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Genome-Wide Identification and Characterization of Apple P_{3A}-Type ATPase Genes, with Implications for Alkaline Stress Responses

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Abstract: The P_{3A}-type ATPases play crucial roles in various physiological processes via the generation of a transmembrane H⁺ gradient (Δp H). However, the P_{3A}-type ATPase superfamily in apple remains relatively uncharacterized. In this study, 15 apple P3A-type ATPase genes were identified based on the new GDDH13 draft genome sequence. The exon-intron organization of these genes, the physical and chemical properties, and conserved motifs of the encoded enzymes were investigated. Analyses of the chromosome localization and ω values of the apple P_{3A}-type ATPase genes revealed the duplicated genes were influenced by purifying selection pressure. Six clades and frequent old duplication events were detected. Moreover, the significance of differences in the evolutionary rates of the P_{3A} -type ATPase genes were revealed. An expression analysis indicated that all of the P_{3A}-type ATPase genes were specifically expressed in more than one tissue. The expression of one P_{3A}-type ATPase gene (*MD15G1108400*) was significantly upregulated in response to alkaline stress. Furthermore, a subcellular localization assay indicated that MD15G1108400 is targeted to the plasma membrane. These results imply that MD15G1108400 may be involved in responses to alkaline stress. Our data provide insights into the molecular characteristics and evolutionary patterns of the apple P_{3A}-type ATPase gene family and provide a theoretical foundation for future in-depth functional characterizations of P_{3A}-type ATPase genes under alkaline conditions.

Keywords: Apple; P_{3A}-type ATPase; evolutionary pattern; expression pattern; subcellular localization; alkaline stress

1. Introduction

Apple (*Malus* × *domestica* Borkh.) is one of the most important fruit crops and is primarily cultivated in arid and semiarid regions worldwide [1-3]. The stress conditions of salt-alkalinized soil are a major limitation to crop production worldwide. According to the statistical analysis by the Food and Agriculture organization (FAO) in 2005, about 830 million hectares of the land throughout the world are affected by salt, over half of which (434 million hectares) are alkaline [4,5]. In China, the characteristics of the northwestern Loess Plateau, which include abundant sunlight, deep soils, and considerable daily temperature variations, make it an ideal region for cultivating apple varieties. However, decreases in rainfall and increases in evaporation have resulted in the salinization and alkalinization of the soil in this region. These changes are detrimental for apple tree growth [1].



Therefore, selecting the optimal apple rootstock that is resistant to salt–alkaline stress is an important goal for apple breeding programs and apple producers.

The salinization and alkalinization of soil are the major global environmental and land resource issues [4,5]. In general, the salinization and alkalinization of soil frequently co-occurring and plant damages induced by the alkalinization (high pH) of soil due to excess NaHCO₃ or Na₂CO₃ are greater than those caused by neutral salts such as NaCl or Na₂SO₄ [6-8]. Salinity stress can seriously influence plant growth. Several studies have revealed that the overexpression of a gene encoding a P-type H⁺-ATPase lacking the autoinhibitory domain can increase the salt tolerance of plants [9]. Subsequent studies confirmed that the abundance of P-type H⁺-ATPases changes in salt-stressed plants [10,11]. Moreover, the posttranslational regulation of P-type H⁺-ATPase activity is essential for the salt tolerance of halophytic species [12]. High pH stress (alkalinization) causes more severe damage on plants by causing root cell injury and death and then leads to the whole plant wilting and even dying. In plants grown in alkaline soil, a deficiency in PKS5 (salt overly sensitive 2-like protein kinase 5) reportedly increases the tolerance to alkaline stress. Moreover, PKS5 can phosphorylate Ser-931 of P-type H⁺-ATPases, thereby inhibiting the enzymatic activity [13]. The P-type H⁺-ATPases activity remains a relatively low level without any stress and increases under saline-alkali stress [14]. In Arabidopsis, the P-type H⁺-ATPase AHA2 can be regulated by the Ca²⁺ Sensor SCaBP3/CBL7 and then alkali tolerance is increased [15]. In apple, the P-type H⁺-ATPases play a key component in regulation rhizosphere acidification [16]. Overall, P-type H⁺-ATPase (P_{3A}-type ATPase) genes are crucial for plant responses to salt-alkaline stress.

