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Full Length Research Paper

Expression of six malate-related genes in pulp during the fruit development of two loquat *(Eriobotrya japonica*) cultivars differing in fruit acidity

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Partial cDNAs encoding phospho*enol*pyruvate carboxylase (PEPC), NADP-malic enzyme (NADP-ME), cytosolic NAD-malate dehydrogenase (cyNAD-MDH), mitochondrial NAD-malate dehydrogenase (mNAD-MDH), tonoplast adenosinetriphosphatase subunit A (V-ATPase A) and tonoplast pyrophosphatase (V-PPiase) were isolated from loquat (*Eriobotrya japonica*) pulp. Thereafter, northern analysis was performed to investigate their expression patterns in pulp during the fruit development of low-acid 'Changhong 3' and high-acid 'Jiefangzhong' cultivars. All gene expressions were developmentally regulated in two cultivars. *NADP-ME, mNAD-MDH, V-ATPase A* and *V-PPiase* displayed basically similar expression patterns in both cultivars, but the expression level of *NADP-ME* in 'Jiefangzhong' was higher, while the expression levels of *mNAD-MDH, V-ATPase A* and *V-PPiase* in 'Changhong 3' was higher. The expression patterns of *PEPC* and *cyNAD-MDH* differed between two cultivars. The activities of PEPC, NADP-ME, cyNAD-MDH, mNAD-MDH, V-ATPase and V-PPiase during the fruit development might be regulated at the gene expression and/or the post-transcriptional level. The possible links between gene expression and malate concentration were also discussed.

Key words: *Eriobotrya japonica* (loquat) fruit, NAD-malate dehydrogenase, NADP-malic enzyme, phospho*enol*pyruvate carboxylase, tonoplast adenosinetriphosphatase, tonoplast pyrophosphatase.

INTRODUCTION

Loquat (*Eriobotrya japonica*) is commercially grown in many countries, including China, Japan, Italy, Brazil, Spain, Northern India, etc. High fruit acidity has been a major factor lowering fruit quality and commodity value in commercial loquat production. Malate and quinate are the major organic acids in loquat pulp. In addition, loquat pulp also contains small quantities of isocitrate, α-ketoglutarate, fumarate, oxaloacetate, tartarate, ferulate, *cis*aconitate and β-coumarate. Loquat pulp acidity is determined mainly by malate concentration, because titratable acidity (TA) correlates highly with malate concentration, but does not correlate with quinate concentration (Chen et al., 2008, 2009).

The final malate concentration in ripe fruit is determined by the balance of malate biosynthesis, degradation and vacuolar storage (Chen et al., 2009; Diakou et al., 2000). The major enzymes potentially involved in fruit malate biosynthesis and degradation include phospho*enol pyruvate carboxylase* (PEPC, EC 4.1.1.31), NAD-malate dehydrogenase (NAD-MDH, EC 1.1.1.37) and NADP-malic enzyme (NADP-ME, EC 1.1.1.40) (Chen et al., 2009; Moing et al., 2000). Malate biosynthesis occurs mainly in the cytosol and is catalysed by PEPC and NAD-MDH

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(Moing et al., 2000). The rapid decrease in malate concentration during fruit ripening has usually been attributed to its degradation by cytosolic NADP-ME (Chen et al., 2009, 2010; Hirai, 1982). A study using two loquat cultivars differing in fruit acidity demonstrated that, NAD-MDH, NADP-ME and PEPC might play significant roles in malate biosynthesis and degradation (Chen et al., 2009). Recent work with 'Jiefangzhong' loquat showed that ethychlozate was very effective in reducing pulp malate concentration, thus, decreasing acidity. The reduction in malate concentration in the ethychlozate-treated pulp was probably caused by decreased malate biosynthesis, as indicated by decreased PEPC and NAD-MDH activities and increased malate degradation, as indicated by increased NADP-ME activity (Chen et al., 2010). Zhao et al. (2007) observed that, the concentration of malate in nectarine (*Prunus persica*) fruit correlated positively with NAD-MDH activity and correlated negatively with NADP-ME activity. By investigating enzyme activities of PEPC and NADP-ME during the fruit development of two apple (*Malus domestica*) genotypes differing in fruit acidity, Yao et al. (2009) proposed that PEPC contributed positively to malate accumulation, while NADP-ME did negatively. However, the changes in the activities of PEPC, NAD-MDH and ME did not parallel the changes in the concentration of malate through the pinea- pple (*Ananas comosus*) fruit development (Saradhuldhat and Paull, 2007). Similar results have been obtained in peach (*P. persica*) fruit (Moing et al. 1998, 2000). The expression of cytosolic NAD-MDH **(**cyNAD-MDH) gene in peach fruit did not correlate with malate changes (Etienne et al., 2002).

