Structure and Expression of Phospho*enol*pyruvate Carboxylase Kinase Genes in Solanaceae. A Novel Gene Exhibits Alternative Splicing[1]

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Phosphorylation of phospho*enol*pyruvate carboxylase (PEPc; EC 4.1.1.31) plays an important role in the control of central metabolism in higher plants. Two *PPCK* (PEPc kinase) genes have been identified in tomato (*Lycopersicon esculentum* cv Alicante), hereafter termed *LePPCK1* and *LePPCK2*. The function of the gene products has been confirmed by transcription of full-length cDNAs, translation, and in vitro assay of kinase activity. Previously studied *PPCK* genes contain a single intron. *LePPCK2* also contains a novel second intron that exhibits alternative splicing. The correctly spliced transcript encodes a functional PEPc kinase, whereas unspliced or incorrectly spliced transcripts encode a truncated, inactive protein. The relative abundance of the transcripts depends on tissue and conditions. Expression of *LePPCK2* was markedly increased during fruit ripening. In ripe Alicante fruit, the locule and seeds contained only the correctly spliced *LePPCK2* transcripts, whereas in ripe fruit of the tomato *greenflesh* mutant, they contained correctly and incorrectly spliced transcripts. Potato (*Solanum tuberosum*) contains genes that are very similar to *LePPCK1*, and *LePPCK2*; *StPPCK2* exhibits alternative splicing. Aubergine (*Solanum melongena*) and tobacco (*Nicotiana tabacum*) also contain a *PPCK2* gene; the sequence of the alternatively spliced intron is highly conserved between all four species. The data suggest that the two *PPCK* genes have different roles in tissue-specific regulation of PEPc and that the alternative splicing of *PPCK2* transcripts is functionally significant.

Phospho*enol*pyruvate carboxylase (PEPc; EC 4.1.1.31) is a ubiquitous enzyme in higher plants. It catalyzes the carboxylation of phospho*enol*pyruvate to form oxaloacetate and inorganic phosphate and plays a range of roles in different tissues. In C_4 and Crassulacean acid metabolism (CAM) plants, a photosynthetic isoform of PEPc catalyzes the primary fixation of CO_2 (O'Leary, 1982; Andreo et al., 1987). In most non-photosynthetic tissue and in C_3 leaves, PEPc is the major anapleurotic enzyme. It allows the replenishment of tricarboxylic acid cycle intermediates to provide the precursors for biosynthetic pathways such as amino acid biosynthesis. It also provides malate in guard cells, legume root nodules, and developing fruit (Andreo et al., 1987; Chollet et al., 1996; Vidal and Chollet, 1997). To fulfill these different functions, PEPc is encoded by a small gene family whose members are expressed in different types of tissue (Lepiniec et al., 1994).

PEPc is strongly regulated by pH and by positive and negative effectors (Glc 6-phosphate and malate, respectively; Andreo et al., 1987). Superimposed on this, reversible phosphorylation of a strictly conserved Ser residue near the N-terminal end of the protein causes a change in the allosteric properties of PEPc. This phosphorylation reduces the sensitivity of the enzyme to its inhibitor, malate, but increases its sensitivity to its activator, Glc 6-phosphate (Chollet et al., 1996; Vidal and Chollet, 1997; Nimmo, 2000). The phosphorylation state of PEPc and, therefore, also its activity increase in response to different signals in different organs, including light in C_4 leaves, a circadian oscillator in CAM leaves, and light plus nitrogen supply in C_3 leaves; these signals control the activity of PEPc kinase (Chollet et al., 1996; Vidal and Chollet, 1997; Nimmo, 2000, 2003).

After the cloning of PEPc kinase genes (termed *PPCK*), it has become clear that PEPc kinase comprises a Ser/Thr kinase catalytic domain with essentially no extensions. It resembles the protein kinase catalytic domain of plant calcium-dependent protein kinases but lacks the C-terminal Ca^{2+}-binding EF hands and the N-terminal extensions of these enzymes (Hartwell et al., 1999a). PEPc kinase is regulated largely at the level of transcript abundance in CAM, C_4, and C_3 plants (Hartwell et al., 1999a, 1999b; Nimmo, 2000, 2003; Taybi et al., 2000; Tsuchida et al., 2001; Fontaine et al., 2002; Nakagawa et al., 2003; Xu et al., 2003).

Recent work in the model C_3 species Arabidopsis showed the existence of two isoforms of PEPc kinase with different expression patterns (Fontaine et al., 2002), and soybean (*Glycine max*) contains at least

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three *PPCK* genes expressed in different organs (Nimmo, 2003; Xu et al., 2003). This raises the possibility that distinct isoforms of the PEPc kinase play different metabolic roles. We have investigated this question in tomato (*Lycopersicon esculentum*) because of the importance of PEPc in the production of organic acids in developing fruit. Malic and citric acid levels increase at the end of the cell division phase of development, peak at the end of the cell expansion phase, and decline during ripening (Varga and Bruinsma, 1986; Guillet et al., 2002). Expression of one PEPc gene, termed *Ppc2*, correlated with high levels of PEPc activity and the accumulation of organic acids during development of tomato fruit (Guillet et al., 2002). This raises the question of whether any *PPCK* gene shows a similar expression pattern.

