A Ma10 gene encoding P-type ATPase is involved in fruit organic acid accumulation in apple

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Summary
Acidity is one of the main determinants of fruit organoleptic quality. Here, comparative transcriptome analysis was conducted between two cultivars that showed a significant difference in fruit acidity, but contained homozygous non-functional alleles at the major gene Ma1 locus controlling apple fruit acidity. A candidate gene for fruit acidity, designated Ma10, was identified. The M10 gene encodes a P-type proton pump, P2+, ATPase, which facilitates malate uptake into the vacuole. The Ma10 gene is significantly associated with fruit malate content, accounting for ~7.5% of the observed phenotypic variation in apple germplasm. Subcellular localization assay showed that the Ma10 is targeted to the tonoplast. Overexpression of the Ma10 gene can complement the defect in proton transport of the mutant YAK2 yeast strain and enhance the accumulation of malic acid in apple callus. Moreover, its ectopic expression in tomato induces a decrease in fruit pH. These results suggest that the Ma10 gene has the capacity for proton pumping and plays an important role in fruit vacuolar acidification in apple. Our study provides useful knowledge towards comprehensive understanding of the complex mechanism regulating apple fruit acidity.

Keywords: Malus domestica, fruit acidity, P-type ATPase, transcriptome analysis, candidate gene association mapping.

Introduction
Sour taste or acidity is an important component of the organoleptic quality of fruits. Fruit acidity is attributed to the accumulation of organic acids whose acidity is commonly associated with their carboxyl group. In general, the predominant organic acids in most fruits are citric and malic acids, with unequal acidic taste. Malic acid has a more prolonged sour sensation in the mouth and thus displays a higher level of sourness compared with citric acid (Ramos Da Conceiao Neta et al., 2019). This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

Vascular storage of organic acids requires many transporters and proton pumps in the vacuolar membrane, such as tonoplast dicarboxylate transporter (tDT) (Emmerlich et al., 2003), alumin-ium-activated malate transporter (ALMT) (Meyer et al., 2011), the vacuolar-type H+-ATPase (V-ATPase) (Aprile et al., 2011) and the vacuolar H+-pumping pyrophosphatase (V-PPhase) (Suzuki et al., 2000). More recently, two additional classes of genes encoding tonoplast transport proteins have also been identified to regulate the accumulation of organic acids in fruit. One is CmPH encoding the PIN H7(auxin transporter and a four amino-acid duplication in the coding region causing the acidity difference between acidic and dessert melons (Cohen et al., 2014). Another is a class of two P-ATPase proton pumps, PH5 and PH1, which directly interact with each other and form a heteromeric complex to promote vacuolar acidification in petunia flower (Faraco et al., 2014; Verweij et al., 2008). Later, a PH5-like H+-ATPase gene has been found to show an extremely low expression in acid-free fruit of orange, thus, it is presumably involved in the regulation of vascular storage of citrate in fruit (Shi et al., 2015). Besides participating in vacuolar acidification, the tonoplast P-ATPase TT13 (ATAH10) can also generate the driving force for the transport of proanthocyanidin precursors to the vacuole in Arabidopsis, (Appelhagen et al., 2015). In addition, increasing evidence shows that transcription factor genes participate in the regulation of the proton pump gene expression. For example, a petunia WRKY gene PH3 and an apple MYB1 gene regulate the transcription of P-ATPase (Verweij et al., 2016) and V-ATPase (Hu
et al., 2016) respectively. An ethylene response factor (ERF) has been shown to interact with V-ATPase to regulate citrate accumulation in citrus fruit (Li et al., 2016a). Overall, the mechanism controlling organic acid metabolism and accumulation in fruits is complex and still remains unclear.

Apple (Malus × domestica Borkh.) is one of the most important fruit trees in temperate regions, and its draft genome has been released (Velasco et al., 2010). In apple fruit, the predominant organic acid is malate that accounts for approximately 90% of the total organic acid content (Ma et al., 2015a). In the past two decades, several quantitative trait loci (QTLs) for apple fruit acidity were identified on linkage groups (LGs) 8, 10, 13, 15, 16, and 17, with one (designated Ma) on the top of LG16 having a major effect (Kenis et al., 2008; Liebhard et al., 2003; Maliepaard et al., 1998; Zhang et al., 2012). Recently, the major QTL in the Ma locus was found to encode aluminium-activated malate transporter (Bai et al., 2012; Khan et al., 2013), and fruit acidity is hypothetically determined by a gene network in which the Ma1 gene is a key mediator (Bai et al., 2015). However, several apple cultivars containing two non-functional mutant alleles of the Ma1 locus also have lower pH values in fruit, which suggests that the Ma1 gene is not the only genetic determinant of fruit acidity in apple (Ma et al., 2015b). Moreover, in addition to the Ma1 gene, another major QTL for fruit acidity was identified on LG8 in different segregating populations (Kenis et al., 2008; Ma et al., 2016; Sun et al., 2015). Hence, our knowledge on genetic basis of the biosynthesis and accumulation of malic acid in apple fruit is still limited.

Transcriptome analysis is becoming an effective and fast method to identify genes controlling the important agronomic traits in fruit crops (Lee et al., 2012; Liu et al., 2009). We recently investigated the genetic diversity for organic acid content in apple germplasm and found that a cultivar Belle de Boskoop accumulates high level of malate in fruit although it contains two homozygous mutant alleles in the Ma1 locus, ma1ma1 (Ma et al., 2015b). In this study, analysis of fruit transcriptome profiles of ‘Belle de Boskoop’ was conducted to identify gene(s) other than Ma1 that are responsible for apple fruit acidity. A gene encoding the P-ATPase proton pump, designated Ma10, was identified to control fruit acidity. This study will facilitate our understanding of the complex gene network regulating fruit acidity in apple.