Although, the exact mechanism of how organic acids cross the tonoplast and are translocated into vacuoles has not been fully understood, tonoplast adenosinetriphosphatase (V-ATPase, EC 3.6.1.3) and tonoplast pyrophosphatase (V-PPiase, EC 3.6.1.1) are believed to play a predominant role in the storage of organic acids in the vacuole by providing driving force for their transport (Sweetman et al., 2009; Terrier et al., 2001). Echevarria et al. (1997) have shown that, a nonacid sweet lime (*Citrus limmetioides*) has a limited *in vivo* capacity for H^+ retention in the vacuole of juice cell, as compared to the more acidic acid lime (*Citrus aurantifolia*). In peach fruit, *PRUpe; AtpvA1* (V-ATPase gene) and *PRUpe; Vp2* (V-PPiase gene) showed a biphasic pattern of transcript accumu- lation consistent with the pattern of malate and citrate accumulation (Etienne et al., 2002). Yao et al. (2009) found that in low-acid apple genotype, *V-ATPase submit A* (*V-ATPase A*) had the highest expression level in 60-days after flowering (DAF) fruit, which also had the highest V-ATPase activity and malate concentration, while the expression level of *V-ATPase A*, the activity of V-ATPase and the concentration of malate in high-acid apple genotype were higher in the ripe fruit than others. In Japanese pear (*Pyrus serotina*) fruit, V-ATPase and V-PPiase activities expressed on a protein basis increased as fruit developed, whereas the ratio of V-PPiase activity to V-ATPase decreased. The activity of V-PPiase was higher than that of V-ATPase through the developmental period. The changes in the levels of polypeptides and mRNA for the catalytic submit of the two enzymes generally paralleled the enzymatic activities except for mRNA level at the cell division stage (Suzuki et al., 2000). In grape (*Vitis vinifera*) berry, the hydrolytic activities of V-ATPase and V-PPiase expressed per berry increased through the development with an acceleration during ripening, as confirmed by analysis of transcript levels and western blotting, whereas, the ratio of V-PPiase activity to V-ATPase activity increased during ripening (Terrier et al., 2001). To date, tonoplasts isolated from all plant species contain both V-ATPase and V-PPiase, with the exception of lemon (*Citrus limon*) where the PPiase is absent (Echevarria et al., 1997; Müller et al., 1996). This absence agrees with the incapacity of the V-PPiase to acidify the vacuolar lumen beyond pH 3.7 (Terrier et al., 2001), since the vacuolar pH in lemon fruit can reach as low as 2.2 (Müller et al., 1996). Previous studies showed that V-PPiase played an important role in the energisation of immature stage and/or mature fruit tonoplast (Terrier et al., 1998). Taken together, it can be speculated that the increase in vacuolar pH during fruit ripening may be due to the replacement of the V-ATPase by V-PPiase.

In this study, we reported for the first time the cloning and characterization of partial cDNAs encoding PEPC, NADP- ME, cyNAD-MDH, mitochondrial NAD-MDH (mNAD-MDH), V-ATPase A and V-PPiase from loquat pulp and presented a comparative study of their expression in pulp during the fruit development of two loquat cultivars differing in fruit acidity. The objective of this study is to elucidate the expression patterns of the six genes during the fruit development and the possible links between gene expression and malate concentration in loquat pulp.

MATERIALS AND METHODS

Plant materials

Two loquat (*Eriobotrya japonica* Lindl.) cultivars, low-acid 'Changhong 3' and high-acid 'Jiefangzhong', were used in this study. All the samples were collected from 18-year-old trees grown in the Loquat Germplasm Repository in the Fujian Fruit Research Institute, Fujian Academy of Agricultural Sciences, Fuzhou, People's Republic of China. The loquat trees were grown at a spacing of 3×4 m and received standard horticultural practice, and disease and insect control.

To measure fruit growth, 6 pieces of fruits for each treatment (cultivar) were collected at midday on a clear day from 50 to 145 DAF, when fruit were ripe. The fresh weight of each fruit was determined. To measure malate and extract RNA, thirty pieces of fruits for each treatment (cultivar) were collected at midday on a clear day from 50 to 145 DAF, when fruit were ripe. Fruits were

Table 1. Degenerate primers used for isolating cDNAs encoding phospho*enol*pyruvate carboxylase (PEPC), NADP-malic enzyme (NADP-ME), cytosolic NAD-malate dehydrogenase (cyNAD-MDH), mitochondrial NAD-malate dehydrogenase (mNAD-MDH), tonoplast adenosinetriphosphatase subunit A (V-ATPase A) and tonoplast pyrophosphatase (V-PPiase) from loquat pulp.