Here, we report on the cloning and expression of two isoforms of PEPc kinase from tomato. The data show one unique feature of PEPc kinase in the Solanaceae. All PEPc kinase genes reported to date contain just one intron, close to the 3′ end of the coding sequence. However, one of the PEPc kinase genes of tomato contains a novel second intron, which is subject to alternative splicing. One transcript encodes a functional PEPc kinase, but two transcripts encode a truncated, inactive protein. Our results show that the relative abundance of the transcripts is dependent on tissue and conditions. Similar genes, also subject to alternative splicing, have been detected in potato (*Solanum tuberosum*), tobacco (*Nicotiana tabacum*), and aubergine (*Solanum melongena*).

RESULTS

Identification of *LePPCK1* and *LePPCK2*

An examination of the expressed sequence tag (EST) database suggested that tomato contains two *PPCK* genes. One putative full-length PEPc kinase EST (GenBank accession no. AW033195), hereafter termed *LePPCK1*, was completely sequenced (GenBank accession no. AF203481). The deduced amino acid sequence (279 residues, 31.4 kD) is aligned with several other PEPc kinase sequences in Figure 1. To examine the structure of the *LePPCK1* gene, we designed PCR primers (see Table I) to amplify genomic DNA. A single band of 1148 bp was generated, cloned, and sequenced (GenBank accession no. AY190084). Inspection of this sequence showed that the coding sequence comprises two exons, with one

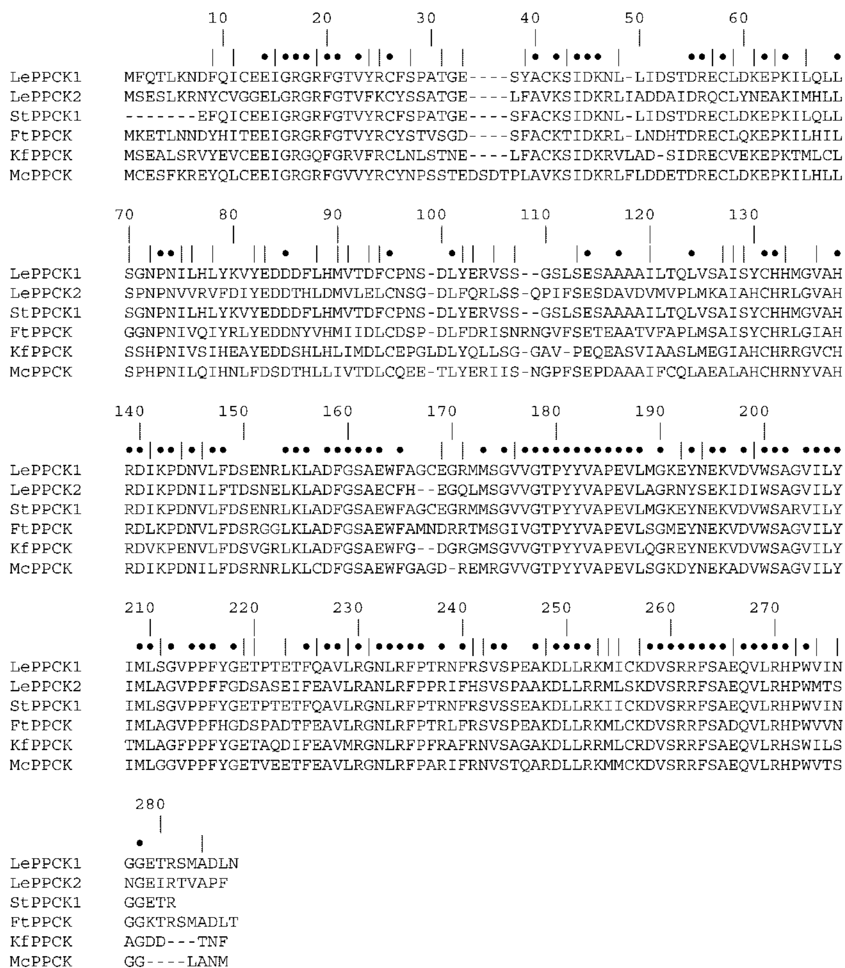

Figure 1. Alignment of deduced *PPCK* amino acid sequences. FtPPCK, *Flaveria trinervia* (AB065100); LePPCK1 and LePPCK2, tomato (this work); KfPPCK, *Kalanchoë fedtschenkoi* (AF162661); StPPCK1, potato (this work); and McPPCK, ice plant (*Mesembryanthemum crystallinum*, AF158091). Dots and vertical lines, Identities and similarities respectively.

Table I. *Primers used in reverse transcription (RT)-PCR analyses and cloning*

Primer Name and Specificity	Sequence
RT-PCR	
LePPCK1, 5′	5′-TCA AAT TTG CGA AGA AAT CG-3′
LePPCK1, 3′	5′-CCT TCT CCT TCT CTC TTC CAC A-3′
LePPCK2, 5′	5′-GAG CTA TTC GCC GTC AAG TC-3′
LePPCK2, 3′	5′-AAA TCA GCC AAT TTC AGT TCG-3′
Le Actin 52, 5′	5′-GAT GCC TAT GTT GGT GAC GA-3′
Le Actin 52, 3′	5′-ATC CTC CGA TCC AGA CAC TG-3′
StPPCK2, 5′	5′-GAG TCA TTC GCC GTC AAG TC-3′
StPPCK2, 3′	5′-AAA TCA GCC AAT TTC AGC TCG-3′
Cloning	
LePPCK2, 5′ FL	5′-AGG AAT TCG GCA CGA GAA A-3′
LePPCK2, 3′ FL	5′-GAC CAG AGT ATA TTG GTG CCT GT-3′
StPPCK1, 5′	5′-ATG TTC CAA ACC TTG AAA AA-3′
StPPCK1, 3′	5′-TCA GTT TAG ATC AGC CAT TG-3′

89-bp intron from base 808 to 896 of AY190084 inclusive (see Fig. 2).

