ORIGINAL PAPER

Isolation and Characterization of A Cytosolic Pyruvate Kinase cDNA From Loquat (*Eriobotrya japonica* Lindl.)

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Abstract Pyruvate kinase catalyzes the final reaction of glycolysis, and plays an important role in controlling glycolytic flux. In this study, a full-length cDNA of a putative *Eriobotrya japonica* Lindl. (loquat) pyruvate kinase, designated *EjPK*, was isolated. The nucleic acid sequence of *EjPK* shares about 78–84 % similarity with already studied plant cytosolic pyruvate kinases. *EjPK* belongs to the cytosolic-1 subgroup of pyruvate kinase, which includes grape, soybean, and citrus cytosolic pyruvate kinases. The cytosolic localization was confirmed by confocal microscopy using transiently expressed 35S:*EjPK*-GFP fusion protein. Real-time RT-PCR indicated that *EjPK* is expressed in loquat leaves, roots, stems, flowers, and fruits. Loquat fruits ripen in two stages: in the first stage, the acid content increases, and in the second stage, acids are consumed and at the same time sugars accumulate. *EjPK* displayed a remarkable expression pattern in the developing fruit as *EjPK* transcripts increased dramatically and transiently during the transition period between the two stages. The burst of *EjPK* mRNA expression had greater intensity in a loquat cultivar displaying higher fruit sugar content than in a cultivar with lower fruit sugar concentration. A potential regulatory role of *EjPK* in loquat fruit ripening is proposed.

Keywords Plant glycolysis · Sugar metabolism · Acid metabolism · Cytosolic pyruvate kinase · Agronomic traits

Introduction

Loquat (*Eriobotrya japonica* Lindl.) belongs to the Rosaceae family and the Maloideae subfamily. This evergreen fruit tree is native to southeastern China and is cultivated in Japan, India, Israel, Brazil and several countries bordering the Mediterranean Basin. Loquat fruit is particularly popular for its succulent flavor and also because of its unusual harvest season, at end of spring and early summer, at which period only a small number of cultivated plants produce palatable fruits (He et al. 2011).

Loquat trees are classified broadly according to the pulp color of their fruits into white-, yellow- and orange-fleshed cultivars. The different flesh colors come from differences in carotenoid content (Zhou et al. 2007; Hasegawa et al. 2010; Fu et al. 2012). Yellow- and orange-fleshed cultivars are more resistant to cold and diseases and produce more fruits than the white-fleshed cultivar. However, white-fleshed fruits are of higher commercial value because their higher sugar/acid concentration ratio makes them more popular, whereas the fruits of the two other cultivars are perceived as having astringent taste (Cañete et al. 2007).

The main sugars in loquat fruits are fructose, glucose, sucrose, and sorbitol (Shaw and Wilson 1981; Hasegawa et al. 2010), and several enzymes related to their accumulation in loquat fruits have been isolated (Bantog et al. 1999; Bantog et al. 2000; Qin et al. 2012). Sorbitol is a product of photosynthesis in Rosaceae and is translocated to sink

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tissues, including fruits (Bieleski and Redgwell 1985; Watari et al. 2004; Liang et al. 2012). Two enzymes related to sorbitol metabolism were identified in loquat fruit: a sorbitol-6-phosphate dehydrogenase (S6PDH), which converts glucose-6-phosphate into sorbitol; and a sorbitol dehydrogenase (SDH), which converts sorbitol into fructose (Bantog et al. 2000). Two acid invertases, which split sucrose into glucose and fructose, have been isolated from loquat fruits (Bantog et al. 1999). Sucrose synthase and sucrose-phosphate synthase, which catalyze the reverse reactions that creates sucrose and sucrose-6-phosphate from glucose and fructose, respectively, have also been evidenced in loquat fruits (Bantog et al. 1999). Finally, we have recently isolated a hexokinase in loquat fruits (Qin et al. 2012) that catalyzes the first step of the glycolysis pathway. Loquat fruit acidity change has been explained mainly by variations in malic acid concentration, and three enzymes that catalyze malic acid biosynthesis and conversion have been isolated: NAD-malate dehydrogenase (MH), NADP-malic enzyme (ME), and phosphoenol pyruvate carboxylase (PEPC) (Chen et al. 2009).

The regulation of the balance between sugar and acid content has not yet been investigated, and the study of this balance in loquat is the focus of the present study. In this regard, the glycolytic pathway is particularly important as it is the main conversion route of sugars into acids. Pyruvate kinase (PK; EC 2.7.1.40) catalyzes the final reaction of the glycolytic pathway by transferring a phosphate group from phosphoenol-pyruvate (PEP) to ADP. This irreversible reaction produces one molecule of ATP and one molecule of pyruvate, which then enters the mitochondrial tricarboxylic acid cycle (TCA). PK controls plant major glycolytic flux from hexose-phosphates to pyruvate (Dennis and Greyson 1987; Oliver et al. 2008), and also has connections with ABA/GA balance and oxidative stress (Zhang et al. 2012a, b)

Two groups of PK isozymes exist in plants: cytosolic PKs (PKcs) and plastidic PKs (PKps) (Plaxton 1996; Givan et al. 1999). The two groups of isozymes differ in their cellular localization, regulatory elements and kinetic characteristics (Smith et al. 2000). PKps are involved in fatty acid biosynthesis in *Arabidopsis thaliana* seeds (Andre et al. 2007; Andre and Benning 2007; Baud et al. 2007). The role of PKcs is not well known, but several studies have revealed their involvement in a range of physiological processes. PKcs play a critical role in the regulation of carbon partitioning and dark respiration in tobacco leaves (Knowles et al. 1998; Grodzinski et al. 1999), and regulate pyruvate level and alternative oxidase level in potato heterotrophic plant tissues (Oliver et al. 2008). Decreased expression of PKc also causes rice dwarfism linked to alteration of monosaccharide

metabolism and sugar transport (Zhang et al. 2012a, b). Another study focused on a fruit PKc during fruit ripening (Law and Plaxton 1997; Turner and Plaxton 2000). The protein of a banana PKc was isolated and extensively characterized in term of post-translational regulations (Turner and Plaxton 2000). Given the essential role of PKcs in glycolysis and in the TCA, we isolated a transcript of a putative PKc from loquat, and investigated its relationship with sugar and acid accumulation during loquat fruit ripening.

Material and Methods

Plant Material

Three 10-year-old *Eriobotrya japonica* Lindl. (loquat), grown in the loquat garden at Tangxi, Hangzhou, China, were chosen randomly to provide materials. Leaves, flowers, roots and fruits at different developmental stages were flash frozen in liquid nitrogen after collection and stored at −80°C until analysis. Loquat fruits were collected over a period of 4 months after flowering, at 60, 83, 100, 117, 125, 135, 142 days after flowering (DAF).

cDNA Cloning and Sequence Analysis

Loquat tissues were ground to a fine powder while still frozen. Total RNA was extracted using a modified CTAB method (Gasic et al. 2004). RNA quality was checked by spectrophotometry and gel electrophoresis. First strand cDNA was synthesized using RevertAid first strand cDNA synthesis kit (Fermentas, http://www.fermentas.com). Degenerate primers were designed according to PKc conserved regions that were identified using an alignment of PK sequences in GenBank (supplemental data 1). The forward degenerate primer was: GAGAACACTGCA(A/G)(T/C)(G/T)CT(T/A)GG(T/C)GAGAGAAA; and the reverse primer was: ACAAAGGA(C/A/T)AG(G/A)GCAATCAT(A/G)TC. A RNA fragment of about 160 bp was amplified by PCR using the primers. For amplification of 5′ end and 3′ end of the putative loquat PK gene, specific primers were designed according to the sequenced fragment, 3′-Full RACE Core Set and 5′-Full RACE kit (TaKaRa, Tokyo. Japan). The primer for 3′ RACE was: 5′-GGATAAGGAAGA-TATTCTGGAATGGGG-3′; the primers for 5′ RACE were: 5′-CGGGCAACCATGAATGAATCTGTC-3′ and 5′-CCCTTGCGTACAAATGAAAGAGC-3′. The PCR products were cleaned and cloned into pUC18-T vector (Sangon, Shanghai, China) and transformed into DH5α competent cells and sequenced by Sangon, Shanghai (GenBank: JF414125). To construct a phylogenetic tree, plant PK amino acid sequences were retrieved from UniProt-KB

(supplemental data 2) (UniProt Consortium 2012). Sequences were aligned using ClustalW (Larkin et al. 2007), and a phylogenetic analysis was carried out using the PhyML program (Guindon et al. 2010) with 100 bootstrap replicates and the JTT evolutionary model (Jones et al. 1992). A model of the three-dimensional structure of the newly discovered PK was generated by homology using Modeller 9v10 (Sali and Blundell 1993).