Enzyme	Forward primers (5′→3′)	Reverse primers (5′→3′)
PEPC	GTGGTACAGTTGGAAGAGGAGG	GTGATATAGGAATCACGCAGCC
NADP-ME	TGGGGATACCTGTAGGGAAACT	TTGTGAGGTTGGGTTGGAGA
cyNAD-MDH	AGTTCGCGTCCTTGTTACTG	CCCAGAGCCCTGTTGTGAT
mNAD-MDH	AATATGATAAGCAACCCAGT	ACTCCATTTTTCCCAAGTCT
V-ATPase A	CTGTAGTTTATGTTGGTTGTGGAG	CGGACTTGTAGAATGGGCAGAAC
V-PPiase	CCTATTTGCCACTGATTTCTTTG	CAGCACTTCCCACACTCTTCA

carefully dissected into pulp, skin and seeds. Then, pulp was immediately frozen in liquid nitrogen and stored at -80°C until used.

Extraction and determination of malate

Malate was extracted according to Chen et al. (2002) with some modifications. About 0.3 frozen pulp was ground in liquid nitrogen with a pestle and mortar, 1.5 ml of ice-cold 4% (v/v) $HClO_4$ was added to the powder and gently pulverized. The mixture was allowed to thaw slowly on ice. The resulting suspension was kept on ice for 30 min then, centrifuged at 20,000 *g* for 10 min at 4°C. One point 2 ml of supernatant was neutralized at 4°C with 5 M K_2CO_3 (approx. 80 µl) and the resulting $KClO_3$ was removed by 10 min centrifugation at 20,000 *g* and 4°C. 12 mg of charcoal (activated, washed with HCl) was added to the supernatant and after 15 min at 4°C, removed again by 10 min centrifugation at 20,000 *g* and 4°C. The supernatant was used for malate assay.

Malate was assayed according to Chen et al. (2002). One ml of reaction mixture contained 50 mM 3-amino-1-propanol-HCl (pH 10), 30 mM glutamate-Na-NaOH (pH 10), 2.7 mM NAD, 1 unit glutamate-oxaloacetate transamimase (GOT, EC 2.6.1.1), 10 unit NAD-MDH and extract.

RNA extraction, cDNA preparation and isolation of partial cDNAs

Total RNA was extracted from loquat pulp using hot borate method (Wan and Wilkins, 1994). 2 µg of total RNA was used for first-strand cDNA synthesis using the AMV RT/PCR kit (Shanghai Shenergy Biocolor Bioscience and Technology Company, Shanghai, People's Republic of China) following the manufacturer's instructions. 2 µl of each resulting cDNA solution was used as a template in a 50 µl polymerase chain reaction (PCR) reaction mixture to amplify partial cDNA using a Tpersonal PCR thermocycler (Biometra, Goettingen, Germany). To isolate cDNAs encoding PEPC, NADP-ME, cyNAD-MDH, mNAD-MDH, V-ATPase A, or V-PPiase, degenerate primers were designed accordingly to conserved regions in the corresponding protein in other plant species. The sequences of the forward (F) and reverse (R) primers used are given in Table 1. The PCR programme used involved an initial denaturation step at 94°C for 2 min, followed by 30 cycles of 94°C for 50 s, 51 to 61°C (depending upon the primers) for 50 s, 72°C for 90 s and a final extension step at 72°C for 10 min. All PCR products were separated by electrophoresis on a 1% (w/v) agarose gel containing ethidium bromide.

Northern analysis

Total RNA was extracted from loquat pulp at various developmental stages using hot borate method (Wan and Wilkins, 1994). Fifteen µg of total RNA was denatured in formaldehyde, separated by electrophoresis on a 1% (w/v) denatured agarose gel containing ethidium bromide, transferred to a Hybond-N^+ membrane (Amersham Pharmacia Biotech, UK) by capillary action. The membrane was hybridized with ^{32}P-labeled probes, which were prepared from the partial cDNAs of PEPC, NADP-ME, cyNAD-MDH, mNAD-MDH, V-ATPase A or V-PPiase isolated in this study, in a commercial high- performance hybridization solution (Innogent Biotech, Shenzhen, P.R. China) following the manufacturer's instructions. Northern analysis was conducted with three replicates using separate three batches of total RNA extracted independently from loquat pulp at various developmental stages.

Statistical analysis

Experiments were performed with 3 or 6 replicates. Results are presented as means ± SD.

