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Two vacuolar proton pumps, *PpVHA-c1* and *PpVHA-c3*, contribute to malate accumulation in peach fruit

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Abstract

Malate is a key determinant of peach (*Prunus persica* L.) fruit acidity and quality. Vacuolar H⁺-ATPase (VHA) contributes to malate accumulation in plants, but its function in peach fruit remains unclear. In this study, we identified 23 *PpVHA* genes in the 'Zhongyoutao 14' (CN14) peach genome and manually adjusted three gene models using transcriptome data. Sequence alignment and phylogenetic analysis revealed that each VHA subunit is encoded by at least one gene in peach. Based on malate content, *PpVHA-c1* and *PpVHA-c3* were selected as candidate genes potentially involved in malate accumulation. Transient transgenic assays revealed that both *PpVHA-c1* and *PpVHA-c3* promote malate accumulation in peach fruit and *Nicotiana benthamiana* leaves. Furthermore, we predicted nine and fourteen transcription factors as potential positive regulators of *PpVHA-c1* and *PpVHA-c3*, respectively. These findings demonstrate that *PpVHA-c1* and *PpVHA-c3* function as positive regulators of malate accumulation, providing a foundation for dissecting the regulatory network controlling VHA-mediated organic acid metabolism in peach fruit.

Keywords Peach, Malate, V-ATPase, *PpVHA-c1*, *PpVHA-c3*

Introduction

Peach (*Prunus persica* L.) belongs to the Rosaceae family and is one of the most economically important fruit trees worldwide. Both external (e.g., shape and size) and

internal (e.g., flesh color, texture, aroma, and flavor) traits are crucial determinants of peach quality (Cao et al. 2021; Yu et al. 2021; Meng et al. 2023; Yu et al. 2018; Zhou et al. 2015). Among them, flavor quality is a critical determinant of consumer preference (Zhang et al. 2003). As the major organic acids in peach fruit, malic acid and citric acid shape sensory perception and define fruit acidity (Cao et al. 2016; Etienne et al. 2002). Beyond their role in flavor, organic acids such as malate and citrate perform essential physiological functions, including pH homeostasis, osmotic adjustment, stress response, and metabolic modulation (Baccichet et al. 2021), thereby contributing to overall peach fruit quality.

Organic acid metabolism in fruits involves complex pathways regulated by multiple enzymes, which balance synthesis, degradation, and storage (Etienne et al. 2013; Mayuoni-Kirshinbaum and Porat 2014). As key intermediates of the TCA cycle, malic acid and citric acid

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function as both metabolic substrates and gluconeogenic precursors (Vuoristo et al. 2016; Wu et al. 2024). Their biosynthesis is initiated by phosphoenolpyruvate carboxylase (PEPC), which catalyzes the first step of carbon fixation (Famiani et al. 2016). The organic acids synthesized in the cytosol are subsequently transported into vacuoles via specific tonoplast transporters. During fruit development, the catabolism of malate and citrate is governed by enzymes such as phosphoenolpyruvate carboxykinase (PEPCK) and NADP-dependent malic enzyme (NADP-ME), which collectively determine final fruit acidity and flavor (Famiani et al. 2016).

Previous research has shown that vacuolar organic acid accumulation is mediated by acid transporters and proton pumps (Verweij et al. 2008). In the cytosol, malic acid and citric acid exist primarily as malate²⁻ and citrate³⁻, respectively. The tonoplast-localized Vacuolar-ATPase (V-ATPase) and V-Pyrophosphatase (V-PPase) proton pumps transport H⁺ ions into the vacuole (Gallois et al. 2009). Plants possess three major classes of proton pumps: V-type (including V-H⁺-ATPase, VHA; and V-H⁺-PPase, VHP), P-type (P-H⁺-ATPase, PHA), and F-type (Martinoia et al. 2007). VHA acts as a fundamental housekeeping enzyme that establishes an electrochemical proton gradient to drive secondary active transport and maintain cellular pH homeostasis (Shibahara et al. 2002). In addition to stabilizing cytoplasmic pH, VHA also participates in diverse stress responses, such as adaptations to salt, cold, and heavy metals (Seidel 2022). Tonoplast-localized VHA, VHP, and PHA collectively regulate cellular acidity and influence multiple aspects of plant growth and development (Liang et al. 2017; Mao et al. 2023). These proton pumps work synergistically to generate the proton motive force required for organic acid transport, with VHA and VHP specifically facilitating malate accumulation (Shiratake and Martinoia 2007). Notably, V-PPase activity is closely associated with sugar accumulation (Hussain et al. 2017). For example, *MdVHPI-2* modulates soluble sugars and organic acids to improve fruit quality in apple (*Malus domestica*) (Xiang et al. 2024). The activity of these proton pumps is precisely controlled at the transcriptional level. The MYB transcription factor MdMYB73 activates the expression of *MdVHA-A*, *MdVHPI*, and *aluminum-activated malate transporter 9* (*MdALMT9*) to modulate cellular pH and malate content (Hu et al. 2017). Similarly, *Citrus* Ethylene Response Factor 13 (CitERF13) interacts with *CitVHA-c4* to regulate citrate accumulation (Li et al. 2016), whereas PbWRKY40 binds to the *PbVHA-B1* promoter to coordinate salt stress responses and organic acid metabolism in pear (*Pyrus betulaefolia*) (Lin et al. 2022). These regulatory networks highlight the integrated control of proton pump activity in plant physiology and stress adaptation.

V-ATPase is a highly conserved ATP-dependent proton pump in eukaryotes and consists of two functional domains: the cytoplasmic V1 domain (comprising eight subunits, A–H) that catalyzes ATP hydrolysis and the membrane-embedded V0 domain (containing five subunits, a, d, c, c', and c'') responsible for proton translocation (Muller and Taiz 2002; Yang et al. 2021; Zhang et al. 2025). As a critical pH regulator, V-ATPase maintains acidification in vacuoles, the Golgi apparatus, and the endoplasmic reticulum, thereby ensuring cellular homeostasis (Nishi and Forgac 2002).

While V-ATPases are known to regulate vacuolar organic acid accumulation in fruits, the function of VHA genes in peach remains poorly characterized. To address this gap, this study aims to identify PpVHA transcripts encoded by the 'Zhongyoutao 14' (CN14) peach genome (Lian et al. 2022) through homology alignment, phylogenetic analysis, and conserved domain analysis, to select candidate genes based on their expression profiles, and to investigate their functions in both peach fruits and *Nicotiana benthamiana* leaves.

