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Genome-Wide Identification and Evolutionary Analysis of AOMT Gene Family in Pomegranate (*Punica granatum*)

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Abstract: Gene duplication is the major resource with which to generate new genes, which provide raw material for novel functions evolution. Thus, to elucidate the gene family evolution after duplication events is of vital importance. Anthocyanin *O*-methyltransferases (AOMTs) have been recognized as being capable of anthocyanin methylation, which increases anthocyanin diversity and stability and improves the protection of plants from environmental stress. Meanwhile, no detailed identification or genome-wide analysis of the AOMT gene family members in pomegranate (*Punica granatum*) have been reported. Three published pomegranate genome sequences offer substantial resources with which to explore gene evolution based on the whole genome. Altogether, 58 identified OMTs from pomegranate and five other species were divided into the AOMT group and the OMT group, according to their phylogenetic tree and AOMTs derived from OMTs. AOMTs in the same subclade have a similar gene structure and protein conserved motifs. The PgAOMT family evolved and expanded primarily via whole-genome duplication (WGD) and tandem duplication. PgAOMTs expression pattern in peel and aril development by qRT-PCR verification indicated that PgAOMTs had tissue-specific patterns. The main fates of AOMTs were neo- or non-functionalization after duplication events. High expression genes of *PgOMT04* and *PgOMT09* were speculated to contribute to "Taishanhong" pomegranate's bright red peel color. Finally, we integrated the above analysis in order to infer the evolutionary scenario of AOMT family.

Keywords: gene duplication; AOMT gene family; anthocyanin methylation; pomegranate; expression pattern; evolutionary scenario

Citation: Zhang, X.; Yuan, W.; Zhao, Y.; Ren, Y.; Zhao, X.; Yuan, Z. Genome-Wide Identification and Evolutionary Analysis of AOMT Gene Family in Pomegranate (*Punica granatum*). *Agronomy* **2021**, *11*, 318. https://doi.org/10.3390/agronomy11020318

Academic Editor: Manish Kumar Pandey
Received: 20 December 2020
Accepted: 8 February 2021
Published: 11 February 2021

1. Introduction

Gene duplication and loss events constitute the main factor of gene family evolution [1]. Whole genome duplication (WGD) and small scale duplication events are the major sources of most duplicates [2]. After duplication events, duplicate copies are a predominant feature of individual genes and whole genomes, while a large fraction of duplications was lost [3]. Some of these duplicates' retention contributes to neo-functionalization, such as increasing metabolic activity, evolving new structure and novel adaptive traits [2,3]. After WGD, the strong biased retention of regulatory and developmental genes has ramifications for evolution in the longer term [4]. These regulatory and developmental genes control multiple reactions in land plants biochemical pathways. Thus, it is significant to elucidate the evolution of the regulatory and developmental gene families after WGD.

Anthocyanins, which are the largest group of flavonoids, are significant pigments to plants. By conferring to the fruit pulp, pericarp, flower and leaf, a red, orange or purple color, anthocyanin, contributes to their appealing appearance [5–8]. Through these bright colors, plants attract the coevolving birds or insects to spread seeds and pollens [9]. Anthocyanins have potential health benefits. Some research suggests that anthocyanins possess antioxidant, antitumor, anti-inflammatory and immunomodulation activity [10].

In addition, anthocyanins play crucial roles in enhancing tolerance in response to multiple abiotic stresses [11]. Like other phenolic compounds, anthocyanins are usually modified by diverse moieties, including acyl, hydroxyl, glycosyl and methyl groups [8,12–14]. These modifications can increase anthocyanin categories and improve its stability.

The methylation of anthocyanin is mediated by *O*-methyltransferases (OMT). OMT family can be divided into three major types based on sequence homology and substrate variance [15]. Type 1 OMTs mediate the methylation of flavonoids, type 2 OMTs mediate the methylation of caffeoyl CoA and type 3 OMTs mediate the methylation of small phenolic compounds. To date, a few of OMTs that were responsible for methylation of anthocyanins were characterized. Anthocyanin *O*-methyltransferases (AOMTs) named FAOMT and VvAOMT were isolated from two grape cultivars and were verified that catalyzed anthocyanin methylation in vitro and planta [16,17]. In the tomato genome, 57 candidates OMT genes were identified, while only one gene (*AnthOMT*) exhibited a strong correlation with the accumulation of methylated anthocyanin using gene silencing tests [18]. Through following *PsAOMT* and *PtAOMT* from purple-flowered and red-flowered Paeonia plants, respectively, and using site-directed mutagenesis, the pair of homologous genes were characterized as being involved in anthocyanin methylation and one key amino acid substitution led to vast difference in catalytic activity between them [8].