Results

Transcriptome sequencing, assembly and functional annotation

Ma1 is a major gene controlling apple fruit acidity, and a single nucleotide substitution of G with A in the last exon causes a premature stop codon, resulting in a loss-of-function allele ma1 (Bai et al., 2012; Khan et al., 2013). Our previous study revealed that two cultivars, ‘Aifeng’ (AF) and ‘Belle de Boskoop’ (BSKP), contain homozygous mutant alleles, ma1ma1, but they show a significant difference in fruit malate accumulation, bearing non-acidic and acidic fruits respectively (Ma et al., 2015b). To identify genes other than Ma1 for fruit acidity, fruit transcriptome was compared between ‘AF’ and ‘BSKP’. Four RNA-seq libraries were constructed and sequenced for fruit samples of ‘AF’ and ‘BSKP’ at two developmental stages, 30 and 90 DAFB. For ease of description, RNA-seq libraries for fruit samples of ‘AF’ at 30 and 90 DAFB were designated AF1 and AF2 respectively, while BSKP1 and BSKP2 representing RNA-seq libraries for fruit samples of ‘BSKP’ at 30 and 90 DAFB respectively (Table 1).

The Q30 value ranged from 90.28% to 90.59%, suggesting the high quality of RNA-seq data. The GC content of RNA-seq reads ranged from 46.28% to 47.05%. The number of raw reads for each RNA-seq library ranged from 57 518 056 to 66 152 318, with an average of 63 037 677. After removing adapter, empty reads and low quality sequences, the number of clean reads per library ranged from 52 344 236 to 59 958 220, and approximately 81.74% of the clean reads were mapped to the apple genome (Velasco et al., 2010). Reads mapped to unique and multiple sites of the reference genome were designated uni- or multi-reads respectively. For each library, approximately 80.7% and 19.3% of the clean reads were identified to be uni-reads and multi-reads respectively.

A total of 31 905 genes were identified in all fruit samples tested. Among these genes, 27 931, 29 391, 29 006 and 26 806 genes were expressed in AF1, AF2, BSKP1 and BSKP2, respectively (Figure 1a). Of the 31 905 genes expressed in fruit, 3249 displayed twofold or greater difference in expression level between any two fruit samples. Cluster analysis of the expression pattern revealed that the 3249 differentially expressed genes could be divided into 26 subgroups, each of which displayed a unique gene expression profile in fruit samples tested (Figure S1).

Identification of candidate gene(s) for apple fruit acidity

In this study, we focused on the identification of candidate genes that were highly expressed in acidic fruit, but lowly expressed in non-acidic fruit. Digital gene expression (DGE) analysis revealed that 562 and 592 genes showed significant down-regulation in fruits of ‘AF’ at 30 and 90 DAFB respectively, compared with fruits of ‘BSKP’ at the same stage (Figure 1b). Of these differentially expressed genes, 175 were down-regulated in fruits of ‘AF’ at both 30 and 90 DAFB compared with ‘BSKP’, thus, designated common down-regulated genes. In addition, our previous study showed that ‘BSKP’ had high levels of malic acid accumulation in fruit throughout the developmental stages, whilst a low level was observed for ‘AF’ (Ma et al., 2015b). This suggests that the candidate genes for fruit acidity were likely to have a consistent expression in fruits of ‘BSKP’ and ‘AF’ during the developmental stages. DGE analysis revealed that 26 933 and 24 315 genes showed no significant difference in expression level between fruit samples of ‘AF’ or ‘BSKP’ at 30 and 90 DAFB respectively. A total of 21 909 genes were consistently expressed in fruits of both ‘AF’ and ‘BSKP’ at the two stages tested. These consistently expressed genes shared 77 common genes (Table S2) with the common down-regulated genes mentioned above, which contain putative candidate genes for fruit acidity.

Subsequently, functional annotation was conducted for these 77 genes based on Arabidopsis thaliana Annotation Database TAIR (https://www.arabidopsis.org/). As a result, four genes (MDP0000193656, MDP0000287416, MDP0000280551 and MDP0000810883) with a potential role in controlling fruit acidity were identified. Among these genes, MDP000193656 and MDP0000287416 were involved in organic acid metabolism, and encoded malic enzyme and pyruvate decarboxylase respectively. MDP0000280551 encoded pyrophosphate-energized proton pump that was reported to participate in regulation of apoplastic pH and auxin transport (Khadilkar et al., 2016). MDP0000810883 encoded P-type ATPase that is well known to mediate vacuole acidification. Since malate is the predominant organic acid in apple fruit and its accumulation is mainly driven by vacuolar storage (Etienne et al., 2013), MDP0000810883, designated Ma10, was deemed to be a strong candidate controlling fruit.
acidity in apple. The expression level of \( \text{Ma10} \) was approximately 10-fold higher in fruits of ‘BSKP’ at both 30 and 90 DAFB than in fruits of ‘AF’ according to the DGE data (Figure 1c).