Materials and methods

Plant materials

Five types of peach germplasms, *P. persica* 'Huangshuimi' (HSM), *P. persica* 'Zhongyoutao 14' (CN14), *P. persica* 'Chunmei' (CM), *P. persica* 'Jin Hui' (JH) and *P. persica* 'Yumeiren' (YMR), were cultivated at the experimental base of Henan Agricultural University (Zhengzhou, China) under standard management conditions. Fruits of CM, JH and YMR were harvested at the S3 stage for transient transformation. *N. benthamiana* plants were subsequently grown at 23 °C under a 16 h light/8 h dark photoperiod for functional assays of PpVHA-c1 and PpVHA-c3.

Identification of PpVHA genes in the CN14 peach genome

Protein sequences of VHA genes from *Arabidopsis thaliana* were downloaded from the NCBI (<https://www.ncbi.nlm.nih.gov/>) and TAIR (<http://www.arabidopsis.org/>) databases. Using these sequences as queries, VHA genes were identified in the CN14 peach genome via BLASTP analysis (E value < 1e⁻⁵). The conserved domains of candidate genes were validated using the NCBI Conserved Domain Database (CDD; <https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>) and the InterPro database (<https://www.ebi.ac.uk/interpro>). The amino acid length, theoretical isoelectric point (pI), and molecular weight of the PpVHA genes were determined using the ExPASy database (<https://www.expasy.org/>). Subcellular localization was predicted using Plant-mPLOC (<http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/>), revealing potential organelle-specific distributions of VHA proteins.

Verification of chromosomal location and gene structure

The genome annotation file of CN14 peach, containing gene location information, was downloaded from the Genome Database for Rosaceae (GDR; <https://www.rosaceae.org/>). The chromosome distributions of the *PpVHA* genes were visualized using TBtools software (v2.476) (Chen et al. 2020). Gene structures were verified against transcriptome data using IGV-sRNA software (v0.6.83) (Feng et al. 2024).

Phylogenetic analysis of *PpVHA* genes

Multiple protein sequence alignments between peach *PpVHA* and *Arabidopsis* VHA were performed using ClustalW (<https://www.genome.jp/tools-bin/clustalw>). A phylogenetic tree was constructed with MEGA11 using the maximum likelihood method and 1000 bootstrap replicates.

Analysis of the *cis*-acting elements of the *PpVHA* gene family

Promoter regions were defined as the 2000 bp sequences upstream of the *PpVHA* genes, extracted from the CN14 peach genome. *Cis*-regulatory elements were predicted using the PlantCARE database (<http://bioinformatics.psb.ugent.be/webtools/PlantCare/html/>) and visualized using TBtools software. Potential transcription factors were predicted via the Plant Transcriptional Regulatory Map database (PlantRegMap; <https://plantregmap.gao-lab.org/>). Correlation coefficients between target genes and putative transcription factors were calculated using the *cor* function in R. The relationships between target genes and transcription factors were visualized using Cytoscape (v3.9.1).

RNA extraction and reverse transcription quantitative PCR (RT-qPCR) analysis

Total RNA was extracted using the FastPure Universal Plant Total RNA Isolation Kit (RC411-01, Vazyme, Nanjing, China). cDNA was synthesized with HiScript III RT SuperMix for qPCR (+ gDNA wiper) (R323-01, Vazyme, Nanjing, China) following the manufacturer's protocols. RT-qPCR was performed on an Applied Biosystems QuantStudio5 (Applied Biosystems, New York, USA) using SupRealQ Purple Universal SYBR qPCR Master Mix (U⁺) (Q412-02, Vazyme, Nanjing, China). The *Actin* gene served as an internal control (Jiang et al. 2022). The primer sequences for *PpVHA-c1* and *PpVHA-c3* are listed in Table S1. Data analysis employed the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Analysis of the *PpVHA* gene expression profile in peach

Transcriptome data from HSM and CN14 fruits at four developmental stages (S1–S4) were used to obtain

fragments per kilobase of exon model per million mapped fragments (FPKM) values for each expressed *PpVHA* gene. The data were normalized across samples and visualized using TBtools software (Chen et al. 2020).

Transient transformation of *PpVHA-c1* and *PpVHA-c3* in peach fruit and *N. benthamiana* leaves

Transient expression assays in peach fruit and *N. benthamiana* leaves were performed as previously described (Wang et al. 2025; Xie et al. 2025). Briefly, a single colony of *Agrobacterium* harboring *PpVHA-c1* or *PpVHA-c3* was selected and cultured in 2 mL of liquid LB medium until the OD₆₀₀ reached approximately 0.8. For injection, at least 10 healthy peach fruits at the S3 stage and *N. benthamiana* leaves were selected and injected using a sterile 1 mL syringe. These materials were subsequently collected for malate measurement.

Malate determination

Malate content was determined using a malic acid assay kit (AKAC015M, Beijing Boxbio Science and Technology Co., Ltd., Beijing, China). Briefly, 0.1 g of freeze-dried peach flesh or *N. benthamiana* leaf powder was used for malic acid extraction, which was subsequently quantified using a microplate reader. All samples were analyzed with three biological replicates.

Statistical analysis

The data are expressed as the mean ± standard deviation (SD) from three independent biological replicates. Significant differences ($P < 0.05$) among treatments were determined using one-way ANOVA followed by Duncan's test.

Results

Identification and classification of VHA gene members in peach

Through homology-based screening using 30 *Arabidopsis thaliana* VHA sequences as queries (Hanitzsch et al. 2007), a total of 23 putative *PpVHA* genes (25 transcripts) were identified that were encoded by the CN14 peach genome, all of which contain conserved VHA protein domains (Table S2). Based on transcriptome data, the gene structures of the *PpVHA* genes were examined and adjusted. Most gene structures remained unmodified (Fig. S1); however, three genes underwent structural revision. Specifically, exon skipping in Ppv2.3G000620 (*PpVHA-A*) and Pp03G032290 (*PpVHA-d*) generated novel transcript variants, whereas erroneous annotation predicted premature termination in Ppv2.3G004040.1 (*PpVHA-D*) (Fig. 1). The sequences of all three genes were subsequently confirmed by PCR cloning and sequencing (Fig. S2). Comprehensive bioinformatic analysis revealed that

