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Genome-Wide In Silico Analysis and Expression Profiling of Phospho*enol*pyruvate Carboxylase Genes in Loquat, Apple, Peach, Strawberry and Pear

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Abstract: Phospho*enol*pyruvate carboxylase (*PEPC*) genes have multiple potential roles in plant metabolism such as regulation and accumulation of organic acids in fruits, movement of guard cells and stress tolerance, etc. However, the systematic identification and characterization of *PEPC* genes in Rosaceae species i.e., loquat, apple, peach, strawberry, and pear are yet to be performed. In present study, 27 putative *PEPC* genes (loquat 4, apple 6, peach 3, strawberry 9, and pear 5) were identified. To further investigate the role of those *PEPC* genes, comprehensive bioinformatics and expression analysis were performed. In bioinformatic analysis, the physiochemical properties, conserved domains, gene structure, conserved motif, phylogenetic and syntenic analysis of *PEPC* genes were performed. The result revealed that the PEPcase superfamily domain was conserved in all examined PEPC proteins. Most of the PEPC proteins were predicted to be localized in cytonuclear. Genomic structural and motif analysis showed that the exon and motif number of each *PEPC* gene ranged dramatically, from 8 to 20, and 7 to 10, respectively. Syntenic analysis indicated that the segmental or whole-genome duplication played a vital role in extension of *PEPC* gene family in Rosacea species. The K_a and K_s values of duplicated genes depicted that *PEPC* genes have undergone a strong purifying selection. Furthermore, the expression analysis of *PEPC* genes in root, mature leaf, stem, full-bloom flower, and ripened fruit of loquat, apple, peach, strawberry, and pear was performed. Some genes were differentially expressed in aforementioned plant tissues, signifying their role in plant metabolism. This study provides the first genome-wide identification, characterization, and expression profiling of *PEPC* gene family in Rosaceae species, and provides the foundation for further functional analysis.

Keywords: gene duplication; *PEPC*; synteny; malic acid; conserved motif; promoter

1. Introduction

Phospho*enol*pyruvate (PEP) carboxylase (PEPC) enzyme can be widely found among bacteria, archaea, green algae, cyanobacteria, protozoa, and vascular plants, while absent

in animals and fungi cells [1,2]. PEPC is used in the primary fixation reaction for photosynthetic CO_2 assimilation in C_4 photosynthesis as well as crassulacean acid metabolism (CAM); which take place in mesophyll cells in the presence of bicarbonate (HCO_3^-) and Mg^{2+} to catalyze an irreversible β-carboxylation reaction of PEP to produce oxaloacetate (OAA) and inorganic phosphate (Pi) [1]. Besides its central role in atmospheric CO_2 fixation during photosynthesis, PEPC is also known to have a wide range of non-photosynthetic activities, such as carbon-nitrogen interaction support and fruit ripening [2], seed germination and formation [2,3], and controlling guard cell metabolism for better stomatal functioning [4]. In addition among non-photosynthetic tissues and in leaves of C_3 plants, PEPC is important for anaplerotic to replenishing tricarboxylic acid (TCA) cycle with intermediates which are consumed in different biosynthetic pathways and N-assimilation [1,2].

PEPCs are important, as they transport organic acids across membranes [5]. With the help of such transporter proteins, organic acids play a critical role in plants' primary metabolism and help plants in adaptation according to changing environments, such as stomatal movement, stress responses, and pH regulation [6–9]. During the past two decades, several experiments have concluded that organic acids specifically malate were released from plant roots to counter the metal toxicity and maintain fruit pH [10,11]. Since the whole-genome sequences of many plant species have been released, various PEPC proteins have been effectively recognized and examined in plants including *Arabidopsis thaliana* [12], *Hordeum vulgare* [13], *Lotus japonicus* [14], *Solanum lycopersicum* [15], *Solanum tuberosum* [16], and *Triticum aestivum* [17]. For instance, three *PEPC* genes (*PPC1- PPC3*) were characterized in *A. thaliana* [12]. Three dicot C_4 *PEPC* (*ppc-A*, *ppc-B*, *ppc-C*) genes were analyzed in *Flaveria* (Asteraceae), with the *ppc-A* gene being identified as the gene recruited for use in the C_4 photosynthetic pathway [18]. The metabolic functions of *PEPC* gene family have rarely been reported in the Rosaceae.

Recently, loquat's (*Eriobotrya japonica* Lindl.) genome was sequenced using 3rd generation sequencing technology via Nanopore and Hi-C technology [19]. The genomes of Rosaceae species including apple (*Malus domestica* Borkh.), peach (*Prunus persica*), strawberry (*Fragaria x ananassa* Duch.) and pear (*Pyrus communis* L.) were also accessible. By utilizing the information available, an opportunity to analyze the *PEPC* gene family in Rosaceae species was undertaken as publishing the first report on *PEPC* gene family members in loquat, apple, peach, strawberry and pear. In this study, we identified 27 *PEPC* genes in the genomes of aforementioned five Rosaceae species; phylogenetic relationships, gene duplication and subcellular localization were investigated, and expression of related genes in roots, stem, leaves, flowers and fruits of aforementioned Rosaceae species was observed. Our findings explore molecular features and evolutionary pattern of *PEPC* gene family and provide groundwork to functionally characterize *PEPC* genes in Rosaceae species.

2. Materials and Methods

2.1. Identification and Characterization of PEPC Genes

The loquat (*Eriobotrya japonica*) genome sequence was downloaded from the GigaScience Database (http://gigadb.org/dataset/view/id/100711, accessed on 22 December 2020) [19]. The apple, peach, and strawberry genome sequences [20] were downloaded from Phytozome (http://phytozome.jgi.doe.gov/pz/portal.html, accessed on 16 June 2021), and the genome sequences of pear [21] was downloaded from the Genome Database for Rosaceae (GDR) (http://www.rosaceae.org/, accessed on 16 June 2021). While, peptide sequences of *Arabidopsis's* [22] *PEPC* genes were retrieved from TAIR (https://www.arabidopsis.org/, accessed on 22 December 2020), and *Arabidopsis's* sequence was used as a query to perform BLAST against the aforementioned species. Furthermore, Pfam online database and HMMER3 software package were used for PEPC domain (PF00311) search and HMM file construction, respectively [23,24]. Using local protein databases, HMM searches were performed of the aforementioned species with HMMER3. Moreover, we checked the physical locations of all candidate *PEPC* genes and rejected redundant sequences with the same chromosome location. For verification of the presence of PEPC domains, all obtained

PEPC protein sequences were analyzed again in the Pfam database via SMART program (http://smart.embl-heidelberg.de/, accessed on 8 January 2021), while proteins lacking PEPC domain were removed.