Gene Expression by Quantitative Real-time RT- PCR and Data Analysis

Real-time RT-PCR primers were designed using Primer3 software (Rozen and Skaletsky 2000). The forward primer for loquat PK was: CATGCACAACACTCAGATTC; reverse primer: TTTCTAGGCTTCAAATCCAC. An actin gene from loquat (GenBank ID: FJ481118) was used as internal standard. The forward primer for actin was: 5′-TGGTCGTACAACAGGTAT-3′ and the reverse primer was 5′-GGGCAACATATGCAAGCT-3′. QuantiTect SYBR Green PCR Kits (Qiagen, Valencia, CA) and Applied Biosystems 7300 Real Time PCR System (ABI, Foster City, CA) were used for gene expression analysis. Three replicates were performed for each sample. All data were analyzed using the statistical software SPSS 14.0 (http://www.spss.com).

Determination of Sugar Content

Loquat fruits were ground and extracted three times, according to Qin et al. (2004). The crude extract was filtered using a Sep-Pak C18 column to remove pigments and other non-sugar compounds and then analyzed by high-performance liquid chromatography (HPLC, Waters 1500, http://waters.com/) equipped with a Sugar-Pak column and a differential refractive index detector (Waters 2414). The mobile phase was distilled water, and the flow rate was 0.6 mL/min. The main soluble sugars identified previously in loquat fruits are fructose, sucrose, glucose and sorbitol (Ding et al. 1998).

Analysis of Acid Content

Fruits were ground and extracted in distilled water, and the extracts were filtered using 0.45 μm membrane filter. pH was titrated using 0.1 mM NaOH. Organic acids were identified by HPLC (LC-20AT system, Shimadzu, Tokyo, Japan) equipped with SPD-20A UV/VIS detector and using a reverse-phase column C18 (Inertsil ODS-SP). The HPLC mobile phase was 0.01 mM NaH_2PHO_4 (pH 2.0), and the flow rate was set to 0.5 mL/min. Two wavelengths, 210 nm and 214 nm, were monitored.

Subcellular Localization of GFP-tagged *EjPK* in Onion Epidermal Cells

To localize the putative loquat pyruvate kinase in cells, the open reading frame (ORF) of the full length cDNA was fused into pCAMBIA-1302 binary vector downstream of the constitutive CaMV 35S and upstream of GFP, creating a 35S: EjPK-GFP fusion protein. The forward primer used for PCR amplification of the ORF was: 5′-CGGGGGACTCTTGACATGGCCAACATAGACATAGAAGGAATACTC-3′; and the reverse primer was: 5′-ACTAGTCAGATCTACCATGGACTTTACCACGCAGATCTTAATAACAGAGGC-3′. The underlined sequences were appended for construction of the expression vector. The PCR products were extracted from gel and the purified cDNA was cloned in the *Nco*I-digested pCAMBIA-1302 using the seamless cloning method (GeneArt Seamless Cloning and Assembly Kit, Invitrogen, Carlsbad, CA). Onion epidermal segments were peeled and placed on a Murashige and Skoog medium (MS) plate. The recombinant pCAMBIA-35S:*EjPK*-GFP plasmids were expressed transiently in onion epidermal cells using PDS-1000/He Biolistic Particle Delivery System (Bio-Rad, Hercules, CA). After 16–24 h, the transformed cells were observed under a Zeiss LSM 510 Meta Confocal Microscope.

Western Blots

Proteins were extracted according to the one step plant protein isolation kit (Sangon, Shanghai, BSP004) and quantified using Bio-Rad protein assay reagent. Bovine serum albumin was used as a quantification standard. Proteins were separated on a 10 % SDS-PAGE and transferred on nitrocellulose membranes. Western blots were carried out using the western blot kit from Sangon (BS393-RB). The membranes were incubated in blocking buffer for 1 h, then probed with primary affinity-purified rabbit antibodies raised against one *Brassica napus* PKc (Smith et al. 2000). Alkaline phosphatase-conjugated anti-rabbit IgG (Bio-Rad) was used as the secondary antibody. DAB peroxidase substrate solution was used to develop the color of the blot.

Results

Identification of Loquat Pyruvate Kinase Gene and Protein Structure Prediction

A full-length cDNA sequence of loquat pyruvate kinase, *EjPK* (GenBank ID: JF414125), was determined using PK specific degenerate primers and PCR RACE. *EjPK* is 1,884 bp in length and displays a 161 bp 5′UTR, a 193 bp 3′UTR, and a 1,530 bp ORF, encoding 510 amino acids with

a calculated molecular weight of 55.245 kDa and a predicted pI of 7.22. *EjPK* nucleotide sequence shares about 78–84 % similarity with PKc genes from castor bean, poplar, grape, *Citrus sinensis*, and lemon. *EjPK* encoded protein shares 78 % to 93 % identity with *Citrus sinensis*, soybean, grape, *Arabidopsis thaliana*, and tobacco (Fig. 1). The *EjPK* protein sequence also shares 47 % identity and 74 % similarity with human M2 pyruvate kinase (Swiss-Prot ID: P14618), whose three-dimensional structure was determined by X-ray crystallography (Dombrauckas et al. 2005; Christofk et al. 2008). It is therefore likely that the two enzymes share a similar fold. As shown in Fig. 2, M2 PK is a homo-tetramer, which has been described as a dimer of dimers (Dombrauckas et al. 2005). Each subunit is composed of four domains, denoted N, A, B and C, as illustrated in Fig. 2b. Domain N is a small α-helical domain located at the center of the tetramer. Domain A is an α8/β8 barrel at the core of the subunit. The active site is a cleft located between domain A and B. The sequence of Domain C, which is the binding site of allosteric effectors, is conserved at only 30 % between the *EjPK* and M2 PK. This low sequence percentage identity might indicate that the two enzymes have different regulatory molecules. Indeed, M2 PK uses fructose-1,6-bisphosphate as an allosteric activator (Dombrauckas et al. 2005), whereas plant cytosolic PK activity is most often influenced by glutamate, aspartate, oxalate and citrate (Ambasht and Kayastha 2002). The only other study that focused on a PKc extracted from fruit, more specifically a banana PKc, showed that the cytosolic pH, glutamate (inhibitor), and aspartate (activator) were the most important regulators, but citrate was found to be only a poor inhibitor (Turner and Plaxton 2000).

Phylogenetic Classification and Cytosolic Localization of *EjPK*

A protein phylogeny of PK was established by Oliver et al. (2008). The two main clades of this phylogeny correspond to PKs located in the cytosol and in plastids, and the cytosolic PKs are further divided into two subgroups, noted cytosolic-1 and cytosolic-2. According to the phylogenetic tree displayed in Fig. 3, *EjPK* belongs to the cytosolic-1 subgroup. The possible cytosolic localization of *EjPK* is also supported by a prediction using WoLF PSORT, which uses signal sequence motifs and other sequence features (Horton et al. 2007). To verify the predicted cytosolic localization, *EjPK* was expressed transiently as a GFP fusion protein in onion epidermal segments. The confocal image in Fig. 4 reveals that the 35S:*EjPK*-GFP fusion protein is expressed mainly in the cytosol, and also at the cell membrane and potentially in the nucleus, but not in plastids.