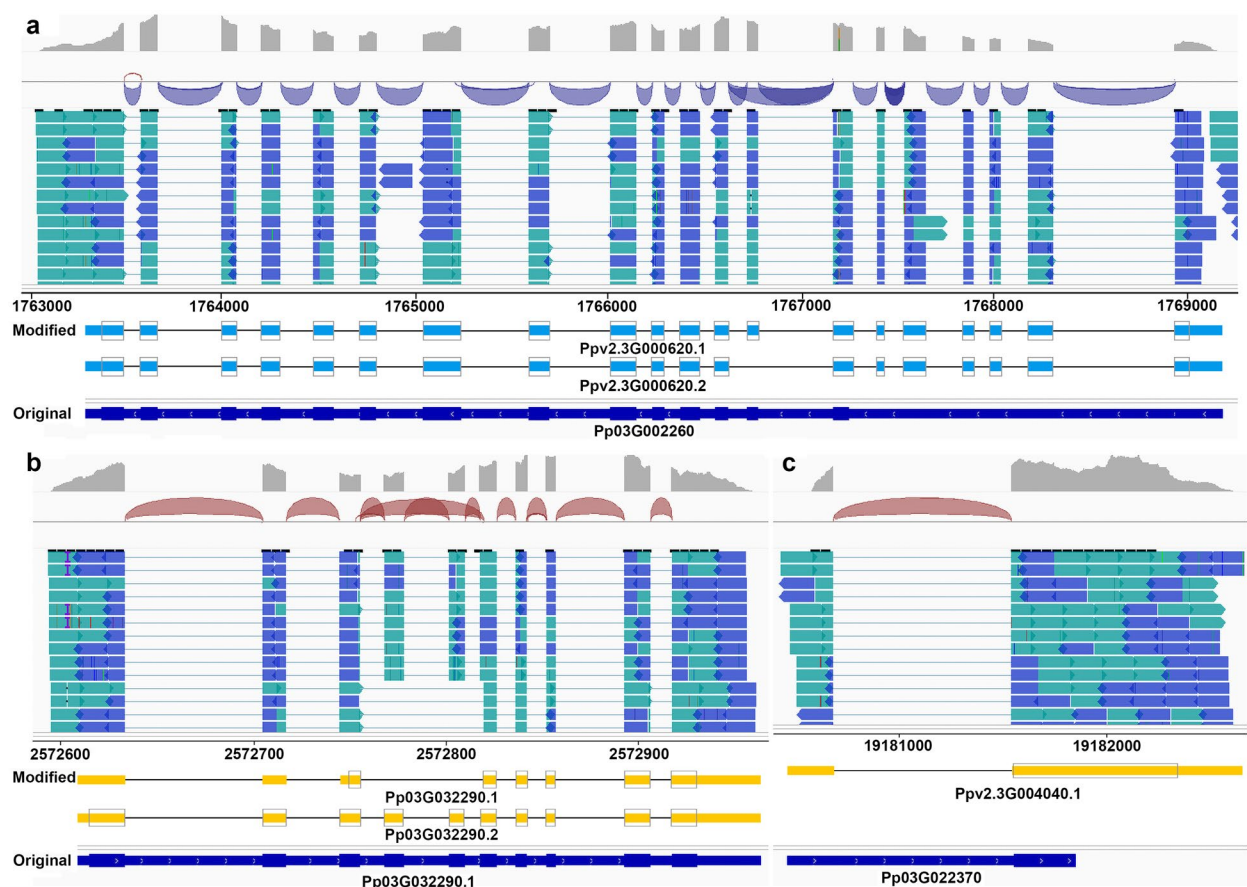


Fig. 1 Vacuolar H⁺-ATPase (*PpVHA*) genes from peach (*Prunus persica*) were adjusted according to the RNA-seq mapping results. **a, b** Each of *Ppv2.3G000620* (**a**) and *Pp03G032290* (**b**) were predicted to produce two transcripts. **c** *Ppv2.3G004040.1* was predicted to terminate earlier

these *PpVHA* genes encode proteins ranging from 70 to 819 amino acids (7.5–93.6 kDa), with pIs between 4.90 and 9.46. The encoded proteins exhibited diverse physicochemical properties: 12 proteins were basic (pI > 7), whereas 13 were acidic (pI < 7), with an average pI of 7.12; 13 proteins were hydrophilic, whereas 12 were hydrophobic. Subcellular localization predictions revealed diverse cellular distributions, including vacuolar (tonoplast), nuclear, plasma membrane, cytoplasmic, mitochondrial, chloroplast, and Golgi apparatus, which is consistent with the known multifunctional roles of V-ATPases in plant cells (Table 1).

Phylogenetic analysis of the *PpVHA* gene family

Using ClustalW-based alignment of full-length protein sequences from *Arabidopsis thaliana* (30 *VHA* transcripts) and peach (25 *VHA* transcripts), a phylogenetic tree was constructed, indicating that the *VHA* family is classified into 13 evolutionarily conserved groups (Fig. 2). The V1 structural domain subunits were distributed into eight distinct clades, including subunits A (2 members),

B (1), C (2), D (1), E (2), F (2), G (3), and H (1). Similarly, the V0 domain subunits were grouped into five branches: a (3 members), c (5), c' (1), d (2), and e (1). Notably, each *VHA* subunit was represented by at least one transcript in peach. Comparative phylogenetic analysis revealed that the *VHA* genes in peach can be systematically classified into 13 V1-domain transcripts and 12 V0-domain transcripts, consistent with the classification of V-ATPase subunits in *Arabidopsis*, demonstrating the conserved evolutionary relationships between these species.

Chromosomal distribution patterns of peach *VHA* genes

Genomic mapping revealed that the 23 identified *PpVHA* genes are distributed across the eight chromosomes of the CN14 peach genome (Fig. 3). Specifically, chromosomes 1 and 3 harbor the highest number of *VHA* genes, with five *PpVHA* genes each, followed by chromosome 6, with four genes; chromosome 8, with three genes; and chromosomes 4 and 5, each containing two genes. Notably, only one *PpVHA* gene was located on each of chromosomes 2 and 7.

Table 1 The gene family of *Vacuolar H⁺-ATPase (PpVHA)* in the ‘Zhongyoutao 14’ (CN14) peach (*Prunus persica*) genome

