



Genome identification and analysis of genes encoding the key enzymes involved in organic acid biosynthesis pathway in apple, grape, and sweet orange

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ABSTRACT

Fruit taste depends on the amounts and types of soluble sugars and organic acids in the fruit. However, a large-scale investigation to identify and compare the genes involved in organic acid biosynthesis across multiple plants has not yet been undertaken. To address this void, we have identified some key enzymes involved in organic acid biosynthetic pathways by comparing the gene copy numbers and related-ESTs (expressed sequence tags) in different fruits. The results show that malic acid is mainly synthesized in mitochondria. Therefore, the malic acid that participates in citric acid biosynthesis, which occurs in glyoxysomes, is transported from the cytoplasm to glyoxysomes to participate in that pathway. The different expression levels of *malate dehydrogenase (MDH)*, *fumarase (FUM)*, *citrate synthase (CS)*, *succinate dehydrogenase (SDH)*, *succinate thiokinase (SAT)*, *NAD-isocitrate dehydrogenase (NAD-ICDH)*, *aconitase (ACO)*, *GDP-D-mannose-3, 5-epimerase (GME)*, *GDP-L-galactose pyrophosphatase (GGalPP)* and *L-galactose dehydrogenase (GalDH)* between apples, grapes, and sweet oranges caused differences in the fruits' predominant organic acids. **MDH**, **FUM**, and **CS** enzymes were found to be the key enzymes in the malic acid and citric acid biosynthetic pathways, while **SDH**, **SAT**, **NAD-ICDH**, and **ACO** enzymes may play key roles in the malic acid–citric acid cycle, whereby citric acid is decomposed and malic acid is created. **GME**, **GGalPP**, and **GalDH** enzymes may play key roles in the Smirnoff–Wheeler pathway for L-ascorbic acid biosynthesis. Because we determined that **L-landH** does not function in converting L-ascorbic acid to L-tartaric acid, the key enzyme involved in that pathway is still unknown. Overall, this study provides a new and quick way to compare the key enzymes involved in specific biosynthetic pathways for many species using publicly available sequence information.

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Fruit ripening is a complex, genetically programmed process that culminates in dramatic changes in the color, texture, flavor, and aroma of a fruit's flesh (Alexander and Grierson, 2002). Soluble sugars and organic acids are important components of fruit taste, and aroma impacts on the overall organoleptic quality of fruit. The taste of fruit depends on the amounts and types of soluble sugars and organic acids harbored by the fruit (Pangborn, 1963). In most fleshy fruits, the main soluble sugars are sucrose (Suc), fructose (Fru), and glucose (Glu), and the main organic acids are malic acid and citric acids, but sorbitol and quinic acid have also been detected at low levels (Moing et al., 1998). Organic acids usually accumulate

in the early stages of fruit development and are used as respiratory substrates during fruit ripening (Diakou et al., 2000). The predominant organic acid in a fruit is different for different fruit species. For example, citrus fruits, strawberries, mangoes, and cranberries contain high levels of citric acid (Sadka et al., 2000; Kallio et al., 2000; Gil et al., 2000; Çelik et al., 2008), while apples, loquats, peaches and grapes have high levels of malic acid (Moing et al., 1998; Chen et al., 2009; Or et al., 2000; Yamaki, 1984).

The organic acid profile of a fruit is generally determined by the balance of acid synthesis, degradation, utilization, and compartmentalization (Diakou et al., 2000; Ruffner et al., 1984). For example, organic acids are intermediates in the ubiquitous tricarboxylic acid (TCA) cycle, which functions in aerobic cellular respiration. The enzymes participating in that biochemical pathway include phosphoenolpyruvate carboxylase (**PEPC**, EC 4.1.1.31),

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citrate synthase (**CS**, EC 4.1.3.7), aconitase (**ACO**, EC 4.2.1.3), malate dehydrogenase (**MDH**, EC 1.1.1.37), and malic enzyme (**ME**, EC 1.1.1.40). Malic acid synthesis mainly occurs in the cytosol and is catalyzed by **PEPC** and NAD-dependent malate dehydrogenase (**NAD-MDH**, EC 1.1.1.37) (Moing et al., 2000). The rapid decrease in the concentration of malic acid during fruit ripening is generally attributed to the degradation of malic acid by cytosolic NADP-dependent malic enzyme (**NADP-ME**, EC 1.1.1.40) (Ruffner et al., 1984; Hirai and Ueno, 1997). Conversely, the biosynthesis and breakdown of citric acid are mediated by mitochondrial **CS**, NAD-dependent isocitrate dehydrogenase (**NAD-IDH**, EC 1.1.1.41) and aconitate hydratase (**ACO**, EC 4.2.1.3), in addition to cytosolic **ACO** (Sadka et al., 2000; Kubo et al., 2002). In tartaric acid synthesis, an L-ascorbic acid substrate is converted to L-tartaric acid by L-idonate dehydrogenase (**L-IdnDH**, EC 1.1.1.264) and other unknown enzymes (Saito and Kasai, 1969).