Genes encoding P-type ATPases are widely distributed and indispensable in most plant species, in which they are involved in transporting diverse small cations and phospholipids [17,18]. The "P-type" in the name of these enzymes is derived from "phosphorylated intermediate" [19–21]. The P-type ATPases consist of only one catalytic subunit, with considerable domain motions during transport. The structural characterization of plant P-type ATPases has revealed the presence of 8–12 transmembrane domains (TMDs), with the C and N termini of these enzymes exposed to the cytoplasm, as well as the phosphorylation and ATP-binding sites [22]. The P-type ATPase gene superfamily is one of the largest plant gene families [21,23]. These genes were divided into five major families (P1, P2, P3, P4, and P5) with subfamilies (P_{1B}, P_{2A}, P_{2B}, P_{3A}, and P_{3B}) based on the sequence identity and ion specificity [24]. For instance, all of the heavy metal pumps that share significant sequence similarities are classified into the P_{1B} subfamily; P_{1A}-ATPase represents the part of the bacterial K⁺ transport system; Ca²⁺ pumps belong to the P2A-ATPases and P2B-ATPases, Na⁺(H⁺)/K⁺ pumps in animals and Na⁺ pumps in fungi are considered as P_{2C}-ATPases and P_{2D}-ATPases, respectively. The plasma membrane H⁺-ATPases, which mediate ATP-dependent H⁺ transport across the plasma membrane or tonoplast, are classified in the P₃ family, P₄-ATPases are considered as putative lipid flippases [21,22,25]. The P_{1B}-ATPase transporters such as ATP7A and ATP7B (involved in the regulation of cytoplasmic copper homeostasis via pumping copper into the endomembrane system or out of cell) and mutation of these two genes cause neuropsychiatric disorders such as Menkes and Wilson disease [26]. The P₃ family comprises two main subfamilies, namely P_{3A} -type and P_{3B} -type [27,28]. The P_{3A} -type ATPase genes normally encode proton pumps that are located in the plasma membrane, where they mediate the transport of H⁺ across the plasma membrane and play crucial role in acidification of the aqueous fraction of the cell wall apoplast. In contrast, Ma10 belongs to the P_{3A}-type ATPase gene family, but the encoded enzyme is localized to the tonoplast, where it promotes the vacuolar acidification of apple fruit [29]. Moreover, PhPH1, which encodes a P-type ATPase belonging to the 3B subfamily in petunia, was believed to comprise only bacterial Mg²⁺ transporters [30]. Furthermore, PhPH1 is present in the tonoplast and cannot transport H⁺ independently, but it enhances the H⁺ transport activity in petunia via its interaction with PhPH5 [28].

Gene duplication and selection are two of the major driving forces of morphogenetic evolution. The duplicated genes are nonfunctional or they evolve novel functions (neofunctionalization) or undergo functional differentiation (subfunctionalization) [2,3,31]. During evolution and domestication,

a large proportion of the duplicated genes become nonfunctional because they accumulate deleterious mutations, whereas other duplicated genes undergo neofunctionalization; they are eventually preserved under positive selection pressure [31,32].

Apple, which is a member of the genus *Malus* in the family Rosaceae, has undergone one autopolyploidization event during its evolution [33,34]. Additionally, a whole genome duplication (WGD) during apple domestication increased the number of chromosomes from nine to seventeen. The duplicated genes arising from the WGD as well as random duplications and segmental duplications exhibited expressional and functional divergence [35]. Thus, deciphering the evolutionary divergence of duplicated apple genes should be investigated during the speciation process.

In this study, the P_{3A} -type ATPase gene family was analyzed using the doubled haploid GDDH13 draft genome [32]. Fifteen P_{3A} -type ATPase genes were identified, and the physical and chemical properties of the encoded enzymes were determined. The chromosomal localization, gene structure, gene loss and duplication, and evolution of the P_{3A} -type ATPase genes were also investigated. Additionally, we analyzed the expression of apple P_{3A} -type ATPase genes in various tissues and in response to alkaline stress. Moreover, the subcellular localization of one P_{3A} -type ATPase (*MD15G1104800*), which is responsive to alkaline stress, was conducted. Our results uncover the molecular characteristics and evolutionary patterns of the P_{3A} -type ATPase gene family and provide the theoretical basis for future in-depth elucidations of the biological functions of P_{3A} -type ATPases under alkaline conditions.

2. Materials and Methods

2.1. Plant Materials and Stress Treatments

The apomictic apple rootstock 'Fupingqiuzi' (*Malus prunifolia* (Willd.) Borkh.) was used in this study. Seedlings were initially cultivated as described by Wen et al. [1], after which the seedlings with 7–8 true leaves were moved to a hydroponics system and cultured according to the previous report by Wen et al. [1]. The hydroponics system included plastic basins ($35 \text{ cm} \times 25 \text{ cm} \times 10 \text{ cm}$) containing 5 L half-strength Hoagland nutrient solution [36] that was continuously aerated with an air pump. To simulate alkaline stress, the pH of the solution was adjusted to 9.96 ± 0.12 by adding 20 mM NaHCO₃ and 20 mM Na₂CO₃ (1:1 volume). The positive and negative controls consisted of seedlings that were not exposed to alkaline stress and the solution under alkaline stress without seedlings, respectively. The solutions were refreshed every 3 days, and the pH of the control and alkaline treatment solutions was adjusted as needed.

2.2. Identification and Phylogenetic Analysis of P_{3A}-Type ATPase Genes

The P_{3A}-type ATPase (*PhPH5*) amino acid sequences in petunia [25] and *Arabidopsis thaliana* [20] were retrieved from the NCBI (https://www.ncbi.nlm.nih.gov/) and The Arabidopsis Information Resource (TAIR) (http://www.arabidopsis.org/) databases, respectively. These sequences were used as queries for identifying homologous genes in basic local alignment search tool (BLAST) searches of the reference genomes of apple [34], pear (*Pyrus communis* L. "Bartlett") [37], peach (*Prunus persica* (L.) Batsch) [38], and black raspberry (*Rubus occidentalis* L.) [39] as described by Tatusov et al. [40] and Ma et al. [2,3]. Briefly, a local BLAST database was performed for each species with the BLAST 2.2.24 program (http://blast.ncbi.nlm.nih.gov/Blast.cgi), after which the putative orthologous genes were identified using an all-against-all protein BLAST search based on the reciprocal best similarity match.

Regarding the phylogenetic analysis, Clustal X2 (http://www.clustal.org/) was performed to align multiple amino acid sequences, after which a phylogenetic tree was generated based on the resulting dataset with MEGA (version 7) software (https://www.megasoftware.net/). Specifically, the neighbor-joining (NJ) and maximum-likelihood (ML) methods were used [41]. The parameters for the NJ method were as follows: positive deletion; bootstrap, 1000 replicates; and p-distance.