The physicochemical properties of PEPC proteins were calculated using ExPASy Proteomics Server (http://web.expasy.org/compute_pi/, accessed on 9 January 2021). The WoLF PSORT web server (https://wolfpsort.hgc.jp/, accessed on 9 January 2021) and CELLO version 2.5, subcellular localization predictor, (http://cello.life.nctu.edu.tw/, accessed on 9 January 2020) were used to predict subcellular localization of PEPC proteins. The 3D-structure models of PEPC proteins were predicted through the online tool i-Tasser (https://zhanggroup.org/I-TASSER/, accessed on 26 June 2021).

2.2. Phylogenetic Analyses

Evolutionary and phylogenetic analyses were performed using Molecular Evolutionary Genetics Analysis X (MEGA-X v10.2.6) [25]. Protein sequence alignment was performed using MUSCLE with default parameters; while *PEPCs*' phylogenetic tree was constructed by neighbor-joining (NJ) bootstrap = 1000x method.

2.3. Gene Structure, Conserved Motif and Promoter Region Analysis of PEPC Genes

By aligning coding sequences with the corresponding genomic sequences exon-intron association of *PEPC* genes was obtained. Conserved motifs of all *PEPC* genes were identified and analyzed using the online MEME suite server (http://meme-suite.org/, accessed on 25 June 2021). The following parameters were set for analysis: maximum numbers of different motifs, 10; minimum width, 10; and maximum width, 50. The promoter region analysis (*cis*-regulatory elements), was performed through the online PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/, accessed on 25 June 2021) and visualized using TBtools software package v0.6655 [26].

2.4. Syntenic Analysis of PEPCs in Five Rosaceae Species

By using TBtools software package v0.6655 [26], the combined gff3-file of the genomes of studied species was used to investigate the distribution and mapping of *PEPC* genes on the chromosomes. By using the MCScanX tool kit [27] duplicated *PEPC* genes were identified in five Rosaceae species. Concisely, protein sequences from those five Rosaceae species were used for BLASTP analysis (http://www.ncbi.nlm.nih.gov/blast/blast.cgi, accessed on 13 June 2021), with less than 1×10^{-5} *E*-value. The BLASTP outputs with gene-location files were used as an input for MCScanX to identify syntenic gene pairs and duplication types with default settings. Circos function in TBtools [26] was used for schematic diagram construction of putative *PEPC* genes duplication, and putative WGD/segmental-duplicated genes or tandem-duplicated genes were connected by links [28].

2.5. K_a and K_s Calculation

MCScanX downstream analysis tools were used to annotate the K_a and K_s substitution rates of syntenic gene pairs. To determine K_a and K_s, KaKs_Calculator 2.0 was used with Nei–Gojobori (NG) method [29,30].

2.6. RNA Isolation and Quantitative RT-PCR Analysis

Five different tissues i.e., stem, root, mature leaf, full-bloom flower, and ripened fruit of loquat, apple, peach, strawberry, and pear were obtained for quantitative RT-PCR evaluation. Total RNA was extracted using a Total RNA kit (TianGen Biotech, Beijing, China). Quantity and quality of RNA were checked through agarose gel electrophoresis as well as by NanoDrop N-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Prime Script RT Reagent Kit with a gDNA Eraser (TaKaRa, Dalian, China) was used to synthesize first-strand cDNA from 1 µg of high-quality total RNA. Real-time qPCR analysis was carried out using high-performance real-time PCR (LightCycler® 96, Roche Applied Science, Penzberg, Germany). The qRT-PCR began with "preincubation" for 5 min

at 95 °C, followed by a "2-step amplification" with 40 cycles of 95 °C for 10 s and 60 °C for 30 s, "melting" at 95 °C for 10 s, 65 °C for 1 min and 97 °C for 1 s, and "cooling" at 37 °C for 30 s. The melting curve was created to identify the amplicon specificity. The relative expression level of each gene was measured according to the cycle threshold (Ct), also known as the $2^{-\Delta\Delta CT}$ method [31]. The validation of $2^{-\Delta\Delta Ct}$ method was carried out by ΔCt variation analysis at different template concentrations [31–33]. All analysis consisted of 3 biological and 3 technical replicates. Following the previous studies actin genes [7,29,34–36], was selected as the control gene. Table 1 shows all the used primers for qRT-PCR analysis.

Table 1. Details about primer sequences of *PEPC* genes of five Rosaceae species.