Fig. 1 Comparison of the predicted amino acid sequence of loquat pyruvate kinase *EjPK* gene with other plant pyruvate kinase (PK) sequences. Identical amino acids are shown on a *black background*. *Eriobotrya japonica*, this work. *Citrus sinensis*, AAY86035; *Glycine max* 1, AAM94348; *G. max* 2, NP_001236056; *Vitus vinifera* 1, CAQ58629; *V. vinifera* 2, XP_002266160; *Solanum tuberosum*, P22200; *Arabidopsis thaliana* 1, NP_196474; *A. thaliana* 2, BAF01483; *Ricinus communis*, XP_002513157; *Capsicum annum*, AAZ86534; *Lilium longiflorum*, AAF44707; *Zea mays*, NP_001149825; and *Populus trichocarpa*, XP_002304847

Tissue Specific Expression of *EjPK* and Comparison Between Cultivars

Real-time RT-PCR was carried out to examine the transcript levels of *EjPK* in young leaves, mature leaves, stems, and flowers of loquat. *EjPK* transcripts were detected in all tissues, and the highest expression level was in young leaves (Fig. 5a). The transcript of *EjPK* was also monitored in fruits produced by the white- and yellow-fleshed cultivars (Fig. 5b). The transcript expression pattern of *EjPK* was similar between the two cultivars: a relatively low expression level before 135 DAF, at which time it suddenly rose to reach a maximum, followed by a slow decline over a week, at which time the fruits start ripening. The maximum level of *EjPK* transcription was four-fold higher at 135 DAF than that at any other dates for the yellow-fleshed fruits and nine-fold higher for the white-fleshed fruits.

PKc protein levels in fruits at different developmental stages were monitored by western blot using a PKc antibody raised against PKc from *Brassica napus* (Smith et al. 2000). The immunoreactive band was about 56 kDa (Fig. 6), which is similar to the predicted size of the *EjPK* encoded protein. Interestingly, protein levels showed similar dynamic changes to the levels of the *EjPK* mRNA monitored during fruit development, *i.e.*, the protein level reached its highest level at 135 DAF, then declined in the mature fruit in both cultivars (Fig. 6). Besides, the protein level of PK at 135 DAF in the white-fleshed cultivar was about twice that in the yellow-fleshed cultivar.

Sugar and Organic Acid Content During Fruit Development

Dynamic changes in fructose, glucose, sucrose, sorbitol and titratable acidity (TA) were monitored during fruit ripening to investigate the potential relationship between sugar and acid accumulation and *EjPK* gene expression. The two cultivars had similar variations of sugar content during fruit development, as shown in Fig. 7. Fructose and glucose were at a low level before 135 DAF, at which point their concentration rose progressively until 142 DAF corresponding to fruit maturity. Sucrose content did not vary and sorbitol content decreased until 117 DAF and then remained relatively constant. The evolution of fruit acidity is shown in

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E.japonica    MANIDIEGILKEL-PN---DGRIPKTKIVCTLGPASRSVPMVELLRAGMNVARFNFSHGTHDYRQETIDNIRTAMYNTQ   76
C.inensis     MANIDIEGLLRDV-PN---DKRLPKTKIVCTLGPASRSVPMLELLRAGMNVARFNFSHGTHYQQETINNIRAAMYNTQ   76
G.max1        MANIDIEGILKQQ-PD---DGRVPKTKIVCTLGPASRSVEMTEKLLRAGMNVARFNFSHGTHDYFQETINNGKTAMHNTG   76
G.max2        MANIDIEGILKQQQPY---DGRVPKTKIVCTLGPASRSVEMTEKLLRAGMNVARFNFSHGTHDYFQETINNNKTAMHNTG   77
V.vinifera1   MANIDIEGILKEL-PN---DGRIPKTKIVCTLGPASRSVPMLEKLLRAGMNVARFNFSHGTHDYFQETIDNIRIAMQNTQ   76
V.vinifera2   MANIDIEGILKEL-PN---DGRIPKTKIVCTLGPASRSVPMLEKLLRAGMNVARFNFSHGTHDYFQETIDNIRIAMQNTQ   76
S.tuberosum1  MANIDIAGIMKDL-PN---DGRIPKTKIVCTLGPASRTVPMLEKLLRAGMNVARFNFSHGTHEYFQETIDNIKIAMQNTQ   76
S.tuberosum2  MANIDIAGIMKDL-PN---DGRIPKTKIVCTLGPASRTVPMLEKLLRAGMNVARFNFSHGTHEYFQETIDNIKIAMQNTQ   76
A.thaliana1   MSNIDIEGILKEL-PN---DGRIPKTKIVCTLGPASRTVSMIEKLLKAGMNVARFNFSHGTHEYFQETIDNIRSAMHNTG   76
R.communis    MANIDIEGILKEL-PE---DGRVPKTKIVCTLGPASRSVPMLEKLLRAGMNVARFNFSHGTHEYFQETINNIRIAMQNTQ   76
C.annuum      MANIDIAGIMKDL-PN---DGRVPKTKIVCTLGPASRSVPMLEKLLRAGMNVARFNFSHGTHEYFQETINNIKIAMQNTQ   76
L.longiflorum MANIDIEGILKEL-P---ADGRVPKTKLVCTLGPSSRSVPMLEKLLRAGMTVARFNFSHGTHEYFQETIDNIRIAMQNTQ   76
A.thaliana2   VSNIDIEGILKEL-PN---DGRIPKTKIVCTLGPASRTVTMIEKLLKAGMNVARFNFSHGTHEYFQETIDNIRTAMQNTQ   76
Z.mays        MANIDMAKILADL-DRGAGDABLPKTKLVCTLGPASRSVPMLEKLLRAGMNVARFNFSHGTHQYFQETIDSIRQAMHNTG   79
P.trichocarpa ---------MEKR-PN--------TKIVCTLGPASRSVPVIEKLLRAGMNVARFNFSHGTHDYFQETIDNIMAAMYNTG   61

E.japonica    IICAVVLDTKGPEIRTGFLKGGKPIQLKEGQEITITTDYSIKGDAEMISVSYRKLAVDLKFRNTLLCADGTITLTVISCD  156
C.inensis     IICAVVLDTKGPEIRTGFLKDGKPIQLKEGQEITVSTDYDFKGNEEMITMSYKKLPVDVKFGNTILLCADGTITLTVISCD  156
G.max1        IICAVVLDTKGPEIRTGFLKDGKPIQLKEGQEITITTDYDIKGDQEMISMSYRKLPVHLKFGNTILCADGTITLTVISCD  156
G.max2        IICAVVLDTKGPEIRTGFLKDGKPIQLKEGQEITITTDYDIKGDPEMISMSYRKLPVHLKFGNTILCADGTITLTVISCD  156
V.vinifera1   IICAVVLDTKGPEIRTGFLKDAKPIQLKEGEEITITTDYSIKGDEKMISMSYRKLPVDLKFGNTILCADGTITLTVISCD  156
V.vinifera2   IICAVVLDTKGPEIRTGFLKDAKPIQLKEGEEITITTDYSIKGDEKMISMSYRKLPVDLKFGNTILCADGTITLTVISCD  156
S.tuberosum1  IICAVVLDTKGPEIRTGFLTDGKPIQLKEGQEITVSTDYTIKGNEEMISMSYKKLVMDLKFGNTILCADGTITLTVISCD  156
S.tuberosum2  IICAVVLDTKGPEIRTGFLTDGKPIQLKEGQEITVSTDYTIKGNEEMISMSYKKLVVDLKFGNTILCADGTITLTVISCD  156
A.thaliana1   IIAAVVLDTKGPEIRTGFLKDGNPIQLKEGQEITITTDYDIQGDESTISMSYKKLPLDVKFGNTILCADGSISLAVISCD  156
R.communis    IICAVVLDTKGPEIRTGFLKDGKPIQLKEGQEITVTTDYSIKGDTDMISMSYRKLPVDVKFGNTILCADGTITLTVISCD  156
C.annuum      IICAVVLDTKGPEIRTGFLKDGKPIQLKEGQEITVSTDYTIKGNVEMISMSYKKLPVDVKFGNTILCADGTITLTVISCD  156
L.longiflorum IIAAVVLDTKGPEIRTGFLKDGKAVQPKEGQEITVSTDYDLKGGGNTITMSYKKLPVDMKFGGTILCADGSISLTVISCD  156
A.thaliana2   IIAAVVLDTKGPEIRTGFLKDGKPIQLKEGQEITITTDYDIKGDEKTISMSYKKLPVDVKFGNTILCADGSISLAVISCD  156
Z.mays        IICAVVLDTKGPEIRTGFLKDGKPIKLTKGQEITVTTDYDIKGDENMIAMSYKKLPVDVKFGNVILCADGTISLAVISCD  159
P.trichocarpa IICAVVLDTKGPEIRTGFLKDGKPIQLNQGQEITISTDYSLKGDENMICMSYKKLAEDVQFGMVILCADGTLSFTVISCD  141