RESULTS

Fruit growth

Increase in fruit fresh weight for 'Changhong 3' and 'Jiefangzhong' displayed a typical single sigmoid growth curve, two periods of slow growth being separated by a period of rapid growth. Fruit fresh weight did not differ between the both cultivars until reaching the rapid growth stage, while at the later stages of fruit development, fruit fresh weight in 'Jiefangzhong' was much higher than that in 'Changhong 3' (Figure 1).

Changes in malate concentration during fruit development

Malate concentration in 'Changhong 3' and 'Jiefangzhong' increased during the early stages of fruit development and decreased at the later stages. At the later stages of fruit development, malate decrease was larger in 'Changhong 3' than that in 'Jiefangzhong' and thus, the former had a lower malate level (Figure 2).

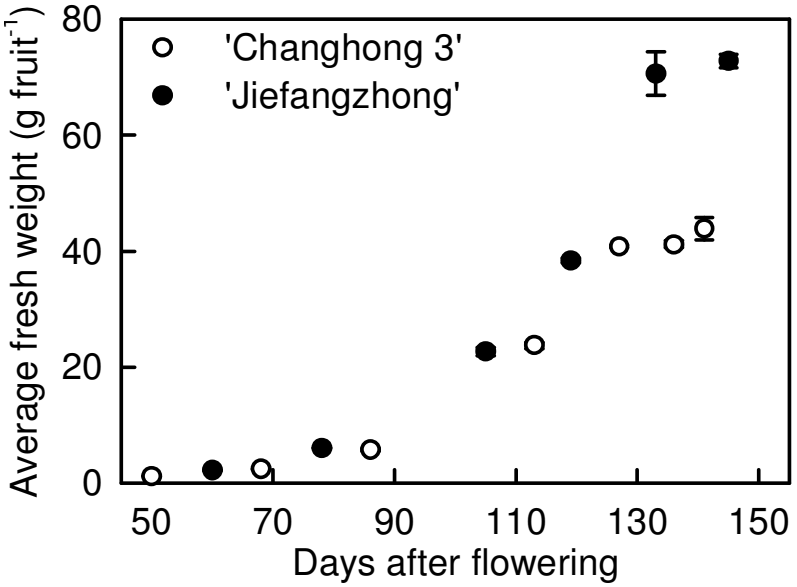

Figure 1. Fruit fresh weight as a function of days after flowering (DAF) for 'Changhong 3' and 'Jiefangzhong' loquat. Each point represents mean ± SD of six replicates.

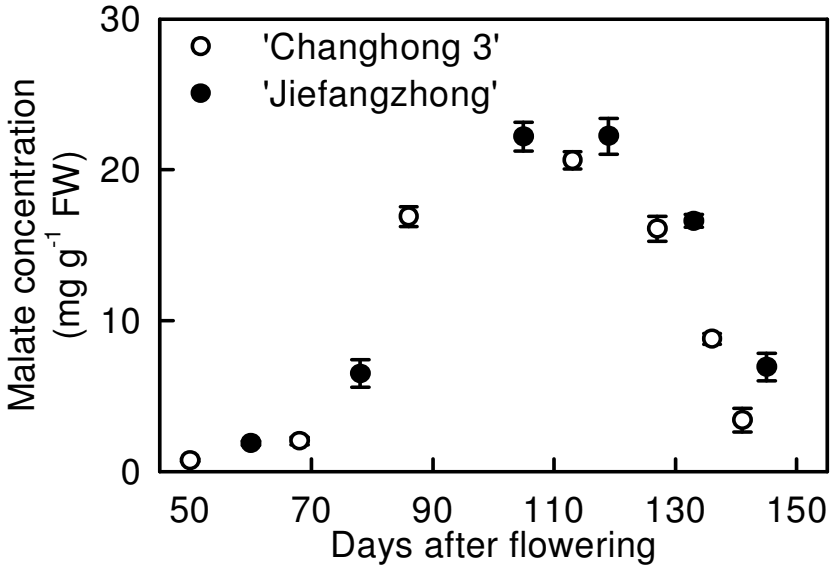

Figure 2. Changes in malate concentration in the pulp during the fruit development of 'Changhong 3' and 'Jiefangzhong' loquat. Each point represents mean ± SD of six replicates.