Gene name	Gene ID	Number of amino acid	Molecular weight (kDa)	Theoretical isoelectric point (pI)	Grand average of hydropathicity	Predicted subcellular location
<i>PpVHA-A1</i>	Ppv2.3G000620.1	623	68.69	5.31	−0.17	Cell membrane; Mitochondrion
<i>PpVHA-A2</i>	Ppv2.3G000620.2	604	66.46	5.08	−0.17	Cell membrane; Mitochondrion
<i>PpVHA-B</i>	Pp08G020140	490	54.54	4.94	−0.234	Cytoplasm
<i>PpVHA-C</i>	Pp03G035920	376	42.56	5.55	−0.307	Vacuole
<i>PpVHA-D</i>	Ppv2.3G004040	261	29.14	9.46	−0.328	Chloroplast; Golgi apparatus; Mitochondrion; Nucleus
<i>PpVHA-E1</i>	Pp05G012480	230	26.20	7.71	−0.447	Nucleus
<i>PpVHA-E2</i>	Pp06G006280	231	26.12	8.78	−0.521	Chloroplast; Nucleus
<i>PpVHA-F1</i>	Pp05G025340	130	14.33	6.83	0.005	Chloroplast
<i>PpVHA-F2</i>	Pp06G040910	130	14.37	6.83	−0.015	Chloroplast
<i>PpVHA-G1</i>	Pp06G012380	110	12.02	9.3	−0.535	Nucleus
<i>PpVHA-G2</i>	Pp03G003680	144	15.79	6.89	−0.354	Nucleus
<i>PpVHA-G3</i>	Pp01G061290	110	12.33	8.92	−0.765	Nucleus
<i>PpVHA-H</i>	Pp02G038660	449	51.06	7.07	−0.107	Nucleus
<i>PpVHA-a1</i>	Pp06G032150	819	93.60	5.82	0.078	Cell membrane
<i>PpVHA-a2</i>	Pp08G012450	814	92.52	6.09	0.062	Cell membrane; Nucleus
<i>PpVHA-a3</i>	Pp01G043890	640	72.88	6.38	0.205	Cell membrane
<i>PpVHA-c1</i>	Pp08G011610	166	16.75	8.62	0.917	Vacuole
<i>PpVHA-c2</i>	Pp01G043750	166	16.75	8.62	0.917	Vacuole
<i>PpVHA-c3</i>	Pp07G009960	166	16.73	8.62	0.933	Vacuole
<i>PpVHA-c4</i>	Pp01G055730	166	16.73	8.62	0.933	Vacuole
<i>PpVHA-c''</i>	Pp01G053080	180	18.41	8.98	1.115	Vacuole
<i>PpVHA-d1</i>	Pp03G032290.1	351	40.60	4.9	−0.152	Cytoplasm
<i>PpVHA-d2</i>	Pp03G032290.2	166	19.68	5.33	−0.286	Vacuole
<i>PpVHA-e</i>	Pp04G001010	70	75.56	7.68	1.487	Cell membrane; Chloroplast; Golgi apparatus; Mitochondrion
<i>PpVHA-c'</i>	Pp04G010750	105	11.51	5.15	0.238	Cell membrane

Differential expression patterns of *PpVHA* genes during peach fruit development

Malate content was measured in the fruits of HSM and CN14 at different developmental stages. The results revealed that malate content was highest at the S1 stage and gradually decreased throughout fruit development (Fig. 4a, b). To further characterize the expression profiles of the 25 *PpVHA* transcripts, the transcriptome data from two different types of peach germplasms, HSM and CN14, were analyzed across four developmental stages (HS1–HS4 and CS1–CS4) (Fig. 4c). The results revealed distinct expression patterns among different *PpVHA* genes. *PpVHA-a2* and *PpVHA-A* exhibited relatively high expression levels during the early developmental stages (CS1–CS2) of CN14, but low expression levels during the late stages (CS3–CS4) and across all stages in HSM fruit (HS1–HS4). *PpVHA-F2*, *PpVHA-D*, and *PpVHA-G1* showed a gradual increase in expression from HS1 to HS3 in HSM fruit, whereas their expression levels remained consistently low at HS4 and across all developmental stages (CS1–CS4)

in CN14. Notably, four genes (*PpVHA-G2*, *PpVHA-c'*, *PpVHA-E1*, and *PpVHA-c'*) were specifically expressed at the HS3 stage in HSM. Interestingly, the expression levels of both *PpVHA-c1* and *PpVHA-c3* showed a general decreasing trend in both HSM and CN14, which was positively correlated with malate content. These results suggest that these two *VHA* genes may be involved in malate accumulation during peach fruit development.

Functional validation of *PpVHA-c1* and *PpVHA-c3* in malate accumulation

To investigate the functional roles of *PpVHA-c1* and *PpVHA-c3* in malate accumulation, transient overexpression and RNA interference (RNAi) assays were conducted in peach fruit and *N. benthamiana* leaves (Fig. 5). Transient overexpression of *PpVHA-c1* and *PpVHA-c3* in CM and JH peach fruits significantly increased the malate content (Fig. 5a–e). Consistent results were also observed in *N. benthamiana* leaves (Fig. 5f–h). Conversely, transient silencing of *PpVHA-c1* and *PpVHA-c3*