The parameters for the ML method were as follows: bootstrap, 1000 replicates; partial deletion; Jones-Taylor-Thornton; and branch-swap filter, very strong.

2.3. Analysis of the Structure, Conserved Motifs, and Transmembrane Domains of P_{3A} -Type ATPases

The exon–intron organization of the P_{3A} -type ATPase genes was visualized with the Gene Structure Display Server 2.0 (http://gsds.cbi.pku.edu.cn/). The coding sequences were aligned to their corresponding genomic sequences. The alignment was manually adjusted, if necessary. The conserved TMDs of the P_{3A} -type ATPases were predicted with the online program TMHMM version 2.0 (http://www.cbs.dtu.dk/services/TMHMM/#opennewwindow).

2.4. Estimation of Gene Duplication and Loss

Gene duplication and loss were identified based on manually checking each clade of the phylogenetic tree. Each clade was considered to include at least one P_{3A} -type ATPase gene from each species. The classification of gene family members in more than one clade and clades with more than one gene family member from each species were assumed to be the result of one or more gene duplications. In addition, gene loss was considered to have occurred if a P_{3A} -type ATPase gene from one or more species was absent in a clade. Gene duplication and loss were verified via searching the GenBank database with the BLASTp algorithm.

2.5. Analysis of Positive Selection

Clustal X2 was performed to align the amino acid sequences encoded by P_{3A} -type ATPase genes, and the resulting dataset together with the corresponding coding sequences were analyzed with PAL2NAL (http://www.bork.embl.de/pal2nal/). The PAL2NAL results were conducted to calculate the ω (dN/dS) values for the different branches derived from the phylogenetic tree based on the branch-specific algorithm in Codeml from PAML (version 4.9) [42]. For the one-ratio model, the ω value is the same for all branches, whereas in the two-ratio model, the ω values vary among different branches (background and foreground branches). The likelihood ration test was used to determine the optimized ω values for both models [43].

The P_{3A} -type ATPase gene coding sequences were aligned with Clustal X2, after which the alignment was manually adjusted. The resulting data were performed to estimate the ω value with the ML method of the KaKs Calculator package (version 2.0) [44]. Variations in the ω values were tested with a pairwise comparison as described by Ma et al. [2,3].

2.6. RNA Extraction and qRT-PCR Analysis

Five apple tissues (flowers at full bloom, roots, mature leaves, ripening fruits, and stems) were analyzed in a quantitative real-time polymerase chain reaction (qRT-PCR) assay. Total RNA was extracted with the RNAprep Pure Plant kit (Tiangen, Beijing, China). The TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix kit (TRANS, Beijing, China) was used to synthesize the first-stand cDNA. The subsequent qRT-PCR assay was completed with the SYBR Green I Master Mix (TaKaRa, Dalian, China) and the 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Relative gene expression levels were determined with the $2^{-\Delta\Delta Ct}$ method [45]. All analyses involved three biological replicates. A previously described actin gene [46] was used as a reference control. Details regarding the qRT-PCR primers are listed in Table S1.

2.7. Measurement of the Solution pH and Analysis of the Transcriptome

After a 15-day cultivation, seedlings were moved to a new hydroponics system, and the solution pH was adjusted to 9.96 \pm 0.12. Root samples were collected at 0, 2, 4, 8, 24, and 48 h and then immediately frozen in liquid nitrogen and then stored at –80 °C for the subsequent RNA extraction, qRT-PCR analysis, and transcriptome sequencing. The RNA extraction and qRT-PCR analysis were

the same as described above. The pH of the solution at various time-points was measured with a pH meter (Denver Instrument, USA). The RNA sequencing (RNA-seq) libraries were constructed as described by Lou et al. [47] by the Biomarker Biotechnology Corporation (Beijing, China). The libraries were sequenced with the HiSeqTM 2000 platform (Illumina, San Diego, CA, USA) (150-bp paired-end reads). Low quality reads, such as adapter sequencing, unknown nucleotides (N) >5%, or Q20 <20%, were removed. The Tophat2 software (version 2.1.1; http://ccb.jhu.edu/software/tophat) was used to map clean reads, which were filtered from the raw reads to the draft genome of the diploid apple cultivar "Golden Delicious" [34]. The potential duplicate molecules were removed based on the aligned records from the aligners in BAM/SAM format. Gene expression was estimated based on the reads per kilobase per million reads (RPKM) by the Cufflinks software (version 2.2.0; http://cole-trapnell-lab.github.io/cufflinks/). The DESeq and Q-value were used to evaluate differential gene expression. Gene abundance differences between samples were calculated based on the ratio of the FPKM values. The threshold of the *p*-value in multiple tests were used to compute the significance of the differences based on the false discovery rate (FDR) control method.

2.8. Subcellular Localization of Apple P_{3A}-Type ATPases in Arabidopsis thaliana Protoplasts

The complete coding region of an apple P_{3A}-type ATPase gene (*MD15G1108400*) was amplified from cDNA derived from the apple rootstock "Fupingqiuzi" with the following primer pair: 5'-ATGGGCCCACTCGTGGATC-3' and 5'-TCAAACTGTATAATGTTGTTGGATTGTA-3'. The PCR product was purified and inserted into the pEASY-Blunt vector (Transgene, Beijing, China). The complete coding sequence was then inserted into the expression vector pHBT-GFP-NOS under the control of the *Cauliflower mosaic virus* 35S promoter, thus producing the constitutive fusion protein MD15G1108400-GFP. The recombinant plasmid was transformed into *A. thaliana* protoplasts according to the PEG-calcium transfection protocol described by [48]. A plasma membrane marker (pm-rk, CD3-1007) labeled with mCherry was used as the control [49]. The corresponding sequence was included in cotransformation experiments with the MD15G1108400-GFP fusion construct. The fluorescence from the GFP in *A. thaliana* protoplasts was detected with the TCS SP8 confocal laser scanning microscope (Leica, Germany) at 12–24 h after the transfection.