Specie	Gene	Forward Primer (5′-3′)	Tm^F (°C)	Reverse Primer (5′-3′)	Tm^R (°C)
Eriobotrya japonica	EVM0016068.1	GCAGTTCTTGGAACCTCTCG	59.8	CCAATTTCCAGATGCTTGGT	57.8
	EVM0004511.1	TTGCTGATGGAAGCCTTCTT	57.8	TGAGACACGAGCCATTCTTG	59.9
	EVM0023388.1	GCAGTTCTTGGAACCTCTCG	55.8	CCAATTTCCAGATGCTTGGT	57.8
	EVM0022212.1	TGGAGCCTCTCGAACTTTGT	58.9	CATTCTTGCCTACGCTCCTC	58.9
	EVM0004523.1 (actin)	GGAGCGTGGATATTCCTTCA	57.8	GCTGCTTCCATTCCAATCAT	55.8
Malus domestica	MD03G1242000	AGGTCACAAGGGATGTTTGC	57.8	ACCGAGAATCACACGGTAGG	59.9
	MD09G1237900	AACAGCCCCATCTGATGTTC	57.8	TGCTGCAGATAAACGACCAG	57.8
	MD11G1261900	GAACCCCGATTTGTCGAGTA	57.8	TGGAACCTTGTTTGTGTCCA	55.8
	MD13G1049200	AACCGCCTGGTTCTGTAATG	57.8	CCATGAGGTTACGCCACTTT	57.8
	MD16G1050300	TTTGCAGAAAGATGCACGAC	59.8	CCGAATATCCAACCATCACC	57.8
	MD17G1230800	AGGTCTTTGCTCCAAAAGCA	58.8	GGAGGAGTCCTTCGGATTTC	59.9
	MD04G1127400 (actin)	CCGTGTTCCCTAGCATTGTT	59.9	CAGGAGCAACACGAAGTTCA	60.0
Prunus persica	Prupe.1G302700	TTTGCAGAAAGATGCACGAC	55.8	TGCTGCAGTAAATCGTCCAG	57.8
	Prupe.3G118300	GTTGAGCTTTTGCAACGTGA	55.8	TTCTTGCTTCCCATTGATCC	55.8
	Prupe.4G166400	ACCTCCCACTCCACAAGATG	59.9	GCAAACATCCCTTGTGACCT	57.8
	Prupe.6G078800 (actin)	GGAGCGTGGTTATTCCTTCA	60.1	TGCAGATTCCATTCCAATCA	60.0
Fragaria ananassa	gene_214.28	TCTTGCAGATTGCTGGACAC	57.8	TGGTCGAACAGTCACATGGT	57.8
	gene_117.42	TCTTGCAGATTGCTGGACAC	55.8	ACCAGGGGCATACTCACTTG	59.9
	gene_89.20	CTTTTGCAGATTGCTGGACA	57.8	GTGGTCGAACAGTCACATGG	58.7
	gene_201.40	CTTTTGCAGATTGCTGGACA	55.8	TGGTCGAACAGTCACATGGT	61.9
	gene_175.31	GCCTGAGAAACAAAGGCAAG	57.8	TTTCACATGGCATTCACGTT	59.9
	gene_78.22	CGGCCTTTCTCTTGTGAGAC	57.8	TCTTCGGTTTTGGGAACATC	59.7
	gene_170.27	GGATCAATGGGAAGCAAGAA	57.8	GGTCCTCCTCCTCTTCCAAC	55.8
	gene_121.5	AAGGGACGTGTGCTTATTGG	57.8	GCTTGTCTCTCACGTCACCA	57.8
	gene_206.27	GCCTGAGAAACAAAGGCAAG	59.9	TTTCACATGGCATTCACGTT	55.8
	gene_191.47 (actin)	TGAACTTCGTGTTGCTCCAG	60.0	ACACCATCCCCAGAGTCAAG	60.0
Pyrus communis	pycom03g18910	TCTTGCAGATTGCTGGACAC	57.8	GCCGGTTTGTTTGATTCACT	55.8
	pycom09g15640	AGATGGTGTTTGCCAAGGTC	57.8	AGGAGTCGCTGCCTCAAATA	57.8
	pycom11g23090	GCAGTTCTTGGAACCTCTCG	59.9	CCAATTTCCAGATGCTTGGT	55.8
	pycom16g04410	AAGTATCGGTCGTGGAGGTG	59.9	CCATGAGGTTACGCCACTTT	57.8
	pycom17g23400	TGGAGCCTCTCGAACTTTGT	57.8	TGCCTGTCTGATTCTTGTCG	57.8
	pycom03g07780 (actin)	ACCACAGCTGAGCGAGAAAT	60.0	ATCATGGATGGCTGGAAGAG	59.9

Before qRT-PCR analysis, the annealing efficiency of all primers was checked through normal PCR and 60 °C was found to be the optimum temperature for all primer pairs. Tm^F—Annealing temperature for forward primer; Tm^R—Annealing temperature for reverse primer.

3. Results

3.1. Identification and Characterization of PEPC Gene Family in Five Rosaceae Species

We found total 27 putative *PEPCs* (loquat 4, apple 6, peach 3, strawberry 9, and pear 5) were identified in the genomes of five Rosaceae species. The details about the location of genes on chromosomes, CDS, and peptide sequence length are shown in Table 2.

We characterized the general information of 27 *PEPCs* in Table 3, which also shows the biochemical and physiological characteristics of respective proteins. PEPC proteins had a length range of 720 to 1144 amino acids, and the molecular weight was predicted from 81.84 kDa to 129.17 kDa. While, theoretical isoelectric point (pI) for PEPC proteins ranged from 5.57 to 7.80, and the grand average of hydropathicity (GRAVY) was noticed as −0.450 to −0.323. Additionally, PEPC proteins show the instability index from 44.36 to 54.34, and the aliphatic index was also noticed as 87.39 to 91.99. All proteins of *PEPC* genes had flexible structures due to the presence of coils (Figure S1). All proteins had at least two large α helices, while β sheets were not common.

Table 2. The basic information about *PEPC* genes in five Rosaceae species.

Specie	Gene	Chromosome	Start Site	End Site	CDS (bps)	Protein Length (A.A.)
Eriobotrya japonica	EVM0016068.1	4	5161178	5166849	2898	965
	EVM0004511.1	11	26908874	26915091	2904	967
	EVM0023388.1	12	33650012	33656070	2898	965
	EVM0022212.1	13	5906340	5912227	2904	967
Malus domestica	MD03G1242000	3	32684130	32689694	2904	967
	MD09G1237900	9	30178715	30185128	2904	967
	MD11G1261900	11	37648686	37655099	2907	968
	MD13G1049200	13	3459038	3465974	3126	1041
	MD16G1050300	16	3556349	3563246	3126	1041
	MD17G1230800	17	27925312	27931528	2904	967
Prunus persica	Prupe.1G302700	1	29707930	29715477	3141	1046
	Prupe.3G118300	3	10180567	10184771	2163	720
	Prupe.4G166400	4	9660509	9667502	2898	965
Fragaria ananassa	gene_214.28	9	21406659	21414433	2898	965
	gene_117.42	10	11752557	11758791	2898	965
	gene_89.20	11	8888390	8894519	2898	965
	gene_201.40	12	20094738	20100872	2898	965
	gene_175.31	21	17493253	17499271	2886	961
	gene_78.22	22	7870362	7870362	2886	961
	gene_170.27	23	16996113	17001913	2886	961
	gene_121.5	23	12176565	12179454	2889	962
	gene_206.27	24	20617837	20624020	2886	961
Pyrus communis	pycom03g18910	3	19727998	19732815	2898	965
	pycom09g15640	9	15712657	15718139	2901	966
	pycom11g23090	11	26092435	26098317	3216	1071
	pycom16g04410	16	2776593	2783601	3435	1144
	pycom17g23400	17	21795080	21800220	2853	950

Genomic structural analysis indicated the number of exons for each *PEPC* gene ranged from 8 to 20. Most of the *PEPC* genes contained 10 exons, while four *PEPC* genes i.e., MD13G1049200, MD16G1050300, Prupe.1G302700, and pycom16g04410, contained 20 exons, whereas Prupe.3G118300 and pycom11g23090 contained 8 and 11 exons, respectively. This proposed that the loss and gain of exons had occurred in the *PEPCs* of five Rosaceae species.

