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Comparative Analysis of the Regulation of Expression and Structures of Two Evolutionarily Divergent Genes for Δ^1-Pyrroline-5-Carboxylate Synthetase from Tomato[1]

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We isolated two tomato (*Lycopersicon esculentum*) cDNA clones, *tomPRO1* and *tomPRO2*, specifying Δ^1-pyrroline-5-carboxylate synthetase (P5CS), the first enzyme of proline (Pro) biosynthesis. *tomPRO1* is unusual because it resembles prokaryotic polycistronic operons (M.G. García-Ríos, T. Fujita, P.C. LaRosa, R.D. Locy, J.M. Clithero, R.A. Bressan, L.N. Csonka [1997] Proc Natl Acad Sci USA 94: 8249–8254), whereas *tomPRO2* encodes a full-length P5CS. We analyzed the accumulation of Pro and the *tomPRO1* and *tomPRO2* messages in response to NaCl stress and developmental signals. Treatment with 200 mM NaCl resulted in a >60-fold increase in Pro levels in roots and leaves. However, there was a <3-fold increase in the accumulation of the *tomPRO2* message and no detectable induction in the level of the *tomPRO1* message in response to NaCl stress. Although pollen contained approximately 100-fold higher levels of Pro than other plant tissues, there was no detectable increase in the level of either message in pollen. We conclude that transcriptional regulation of these genes for P5CS is probably not important for the osmotic or pollen-specific regulation of Pro synthesis in tomato. Using restriction fragment-length polymorphism mapping, we determined the locations of *tomPRO1* and *tomPRO2* loci in the tomato nuclear genome. Sequence comparison suggested that *tomPRO1* is similar to prokaryotic P5CS loci, whereas *tomPRO2* is closely related to other eukaryotic P5CS genes.

Water stress can be imposed by high salinity, dehydration, or freezing, which are environmental conditions that lead to the loss of water from cells. Water stress triggers the accumulation of Pro in a wide variety of species in all biological kingdoms (Paleg and Aspinall, 1981; Gilles et al., 1987; Csonka and Hanson, 1991). It has been suggested that the accumulation of Pro contributes to the maintenance of proper balance between extracellular and intracellular osmolality under conditions of water stress. Direct evidence supporting this hypothesis was provided by the fact that mutations that resulted in high level Pro overproduction conferred increased osmotic stress tolerance in *Salmonella typhimurium* (Csonka, 1981). High-level expression of P5CS, a bifunctional enzyme that catalyzes the first and second reactions of Pro biosynthesis, has been reported to result in increased salinity stress tolerance in transgenic tobacco plants (Kishor et al., 1995). However, the significance of Pro accumulation is still controversial (Verma and Hong, 1996; Hare and Cress, 1997), and other functions have been proposed for this response, such as free radical scavenging, nitrogen storage, or pH regulation (Stewart and Hanson, 1980; Delauney and Verma, 1993).

Pro is synthesized by the following four reactions: (a) ATP-dependent phosphorylation of glutamate to γ-glutamyl phosphate, catalyzed by GK; (b) reduction of γ-glutamyl phosphate by NADPH to γ-glutamyl semialdehyde, mediated by GPR; (c) spontaneous cyclization of γ-glutamyl semialdehyde to P5C; and (d) NADPH-dependent reduction of P5C to Pro, carried out by P5C reductase. In addition to this so-called "glutamate pathway" of Pro synthesis, an alternate route to Pro has been suggested, involving the conversion of Orn to P5C by Orn-δ-amino transferase. There are contradictory conclusions in the literature concerning the importance of the latter pathway during salinity stress. Whereas Delauney et al. (1993) found that the level of the Orn-δ-amino transferase mRNA was markedly decreased by high salinity, Roosens et al. (1998) observed that this message was induced by the same stress in Arabidopsis. Isotope-tracing studies suggested that the pathway via Orn is not important for Pro synthesis during osmotic stress in tomato (*Lycopersicon esculentum*) (Rhodes et al., 1986).

The finding that water stress increases the accumulation of Pro in numerous plant species, together with the demonstration that it is possible to enhance osmotic stress tolerance in bacteria by Pro overproduction provided the motivation for the cloning of genes of the Pro biosynthetic pathway from plants. Genes specifying GK have been cloned from moth bean (*Vigna aconitifolia*), Arabidopsis, rice (*Oryza sativa*), and tomato (Hu et al., 1992; Savouré et al., 1995; Yoshiba et al., 1995; Maggio et al., 1996; García-Ríos et al., 1997; Igarashi et al., 1997; Strizhov et al., 1997). The genes that were cloned from moth bean, Arabidopsis, and rice encode a P5CS made up of a hybrid GK and GPR.

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Abbreviations: GK, γ-glutamyl kinase; GPR γ-glutamyl phosphate reductase; IPTG, isopropyl-β-D-thiogalactopyranoside; P5C, Δ^1-pyrroline-5-carboxylate; P5CS, Δ^1-pyrroline-5-carboxylate synthetase; RFLP, restriction fragment-length polymorphism.

High salinity or dehydration results in increased accumulation of Pro in Arabidopsis, rice, and moth bean roots, and has been shown to be accompanied by an increase in the P5CS message level (Hu et al., 1992; Savouré et al., 1995; Yoshiba et al., 1995; Igarashi et al., 1997).

The induction in the level of P5CS mRNA has been determined to be 7- to 8-fold in Arabidopsis (Savouré et al., 1995; Yoshiba et al., 1995). Genes for the last enzyme of Pro biosynthesis, P5C reductase, were cloned from soybean, Arabidopsis, and pea (Delauney and Verma, 1990; Williamson and Slocum, 1992; Verbruggen et al., 1993), and it was observed that osmotic stress resulted in a similar increase in the P5C reductase message level as were seen for the P5CS message. The observation that salinity or dehydration stress stimulated the accumulation of the transcripts for the Pro-biosynthetic genes has been interpreted to mean that the transcriptional control of these genes is important for the regulation of Pro synthesis by osmotic stress. However, 50-fold overproduction of P5C reductase in transgenic tobacco plants did not lead to increased Pro accumulation (Szoke et al., 1992), indicating that the much smaller induction of P5C reductase in NaCl-stressed plants is not likely to be of significance for the Pro accumulation.

Plant species exhibit substantial variation both in the relative increases and final levels of Pro attained in response to osmotic stress (Delauney and Verma, 1993). Arabidopsis, pea, and rice, which have been used to probe the importance of transcriptional control for Pro synthesis, are in fact not the best representatives of Pro accumulators. These plants accumulate only approximately 2 to 6 μmol Pro/g fresh weight in response to NaCl stress (Williamson and Slocum, 1992; Savouré et al., 1995; Peng et al., 1996; Igarashi et al., 1997). Thus, unless it is highly concentrated in specific subcellular compartments or organelles, Pro at such low overall concentrations would not be expected to be a substantial determinant of the osmotic potential of the whole cells (Blum et al., 1996; Sharp et al., 1996). However, plants in the family Solanaceae have been found to contain much higher levels of Pro (Treichel et al., 1984; Handa et al., 1986; Rhodes et al., 1986; Delauney and Verma, 1993). The levels of this imino acid can be regulated over 300-fold in tomato tissue-culture cells by osmotic stress (Handa et al., 1986; Rhodes et al., 1986). ^{15}N-isotope-tracing experiments indicated that this increase in the Pro pool in cultured tomato cells upon osmotic stress was primarily due to a 10-fold increase in the rate of Pro synthesis via the glutamate pathway. Therefore, if transcriptional regulation of P5CS is important for the control of Pro synthesis by water stress, as has been suggested for Arabidopsis and rice, then one might expect that the Solanaceae, which accumulate much more robust levels of Pro under osmotic stress, would be more suitable for the study of this effect than the model species studied thus far.

For the above reasons, we cloned the genes that specify the first and second enzymes of Pro biosynthesis in tomato. We obtained two distinct clones, *tomPRO1* and *tomPRO2*. The *tomPRO1* clone was isolated from a tomato cDNA library by complementation of GK (*proB*) and GPR (*proA*) mutations in *Escherichia coli* (García-Ríos et al., 1991). Surprisingly, this locus proved to have an unusual structure, in that it contains two open reading frames that encode GK and GPR, arranged as a dicistronic operon (García-Ríos et al., 1997). The *tomPRO2* locus was cloned by hybridization to a fragment of the first P5CS gene cloned from Arabidopsis (see below). Like the P5CS genes from Arabidopsis, moth bean, and rice, *tomPRO2* specifies a hybrid GK-GPR as a single polypeptide.