Isolation of partial cDNAs encoding PEPC, NADP-ME, cyNAD-MDH, mNAD-MDH, V-ATPase A and V-PPiase

Partial cDNAs encoding PEPC, NADP-ME, cyNAD-MDH, mNAD-MDH, V-ATPase A and V-PPiase were isolated from loquat pulp using reverse transcriptase (RT)-PCR. Characteristics of the partial cDNAs isolated are presented in Table 2. The deduced amino acid sequences of the six cDNA fragments were aligned with their known counterparts in other higher plant species using the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) software (http://www.ncbi. nlm.nih.gov/) and verified that, they encoded enzymes PEPC, NADP-ME, cyNAD-MDH,

Table 2. Characteristics of partial cDNAs encoding phospho*enol*pyruvate carboxylase (PEPC), NADP-malic enzyme (NADP-ME), cytosolic NAD-malate dehydrogenase (cyNAD-MDH), mitochondrial NAD-malate dehydrogenase (mNAD-MDH), tonoplast adenosinetriphosphatase subunit A (V-ATPase A) and tonoplast pyrophosphatase (V-PPiase) isolated from loquat pulp.

Enzyme	Accession no.	cDNA fragment size (bp)	Amino acid sequences with highest identity		
			Origin	Accession no.	Identity (%)
PEPC	EF523436	772	*Malus domestica*	EU315246.1	97
NADP-ME	EF523435	743	*M. domestica*	DQ280492.1	96
cyNAD-MDH	EF666048	483	*M. domestica*	DQ221207.1	98
mNAD-MDH	EU268881	485	*Arabidopsis lyrata*	XM_002894384.1	90
V-ATPase A	EF666049	804	*Pyrus communis*	AB189963.1	100
V-PPiase	EF666050	776	*P. communis*	AB097115.1	99

mNAD-MDH, V-ATPase A and V-PPiase, which shared 87 to 97%, 80 to 96%, 78 to 98%, 86 to 90%, 94 to 100% and 79 to 99% identity, respectively, with the corresponding protein sequences in other plant species.

Expression analysis of the genes encoding PEPC, NADP-ME, cyNAD-MDH, mNAD-MDH, V-ATPase A and V-PPiase during fruit development

Figure 3 shows the expression patterns of PEPC, NADP-ME, cyNAD-MDH, mNAD-MDH, V-ATPase A and V-PPiase in loquat pulp during fruit development. PEPC expression in 'Changhong 3' increased from 50 to 113 DAF, then, decreased as fruit developed, whose level was very weak when fruit was ripe. In 'Jiefangzhong', PEPC expression decreased at the early stages of fruit development and increased at the later stages except a slight decrease at the last sampling date.

NADP-ME expression increased from the first to the second sampling date, decreased from the second to the third sampling date and then, increased as the fruit developed except for a decrease in 'Changhong 3' at the last sampling date. The expression level of NADP-ME in 'Jiefangzhong' was somewhat higher than that in 'Changhong 3'.

The expression of cyNAD-MDH in 'Changhong 3' decreased from 50 to 68 DAF then, increased through the rest of the sampling dates. This gene's expression in 'Jiefangzhong' increased from 60 to 78 DAF, decreased from 78 to 105 DAF then, increased with fruit development except for a slight decrease at the last sampling date.

The expression of mNAD-MDH in both cultivars decreased at the early stages of fruit development and increased at the later stages except for a decrease in 'Jiefangzhong' at 145 DAF. The expression level of mNAD-MDH in 'Changhong 3' was higher than that in 'Jiefangzhong'. The expression patterns of V-ATPase A and V-PPiase in both cultivars were basically similar. Their expression decreased at the early stages of fruit development and increased at the later stages except for a slight decrease in 'Changhong 3' at 141 DAF. Their expression levels in 'Changhong 3' were higher than those in 'Jiefangzhong'.

DISCUSSION

The concentration of malate in the pulp of 'Changhong 3' and 'Jiefangzhong' increased at the early stages of fruit development and decreased at the later stages (Figure 2), as previously found for loquat (Amorós et al., 2003; Chen et al., 2009), apple (Zhang et al., 2010), peach (Etienne et al., 2002; Zhao et al., 2007) and grape (Famiani et al., 2000; Terrier et al. 2001). At the later stages, the loss of malate due to metabolic degradation and/or decreased biosynthesis was larger in 'Changhong 3' than that in 'Jiefangzhong' and the concentration of malate was lower in the former (Figure 2). The lower TA in the ripe pulp of 'Changhong 3' (Chen et al., 2008, 2009) may be related to the fact that it contains a lower concentration of malate, because loquat pulp TA correlates highly with malate concentration (Chen et al., 2009). It is worth mentioning that, the lower concentration of malate in the ripe pulp of 'Changhong 3' might not result from less dilution due to fruit growth, since the increase in fruit fresh weight for 'Jiefangzhong' was higher than that for 'Changhong 3' at the later stages of fruit development (Figure 1).