Subcellular localization exploration validated that 27 PEPC proteins were located in the nucleus, mitochondria, vacuole, chloroplast, endoplasmic reticulum, plasma membrane, and Golgi apparatus (Figure 1A). Additionally, among 27 PEPC proteins, 2 putative functional domains were identified, and the PEPcase superfamily domain was present in all PEPC proteins (Figure 1B).

Table 3. Summary information of physiological and biochemical properties, and structural analysis of the PEPC proteins in five Rosaceae species.

Gene	MW (kDa)	pI	Instability Index	Aliphatic Index	GRAVY	Introns	Exons
EVM0016068.1	110,093.82	6	48.17	91.77	−0.377	10	10
EVM0004511.1	110,394.49	6.54	45.48	89.17	−0.404	9	10
EVM0023388.1	110,081.82	6.04	46.83	90.87	−0.391	10	10
EVM0022212.1	110,182.06	5.97	45.78	88.98	−0.387	9	10
MD03G1242000	110,304.06	6	47.91	91.69	−0.379	10	10
MD09G1237900	110,536.41	6.05	45.27	88.87	−0.416	10	10
MD11G1261900	110,608.47	6	47.3	91.19	−0.382	10	10
MD13G1049200	117,260.81	6.51	52.76	87.87	−0.433	19	20
MD16G1050300	117,245.63	6.46	54.32	87.39	−0.45	20	20
MD17G1230800	110,225.08	5.97	45.78	88.87	−0.392	10	10

Table 3. *Cont.*

Gene	MW (kDa)	pI	Instability Index	Aliphatic Index	GRAVY	Introns	Exons
Prupe.1G302700	117,827.73	6.81	52.29	87.72	−0.413	20	20
Prupe.3G118300	81,846.16	5.57	47.39	88.74	−0.426	8	8
Prupe.4G166400	110,062.78	6.04	47.29	91.98	−0.387	10	10
gene_214.28	110,219.73	5.7	46.74	89.56	−0.387	10	10
gene_117.42	110,245.92	5.81	47.07	89.46	−0.384	10	10
gene_89.20	110,216.86	5.89	46.99	89.56	−0.392	10	10
gene_201.40	110,217.8	5.8	47.19	89.56	−0.391	10	10
gene_175.31	109,447.16	5.86	45.34	88.92	−0.391	10	10
gene_78.22	109,389.18	5.76	44.72	89.52	−0.367	10	10
gene_170.27	109,374.06	5.86	45.07	88.71	−0.389	10	10
gene_121.5	109,658.5	6.49	44.89	87.61	−0.423	10	10
gene_206.27	109,402.08	5.86	45.11	88.71	−0.389	10	10
pycom03g18910	110,058.82	6.04	46.84	91.88	−0.376	9	10
pycom09g15640	110,386.36	6.24	45.1	89.27	−0.411	9	10
pycom11g23090	122,259.06	6.15	49.43	91.99	−0.323	10	11
pycom16g04410	129,170.71	7.8	54.34	89.66	−0.4	20	20
pycom17g23400	108,062.61	5.79	44.36	90.46	−0.354	10	10

MW: Molecular weight of the amino acid sequence; pI: Theoretical isoelectric point; GRAVY: Grand average of hydropathicity.

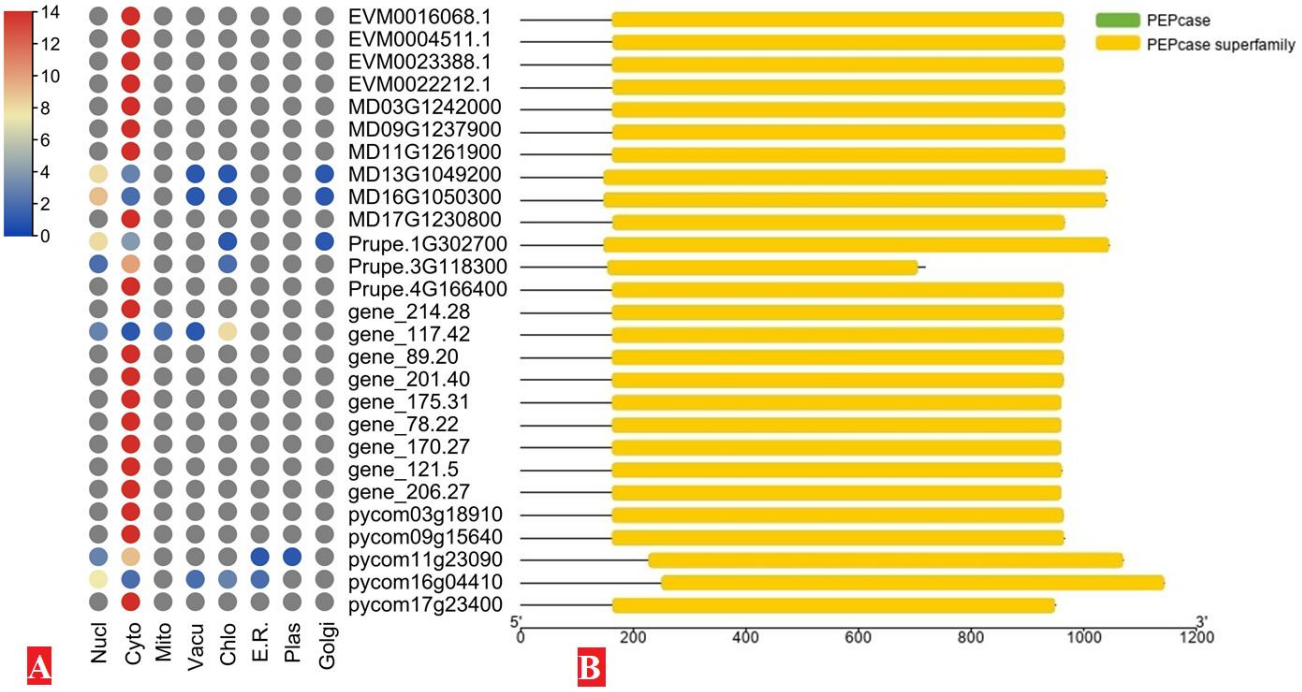

Figure 1. (**A**) Prediction of subcellular localization of PEPC proteins of five Rosaceae species. Higher signal levels are shown in red, lower signal levels are denoted in blue, while the white color represents no available data. Abbreviations: Nucl—Nucleus; Cyto—Cytonuclear; Mito—Mitochondria; Vacu—Vacuole; Chlo—Chloroplast; E.R.—Endoplasmic reticulum; Plas—Plasma membrane; Golgi—Golgi apparatus. (**B**) Conserved domain query of *PEPC* genes in five Rosaceae species genomes.