Because mitochondria and chloroplasts, like prokaryotes, are able to translate polycistronic messages, we considered the possibility that the *tomPRO1* might be present on a plastid genome. To test this, we carried out RFLP mapping of *tomPRO1* and *tomPRO2* loci and demonstrated that both are present in the tomato nuclear genome. To our knowledge, this is the first example of a polycistronic locus mapped in plant nuclear genome. We also used these clones to probe the transcriptional regulation of the corresponding genes by osmotic stress. Our major finding was that transcriptional induction is not likely to be important for the regulation of Pro synthesis by osmotic stress in tomato, despite the fact that this plant accumulates Pro to much higher levels than Arabidopsis, pea, and rice.

MATERIALS AND METHODS

Isolation of *tomPRO2* cDNA

A 1.6-kb fragment of the *AtP5CS1* gene was generated by García-Ríos (1995) by PCR amplification of Arabidopsis total DNA with primers designed from highly conserved sequences in the *tomPRO1* clone (García-Ríos et al., 1997), the moth bean P5CS gene (Hu et al., 1992), and a partial sequence of the *AtP5CS2* gene (Strizhov et al., 1997; L. Szabados, personal communication). The two primers were 5′-GATGCTCATTTATGGGCTCC-3′ (specifying amino acids corresponding to residues 283–288 of the *tomPRO2* product) and 5′-CCATTCTGCTCCAAATCTTT-3′ (complementary to sequences specifying amino acids corresponding to residues 553–558 of the *tomPRO2* product). The amplified fragment was radiolabeled and used to probe a tomato (*Lycopersicon esculentum* cv Ailsa Craig) cDNA library in λgt10 (kindly provided by Dr. G. Martin; described in Martin et al. [1993]). Hybridization of the plaque blots on Hybond N$^+$ membranes (Amersham) was performed in 6× SSC and 1% SDS at 42°C. The blots were washed with 0.5× SSC and 0.1% SDS at 60°C. Among the positive clones, the one with the longest insert was subcloned into the *Eco*RI site of pBluescript SKII(−) (Stratagene) and sequenced using an automated fluorescence sequencer (Applied Biosystems).

Plant Materials

Tomato seeds were planted on 3M paper immersed with 0.25× Murashige and Skoog solution (JRH Biosciences, Lenexa, KS) under continuous light at 25°C. About 3 weeks after germination, the seedlings were transplanted to plastic containers filled with one-half-strength Hoagland solution and maintained hydroponically in a greenhouse under natural light. Leaf and root samples were taken at d 2, 6, 16, and 31 after the initiation of NaCl treatment. Various tis-

sues were collected from nonstressed hydroponic plants. Tomato tissue-culture cells were grown in the normal liquid medium (S0 cells) or in the medium containing 15 g/L NaCl (S15 cells), as described by Hasegawa et al. (1980).

Measurement of Pro Content in Tissues

Frozen materials were ground with a mortar and pestle in methanol:chloroform:water (12:5:1, v/v), and Pro content was determined by the acid ninhydrin procedure as described in Troll and Lindsley (1955).

Analyses of RNA

Total RNA was obtained by the LiCl-precipitation method as described in Nagy et al. (1988). For northern-blot analysis, 20 μg of total RNA was electrophoresed on a formaldehyde-agarose gel (1.2% agarose). After electrophoresis, the analysis was carried out by the standard protocol (Sambrook et al., 1989) on Hybond N^+ membrane (Amersham). For use as probes of northern blots, fragments containing nucleotides 1 to 899 of the *tomPRO1* cDNA clone plus an additional 90 bp derived from the multicloning site in a vector or the full-length *Eco*RI fragment of *tomPRO2* cDNA were amplified with PCR and labeled with [α-^{32}P]dATP and/or dCTP by a random-primer reaction according to the manufacturer's instructions (Amersham). After hybridization, filters were washed three times for 20 min with 0.1× SSC and 0.1% SDS at 42°C. RNase protection analyses were performed as described previously (García-Ríos et al., 1997) with 80 μg of total RNA, and probed with an antisense riboprobe from *tomPRO1* cDNA using the HybSpeed RPA kit (Ambion, Austin, TX). Levels of mRNA were quantified by scanning of the autoradioagrams with a densitometer (Molecular Dynamics, Sunnyvale, CA). To correct for loading differences in the northern blots (Figs. 1B and 2B), the blots were also probed with a fragment containing the 18S and 25S rRNA genes of flax (obtained from Dr. Joel Gaffe, Purdue University), and the densitometer readings for the *tomPRO2* signal for each sample were normalized to the total 25S and 18S rRNA signal.

Computer Analyses

Analyses of nucleotide and amino acid sequences were carried out with programs in the Genetics Computer Group (GCG) package of the University of Wisconsin, Madison, through a UNIX system. Comparisons against sequences in GenBank and amino acid sequence alignments were performed using the GAP and PILEUP programs, respectively. The codon usage table was derived by the CODONFREQUENCY program, and the codon usage tables for low- and high-expression genes in *Escherichia coli* and for genes of tomato were also supplied by the GCG package. For constructing phylogenetic trees, the neighbor-joining method was performed on the amino acid-composition data using the SEQBOOT, PROTDIST, NEIGHBOR, and CONSENSE tools from the PHYLIP program (Phylogeny Inference Package, version 3.5c, 1993; J.

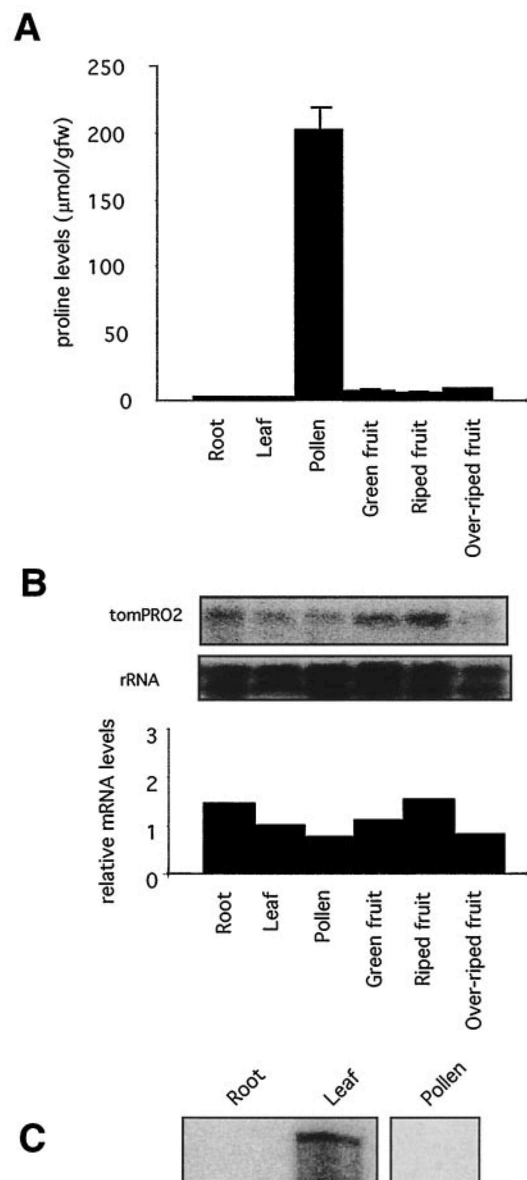

Figure 1. Relationship between Pro accumulation and mRNA levels in unstressed tomato tissues. A, Pro levels in different tissues from unstressed tomato. Pro values are means ± SE (n = 3). gfw, Grams fresh weight. B, Relative RNA levels in different tissues from unstressed tomato. Same materials as in A were used to extract total RNA. The top panel shows the result of northern-blot analysis, which was carried out using 20 μg of total RNA and probed with *tomPRO2* cDNA, as described in "Materials and Methods." The rDNA probe was used as a control for sample loading. RNA levels in each sample were quantified by densitometric scanning of the autoradiograms and normalized to the respective rRNA signal. The bottom panel shows normalized levels of the *tomPRO2* mRNA in the indicated tissues compared with leaves, where the value for leaves has been set to 1.0. C, RNase-protection assay of *tomPRO1* in roots, leaves, and pollen from unstressed tomato. RNase-protection analyses were performed using 80 μg of total RNA with a riboprobe from *tomPRO1* cDNA, as described in "Materials and Methods."

