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Cloning of a polycistronic cDNA from tomato encoding γ-glutamyl kinase and γ-glutamyl phosphate reductase

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(osmoregulation/compatible solutes/water stress/translational control)

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ABSTRACT **We isolated from a tomato cDNA library the tom*PRO1* locus, which encodes γ-glutamyl kinase (GK) and γ-glutamyl phosphate reductase (GPR). This locus is unusual among eukaryotic genetic elements because it contains two open reading frames, and thus resembles prokaryotic polycistronic operons. The first open reading frame, specifying GK, is terminated by a TAA codon, which is followed by five nucleotides, an ATG translation initiation codon, and the second open reading frame, encoding GPR. DNA sequence analysis of fragments obtained by PCR amplification confirmed that the internal TAA and neighboring sequences are present in the endogenous tom*PRO1* sequence in tomato. We demonstrated with RNase protection assays that the tom*PRO1* locus is transcribed in tomato tissue culture cells, into a product that contains the internal stop codon. In *Escherichia coli*, tom*PRO1* directed the synthesis of two proteins, a 33-kDa GK and a 44-kDa GPR. Antibodies against the 44-kDa GPR purified from *E. coli* recognized a 70-kDa product in tomato tissue culture cells and a 60-kDa product in leaves and roots. These results suggest that in tomato tissues, GPR is made as part of a longer polypeptide by some translational mechanism that enables bypass of the internal stop codon, such as frameshifting or ribosome hopping. The tom*PRO1* locus may be the first example of a nuclear genetic element in plants that encodes two functional enzymes in two distinct open reading frames.**

Organisms generally respond to high osmolality or desiccation by increasing the intracellular concentrations of small molecules known as compatible solutes (1, 2). This response enables cells to maintain proper balance between the intracellular and the extracellular water potential. One of the compatible solutes accumulated in response to osmotic stress by a variety of organisms in the bacterial, plant, and animal kingdoms is proline (3, 4). This imino acid is synthesized by three enzymes: γ-glutamyl kinase (GK), γ-glutamyl phosphate reductase (GPR), and Δ^1-pyrroline-5-carboxylate reductase. There is evidence that the first two enzymes, which together carry out the Δ^1-pyrroline-5-carboxylate synthetase (P5CS) reaction, form a complex (5). To characterize the regulation of proline biosynthesis by osmotic stress in tomato (*Lycopersicon esculentum*), which accumulates high concentrations of proline in response to osmotic stress (6), we isolated a DNA fragment encoding GK from this plant. The clone, designated as tom*PRO1*, contains coding information not only for GK but also for GPR. However, DNA sequence analysis of this clone revealed a very surprising result — GK and GPR are specified by two distinct open reading frames that are arranged in a manner similar to polycistronic operons in prokaryotes. This organization is unusual because nuclear mRNAs in eukaryotes are almost exclusively monocistronic (7). The organization of the tom*PRO1* locus differs from genes for P5CS that have been cloned from *Vigna aconitifolia* (mothbean) and *Arabidopsis thaliana* (8–10) because in the latter two plants, P5CS is made as a hybrid GK and GPR protein, encoded by a standard single open reading frame.

Here, we describe the cloning of the tom*PRO1* locus. Portions of this work have been reported in preliminary form elsewhere (11–13).

MATERIALS AND METHODS

Isolation of tom*PRO1* Clones. A λgt11 poly(T)-primed cDNA library of poly(A)$^+$ RNA from breaker stage tomato fruit (*L. esculentum*, var. Ailsa Craig) was obtained from R. Fisher (University of California, Berkeley). The library, which contained ≈10^6 independent clones, was amplified to 10^9 pfu/ml. *Escherichia coli* strains G13 (*proB leuB6 thr thi-1 lac rpsL* F$^-$ λ$^-$) and G9 (*proA leuB6 thr thi-1 lac rpsL* F$^-$ λ$^-$) (14) were grown in Luria–Bertani broth (15) plus 0.2% maltose to a density of ≈4×10^8 cells/ml, and 1 ml cultures were infected with ≈10^8 phage from the library (15). Pro$^+$ transductants were selected at 30°C on solid minimal medium 63 (16) containing 10 mM glucose, 0.2 mM threonine, 0.2 mM leucine, and 0.05 mM thiamine·HCl. In two independent infections, we obtained nine Pro$^+$ transductants with strain G13 and four with G9. High titer phage λ stocks were prepared from these lysogens by heat induction, and phage DNA was isolated as described (15). Inserts from two of these phages, tom*PRO1–1* and tom*PRO1–7*, which were isolated by complementation of the *proB* mutation in strain G13, and one, tom*PRO1–8*, which was obtained by complementation of the *proA* mutation in strain G9, were characterized in detail. The size of the tom*PRO1–1* insert was 2.9 kilobase pairs (kbp) and that of the inserts in tom*PRO1–7* and tom*PRO1–8* was 3.7 kbp. The three inserts had the identical restriction map for an internal 2.9 kbp (data not shown), suggesting that they probably originated from the same genetic locus. The nucleotide sequence of both strands of the tom*PRO1–1* insert and parts of the tom*PRO1–7* and tom*PRO1–8* inserts were determined by the method of Sanger *et al.* (17).