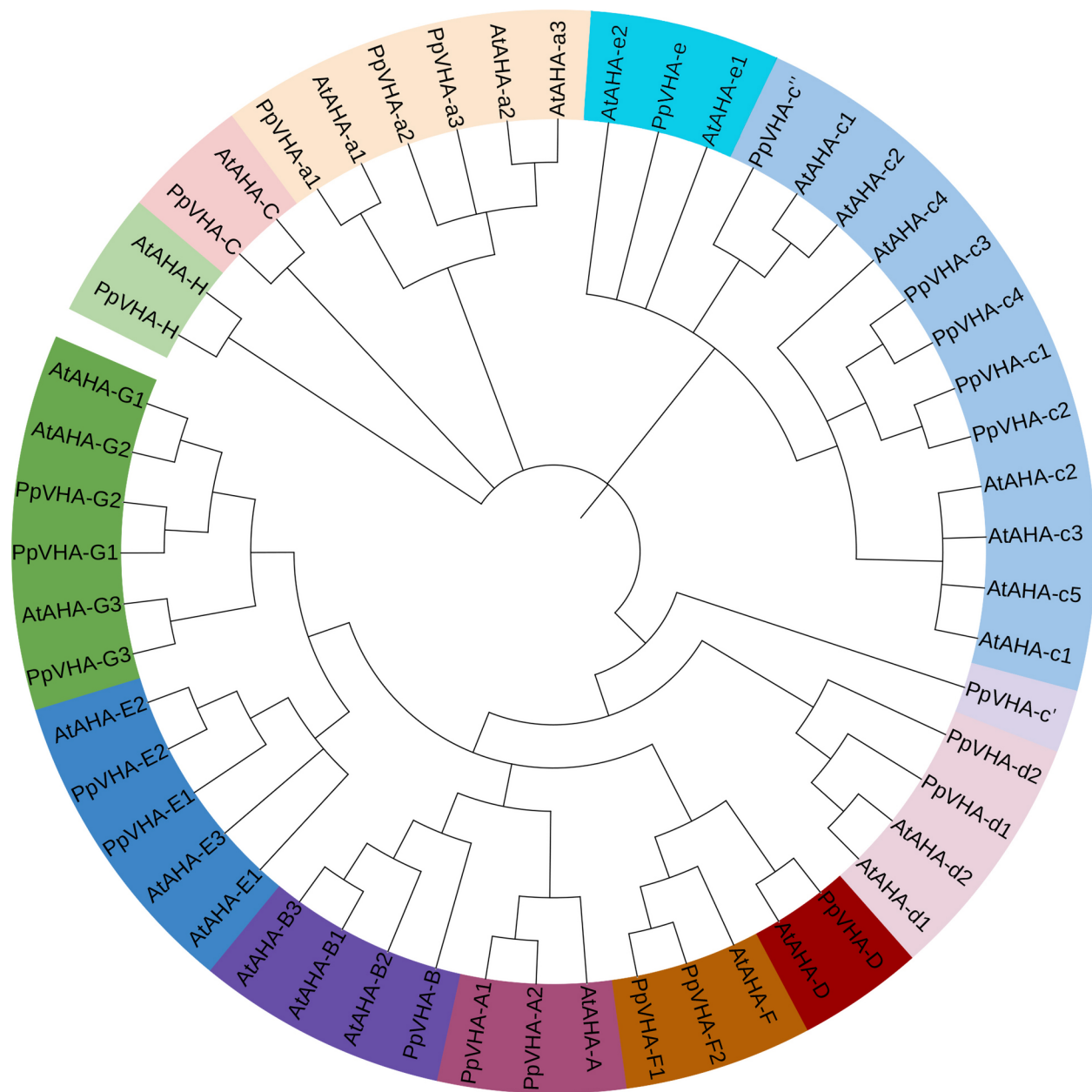


Fig. 2 Phylogenetic analysis of Vacuolar H⁺-ATPase (VHA) proteins from the genome of *Arabidopsis thaliana* and the 'Zhongyoutao 14' (CN14) peach (*Prunus persica*). Twenty-five *PpVHA* transcripts were classified into 13 groups, with different colors indicating different subunits

in YMR peach fruits significantly reduced the malate concentration (Fig. 5g–h). These results indicate that *PpVHA-c1* and *PpVHA-c3* play crucial roles in regulating malate accumulation in peach fruit.

Prediction and analysis of *cis*-acting elements of the *PpVHA* gene promoter

The predicted *cis*-acting elements located 2,000 bp upstream of the translational start codons in the 23 *PpVHA* genes were predicted using the PlantCARE

database (Fig. S3). The results revealed that *PpVHA* promoters may contain diverse regulatory elements, including photoresponsive elements, hormone-responsive elements (MeJA, abscisic acid, and gibberellin), and elements related to meristem development, cell cycle regulation, low-temperature response, and defense mechanisms. These findings suggest that the *VHA* gene family is subject to complex transcriptional regulation in response to both developmental and environmental signals (Fig. S3).

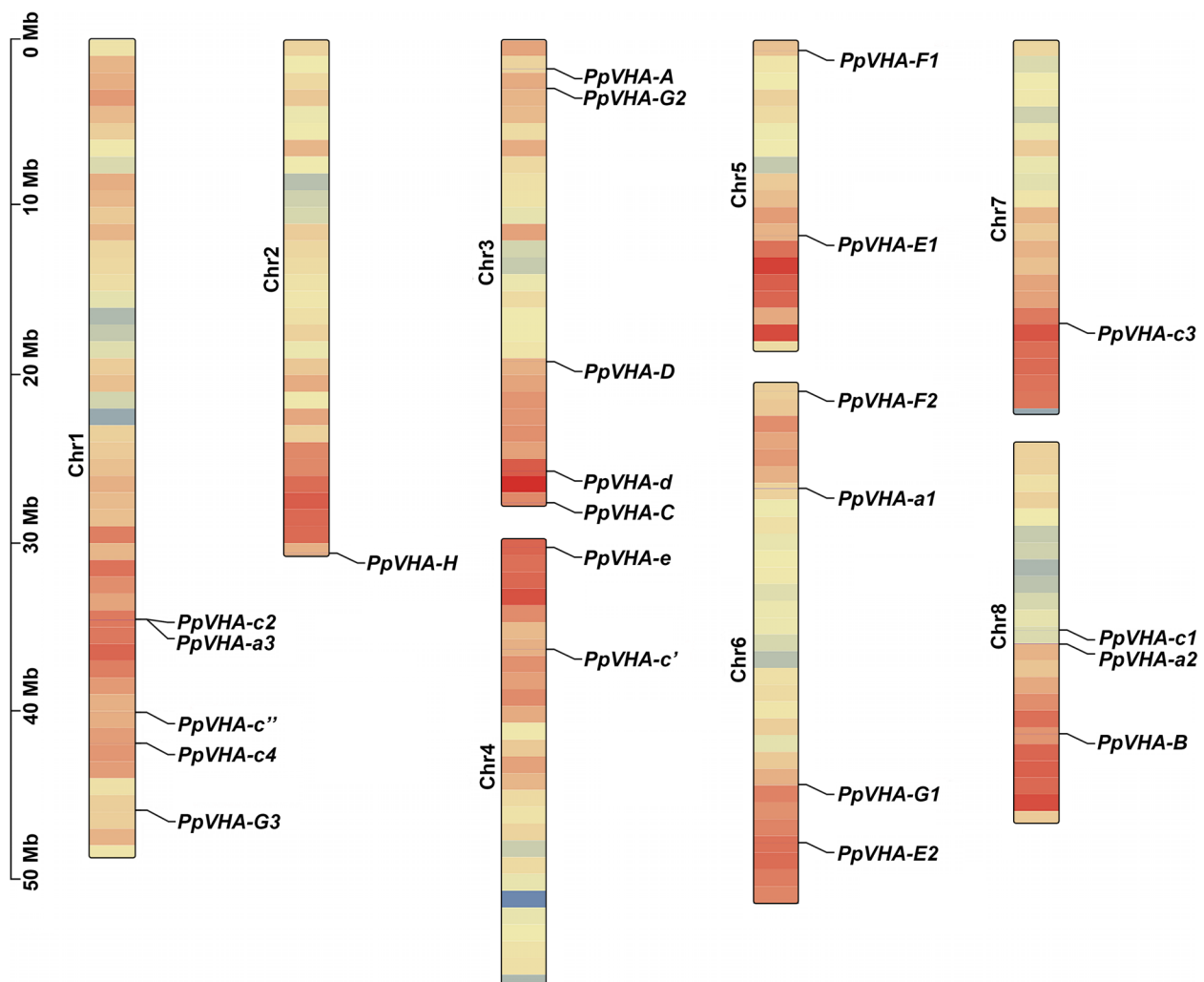


Fig. 3 Chromosomal locations of Vacuolar H^+ -ATPase (*PpVHA*) genes on the eight chromosomes of peach (*Prunus persica*). The scale bar on the left indicates chromosome length in megabases. The gradient color of chromosomes represents gene distribution density

To further identify transcription factors potentially regulating *PpVHA-c1*, *PpVHA-c3* and malate accumulation, transcription factor binding sites were predicted using the PlantRegMap database. The results revealed 310 and 234 potential TF binding sites in the promoters of *PpVHA-c1* and *PpVHA-c3*, corresponding to 128 and 134 transcription factors, respectively (Table S3). Nine TFs for *PpVHA-c1* and fourteen TFs for *PpVHA-c3* exhibited high Pearson correlation coefficients ($|r| > 0.80$) with their respective target genes and were therefore considered potential positive regulators (Fig. 6a; Table S4). Among the nine candidate regulators of *PpVHA-c1*, two belong to the TCP family and two belong to the MYB family, and one each belongs to the bZIP, Dof, NAC, MYB-related and WRKY families. The candidate regulators of *PpVHA-c3* include three NAC TFs, two each from the MYB-related and HSF families, and one each from the bZIP,

Dof, ERF, GATA, MADS, and SRS families. Notably, one NAC TF (Ppchr02G027880) was predicted to potentially regulate both *PpVHA-c1* and *PpVHA-c3* (Fig. 6b). The relative expression levels of *PpVHA-c1*, *PpVHA-c3*, and selected potential transcription factors were further analyzed in HSM and CN14 fruits at four developmental stages. Three transcription factors were significantly positively correlated with *PpVHA-c1*, whereas two were significantly positively correlated with *PpVHA-c3* (Fig. 6c). These transcription factors may play important roles in regulating malate accumulation in peach fruit.