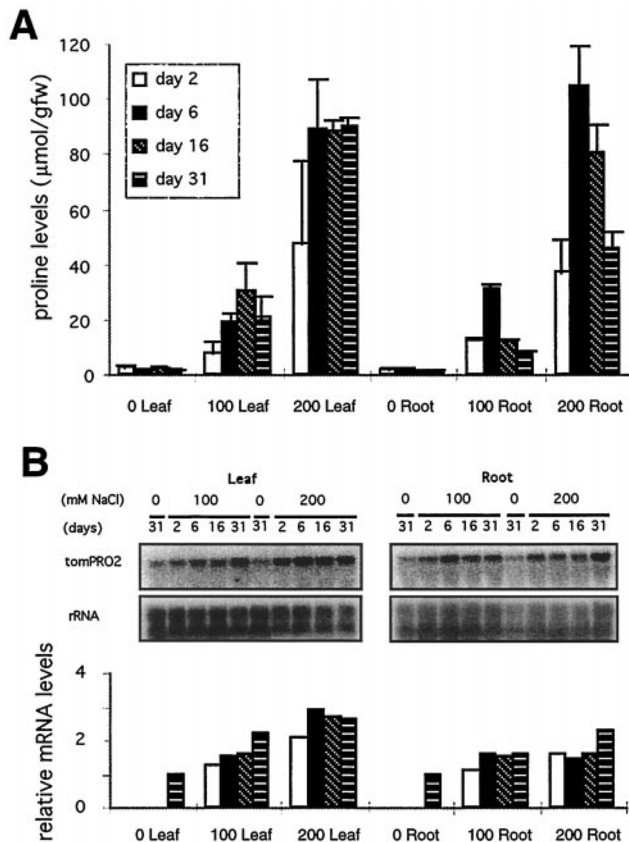

Figure 2. Relationship between Pro accumulation and *tomPRO2* mRNA levels in response to NaCl stress in tomato leaves and roots. A, Pro accumulation in leaves and roots of hydroponic tomato after 2, 6, 16, and 31 d of treatment with 0, 100, and 200 mM NaCl. Pro values are means ± SE ($n = 3$). gfw, Grams fresh weight. B, Relative RNA levels in leaves and roots of hydroponic tomato. The conditions for treatment were the same as in A. The top panel represents the result of northern-blot analysis, which was performed using total RNA (20 µg) probed with *tomPRO2* cDNA. The rDNA probe was used as a control for sample loading, and the results were measured, as described in the Figure 1B legend. The relative *tomPRO2* mRNA levels are shown in the bottom panel, with the message set to 1.0 for the 31-d samples in the untreated leaves and roots, respectively.

Felsenstein, Department of Genetics, University of Washington, Seattle). Bootstrapping was performed with 100 replicates. Distances were calculated using the Dayhoff PAM matrix option of PROTDIST. Abbreviations and accession numbers are: *tomPRO2*, tomato P5CS, U60267; Arabid, Arabidopsis P5CS, D32138; ArabidB, Arabidopsis P5CS2, X86778; Vigna, *V. aconitifolia* P5CS, M92276; Medicago, *Medicago sativa* P5CS, X98421; Rice, *Oryza sativa* P5CS, D49714; Homos, *Homo sapiens* P5CS, X94453; Cele, *Caenorhabditis elegans* P5CS, Z50797; Yeast, *Saccharomyces cerevisiae* GK and GPR, P32264 and X90565; Coryne, *Corynebacterium glutamicum* GK and GPR, U31230 and X82929; Bacsub, *Bacillus subtilis* GK and GPR, P39820 and P39821; Tthermo, *Thermus thermophilus* GK and GPR, D29973; Trepone, *Treponema pallidum* GK and GPR, U61535; Haein, *Hemophilus influenzae* GK and GPR, P43763 and U32804; Serma, *Serratia marcescens* GK and GPR, P17856 and P17857; Ecoli, *E. coli* GK and GPR, P07005 and P07004; Synecho, *Synechocystis* sp. GK and GPR, D90903 and D64001; Strept, *Streptococcus thermophilus* GK and GPR, X92418; tomPRO1, tomato GK and GPR, U27454.

Complementation of Pro Auxotrophy in *E. coli*

For the construction of a Pro auxotrophic derivative (KC1325) of *E. coli* strain BL21(DL3)pLysS (Novagen, Madison, WI), the *proB1658*::Tn*10* insertion, which is polar on *proA* (Mahan and Csonka, 1983), was transduced with P1 phage (Miller, 1972) into BL21(DE3)pLysS, selecting Tet^r progeny. Strain KC1325 was not able to grow without Pro, but the parental strain was, confirming that KC1325 is a Pro auxotroph. A *tomPRO2* fragment (nucleotides 44–2197) containing a complete open reading frame was amplified by PCR with the primers 5′-TTCCATGGAGACAGTTGATTCAACTCG-3′ and 5′-TTGGATCCATCACCCTTGCTGAGTAAGGT-3′ (which contain *Nco*I and *Bam*HI restriction enzyme sites, respectively), and the fragment was cloned between the *Nco*I and *Bam*HI sites of pET32a vector (Novagen) to yield pET32PRO2, resulting in a fusion protein of *tomPRO2* with an N-terminal extension from Trx-, His-, and S-tag sequences. Construction of pPRO1, which carries the *tomPRO1* cDNA in the *Eco*RI site of pBluescript KSII(+) (pKS; Stratagene), has been described by García-Ríos et al. (1997). Plasmids pPRO1, pET32PRO2, pKS, and pET32a were electroporated into KC1325, respectively. Complementation tests were carried out at 37°C on solid medium 63 (Cohen and Rickenberg, 1956) containing 10 mM Glc, 0.05 mM thiamine-HCl, and 1 mM IPTG, with or without 1 mM Pro.

Expression of Recombinant Proteins

For *tomPRO1* expression, *E. coli* strain HB101 (Δ*proBA leu thi-1*) was transformed with pPRO1. The transformants were grown in Luria-Bertani broth with ampicillin (100 µg/mL) at 37°C for 10 h. pET32PRO2 was used for the transformation of the strain KC1325. Production of a recombinant protein for *tomPRO2* was induced by 1 mM IPTG at 25°C for 17 h, based on the manufacturer's instructions (Novagen). Cells were collected and resuspended in a 125 mM Tris-HCl (pH 6.8), 4% SDS, 5% β-mercapthoethanol, and 20% glycerol. Total crude extracts were separated by 12% or 10% SDS-PAGE and then visualized with Coomassie brilliant blue R250 as in Sambrook et al. (1989).

RFLP Mapping

RFLP linkage analyses were performed utilizing F_2 progeny from the cross between *L. esculentum* and *Lycopersicon pennellii*. DNA samples from 67 of the F_2 progeny had been digested by various restriction enzymes, separated by electrophoresis, and transferred to Hybond N^+ membranes. These membranes, which had been used previously for the mapping of numerous other markers (Tanksley et al., 1992), were generously provided by Dr. G. Martin (Purdue University). RFLP markers were also collected and sup-

plied by Dr. G. Martin. ^{32}P-labeled probe preparation and DNA gel-blot analyses were basically the same as for the RNA gel-blot analyses, except that they were washed with 0.2× SSC, 0.1% SDS at 25°C or with 0.1× SSC and 0.1% SDS at 42°C. Multipoint linkage analyses were performed using the MapMaker program (version 2.0, Lander et al., 1987). Recombination frequencies from multipoint analysis were converted into map distances (in centiMorgans [cM]) using the mapping function of Kosambi (1944).