3.2. Phylogenetic Analysis of PEPC Genes in Five Rosaceae Species

Utilizing multiple sequence alignment tools among the protein sequences of *E. japonica* and four other plant species *(M. domestica, P. persica, F. ananassa, and P. cummunis)*, a phylogenetic tree was created by following neighbor joining (NJ) method. Results demonstrated

that all studied *PEPCs* among five species were clustered into three discrete subgroups (A–C) (Figure 2). All *EjPEPC* genes were allocated in subgroups A and B.

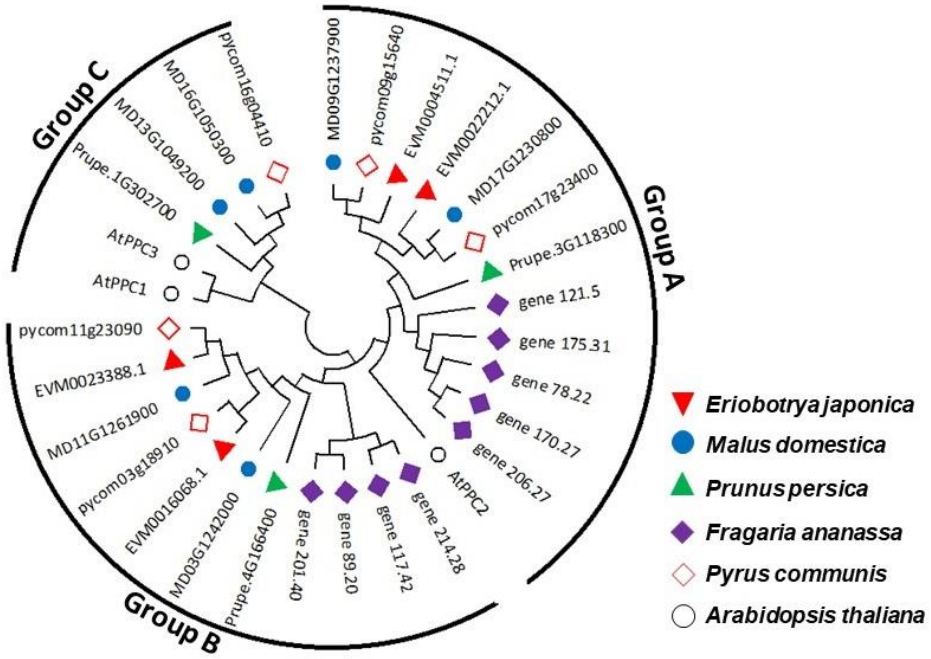

Figure 2. Phylogenetic tree analysis of *PEPC* genes in five Rosaceae species using the neighbour-joining (NJ) method.

3.3. Conserved Motif Analysis of PEPC Genes of Five Rosaceae Species

By utilizing online servers of MEME, the distribution of conserved motifs for *PEPC* genes was thoroughly assessed; a range from 7 to 10 presumed conserved motifs was acknowledged among PEPC proteins. The majority of *PEPCs* contained 10 motifs, while one *PEPC* gene i.e., Prupe.3G118300 contained 7 motifs. Figure 3 shows the distribution of conserved motifs. Thus, it can be assumed that during the evolutionary process *PEPCs* of five Rosaceae species evidently exhibited extreme conservation.

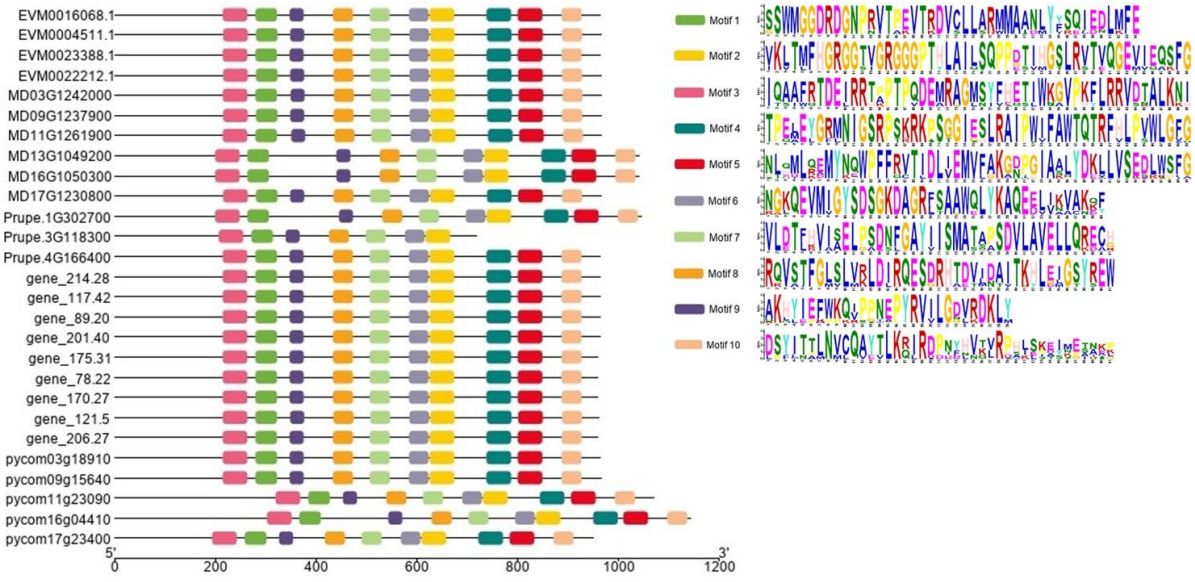

Figure 3. Conserved motifs identified in *PEPCs* of five Rosaceae species.

3.4. Promoter Region Analysis of PEPCs in Five Rosaceae Species

To further investigate the transcriptional mechanism of *PEPCs*, 1000 bps from the upstream region of *PEPCs* were subjected to promoter analysis (Figure 4). Several plant growth hormones related *cis*-elements (i.e., ABRE, AuxRE, CGTCA-motif, GARE-motif, TCA-element, TGACG-motif, and TGA-element) were detected in the promoter regions of *PEPCs*. These *cis*-elements were responsible for the regulation of abscisic acid, auxins, methyl jasmonate, gibberellins, and salicylic acid. Besides, stress response *cis*-elements i.e., LTR and TC-rich repeats were also identified in several genes. The ACE, AE-box, GA-motif, Gap-box, GATA-motif, GATT-motif, I-box, LAMP-element, TCCC-motif, and TCT-motif were found as light-responsive *cis*-elements. Apart from the aforementioned *cis*-elements, CAT-box was also identified, meristem expression.