**Expression profiling of \( \text{Ma10} \) in apple fruits at different developmental stages**

qRT-PCR was conducted to investigate the expression profiles of \( \text{Ma10} \) in fruits of five cultivars, ‘Aifeng’, ‘Belle de Boskoop’, ‘Zhumary, Xinnongjin’, and ‘Yanhongmei’, at three developmental stages, with \( \text{ma1ma1} \), \( \text{ma1ma1} \), \( \text{Ma1ma1} \), \( \text{Ma1ma1} \) and \( \text{Ma1 Ma1} \) in the \( \text{Ma} \) locus respectively (Figure 2). These five cultivars have similar ripening periods, with maturation occurring about 90 days after full bloom. Similar to the result of DGE mentioned above, the \( \text{Ma10} \) gene was consistently highly expressed in fruit of ‘BSKP’ at the three stages tested, whilst its transcript accumulation was extremely low in fruit of ‘AF’ throughout the developmental stages. In addition, the \( \text{Ma10} \) gene showed an increase in expression level in fruits of ‘Xinnongjin’ throughout the developmental stages. In contrast, the expression level of the \( \text{Ma10} \) gene showed a decrease in fruits of ‘Zhumary’ and ‘Yanhongmei’ at 90 DAFB, and had a peak in fruits at 60 DAFB.

We also investigated the correlation between transcript level of \( \text{Ma10} \) and malic acid content. Transcript accumulation patterns were very similar between ‘Yanhongmei’ and ‘Zhumary’, and malic acid contents were also similar. Similarly, a consistency between transcript level and malic acid content was observed in fruits of ‘AF’ and ‘BSKP’ throughout the developmental stages. For these four cultivars, the expression level of \( \text{Ma10} \) showed a consistency with malic acid content in fruits during the late developmental stages. However, although transcript level was higher in fruit of ‘Xinnongjin’ than in fruit of ‘BSKP’, at late developmental stages, ‘Xinnongjin’ had a very low level of malic acid accumulation in fruit throughout the developmental stages. This inconsistency could be due to the fact that genes other than \( \text{Ma10} \) are also involved in the regulation of malic acid accumulation in apple fruit.

**Gene family encoding P-type ATPase in the apple genome**

The amino acid sequence of the \( \text{Ma10} \) gene was BLASTed against the genome sequences of apple (Velasco et al., 2010), and fifteen homologs were identified on chromosomes 2, 4, 5, 6, 8, 9, 11, 13, 15 and 17 or among the unanchored sequences (Figure S2A). Phylogenetic analysis revealed that these homologs were divided into two gene families, designated P3A-I and P3A-II (Figure S2B). The P3A-II family could be further divided into two subfamilies, P3A-IIa and P3A-IIb. The P3A-I family consisted of two homologs, MDP0000303799 and MDP0000290422. The P3A-IIa subfamily included seven homologs, MDP0000303799 and MDP0000290422.
MDP0000810883 (Ma10), MDP0000158160, MDP0000266132, MDP0000215211 and MDP0000157578, whereas, the P-3A-IIb subfamily contained seven homologs, MDP0000249645, MDP0000162032, MDP0000150049, MDP0000259837, MDP0000136397, MDP0000195785 and MDP0000181085. Moreover, the P-ATPase genes showed a great variation in genomic structure, with the number of exons ranging from 14 to 22 (Figure S3). For example, MDP0000290422 comprised 22 exons spanning approximately 8.8 kb in physical length, whilst MDP0000249645 consisted of 14 exons spanning approximately 4.1 kb in size.

Expression profiles of the P-ATPase genes were investigated in different tissues, including flower, leaf, root and mature fruit (Figure 3). Of these P-ATPase genes, four were not expressed in any tissues tested, including MDP0000290422 and MDP0000249645. In contrast, six genes, MDP0000158160, MDP0000157578, MDP0000136397, MDP0000195785, MDP0000266132 and MDP0000215211 were expressed in all the tissues tested. Four genes, MDP0000152620, MDP0000277881, MDP0000150049 and MDP0000259837, were exclusively expressed in the root. The remaining two genes, MDP0000303799 and MDP0000810883, were mainly expressed in the root and fruit. Overall, the P-ATPase genes showed relatively higher levels of expression in the root. It is worth noting that only the Ma10 gene (MDP0000810883) out of the eight P-ATPase genes that were expressed in fruit was found to be a candidate controlling malic acid content based on comparative transcriptome analysis. Hence, the Ma10 gene was further subjected to functional analysis.

Association analysis between malic acid content and Ma10 in apple fruits

Two adjacent polymorphic SNP loci, A/T and A/G, located 4328 and 4296 bp upstream of the Ma10 translational start site were identified (Figure 4a) and subsequently used to screen a collection of 353 Malus accessions using PCR-direct sequencing method (Figure 4b). For each SNP locus, two homozygous genotypes and one heterozygous genotype were identified. The two SNPs formed four haplotypes, T-G, A-A, A-G and T-A, with frequencies of 0.66, 0.31, 0.02 and 0.01 respectively. Candidate gene-based association analysis showed that the A/T and A/G SNPs were both significantly associated with malic acid content, with P-values of \(1.63 \times 10^{-5}\) and \(1.06 \times 10^{-6}\), and accounted for 7.93% and 7.43% of the observed phenotypic variation respectively. Mean values of fruit malic acid content for A/A, A/T and T/T genotypes at the A/T locus were 8.08, 4.66 and 3.59 mg/g FW, while 7.74, 5.03 and 3.61 mg/g FW for A/A, A/G and G/G genotypes at the A/G locus respectively. Significant difference in malic acid content was observed among the three genotypes for both A/T and A/G SNP loci (Figure 4c). These results further confirm that the Ma10 gene is a candidate controlling malic acid accumulation in apple fruit.