2.9. Data Analysis

The statistical analysis was performed using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). Two-tailed tests were used to estimate the significant difference values. Unless otherwise stated, differences were significant at p < 0.01.

3. Results

3.1. Identification, Characterization, and Genomic Distribution of Apple P_{3A}-Type ATPase Genes

Fifteen putative P_{3A} -type ATPase genes were identified in the draft genome of the diploid apple cultivar "Golden Delicious" (Table S2). The physical and chemical properties of the P_{3A} -type ATPases were characterized with ProtParam (https://web.expasy.org/protparam/), and the results are presented in Table 1. The P_{3A} -type ATPases consisted of 930 (MD17G1220200) to 1032 (MD15G1108400) amino acids, with predicted molecular weights ranging from 102.12 kDa to 113.75 kDa. The theoretical isoelectric point ranged from 5.37 (MD17G1155800) to 8.64 (MD17G1220200) and the grand average of hydropathicity (GRAVY) ranged from 0.02 (MD15G1108400) to 0.19 (MD03G1197800). Additionally, the instability index of the P_{3A} -type ATPases ranged from 32.68 (MD09G1223600) to 40.21 (MD15G1108400) and the aliphatic index ranged from 100.4 (MD15G1108400) to 107.25 (MD03G1197800). We also predicted the conserved TMDs of the P_{3A} -type ATPases. The results indicated that all P_{3A} -type ATPase gene family members encode 7–10 TMDs (Figure S1), with most encoding eight or nine TMDs.

Gene ID	Animo Acids	MW (kDa)	pI	GRAVY	Instability Index	Aliphatic Index	TMD
MD17G1155800	950	104.38	5.37	0.15	38.08	106.37	8
MD09G1171300	948	104.45	5.50	0.15	36.68	107.10	9
MD15G1108400	1032	113.75	6.86	0.02	40.21	100.40	8
MD08G1130200	954	105.03	7.24	0.04	34.55	101.45	10
MD13G1247200	955	105.21	6.51	0.09	36.47	101.27	8
MD06G1186400	956	105.26	5.99	0.12	38.33	105.33	8
MD16G1254200	955	105.01	6.13	0.09	36.11	103.11	9
MD14G1192600	956	105.18	6.10	0.11	38.51	104.62	8
MD02G1011600	947	103.68	6.05	0.09	34.82	101.44	9
MD09G1223600	954	104.81	6.17	0.07	32.68	103.14	9
MD17G1046600	956	105.31	6.45	0.12	35.64	104.01	8
MD02G1216000	991	108.87	7.06	0.08	34.10	101.40	8
MD04G1058300	973	106.86	6.25	0.17	35.45	107.07	9
MD03G1197800	966	105.84	6.17	0.19	34.59	107.25	7
MD17G1220200	930	102.12	8.64	0.07	33.80	103.84	8

Table 1. Detailed information of physiological and biochemical characteristics of the apple P_{3A}-Type ATPases proteins.

MW: Molecular weight, pI: theoretical isoelectric point, GRAVY: grand average of hydropathicity, TMD: Transmembrane domains.

An analysis of the genomic distribution of the 15 P_{3A} -type ATPase genes (Figure 1A) revealed that 11 of these genes were located on four homologous pairs of chromosomes (13 and 16, 9 and 17, 8 and 15, and 6 and 14). Additionally, chromosome 2 included two P_{3A} -type ATPase genes, whereas chromosomes 3 and 4 had one P_{3A} -type ATPase gene each. Chromosome 17 carried the most P_{3A} -type ATPase genes (three), followed by chromosomes 9 and 2 (two) and eight chromosomes with one P_{3A} -type ATPase gene. Moreover, the WGD and segmental duplication that occurred during apple domestication were important events that contributed to the expansion of the apple gene family [30,31]. Many of the duplicated genes were retained within the apple genome [2]. To determine whether the P_{3A} -type ATPase genes were influenced by diverse selection pressures and evolutionary rates, we estimated the selection pressure and evolutionary rates based on Ka (nonsynonymous mutation), Ks (synonymous mutation), and the Ka/Ks ratio (ω). In general, $\omega > 1$ indicates positive selection, $\omega < 1$ corresponds to purifying selection, and $\omega = 1$ suggests neutral evolution. Thus, we examined the selection pressures on the duplicated P_{3A} -type ATPase genes. The ω values for each duplicated gene pair are provided in Figure S2. The ω value of all P_{3A} -type ATPase gene pairs was less than 1, indicating the genes evolved under strong purifying selection pressure during apple domestication.

To more thoroughly characterize the exon/intron organization of the P_{3A} -type ATPase genes, exon–intron diagrams were generated according to the coding and genomic sequences (Figure 1B). The number of exons ranged from 13 to 21. Of the 15 analyzed P_{3A} -type ATPase genes, five contained 20 exons, three contained 16 exons, three contained 21 exons, two (*MD09G1223600* and *MD17G1220200*) contained 14 exons, one (*MD02G1011600*) contained 13 exons, and one (*MD15G1108400*) contained 17 exons. These findings suggested that P_{3A} -type ATPase genes gained and lost exons during apple domestication.