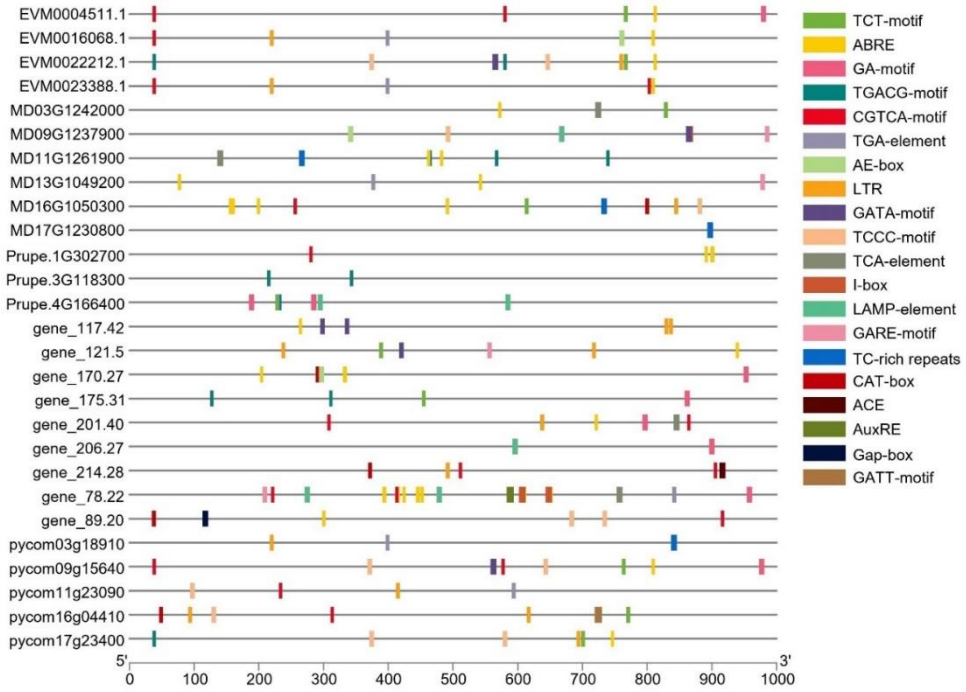

Figure 4. The *cis*-regulatory elements detected in the promoter sequences of *PEPC* genes in five Rosaceae species.

3.5. Syntenic Analysis of PEPC Genes in Five Rosaceae Species

The gene duplication among *PEPC* genes of five Rosaceae species is presented in Figure 5. Among these genes, in *E. japonica*, all 4 genes were located on different chromosomes i.e., chromosome 4, 11, 12, and 13. In *M. domestica*, all 6 genes were located on different chromosomes i.e., 3, 9, 11, 13, 16, and 17. Similarly, in *P. persica*, 3 *PEPC* genes were located on chromosome 1, 3, and 4. In *F. ananassa*, chromosome 9, 10, 11, 12, 21, 22, and 24 had one *PEPC* gene, each, while two genes were located on chromosome 23. In *P. communis*, all *PEPC* genes were found on different chromosomes i.e., 3, 9, 11, 16, and 17. A total of 16 (59.25%) *PEPC* genes in five Rosaceae species revealed WGD/segmental duplication. By this it can be suggested that WGD/segmental duplication is a significant step for the expansion of *PEPC* gene family in Rosaceae species, because of the duplication process retention of several duplicated genes take place in the genome. For the estimation of evolution rate and selective pressure, K_a (nonsynonymous)/K_s(synonymous) ratio (ω) was used [37]. Table 4 shows analyzed evolutionary pattern among the genomes of studied species, the ω values of gene duplication pairs were calculated to observe and understand selective pressures upon gene duplication, ω value for all *PEPC* gene pairs was observed to be less than 1, showing the purifying selection has made a strong influence for *PEPC*

evolution occurrence. Thus, it can be concluded that during the domestication of Rosaceae species evolutionary pattern of *PEPCs* was conserved.

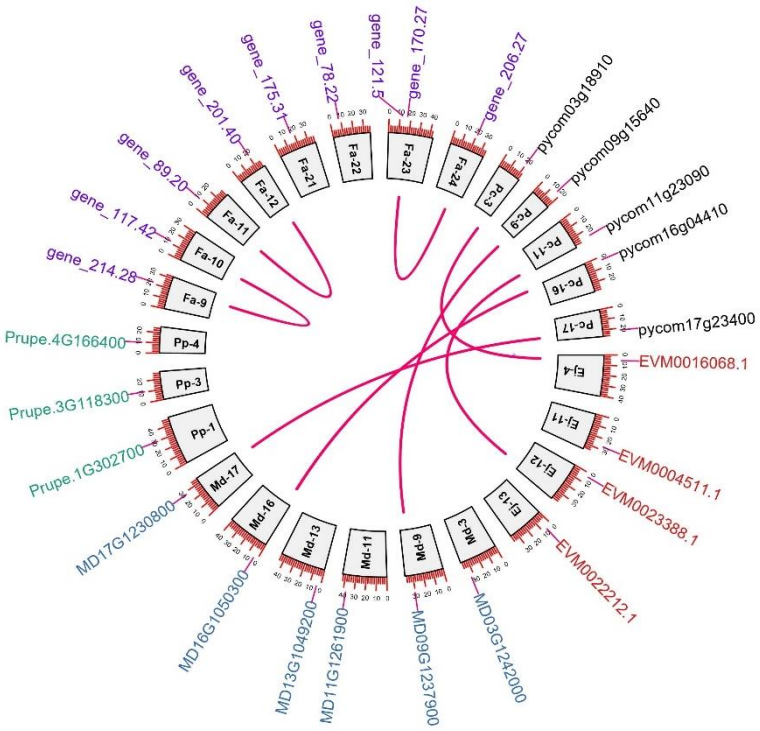

Figure 5. Chromosomal distribution and gene duplication of the *PEPC* genes in five Rosaceae species. Gene IDs of loquat, apple, peach, strawberry, and pear are labeled in red, blue, green, purple, and black colors, respectively. Red lines represent the putative WGD/segmental-duplication of genes.

Table 4. The K_a (nonsynonymous), K_s (synonymous), and K_a/K_s ratio of duplicated *PEPC* genes in five Rosaceae species.