RESULTS

Isofunctional Enzymes Catalyzing the First Step of Pro Biosynthesis Are Specified by Two Distinct Genes, *tomPRO1* and *tomPRO2*, in Tomato

We isolated a polycistronic *tomPRO1* locus in tomato, which specifies GK and GPR divided by an internal stop codon in a single gene (García-Ríos et al., 1997). Because the accumulated sequence information indicated that the first step of Pro biosynthesis is mediated by a bifunctional P5CS in other higher eukaryotes, we screened a tomato cDNA library with a genomic DNA fragment from Arabidopsis encoding P5CS. We identified several inserts that strongly hybridized to this probe, the longest of which was selected for further analysis. Nucleotide sequencing of this clone, which was designated *tomPRO2*, revealed that it contained a single, long open reading frame, flanked by 43- and 42-bp untranslated regions at the 5′ and 3′ ends, respectively. The predicted amino acid sequence of *tomPRO2* indicated that it consists of a GK-GPR hybrid as a monocistron, having an overall 76% identity at the amino acid level to Arabidopsis P5CS. However, *tomPRO2* shows only 35% identity to *tomPRO1* products, suggesting that *tomPRO2* represents a homolog of the Arabidopsis P5CS gene.

The Levels of Pro and of the P5CS Message in Various Tissues in Unstressed Tomato Plants

In tomato plants grown under nonstressed conditions, Pro was present in the range of 1 to 7 μmol/g fresh weight in roots, leaves, and fruits of various stages (Fig. 1A). However, in accord with previous reports that pollen are rich in free Pro (Khoo and Stinson, 1957; Hong-qi et al., 1982), we found that the level of this imino acid was 200 μmol/g fresh weight in tomato pollen. We examined the accumulation of *tomPRO1* and *tomPRO2* mRNAs in the different tissues in the unstressed plants using northern-blot analyses with the *tomPRO1* and *tomPRO2* cDNA inserts as the probes. The *tomPRO2* message was readily observable in all tissues, but the *tomPRO1* message was not detectable by northern blots (data not shown), indicating that *tomPRO1* is not expressed at all or at a substantially lower level than the *tomPRO2* gene in the tissues analyzed. As shown in Figure 1B, the level of the *tomPRO2* mRNA, normalized to the rRNA level to correct for loading errors, was nearly the same in all of the tissues, including pollen. Whereas the *tomPRO1* message was not observable with northern blots, using the more sensitive RNase-protection assay, we were able to observe it in leaves (Fig. 1C). The *tomPRO1* transcript was undetectable in roots and pollen even with this assay. Thus, our data indicate that the high-level accumulation of Pro in pollen was not correlated with a detectable induction in the levels of the *tomPRO1* and *tomPRO2* transcripts.

Pro Is Accumulated to High Levels in NaCl-Stressed Plants

We examined the effect of NaCl stress on Pro levels in hydroponically grown tomato plants (Fig. 2A). Treatment with 100 mM NaCl elicited an approximately 15-fold increase in the level of Pro accumulation in both leaves and roots, and treatment with 200 mM NaCl resulted in 60- and 80-fold increases in these tissues, respectively. In leaves the highest level of Pro was reached after 6 d, and was maintained until 31 d after treatment; in roots, Pro decreased to about one-half of the highest level by this time. The more rapid disappearance of Pro in roots compared with leaves in NaCl-treated plants could reflect a more severe osmotic stress in leaves because of transpiration and/or slower osmotic adjustment than in roots.

The same plants that were used for Pro measurement were also subjected to northern analysis to determine the effect of osmotic stress on the accumulation of the P5CS transcripts. As before, the *tomPRO2* mRNA was much more abundant than the *tomPRO1* mRNA, which was not detectable with northern analysis in any of the tissues in the NaCl-stressed plants (data not shown). NaCl stress resulted in some increase in the accumulation of the *tomPRO2* transcript. However, the *tomPRO2* message level, normalized to the rRNA signal, increased only about 2-fold after 2 d of treatment with 100 and 200 mM NaCl in both leaves and roots, and remained at almost the same level for 31 d after the treatment (Fig. 2B). Although Pro accumulation in leaves and roots treated with 200 mM NaCl was 3- to 7-fold higher throughout the entire course of the treatment than in the same tissues treated with 100 mM NaCl (Fig. 2A), we have no evidence that the level of the *tomPRO2* mRNA is altered substantially by either the severity or duration of osmotic stress (Fig. 2B). Thus, our results show that the *tomPRO2* transcript level was induced much less by NaCl in tomato than in Arabidopsis, rice, and moth bean (Hu et al., 1992; Savouré et al., 1995; Yoshiba et al., 1995; Igarashi et al., 1997; Strizhov et al., 1997), even though tomato accumulated >15-fold higher levels of Pro than those other plants. Thus, our results suggest that control of the accumulation of the *tomPRO2* message level is probably not important for the regulation of Pro synthesis by NaCl stress.

Effect of NaCl Stress on the Pro Levels and the Accumulation of the P5CS Transcripts in Tissue-Culture Cells

We also measured the Pro levels in normal and NaCl-adapted tissue-culture cells. As shown in Figure 3A, cells grown in normal medium (S0) had a very low level of Pro, whereas cells grown in medium containing 15 g/L NaCl (S15) had an approximately 30-fold higher level of this

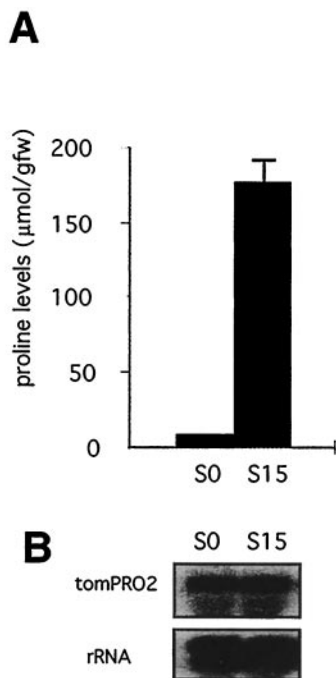

Figure 3. Relationship between Pro accumulation and mRNA levels in tomato tissue-culture cells. A, Comparison of Pro levels in tissue-culture cells grown in normal medium (S0) and in medium containing 15 g/L NaCl (S15). Pro values are means ± SE ($n = 3$). gfw, Grams fresh weight. B, Relative RNA levels in the tissue-culture cells S0 and S15. Same materials as in A were used to extract total RNA. The analysis was carried out using 20 μg of total RNA and probed with *tomPRO2* cDNA. The rDNA probe was used as a control for sample loading.

imino acid. Despite this difference in the Pro content, the level of the *tomPRO2* message was essentially the same in the two types of cells, as detected by northern blotting (Fig. 3B), indicating that the 30-fold increase in the Pro levels seen in tissue-culture cells adapted to 15 g/L NaCl occurred without any notable change in the accumulation of the *tomPRO2* message. The *tomPRO1* mRNA was not detectable by northern-blot analysis in either of the cells, but as reported earlier (García-Ríos et al., 1997), the more sensitive RNase-protection assays demonstrated that the S15 cells had a 4-fold higher level of the *tomPRO1* message than the S0 cells. However, because of the low abundance of the *tomPRO1* message even in the NaCl-adapted cells, the induction of this message probably is not sufficient to account for the elevation in the Pro pools size in the tissue-culture cells.

The *tomPRO1* and *tomPRO2* Loci Are Structurally Different and May Have Evolved from Separate Ancestral Genes

In addition to the different levels of expression of *tomPRO1* and *tomPRO2* described above, the two cDNAs have remarkable structural differences. The *tomPRO1* has a dicistronic structure (García-Ríos et al., 1997), which is unusual in eukaryotes (Kozak, 1986). Thus far, all genes encoding GK and GPR in bacteria and yeast have been reported as separate genes, organized as an operon in most bacteria. In contrast, *tomPRO2* encodes a bifunctional enzyme formed by a hybrid of GK and GPR without an intervening stop codon, similar to genes encoding a hybrid GK and GPR, or P5CS, that have been cloned from several higher eukaryotes, such as *C. elegans, H. sapiens,* and higher plants (Hu et al., 1992; Savouré et al., 1995; Yoshiba et al., 1995; Liu et al., 1996; Igarashi et al., 1997).