Figure 1. Genes encoding P_{3A} -Type ATPase genes in apple genome. (**A**) Chromosomal localization of apple P_{3A} -Type ATPase. (**B**) Exon/intron organization of apple P_{3A} -Type ATPase. Yellow boxes indicate exons; black line indicates introns.

3.2. Phylogenetic Relationship Analysis, Gene Duplication, and Gene Loss of the P_{3A} -Type ATPase Gene Family in Four Rosaceae Species

To analyze the evolutionary relationships among P_{3A} -type ATPase genes from apple, peach, black raspberry, and pear, we constructed a phylogenetic tree of P_{3A} -type ATPase genes with the NJ and ML methods, which yielded very similar topologies (Figure 2). The results indicate that the P_{3A} -type ATPase genes are classified into six clades. All clades were clustered with bootstrap values greater than 50%, and the P_{3A} -type ATPase genes from each species were clearly separated in each clade. The 15 P_{3A} -type ATPase genes were grouped into clades as follows: clades I (two genes), IIa (one gene), IIb (three genes), IIc (two genes), IId (three genes), and IIe (four genes).



Figure 2. Phylogenetic tree derived from amino acid sequences of P_{3A} -Type ATPase genes in Rosacease genomes based on NJ method. The red triangle and square represent recent and old duplication events. Gene loss is shown by the red dotted line. All the bootstrap values are higher than 50% and indicated near branched lines.

Gene duplication events were classified as recent or old based on the evolutionary timeline [50]. Gene duplications that occurred within species were considered recent duplications, whereas gene duplications that occurred prior to the species radiation of Rosaceae were categorized as old duplications [51]. An examination of P_{3A} -type ATPase genes revealed two recent duplications and seven old duplications, indicating that all of the P_{3A} -type ATPase genes, except for *PCP019260.1*, *PCP019257.1*, and *PCP019253.1*, existed prior to the species radiation of Rosaceae. Moreover, old duplications were detected in clades IIb, IId, and IIe, implying these clades underwent at least one duplication event before the species radiation of Rosaceae. Furthermore, a recent loss of a *Rubus occidentalis* gene was detected in clade IIa (Figure 2). There was no evidence of an old gene loss event for the P_{3A} -type ATPase genes.

3.3. Estimation of Selection Pressures Affecting P_{3A}-Type ATPase Genes in Four Rosaceae Species

To assess whether the P_{3A} -type ATPase genes in each clade are controlled by different evolutionary constraints, the pairwise ω (d_N/d_S) values within each clade for all tested P_{3A} -type ATPase genes were estimated using pairwise comparison model (Table S3). In addition, we compared the pairwise ω values of the P_{3A} -type ATPase genes in each clade. The following pattern for the average ω values of the P_{3A} -type ATPase genes among six clades was observed: clade I > clade IIc > clade IIa > clade IIe > clade IIb (Figure 3). However, although the P_{3A} -type ATPase genes in clade I had the highest ω values (p < 0.01), the differences in the ω values for the P_{3A} -type ATPase genes among the other clades were not significant (Figure 3). Overall, the pairwise ω values indicated a significant difference in the evolutionary rates of the P_{3A} -type ATPase genes in each clade. Additionally, the ω values of members of the same clade were less than 1, indicating that the genes had evolved mainly under strong purifying selection pressure.



Figure 3. Mean ω values of P_{3A}-Type ATPase genes in different clades. Different lowercase letters represent significant differences among clades (*t*-test, LSD test at *p* < 0.01). Error bars show the SE of means.

To clarify the evolutionary rate of each P_{3A} -type ATPase clades, which was derived from a recent common ancestor, the branch-specific model of PAML was used to calculate the ω values (Ka/Ks) for each P_{3A} -type ATPase clades. As presented in Table 2, the one-ratio model was favored for two pairs of P_{3A} -type ATPase clades, namely IId and IIe, as well as IIc and IId + IIe (p > 0.05). The two-ratio model was favored for the other pairs of P_{3A} -type ATPase clades (p < 0.01).

Clades		Null Hypothesis		Alternative Hypothesis			LRT	
Α	В	–In L	ω	–In L	ω1	ω2	Statistic	p
IId	IIe	6436.65	0.14	6435.15	0.15	0.12	3.00	>0.05
IIc	(IId+IIe)	8149.43	0.14	8148.49	0.17	0.14	1.88	>0.05
IIb	(IIc+IId+IIe)	10,632.66	0.13	10,624.97	0.15	0.09	15.38	< 0.01
IIa	(IIb+IIc+IId+IIe)	10,466.18	0.13	10,458.89	0.15	0.10	14.58	< 0.01
Ι	(IIa+IIb+IIc+IId+IIe)	10,176.17	0.18	10,145.07	0.51	0.15	62.20	< 0.01

Table 2. The statistic and parameters from Branch model of PAML based on likelihood-ratio test (LRT).