Subcellular localization of Ma10 and its functional analysis through overexpression in yeast, tomato and apple callus

Similar to tonoplast P-type ATPase proteins AtAHA10 and PhPH5, Ma10 contained four putative transmembrane domains (Figure S4). To further determine subcellular localization of Ma10, the Ma10-GFP fusion construct under the control of CaMV 35S promoter was transiently expressed in Arabidopsis mesophyll protoplasts. It was also found that the Ma10-GFP fusion protein resided in the tonoplast (Figure 5a,b,c). To clarify tonoplast location of Ma10, co-localization was conducted using the standard vacuolar marker, Vac-rk (CD3-975) (Nelson et al., 2007; Figure 5d). The GFP fluorescence of Ma10-GFP is completely merged with mCherry fluorescence of the tonoplast marker Vac-rk (Figure 5e). Taken together, these results indicate that Ma10 is located in the tonoplast.

To examine whether Ma10 has proton pumping activity, it was expressed in the YAK2 yeast strain, which has two disrupted
plasma membrane H^+-ATPase genes, \textit{PMA1} and \textit{PMA2}, and contains a plasmid carrying the yeast \textit{PMA1} gene under the control of a galactose-inducible \textit{GAL1} promoter (de Kerchove d’Exaerde \textit{et al.}, 1995). Both the \textit{YAK2} strain and the transgenic strain expressing \textit{Ma10} were able to grow on acidic medium containing 2% galactose (Figure 6a). However, \textit{YAK2} cells were unable to grow on acidic medium lacking galactose. By contrast, expression of \textit{Ma10} sustained growth on non-permissive medium (pH 6.0, 2% glucose) (Figure 6b), which suggests that \textit{Ma10} is able to complement the defect in proton transport of the \textit{YAK2} yeast strain. The ability to restore \textit{YAK2} growth on acidic media demonstrates that \textit{Ma10} has proton pumping activity. In addition, \textit{Ma10} rescued \textit{YAK2} cells less efficiently than yeast \textit{PMA1} (Figure 6). This might be due to the fact that the subcellular localization of plant tonoplast protein expressed in yeast is not only restricted to the vacuolar membrane, but also partially present on plasma membrane (Marinova \textit{et al.}, 2007; Verweij \textit{et al.}, 2008).

To further confirm whether overexpression of the \textit{Ma10} gene has proton pumping activity, it was transformed into tomato. Three transgenic lines were generated and there was no significant difference in fruit size between transgenic lines and wild type (Figure 7a). Semi-quantitative RT-PCR analysis revealed that the \textit{Ma10} gene was highly expressed in fruits of transgenic lines (Figure 7b). The pH values in transgenic and wild-type fruits are shown in Figure 7c. The average pH value in transgenic fruits was 3.98, which was significantly lower than the pH value in wild-type fruits (pH 4.18). Overall, transgenic fruits were significantly more acidic than wild-type fruits. These results suggest that the \textit{Ma10} gene plays an important role in organic acid accumulation in tomato fruit, leading to a decrease in the pH value.

To validate the function of \textit{Ma10} gene in apple malic acid accumulation, \textit{Ma10} was transformed into the callus of apple cv. Orin (Figure 8a). Three transgenic lines were generated and validated by PCR amplification (Figure 8b). Real-time PCR assay clearly indicated that the transcript level of \textit{Ma10} was significantly

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{expression_profiling}
\caption{Expression profiling of the apple \textit{P_{3A}}-ATPase genes in four tissues, including full-bloom flower, mature leaf, ripening fruit and root. The values represent the average of three biological replicates.}
\end{figure}
Figure 4  Characterization of the Ma10 gene responsible for fruit acidity in apple. (a) Gene structure of the Ma10 gene and two adjacent SNP loci, A/T and A/G, located 4328 and 4296 bp upstream of the start codon respectively. (b) An example of genotyping of the apple population using molecular tag of the Ma10 gene. (c) Mean values of malic acid content in mature fruits of different genotypes that are classified according to the A/T and A/G SNPs of the Ma10 gene in apple. Different lowercase letters indicate significant differences among genotypes (t-test, LSD test at \( P < 0.01 \)). Error bars show the SE of the mean. FW, fresh weight.

Figure 5  Subcellular localization of the Ma10-GFP fusion protein in Arabidopsis mesophyll protoplast. (a) The fluorescence of Ma10-GFP fusion protein; (b) Chloroplasts show auto fluorescence; (c) Optical photomicrographs (bright field); (d) The fluorescence of Vac-rk (vacuolar marker) protein; (e) An overlay of bright and fluorescence illumination.
involved in the regulation of apple fruit acidity

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evolutionarily conserved throughout plant evolution (Kriegel et al., 2015; Lobit et al., 2000), grape (Terrier et al., 2000), peach (Lobit et al., 2001), and pear (Suzuki et al., 2008), which suggests that it is a good candidate for apple fruit acidity. It is worthy of further study to ascertain whether MDP0000319016 can physically interact with the Ma10 gene to increase Ma10 H\(^+\) transport activity.