E.japonica    AAAGTVRCRCENTAMLGERKNVNLPGVVVDLPTLTEKIKEDILEWGVPNIDMIALSFVRKGSDLVNVRKVIGPHAKNIQ  236
C.inensis     PKSGTVRCRCENTAMLGERKNVNLPGVVVDLPTLTEKIKEDILQWGVPNNIDMIALSFVRKGSDLVNVRKVEPHAKTTQ  236
G.max1        PDAGTVRCRCENTATLGERKNVNLPGVVVDLPTLTEKIKEDILGWGVPNIDMIALSFVRKGSDLVNVRKVIGPHAKNIQ  236
G.max2        PDAGTVRCRCENTATLGERKNVNLPGVVVDLPTLTEKIKEDILGWGVPNIDMIALSFVRKGSDLVNVRKVIGPHAKNIQ  237
V.vinifera1   LGAGTVRCRCENTATLGERKNVNLPGVVVDLPTLTEKIKEDILGWGVPNNIDMIALSFVRKGSDLVNVRKVIGSHAKRIQ  236
V.vinifera2   LGAGTVRCRCENTATLGERKNVNLPGVVVDLPTLTEKIKEDILGWGVPNNIDMIALSFVRKGSDLVNVRKVIGSHAKRIQ  236
S.tuberosum1  PPSGTVRCRCENSATLGERKNVNLPGVVVDLPTLTEKIKEDILEWGVPNIDMIALSFVRKGSDLVNVRKVIGPHAKRIQ  236
S.tuberosum2  PPSGTVRCRCENSAMLGERKNVNLPGVVVDLPTLTEKIKEDILEWGVPNIDMIALSFVRKGSDLVNVRKAIGPHAKRIQ  236
A.thaliana1   PESGTVRCRCENTAMLGERKNVNLPGVVVDLPTLTEKDIIEDILGWGVPNIDMIALSFVRKGSDLVHVRKVIGPHAKHIQ  236
R.communis    PEAGTVRCRCENTAMLGERKNVNLPGVVVDLPTLTEKIKEDILGWGVPNKIDMIALSFVRKGSDLVNVRKVIGPHAKHIQ  236
C.annuum      PAAGTVRCRCENTATLGERKNVNLPGVVVDLPTLTEKIKEDILGWGVPNIDMIALSFVRKGSDLVNVRKVIGPHAKRIQ  236
L.longiflorum PDAGTVRCRCENTALLGERKNVNLPGVVVDLPTLTEKIKEDILEWGVPNNIDMIALSFVRKGSDLVNRQVIGSHAKNIK  236
A.thaliana2   PNAGTVIRCRCENTAMLGERKNVNLPGVVVDLPTLTEKDVEDILKWGVPNNIDMIALSFVRKGSDLVNVRKVIGSHGKSIM  236
Z.mays        PDAGTVRCRCENTAMLGERKNCNLPGIVVDLPTLTEKIKEDILGWGVPNIDMIALSFVRKGSDLVTVRQVLGQHARRIK  239
P.trichocarpa TEAGLVRCRCENSAVLGERKNVNLPGVIVDLPTLTEKIKEDILANGVPNIDMIALSFVRKGSDLVEVRKILGKHARNIL  221

E.japonica    LMSKVENQEGVINFDEILRETDSFMVARGDLGMEIPVEKIFLAQKMIYKCNLVGKEVVTATQMLESMIKSPRPTRAEAT  316
C.inensis     LMSKVENQEGVVNFDDILRETDSFMVARGDLGMEIPVEKIFLAQKMIYRICLVGKEVVTATQMLESMIKSPRPTRAEAT  316
G.max1        LMSKVENQEGVLNFDEILRETDAFMVARGDLGMEIPVEKIFLAQKMIYKCNLVGKEVVTATQMLESMIKSPRPTRAEAT  316
G.max2        LMSKVENQEGVLNFDEILRETDAFMVARGDLGMEIPVEKIFLAQKMIYKCNLVGKEVVTATQMLESMIKSPRPTRAEAT  317
V.vinifera1   LMSKVENQEGVINFDEILRETDSFMVARGDLGMEIPVEKIFLAQKMIYKCNLVGKEVVTATQMLESMIKSPRPTRAEAT  316
V.vinifera2   LMSKVENQEGVINFDEILRETDSFMVARGDLGMEIPVEKIFLAQKMIYKCNLVGKEVVTATQMLESMIKSPRPTRAEAT  316
S.tuberosum1  LMSKVENQEGVINFDEILRETDSFMVARGDLGMEIPVEKIFLAQKMIYKCNLAGKEVVTATQMLESMIKSPAPTRAEAT  316
S.tuberosum2  LMSKVENQEGVINFDEILRETDSFMVARGDLGMEIPVEKIFLAQKMIYKCNLAGKEVVTATQMLESMIKSPRPTRAEAT  316
A.thaliana1   LM--LRTRKVLLTLMRSCGETDSFMVARGDLGMEIPVEKIFLAQKLMIYKCNLAGKEVVTATQMLESMIKSPRPTRAEAT  316
R.communis    LM--LRTRKVLLTLMRSCGETDSFMVARGDLGMEIPVEKIFLAQKLMIYKCNLAGKRVVTATQMLESMIKSPRPTRAEAT  314
C.annuum      LMSKVENQEGVVNFDEILRETDSFMVARGDLGMEIPVEKIFLAQKMIYKCNLAGKEVVTATQMLESMIKSPRPTRAEAT  316
L.longiflorum LMSKVENQEGVVNFDEILKETDAFMVARGDLGMEIPVEKIFLAQKLMIYICNLAGKEVVTATQMLESMIKSPRPTRAEAT  316
A.thaliana2   LMSKVENQEGVLNFDEILRETDAFMVARGDLGMEIPVEKIFLAQKIMIYKCNLAGKEVVTATQMLESMIKSPRPTRAEAT  316
Z.mays        LMSKVENQEGVVNFDEILRETDAFMVARGDLGMEIPVEKIFLAQKMIYKCNIAGKEVVTATQMLESMIKSPRPTRAEAT  319
P.trichocarpa LMSKVENQEGVANFDDILANSDAFMVARGDLGMEIPIEKIFLAQKMIYKCNIQGKEVVTATQMLESMIKAPRPTRAEAT  301