3.4. Analysis of P_{3A}-Type ATPase Gene Expression in Various Apple Tree Tissues

An examination of the P_{3A} -type ATPase gene expression patterns in five apple tissues (roots, stems, mature leaves, flowers at full bloom, and ripening fruits) (Figure 4, Table S4) revealed significant differences among the tested tissues and four distinctive expression patterns were identified. Nine genes were expressed in all five tested tissues. Additionally, eight genes were most highly expressed in ripening fruits, with a relative expression level greater than 1.0. Similarly, 12, 15, 8, and 10 apple P_{3A} -type ATPase genes were most highly expressed in the mature leaves, flowers at full bloom, roots, and stems, respectively. Furthermore, two genes (*MD03G1197800* and *MD02G1011600*) were specifically expressed in the mature leaves and flowers at full bloom, suggesting these genes play crucial roles in mature leaves and flowers.



Figure 4. Expression profiles of apple P_{3A} -Type ATPase genes in five kinds of tissue, Fruit (ripening fruit), Leaf (mature leaf), Flower (full-bloom flower), Root, and Stem. The heat map was generated via Mev software using relative expression data, which are expressed as normalized log^2 transformed values. The hierarchical cluster indicates the differential expression pattern.

3.5. Transcriptome Analysis and Identification of Candidate Genes Responsive to Alkaline Stress

A transcriptome analysis of *M. prunifolia* roots exposed to alkaline conditions for 0 or 24 h was completed to identify candidate genes responsive to alkaline stress. Six RNA-seq libraries were constructed, with the libraries representing samples collected at 0 and 24 h designated as C and T, respectively (Table S5). The Q20 and Q30 value ranged from 97.41% to 97.50% and 91.54% to 91.71%, respectively, indicating the high quality of the RNA sequencing data. The GC content of the RNA

sequencing reads ranged from 46.78% to 47.62%. The number of clean reads for each library ranged from 43,022,734 to 57,795,922, with an average of 49,625,614. The size of the clean reads for each sample ranged from 6.44 Gb to 8.65 Gb, with an average of 7.42 Gb. Approximately 60% of the clean reads were mapped to the draft genome of the diploid apple cultivar "Golden Delicious" [34].

Of the 34,095 genes identified as expressed in the tested root samples, 492 exhibited 2-fold or greater differences in expression levels between the roots collected at 0 and 24 h after initiating the alkaline treatment. Gene expression analysis indicated that the expression levels of 245 and 247 genes were significantly up- and downregulated, respectively, in the roots between the 0 h and 24 h time-points (Figure 5A). Additionally, the pH of the hydroponics solution was determined at 0 and 24 h after initiating the alkaline treatment (Figure 5B). The average pH of the hydroponics solution was significantly lower at 24 h than at 0 h. Thus, we concentrated on the candidate genes that were expressed in the roots at high and low levels at 24 and 0 h, respectively. We subsequently functionally annotated the 245 upregulated genes based on the TAIR database (https://www.arabidopsis.org/). One gene (*MD15G1104800*) encoding a P_{3A} -type ATPase that mediates H⁺ transport activity was identified. The MD15G1104800 expression level was approximately 2.5-fold higher in the roots collected at 24 h than in the roots collected at 0 h (Figure 5C).



Figure 5. Transcriptome analysis of roots of *Malus prunifolia* (Willd.) Borkh. under alkaline treatment at 0 h (C) and 24 h (T). (A): Hierarchical clustering which represents the differential expression pattern indicated up- and downregulated genes. The numbers 1, 2, and 3 represent three biological replicates. (B): The change of hydroponics solution pH under alkaline stress. C and T represent the treat time 0 h and 24 h. (C): Digital expression of candidate gene *MD15G1108400* based on transcriptome data under alkaline treatment at 0 h (C) and 24 h (T). The signification differences (*t*-test, LSD test at *p* < 0.01) among C and T were indicated by different lowercase letters.

3.6. Expression Profiles of Apple P_{3A} -Type ATPase Genes in the Roots Under Alkaline Conditions

A qRT-PCR assay was completed to investigate the expression profiles of apple P_{3A} -type ATPase genes in the roots under the control and alkaline condition (Figure 6). The expression of six apple P_{3A} -type ATPase genes was undetectable in the roots at all examined time-points. The expression levels of nine P_{3A} -type ATPase genes remained almost unchanged throughout all the examined time-points under the control condition. In addition, all the expressed P_{3A} -type ATPase genes exhibited a higher expression level throughout all the examined time-points under the alkaline stress, compared to control, excepted at 8 h and 48 h of the *MD16G1254200*, 2 h of *MD14G1192600*, and 48 h of *MD06G1186400* and *MD13G1247200*.



Figure 6. Expression profiling of the apple P_{3A} -Type ATPase genes in roots of *Malus prunifolia* (Willd.) Borkh. under control and alkaline condition at different treatment times. The values indicate the average of three biological replicates. Error bars indicate the SD of the mean, and the double asterisks show significant differences between control and treatments (*t*-test, *p* < 0.01).

Under the alkaline condition, three apple P_{3A} -type ATPase genes (*MD08G1130200*, *MD09G1171300*, and *MD17G1046600*) were similarly expressed, with peak expression levels in the roots at 24 h, which was followed by a significant decrease at 48 h. The expression levels of two P_{3A} -type ATPase genes (*MD14G1192600* and *MD17G155800*) increased from 0 to 8 h and then decreased from 8 to 48 h. In contrast, complex and diverse expression trends were observed for *MD06G1186400*, *MD13G1247200*, and *MD16G1254200* under alkaline conditions; however, the expression levels of all three genes were lowest at 48 h. The expression of another P_{3A} -type ATPase gene (*MD15G1108400*) was highly upregulated in response to alkaline stress.