Discussion

Ma10 gene encodes a tonoplast P\(_{3\alpha}\) H\(^+\)-ATPase that is involved in the regulation of apple fruit acidity

In apple, fruit acidity is linked to the capacity to accumulate malic acid in vacuoles. The movement of malic acid from cytoplasm to vacuole is against an electrochemical potential gradient across the tonoplast (\(\Delta V_p\)), which requires an energy-dependent transport. This active transport is accomplished by two distinct types of proton pumps, V-ATPase and V-PPase, both of which are highly evolutionarily conserved throughout plant evolution (Kriegel et al., 2015; Lobit et al., 2006). V-ATPase uses the energy from ATP hydrolysis to produce an electrochemical proton gradient, whilst V-PPase uses inorganic pyrophosphate (PPi) as an energy source. These two types of proton pumps have been reported in a variety of fruit crops, such as apple (Hu et al., 2016), pear (Suzuki et al., 2000), grape (Terrier et al., 2001), and peach (Lobit et al., 2006). However, there have been few reports on the role of P-type ATPase (P-ATPase) in fruit acidity although P-ATPases constitute a large superfamily of membrane proteins. The P-ATPase superfamily can be divided into five subfamilies that translocate distinct cations (Axelsen and Palmgren, 1998).

In this study, we reveal that the \(P_{3\alpha}\)-ATPase genes can be divided into two types, I and II. The apple genome has two type I \(P_{3\alpha}\)-ATPase genes, which showed no expression in any tissues tested or no difference in expression between ‘AF’ and ‘BSKP’. Hence, both these two type I \(P_{3\alpha}\)-ATPase genes are unlikely to be candidates for malate accumulation in apple fruit. By contrast, a type II \(P_{3\alpha}\)-type ATPase gene, Ma10, was showed to be involved in the vacuolar acidification of apple fruit. P-type ATPases are widely assumed to localize to the plasma membrane and use ATP as an energy source to secrete protons out of the cytoplasm into the apoplast (Gaxiola et al., 2007; Schumacher, 2014). However, the subcellular localization assay indicates that Ma10 resides in the tonoplast. This result is consistent with the recent finding that PH5 homologs evolved from plasma membrane \(P_{3\alpha}\)-ATPases, but changed from the plasma membrane into tonoplast proteins due to an N-terminal tonoplast-sorting sequence acquisition before angiosperms appeared (Li et al., 2016b). Organic acid uptake is known to be driven by the electrical component of the electrochemical potential (Martinouta et al., 2000). Functional complementation of the YAK2 yeast strain suggests that the Ma10 gene has the capacity for proton pumping, and its ectopic expression in tomato significantly increases organic acid accumulation in fruit. These results indicate that the Ma10 proton-motive force is able to enhance the influx of protons, which not only reduces the vacuolar pH, but also provides a driving force for additional organic acid uptake. In addition, the Ma10 gene is significantly associated with fruit malic acid content in apple germplasm. Taken together, all the above results indicate that the Ma10 gene encodes a tonoplast \(P_{3\alpha}\) H\(^+\)-ATPase controlling vacuolar acidification in apple fruit.

We compared the physical location between Ma10 and previously reported QTLs for apple fruit acidity, and found that Ma10 is physically close to the fruit acidity QTL on chromosome 17 (Kenis et al., 2008). This suggests that the Ma10 gene could be the candidate gene within a QTL interval that is associated with apple fruit acidity. In addition, the \(P_{3\alpha}\) H\(^+\)-ATPase PH1 can interact with the \(P_{3\alpha}\) H\(^+\)-ATPase PH5 to boost PH5 H\(^+\) transport activity (Faraco et al., 2014). There are two PH1 homologs in the apple genome, MDP0000254558 and MDP0000319016, which are located on chromosomes 12 and 15 respectively (Li et al., 2016b). Interestingly, MDP0000319016 is located in the middle of chromosome 15 that harbours a QTL for titratable acidity (Kenis et al., 2008), which suggests that it is a good candidate for apple fruit acidity. It is worthy of further study to ascertain whether MDP0000319016 can physically interact with the Ma10 gene to increase Ma10 H\(^+\) transport activity.
It is worth noting that five homologs of *Ma10* are located on chromosomes that harbour QTLs for fruit acidity. Of these homologs, two (MDP0000181085 and MDP0000152620) and one (MDP0000259837) are not expressed in all tissues tested or exclusively expressed in root. The remaining two, MDP0000195785 and MDP0000136397, are located on the top of chromosome 15, which is distant from the QTL interval controlling fruit acidity (Kenis et al., 2008). Therefore, these *Ma10* homologs are unlikely to be candidates for the QTLs controlling apple fruit acidity.

**Duplication and expression divergence of P3A-ATPase genes in the apple genome**

Gene duplication is a major driving force in evolution of genome and genetic systems and an important mechanism for the emergence of new genes and biological adaptation because over 90% of eukaryotic genes arise from gene duplication (Teichmann and Babu, 2004). Apple is a diploid, with an allopolyploid origin. Therefore, the emergence of new genes and biological adaptation because over 90% of eukaryotic genes arise from gene duplication (Teichmann and Babu, 2004). Apple is a diploid, with an allopolyploid origin. However, more studies are still needed to clarify whether this gene expression divergence is caused by subfunctionalization and/or neofunctionalization after gene duplication.

**Genetic complexity of organic acid accumulation in apple fruit**

Apple fruit acidity is a quantitative trait and genetic mapping studies have revealed at least nine QTLs for fruit acidity in apple. However, only one candidate gene for fruit acidity, termed *Ma1*, was identified within the QTL interval on LG16 and functions as a malate channel to facilitate malate uptake into the vacuole (Bai et al., 2012; Ma et al., 2015b). Although *Ma1* is a major gene for fruit acidity, its mutation does not result in low content of organic acids in fruit of certain cultivars such as Belle de Boskoop (Ma et al., 2015b). Here, we show the *Ma10* gene encoding P3A-ATPase, which functions as a proton pump to regulate vacuolar acidification of apple fruit. Thus, there are at least two distinct malate translocation systems, a vacuolar ALMT channel and a P3A-ATPase proton pump, which are crucial for malate uptake into the vacuole in apple.