The *LePPCK1* cDNA was subcloned behind a T3 promoter in pBluescript. To test whether the full-length cDNA was functional, the plasmid was linearized with *Not*I, transcribed, and translated. The translation product was then assayed for PEPc kinase activity. Figure 3 shows that the translation product was of the expected size (31 kD) and was able to phosphorylate PEPc.

A second putative full-length tomato PEPc kinase EST (GenBank accession no. AW223421), hereafter termed *LePPCK2*, was also completely sequenced (data not shown; see below). Inspection of this sequence showed that the cDNA contains an insertion of 138 bp near the middle of the coding region relative to other PEPc kinase cDNAs. The insertion also included an in-frame stop codon. To examine the structure of the gene, PCR primers were designed to

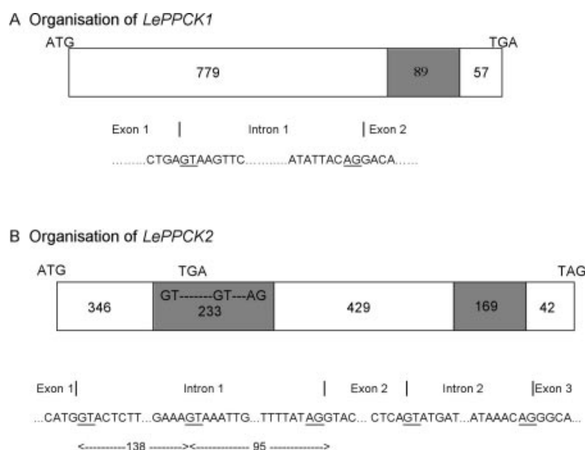

Figure 2. Organization of the *LePPCK1* and *LePPCK2* genes. White boxes, Exons; shaded boxes, introns. A, *LePPCK1*; B, *LePPCK2*. The numbers show the lengths of exons and introns in base pairs. The sequences round the splice sites are shown in full; the intron start GT and end AG sequences are underlined.

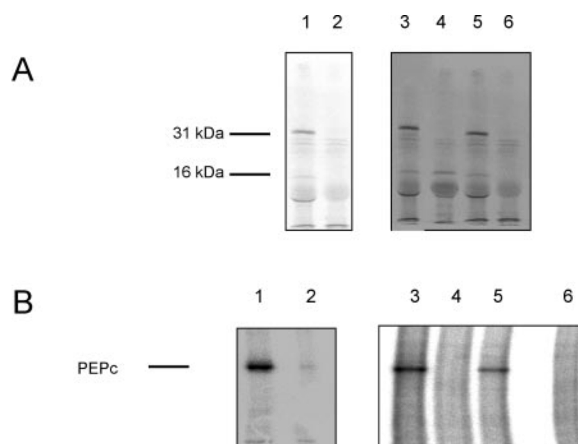

Figure 3. Functional analysis of *LePPCK* cDNAs. A, Phosphor images of [^{35}S]Met-labeled products from in vitro translation of RNA samples, separated on a 12.5% (w/v) SDS polyacrylamide gel. B, Phosphor images of immunoprecipitated, ^{32}P-labeled PEPc from assays of the PEPc kinase activity of translation products, separated on an 8% (w/v) SDS polyacrylamide gel. The bar indicates PEPc. In each panel, the lanes are: 1, RNA transcribed from the cDNA clone of *LePPCK1*; 2, no RNA control; 3, RNA transcribed from the cDNA clone of *LePPCK2* transcript 1; 4, RNA transcribed from the cDNA clone of *LePPCK2* transcript 2; 5, RNA transcribed from the cDNA clone of *K. fedtschenkoi PPCK* as a positive control; and 6, no RNA control.

amplify cDNA (from tomato fruit) and genomic DNA, and the products were sequenced. Analysis of these sequences revealed that the *LePPCK2* gene (GenBank accession no. AY188444) contains two introns (Fig. 2). Like other PEPc kinase genes, one is located close to the 3′ end of the coding sequence (bases 1,044–1,212 of AY188444). The second intron (bases 372–604 of AY188444) is located close to the middle of the coding region and possesses two alternative 5′ splice start sites (bases 372 and 510, see Fig. 2). The in-frame stop codon is between these sites (bases 450–452 of AY188444). Hence, splicing can give rise to a transcript that encodes a functional PEPc kinase (transcript 1, the full sequence of which is GenBank accession no. AY187634), an incorrectly spliced transcript with a premature stop codon (transcript 2), and an unspliced transcript with a premature stop codon (transcript 3). Transcripts 2 and 3 both encode a truncated, nonfunctional PEPc kinase. As shown in Table II, ESTs corresponding to each transcript have been detected. The deduced amino acid sequence encoded by transcript 1 (278 residues, 31.1 kD) is shown in Figure 1. There is 61% sequence identity between the two tomato PPCK proteins.