E.japonica    DVANAVLDGTDCVMLSGESAAGSYELAVKIMARICIEAESSLDYRAIFKEMIKSABLPMSPL-ESLASSAVRTANKARA  395
C.inensis     DVANAVLDGTDCVMLSGESAAGAYELAVKIMARRICIEAESSLDYRAVFKEMIRSTBLPMSPL-ESLASSAVRTANKARA  395
G.max1        DVANAVLDGTDCVMLSGESAAGAYELAVKIMARICIEAESSLDYGAIFKEMIRSTBLPMSPL-ESLASSAVRTANKAKA  395
G.max2        DVANAVLDGTDCVMLSGESAAGAYELAVKIMARICIEAESSLDYGAIFKEMIRSTBLPMSPL-ESLASSAVRTANKAKA  396
V.vinifera1   DVANAVLDGTDCVMLSGESAAGAYELAVKIMARICIEAESSLDYAAIFKEMIRSTBLPMSPL-ESLASSAVGTANKAKA  395
V.vinifera2   DVANAVLDGTDCVMLSGESAAGAYELAVKIMARICIEAESSLDYAAIFKEMIRSTBLPMSPL-ESLASSAVGTANKAKA  395
S.tuberosum1  DVANAVLDGTDCVMLSGESAAGAYELAVKISRICIEAESSLDNEAIFKEMIRCTBLPMSPL-ESLASSAVRTANKARA  395
S.tuberosum2  DVANAVLDGTDCVMLSGESAAGAYELAVKISRICIEAESSLDNEAIFKEMIRCTBLPMSPL-ESLASSAVRTANKARA  395
A.thaliana1   DVANAVLDGTDCVMLSGESAAGAYKVMAVKIMARICIEAESSLDYNTIFKEMIRATBLPMSPL-ESLASSAVRTANKAKA  395
R.communis    DVANAVLDGTDCVMLSGESAAGAYELAVKIMARICIEAESSLDYGAIFKEMIRCTBLPMSPLDESSIISCPHGLTKLKQ  393
C.annuum      DVANAVLDGTDCVMLSGESAAGAYELAVKVMAKICIEAESSLEYEAIFKEMIRCTBLPMSPLDESSIISCPHGLTKLKQ  396
L.longiflorum DVANAVLDGTDCVMLSGESAAGAYELAVKVMAKICIEAESSLDYDAIFKEMIRSGEBLPMSPL-ESLASSAVRTANKAKA  395
A.thaliana2   DVANAVLDGTDCVMLSGESAAGAYELAVKIMARICIEAESSLDYNTIFKEMIRATBLPMSTL-ESLASSAVRTANKAKA  395
Z.mays        DVANAVLDGTDCVMLSGESAAGAYEVAVKIMARICIEAESSLDHEAVFKAMIRSAPLPMSPL-ESLASSAVRTANKAKA  398
P.trichocarpa DVANAVLDGTDCVMLSGETAAGAYELAVQTKAKICVEAESTLDGDVEKRTTKHSEVPMSPL-ESLASSAVRTANSVKA  380

E.japonica    KLIVVLTRGGSTARLVAKYRBAVPILSVVVPVLTTDFDWTCSDBAPARHSLIYRGLIPLDAEGSAKATDABSTEVILEA  475
C.inensis     KLIVVLTRGGTTARLVAKYRBAVPILSVVVPVLTTDFDWTCSDBTPARHSLIYRGLIPIIAEGSAKATDABSTEVILEG  475
G.max1        KLIVVLTRGGTTARLVAKYRBAVPILSVVVPVLSTDFDWTCSDBTPARHSLIYRGLIPIIAEGSAKATDABSTEVILEA  475
G.max2        KLIVVLTRGGTTARLVAKYRBAVPILSVVVPVLSTDFDWTCSDBTPARHSLIYRGLIPIIAEGSAKATDABSTEVILEA  476
V.vinifera1   KLIVVLTRGGTTARLVAKYRAVPILSVVVPLLTTDFDWTCSDBAPARHSLIYRGLIPIIAEGSAKATDABSTEVILEA  475
V.vinifera2   KLIVVLTRGGTTARLVAKYRAVPILSVVVPLLTTDFDWTCSDBAPARHSLIYRGLIPLLGEGSAKATDABSTEVILEA  475
S.tuberosum1  KLIVVLTRGGSTARLVAKYRBAVPILSVVVPVLTTDFDWSISDBTPARHSLVYRGLIPLLGEGSAKATDSBSTEVILEA  475
S.tuberosum2  KLIVVLTRGGSTARLVAKYRBAVPILSVVVPVMTTDFDWSCSDBTPARHSLIYRGLIPMIAEGSAKATDSBATEVILEA  475
A.thaliana1   KLIIVLTRGGSTANLVAKYRBAVPILSVVVPVLTTDFDWTCSDBTPARHSLIYRGLIPIIPMDAEGSAKATDSBATEVILEA  475
R.communis    KLIVVLTRGGTTARLVAKYRBAVPILSVVVPVLTTDFDWTCSDBTPARHSLIYRGLIPIIAEGSAKATDABSTEVILEA  473
C.annuum      NSIVVLTRGGSTARLVAKYRBAVPILSVVVPVLTTDFDWSISDBTPARHSLVYRGLIPIIAEGSAKATDSBSTEVILEA  476
L.longiflorum ALLIVVLTRGGTTARLVAKYRBAVPILSVVVPVLTSDFDWNVSDBSPARHSLIYRGLIPLLAEGSAKATDSBSTEVILEA  475
A.thaliana2   KLIIVVLTRGGTTARLVAKYRBAVPILSVVVPVFTSDFDWISNGCSBLSPARHSLIYRGLIPVLGEGSAKATDSBSTEEIIES  475
Z.mays        ALIVVLTRGGSTARLVAKYRBVPILSVVVPVLTTDFDWTISDBSPARHSLIYRGLIPLIAEGSAKATDSBSTEVILEA  478
P.trichocarpa SIIIVMLTRGGSTARLVAKYRGMPILSVVVPEIQTDFDWSCSDBAPARHSLIFRGLIPVLYAGSAKASHABTTEEALDF  460

E.japonica    ALKSATERGLCTPGDAVVALHRIGVASVIKICVVK  510
C.inensis     ALKSAIEKGLCSPGDAVVALHRIGVASVIKICIVK  510
G.max1        ALKSATQRGLCKPGDAVVALHRIGTASVIKICIVK  510
G.max2        ALKSATQRGLCKPGDAVVALHRIGTASVIKICIVK  511
V.vinifera1   ALKSATGKGLCKPGDAVVVALHRIGVASVIKICIVK  510
V.vinifera2   ALKSATGKGLCKPGDAVVALHRIGVASVIKICIVK  510
S.tuberosum1  ALKSAVTRGLCKPGDAVVALHRIGSASVIKICVVK  510
S.tuberosum2  ALKSAVTRGLCKPGDAVVALHRIGVASVIKICIVLK  510
A.thaliana1   ALKSATQRGLCNRGDAIVALHRIGAASVIKICVVK  510
R.communis    ALKSATERGLCKAGDAVVALHRIGAASVIKICIVK  508
C.annuum      SLKSATEKGLCQPGDAVVALHRIGAASVIKICIVK  511
L.longiflorum ALKSATARRLCKPGDSIVALHRIGVASVIKICVVK  510
A.thaliana2   ALKSATEKGLCNHGDAVVALHRIGVASVIKICVVK  510
Z.mays        ALKSAVQKQLCKPGDAIVALHRIGVASVIKICIVK  513
P.trichocarpa ALQHAKGKGLCRTGDPVVALHRVGTASVIKIINVK  495
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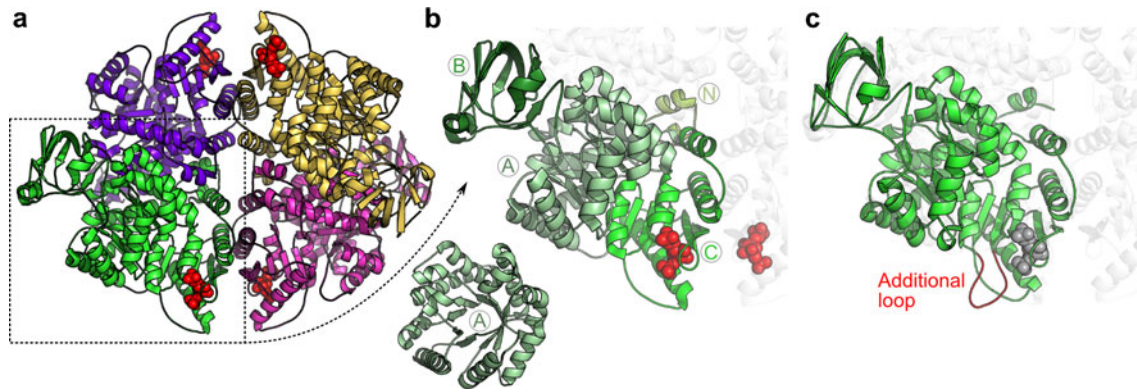
Fig. 2 Structure of human M2 PK and predicted structure of loquat PK. **a** Overall representation of human PK (Protein Data Bank identifier 3me3), which is a homo-tetramer. Each chain is shown using a different *color*, and the fructose-1,6-bisphosphate molecules are shown in *red*. **b** Identification of the four domains (*N*, *A*, *B* and *C*) that constitute each subunit of human M2 PK. *Inset at bottom of figure* α/β-Barrel structure of domain A. **c** Homology model of the loquat