Discussion

Peach is among the most economically important fruit trees worldwide and is highly favored by consumers because of its pleasant flavor. Organic acids play important roles in fruit flavor development. As in pear, apple

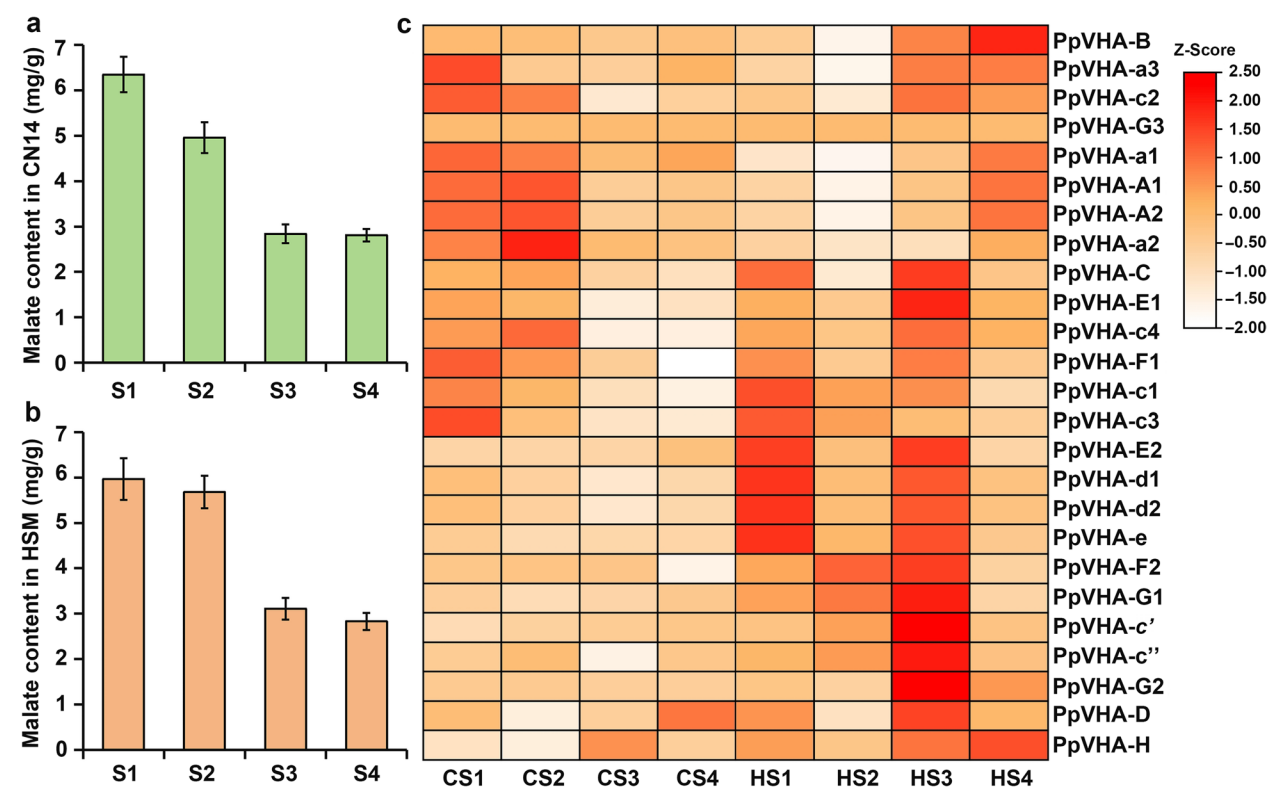


Fig. 4 Malate content and expression levels of 25 *Vacuolar H⁺-ATPase* (*PpVHA*) transcripts in ‘Huangshuimi’ (HSM) and ‘Zhongyoutao 14’ (CN14) peach (*Prunus persica*) fruits during development. **a, b** Malate content in CN14 (**a**) and HSM (**b**) peach fruit. **c** Expression levels of *PpVHA* transcripts in HSM and CN14 peach fruits. CS1–CS4, fruit at S1–S4 stages of CN14 peach; HS1–HS4, fruit at S1–S4 stages of HSM peach

and citrus fruits, malate and citrate are the predominant organic acids in peach fruits (Ma et al. 2018; Shi et al. 2019; Zheng et al. 2020). Notably, considerable variation in total acidity is often observed among different cultivars and across developmental stages.

Plant V-ATPase is a major active proton pump composed of V1 and V0 complexes. *VHA* genes encoding each subunit have been identified in *Arabidopsis*, tomato, and sweet orange (Coker et al. 2003; Hanitzsch et al. 2007; Hu et al. 2012; Shi et al. 2018; Zhang et al. 2025). For instance, in *Arabidopsis*, five genes encode subunit c; three genes encode subunits B, E, G, and a; two genes encode subunits c', d, and e; and one gene encodes subunits A, C, D, and f (Hanitzsch et al. 2007). In sweet orange, four genes encode subunit c, whereas two genes encode subunits E, F, H, and a (Shi et al. 2018). In this study, 23 *PpVHA* genes were identified in the CN14 peach genome through homology alignment, and their gene models were validated using transcriptome mapping data. Each of the subunits B, C, D, H, c', e, and c' was represented by at least one gene; subunits A, E, F, and d were each encoded by two genes; subunits G and a were encoded by three genes; and subunit c was encoded by four genes. These findings indicate that the number of

genes encoding each *VHA* subunit is largely conserved across plant species, with each subunit being encoded by at least one gene in most species.