LePPCK2 transcript 1 was positioned behind a T7 promoter in pCR4-TOPO, and *LePPCK2* transcript 2 was positioned behind a T3 promoter in pBluescript. The clones were linearized, transcribed, and translated. The translation products were then assayed for PEPc kinase activity. Figure 3 (lane 3) shows that the product from transcript 1 was of the expected size (31 kD) and was able to phosphorylate PEPc as effectively as did *K. fedtschenkoi* PEPc kinase generated in

Table II. *Origin of PPCK ESTs*

The Institute for Genomic Research tentative consensus sequences TC125298, TC117287, TC117288, and TC117289 refer to *LePPCK1*, *LePPCK2* transcript 1, *LePPCK2* transcript 2, and *LePPCK2* transcript 3, respectively (see http://www.tigr.org/tdb/tgi/lgi/), whereas TC67959, TC59893, and TC59894 refer to *StPPCK1*, *StPPCK2* transcript 2, and *StPPCK2* transcript 3, respectively (see http://www.tigr.org/tdb/tgi/stgi/).

GenBank Accession No., Source of Clone
LePPCK1
AI774158, leaf, *Pseudomonas syringae* pv. tomato resistant
AW033195, callus
AW933544, green fruit
BE431605, breaker fruit
BE459112, developing immature green fruit
BF096819, nutrient-deficient roots
BG129025, shoot meristem
StPPCK1
BG887336, dormant tuber
BG887353, dormant tuber
BG888336, dormant tuber
BQ045511, leaves challenged with *Phytophthora infestans*, incompatible interaction
LePPCK2
AW222608, red ripe fruit
AW223421, red ripe fruit, transcript 2
AW441584, red ripe fruit
AW442172, red ripe fruit
AW738217, flower buds, anthesis
BE462009, breaker fruit, transcript 2
BF112946, breaker fruit, transcript 3
BG127024, shoot meristem, transcript 3
BI205375, suspension culture
BI934398, flower, anthesis, transcript 2
BM409042, breaker fruit, transcript 2
BM409651, breaker fruit, transcript 1
BM411455, breaker fruit
BM536300, breaker fruit, transcript 2
StPPCK2
BG594065, sprouting eyes, transcript 3
BG594668, sprouting eyes, transcript 2
BQ505603, mixed tissues, transcript 3
BQ505604, mixed tissues
BQ505884, mixed tissues, transcript 2
BQ505885, mixed tissues, transcript 2

a similar way (lane 5). Transcript 2 consistently directed the synthesis of a 16-kD protein (compare Fig. 3, lane 4 with the no RNA control lane 6 in A) that did not phosphorylate PEPc. The size of this protein was as expected from the position of the in-frame stop codon in *LePPCK2* transcript 2. A slightly smaller band can be seen in the translation products from the full-length PEPc kinase clones (lanes 1, 3 and 5). This presumably results from either breakdown of the major product or premature termination of translation.

PEPc Kinase Expression in Different Tissues of Tomato

PEPc kinase is expressed at a low level in C_3 plants, with the exception of legume root nodules (Nakagawa et al., 2003; Xu et al., 2003). The expression of both tomato PEPc kinases was studied, therefore, by semiquantitative RT-PCR rather than by northern blotting. The primers used for the two *PPCK* genes and for *actin52* as a constitutive control are shown in Table I. We investigated developing fruit at several stages: young leaves, mature leaves, flowers, roots, and seedlings harvested in the light and dark. The cycle number of 35 was chosen to ensure that bands could be detected in all samples; similar band patterns were detected with lower cycle numbers (data not shown). Although there is some variation in the intensities of the *actin52* bands, it is clear that *LePPCK1* transcripts were present in all of the samples studied, lowest in expansion phase 1 fruit, and highest in mature leaves. Expression in seedlings was enhanced in the light (Fig. 4A, compare lane 10 with 11). The expression of *LePPCK2* showed three interesting features (Fig. 4B). First, it was strongly increased during fruit ripening. Second, as for *LePPCK1* expression, transcript abundance was increased in the light (Fig. 4B, compare lane 10 with 11). Third, the relative abundance of the three transcripts appeared to depend upon both tissue and conditions. In ripening fruit and illuminated seedlings, transcripts 2 and 3 were more abundant than transcript 1. In roots and in seedlings in the dark,

