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Divergent Fructokinase Genes Are Differentially Expressed in Tomato[1]

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Two cDNA clones (Frk1 and Frk2) encoding fructokinase (EC 2.7.1.4) were isolated from tomato (*Lycopersicon esculentum*). The Frk2 cDNA encoded a deduced protein of 328 amino acids that was more than 90% identical with a previously characterized potato (*Solanum tuberosum*) fructokinase. In contrast, the Frk1 cDNA encoded a deduced protein of 347 amino acids that shared only 55% amino acid identity with Frk2. Both deduced proteins possessed an ATP-binding motif and putative substrate recognition site sequences identified in bacterial fructokinases. The Frk1 cDNA was expressed in a mutant yeast (*Saccharomyces cerevisiae*) line, which lacks the ability to phosphorylate glucose and fructose and is unable to grow on glucose or fructose. Mutant cells expressing Frk1 were complemented to grow on fructose but not glucose, indicating that Frk1 phosphorylates fructose but not glucose, and this activity was verified in extracts of transformed yeast. The mRNA corresponding to Frk2 accumulated to high levels in young, developing tomato fruit, whereas the Frk1 mRNA accumulated to higher levels late in fruit development. The results indicate that fructokinase in tomato is encoded by two divergent genes, which exhibit a differential pattern of expression during fruit development.

Many studies have focused on elucidating sugar transport, metabolism, and starch biosynthesis in sink tissues. In tomato (*Lycopersicon esculentum*) starch accumulates during the early stage of fruit development and then decreases during maturation (Yelle et al., 1988). Suc synthase has been proposed to be the primary enzyme in the metabolism of imported Suc during the early developmental stages, and localization of its mRNA suggests a relationship between Suc synthase and starch granule accumulation (Wang et al., 1993, 1994). In addition, invertase has been identified in the intercellular fraction, and it is suggested that hexose produced by invertase is actively imported across the plasma membrane (Sato et al., 1993; Ruan and Patrick, 1995). Fru derived from the activity of invertase or Suc synthase must be phosphorylated by fructokinase or hexokinase for further metabolism. Because the affinity of fructokinase for Fru is much higher than that of hexokinase (Renz and Stitt, 1993), fructokinase is likely to be of primary importance in Fru metabolism.

Fructokinase has been characterized from some sink tissues such as potato (*Solanum tuberosum*) tubers (Gardner et al., 1992; Renz and Stitt, 1993), pea seeds (Copeland et al., 1978), taproots of sugar beet (Chaubron et al., 1995), avocado fruit (Copeland and Tanner, 1988), maize kernels (Doehlert, 1990), and tomato fruit (Martinez-Barajas and Randall, 1996). In potato tubers a cDNA encoding fructokinase has been cloned (Smith et al., 1993) and its physiological role in starch synthesis has been investigated (Taylor et al., 1995). Fructokinase and Suc synthase are coordinately expressed in potato tubers. Because Suc synthase is inhibited by Fru, fructokinase could be important in maintaining carbon flux through Suc synthase to starch (Ross et al., 1994). In tomato fruit starch and hexose accumulate at the early stage of growth, but in later stages of development starch is depleted and only hexose continues to accumulate (Yelle et al., 1988). Fructokinases have been purified from this early stage of developing tomato fruit and shown to be composed of at least two isoenzymes, distinguishable by pI and kinetic properties (Martinez-Barajas and Randall, 1996). To further elucidate the role of Fru metabolism in developing tomato fruit, we have cloned tomato fruit fructokinase cDNAs and analyzed their expression throughout development. Two divergent putative fructokinase cDNAs were identified, suggesting the presence of distinct fructokinase isoforms that are differentially expressed and may play distinct metabolic roles.