pyruvate kinase (*green*) shown superimposed on M2 PK structural template (*transparent*) (3me3), which was used as the only template. The homology model was generated using Modeller 9v10 (Sali and Blundell 1993). Domain C, to which the allosteric regulator of M2 PK binds, displays significant sequence difference with *EjPK*, possibly indicating a difference in regulatory molecules. A loop of *EjPK* (*red*) is much larger and differs in sequence from that in M2

Fig. 8. TA increased gradually until 135 DAF, and then declined for both cultivars. A similar trend was observed for malic acid, which was the most concentrated organic acid monitored in our study. Oxalacetic acid, quinic acid, and succinic acid, were 10 to 60 times less concentrated than malic acid and their concentration decreased slightly or was constant during fruit development.

Discussion

In this study, a putative loquat PK full-length cDNA called *EjPK* was isolated. Its predicted amino acid sequence shares high similarity with other plant PK sequences, including PKs isolated from *Arabidopsis thaliana*, potato and poplar (Oliver et al. 2008; Zhang et al. 2012a, b). *EjPK* probably

Fig. 3 Phylogenetic tree of some plant PKs. The sequences used to build the tree include those of EjPK and of the PKs whose subcellular localization is known (classified by Oliver et al. 2008). The tree was generated from the amino acid sequences using the maximum likelihood program PhyML (Guindon et al. 2010) with 100 bootstrap replicates and the JTT evolutionary model. Internal nodes supported by >70 replicates are shown. The tree representation was generated using figtree
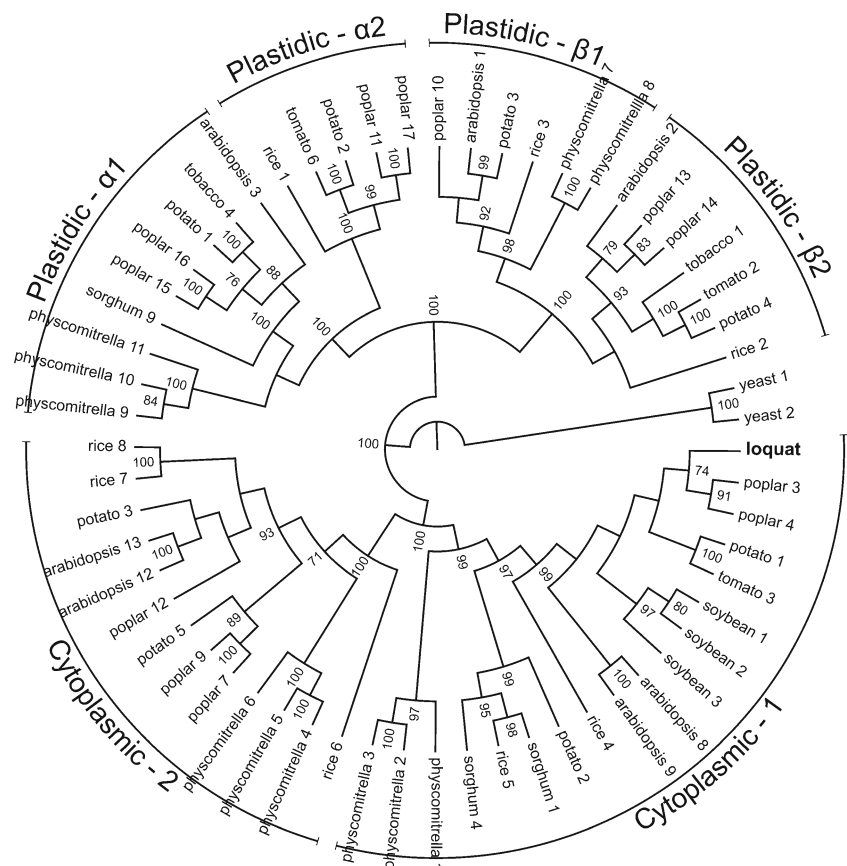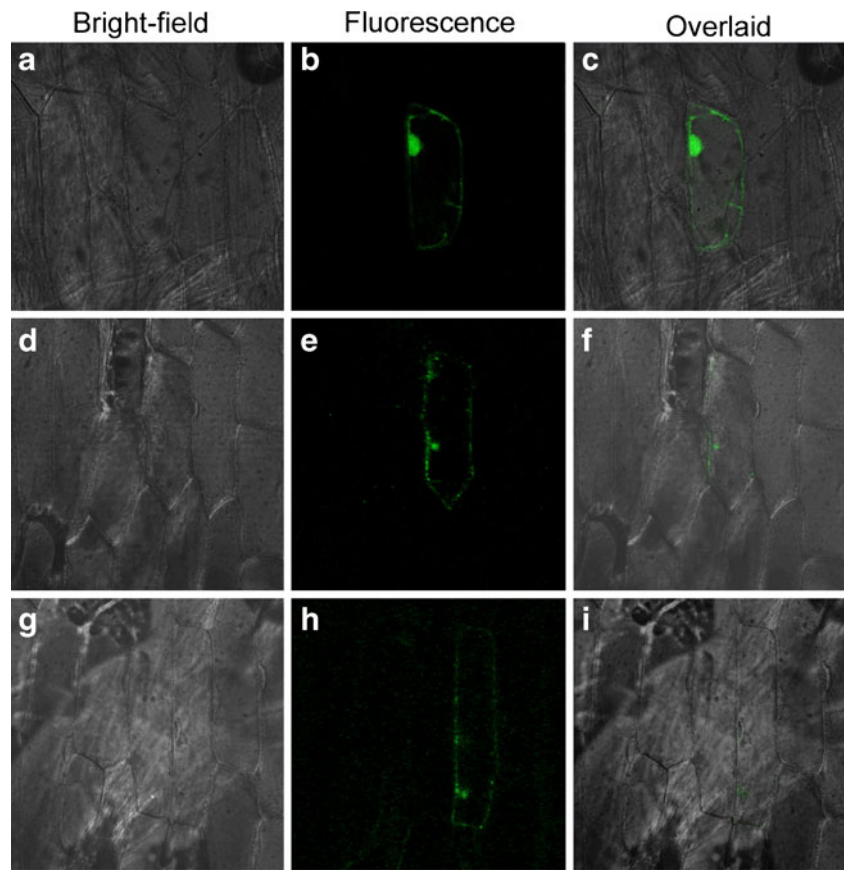
adopts a similar 3D structure to that of other PKs, and the closest PK with known 3D structures share >60 % identity in its core domain with *EjPK* but only 30 % identity for the regulatory domain, which is consistent with a difference of allosteric effectors. Phylogenetic analysis indicates that *EjPK* belongs to the cytosolic-1 subgroup, which includes PKcs from grape, soybean, and citrus. The cytosolic localization of *EjPK* was also predicted using the WoLF PSORT bioinformatics tool, and a 35S:*EjPK*-GFP fusion protein was observed by confocal microscopy to be localized in the cytosol.