RNase Protection Analysis. Tomato tissue culture cells (cv. VFNT Cherry) were grown in normal tissue culture medium (S0 cells) and in medium containing an additional 15 g/liter NaCl (S15 cells) (18). Total RNA was obtained by the LiCl

Abbreviations: GK, γ-glutamyl kinase; GPR, γ-glutamyl phosphate reductase; kbp, kilobase pair.

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precipitation method described (19). We used three RNase protection probes, specific for the 5′, middle, and 3′ portions of the tom*PRO1* transcript. These probes, which carried nucleotides 1–363, 826–1,558, and 1,674–2,155, respectively, from the antisense strand of the clone, were labeled throughout with CTP-α-^{32}P in T7 polymerase reactions (MAXIscript; Ambion, Austin, TX), in which the templates were derivatives of plasmid Bluescript IIKS+ (pKSII+; Stratagene) containing the above tom*PRO1* sequences (11). RNase protection assays were carried out with 80 μg total RNA with the HybSpeed RPA kit (Ambion).

Western Blot Analysis. GPR was purified to near homogeneity from a derivative of *E. coli* strain HB101 (Δ*proBA leu thi-1*) carrying the tom*PRO1–1* insert on pKSII+ (13). The purified GPR was used to immunize chickens, and antibodies were obtained from eggs as described (20). Cultured tomato cells and whole plant tissues were frozen in liquid nitrogen, ground with mortar and pestle, and extracted with 50 mM NaH_2PO_4 (pH 7.0) containing 10 mM EDTA, 0.1% Triton X-100, 0.1% sodium lauryl sarcosine plus 10 mM 2-mercaptoethanol. Proteins in crude extracts were separated on 4–20% polyacrylamide gradient-SDS gels, transferred to Immobilon poly(vinylidene difluoride) membranes (Millipore), and probed with the polyclonal antibodies as described (21).

Coupled GK/GPR Assay. *E. coli* strains carrying the cloned tom*PRO1–1* insert were grown to saturation overnight in Luria–Bertani broth with selective antibiotics, pelleted by centrifugation, taken up at 20-fold concentration in 50 mM Hepes·KOH (pH 7.2) containing 2 mM dithiothreitol, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1 mM benzamidine, and lysed by two passages through a French Press (SLM-Aminco, Urbana, IL) at 95 atm (1 atm = 101.3 kPa). The P5CS assay, which is the ATP- and NADPH-dependent reduction of glutamate to γ-glutamic semialdehyde, was carried out in 100 mM Tris·Cl (pH 7.2), 25 mM $MgCl_2$, 75 mM Na·glutamate, 5 mM ATP, 0.4 mM NADPH, and 10–50 μg crude extract protein at 25°C. The reaction velocity was measured as the rate of consumption of NADPH, monitored as decrease in absorption at 340 nm as a function of time.

RESULTS

Isolation of the tom*PRO1* Clones. The tom*PRO1* clones were obtained from a cDNA library of tomato by complementation of *proB* and *proA* mutations of *E. coli* as described. In Southern blots, the tom*PRO1–1* clone hybridized under conditions of high stringency (4 washes for 15 min with 0.1× SSC + 0.1% SDS at 65°C) as a single-copy sequence to total DNA from several cultivars of domestic tomato (*L. esculentum*), the wild tomato (*Lycopersicon pennellii*), and the related species, tobacco (*Nicotiana tabacum*) (data not shown). The clone did not show any specific hybridization to total DNA from rice, maize, sorghum, and *E. coli* under the same conditions (data not shown).