Our results showed that, the developmental changes in mRNA level of PEPC in 'Changhong 3' (Figure 3) correlated well with the changes in the activity of PEPC (Chen et al., 2009) and the concentration of malate (Figure 2), meaning that the control of malate accumulation and PEPC activity during the fruit development of 'Changhong 3' loquat might involve the developmental regulation of PEPC expression. Similar results have been obtained for apple fruit (Yao et al., 2009) and grape berry (Or et al., 2000). However, the expression of PEPC in 'Jiefangzhong' (Figure 3) displayed

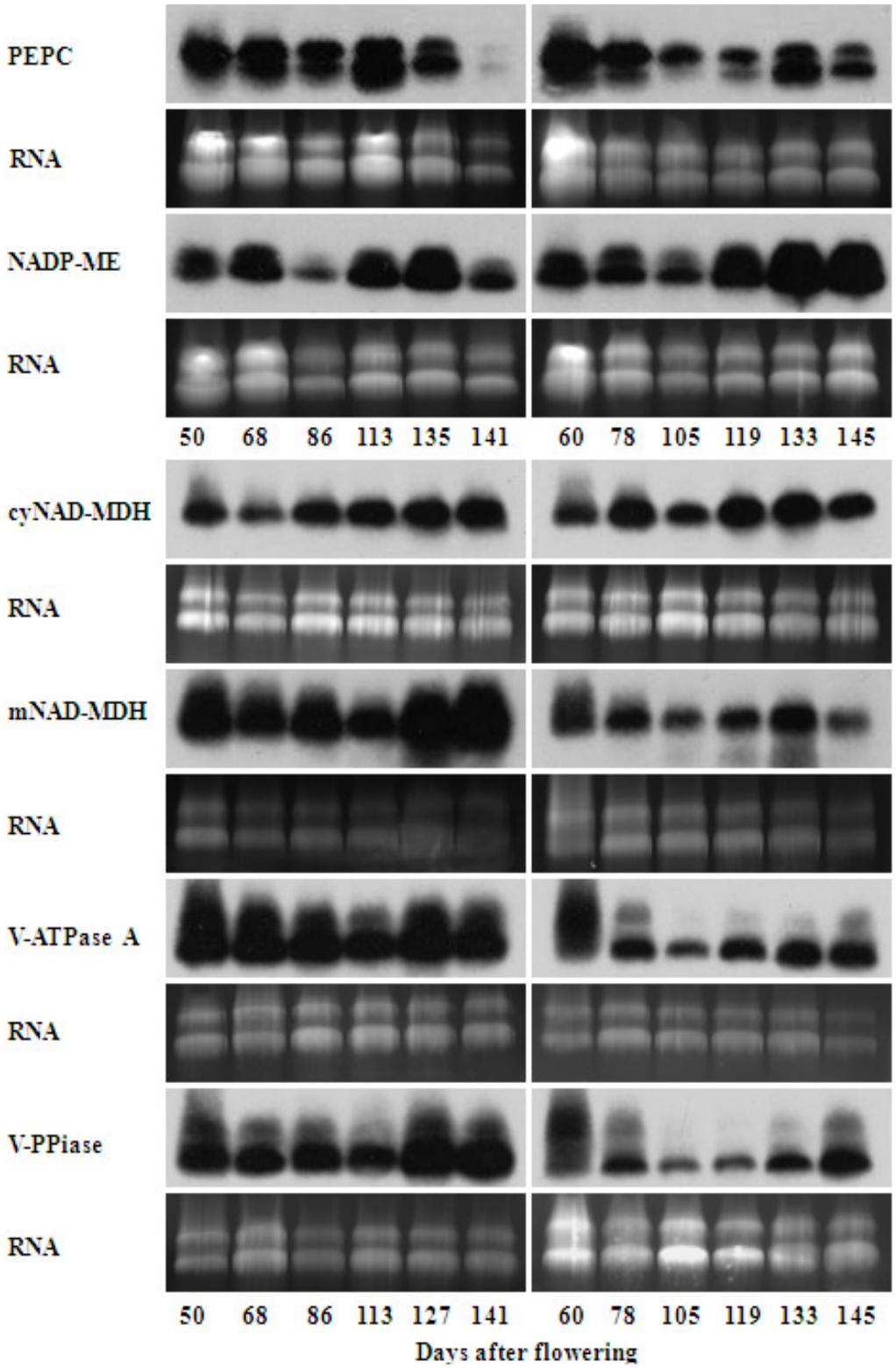

Figure 3. Northern analysis of phospho*enol*pyruvate carboxylase (PEPC), NADP-malic enzyme (NADP-ME), cytosolic NAD-malate dehydrogenase (cyNAD-MDH), mitochondrial NAD-malate dehydrogenase (mNAD-MDH), tonoplast adenosinetriphosphatase subunit A (V-ATPase A) and tonoplast pyrophosphatase (V-PPiase) genes in the pulp during the fruit development of 'Changhong 3' (Left) and "Jiefangzhong' (Right) loquat. Each lane was loaded with 15 µg of denatured total RNA.