EjPK mRNA was found expressed in all examined loquat tissues, including leaves, roots, stem, flowers and fruits. Expression of *EjPK* transcripts was four to five times higher in young leaves than in the mature leaves, roots, stems or flowers, indicating a possible up-regulation in developing tissues. Plants have multiple PKs, which have complex pattern of expression in different tissues and at different development stage (McHugh et al. 1995; Knowles et al. 1998; Plaxton and Podestá 2006; Zhang et al. 2012a, b). In this study, we discovered a remarkable expression pattern of *EjPK* during fruit development. At an initial stage of

Fig. 5 Evolution of *EjPK* expression level in loquat tissues detected by real-time RT-PCR. **a** *EjPK* expression level in fruits at different developmental stages. **b** *EjPK* expression level in young leaves, mature leaves, roots, stem and flowers. Loquat actin was used as an internal reference. Statistics for each data point were based on at least three measurements
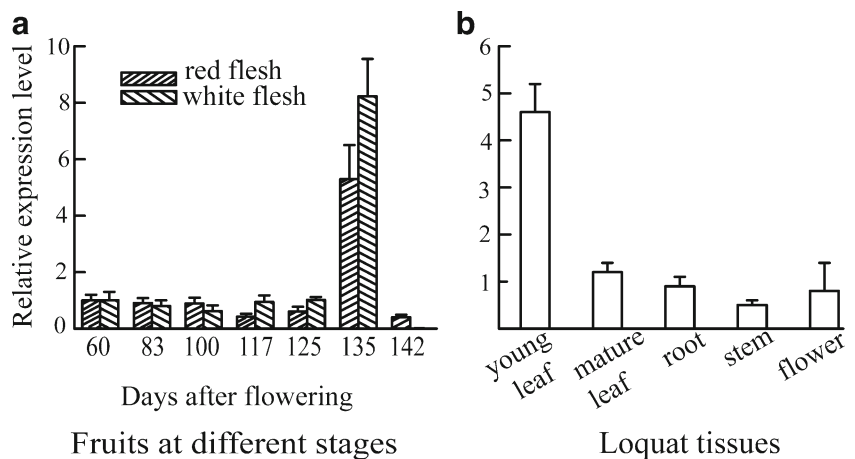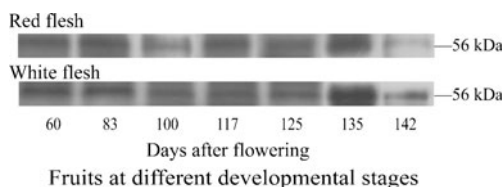
Fig. 6 Western blot of loquat fruit extracts probed with an anti-PKc antibody. Loquat fruits of red and white flesh cultivars were sampled on several days after flowering (DAF) and the level of PKc protein was assessed by western blot analysis. Proteins were extracted and separated on a 10 % SDS-PAGE and transferred to nitrocellulose membranes. Each lane contained 4 μg extracted protein. Immunoblotting was carried out using affinity-purified rabbit antibodies against *Brassica napus* PKc (Smith et al. 2000). The secondary antibody was alkaline phosphatase-conjugated (Bio-Rad, Hercules, CA)

loquat fruit development, *EjPK* was expressed at the same basal level as in mature leaves and roots, but a dramatic five-fold up-regulation was observed at 135 DAF, followed, a week later, by a dramatic drop to a low level of expression. To the best of our knowledge, a similar burst of PKc has never been described previously. We have then established that, after *EjPK* transcript up-regulation, acids stopped accumulating and were consumed, while the level of monosaccharides, including fructose and glucose, increased four to five times.

The concentration of sugars, acids and *EjPK* transcripts displayed pronounced differences between the two cultivars. It has been suggested that increasing levels of fructose in loquat fruits is caused by sorbitol conversion into fructose (Hirai 1980; Bantog et al. 2000; Hasegawa et al. 2010). Bantog et al. (2000) identified an increased concentration of the conversion enzymes SDH and S6PDH during the second stage of maturation, with a level of SDH being constantly higher than S6PDH (Bantog et al. 2000). Our study showed that the sorbitol level remained relatively constant while fructose was accumulating. In the context of agricultural production, it has been suggested that

fructose may be generated from sorbitol after fruit harvest (Hasegawa et al. 2010), and this conversion may therefore have occurred after our measurements stopped. The concentration of sorbitol was slightly higher for the white-flesh cultivar at the end of the measurement period, which could contribute to the higher sugar content of the marketed fruit. Additionally, the fructose content was 10 mg g^{-1} WF higher in fruits of the white-fleshed cultivar at the end of the experiment, whereas the sucrose content was only 1 mg g^{-1} WF higher in the yellow-fleshed cultivar fruit than in the white-fleshed cultivar fruit. TA content was only 0.2 % higher in the white-fleshed cultivar. Interestingly, the burst of transcript of *EjPK* at 135 DAF was noticeably higher for the white-fleshed cultivar than for the yellow-fleshed cultivar.

Using previous studies on loquat fruit metabolism (Chen et al. 2009; Qin et al. 2012), we suggest possible links between *EjPK* transcript levels and acids and sugar concentration. Chen et al. (2009) studied the concentration of acids in loquat fruits during fruit development for both the yellow- and white-fleshed fruit cultivars (Chen et al. 2009). Similarly to this study and others (Ding et al. 1998; Hasegawa et al. 2010), Chen et al. (2009) discovered that the variation in TA was due mainly to malic acid; they also evidenced a correlation between the level of three enzymes: MH, ME and PEPC, with malic acid concentration. PEPC is involved in several metabolic pathways (Wang et al. 2012). The metabolic link between PKc and the three enzymes MH, ME and PEPC is illustrated in Fig. 9a. The evolution of the concentration of the different acids measured by Chen et al. (2009) was similar to our measurements, allowing the combination of their observations with ours to provide a more complete figure of acid and sugar evolution during loquat fruit maturation. At the initial stage, malic acid concentration increases because the PEPC and MH are up-regulated whereas the activity of ME is low (Fig. 9b). At the