Thus far, little effort has been made to use genome-wide mining to compare the genes involved in organic acid metabolism across multiple plant species, likely because of limited access to sequence information and insufficient bioinformatics tools. Now though, the entire genomes of more than 30 plants have been completely sequenced (http://www.mgrc.com.my/list_eukaryotic_genomes.shtml), and the genome sequences and predicted genetic information for many fruits are freely available. Another other important available resource for such plants are expressed sequence tags (ESTs), which are widely used in identifying genes, constructing genetic linkage maps, annotating genome sequences, developing DNA markers, and analyzing gene expression profiles (Graham et al., 2004; Toulza et al., 2010; Chen et al., 2010; Wongsurawat et al., 2010; May et al., 2008; Johnson et al., 2010; Murray et al., 2005; Huang et al., 2012). As of January 1 2013, a total of 74,186,692 ESTs had been deposited into the expressed sequence tags database, called dbEST, run by the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/dbEST/dbEST_summary.html). Many ESTs have been identified for a variety of plants, such as *Vitis vinifera* (446,664 ESTs), *Malus × domestica* (325,020 ESTs), *Citrus sinensis* (214,598 ESTs), and *Prunus persica* (79,824 ESTs).

In the present work, we sought to investigate the gene copy number, related ESTs number and sequence similarity in the genes involved in the organic acid biosynthetic pathways for different fruits by comparing the genome sequences and ESTs of apples, grapes and sweet oranges. The objective of this study was to determine the key genes involved in the organic acid metabolism in these fruits by analyzing their entire genomes.

1. Materials and methods

1.1. Preparation of the data and analysis tools

Two sequence formats of ESTs (the EST-GenBank format and the EST-FASTA format) for apples (*M. domestica*), grapes (*V. vinifera*), and sweet oranges (*C. sinensis*) were downloaded from the NCBI's dbEST (<http://www.ncbi.nlm.nih.gov/nuclest/>).

The predicted genetic sequences (protein and nucleotide sequences) were downloaded from the Orange (*C. sinensis*) Genome Annotation Project Database (<http://citrus.hzau.edu.cn/orange/>), the Genome Database for Rosaceae (<http://www.rosaceae.org/>), and the Genoscope (<http://www.genoscope.cns.fr/externe/GenomeBrowser/Vitis/>) (Jaillon et al., 2007; Velasco et al., 2007; Xu et al., 2013). Target sequences of enzyme-encoded genes were downloaded from The Arabidopsis Information Resource database (TAIR, <http://www.arabidopsis.org/>) and the NCBI nucleotide database (<http://www.ncbi.nlm.nih.gov/nuclest/>). The Basic Local Assignment Search Tool (BLAST,

v2.2.27) was downloaded from NCBI (<ftp://ftp.ncbi.nlm.nih.gov/blast/executables/blast+/LATEST/>).

1.2. Isolation of organic acid biosynthesis-related homologous genes

To identify the homologous genes in the organic acid biosynthetic pathways of apples, grapes, and sweet oranges, we used the genetic sequences of *Arabidopsis thaliana* and other species as queries to search whole genome protein datasets using the protein BLAST tool. The search parameters were as follows: *E*-value $>1 \times 10^{-10}$ and score >100 . Using manual correction (compare the *E*-value and score) and analyzing the conserved domains (batch CDD search tool, <http://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi>), we identified the predicted genes of these three fruits.

1.3. Analysis of organic acid biosynthesis-related EST sequences

The protein sequences of candidate genes in apples, grapes, and sweet oranges were used as queries to search for organic acid biosynthesis-related EST sequences in each plant EST database. The alignment tool was tblastn, and the alignment parameters were as follows: score >100 and *E*-value $>1 \times 10^{-20}$. The tissue types associated with the selected ESTs were obtained from the EST-GenBank format file, which was downloaded from NCBI's dbEST.

1.4. Sequence alignment and phylogenetic tree construction

The selected apple, grape, sweet orange, and target protein sequences were first aligned using the MEGA 6.0 software with ClustalW method (Tamura et al., 2013). Phylogenetic tree construction was performed with neighbor joining (NJ) approach using the aligned file.

2. Results

2.1. Identification of genes and related ESTs involved in the citric acid and malic acids biosynthetic pathways in apples, grapes, and sweet oranges

Based on previous reports (Badejo et al., 2007; Upadhyaya et al., 2009; Melino et al., 2009; Agius et al., 2003; Wheeler et al., 1998; Wolucka and Van Montagu, 2003; Lorence et al., 2004; Ma et al., 2001), malic acid, citric acid and tartaric acid are the main types of organic acids present in apples, grapes, and sweet oranges, and they are synthesized and decomposed by a series of key enzymes (Fig. 1). More than 86,000 genes were annotated from the genome sequences of these three fruits, namely 30,294 genes in the apple genome, 26,346 genes in the grape genome, and 29,445 genes in the sweet orange genome. From the TAIR and NCBI databases, we downloaded a total of 75 genes from *A. thaliana* and *V. vinifera* that are involved in the organic acid biosynthetic pathways, and these sequences were used to search for homologous genes in our target fruits.

In the biosynthesis and degradation pathways for citric acid and malic acid, **PEPC** was found to be the first key enzyme. Three **PEPC**-encoding genes were identified in apples, while only two **PEPC**-encoding genes were identified in grapes and sweet oranges (Table 1, details in Table S1). The EST hit numbers showed that **PEPC** expression in apples (69 ESTs) and grape (66 ESTs) was higher than in sweet orange (17 ESTs) (Table 2, details in Table S2). Malic acid was found to be synthesized by **MDH** and fumarase (**FUM**). Only one **MDH**-encoding gene was found in apples, grapes, and sweet oranges, but two **FUM**-encoding genes were identified in grapes, whereas one **FUM**-encoding gene was identified in both

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