MATERIALS AND METHODS

PCR Amplification of cDNA and Sequencing

Amino acid alignment of fructokinase sequences from potato (*Solanum tuberosum*) (Smith et al., 1993) and three bacteria (Blatch et al., 1990; Aulkemeyer et al., 1991) were used to identify two conserved domains for the construction of degenerate PCR primers. The conserved domain sequences correspond to amino acids 43 to 49 and 252 to 258 of potato fructokinase (Fig. 1). These amino acid sequences were used to design the 5′ PCR primer (5′-GGIGGIGCICCIGCIAA[CT]GT-3′) and the 3′ PCR primer (5′-[AG]TCNCCNGCNCCNGTNGT[AG]TC-3′), where I = inosine and N = all from nucleotides. PCR was carried out with the tomato (*Lycopersicon esculentum*) cDNA library from ripe fruit (DellaPenna et al., 1986). Amplifications

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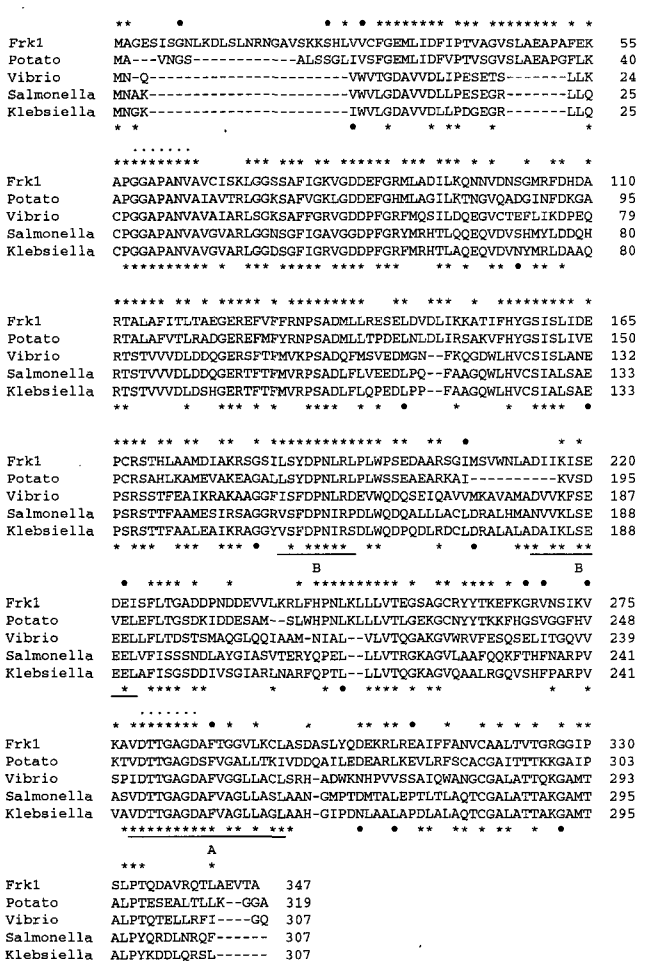

Figure 1. Sequence analysis of the Frk1 cDNA and alignment of its deduced amino acid sequence with potato (Smith et al., 1993), *Vibrio alginolyticus* (Blatch et al., 1990), *Klebsiella pneumoniae*, and *Salmonella typhimurium* (Aulkemeyer et al., 1991) fructokinases. Asterisks indicate identical residues between Frk1 and potato fructokinase (above) and between Frk1 and bacterial fructokinase (below). Proposed sequence domains involved in ATP binding (A) and substrate recognition (B) are underlined. Conserved amino acid sequences corresponding to PCR primers are indicated by dots.

were for 40 cycles, each consisting of 1 min at 94°C, 1 min at 45°C, and 1 min at 72°C. The resulting approximately 680-bp DNA fragment was gel-purified and cloned into the pCRII (Invitrogen, San Diego, CA) plasmid vector, according to the manufacturer's instructions. DNA sequences were determined by the dideoxy chain termination method using Sequenase, version 2.0 (United States Biochemical). Several PCR products derived from ripe fruit cDNA were sequenced, and one was identified as Frk1.

cDNA Isolation and Characterization

A tomato root cDNA library in λZAP II was screened with the Frk1 PCR product described above or with the potato fructokinase cDNA (kindly provided by Dr. H. Davies, Scottish Crop Research Institute, Dundee, UK). Probes were prepared by random priming with [α-^{32}P]dATP, and hybridization was carried out in 50% (v/v) formamide, 6× SSPE, 5× Denhardt's reagent, 0.5% (w/v) SDS, and 100 μg/mL denatured salmon sperm DNA at 42°C (Frk1) or 37°C (Frk2). Filters were washed in 0.5× SSC, 0.1% SDS at 58°C. pBluescript containing a cDNA insert was excised from selected clones and completely sequenced on both strands. Alignment of the Frk1 and Frk2 deduced amino acid sequence with other fructokinase sequences was carried out using the MacDNASIS Pro program (version 3.5, Hitachi Software, San Bruno, CA).