We found that the inserts in the phage could correct the proline auxotrophy of *E. coli* strains carrying a deletion of both *proB* and *proA* genes. This result suggested that the inserts probably specify both GK and GPR. We determined the sequence of the entire 2.9-kbp tom*PRO1–1* insert (GenBank no. U27454) and parts of the tom*PRO1–7* and *PRO1–8* inserts; for comparable regions, sequences of the independent tom*PRO1–1*, tom*PRO1–7*, and tom*PRO1–8* clones were identical. Comparison with sequences in the GenBank database revealed that between positions 279 and 1,091, the tom*PRO1–1* clone contains an 813-bp coding sequence whose predicted product shows a 54–56% amino acid sequence similarity to the GK domain of the P5CS of *V. aconitifolia* and *A. thaliana*, and between positions 1,100 and 2,341, it contains a 1,242-bp coding sequence having comparable similarity to the GPR domain of the P5CS proteins of these two plants (Fig. 1). The tom*PRO1*-encoded GK exhibits a 54–63% amino acid sequence similarity to GKs from a number of diverse bacteria (Fig. 2). However, there is an important difference between the GKs from tomato and the latter organisms: the tomato GK lacks ≈100 C-terminal residues that are present in all the bacterial GKs. Although this tail is highly conserved (46–72% amino acid sequence similarity, 27–59% identity) among the bacterial GKs, its function is not clear. Because the tomato GK is sensitive to feedback inhibition by proline (see below) and can function together with either the *E. coli* or the tomato GPR to synthesize proline, evidently the tail is not necessary for enzymatic activity or allosteric control of GK, or its interaction with GPR.

There Is a Termination Codon Within the tom*PRO1* Locus. DNA sequence analysis revealed that the tom*PRO1* clones contain an internal in-frame TAA codon, located precisely at

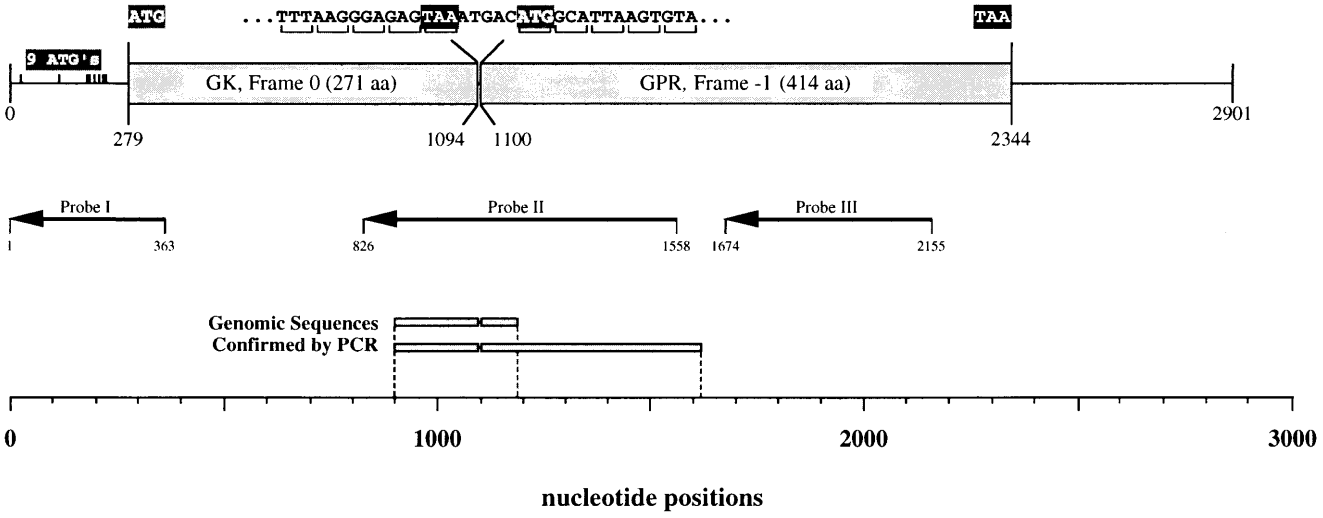

FIG. 1. Schematic representation of the structure of the tom*PRO1–1* cDNA clone. The complete sequence is available from GenBank (accession no. U27454). The open reading frame specifying GK is initiated at position 279 and terminated at the TAA at positions 1,092–1,094; the open reading frame encoding GPR is initiated at position 1,100 and terminated at the TAA at positions 2,342–2,344. There are nine additional ATG codons in the 5′ leader upstream of the GK reading frame, at positions 27–29, 116–118, 182–184, 188–190, 199–201, 210–221, 221–223, 224–226, and 227–229. The rectangles immediately above the nucleotide position scale line indicate two fragments obtained with PCR amplification of DNA from tomato tissue cells; they contained nucleotides 898–1,188 and 898–1,617, and their sequence was identical to that of the corresponding regions of the tom*PRO1–1* cDNA clone.