Gene 1	Gene 2	K_a	K_s	K_a/K_s (ω)	Selection	Duplication Mode
MD09G1237900	pycom09g15640	0.004976	0.036138	0.137707	Purifying	Segmental
MD17G1230800	pycom17g23400	0.011043	0.056552	0.195266	Purifying	Segmental
gene_170.27	gene_206.27	4.54E-04	0.030053	0.015101	Purifying	Segmental
gene_214.28	gene_117.42	0.003619	0.023942	0.151148	Purifying	Segmental
gene_89.20	gene_201.40	4.52E-04	0.001466	0.308365	Purifying	Segmental
EVM0016068.1	pycom03g18910	0.003629	0.099771	0.036372	Purifying	Segmental
EVM0023388.1	pycom11g23090	0.003855	0.064843	0.059445	Purifying	Segmental
MD16G1050300	pycom16g04410	0.003784	0.051872	0.07294	Purifying	Segmental

3.6. Expression Patterns of PEPC genes in Five Rosacea Species

Further, we evaluated the relative expressions of *PEPC* genes in five different tissues of loquat, apple, peach, strawberry, and pear (Figure 6). Relative expression of *PEPC* genes showed noteworthy differences among all selected tissues for analysis in all species. Briefly, among 4 loquat *PEPC* genes, expressions of 3 were noticed as relatively less (i.e., EVM0016068.1, EVM0004511.1, and EVM0022212.1), ranging from 0.46 to 3.2. Briefly, two loquat PEPC genes (EVM0016068.1 and EVM0023388.1) showed high expression levels in root and leaf tissues; while, EVM0004511.1 was highly expressed in flower, leaf, and stem. EVM0022212.1 showed maximum expression in leaf tissues. Among 6 apple *PEPCs*, MD09G1237900 showed higher expressions in leaves and roots, while maximum expressions of MD11G1261900 and MD13G1049200 were observed in leaves and roots, respectively. In case of peach *PEPCs*, 2 genes (Prupe.1G302700 and Prupe.4G166400) showed high relative expressions in stem, leaves, flowers and fruits. Among 9 strawberry *PEPCs*,

only 5 were differentially expressed in different plant tissues. The maximum transcript level (4.14) was showed by gene_175.31 in stem tissues followed by gene_206.27 in stem and leaf tissues. The gene_214.28 was only expressed in roots among all examined plant tissues. Among 5 *PcPEPCs*, 2 genes (pycom09g15640 and pycom16g04410) showed high transcript level in roots, while pycom17g23400 exhibited maximum relative expression in stem. The relative expressions of 6 *PEPC* genes (i.e., MD17G1230800, Prupe.3G118300, gene_89.20, gene_78.22, gene_170.27, gene_121.5) were not detected.

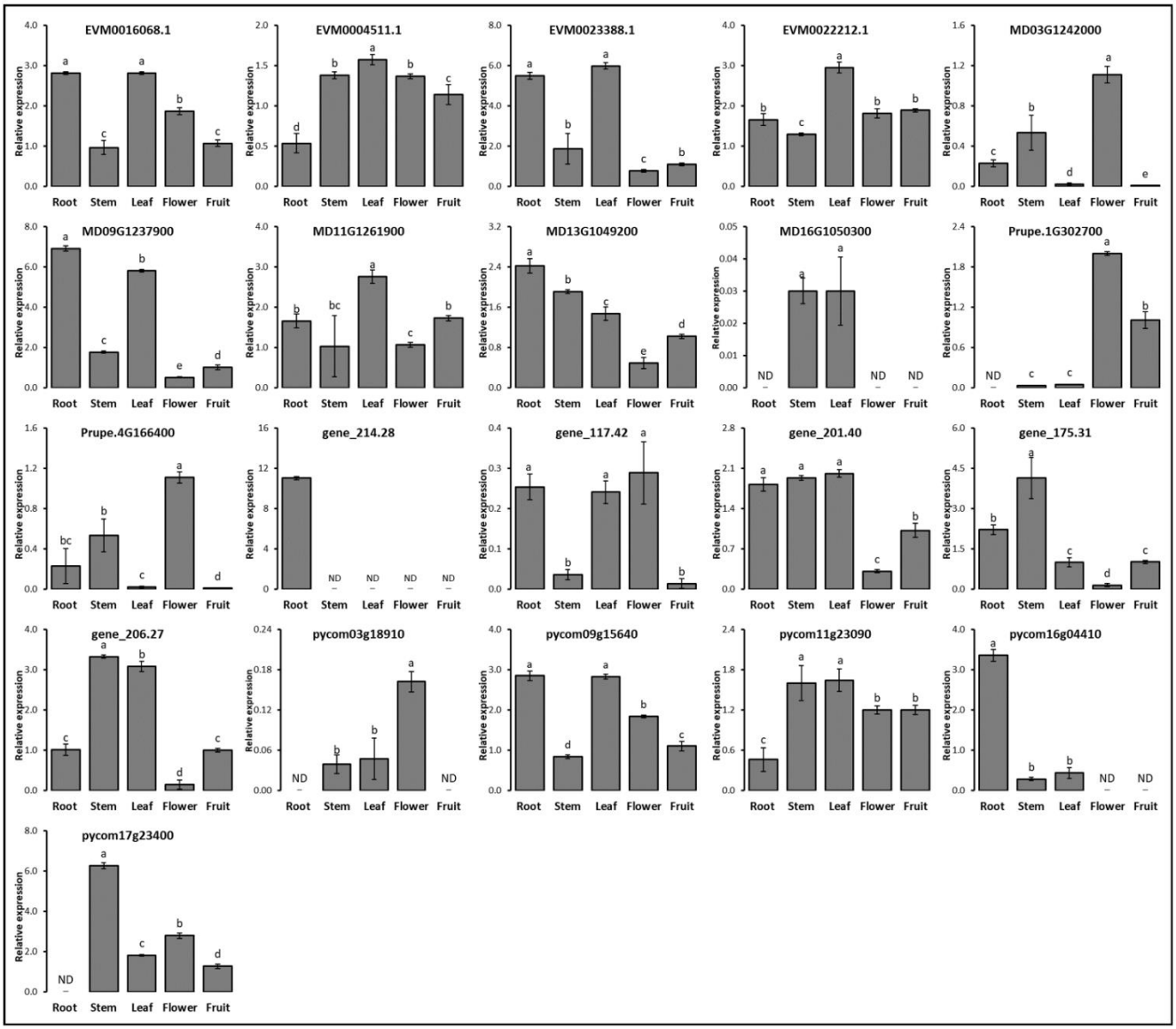

Figure 6. Relative expressions of *PEPCs* in different tissues of five Rosaceae species i.e., loquat, apple, peach, strawberry, and pear. Same letters indicate non-significant difference among treatments according to Fisher's least significant difference (LSD) test, when $p \leq 0.05$. Vertical bars indicate mean ± standard error (3 biological and 3 technical replicates). ND—not detected.