Yeast Strain and Media

The yeast (*Saccharomyces cerevisiae*) strain used was DFY632-MATa, ura3-52, hxk1::LEU2, hxk2::LEU2, glk1::LEU2, lys1-1, leu2-1 (Walsh et al., 1991). Yeast cells were grown on YEPG medium consisting of 1% yeast extract (Difco, Detroit, MI), 2% Bacto Peptone (Difco), and 110 mM (2%) Gal (Sherman et al., 1986). Selective medium for uracil auxotrophic growth (−uracil+sugar) contained 0.5% ammonium sulfate, 0.17% yeast nitrogen base without amino acids (Difco), 0.2% casamino acids (Difco), 0.004% adenine (Sigma), 0.008% Trp (Sigma), and 110 mM Gal, Fru, or Glc.

Yeast Transformation

A yeast shuttle vector (pFL61) containing the URA3 gene as a selective marker and the constitutive phosphoglycerate kinase promoter and terminator (Minet et al., 1992) were used for transformation. Frk1 cDNA was cloned downstream of the phosphoglycerate kinase promoter in pFL61 (pFL61-Frk1). Yeast transformations were carried out by growing DFY632 cells in the YEPG liquid medium to mid-logarithmic phase, treating the cells with lithium acetate according to Ito et al. (1983), and selecting for transformants on −URA+Gal plates.

Protein Extraction and Hexose Kinase Activity

DFY632 yeast cells transformed with either pFL61 or pFL61-Frk1 were grown in 40 mL of −URA+Gal liquid medium for 72 h to approximately 5×10^7 cells/mL. Cells were centrifuged for 5 min at 6000 rpm, washed twice with water, and resuspended in 0.5 mL of water. Two-hundred-fifty microliters of the cells was extracted two times with 500 μL of the extraction buffer (50 mM Hepes, pH 7.5, 1 mM EDTA, and 1 mM PMSF) by vortexing with 250-μL glass beads. Following vortexing for 3 × 30 s, the mixture was centrifuged for 5 min at 12,000*g* and 4°C, and the supernatant was brought to 80% ammonium sulfate saturation. After centrifugation at 12,000*g* and 4°C, the pellet was resuspended in 0.5 mL of the washing buffer (50 mM Hepes, pH 7.5, 1 mM EDTA, and 1 mM DTT), desalted on a Sephadex G-25 column (Sigma), and used as the crude enzyme extract for subsequent enzymatic analysis.

Hexose kinase activity was measured by an enzyme-linked assay according to a modification of the method of Huber and Akazawa (1985). Assays contained, in a total volume of 1 mL, 30 mM Hepes-NaOH (pH 7.5), 1 mM $MgCl_2$, 0.6 mM EDTA, 9 mM KCl, 1 mM NAD, 1 mM ATP, and 2 units of NAD-dependent Glc-6-P dehydrogenase. For

the assay of Glc phosphorylation, the reaction was initiated with 2 mM Glc. For the assay of Fru phosphorylation, 2 units of phosphoglucoisomerase were added and the reaction was initiated with 2 mM Fru. Reactions were carried out at 37°C and A_{340} was monitored continuously.

RNA Analysis

Tissues of tomato (cv T5) were collected from mature plants grown in a greenhouse, frozen in liquid N_2, and stored at −80°C. Total RNA was isolated from fruit by the hot borate method of Wan and Wilkins (1994) and from vegetative tissues by the phenol/SDS method (Ausubel et al., 1995). Poly(A)$^+$ RNA was purified by Oligotex-dT (Qiagen, Chatsworth, CA), and 1 μg from each sample was subjected to electrophoresis through a 1.2% agarose, 10% (v/v) formaldehyde denaturing gel and transferred to a Hybond-N membrane (Amersham). The resulting blots were hybridized with the Frk1 PCR product or the entire insert of Frk2 cDNA, as described above for screening, and washed in 0.5× SSC, 0.1% SDS at a stringency of Tm −25°C. The blots were exposed to x-ray film (DuPont) with an intensifying screen at −80°C for 2 d.