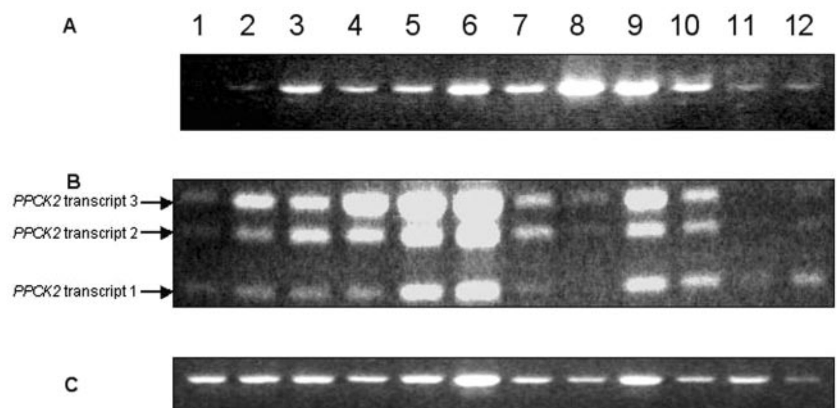

Figure 4. Expression pattern of *LePPCK* genes. This shows the products obtained from RT-PCR (35 cycles). A, *LePPCK1*; B, *LePPCK2*; C, *Actin52*. Lane 1, Expansion phase 1 fruit; lane 2, expansion phase 2 fruit; lane 3, green fruit; lane 4, breaker fruit; lane 5, pink fruit; lane 6, ripe red fruit; lane 7, young leaves; lane 8, mature leaves; lane 9, flowers, lane 10, seedlings in light; lane 11, seedlings in dark; lane 12, roots.

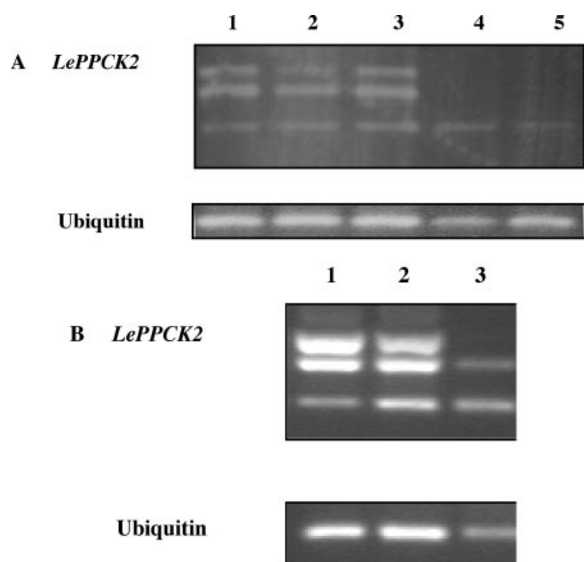

Figure 5. Expression pattern of *LePPCK2* in fruit compartments. This shows the products obtained from RT-PCR (35 cycles) of compartments from ripe red fruit. A, *LePPCK2* and ubiquitin from wild-type plants. Lane 1, Skin; lane 2, outer pericarp; lane 3, inner pericarp; lane 4, gel; lane 5, seeds. B, *LePPCK2* and ubiquitin from the *gf* mutant. Lane 1, Skin; lane 2, pericarp; lane 3, locule plus seeds.

transcript 1 was the major species. Differences in the relative abundance of the transcripts are further illustrated in Figure 5A, which shows that the skin, outer pericarp, and inner pericarp of ripe fruit contain all three transcripts, whereas in the locule and seeds, only transcript 1 is detectable.

In view of the developmental control of expression exhibited by *LePPCK2* and the positional control of splicing shown in Figures 4 and 5A, we examined the expression of this gene in the *gf* (greenflesh) tomato mutant, in which the breakdown of chlorophyll during fruit ripening is markedly reduced (Akhtar et al., 1999). One notable difference was observed between *gf* and Alicante: In the *gf* mutant, the splicing pattern of *LePPCK2* was affected such that the seeds and locule of ripe *gf* fruit contained both transcripts 1 and 2 (Fig. 5B), whereas Alicante contained only transcript 1 (Fig. 5A).

Identification of Potato, Tobacco, and Aubergine PEPc Kinases

Further analysis of the EST database revealed two putative potato PEPc kinase genes. As summarized in Table II, several ESTs closely resemble *LePPCK1*; none of these, however, are full length. Other ESTs resemble either transcript 2 or 3 of *LePPCK2*. To examine the structure of the corresponding genes, hereafter termed *StPPCK1* and *StPPCK2*, we designed PCR primers (see Table I) to amplify cDNA (from mature leaves) and genomic DNA. Using *StPPCK1* primers, a partial genomic sequence of 913 bp was cloned and sequenced (GenBank accession no. AY219178); this contains only one intron as for *LePPCK1* (bases 765–877 of AY219178). Comparison of the deduced amino acid sequences of *StPPCK1* and *LePPCK1* shows 98.5% identity over 265 amino acids (see Fig. 1). To test whether *StPPCK2* exhibits alternative splicing, we designed primers to the region flanking its putative additional intron based on the sequence of the ESTs BG594065 and BG594668. Using these, we amplified three bands of the predicted mobilities from potato leaf cDNA but only one from genomic DNA (Fig. 6A), indicating that *StPPCK2* does contain an additional intron that is subject to alternative splicing. The sequence of the partial genomic fragment of *StPPCK2* (555 bp, GenBank accession no. AY293738) matches the sequences of the ESTs corresponding to *StPPCK2* (Table II) almost exactly (not illustrated).