Fig. 7 Evolution of fructose, glucose, sucrose and sorbitol content in fruits at various time of their development. White-fleshed and yellow-fleshed varieties are indicated in the graphs. Statistics for each data point were based on at least three measurements. The quantification of sugar is given in milligram per gram fresh weight (FW) and was evaluated by HPLC
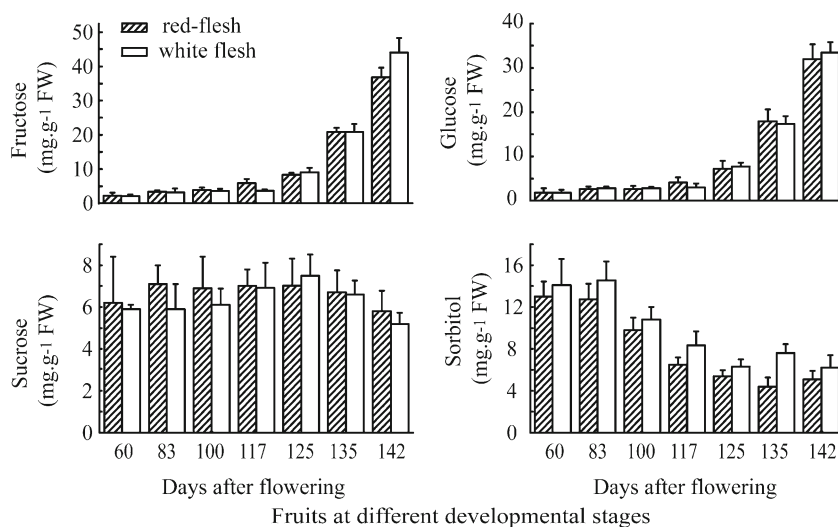
Fig. 8 Evolution of organic acid content in fruits at various times of development. White-fleshed and yellow-fleshed varieties are indicated in the graphs. Statistics for each data point were based on at least three measurements. Titratable acidity (TA) was measured using 0.1 mM NaOH. The quantity of each acid is given in milligram per gram FW and was evaluated by HPLC
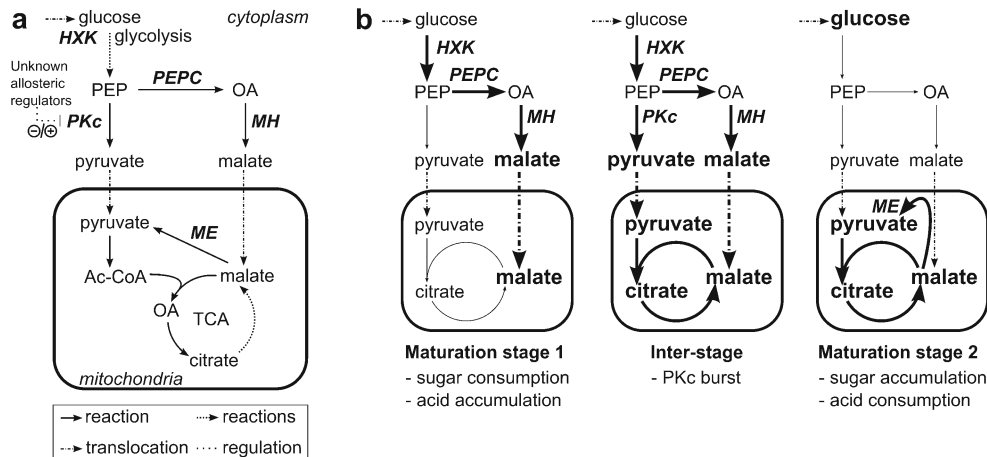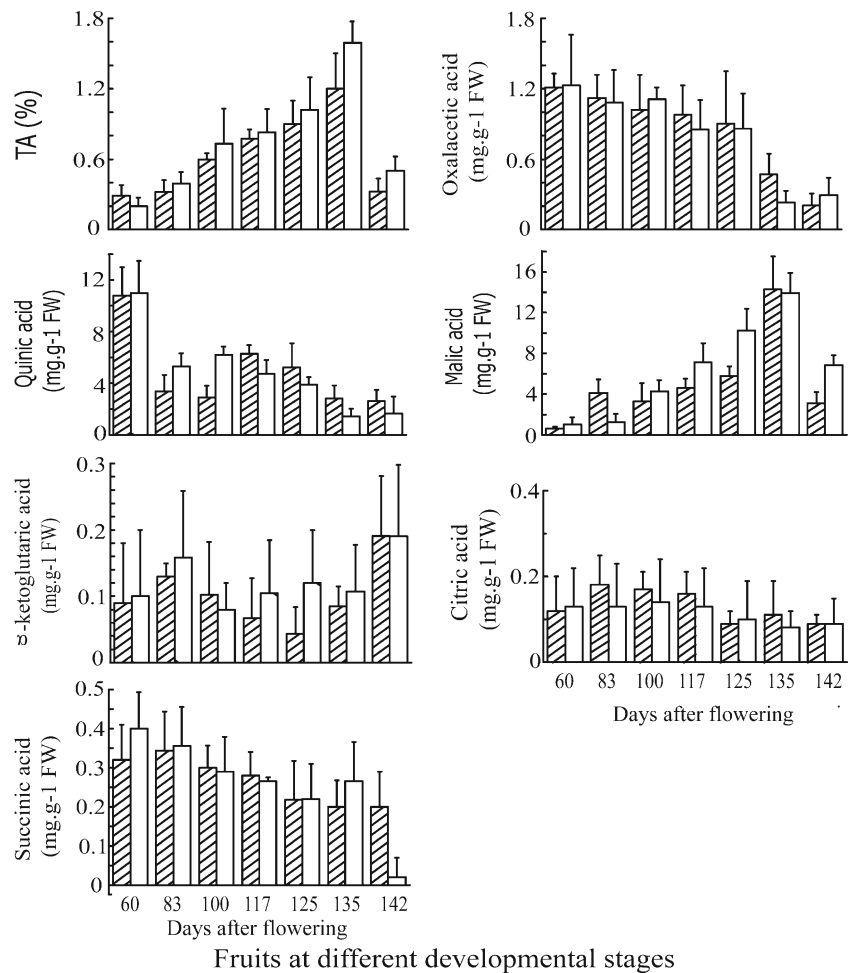

Fruits at different developmental stages

Fig. 9 **a** Sugar and acid metabolic pathways affected during loquat fruit ripening. **b** Potential change of metabolic pathways at different development stage of fruit ripening. *HXK* Hexose kinase, *PEP* phospho-enol-pyruvate, *PEPC* phospho-enol-pyruvate-carboxylase, *OA* oxaloacetate, *ME* malic enzyme, *Ac-CoA* acetyl-coenzyme A, *TCA* tricarboxylic acid cycle. Two development stages of fruit ripening have been identified in previous studies (Chen et al. 2009; Ding et al. 1998; Hasegawa et al. 2010; Qin et al. 2012) and in this study: an initial acid accumulation stage and a later sugar accumulation stage.

The transient burst of *EjPK* discovered in this study occurs at the transition between the two loquat fruit development stages. The influence of unknown allosteric regulators has been represented only for PKc, which is the focus of this study, but the other enzymes on this diagram are also potentially under the influence of post-translational regulators. A figure from Turner and Plaxton (2000) was inspirational to the organization of several elements in panel **a**. In panel **b**, molecules written in *bold font* are, or are likely to be, present in high concentration

maximum of acidity, PKc is suddenly and transiently up-regulated, which might result in an increased consumption of PEP and increased production of pyruvate. Increased levels of translation and expression of PKc is not sufficient to support this hypothesis of increased activity because many type of post-translational regulation exist for PKc. This increased PKc activity would have to be tested to partly validate this working model. In the last stage of maturation, the activities of PEPC and MH decrease and the concentration of ME increases. In this last stage, the malic acid is probably converted into pyruvate by ME and both pyruvate and malic acid feed the TCA, reducing the need to use glycolysis as a carbon source. Indeed, we recently evidenced that a loquat hexokinase, *EjHXK*, was highly expressed in the acid production phase but was repressed in the final acid consumption stage, indicating that glycolysis enzymes are down-regulated (Qin et al. 2012). In the second maturation stage, glucose and fructose increase dramatically. Fructose and glucose are probably synthesized from sucrose, whose concentration remains constant despite being continuously conveyed to the fruit from photosynthetic organs (Patrick and Offler 1996). This hypothesis is supported by the increased concentration of invertases in the second maturation stage (Bantog et al. 1999).

The influence of PKc in fruit maturation is still unclear, but the transient expression of *EjPK* occurring at the same time as the change of metabolic regime observed in loquat fruit indicates a possible link with fruit acid and sugar levels. The regulation of plant PK is still poorly understood (Zhang et al. 2012a, b), and the origin of the burst of PKc is unknown. Nevertheless, the in vitro study of Turner and Plaxton (2000) revealed that some of the allosteric effectors of another PKc present in fruit ripening are glutamate and aspartate but that citrate was a poor inhibitor. Future investigations will focus on discovering the regulatory mechanisms that control the levels of PKc, ME, MH and PEPC enzymes during the two development stages and during the transition period between stages. It is tempting to speculate that the depletion of PEP or an increase level of pyruvate resulting from the sudden burst of PKc might influence the activity of PEPC and MH and that oxaloacetate might negatively regulate ME. Further investigations are carried out in our laboratories to test these hypotheses.

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