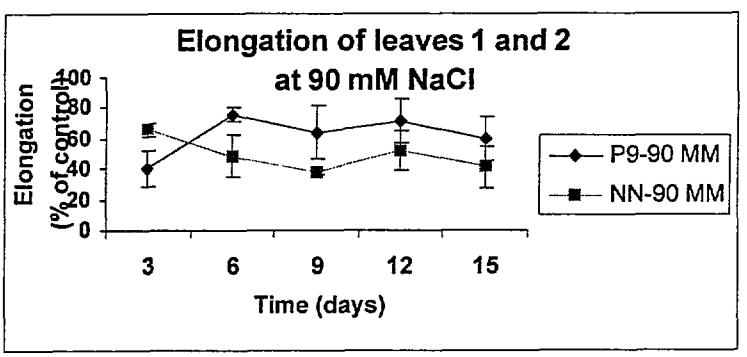


Figure 13: Elongation of leaves 1 and 2 of transgenic (P9) and wild type (NN) plants at saline growth conditions (90 mM NaCl) during the two weeks experiment.

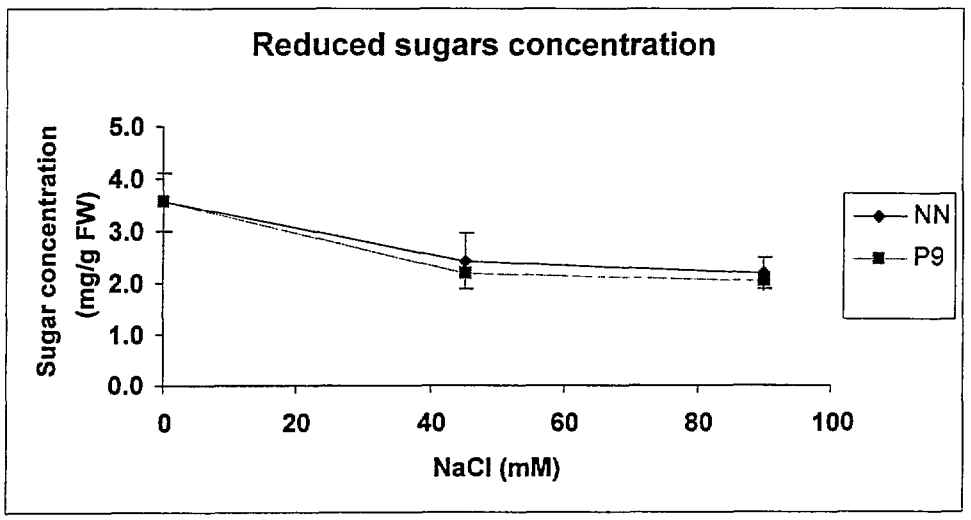


Figure 5: Evaluation of reduced sugars concentrations under salinity stress