To check other members of the Solanaceae, we amplified and sequenced a 553-bp fragment of aubergine genomic DNA (GenBank accession no. AY236482) and a 538-bp fragment of tobacco genomic DNA (GenBank accession no. AY347261) using the primers designed for RT-PCR of *LePPCK2* (Table I) that span the unusual intron in *LePPCK2*. The resulting sequences are very similar to the sequences of the *LePPCK2* and *StPPCK2* genes. Figure 7 shows a comparison of the intron sequences, which comprise 233, 234, 232, and 217 bp in tomato, potato, aubergine, and tobacco, respectively. The two donor splice sites and the acceptor site are conserved. All four sequences contain at least one in-frame stop codon 70 to 80 bp into the intron (bold in Fig. 7); aubergine has two closely adjacent stop codons. The tobacco intron has a deletion of 18 bp relative to the other introns just 3′ to the second ("incorrect") donor splice site. Using the same primers, we amplified three bands of the predicted mobilities from tobacco flower and leaf cDNA but only one from genomic DNA (Fig. 6B), indicating that the tobacco *PPCK2* gene does contain an additional intron that is subject to alternative splicing. As with tomato, the relative abundance of the three transcripts differs between organs (Fig. 6B). The data argue strongly that both aubergine and tobacco contain a gene equivalent to the *PPCK2* genes of potato and tomato.

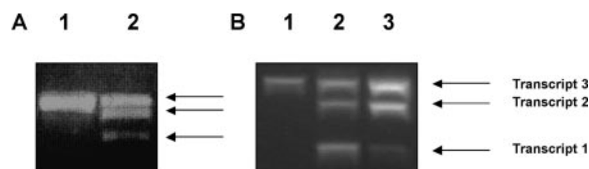

Figure 6. Analysis of potato and tobacco *PPCK2* transcripts. A, Potato; lane 1, products obtained from PCR of genomic DNA; lane 2, products obtained from RT-PCR of mature leaf RNA (35 cycles). B, tobacco; lane 1, products obtained from PCR of genomic DNA; lane 2, products obtained from RT-PCR of flower RNA; lane 3, products obtained from RT-PCR of mature leaf RNA (33 cycles). The arrows indicate (from top to bottom) transcripts 3, 2, and 1.

Figure 7. Sequences of the unusual *PPCK2* intron. Tomato, Le; potato, St; aubergine, Sm; tobacco, Nt. The in-frame stop codons are in bold. The sequences conserved around the strong internal splice site used in *LePPCK2* transcript 2 are underlined, with the intron start GT in italics.

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Le    GTACTCTTCC AGATCACCAA AATCTCACTC GTTGCTGATC AAAATGGGGA AATTTT-ACT
St    GTACTCTTCC AGATCACCAA AATCTCACTC GTTGCTGATC AAAATGGGGA AATTTTTACT
Sm    GTACTCTTCC AGATTACCAA AATCTCACTC GCTGCTGATC ATAAAAGGGG AATTTT-ACT
Nt    GTACTCTTCC AGATCACCAC AATCTCACTC GTTGCTGATC AAAAAGGGGA AATTTT-ACT

Le    TTTGCTTCGT AAAATGGCTT GAATCAGGAA CGATCTACGA TGCAGCACGT TGTTGAATTT
St    TTTGCTTCGT AAAATGGCTT GAATCAGGAG CGATCTACGA AGCAGCACGT TGTTGAATTT
Sm    TTTCCTTCGT TAAATGGCTT GAATCAGGAA CGATCTACGA AGGAGCATCT TGTTGAATTT
Nt    TTTGCTTCGT ATAATGGCTT GAATCAGGAA CGATCTACGA AGCAGCACCT TGTTGAATTT

Le    TCGAAAATTG GAGGAGAAAG TAAATTGTAA AGCTAAGCGA AGTAGAATTC ATAATCGGCT
St    TCGAAAATTG GAGGAGAAAG TAAATTGTAA AGCTAAGCGA AGTAGAATTC ATAATCGGCT
Sm    TGGAAAATTG GAGGAGGAAG TAA-TTGTAA AGCTAAGCGA AGTAGAATTT ATAATCGGCT
Nt    TCGAAAATTG GAGGAGAAAG TAAAT----- ---------- ---AGAATTC ATAATCGGCT

Le    TTT--ATTTT AGTTTTTCGT CATTTCTGAT AAATTTTATG CTTAATGTTTT TATAG
St    TTT--ATTTT AGTTTTTCGC CATGTCTGAT AATTTTTCTG CCTAATGTTTG TATAG
Sm    TTTAT-TTTA GGTTTTCGGT CATCTCTGAC AA-TTTTCTG TATAATGTTTG TACAG
Nt    TTTTTATTTT AGTTTTTCGT AATTACTGAT AAATTTTCTG CATAATGTATG TATAG
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DISCUSSION

The results presented here demonstrate that tomato contains at least two genes that encode functional PEPc kinases. *LePPCK1* is similar to other reported PEPc kinase genes (Hartwell et al., 1999a; Taybi et al., 2000; Tsuchida et al., 2001; Fontaine et al., 2002; Nakagawa et al., 2003; Nimmo, 2003; Xu et al., 2003) in that it encodes a protein kinase catalytic domain with minimal N- and C-terminal extensions and contains one small intron toward the 3′ end of the coding region. *LePPCK2* has one major difference from other *PPCK* genes studied to date, namely the presence of an additional intron that exhibits alternative splicing. Our data show that three types of transcript can be detected, corresponding to correctly spliced, incorrectly spliced, and unspliced transcripts. The correctly spliced transcript encodes a functional PEPc kinase very similar to the enzyme from other species, whereas the other two encode truncated, nonfunctional proteins. It remains to be seen whether the truncated product accumulates in cells. A similar gene has been detected in potato, aubergine, and tobacco (Figs. 6 and 7). To date, this unusual *PPCK* gene with alternative splicing has been found only in the Solanaceae.