DNA Analysis

Genomic DNA was prepared from tomato roots using the method of Murrey and Thompson (1980), and 7.4-μg samples were digested with the indicated restriction enzymes, fractionated on 0.8% agarose gels, and transferred to a Hybond-N membrane. The resulting blot was hybridized with the Frk1 PCR product, 683-bp of *Eco*RI fragments from Frk2 cDNA, or the entire insert of potato fructokinase cDNA at 37°C in the hybridization solution described above, washed in 0.5× SSC, 0.1% SDS at 56°C, and then exposed to x-ray film with an intensifying screen at −80°C for 1 d.

RESULTS

Cloning of Frk1 and Frk2 cDNA

The complete nucleotide sequence of the Frk1 cDNA, which contains 1887 bp plus a poly(A) tail, is accessible through GenBank. The cDNA contained an open reading frame that encoded a protein of 347 amino acids with a calculated molecular mass of 37,308 D. The ATG triplet beginning at nucleotide position 271 was assigned as the likely site of translation initiation because there was an in-frame termination codon (TGA) at positions 220 to 222, and 6 of 9 nucleotides surrounding the ATG triplet were identical to the proposed consensus sequence (AACAATGGC) for plant initiation codons (Lutcke et al., 1987). A putative polyadenylation signal was located 31 bp upstream from the polyadenylation start site.

The deduced amino acid sequence of Frk1 was 28 amino acids longer than that of the potato fructokinase cDNA (Smith et al., 1993). The amino acid sequence identity between Frk1 and potato fructokinase was only 55% (Fig. 1), but several domains implicated in fructokinase function (Fig. 1, domains A and B) were conserved between the Frk1 sequence and other fructokinases. There was no sequence similarity between Frk1 and Arabidopsis hexokinase (Dai et al., 1995), an enzyme that phosphorylates both Glc and Fru.

The complete Frk2 cDNA nucleotide sequence made up of 1261 bp plus a poly(A) tail is accessible through GenBank. The cDNA contained an open reading frame that encoded a predicted protein of 328 amino acids with a calculated molecular mass of 34,761 D. The ATG triplet beginning at nucleotide 62 was assigned as the likely site of translation initiation because there was a termination codon (TAG) at positions 29 to 31, and 7 of 9 nucleotides surrounding the ATG triplet were identical to the proposed consensus sequence for plant initiation codons, as described above. An AATAAA motif was located at 71 bp upstream from the polyadenylation site, and a GT-rich sequence was also located upstream from the motif in the 3′ untranslated region. In plant mRNAs an AAUAAA-like sequence is typically located 10 to 30 nucleotides upstream from the polyadenylation site and a GU-rich sequence is typically located upstream from the polyadenylation signal (Wu et al., 1995). Because there were no other AATAAA-like sequences between the AATAAA motif and the polyadenylation site in the 3′ untranslated region of Frk2 cDNA, it is appears that the Frk2 transcript has a polyadenylation signal unusually far from the polyadenylation site. The deduced amino acid sequence of Frk2 is 93% identical to that of potato fructokinase (Fig. 2) but only 57% identical to tomato Frk1.

Identification of Frk1 cDNA

The potato fructokinase cDNA was demonstrated to encode an authentic fructokinase by assay of its in vitro transcription and translation product (Taylor et al., 1995). The high sequence similarity between tomato Frk2 and the potato fructokinase cDNA (93% identity; Fig. 2) strongly suggests that Frk2 encodes an authentic fructokinase. To determine whether the Frk1 cDNA also encodes an authentic but divergent fructokinase isoform, we cloned the Frk1

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          ***** * **************************************************
Frk2      MAVNG-ASSSGLIVSPGEMLIDFVPTVSGVSLAEAPGFLKAPGGAPANVAIAVTR   54
potato    MAVNGSALSSGLIVSPGEMLIDFVPTVSGVSLAEAPGFLKAPGGAPANVAIAVTR   55

          ******************************** ***********************
Frk2      LGGKSAFVGKLGDDEFGHMLAGILKTNGVQAEGINFDKGARTALAFVTLRADGER  109
potato    LGGKSAFVGKLGDDEFGHMLAGILKTNGVQADGINFDKGARTALAFVTLRADGER  110