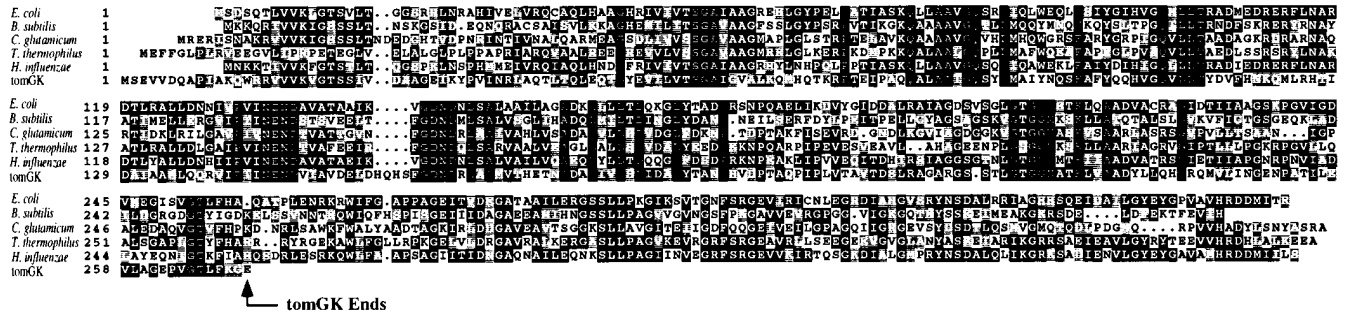

FIG. 2. Comparison of the GK specified by the tom*PRO1* clone (tomGK) with bacterial GKs. The amino acid sequence of the GK encoded by tom*PRO1* was aligned to the indicated bacterial GKs with the PILEUP program, and the results displayed with the BOXSHADE program. Letters in the black and gray backgrounds denote identical and similar residues, respectively. Sequences of GKs from the various bacteria are available from GenBank under the following accession numbers: *E. coli*, P07005; *Bacillus subtilis*, P39820; *Corynebacterium glutamicum*, U31230; *Thermus thermophilus*, D29973; *Haemophilus influenzae*, P431763.

the 3′ end of the GK reading frame (positions 1,092–1,094; Fig. 1). This internal TAA codon was present in the tom*PRO1–1*, tom*PRO1–7*, and tom*PRO1–8* clones. Five nucleotides downstream from this translation termination codon, there is a potential translation initiation ATG codon, followed by the open reading frame specifying GPR. Because there are five nucleotides between the TAA and ATG codons, the GPR reading frame is offset by a −1 frameshift with respect to the GK reading frame.

To investigate whether the internal TAA codon is present in the genomic tom*PRO1* sequence in tomato, we amplified segments of total DNA of tomato tissue culture cells with PCR using primers derived from the sequence of the tom*PRO1* cDNA clones. The sequences of a 281-bp fragment containing nucleotides from positions 898–1,188 and of a 720-bp fragment containing nucleotides from 898 to 1,617 (Fig. 1) obtained in this amplification were identical to that of the cDNA clones, including the TAA codon between the GK and GPR coding sequences. This result demonstrates that the TAA codon is present in the cellular copy of tom*PRO1* and that there are no introns in this segment of this genetic element. It should be noted that our use of tissue culture cells as the source of the tomato genomic DNA minimized the risk that the amplified sequences were derived from a bacterial contaminant.

Another unusual feature of the tom*PRO1* clone is that it contains a 278-bp 5′ leader sequence upstream of the GK open reading frame, in which there are nine ATG codons (Fig. 1). These are followed closely by in-frame translation termination codons, so even if they are recognized as translation start sites, they could generate peptides consisting of a maximum of 26 amino acids. Although nuclear mRNAs are generally monocistronic (7), there are a few other eukaryotic mRNAs in which there are ATG codons before the main coding information, including the 35S RNA of Cauliflower mosaic virus (22), the *Antennapedia* homeotic gene of *Drosophila melanogaster* (23), and the *GCN4* gene of *Saccharomyces cerevisiae* (24). The short open reading frames in the 5′ leaders of these genetic elements are involved in the regulation of translation initiation of the structural genes, but we have no information on the function of the 5′ leader of tom*PRO1*.

Products of the tom*PRO1* Clone in *E. coli*. Previously, we observed that in *E. coli* maxi cells, the tom*PRO1–1* clone produced two proteins, having masses of ≈33 and ≈44 kDa (11). This result suggests that the tom*PRO1–1* clone functions as a polycistronic locus in *E. coli*. We purified both proteins to near homogeneity from *E. coli* (data not shown), and determined their N-terminal amino acid sequences. The experimentally determined sequence of the first six residues of GK was SEVVDQ. Except for the lack of an N-terminal methionine (which is often removed in *E. coli*), this sequence matches completely that predicted from the DNA sequence at the start of the GK reading frame of tom*PRO1–1* clone (nucleotide positions 282-299). The sequence ALSVQEMGQRAKKATAQVA was obtained for the first 19 residues of GPR, in agreement with the DNA sequence at the N terminus of the GPR reading frame (nucleotide positions 1,103–1,159; see Fig. 1). The fact that in *E. coli* the tom*PRO1–1* clone directs the synthesis of two proteins whose masses and N-terminal amino acid sequences are consistent with the DNA sequence demonstrates that in this prokaryotic host, the ATG triplets (that is, AUG in mRNA) at positions 279 and 1,100 are indeed used as the translation start sites of GK and GPR, respectively, and that the TAA (UAA) codon at positions 1,092–1,094 is recognized as the translation termination signal for GK.