a quite different way with the activity of PEPC (Chen et al., 2009) during fruit development. The discrepancy between PEPC expression and PEPC activity might result from post-transcriptional regulation of PEPC.

Taureilles-Saurel et al. (1995a) showed that cyNAD-MDH accounted for 70 to 80% of total NAD-MDH activity in grape fruit. Similar ratio of cyNAD-MDH to total activity was observed in both sweet lemon (*C. limettioides*) and sour lemon (*C. limon*) fruits (Abou-Zamzama and Wallacea, 1970). Thus, it is likely that cyNAD-MDH activity constitutes the major part of NAD-MDH activity in loquat pulp. Recent work from our laboratory showed that, the concentration of malate in pulp of 'Changhong 3' and 'Jiefangzhong' increased linearly with increasing total activity of NAD-MDH (Chen et al., 2009) and that spraying ethychlozate was very effective in reducing the total activity of NAD-MDH in pulp of 'Jiefangzhong' thus, decreasing malate concentration (Chen et al., 2010). These findings suggest that cyNAD-MDH may play important roles in malate biosynthesis. Northern analysis showed that, the developmental changes in the expression level of cyNAD-MDH in both cultivars (Figure 3) did not parallel with the changes in enzyme activity (Chen et al., 2009) and malate concentration (Figure 2). Similar results have been obtained in fruit mesocarp of two peach cultivars, normal-acid 'Fantasia' and low-acid 'Jalousia' (Etienne et al., 2002) and for 'Cabernet Sauvignon' grape berry (Deluc et al., 2007). The discrepancy between cyNAD-MDH expression and enzyme activity may by explained by regulation at post-transcriptional level.

Our finding that the transcript level of mNAD-MDH was higher in the young fruits of both cultivars at the first sampling date, declined at the early stages of fruit development of which malate accumulated and increased at the later stages except for a decrease in 'Jiefangzhong' at the last sampling date of which malate declined, (Figure 3); this agrees with the results obtained for grape berry (Or et al., 2000). A similar expression pattern was also observed during the fruit development of strawberry (*Fragaria ananassa*), but malate concentration increased as fruit developed (Iannetta et al., 2004). It has been suggested that, mNAD-MDA might play a role in pre-veraison respiration of grape berries (Sweetman et al., 2009). A high level of mNAD-MDH expression at the early stages of loquat fruit development may be due to the high rate of respiration observed in the young fruit (Amorós et al., 2003). It was proposed that, mNAD-MDH could be involved in malate degradation during fruit ripening (Or et al., 2000; Taureilles-Saurel et al., 1995b). Thus, the observed higher mRNA level of mNAD-MDH in the low-acid 'Changhong 3' at the later stages of fruit development, when compared with the high-acid 'Jiefangzhong' (Figure 3) might be one cause as to why the concentration of malate in the ripe pulp of 'Changhong 3' was lower.

The developmental changes in the mRNA level of NADP-ME (Figure 3) and the enzyme activity (Chen et al., 2009) in 'Changhong 3' and 'Jiefangzhong' loquat followed a similar pattern, but the expression level of NADP-ME in 'Jiefangzhong' was somewhat higher than that in 'Changhong 3' through the fruit development (Figure 3), while this enzyme activity in 'Changhong 3' was higher at the later stages (Chen et al., 2009). These findings indicated that, the activity of NADP-ME during the fruit development of 'Changhong 3' might be regulated at the post-transcriptional level. Yao et al. (2009) proposed that NADP-ME activity was regulated by other genes or at post-translational level.