To further characterize the P_{3A}-type ATPase genes responsive to alkaline stress, the average pH of the hydroponics solution was determined with a pH meter (Figure 7). The average pH of the hydroponics solution with the apple rootstock "Fupingqiuzi" decreased from 0 to 48 h. The average pH in the absence of the apple rootstock was similar but decreased a little following the alkaline treatment. To clarify the relationship between P_{3A}-type ATPase gene expression and the pH of the hydroponics solution, Spearman correlation coefficients for the P_{3A}-type ATPase gene expression levels and the hydroponics solution pH were calculated. The results indicated that only *MD15G1108400* expression was significantly negatively correlated with the hydroponics solution pH ($R^2 = 0.90$, p < 0.01), implying that *MD15G1108400* is important for responses to alkaline stress.



Figure 7. The time course of in hydroponics solution pH under alkaline stress for 48h. The points, triangles, and squares represent mean value \pm SD of three biological repeats. CK: the hydroponics solution with the apple rootstock without alkaline stress.

3.7. Subcellular Localization of MD15G1108400

To determine the subcellular localization of the enzyme encoded by *MD15G1108400*, an MD15G1108400-GFP fusion construct was transiently expressed in *A. thaliana* mesophyll protoplasts. Confocal microscopy indicated that the MD15G1108400-GFP fusion protein was targeted to the plasma membrane (Figure 8a). To further clarify the plasma membrane localization of MD15G1108400, a colocalization experiment was conducted with the pm-rk standard plasma membrane marker (Figure 8d). The fluorescence of the MD15G1108400-GFP fusion protein completely overlapped the mCherry fluorescence of the plasma membrane marker (Figure 8e). These results indicated that MD15G1108400 is localized to the plasma membrane.



Figure 8. Subcellular localization of the MD15G1108400-GFP fusion protein in *Arabidopsis* mesophyll protoplast. From **a** to **e**: (**a**) MD15G1108400-GFP fusion protein fluorescence, (**b**) chlorophyll auto-fluorescence, (**c**) optical photomicrographs (bright field), (**d**) pm-rk (plasma membrane marker: CD3-1007) protein fluorescence, and (**e**) overlay of bright and fluorescence illumination.

4. Discussion

The P_{3A} -type ATPase genes are important for various physiological processes, including plant growth, stomatal movement, and stress responses. With the release of whole genome sequences, members of the P_{3A} -type ATPase gene family have been identified and investigated in many species.

For example, 11 P_{3A} -type ATPase genes were identified in the model plant species *A. thaliana* [52]. A high-quality de novo "GDDH13" draft genome sequence was recently released [34], which enabled the identification and investigation of the P_{3A} -type ATPase gene family. In this study, 15 members of the P_{3A} -type ATPase gene family were identified in the GDDH13 apple reference genome and then comprehensively characterized. The resulting data provide the foundation for future investigation on the roles for the identified apple genes.

Domesticated apple is an autopolyploid species with 34 diploid chromosomes [33,34]. During apple domestication, a WGD as well as segmental and random duplications altered the apple genome [2,33,34,53]. In this study, we identified 15 P_{3A} -type ATPase genes, 11 of which were detected on eight pairs of homologous chromosomes (Figure 1A). For instance, chromosomes 6 and 14 (homologous pair) contain one P_{3A} -type ATPase gene. Similar results were obtained for the other homologous chromosome pairs (13 and 16, 9 and 17, and 8 and 15) (Figure 1A). These findings indicate that the duplication of the apple P_{3A} -type ATPase genes is related to the WGD. Moreover, the tandem duplication of P_{3A} -type ATPase genes was not observed. A comparison of chromosomes 9 and 17 revealed that one P_{3A} -type ATPase gene on chromosome 17 was not detected on chromosome 9. Similar findings were obtained for homologous chromosome pairs 2 and 15, 3 and 11, and 4 and 12 (Figure 1A) [2,33,34,53]. Thus, the duplicated genes resulting from a WGD rapidly diverged and many were subsequently lost.

In this study, a phylogenetic analysis grouped 15 apple P_{3A} -type ATPase genes and the P_{3A} -type ATPase genes from three other Rosaceae species into six subclades (Figure 2). Specifically, the apple P_{3A} -type ATPase genes were clustered with the P_{3A} -type ATPase genes from pear (*P. communis* L. "Bartlett"), indicating an intimate genetic relationship between P_{3A} -type ATPase genes from apple and pear. Apple, pear, and peach genomes contain 15, 19, and 15 P_{3A} -type ATPase genes, respectively, despite the fact that the apple genome (approximately 750 Mb) and pear genome (approximately 512 Mb) are more than 2-times larger than the peach genome (approximately 256 Mb) [38]. The similarity in the number of P_{3A} -type ATPase genes. To more comprehensively investigate the evolution of P_{3A} -type ATPase genes, the gene structures and encoded conserved motifs were studied. The number of exons and the conserved motifs were similar for most of the analyzed P_{3A} -type ATPase genes (Figure 1B, Figure S3), and the ω values for the duplicated genes derived from polyploidization events were less than 1 (Figure S2). These results imply the apple P_{3A} -type ATPase genes evolved slowly and under purifying selection pressure during domestication.