The two products of the tom*PRO1–1* clone, as synthesized in *E. coli*, had functional GK and GPR, that is P5CS activity, which was sensitive to inhibition by proline (Fig. 3). Inhibition by proline was dependent on the glutamate concentration: in the presence of 75 mM glutamate, the activity was inhibited 50% by ≈0.07 mM proline (Fig. 3), whereas in the presence of 10 mM glutamate, 50% inhibition was obtained with only 0.02

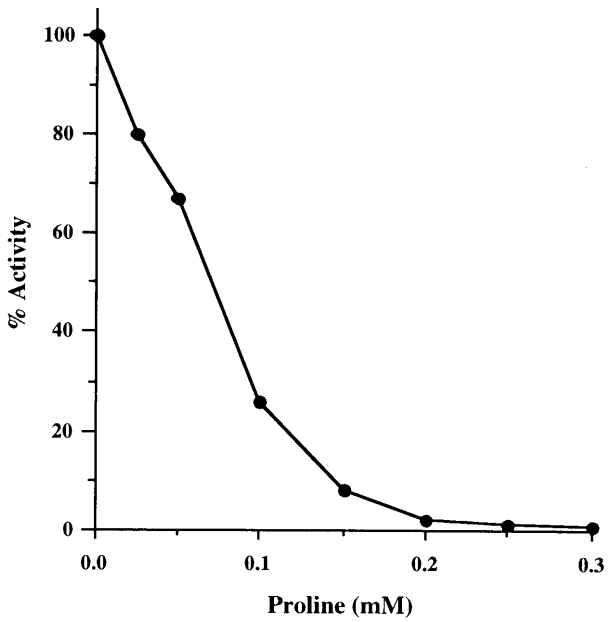

FIG. 3. Inhibition of the Δ^1-pyrroline-5-carboxylate synthetase (P5CS) activity of GK/GPR products of the tom*PRO1* clone. P5CS activity was assayed in crude extracts of *E. coli* strains carrying the tom*PRO1–1* clone in pKSII+. Assays were carried out in the presence of the indicated concentrations of proline, as described in *Materials and Methods*. Results are expressed as percent of specific activities in the absence of proline, which was 13 nmol $(min)^{-1}$·(mg total protein)$^{-1}$.

mM proline (data not shown). The target of the inhibition by proline is the GK component of the coupled activity (data not shown).

Detection of the tom*PRO1* Message in Tomato Cells. We carried out RNase protection assays to analyze the level of the tom*PRO1* mRNA in tomato tissue culture cells. A probe that was complementary to nucleotides 1–363 from the antisense strand of the tom*PRO1* clone (Probe I; Fig. 1) was protected by total RNA from tomato tissue culture cells grown in the normal medium or in medium containing 15 g/liter NaCl (Fig. 4*A*). Because protection of the probe is dependent on precise hybridization with complementary sequences, the results of the RNase protection assays demonstrate that the tom*PRO1* clone is expressed in the tomato tissue culture cells. Densitometry indicated that the level of the tom*PRO1* transcript was ≈4-fold higher in S15 cells than in S0 cells. Thus, similar to the results seen with the P5CS message in *V. aconitifolia* (8) and *A. thaliana* (9, 10), osmotic stress caused an increase in the accumulation of the tom*PRO1* mRNA in tomato tissue culture cells. We also detected the tom*PRO1* transcript in tomato leaves with Probes I and III; the intensity of the signals was similar with the two probes, suggesting that there were no differences in the stability of the 5′ and 3′ portions of the message (data not shown).