          ********************* ************************** ** ********
Frk2      EFMFYRNPSADMLLTPAELNLDLIRSAKVFHYGSISLIVEPCRAAHMKAMEVAKE  164
potato    EFMFYRNPSADMLLTPDELNLDLIRSAKVFHYGSISLIVEPCRSAHLKAMEVAKE  165

          ********************* * ** * *       •  ************** ****
Frk2      AGALLSYDPNLRLPLWPS-AEEAKKQIKSIWDSADVIKVSDVELEFLTGSNKIDD  218
potato    AGALLSYDPNLRLPLWSSEAE-ARK--------A--IKVSDVELEFLTGSDKIDD  209

          ********************************** ***********************
Frk2      ESAMSLWHPNLKLLLVTLGEKGCNYYTKKFHGTVGGFHVKTVDTTGAGDSFVGAL  273
potato    ESAMSLWHPNLKLLLVTLGEKGCNYYTKKFHGSVGGFHVKTVDTTGAGDSFVGAL  264

          ******** ************************************ ************
Frk2      LTKIVDDQTILEDEARLKEVLRFSCACGAITTTKKGAIPALPTASEALTLLKGGA  328
potato    LTKIVDDQAILEDEARLKEVLRFSCACGAITTTKKGAIPALPTESEALTLLKGGA  319
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Figure 2. Sequence analysis of the Frk2 cDNA and alignment of its deduced amino acid sequence with potato fructokinase (Smith et al., 1993). Asterisks indicate identical residues between Frk2 and potato fructokinase.

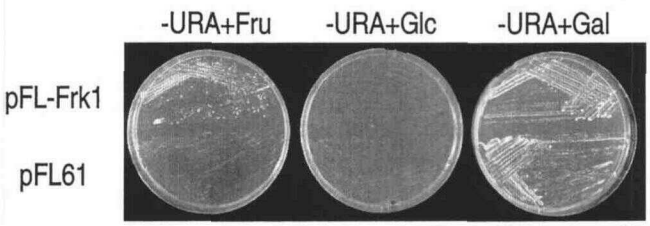

Figure 3. The growth of yeast triple-mutant cells transformed with pFL61-Frk1 on selective media. The Frk1 cDNA was subcloned to pFL61 and expressed in the yeast cells. Yeast cells transformed with pFL61 were used as a control. The amount of 110 mM of either Gal, Fru, or Glc was added to selective media (−URA) for uracil auxotrophic strains.

cDNA into a yeast expression vector pFL61 and expressed the cloned cDNA in DFY632 triple-mutant yeast cells, which are unable to phosphorylate either Glc or Fru (Walsh et al., 1991). As shown in Figure 3, cells with pFL61 containing Frk1 cDNA (pFL61-Frk1) grew on Fru but not on Glc, indicating that the product of Frk1 cDNA phosphorylates Fru but not Glc. Protein extracts from cells transformed with pFL61-Frk1 phosphorylated Fru but not Glc, whereas protein extracts from cells transformed with pFL61 as a control were unable to phosphorylate either Fru or Glc (Table I).

Analysis of Fructokinase mRNA

The 1.9-kb Frk1 mRNA was detected in leaves, stems, roots, and fruit, and its levels were similar in all of the three vegetative tissues (Figs. 4 and 5). Although the 1.2-kb Frk2 mRNA was also detected in all of the organs that were examined, the abundance of the Frk2 mRNA was much lower in the leaves than in the stems and roots. In developing fruit the level of Frk1 mRNA was low in young green fruit, increased in immature green fruit, and remained at relatively high levels throughout ripening (Fig. 5). In contrast, the levels of Frk2 mRNA were very high in young developing fruit but declined to much lower levels in mature green fruit and continued to decline during the early stage of fruit ripening. The highest levels of Frk2 mRNA corresponded to the developmental period of starch accumulation and corresponding high levels of Suc synthase activity (Robinson et al., 1988; Yelle et al., 1988).

Table I. *Hexose kinase activity in a crude enzyme extract from yeast cells transformed with pFL61-Frk1*

Enzyme activity was measured in the presence of 2 mM sugar. A transformant with pFL61 was used as a control. Each value is an average of two or three independent measurements.