4. Discussion

PEPCs are functionally so much important for the regulation of several physiological processes, such as guard-cells movement, fruit acidity, metal-toxicity tolerance, and mineral nutrition. However, a comprehensive analysis of *PEPC* genes in Rosaceae species has not been reported yet. In present study, 4, 6, 3, 9 and 5 (total 27) *PEPCs* were identified in the genomes of loquat [19], apple [20], peach [38], strawberry [39] and pear [21], respectively.

In previous studies, *PEPC* family members were studied in different species [12–17]. The conserved domain analysis performed with PEPC proteins of five Rosaceae species revealed that all genes had similar conserved domains (Figure 2).

The current investigation identified 27 *PEPC* genes in five Rosaceae species. Through phylogenetic analysis, all genes were classified into three subgroups (Figure 3), while, the presence of *EjPEPC* and *MdPEPC* genes in the same group of classification denotes that *PEPC* genes from both species have a close relationship. Novelty in protein functioning induced owing to the result of evolution is primarily due to the gene duplication; which resulted in the loss of functionality for some genes while enhancing protein function for others [40]. Characterization of *PEPC* gene family in Rosaceae species was done to evaluate their conserved motifs as well as genomic structure, which showed that the *PEPC* genes almost retained similar exons and conserved motifs (Figure 3). The value of ω is known to be important for diversity measurement [7,41], as we observed lesser than 1 ω value for all the duplicated *PEPC* gene pairs (Table 2), exhibiting the evolutionary rate was slow and purifying selection during evolution was mainly focused.

Elaborating the functional importance of *PEPC* genes, it was reported to have plant abiotic stress response mechanism among different species, such as chilling injury and salt stress, induced *PEPC* gene expression in sorghum, wheat, and Arabidopsis [42–44]; while, a study conducted on rice overexpressing *PEPC* transgenic lines, were noticed with increased photosynthetic rate under temperature and high light stress conditions [45]. In this study, it was observed in the promoter regions of most *PEPC* genes to have 12 stress-responsive *cis*-regulated elements such as ABA, MeJA, cold, heat, light, and meristem-related elements, which indicates *PEPCs* could also be potentially important for phytohormone and stress responses among Rosaceae species (Figure 5).

For Rosaceae species, identified *PEPC* genes are diverse which may arise from ancient polyploidy as well as recent WGD events. The number of *PEPCs* among apple and strawberry were noticed almost double among the other three Rosaceae species, indicates they have gone through a recent lineage-specific WGD duplication [46]. Different modes of duplication are key characters for the evolutionary process of a eukaryotic genome to enhance the function of some genes for the betterment of a species as well as deletion of other non-essential genes, such as WGD or segmental duplication, tandem and dispersed duplication [47,48]. However, segmental duplication, which is more like a small-scale duplication, is difficult to distinguish from WGD [49]. There are some reported examples which likely to expand by duplication, such as *HCT* (Hydroxycinnamoyl Transferase) and *CPA* (Cation Proton Antiporters) [50,51]. The term tandem duplicates which are adjusted to one another on chromosomes are known as paralogs which may be derived from illegitimate chromosomal recombination [52]. Two large gene families, *AP2/ERF* and *WRKY*, expanded primarily through tandem duplication [53,54]. It was reported by several studies that Rosaceae species like apple, pear, and loquat underwent at least a double duplication process [19,20,55]. In the present study, results of synteny analysis also verified that Rosacea species likely derived as a result of segmental duplication which caused the expansion of the *PEPC* gene family members. The 16 *PEPC* genes exhibited WGD/segmental duplication in the genomes of examined Rosaceae species (Figure 6). These results indicated that the duplication of *PEPC* genes is related to WGD/segmental duplication during the process of speciation and domestication.

PEPC genes have been reported among several tissues, including seeds of barley (*H. vulgare*) and castor (*R. communis*) [13], seedlings and cell cultures of *A. thaliana*, tomato (*S. lycopersicum*) and potato (*S. tuberosum*) [15,56], root nodules of soybean (*G. max*) and lotus (*L. japonicas*) [14,57]. Besides, PEPC proteins are also integral for the non-photosynthetic process like seed germination, fruit ripening, seed formation, and guard cell movement during stomatal opening [2]. In present investigation, we also noticed that EVM0004511.1 has a higher expression level in stem and leaf, but not in root and flower. Besides, other *EjPEPCs* (EVM0016068.1 and EVM0023388.1) had lower relative expression in flower tissues (Figure 6), which is consistent with the earlier findings in Arabidopsis *BTPC*, to have

lower expression levels in flower and silique [12]. Almost all *PEPC* genes were predicted to be localized in cytonuclear region, while the higher genetic expressions of EVM0016068.1, EVM0004511.1, EVM0023388.1, EVM0022212.1, MD09G1237900, MD11G1261900, MD16G1050300, gene_117.42, gene_201.40, gene_206.27, pycom09g15640 and pycom11g23090 were recorded in leaves and stem, indicating their possible role in improving plant growth and development [58]. All these findings indicate that *PEPCs* have various physiological roles in plant species and proposed that members of *PEPC* gene family may be considered important factors in regulating variety of functions in Rosaceae species.

5. Conclusions

In present study, 27 *PEPC* genes were identified in five Rosaceae species i.e., loquat, apple, peach, strawberry, and pear. All *PEPC* genes were subjected to conserved domains, gene structure, conserved motif, phylogenetic, syntenic, and expression analysis. Based on the subcellular localization analysis, it was predicted that all PEPC proteins were localized in cytonuclear region. Syntenic analysis revealed that WGD/segmental duplication played an important role in the expansion of *PEPC* gene family in Rosacea species. The K_a and K_s values of duplicated genes indicated that *PEPC* genes had undergone a strong purifying selection. In addition, as the result of expression analysis, some genes were differentially expressed in different plant tissues i.e., root, mature leaf, stem, full-bloom flower, and ripened fruit. This study laid the basis for studying the roles of *PEPC* genes in developmental processes of Rosaceae species and provide the foundation for further functional analysis such as overexpression, knockout via CRISPR/Cas9 systems, etc.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agronomy12010025/s1, Figure S1: The predicted structures (3D) of PEPC proteins in five Rosaceae species.

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