The vacuole accounts for up to 90% of the total cell volume and stores the majority of organic acids and other metabolites. Previous studies have shown that V-ATPase functions as a key proton pump and plays a crucial role in organic acid accumulation (Hu et al. 2012). Several studies have further highlighted the involvement of *VHA* in regulating organic acid accumulation in fruits. For example, *MdVHA-B1*, *MdVHA-B2*, *MdVHA-E2*, and *MdVHA-A* are associated with malate accumulation in apple (Hu et al. 2017; Yao et al. 2009). *ChVHA-V0-a3* and *ChVHA-V1-G3* are involved in malate metabolism in *Cerasus humilis* (Zhang et al. 2025). *CitVHA-c4* contributes to citric acid accumulation in citrus (Li et al. 2016). *PbVHA-B1* may promote organic acid accumulation in pear (Lin et al. 2022). In our study on the two peach cultivars, the malate content decreased during fruit development, and the expression patterns of 25 *PpVHA* transcripts were analyzed. Nine transcripts exhibited relatively high expression levels at the CS1 and CS2 stages in CN14 fruit, whereas three were specifically expressed at the HS1 and HS2 stages in HSM fruit. The

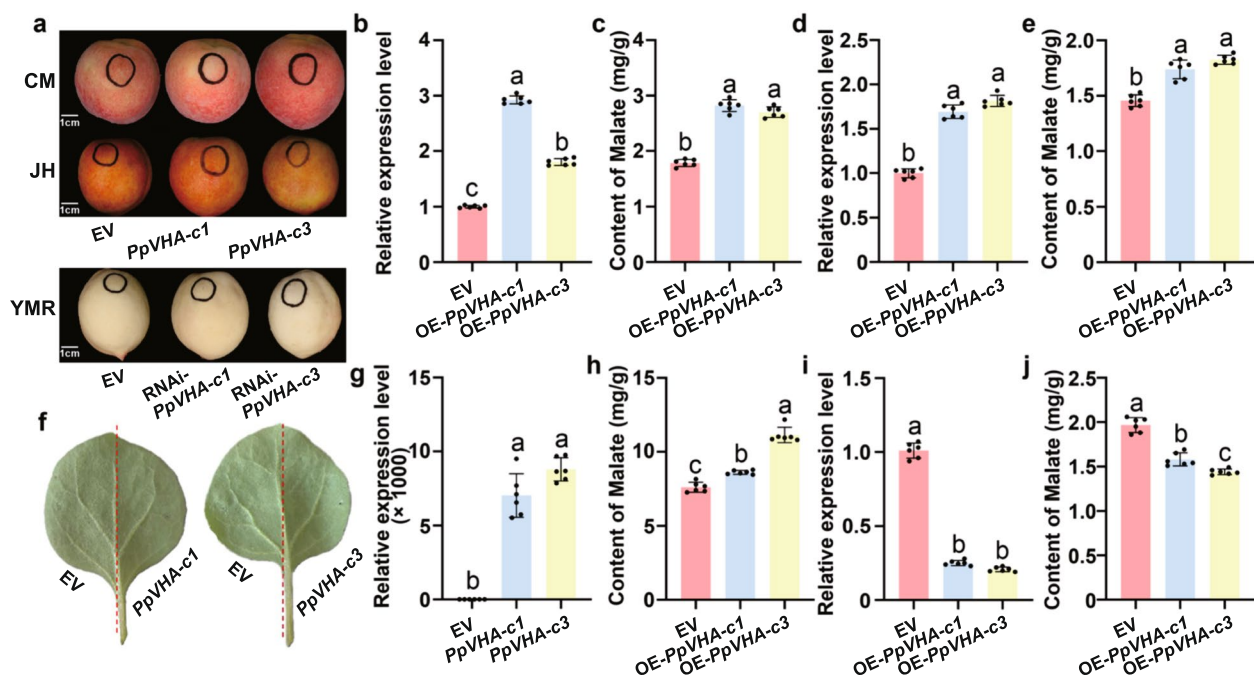


Fig. 5 Effects of Vacuolar H^+ -ATPase 1 (*PpVHA-c1*) and $V-H^+$ -ATPase 3 (*PpVHA-c3*) from peach (*Prunus persica*) on malate accumulation. **a** Transient overexpression of *PpVHA-c1* and *PpVHA-c3* in 'Chunmei' (CM) and 'Jin Hui' (JH) peach fruit, and RNAi of *PpVHA-c1* and *PpVHA-c3* in 'Yumeiren' (YMR) fruits. **b, c** Expression levels of *PpVHA-c1* and *PpVHA-c3* (**b**) and malate concentrations (**c**) in CM peach fruit. **d, e** Expression levels of *PpVHA-c1* and *PpVHA-c3* (**d**) and malate concentrations (**e**) in JH peach fruit. **f** Transient expression of *PpVHA-c1* and *PpVHA-c3* in *Nicotiana benthamiana* leaves. **g, h** Expression levels of *PpVHA-c1* and *PpVHA-c3* (**g**) and malate concentrations (**h**) in *N. benthamiana* leaves. **i, j** Expression levels of *PpVHA-c1* and *PpVHA-c3* (**i**) and malate concentrations (**j**) in YMR peach fruit. Different letters denote significant differences according to Duncan's multiple range test ($P < 0.05$)

expression levels of *PpVHA-c1* and *PpVHA-c3* were significantly correlated with malate accumulation. Transient overexpression and RNAi experiments confirmed that *PpVHA-c1* and *PpVHA-c3* function in regulating malate accumulation in both peach fruit and *N. benthamiana*. Collectively, these findings indicate that *VHA* genes play important roles in organic acid accumulation in fruits.

Previous studies have shown that the expression levels of certain *VHA* genes are closely associated with organic acid accumulation. For instance, the expression level of the *VHA-A* gene is significantly higher in a high-acid loquat cultivar than in low-acid cultivars (Yang et al. 2021). Similarly, in citrus, the expression levels of several *VHA* genes (*V1-A*, *V1-C*, *V1-E1*, *V1-H2*, *V0-a2*, *V0-c4*, and *V0-d*) are significantly lower in low-acid cultivars ('HongAnliu') than in high-acid cultivars ('Anliu') (Shi et al. 2018). Several transcription factors that play crucial roles in regulating *VHA* gene expression have been identified in apple and citrus (Hu et al. 2017, 2016, 2012; Li et al. 2016; Martinez and Rao 2012). In apple, MdMYB73 increases malate accumulation by upregulating the expression levels of *MdVHA-A*, *MdVHP*, and *MdALMT9*. Furthermore, MdCibHLH1 interacts with MdMYB73 to enhance

the transcriptional activation of these target genes (Hu et al. 2017). In addition, MdMYB1 and MdMYB10 directly bind to the promoters of *MdVHA-B1*, *MdVHA-B2*, and *MdVHA-E2*, thereby promoting their transcription and increasing malate accumulation (Hu et al. 2016). In citrus, CitERF13 contributes to citric acid accumulation by regulating *CitVHA-c4* expression (Li et al. 2016). In this study, several transcription factors were identified as highly correlated with *PpVHA-c1* and *PpVHA-c3* through transcription factor prediction, transcriptome analysis and RT-qPCR validation, suggesting their potential role as regulators of malate metabolism in peach.