Gene duplication is considered as the major driving force for the development of novel genetic functions enabling plants to adapt to environmental conditions [31,32]. The expression levels of most duplicated genes are highly diverse [35]. In the current study, we examined the expression patterns of apple P_{3A} -type ATPase genes. Diverse expression patterns were observed for two homologous P_{3A} -type ATPase genes (*MD09G1223600* and *MD17G1220200*) located on chromosomes 9 and 17 (homologous pair) (Figures 1A and 4). These two genes were expressed in ripening fruits, mature leaves, flowers at full bloom, and stems. However, *MD09G1223600* was most highly expressed in flowers at full bloom, whereas *MD17G1220200* was most highly expressed in stems. Similar expression patterns were observed for the following homologous gene pairs: *MD08G1130200* and *MD15G1108400*, *MD06G1186400* and *MD14G1192600*, *MD09G1171300* and *MD17G1155800*, and *MD16G1254200* and *MD13G1247200*. Thus, the expression patterns of the duplicated P_{3A} -type ATPase genes resulting from a WGD were conserved during apple domestication. This finding is consistent with the results of a previous study that confirmed that the P_{3A} -type ATPase gene expression levels are surprisingly consistent regardless of the environmental conditions (e.g., nutrient availability) [54].

A previous investigation proved that P_{3A} -type ATPase genes are crucial for various physiological processes such as salt tolerance, stomatal opening, etc., because they generate a transmembrane H⁺ gradient (ΔpH) [52]. Alkaline stress is characterized by a high soil pH due to the hydrolysis of Na₂CO₃ and NaHCO₃ [55]. Plants must be able to mitigate the adverse effects of alkaline soil because

they are sessile organisms. Thus, plants have evolved several alkaline stress tolerance mechanisms, including the accumulation of osmoprotectants to facilitate water uptake and the secretion of organic acids to acidify the rhizosphere [56–59]. In plants, the acidification of the rhizosphere was related with an increase in P_{3A}-type ATPase activity to extrude protons. P_{3A}-type ATPase genes play crucial roles in transporting protons and pumping H⁺ out of the roots, thus plants can be able to acidify the root apoplast and increase the tolerance to environmental stress [60-62]. In the current study, we investigated the responses of P_{3A} -type ATPase genes to alkaline stress. We determined that the expression level of one P3A-type ATPase gene (MD15G1108400) is highly correlated with the pH of a hydroponics solution (p < 0.01). All P_{3A}-type ATPase genes are considered to be important for transporting H⁺ [63]. A P_{3A}-type ATPase Ma10 which located to the tonoplast, has the capacity for proton pumping and plays a vital role in vacuolar acidification of apple fruit [29]. Moreover, gene functions are closely associated with the subcellular localization of the encoded protein [2]. The subcellular localization of the MD15G1108400-GFP fusion protein revealed it is present in the plasma membrane (Figure 8). Thus, we speculate that MD15G1108400 is involved in plant responses to alkaline stress. In plants, the P_{3A}-type ATPase genes induced acidification of the cell wall apoplast by H⁺ excretion play critical roles in plant growth, and two proton pumps (AtAHA1 and AtAHA2) which belong to the P_{3A} -type ATPase gene family are necessary to regulate apoplastic pH [64]. Therefore, we hypothesize that the P3A-type ATPase encoded by MD15G1108400 can enhance the transport of H⁺ across the plasma membrane, thereby decreasing the rhizosphere pH to protect plants from alkaline stress.

5. Conclusions

In this study, the evolutionary pattern of the P_{3A} -type ATPase gene in apple was investigated. In general, a total of 15 P_{3A} -type ATPase genes were identified in the draft genome of the diploid apple cultivar "Golden Delicious". The chromosomal localization indicated that gene duplication of apple P_{3A} -type ATPase is related to the WGD and many duplicated genes from a WDG were lost. The ω value indicated that the duplicated P_{3A} -type ATPase genes evolved under strong purifying selection pressure during apple domestication.

The phylogenetic relationship analysis was performed using the amino acid sequences of P_{3A} -type ATPase genes from four Rosaceae species, and the P_{3A} -type ATPase genes are classified into six clades. Base on the phylogenetic tree, two recent duplications, seven old duplications, and one recent loss of a *Rubus occidentalis* gene were identified. In addition, significant differences were observed in the evolutionary rate of the P_{3A} -type ATPase genes' members and clades. Furthermore, expression profiling of P_{3A} -type ATPase genes exhibited significant divergence among five apple tissues. The expression of one gene, *MD15G1104800*, which located to the plasma membrane, was highly upregulated in response to alkaline stress and was significantly negatively correlated with the hydroponics solution pH. Thus, our results indicated that *MD15G1104800* is a likely candidate gene involved in alkaline stress.

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4907/11/3/292/s1, Figure S1: A schematic diagram showing the conserved transmembrane domain of P_{3A} -Type ATPase. TMD: transmembrane domain. The dotted line represents the boundary of N-terminal and C-terminal, **Figure S2**: Estimation of ω values of apple P_{3A} -Type ATPase genes using the ML method, Table S1: Primer sequences used for quantitative real-time PCR. Table S2: The CDS sequences and deduced amino acid sequences of P_{3A} -type ATPase genes. Table S3: Estimation of ω values of P_{3A} -Type ATPase gene using the ML method. The ω value higher than one is highlighted in black bold. Table S4: Expression levels of apple P_{3A} -Type ATPase genes in five different tissues from apple, Table S5: Summary of RNA-seq data for roots of *Malus prunifolia* (Willd.) Borkh. under alkaline treatment at 0 h (C) and 24 h (T).

Author Contributions: B.M., Y.Y. and F.M. conceived and designed the experiments. B.M., M.G. and L.Z. performed the experiments. Y.Y., H.Z., L.Z., J.S. and C.L. analyzed the data. B.M. and Y.Y. wrote the manuscript. M.L. and F.M. revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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