Although the three independent cDNA clones of tom*PRO1* that we characterized contain the internal TAA codon, there could be derivatives of the tom*PRO1* transcript from which the corresponding UAA codon has been removed by splicing or other forms of editing. To test this possibility, we carried out RNase protection assays with a probe complementary to the GK-GPR intercistronic region (Probe II: nucleotides 826-1,558; Fig. 1). We observed a single protected fragment with this probe (Fig. 4*B*), whose length of 733 nucleotides is consistent with protection of this probe throughout the entire length of sequences that were complementary to the expected message. Thus, the only detectable form of the tom*PRO1* message was identical in its sequence in the GK–GPR junction to that predicted from the sequence of the cDNA clones, which is inconsistent with splicing as the mechanism for the bypass of the internal UAA codon. However, strictly speaking, we cannot rule out the possibility that there could be edited forms of the transcript present below the level of detection (estimated to be <5% from reconstruction experiments), which could be translated into a hybrid GK–GPR.

Western Blot Analysis of the tom*PRO1* Products in Plants. We generated polyclonal antibodies against GPR that had been purified from *E. coli* carrying this clone (see *Materials and Methods*). In Western blot analyses of extracts of *E. coli* strains carrying the tom*PRO1* clone, the antibodies recognized a product having an expected mass of ≈44 kDa (Fig. 5). However, in extracts of various tomato tissues, the antibodies recognized two polypeptides, whose molecular masses were ≈70 and ≈60 kDa (Fig. 5). The 70-kDa product was the most prominent in tissue culture cells, whereas the 60-kDa product was present in flower, fruit, leaf, and especially abundant in root tissues. We also detected the 70-kDa antigenic product in tissue culture cells with three independent monoclonal antibodies generated against the 44-kDa GPR, but the intensity of the signal with the latter antibodies was not as strong as with the polyclonal preparation (data not shown).

DISCUSSION

The fact that the tom*PRO1* clones, which encode two separate enzymes, GK and GPR, in two reading frames, were isolated from a tomato cDNA library by functional expression in *E. coli* might raise the concern that the clones could have originated from a prokaryotic contaminant, rather than from tomato cells. However, the demonstration of this locus in the tomato genome with Southern and PCR analyses and, especially, the detection of the tom*PRO1* transcript in tomato tissues provided strong evidence that the clones were derived from tomato. Southern blots additionally demonstrated that the tom*PRO1* locus is present in single copy per haploid tomato genome, indicating that it is contained in the nuclear, rather than in the choloroplast or mitochondrial, genome. The result, that the tom*PRO1*-encoded GK is ≈100 amino acids shorter than all known prokaryotic GKs (Fig. 2), is further evidence against the possibility that the clone arose from a prokaryotic source. Finally, the GK and GPR produced from tom*PRO1* in