Several lines of evidence suggest that this alternative splicing of *PPCK2* transcripts may be functionally significant. First, the nucleotide sequence of the intron is highly conserved between four members of the Solanaceae (see Fig. 7); moreover, the intron and exon sequences are equally similar. For example, the aubergine and tomato sequences are 94% identical over 321 bases of exon sequence (not shown) and 92% identical over 233 intron bases (Fig. 7). The conservation of intron sequence in the *PPCK2* genes of the four species includes the splice sites as shown both directly (Fig. 7) and functionally (Fig. 6); it also covers the presence of in-frame stop codons that would lead to premature truncation of the gene product (although in aubergine there are actually two in-frame stop codons as shown in Fig. 7). The major difference between the intron sequences is an 18-bp deletion in the tobacco sequence that is downstream of both the internal stop codon and the "incorrect" donor splice site. Second, as noted below, the relative abundance of the three transcripts in tomato depends on tissue and conditions. Third, transcripts 2 and 3 actually predominate in some samples even though they contain a premature in-frame stop codon, which often leads to instability of the mRNA (Abler and Green, 1996; Brown and Simpson, 1998). Taken separately, these arguments are not conclusive; together, they suggest strongly that the alternative splicing is significant.

Various possible roles for this alternative splicing can be envisaged. First, it could allow control of the abundance of the functional transcript 1, either in the context of tissue specificity or in response to a signal; a complex example of such behavior is found in the alternative processing of the Arabidopsis FCA gene (Macknight et al., 2002; Quesada et al., 2003). For example, the relative abundances of the three *PPCK2* transcripts are very different in tomato roots, seeds, and locule (which contain mainly transcript 1) compared with leaves (which contain mainly transcripts 2 and 3; Figs. 4 and 5). In seedlings, light not only increases the expression of *LePPCK2* overall but also reduces the relative abundance of transcript 1 (Fig. 4). One of the major metabolic changes that occur during fruit ripening is the breakdown of chlorophyll. Chlorophyll breakdown is reduced in the *gf* mutant, whereas several other parameters, such as accumulation of carotenoids, are not affected (Akhtar et al., 1999). The relative abundance of *LePPCK2* transcripts is altered in the *gf* mutant (Fig. 5), emphasizing that the alternative splicing is dependent on metabolic context.

It is possible that cells contain limiting amounts of a splicing factor that is essential for the production of transcript 1; if so, roots must contain more of this factor than, for example, leaves. Requirement for a specific factor to give correct splicing would be consistent with the sequence of the intron. Plant introns are AU and particularly U rich (Brown and Simpson, 1998). For example, the 89-bp intron in *LePPCK1* mRNA contains 15 A and 47 U residues (70% AU).

Incorrect processing of the unusual intron in *LePPCK2* mRNA removes a sequence of 95 bases that is 75% AU. In contrast, correct processing removes an additional 138 bases that is only 61% AU. Thus, the intron is more AU rich than the open reading frame (52% AU), but selection of the correct 5′ splice site must be made in the context of an uncommonly low AU content downstream of the site.

In a second potential role of the alternative splicing, it is possible that the truncated protein expressed in vitro from *LePPCK2* transcripts 2 and 3 (Fig. 3) may accumulate in vivo and play a functional role. This truncated protein would extend just beyond the N-terminal ATP-binding domain of PEPc kinase; it may be able to fold stably, to bind ATP, and/or to interact with other proteins such as PEPc. Hence, this truncated protein could play an unsuspected role in the control of the phosphorylation state of PEPc. It clearly will be important to assess whether the truncated protein does accumulate in cells. However, it must be pointed out that PEPc kinase is a very low-abundance protein. For example, even in highly illuminated maize leaves, a rich source, it comprises less than 1 in 10^6 of soluble protein (Saze et al., 2001); our attempts to detect native PEPc kinase immunochemically in tomato tissue extracts have proved negative (data not shown).

To gain information about the possible functions of the two tomato *PPCK* genes, we examined their tissue expression patterns. Our RT-PCR data agree with the distribution of ESTs between tissues (Table II); both approaches show that *LePPCK1* is expressed in many organs, whereas *LePPCK2* is predominantly expressed in ripening fruit. This would suggest that *LePPCK1* encodes a housekeeping kinase, mainly involved in ensuring the replenishment of the TCA cycle, whereas the main role of the kinase encoded by *LePPCK2* is in late ripening. ESTs corresponding to the two potato *PPCK* genes are found in different libraries (Table II). Following the arguments of Ronning et al. (2003), this implies that the two genes have different functions; for example, *StPPCK1* seems to be expressed in dormant tubers, whereas *StPPCK2* is expressed in sprouting eyes.