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*Ma10* regulates apple fruit acidity

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The *Ma10* gene is associated with fruit malate content in apple germplasm. According to the A/T or A/G SNPs in the promoter of *Ma10*, three genotypes were identified in all the apple accessions tested. The mean values of malic acid content in mature fruits showed significant differences among the three genotypes and displayed the following patterns: AA > AT > TT or AA > AG > GG. Previous studies reveal three genotypes in the *Ma1* locus, *Ma1 Ma1*, *Ma1ma1* and *ma1ma1*, based on an A/G SNP 252 bp upstream of the stop codon of the *Ma1* gene. All the
apple accessions tested in this study can be divided into nine genotypes based on the presence of Ma1 and Ma10 alleles. When the Ma10 locus is A/A at the A/T or A/G loci, the dominant homozygous Ma1 Ma1 genotype had the highest fruit malate content (Table S3). This suggests the possibility of an interaction between the Ma10 proton pump and the Ma1 transport channel. To address this hypothesis, the expression level of Ma1 was also evaluated in transgenic and wild-type apple calli. Similar to Ma10, Ma1 showed significantly higher expression in transgenic calli than in wild-type calli (Figure S5). This suggests that transport of protons into the vacuole by Ma10 enhances the electrochemical gradient across the vacuole membrane, which in turn activates the Ma1 channel to increase the malate uptake into the vacuole. Once the malate dianion is transported into acidic vacuole, they immediately become protonated and accumulate in the vacuole. Thus, we propose a model of malic acid accumulation through the interaction between the Ma10 and Ma1 proteins in apple (Figure 9). Low expression of Ma10 is unfavourable to activate the Ma1 channel to induce malate uptake into the vacuole.

In addition, evidence for interaction between proton pump genes has been reported. For example, P3α-ATPase is able to interact with P3β-ATPase to increase vacuolar acidification (Eisenach et al., 2014; Faraco et al., 2014). The combined activity of V-ATPase and V-PPase is required for vacuolar acidification, and the presence of the V-PPase is crucial for increase in V-ATPase activity during cold acclimation (Kriegel et al., 2015). In addition, transcription factors, such as WRKY, ERF and MYB genes, have been shown to interact with proton pump genes (Hu et al., 2016; Li et al., 2016a; Quattrocchio et al., 2006; Verweij et al., 2016). Hence, fruit vacuolar acidification appears to be genetically complex and probably regulated by complex gene regulation networks.

Material and methods

Plant materials

The population of apple germplasm used in this study is maintained at the Xingcheng Institute of Pomology of the Chinese Academy of Agricultural Sciences, Xingcheng, Liaoning, China. The population consisted of the 353 apple accessions as reported in our previous study (Ma et al., 2015a), and their leaf DNA samples together with database of the malic acid content in mature fruits were used for candidate gene-based association mapping in this study. In addition, five cultivars, ‘Belle de Boskoop’, ‘Aifeng’, ‘Zhumary’, ‘Xinnongjin’ and ‘Yanhuongmei’, were selected for RT-PCR assay and/or transcriptome sequencing. Fruit samples were collected at 30, 60 and 90 days after full bloom (DABF). Fruit samples were peeled, and pulps were cut into small pieces, immediately frozen in liquid nitrogen and then stored at −75 °C for later use.

RNA-seq library construction and sequencing

A total of 3 μg RNA per sample were subjected to RNA-seq library construction using NEBNext Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA) according to the manufacturer’s instructions. Briefly, the poly-T oligo-attached magnetic beads were used to extract mRNA molecules from total RNA, and the purified mRNA was fragmented using divalent cations at elevated temperature in NEBNext First Strand Synthesis Reaction Buffer (5×). The random hexamer primer and M-MuLV reverse transcriptase (RNase H−) were used to synthesize the first strand cDNA, and the second strand cDNA was subsequently synthesized using DNA Polymerase I and RNase H. The overhangs were converted into blunt ends using exonuclease/polymerase. Following adenylation of 3′ ends of cDNA fragments, NEBNext Adaptor with hairpin loop structure were ligated to prepare for hybridization. The fragments with 150–200 bp in length were purified with AMPure XP system (Beckman Coulter, Beverly, USA), and then PCR amplification was performed to enrich the purified fragments. Finally, the PCR products were purified with the Agencourt AMPure XP kit, and library quality was evaluated using the Agilent Bioanalyzer 2100 system. A cBot cluster generation system was used to perform the clustering of the index-coded samples based on TruSeq PE Cluster Kit v3-cBot-HS (Illumina) according to the manufacturer’s instructions. The library sequencing was performed using the Illumina HiSeq 2000 platform (Illumina, San Diego, CA, USA) with 100-bp paired-end reads.

Sequence analysis and mapping of clean reads

Raw data were filtered through in-house Perl scripts, and clean reads were obtained by removing adapter sequences, empty reads and low quality sequences. The apple draft genome (Velasco et al., 2010) was downloaded from Genome Database for Rosaceae (https://www.rosaceae.org). An index of the reference genome was built using Bowtie v2.0.6 (Langmead et al., 2009), and clean reads were aligned back to the apple draft genome using TopHat v2.0.9 (Trapnell et al., 2009).