Plasmid	Substrate	Activity
		nmol mg^{-1} protein min^{-1}
pFL61-Frk1	Fru	127
	Glc	0
pFL61	Fru	0
	Glc	0

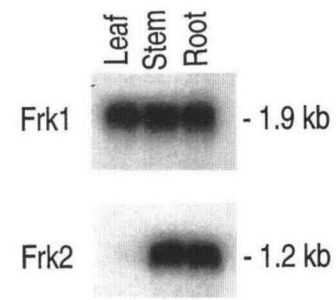

Figure 4. Northern-blot analysis of fructokinase RNA in tomato vegetative tissues. Each lane was loaded with 1 μg of poly(A) RNA isolated from leaves (Leaf), stems (Stem), and roots (Root). The blot was probed with the Frk1 PCR fragment or the Frk2 cDNA and then washed in 0.5× SSC at a stringency of Tm −25°C.

DNA Gel-Blot Analyses

Southern-blot analyses were carried out on tomato genomic DNA digested with three restriction enzymes (Fig. 6). The Frk1 probe hybridized to a single band in each restriction digest, suggesting the presence of a single gene. The Frk2 cDNA probe hybridized to single restriction fragments that were distinct from those identified by Frk1. The potato fructokinase cDNA hybridized to the same genomic restriction fragments identified by Frk2. The results suggest that, in tomato, there are two distinct fructokinase genes, which are sufficiently divergent that they fail to cross-hybridize. Based on hybridization of Southern blots with the potato fructokinase cDNA, Frk2 appears to represent the tomato homolog of this previously characterized fructokinase gene.

DISCUSSION

Fructokinase has been purified from developing potato tubers (Gardner et al., 1992), sugar beet taproots (Chaubron et al., 1995), and tomato fruit (Martinez-Barajas and Randall, 1996), indicating a subunit size of 35 to 38 kD. The calculated molecular mass of Frk1 was similar to those of

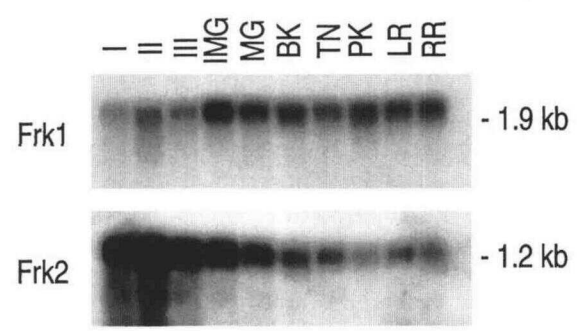

Figure 5. Northern-blot analysis of fructokinase RNA in developing tomato fruit. Each lane was loaded with 1 μg of poly(A) RNA isolated from developing fruit tissues, which are young, green fruit of 0.5 to 1.5 cm diameter (I), 2 to 3 cm diameter (II), 4 to 6 cm diameter (III), immature green (IMG), mature green (MG), breaker (BK), turning (TN), pink (PK), light red (LR), and ripe red (RR). The blot was probed with the Frk1 PCR fragment or the Frk2 cDNA and washed in 0.5× SSC at a stringency of Tm −25°C.

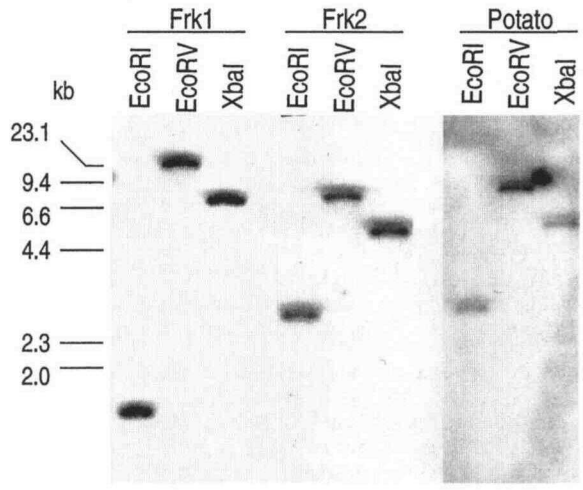

Figure 6. Tomato genomic DNA gel-blot analysis of fructokinase. Genomic DNA (7.4 μg/lane) was digested with the indicated restriction enzymes. The blot was probed with the Frk1 PCR fragment, the Frk2 cDNA, or the potato fructokinase cDNA, and washed in 0.5× SSC at 56°C.