Recently, Guillet et al. (2002) have reported the cloning and expression analysis of two PEPc genes from tomato (*Ppc1* and *Ppc2*). The *Ppc1* gene product is thought to be anapleurotic, whereas expression of *Ppc2* is increased during the cell expansion phase of fruit development, before breaker phase. *Ppc2* transcripts in unripe fruit were detected in the vacuolated cells in the pericarp, enlarging cells near the vicinity of the seeds and in the periphery of the vascular bundles. In ripening fruit, both expression of *Ppc2* and PEPc activity drop considerably. Guillet et al. (2002) suggested that the *Ppc2* gene product is probably involved in accumulation of malate, aiding cell expansion. Hence, the observation that *LePPCK2* is expressed during ripening is surprising because it occurs at a time when the PEPc activity and malate content of the fruit are falling. It is possible that the *LePPCK2* gene product either phosphorylates another PEPc or phosphorylates a different protein. In any event, it will be important to determine the precise spatial distribution of the functional and nonfunctional *LePPCK2* transcripts and compare these with the distribution of *Ppc2* transcripts.

MATERIALS AND METHODS

Plant Material

Tomato (*Lycopersicon esculentum* cv Alicante) plants were grown from seed in bedding compost (William Sinclair Horticulture Ltd., Lincoln, UK) in a greenhouse under a 16-h photoperiod supplemented with mercury vapor lamps (300 μmol m^{-2} s^{-1}). Plants were repotted at 4 weeks and were watered every 2 d. Tomato fruits were allowed to ripen on the vine. RNA was isolated from quartered segments. For localization studies, fruit were collected at the ripe red stage and separated into skin, outer pericarp, inner pericarp, locule, and seeds. Young leaves were harvested as the primary leaf 14 d after sowing, whereas mature leaves were harvested after 6 weeks. Seedlings (8 d old) were harvested after either 10 h of darkness or 3 h of light.

Isolation of RNA and DNA

Plant material was frozen under liquid nitrogen and stored at −70°C. Frozen plant tissue (2–3 g) was ground to a fine powder using an autoclaved mortar and pestle. RNA was then isolated according to the protocol of Chang et al. (1993), with the following modifications. Ten milliliters of extraction buffer was used for 2 to 3 g of tissue. Chloroform was used rather than chloroform:isoamyl alcohol. After precipitation with LiCl, the pellet was resuspended in 500 μL of 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA and extracted once with an equal volume of chloroform. Sodium acetate (50 μL of a 3 M solution [pH 5.2]) was added to the aqueous layer, followed by 2.5 volumes of ice-cold ethanol. RNA was allowed to precipitate overnight at 4°C. The quantity and purity of the RNA was determined spectrophotometrically according to the method described by Sambrook et al. (1989). Intactness of the RNA was determined by detection of ribosomal RNA bands after agarose gel electrophoresis. A DNase treatment (DNA-free, Ambion, Huntingdon, UK) was used to ensure elimination of contaminating DNA from RNA preparations.

Genomic DNA was extracted using a DNA isolation kit (PUREgene DNA isolation kit, Gentra Systems, Gentra Systems, Minneapolis). The quantity and purity of the DNA was determined spectrophotometrically according to the method described by Sambrook et al. (1989).

RT-PCR and PCR

The RNA samples (100 ng) were mixed with 0.25 μM oligo(dT) for 5 min at 70°C and cooled at 4°C for 5 min. RT was carried out in a reaction mixture (50 μL) containing avian myeloblastosis virus reverse transcriptase buffer, 1 mM dNTPs, 1 unit μL^{-1} RNase inhibitor, and 0.4 units μl^{-1} avian myeloblastosis virus reverse transcriptase (all from Promega, Madison, WI). The reaction was performed at 48°C for 45 min. The enzyme was then heat inactivated at 95°C for 5 min, and the samples were used directly for PCR.

PCR reactions were performed using 2.5 μL of each cDNA sample in a reaction mixture (25 μL) containing 12.5 μL of 2× Reddy Mix (Abgene, Epsom, UK) and 0.5 μM of the 5′ and 3′ primers. The primer sequences are shown in Table I. The PCR reactions were conducted in a programmable thermocycler (PCR Sprint, Hybaid, Ashford, UK). The reaction conditions for the amplification of *LePPCK1* were an initial denaturation step of 94°C for 5 min, 35 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 60 s, and a final extension step of 72°C for 5 min. The reaction conditions for the amplification of *LePPCK2* were an initial denaturation step of 94°C for 5 min, 35 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 60 s, and a final extension step of 72°C for 5 min. Primers for either *Actin52* or ubiquitin were used as a constitutive control in both conditions. After amplification, reac-

tions were resolved by electrophoresis on a 1% (w/v) agarose gel and stained with ethidium bromide.

Cloning

PCR fragments were extracted from an agarose gel by purifying with QIAquik gel extraction kit (Qiagen USA, Valencia, CA) and cloned directly into pCR4-TOPO vectors using the TOPO TA cloning kit with one shot cells (Invitrogen, Carlsbad, CA). All clones were sequenced using universal primers (MWG-Biotech, Ebersberg, Germany).

Assessment of PEPc Kinase Activity Encoded by cDNAs

This was carried out as described previously (Fontaine et al., 2002). In outline, plasmids were linearized and transcribed from either a T3 or T7 promoter as appropriate. The transcripts were translated in a rabbit reticulocyte lysate system using [^{35}S]Met, and the PEPc kinase activity of the translation products was assayed (Hartwell et al., 1996). Figure 3 shows phosphor images of ^{35}S incorporated into protein and ^{32}P incorporated into PEPc after SDS-PAGE.

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