Differential gene expression analysis

Gene expression level was estimated using RPKM values (Reads Per Kilobase of exon model per Million mapped reads). HTSeq v0.5.4p3 was used to count the number of clean reads that were mapped to each gene. The read counts of each sequenced library
were standardized by scaling the number of reads in a given
library to a common value using the program edgeR version
2.6.10 (Robinson et al., 2010). Differential expression analysis
was conducted using the DESeq R package (1.12.0; TNLIST,
Beijing, China). The P-values were adjusted based on the method
described by Benjamini and Hochberg (1995), and Q-value of
0.005 and log2-fold change of 1 were set as the threshold for
significantly differential gene expression. Differentially expressed
genes were compared against NCBI RefSeq nucleotide database,
UniProt protein and Swiss-Prot databases, and then annotated
based on the BLAST results, followed by the pathway annotation
pipelines, including KEGG (www.genome.jp/kegg/) and GO
(http://www.geneontology.org).

RNA isolation and quantitative real-time RT-PCR
(qRT-PCR) analysis

Total RNA was isolated using the RNAprep pure Plant kit (Tiangen,
Beijing, China) according to the manufacturer’s instructions, and
approximately 3 µg of total RNA per sample was used for cDNA
synthesis using TransScript One-Step gDNA Removal and cDNA
Synthesis SuperMix (TRANS, Beijing, China) following the manu-
facturer’s instructions. A SYBR Green-based real-time PCR assay
was performed in a total volume of 20 µL reaction mixture
containing 10 µL of 2× SYBR Green I Master Mix (Takara, Dalian,
China), 0.2 µM of each primer and 100 ng of template cDNA. All
the qRT-PCR amplifications were performed using Applied Biosys-
tems 7500 Real-Time PCR Systems (Applied Biosystems, Foster
City, CA). Melting curve analysis was performed at the end of 40
cycles to identify specific amplification products. An actin gene
described in a previous study (Ma et al., 2015b) was used as a
constitutive control. Negative control was also run for each sample,
and each treatment consisted of three biological replicates. Primer
sequences for qRT-PCR analysis are listed in Table S1.

Phylogenetic and statistical analyses

Amino acid sequences were aligned using CLUSTAL X. The
resulting data matrix was subjected to phylogenetic tree analysis
with MEGA software version 6 using Neighbor-Joining (NJ)
method, with the following parameters: bootstrap, 5000 repli-
cates; model, p-distance; and gaps/missing data treatment,
pairwise deletion. All statistical analyses were carried out using
SPSS statistics 17.0 (SPSS Inc., Chicago, Illinois), and significant
difference values were estimated using two-tailed test.

Development of molecular tag of Ma10 and marker-trait
association

Promoter sequences of Ma10 gene were retrieved from the draft
genome of ‘Golden Delicious’ (Velasco et al., 2010) to design PCR
primers to amplify ‘Belle de Boskoop’ and PCR products were
sequenced using forward primer. Single nucleotide polymorphisms
(SNPs) with primer sequences of 5'-CATATGGGATCCCTCTCGCT-3'/
5'-TGGATGATGCATCCACCCT-3' were successfully developed
to screen apple germplasm. Candidate gene-based association mapping
was performed with the software package TASSEL version 5.0
using a mixed linear model (Bradbury et al., 2007). The apple
germplasm collection and population structure such as the Q matrix
and the K matrix were the same as described by Ma et al. (2015b).
Candidate gene association analysis was performed using a mixed
linear model (MLM). The criterion for marker–trait association
was set at P ≤ 0.05 according to the false discovery rate (Benjamini
and Yekutieli, 2001).

Subcellular localization of Ma10 in Arabidopsis
protoplast

The entire coding region of Ma10 was amplified from cDNA
templates in fruits of ‘Belle de Boskoop’ using a pair of primers,
5'-CGATTACAGCTAGATGAGA-3'/5'-TAAACAGTGTAC
ATTGCTTGGCTGAA-3'. RT-PCR products were purified
and inserted into the TA cloning vector pEASY-T1. Later, the full
CDS of Ma10 was amplified and inserted into pHBT-GFP-NOS
vector under the control of the cauliflower mosaic virus (CaMV)
35S promoter using a pair of primers, 5'-GCTTGAATTCTG-
CAGTGGGATGAGAAGGCTGCTG-3' and 5'-GCCCTTGCG
TCACATCGGACATCCACAGCTGTATGCTCGGA-3'—which
added SacI and BamHI sites at the 5' and 3' ends respectively.
As a result, a construct constitutively expressing the fusion protein
Ma10-GFP (fluorescent protein) was generated and co-trans-
fected with the vascular membrane marker (vac-rk CD3-975)
that contained the mCherry reporter gene (Nelson et al., 2007).
PEG-calcium transfection was conducted using Arabidopsis mes-
yophyll protoplasts, which were isolated from well-expanded
leaves of 3–4 week old seedlings using the protocol described
by Yoo et al. (2007). Detection of green fluorescent protein in
Arabidopsis protoplast was performed at 12–24 h after transfec-
tion using a confocal laser scanning microscope Leica TCS SP8
(Leica, Germany). Different filter settings used to measure the
GFP and mCherry fluorescence and chlorophyll autofluorescence
were 488, 651 and 750 nm respectively.