Conclusion

In this study, a total of 23 *PpVHA* genes were identified in the CN14 peach genome and classified into 12 subgroups based on phylogenetic analysis. Their gene structures were manually adjusted using transcriptome data. Based on phylogenetic relationships and sequence alignment, each *VHA* subunit was found to be represented by at least one transcript. The expression levels of *PpVHA-c1* and *PpVHA-c3* decreased during fruit

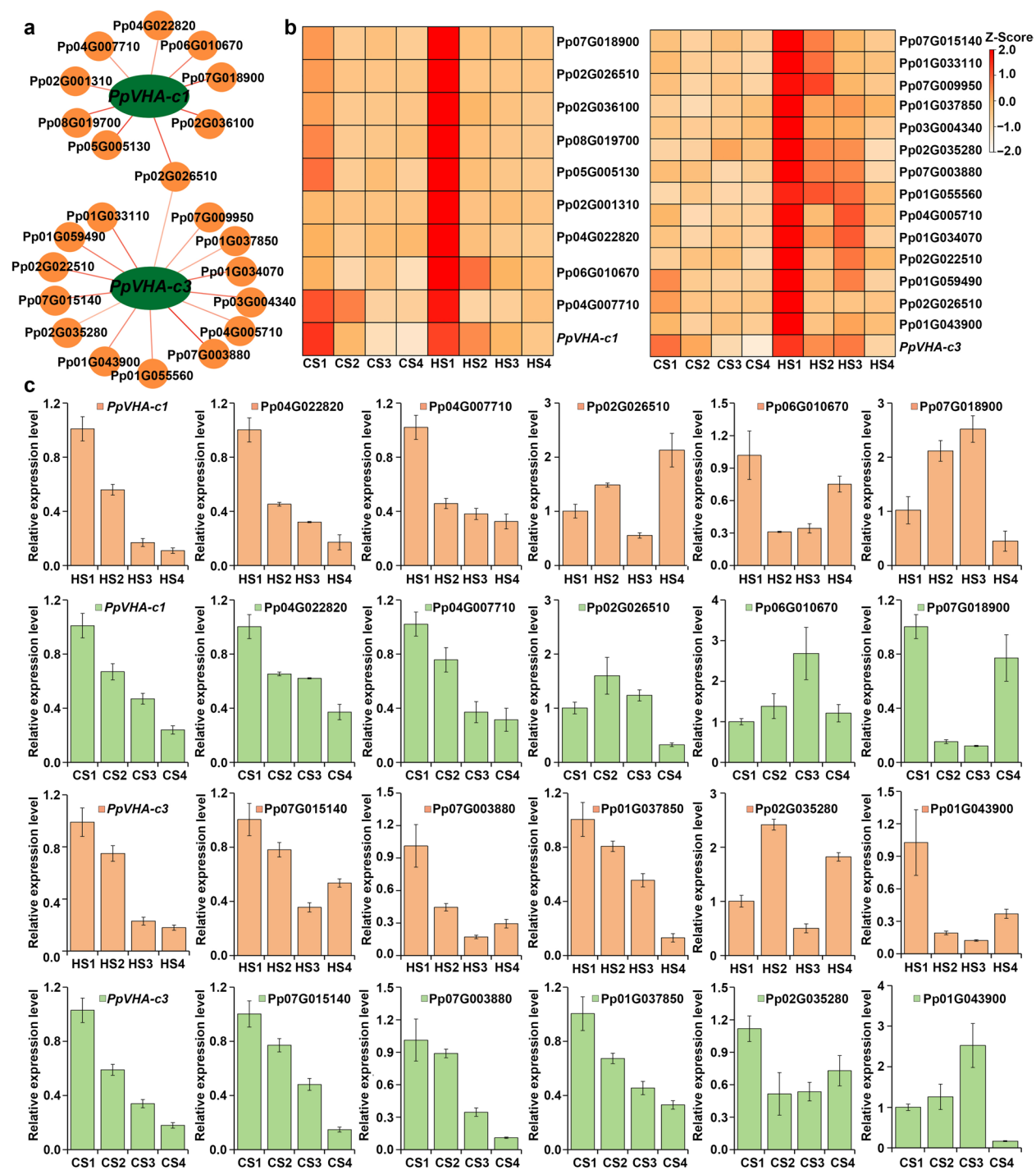


Fig. 6 Relationships between Vacuolar H^{+} -ATPase *c1* (*PpVHA-c1*) and $V-H^{+}$ -ATPase *c3* (*PpVHA-c3*) from peach (*Prunus persica*) and their potential TFs based on transcriptome data. **a** Correlations of *PpVHA-c1* and *PpVHA-c3* with their candidate TFs. **b** Expression levels of *PpVHA-c1* and *PpVHA-c3* and their potential regulatory transcription factors. CS1–CS4, fruit at S1–S4 stages of ‘Zhongyoutao 14’ (CN14) peach; HS1–HS4, fruit at S1–S4 stages of ‘Huangshuimi’ (HSM) peach. **c** Relative expression levels of *PpVHA-c1* and *PpVHA-c3* and their potential regulatory transcription factors determined by RT-qPCR

development in both CN14 and HSM cultivars, suggesting their potential involvement in organic acid accumulation. Functional analysis further revealed that *PpVHA-c1* and *PpVHA-c3* regulate malate accumulation in peach fruit. This study provides a solid foundation for understanding the roles of *VHA* genes and their regulatory mechanisms in organic acid metabolism and fruit quality development in peach.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1007/s44281-026-00108-8>.

Supplementary Material 1. Figure S1. Gene models were verified based on transcriptome data. Most gene models were consistent with transcriptome mapping results. Figure S2. The adjusted predicted transcript sequences were verified by sequencing the positive clones. *PpVHA*, *Prunus persica* Vacuolar H⁺-ATPase. Figure S3. The *cis*-acting elements were predicted of 23 *PpVHA* promoters using PlantCARE.

Supplementary Material 2. Table S1. Primer information used in this study. Table S2. *VHA* genes in *Arabidopsis thaliana* and 'Zhongyoutao 14' (CN14) peach genome. Table S3. Potential TFs might regulate *PpVHA-c1* and *PpVHA-c3* genes. Table S4. The correlation coefficient between target genes and transcription factors (> 0.8).

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Not applicable.

Authors' contributions

BT, JCF and HPZ designed the research. BBF and QQL performed the experiments with the contributions from KXL, DSW, YJD, and XDL. WW, LLZ, XY and JDL analyzed the data. BBF and QQL wrote the manuscript. BT and HPZ supervised the research. XBW, XBX and JC provided the study materials. BT and JCF finished the revised work. All authors read and approved the final manuscript.

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Data availability

Data will be made available on request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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