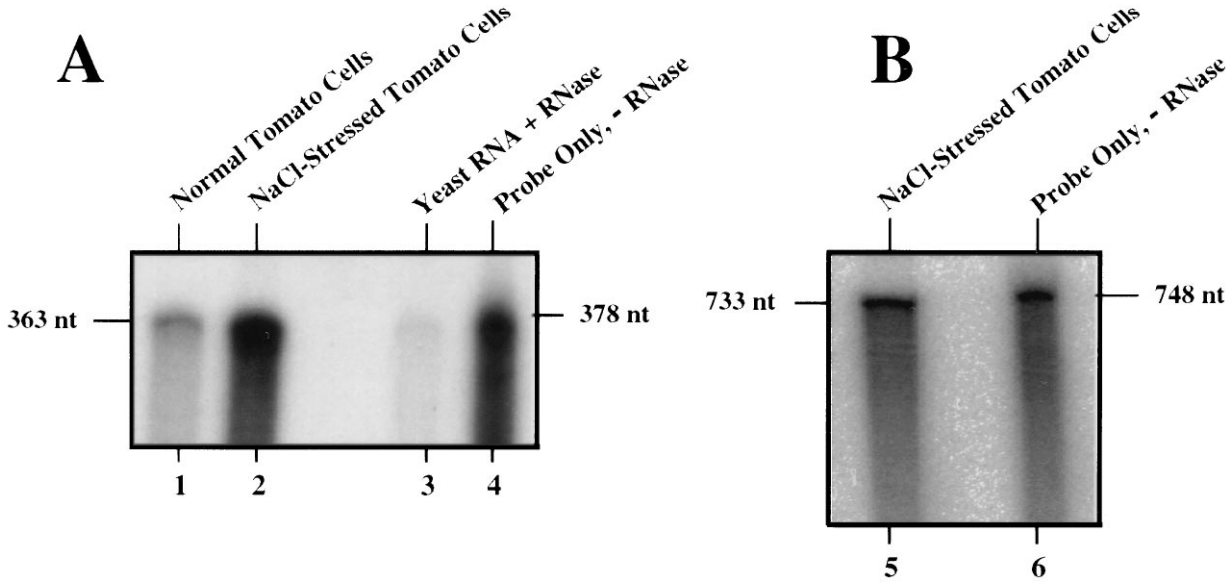

FIG. 4. RNase protection analysis of tom*PRO1* transcript. (*A*) Probe was a 378-nt riboprobe containing nucleotides 1–363 from the antisense strand of the tom*PRO1–1* cDNA clone plus an additional 15 non-complementary nucleotides from linkers (Probe I in Fig. 1). Lanes: 1, RNA from normal (S0) cells; 2, RNA from NaCl adapted (S15) cells; 3 and 4, Controls, containing 50 μg yeast RNA with and without RNase, respectively. (*B*) Probe was a 748 nt riboprobe containing nucleotides 826-1,158 from the tom*PRO1* antisense strand plus 15 non-complementary nucleotides from linkers (Probe II in Fig. 1). Lanes: 5, RNA from S15 cells; 6, control containing 50 μg yeast RNA without RNase. Conditions for RNase protection assays are described in text. The protected probes (lanes 1, 2, and 5) were 15 nucleotides shorter than the respective full length probes (lanes 4 and 6), because of loss of the 15 non-complementary nucleotides.

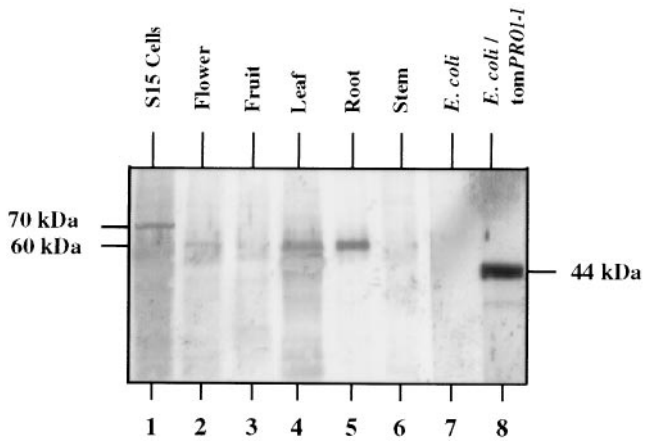

FIG. 5. Western blot analysis of proteins of tomato tissues with antibodies against the GPR product of the tom*PRO1* locus. Extracts of the indicated tomato tissues were subjected to electrophoresis on 4–20% polyacrylamide gradient SDS gels and probed with polyclonal antibodies against GPR. Antibodies were obtained from eggs of a chicken that was injected with a highly purified preparation of GPR, synthesized in *E. coli* from the tom*PRO1–1* clone as a 44-kDa product. Lanes: 1 (S15 cells), proteins from tissue culture cells (cv. VFNT Cherry) adapted to 15 g/liter NaCl; 2–6, proteins from the indicated tissues of tomato plants (cv. Rutgers); 7, negative control, containing an extract of *E. coli* strain JM109 (21); and 8, positive control, containing extract from a derivative of strain JM109 carrying the tom*PRO1–1* clone on pKSII+. Lanes 1–4 and 6 were loaded with 25 μg protein, lane 5 with 5 μg protein, and lanes 7 and 8 with 0.5 μg protein. The specific immunoreactive 70-kDa protein (lane 1), 60-kDa protein (lanes 2–5), and 44-kDa protein (lane 8) were absent when the extracts were probed with pre-immune antibodies (data not shown).

E. coli are enzymatically active components of a proline-inhibitable P5CS (Fig. 3). Together, these data suggest that tom*PRO1* is not a pseudogene, but a functional locus, whose product could participate in proline biosynthesis in tomato.

In eukaryotes, translation of nuclear mRNAs is usually initiated at the AUG codon closest to the 5′ end and does not proceed past the first in-frame translation termination codon (7). There are, however, a number of notable exceptions to this generalization. A limited number of translation stop codons can be misread at a low frequency by some amino acyl tRNA (reviewed in ref. 25). Because the GK and GPR coding sequences of the tom*PRO1* clone are separated by five nucleotides, this mechanism could not be used to produce a hybrid GK–GPR polypeptide.

In *Caenorhabditis elegans*, some messages are first synthesized as polycistronic precursors and are converted by *trans*-splicing to monocistronic derivatives before translation (26). The 35S RNA of Cauliflower mosaic virus contains seven long coding sequences, whose translation is mediated by internal initiation at the beginning of each coding sequence (22). The mammalian growth/differentiation factor 1 (GDF1) is specified by a bicistronic mRNA that contains two long open reading frames, separated by a 269–401 nucleotide spacer (27); the mechanism for the translation of the second reading frame, which encodes GDF1, is not known.