Table I shows that the predicted amino acid sequence of *tomPRO2* has greater than 76% identity with other plant P5CS, but shows a much lower identity (35%–44%) with the *tomPRO1* product and the bacterial and yeast GK and GPR enzymes. In contrast, the predicted *tomPRO1* product has a low amino acid identity (32%–47%) with GK and GPR from yeast and prokaryotes and with P5CS from all other eukaryotes. Although the *tomPRO1* product has a similar homology to the corresponding proteins from different organisms, sequence alignment shown in Figure 4 revealed that *tomPRO1* is closer to bacterial GK and GPR than to P5CS of plants. Some regions that are highly conserved in P5CS proteins from plants are either missing, divergent, or carry insertions in *tomPRO1* and in bacterial GK and GPR (e.g. amino acids 71–83, 151–161, and 194–219 in GK, and amino acids 426–451, 476–497, and 571–577 in GPR, where the numbers of amino acid residues are based on *tomPRO1* sequences). However, the GK part of *tomPRO1* also shows a striking difference from most bacterial GKs, because it lacks an approximately 100-amino acid C-terminal tail that is conserved in most other prokaryotic GKs (represented

Table I. *Comparison of predicted amino acids from various GKs, GPRs, and P5CSs*

Name[a]	*tomPRO1*	*tomPRO2*
	%	
tomPRO2	35/57[b]	100
Arabid	34/56	76/87
ArabidB	34/56	76/88
Vigna	32/54	76/88
Medicago	33/56	77/87
Rice	34/55	76/85
Homos	34/56	48/66
Cele	32/56	47/66
Yeast	37/59	39/59
Coryne	40/61	34/57
Bacsub	39/56	35/56
Tthermo	39/61	35/56
Trepone	41/60	35/55
Haein	39/60	37/59
Serma	40/60	38/59
Ecoli	40/61	39/60
Synecho	41/62	44/62
Strept	47/65	37/59
tomPRO1	100	35/57

[a] Comparison was done by the GAP program of the GCG. Abbreviations and accession numbers for these proteins are as in "Materials and Methods." [b] Each number is depicted as identity/similarity.

Figure 4. Amino acid sequence comparison of *tomPRO1*, *tomPRO2*, and other related genes. Predicted amino acid sequences of *proB*, *proA*, and *P5CS* genes were aligned using the multiple alignment program PILEUP, and the results were highlighted with the BOXSHADE program. Letters in the black and gray backgrounds indicate identical and similar residues, respectively. Representative regions that are highly conserved in P5CS proteins in plants but are either missing, divergent, or carry insertions in GK and GPR for *tomPRO1* and in bacterial GK and GPR are underlined. Extended C-terminal tails of GK, which are conserved in most of prokaryotic GK, are shown by a dashed line. Abbreviations and accession numbers are provided in "Materials and Methods."

by *E. coli* GK, amino acids 259–367, and *B. subtilis* GK, amino acids 256–354 [García-Ríos et al., 1997]). At present, *S. thermophilus* is the only exception among bacteria that also lacks this C-terminal tail in GK. The *tomPRO1* product has the closest sequence similarity to the GK and GPR from the latter organism (Table I).

We compared codon usage of *tomPRO1* and *tomPRO2* with the average codon usage of tomato genes and of genes expressed at low or high levels in *E. coli*. The *tomPRO2* codon usage agrees well with the preference of average codon usage from tomato genes, whereas *tomPRO1* codon usage deviates from the usage of tomato genes (Table II), and in fact, appears to be between the preference in genes in tomato and in *E. coli* (results not shown).

Because of the intriguing differences in nucleotide and predicted amino acid sequences of *tomPRO1* and *tomPRO2*, the relationship of these two loci to other GK, GPR, and P5CS from various species was examined further by a phylogenetic analysis. A phylogenetic tree was constructed by the neighbor-joining method from a highly conserved region of GK (amino acids 84–149 of *tomPRO1*). Figure 5A revealed that P5CS proteins from higher eukaryotes are clearly monophyletic, and that *tomPRO2* was tightly clustered as a member of plant P5CS within this group. On the other hand, *tomPRO1* appeared together with *T. pallidum* and *S. thermophilus* within a bacterial group. This trend is also true for the phylogenetic tree constructed from a highly conserved region of GPR (361–436 amino acids of *tomPRO1*) as shown in Figure 5B. Whereas the GPR domain of *tomPRO2* grouped with the corresponding regions of other eukaryotes, the *tomPRO1*-encoded GPR clustered with bacterial ones. These results suggest that *tomPRO1* and *tomPRO2* were probably incorporated into the tomato genome separately during evolution.

tomPRO1 and *tomPRO2* Encode Functional Enzymes Catalyzing GK and GPR Activities

We demonstrated that the *tomPRO2* gene encodes a functional P5CS by the fact that it can complement both a GK and a GPR defect in *E. coli*. As shown in Figure 6B, the plasmid carrying the *tomPRO2* fragment could complement the polar *proB1658*::Tn*10* mutation, whereas the vector by itself was unable to do so. In accord with our earlier observations (García-Ríos et al., 1997), the *tomPRO1* cDNA clone inserted pBluescript KSII(+) could likewise comple-

Table II. *Comparison of codon frequency usage in tomPRO1, tomPRO2, and general tomato genes*

Values are given of each codon in each amino acid. Trp, Met, and stop codons are not included.

Amino Acid	Codon	*tomPRO1*	*tomPRO2*	Tomato
			%	
Arg	CGA	14	12	9
	CGC	29′ [a]	5	7
	CGG	43″ [b]	5	4
	CGU	14	29′	18
	AGA	0	36″	37″
	AGG	0	14	24′
Leu	CUA	7	10	9
	CUC	4	11	13
	CUG	10	14	7
	CUU	7	35″	30″
	UUA	28′	12	12
	UUG	42″	19′	30″
Ser	UCA	28′	21	26″
	UCC	0	7	13
	UCG	10	5	7
	UCU	14	28′	25′
	AGC	3	7	13
	AGU	45″	33″	16
Thr	ACA	24	35′	32′
	ACC	27′	9	22
	ACG	41″	6	8
	ACU	8	50″	38″
Pro	CCA	56″	40′	47″
	CCC	7	15	13
	CCG	22′	0	6
	CCU	15	45″	35′
Ala	GCA	13	32′	30′
	GCC	31′	13	18
	GCG	34″	4	5
	GCU	21	51″	46″
Gly	GGA	10	44″	38″
	GGC	27	22	13
	GGG	29′	11	11
	GGU	34″	24′	38″
Val	GUA	7	17	14
	GUC	24′	7	16
	GUG	48″	33′	25′
	GUU	20	43″	45″
Lys	AAA	58	55	46
	AAG	42	45	54
Asn	AAC	52	33	45
	AAU	48	67	55
Gln	CAA	76	44	64
	CAG	24	56	36
His	CAC	20	38	39
	CAU	80	63	61
Glu	GAA	76	51	52
	GAG	24	49	48
Asp	GAC	21	22	33
	GAU	79	78	67
Tyr	UAC	13	19	53
	UAU	87	81	47
Cys	UGC	0	43	46
	UGU	100	57	54
Phe	UUC	15	22	44
	UUU	85	78	56
Ile	AUA	4	25	18
	AUC	16	23	29
	AUU	80	52	52

[a] ′, Second-most frequently used codon. [b] ″, Most frequently used codon.