the polypeptides purified from these previously characterized plant fructokinases. The predicted amino acid sequence of Frk1 showed 55% identity to potato fructokinase and no similarity to hexokinase from Arabidopsis (Dai et al., 1995). Three regions have been proposed to be conserved functional domains in bacterial fructokinases (Fennington and Hughes, 1996), and each domain is indicated in Figure 1. The proposed ATP-binding motif (domain A in Fig. 1), which contains the GD motif essential for activity, was conserved in the Frk1 sequence and, indeed, the identity of Frk1 with other bacterial fructokinases over this region was higher than that of potato fructokinase. In addition to the ATP-binding motif, there are two additional motifs (domain B in Fig. 1) conserved in all other fructokinases, which together constitute an approximately 100-amino acid domain (Fennington and Hughes, 1996). Because domain B is unique to fructokinases and is not conserved in other sugar kinases, it is proposed to represent the Fru substrate recognition site. Potato fructokinase contains a 10-amino acid gap between the two regions of domain B, whereas Frk1 is highly homologous with bacterial fructokinases in this region. These sequence comparisons strongly suggest that the Frk1 cDNA identified here encodes a novel, higher-plant fructokinase.

Yeast possesses three enzymes that are capable of phosphorylating hexoses. Two enzymes, hexokinase 1, coded by HXK1, and hexokinase 2, coded by HXK2, phosphorylate either Glc or Fru, whereas glucokinase 1, encoded by GLK1, phosphorylates only Glc (Fraenkel, 1982). The triple-mutant cells hxk1, hxk2, and glk1 are unable to phosphorylate Glc or Fru and therefore do not grow on Glc or Fru (Fraenkel, 1982; Walsh et al., 1991). The mutant cells transformed with the vector containing Frk1 cDNA grew on Fru but not on Glc, and the protein extracts from those cells showed fructokinase activity. Therefore, the product of Frk1 encodes an authentic fructokinase enzyme.

The early stage of tomato fruit growth is characterized by high levels of starch accumulation and Suc synthase activity (Yelle et al., 1988). In addition, the spatial expression of Suc synthase mRNA correlates with starch granule accumulation (Wang et al., 1994). The Frk2 mRNA is also expressed at high levels during this same developmental stage. Therefore, Frk2 may play a role in phosphorylating Fru that is formed by Suc synthase, thus maintaining Suc synthase activity, which is inhibited by free Fru, and maintaining carbon flux to starch biosynthesis. Transgenic plants expressing antisense RNA of fructokinase could be useful in clarifying the relationship between fructokinase, Suc synthase, and starch biosynthesis in much the same way that the role of Suc synthase in starch biosynthesis has been demonstrated in potato tubers by transgenic expression of Suc synthase antisense RNA (Zrenner et al., 1995).

Two distinct fructokinase cDNAs were cloned and characterized in tomato. Frk2 has a very high homology with fructokinase previously cloned from potato and showed a similar pattern of expression with potato, i.e. low abundance of transcript in leaves and high abundance in the early stages of storage organ development. In contrast, Frk1 showed much lower sequence similarity with potato fructokinase and was differentially expressed relative to Frk2. To our knowledge, this type of fructokinase gene has not been previously described in plants. There are many reports suggesting the presence of multiple fructokinase isoforms in plants. Two or three fructokinases have been separated by ion-exchange chromatography in potato (Gardner et al., 1992; Renz and Stitt, 1993), spinach (Schnarrenberger, 1990), barley (Baysdorfer et al., 1989), avocado (Copeland and Tanner, 1988), maize (Doehlert, 1989), and tomato fruit (Martinez-Barajas and Randall, 1996), and some show differences in their specificity for nucleotide triphosphates and/or in their substrate inhibition by Fru. It is likely that the products of the two divergent genes we have identified in tomato correspond to distinct fructokinase isoforms, although they do not appear to correspond to the two tomato fruit fructokinase isoenzymes described by Martinez-Barajas and Randall (1996). Our preliminary evidence indicates that, unlike the fructokinase isoenzymes purified from young tomato fruit (Martinez-Barajas and Randall 1996), Frk1 and Frk2 exhibit dramatic kinetic differences, especially with regard to Fru inhibition.

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The GenBank accession numbers for the nucleotide sequences described in this article are U64817 (Frk1) and U64818 (Frk2).

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