Termination codons can be eluded by translational mechanisms, such as a frameshift of a single nucleotide in either the −1 or +1 direction (28, 29) or by hopping of the ribosomes over longer stretches of nucleotides (30). Translational frameshifting is best documented in viruses and retrotransposons, but recently it has also been demonstrated in a nuclear gene (31). Frameshifting in the −1 direction is usually mediated by two elements: a so-called "slippery sequence" upstream of the termination codon, and a tertiary structure or "pseudoknot" in the RNA, immediately downstream from the slippery sequence. Slippery sequences generally consist of a heptanucleotide sequence X-XXY-YYZ (28). It has been proposed that the RNA tertiary structures cause pausing of the ribosomes, which directs them to change the reading frame at the slippery sequence.

In *E. coli*, GPR was synthesized from the tom*PRO1* clone as a ≈44-kDa product. However, antibodies against this protein recognized a ≈70-kDa product in tomato tissue culture cells and a ≈60-kDa product in leaves, flowers, fruits, and roots (Fig. 5). The 60-kDa product was present at much higher levels in roots than in other tissues. We do not have any information concerning the relationship of the two antigenically active polypeptides. Assuming that the signals detected on the Western blots were due to authentic tom*PRO1* products rather than to unrelated proteins exhibiting adventitious cross-reactivity, there are several possible mechanisms that could account for their synthesis. The fact that we could not detect a 44-kDa product in plant tissues with the antibodies against GPR suggests that, unlike in *E. coli*, translation initiation probably does not occur efficiently at the internal AUG codon in plant cells. However, we cannot rule out the possibility that translation can be initiated at this site, but the 44-kDa product is converted by some covalent modification to the 60-kDa and 70-kDa forms in the whole plant tissues and in tissue culture cells, respectively. The fact that we detected only one species of mRNA, which had the same sequence in the GK–GPR junction as the cDNA clones, provides evidence against the removal of the UAA codon from the message by splicing or other form of editing, and therefore suggests that the UAA is bypassed by some translational mechanism, such as frameshifting or ribosome hopping. In the absence of information about the amino acid sequences of the 70-kDa and the 60-kDa proteins detected by the antibodies, it is not clear whether the former is the primary translation product, and is converted to the latter by proteolysis, or whether the two proteins represent different translational products which might be generated by different ribosomal hopping events. A GK–GPR hybrid of 70-kDa mass could be generated by a −1 frameshift. If a −1 frameshift does occur, then it must do so between positions 1,086 and 1,091, because there is a UAA codon in the same reading frame as GPR at positions 1,082–1,084 (Fig. 1). The interval between this UAA and the one at the end of GK contains the heptamer G-GGA-GAG (positions 1,085–1,091), which differs from a canonical slippery sequence by one base (G instead of A at position 1,089). Although the mouse mammary tumor virus serves as a precedent for a −1 frameshift occurring at a slippery sequence which is also deviates from canonical slippery sequences by one nucleotide (29), we have no evidence whether the above sequence in tom*PRO1* can direct a −1 frameshift. The tom*PRO1* message does not contain a recognizable stem and loop or pseudoknot structure close to the GK-GPR junction. Because there are examples of frameshifting without recognizable slippery sequences or RNA tertiary structures (28, 29), the lack of these structures in tom*PRO1* does not rule out −1 frameshifting altogether. However, at present, we cannot dismiss other translational mechanisms for bypass of the internal UAA codon in tom*PRO1*, such as ribosome hopping.

Whether or not the UAA codon at the end of the GK reading frame in the tom*PRO1* transcript can be bypassed in tomato to synthesize GPR, it would be expected that the tom*PRO1* locus should be able to direct the synthesis of an enzymatically active, 33-kDa GK. We have not yet obtained antibodies against GK, which would enable us to test whether a 33-kDa GK protein is synthesized in addition to the 60-kDa and 70-kDa products, and if so, whether its synthesis is under osmotic control. These antibodies would also enable us to determine whether the longer products that are recognized by the antibodies against GPR also contain a GK domain. We have recently discovered that tomato contains a second locus, tom*PRO2*, which also specifies P5CS, but as a single hybrid

polypeptide (32). This protein, as synthesized in *E. coli*, is recognized by the monoclonal and polyclonal antibodies against the tom*PRO1*-encoded GPR with <1,000-fold and <20-fold efficiency, respectively. If a 33-kDa GK subunit is made from the tom*PRO1* locus, conceivably it could have a catalytic or regulatory role in proline syntheis via its enzymatic activity or via interactions with the P5CS synthesized from tom*PRO2* or from tom*PRO1* by bypass of the stop codon. Our next experimental goals will be to determine the mechanisms for the bypass of the stop codon in the tom*PRO1* locus, and to elucidate whether this stop codon has any role in the regulation of proline synthesis.

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