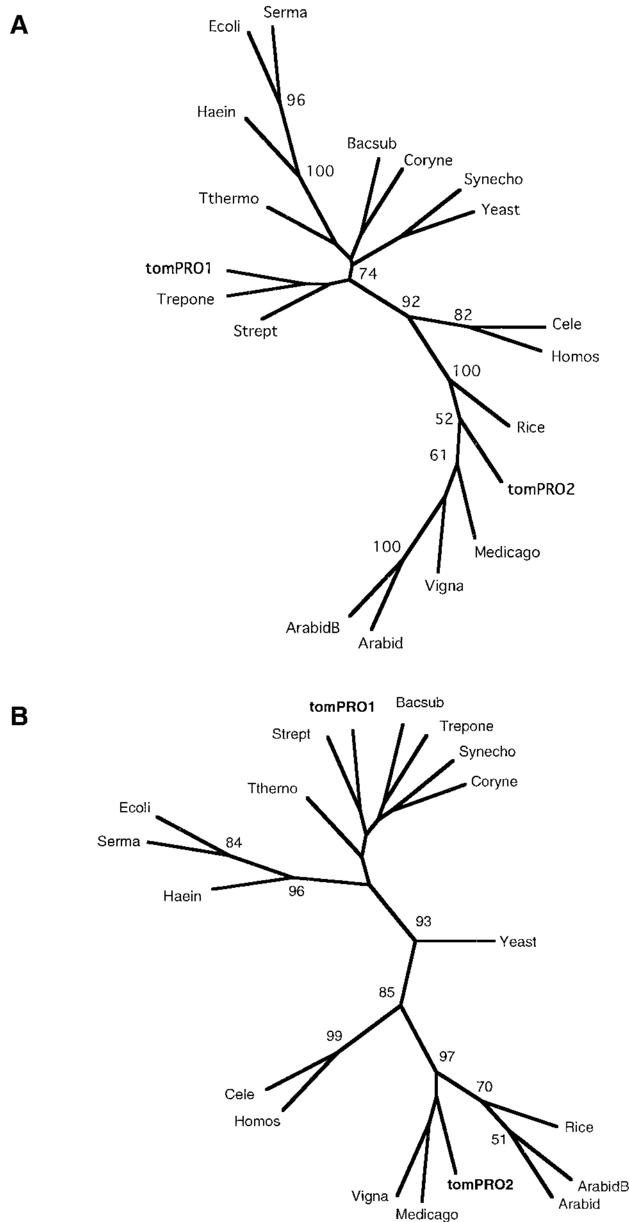

Figure 5. Possible evolutionary relationship among the GK (A) and the GPR (B) proteins. The phylogenetic tree was generated using the PHYLIP program (Felsenstein, 1993). Numbers are bootstrap values given as percentages, and only 50% or greater values are indicated at a node. Abbreviations and accession numbers are as described in "Materials and Methods."

ment the Pro auxotrophic mutation in KC1325 (Fig. 6B). All strains could grow on the medium containing Pro (Fig. 6A). These results demonstrate that although both *tomPRO1* and *tomPRO2* show only 35% amino acid sequence identity, they both specify functional GK and GPR.

We verified with SDS-PAGE analysis that the *tomPRO2* gene directs the synthesis of a single polypeptide of the expected mass of 98 kD in *E. coli* (Fig. 6C), whereas *tomPRO1* directs the synthesis of two polypeptides: the approximately 33-kD GK and the approximately 44-kD GPR, in accord with our previous report (García-Ríos et al., 1997) that *tomPRO1* is recognized as a polycistronic locus in *E. coli*.

tomPRO1 and *tomPRO2* Are Located at Different Loci within the Tomato Nuclear Genome

Restriction fragments of total tomato DNA were probed with sequences from the GK region of *tomPRO1* and with full-length *tomPRO2*. Probes made from both clones hybridized with an efficiency of less than 10 copies per haploid genome (data not shown). This result suggested that each locus is present in the nuclear genome but not in an organelle genome, which is present at a much higher copy number (>50 copies).

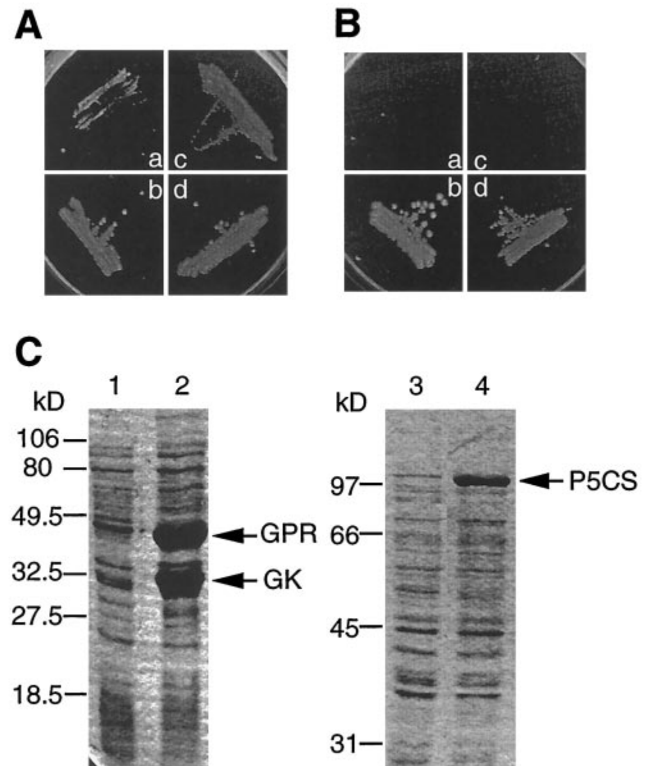

Figure 6. Complementation of a *proBA* mutation by *tomPRO1* and *tomPRO2*, and their products expressed in *E. coli*. A and B, Expression vectors containing the *tomPRO1* and *tomPRO2* cDNA clones were introduced into strain KC1325 (a derivative of BL21[DL3]pLysS carrying the *proB1658*::*Tn10* insertion, which is polar on *proA*). a, KC1325 harboring the vector, pKS only. b, KC1325 harboring pPRO1. c, KC1325 harboring pET32a only. d, KC1325 harboring pET32PRO2. Strain KC1325 containing each plasmid was streaked on minimal M63 medium containing Glc, thiamine, and IPTG with (A) and without (B) Pro, and incubated for 2 d at 37°C. All strains could grow on the media supplemented with Pro (A). C, Total cell extracts from either *E. coli* strain HB101, containing pKS (lane 1) and pPRO1 (lane 2), or strain KC1325, containing pET32a (lane 3) and pET32PRO2 (lane 4), were analyzed by SDS-PAGE. The gels were stained with Coomasie brilliant blue. *tomPRO1* products are indicated as GK and GPR, and the *tomPRO2* product as P5CS. Numbers at left refer to size standards (in kD).

We mapped both genes using a segregating population of progeny from the cross *L. esculentum* × *L. pennellii*. As shown in Figure 7, the *tomPRO1* locus was mapped to a region of 2.6 cM adjacent to the TG33 locus on chromosome 2. Mapping of the *tomPRO1* locus to chromosome 2 supports unambiguously our conclusion that a polycistronic locus is present in the tomato nuclear genome, as opposed to being in a chloroplast or mitochondrial genome, which would not only be in a much higher copy, but would also be maternally inherited. The *tomPRO2* locus proved to be present in a region of approximately 1 cM between the TG228 and CT92 markers on chromosome 8 (Fig. 7B, left panel). Southern analysis with a *tomPRO2* probe at a milder stringency (Fig. 7B, right panel) revealed additional bands that appeared homologous to *tomPRO2,* which did not hybridize to a *tomPRO1* probe under these conditions. These *tomPRO2*-related bands were mapped to chromosome 6.

DISCUSSION

Pro Accumulation Is Not Correlated with the *tomPRO1* and *tomPRO2* Message Levels in Tomato

It has been proposed that transcriptional control of the P5CS gene, which encodes a bifunctional enzyme catalyzing the first and second reactions of Pro synthesis, is important for the regulation of accumulation of this imino acid during osmotic stress in plants. This conclusion was based on the observation that NaCl stress increased the P5CS transcript level in moth bean, Arabidopsis, and rice (Hu et al., 1992; Savouré et al., 1995; Yoshiba et al., 1995;