Complementation analysis of the yeast mutant YAK2

Yeast expression vector pGADT7 AD was digested with HindIII
and then ligated with a multiple cloning site (MCS) from pUC19
vector, generating a new yeast expression vector H401. The full
coding region of Ma10 was amplified from fruit cDNAs of ‘Belle
de Boskoop’ using a pair of primers, 5'-CGCTTGAATTCTG-
CAGTGGGATGAGAAGGCTGCTG-3' and 5'-GCCCTTGCG
TCACATCGGACATCCACAGCTGTATGCTCGGA-3'—which
added XbaI and BamHI sites at the 5' and 3' ends respectively.
PCR products were digested with XbaI and BamHI and then inserted
into the H401 vector under the control of the ADH1 promoter, generating a construct
H401-Ma10. Subsequently, H401-Ma10 was transformed to
YAK2 cells using the polyethylene glycol–lithium acetate method
(Gietz and Woods, 2002), and the transformed cells were grown
on the plates with pH 6.0 containing 2% galactose. The YAK2
cell was provided by Dr. Francesca M. Quattrocchio in VU
University, Amsterdam, Netherlands. To avoid any false positives,
positive colonies were confirmed again using the PCR sequencing
approach.

Functional analysis of Ma10 in transgenic tomato and
apple callus

A pair of primers, 5'-CGCCGTTTTGTAACGATAGAGAGAGAG
GATGAGA-3' and 5'-CGCCGTTTTGTAACGATAGAGAGAG
GATGAGA-3', which added PacI and NotI sites at the 5' and 3' ends
respectively, was designed to amplify the whole coding sequence
of Ma10 using cDNA templates from fruit of ‘Belle de Boskoop’. PCR
products were digested with PacI and NotI and then inserted into
the expression vector pMDC83, generating a construct pMDC83-
Ma10. The pMDC83-Ma10 construct was introduced into Agrobac-
terium tumefaciens strain GV3101 by electroporation, and the
nucleotide sequence of inserted fragments were validated by the
PCR sequencing method.

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The pMDC83-Ma10 construct was transformed into tomato (Lycopersicon esculentum Mill. cv. Money Maker) using the Agrobacterium-mediated leaf disc infiltration method (Frary and Earle, 1996). Transformants were selected on MS medium containing 50 μg/mL of hygromycin B. The hygromycin-resistant seedlings were transferred into soil and cultivated in greenhouse. Positive T1 plants were assayed again with the PCR sequencing method, and the expression of MaTo1 in fruit was validated using RT-PCR (35 cycles) with the same primers as qRT-PCR. Red fruit samples of transgenic plants were randomly harvested, and each transgenic line had three replicates, consisting of about 10 fruits. Fruit juice of each replicate was filtered with gauze and then transferred into 15 mL tubes. After centrifugation at 5000 g for 15 min, the supernatants were immediately used to measure fruit pH with a pH metre (DENVER INSTRUMENT, USA).

The pMDC83-Ma10 construct was introduced into Agrobacterium tumefaciens strain LBA4404 to conduct apple callus transformation using the protocol described by Li et al. (2002). The transgenic apple calli were validated by PCR amplification using a pair of primers, 5′-TTGCGCAATGAGGAGGAAAGAAACACGTGTCTGGCTGCCC-3′, with forward primer spanning exons 4 and 5, while reverse primer primer spanning exon 10. Measurement of malic acid content in transgenic and wild-type calli was conducted following the procedure described by Ma et al. (2015a). Briefly, approximately 0.1 g of calli was ground into powder in liquid nitrogen, homogenized with 1.0 mL dH2O and incubated at room temperature for 5 min. The mixture was then centrifuged at 12 000 rpm for 15 min at 4 °C. The supernatant was passed through a SEP-C18 cartridge (Supel clean ENV1 C18 SPE), and filtered through a 0.45 μm Sep-Pak filter. The supernatants were subjected to measure malic acid content using high performance liquid chromatography. Organic acid concentration was expressed in mg/g FW (fresh weight).

Acknowledgements
This work was supported by grants from the National Natural Science Foundation of China (Grant Nos. 31420103914 and 31372048) and the Overseas Construction Plan for Science and Education Base, China-Africa Center for Research and Education, Chinese Academy of Sciences (Grant No. SAJC201327). We thank Dr. Schuyler S. Korban for revising the manuscript.

Conflict of interest
The authors declare no conflict of interest, in accordance with the policy described in the Instructions for Author (http://onlinelibrary.wiley.com/journal/10.1111/%28ISSN%2921467-7652/homepage/ForAuthors.html).

Author contributions
Y.H. and B.M. conceived and designed the experiments. B.M., L.L., T.F., Q.P. and H.Z. performed the experiments. B.M. and H.Z. wrote the paper. C.O. revised the manuscript.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Cluster analysis of expression profiles of genes that show differential expression between any two fruit samples.

Figure S2 Genes encoding P₃₆₅-ATPase in the apple genome.
Figure S3 Schematic overview of genomic structure of sixteen Ma10 homologs in apple. The black box and single line indicate exon and intron respectively.

Figure S4 Alignment of amino acid sequences of Ma10 and its two homologs, AtAHA10 in Arabidopsis and PhPH5 in petunia. Four putative transmembrane domains that were identified in the N-terminal region using TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/) were highlighted in black background.

Figure S5 Analysis of gene expression of Ma1 in apple calli using qRT-PCR. CK indicates wild type, while T1, T2 and T3 represent transgenic lines. Asterisks indicate significant differences between transgenic lines and wild type (t-test, \( P < 0.01 \)).

Table S1 Primers used for qRT-PCR analysis in apple.

Table S2 The 77 common down-regulated genes in apple cultivars AF and BSKP.

Table S3 Mean values of malic acid contents (mg/g FW) in mature fruits of different genotypes that are classified according to the A/T SNP of the Ma10 gene and the A/G SNP of the Ma1 gene in apple.