Northern analysis showed that, the mRNA levels of V-ATPase A and V-PPiase in both cultivars decreased at the early stages of fruit development and increased at the later stages (Figure 3), which is in agreement with the results obtained for Japanese pear fruit (Suzuki et al., 2000). Similar results have been obtained for *PRUpe; AtpA1* and *PRUpe; Vp1* (V-PPiase gene) of 'Jalousia' peach fruit, but the transcript of *PRUpe; Vp2* generally increased as fruit developed. In 'Fantasia' peach, *PRUpe; AtpA1* (*PRUpe; Vp1*) expression generally increased (decreased) as fruit developed, while the transcript of *PRUpe; Vp2* increased from 20 to 45 DAF, decreased from 45 to 102 DAF and then, increased at the last sampling date (Etienne et al., 2002). Yao et al. (2009) showed that, V-ATPase A expression in the low acid 'Fuji' apple increased from 30 to 60 DAF then, decreased as fruit developed, while the high-acid 'Fuji' apple had the highest expression level at the last sampling date. In European pear (*Pyrus communis*) and grape, V-PPiase expression increased during fruit development (Suzuki et al., 1999; Terrier et al., 2001). In *Citrus unshiu*, the mRNA level of V-ATPase A increased in both the edible parts and the peel during growth and maturity (Takanokura et al., 1998). As mentioned earlier, the mRNA levels of V-ATPase A and V-PPiase in most fruits increased during ripening, whether their levels increased at the early stages or decreased.

Previous studies with apple, grape and pear showed that the changes in the expression levels of V-ATPase A and V-PPiase matched well with the enzymatic activities at most stages of fruit development (Suzuki et al., 2000; Terrier et al., 2001; Yao et al., 2009). Therefore, it is likely that the activities of V-ATPase and V-PPiase in 'Changhong 3' and 'Jiefangzhong' increased at the later stages of fruit development. The fall in malate concentration in loquat pulp may not be due to a progressive impairment of H^+-pumping. Previous study with grape showed that, the increase of V-ATPase and V-PPiase activities during the berry ripening might be necessary to compensate for the proton leakage, as the passive permeability ('leakiness') of the tonoplast to protons also increased through the ripening (Terrier et al., 2001). Echevarria et al. (1997) observed that, the tonoplast vesicles isolated from acid lime

fruits had a lower passive permeability than those from sweet lime. Therefore, we concluded that the low-acid 'Chang- hong 3' might had a higher tonoplast leakage than the high-acid 'Jiefangzhong', as the transcript levels of V-ATPase A and V-PPiase were higher in the former. The difference in malate concentration may result from a difference in tonoplast leakage between the two cultivars. Etienne et al. (2002) observed that at the later stages of peach fruit development, the relative units (normalized against rRNA) of *PRUpe; AtpA1* in normal-acid cultivar 'Fantasia' were higher than those in low-acid cultivar 'Jalousia', whereas, the relative units of *PRUpe; Vp1* and *Vp2* in the former were lower, supporting the speculation that the increase in vacuolar pH during ripening may be due to the replacement of V-ATPase by V-PPiase. The patterns of malate accumulation in 'Changhong 3' and 'Jiefangzhong' through the fruit development could be explained in this way, since the changes in the ratio of V-ATPase A mRNA to PPiase mRNA in the two cultivars corresponded with the changes in malate concentration (data not shown). However, the difference in malate con- centration between the two cultivars during ripening could not be explained in this way, since the ratio in 'Jiefang- zhong' was not higher than that in 'Changhong 3' (data not shown).

Conclusions

In this study, we isolated for the first time partial cDNAs encoding PEPC, NADP-ME, cyNAD-MDH, mNAD-MDH, V-ATPase A and V-PPiase from loquat pulp. The expression of all six genes was developmentally regulated in low-acid ('Changhong 3') and high-acid ('Jiefangzhong') cultivars. NADP-ME, mNAD-MDH, V-ATPase A and V-PPiase displayed basically similar expression patterns in both cultivars, but the expression level of NADP-ME in high-acid cultivar was somewhat higher than that in low-acid one, while the expression levels of mNAD-MDH, V-ATPase A and V-PPiase were higher in low-acid cultivar. In contrast, the expression patterns of PEPC and cyNAD-MDH differed between low-acid and high-acid cultivars. The activities of PEPC, NADP-ME, cyNAD-MDH, mNAD-MDH, V-ATPase A and V-PPiase during the fruit development might be regulated at the gene expression and/or the post-transcript level.

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Abbreviations

PEPC, phospho*enol*pyruvate carboxylase; **NAD-MDH,** nicotinamide adenine dinucleotide -malate dehydrogenase; **NADP-ME,** nicotinamide-adenine dinucleotide phosphate -malic enzyme; **V-ATPase,** tonoplast adenosinetriphosphatase; **V-PPiase,** tonoplast pyrophosphatase; **TA,** titratable acidity; **DAF,** days after flowering; **mNAD-MDH,** mitochondrial nicotinamide adenine dinucleotide -malate dehydrogenase; **PCR,** polymerase chain reaction.

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