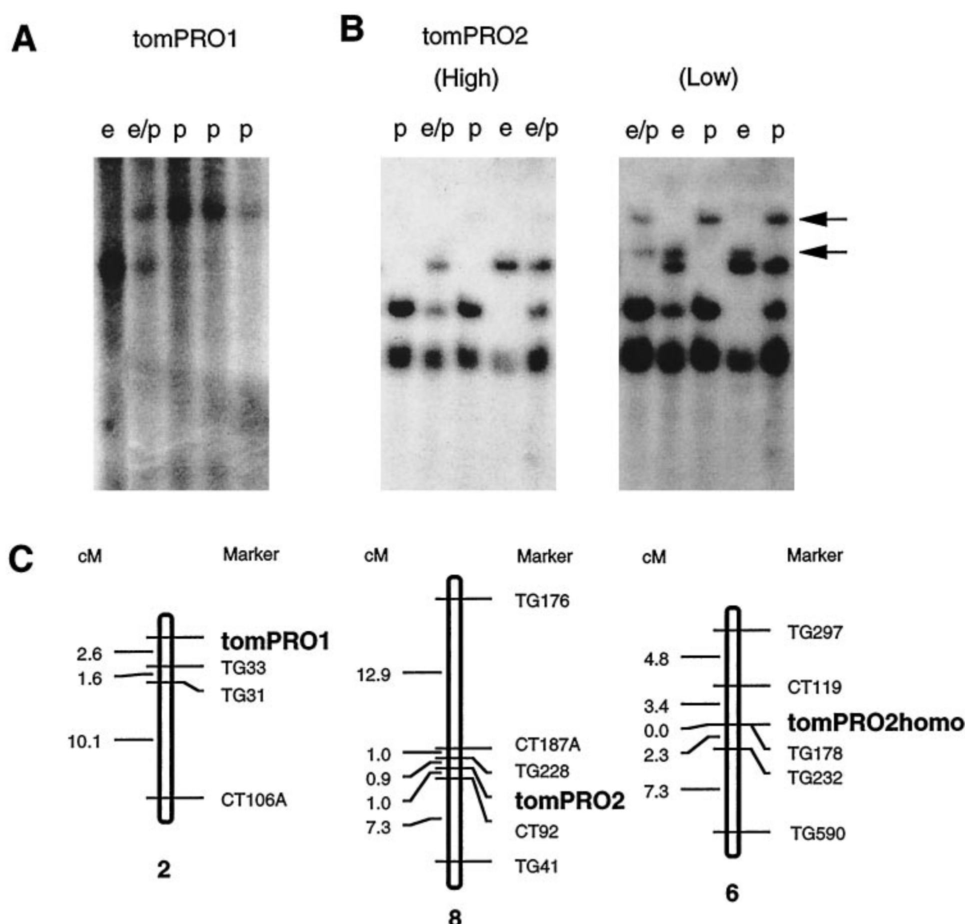

Figure 7. Mapping of *tomPRO1*, *tomPRO2*, and *tomPRO2* homolog in tomato nuclear genome. A and B, Southern-blot analyses of total DNA from the F_2 population of crosses between *L. esculentum* and *L. pennellii*. Hybridizations were performed with the GK part of the *tomPRO1* cDNA fragment (A) and the full length of *tomPRO2* cDNA as the probes (B) at a high-stringency wash condition, 0.1× SSC, 0.1% SDS, at 42°C (High), and at a low-stringency wash condition, 0.2× SSC, 0.1% SDS, at 25°C (Low). Two bands that appeared at a low-stringency condition are depicted by arrows. This figure shows a representative portion of the blots, in which a total of 67 of the F_2 populations were used for the RFLP mapping. Shown at the top of each lane is the RFLP pattern representative for the *L. esculentum* homozygote (e), the *L. pennellii* homozygote (p), or their heterozygote (e/p). C, Map position of *tomPRO1*, *tomPRO2*, and *tomPRO2* homologs on the tomato chromosome. The maps were drawn by segregation analysis of RFLPs based on data by Tanksley et al. (1992). The map distances (in cM) are indicated on the left. Maps are not drawn to scale. *tomPRO1*, *tomPRO2,* and *tomPRO2* homologs (tomPRO2homo) were located on chromosomes 2, 8, and 6, respectively.

Igarashi et al., 1997). In rice and Arabidopsis, the increases in the Pro levels were accompanied by coordinate increases in the P5CS transcript levels. (The accumulation of Pro was not monitored in moth bean during the course of induction of the P5CS message [Hu et al., 1992].) Arabidopsis has two P5CS isoenzymes, encoded in the *AtP5CS1* and *AtP5CS2* genes (Savouré et al., 1995; Yoshiba et al., 1995; Strizhov et al., 1997; Zhang et al., 1997). *AtP5CS1*, which was estimated to synthesize about 60% to 80% of the total P5CS mRNA (Strizhov et al., 1997), exhibited up to an 8-fold induction upon osmotic stress (Savouré et al., 1995; Yoshiba et al., 1995; Strizhov et al., 1997), whereas *AtP5CS2* exhibited a $<$4-fold regulation (Strizhov et al., 1997; Zhang et al., 1997). Because tomato accumulates much more Pro than Arabidopsis or rice, our initial hypothesis had been that tomato might show an even more sensitive regulation of P5CS transcript accumulation than the other two plants. To test whether this is the case, we determined the effect of NaCl stress on the accumulation of the *tomPRO1* and *tomPRO2* transcripts. We also determined whether there is a special transcriptional regulation of these two loci in pollen, which contain very high levels of Pro.

Although NaCl stress was shown to cause some Pro accumulation in rice and Arabidopsis, this metabolite reached only a maximum of 2 and 6 μmol Pro/g fresh weight in these two species (Savouré et al., 1995; Igarashi et al., 1997; Zhang et al., 1997). In contrast, we found that tomato accumulated to 90 and 105 μmol Pro/g fresh weight in leaves and roots, respectively, after 6 d of treatment with 200 mM NaCl (Fig. 2), representing a 60- to 80-fold increase over the level in unstressed plants. Surprisingly, in view of the results reported for Arabidopsis and rice, there was only about a 2- to 3-fold change in the *tomPRO2* transcript level throughout the entire time course of NaCl treatment. In roots the accumulation of Pro was maximal at d 6 of NaCl treatment, after which it declined gradually, but this was not reflected by a decrease in the *tomPRO2* message level (Fig. 2). The Pro pool size was 30-fold higher in the NaCl-adapted S15 tomato tissue-culture cells than in the control, unadapted S0 cells (Fig. 3). Despite this large difference in Pro content, the *tomPRO2* message was present at similar levels in the two types of cells.

Previously, we showed that the level of the *tomPRO1* message in the S15 cells was approximately 4-fold higher than in the S0 cells (García-Ríos et al., 1997). However, because of the low level of this transcript even in the NaCl-stressed cell line, this induction of the transcript probably is not sufficient to account for the increase in the Pro content. The highest level of Pro in all tissues tested was found in pollen of unstressed plants. (We did not measure the Pro content in pollen of NaCl-stressed plants.) The *tomPRO2* message level, however, was unchanged compared with other tissues, and the *tomPRO1* message was undetectable in pollen. These results indicate that in tomato, the large increases in Pro levels in response to NaCl stress or pollen-specific developmental signals are brought about without substantial increases in the levels of the *tomPRO1* and *tomPRO2* messages.

Because we only determined the steady-state accumulation of these messages, we cannot infer that *tomPRO1* and *tomPRO2* are transcribed constitutively. In principle, it is conceivable that changes in the rates of synthesis of these transcripts could be compensated for by comparable changes in their turnover. Zhang et al. (1997) demonstrated with a GUS reporter fusion that the 2- to 4-fold increase in the level of the *AtP5CS2* message after dehydration or NaCl stress in transgenic Arabidopsis and tobacco plants was the result of transcriptional induction. However, all of the other studies on the regulation of the accumulation of the P5CS messages in Arabidopsis and rice (Savouré et al., 1995; Yoshiba et al., 1995; Igarashi et al., 1997; Strizhov et al., 1997) involved only measurements of the steady-state levels of these messages, and, therefore, direct evidence is lacking that the increases in the accumulation of these transcripts upon water stress are necessarily brought about by induction of transcription initiation.

Aside from control of the accumulation of P5CS message (at the level of synthesis or turnover), there are several other possible mechanisms for the control of Pro biosynthesis. The enzyme for the first step, GK, is sensitive to feedback regulation by Pro (Hu et al., 1992; García-Ríos et al., 1997). However, there may be important differences in the allosteric properties of the enzymes in tomato and other plants, as indicated by the observations that the activity of the *tomPRO1*-encoded P5CS was inhibited 50% by 0.07 mM Pro (García-Ríos et al., 1997), whereas 5 mM Pro was required to elicit 50% inhibition of the GK activity of moth bean P5CS (Zhang et al., 1995). We have not been successful in measuring the kinetic properties of *tomPRO2* product because of difficulties in obtaining this enzyme in a soluble form. However, we have preliminary evidence that this enzyme, which is more similar in its amino acid sequence to the moth bean P5CS than to the *tomPRO1* product, is also sensitive to feedback inhibition by Pro. It is possible that the regulation of synthesis of Pro in tomato is effected by relief of allosteric inhibition of the activities of the *tomPRO1* and *tomPRO2* products under NaCl or dehydration stress. Tomato may have an additional gene related to *tomPRO2* (Fig. 7), for which we have no sequence information. If it proves to be related to P5CS, it could participate in the regulation of Pro synthesis.

The accumulation of Pro could also be regulated by changes in the rate of its catabolism to glutamate by the combined action of Pro dehydrogenase and P5C dehydrogenase. However, because Pro dehydrogenase is a mitochondrial enzyme (Kiyosue et al., 1996), effective catabolism of Pro would presumably require transport of Pro from the cytosol to the mitochondria. In Arabidopsis NaCl stress or dehydration down-regulates the accumulation of the message for Pro dehydrogenase (Kiyosue et al., 1996; Peng et al., 1996; Verbruggen et al., 1996). Although repression of the synthesis of Pro dehydrogenase could have a role in the long-term regulation of Pro accumulation in response to water stress, the effect of water stress on the activity or stability of Pro dehydrogenase itself has not been determined in Arabidopsis. Repression of transcription of the gene for Pro dehydrogenase would be an efficient mechanism for increasing the Pro pools size only if

this response is accompanied by a simultaneous inactivation or turnover of preexisting Pro dehydrogenase molecules. Direct evidence on the relative contributions of the biosynthetic and catabolic pathways for the regulation of Pro pool size was provided in cultured tomato cells by the ^{15}N-isotope-tracing experiments of Rhodes et al. (1986). These studies indicated that the 300-fold increase in the Pro accumulation resulting from 25% PEG stress was mainly due to a 10-fold increase in the rate of biosynthesis and provided no evidence that the rate of Pro catabolism was inhibited under these conditions.

Changes in the intracellular Pro levels could also be accomplished by translocation of this metabolite between different tissues or cell compartments. Two genes, *ProT1* and *ProT2*, which encode closely related Pro-transport proteins, have been cloned from Arabidopsis (Rentsch et al., 1996). Accumulation of the *ProT2* message was strongly elevated by NaCl stress, indicating that control of the synthesis of Pro-transport proteins also could be involved in the regulation of the cellular Pro pool sizes.

Two Evolutionarily Distinct Genes Are Present in the Tomato Nuclear Genome

We showed that the *tomPRO1* and *tomPRO2* loci are present in the tomato nuclear genome. Comparison of protein sequence, codon usage, and phylogenetic analysis suggested that *tomPRO2* is in a tight group containing the P5CS proteins from plants and other higher eukaryotes. In contrast, *tomPRO1* has several unique features that distinguish it from the eukaryotic P5CS group. First, *tomPRO1* did not show a high identity to eukaryotic P5CS (32%–35% at the amino acid level). A comparison of codon usage of *tomPRO1* with genes from *E. coli* or tomato suggested that the codon usage of *tomPRO1* is not typical for either *E. coli* or tomato genes, but something in between. Second, *tomPRO1* has a dicistronic structure, similar to polycistronic operons found in bacteria, and in fact, *tomPRO1* is recognized as a dicistronic operon in *E. coli* (Fig. 6C). Third, phylogenetic analysis placed *tomPRO1* within the same clade of prokaryotes, separate from the other eukaryotic P5CS genes. These characteristics suggest that *tomPRO1* and *tomPRO2*, which are both nuclear loci, might have different origins. We present alternative hypotheses for the possible origin of the *tomPRO1* locus, but we would like to emphasize that at this stage we do not have sufficient data to distinguish among these speculative alternatives.

There are several reports in which isozymes are encoded by multigene families in nuclear genomes, as exemplified by the existence of dual genes for P5CS in Arabidopsis (Strizhov et al., 1997). Multigene families may be derived by gene duplication or by gene conversion from a single gene. It is, however, unlikely that the *tomPRO1* and *tomPRO2* loci arose in tomato by such mechanisms, because of the difference in their coding regions. The prokaryotic features of *tomPRO1* are consistent with the notion that it may have been acquired by organelle-to-nucleus gene transfer, or by uptake of DNA of prokaryotic origin into the nuclear genome. According to the theory of endosymbiosis, mitochondria and chloroplasts originated from once free-living eubacteria (Gray, 1989), followed by the loss of genes from the organellar genomes or transfer to the nucleus (Weeden, 1981; Palmer, 1985). The *tufA* gene, encoding the chloroplast protein synthesis elongation factor Tu in Arabidopsis, and the *rpl22* gene, encoding chloroplast ribosomal protein CL22, are examples of genes that were transferred from the chloroplast genome to the nucleus (Baldauf and Palmer, 1990; Gantt et al., 1991). There are two isoenzymes of glyceraldehyde-3-phosphate dehydrogenase in tobacco and maize, one found in the chloroplasts and the other in the cytosol. Although both of these isoenzymes are encoded in nuclear genome, they display sequence divergence corresponding to the prokaryotic/eukaryotic separation (Shih et al., 1986; Brinkmann et al., 1987). These examples support the endosymbiotic theory of chloroplast evolution, with subsequent transfer of genes from the endosymbiont to the host nucleus.

An alternative explanation for the origin of *tomPRO1* is that it may have been acquired from a bacterium or virus by a horizontal gene transfer. This mechanism has been invoked to explain the close homology between vertebrate hemoglobin genes and the leghaemoglobin gene from legume (Lewin, 1981). Some soil bacteria (e.g. the genus *Rhizobium*) have two forms of Gln synthetase, a prokaryotic type and a eukaryotic type. It has been proposed that the eukaryotic-type genes may have been incorporated by a horizontal transfer from a host plant to symbiont bacteria (Carlson and Chelm, 1986; Smith et al., 1992).

It is unlikely that *tomPRO1* has been translocated from an organelle to the nucleus, because if *tomPRO1* originated from organelles, then there should be *tomPRO1* homologs in other plants. However, we did not find any homologs in organellar genomes using a computer search. This is also supported by the results of Southern-blot analysis that sequences homologous to *tomPRO1* are present in species of the Solanaceae family, such as tobacco, potato, and two wild species of tomato (*L. pennellii* and *Lycopersicon cheesmanii*), but could not be detected in rice and maize (García-Ríos, 1995). These results suggest that horizontal gene transfer may have been responsible for the integration of the *tomPRO1* gene into the nuclear genome after the divergence of dicots and monocots, but before divergence of the family Solanaceae. An examination of the subcellular localization of the *tomPRO1* product and a more detailed search of *tomPRO1* homologs in other plants may lead to clues as to the origin of *tomPRO1*, as well as to the mechanism of its transfer. There is little evidence about the possibility that bacteria or viruses could be responsible for the introduction of the *tomPRO1* gene into the tomato genome. However, because of its close sequence similarity to the *S. thermophilus proBA* and the common lack of the C-terminal 100-amino acid tail in the GK region, the *tomPRO1* locus may have been derived from a bacterium related to *S. thermophilus.*

Although we found evidence for *tomPRO1*-like genes in some other Solanaceae (see above), it is not clear whether this locus is present in other plants. The *tomPRO1* clone and the P5CS clone from moth bean (Hu et al., 1992) were isolated by complementation of a *proB* point mutation in *E. coli*, but all subsequent plant P5CS clones, including

tomPRO2, were isolated on the basis of sequence homology with the P5CS gene family. It is possible that homologs of *tomPRO1* might be present in other plants, but because of the sequence divergence between *tomPRO1* and the other plant P5CS clones, it is unlikely that the former type of gene could be cloned by sequence hybridization with P5CS clones.

There are few reports of the coexistence of prokaryotic and eukaryotic forms of a gene in a single genome. The coexistence of dual genes specifying isoforms of enzymes in one organism may serve two functions. Multiple copies of genes could satisfy a need for high amounts of a particular gene product, or they could provide an efficient means for differential regulation of gene-expression development in response to different factors (Long and Dawid, 1980). It seems likely that *tomPRO1* and *tomPRO2* will fit into the latter type of gene family, because of their distinct pattern of expression. Because the *tomPRO2* message was much more abundant than the *tomPRO1* in all tissues under the conditions we tested, it is likely that *tomPRO2* may have the predominant responsibility for Pro production in these situations, and it is possible that the expression of the *tomPRO1* gene might be restricted to very specific cell types or developmental stages. The significance of the existence of *tomPRO1* and the coexistence of *tomPRO1* and *tomPRO2* at this time remains elusive.

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