Pomegranate (*Punica granatum*) has achieved considerable attention due to its high antioxidant activity, soft seed characteristic, rich color in peel and aril (the edible part of pomegranate) and active pharmaceutical ingredients [19–21]. In addition, part of the important stress relevant and nutritionally beneficial secondary metabolites of pomegranate was found in its seed oil [19,22]. Indeed, lipid droplets that make up the seed oils are an ancient response mechanism and these droplets can accumulate secondary metabolism [23]. Pomegranate peel and aril color, as a consequence of the anthocyanin accumulation, is a crucial feature in determining its fruit commodity value. Moreover, the high anthocyanin content and biological activities in fruit indicate that their consumption would be of great benefit to health, including such afflictions as the effect of colon cancer, breast cancer, lung cancer and gastric cancer and that they could be used to produce functional foods [24,25]. While phenylpropanoid metabolisms and anthocyanin production is not limited to pomegranate, indeed, the molecular mechanisms that regulate this process are found across land plants, and probably even in the algal relatives of land plants [26–28].

Previous studies found that pomegranate has experienced a paleohexaploidy event shared with eudicots and a paleotetraploidy event shared with *Eucalyptus grandis* [20]. These two duplication events resulted in varying degrees expansion of the pomegranate multiple gene families. Yuan et al. [20] constructed a phylogenetic tree with six species of AOMT genes and put forward the assumption that high copy number of AOMTs were responsible for producing anthocyanin. In addition, nine AOMT genes were identified in pomegranate. The diverse AOMTs may have a link with pomegranate distinct colors [20].

In this study, we carry out an analysis of AOMT genes, a gene family involved in anthocyanin methylation, to explore the evolution scenario of the gene family after duplication events. A total of 58 putative OMT genes were identified from pomegranate, *Durio zibethinus*, *Citrus sinensis*, *Arabidopsis thaliana*, *Malus domestica* and *Vitis vinifera* and were divided into two classes (the OMT group and AOMT group) through phylogenetic analysis. We present detailed analyses of OMTs, including gene structure, conserved motif distribution, expression pattern and gene duplication.

2. Materials and Methods

2.1. Sample Collection

The pomegranate "Taishanhong" (2n = 2x = 18) has been widely cultivated in China as an important commercial cultivar because of its large body, bright red peel color and sweet taste [20]. In our test, pomegranate materials were obtained from the pomegranate germplasm resource nursery of Nanjing Forestry University.

In order to verify the reliability of the AOMT candidate gene sequences, the results of RT-PCR and gene cloning were compared with the predicted genes. Fresh young leaves of pomegranate were selected as materials and stored at −80 °C after liquid nitrogen freezing.

In order to study the differential expression of AOMT genes in peel and aril at different stages during development, the fruits of four different development stages (July, August, September and October) were selected at random. The peel and aril were separated and stored at −80 °C after liquid nitrogen freezing.

2.2. Data Sources and Gene Identification of AOMTs

Pomegranate genome (ASM220158v1) was downloaded from NCBI (https://www.ncbi.nlm.nih.gov) [20]. *A. thaliana* AOMT protein sequences were downloaded from the *A. thaliana* database (http://www.arabidopsis.org). *M. domestica*, *C. sinensis* and *V. vinifera* AOMT protein sequences were downloaded from Phytozome and *D. zibethinus* was downloaded from NCBI. Detailed information of the species above was listed in the Supplementary S1.

Using HMMER v3.1b1 to search for sequences with AOMT domains according to the downloaded PFAM (the protein families database) file (PF01596, e-value $\leq 10^{-10}$) [29]. The protein domains were detected by CDD [30] (https://www.ncbi.nlm.nih.bov/cdd) and SMART [31] (http://smart.embl-heidelberg.de). The sequences with no AOMT domain or incomplete domain were removed. If there was a redundant sequence, the longest one was retained.

2.3. Phylogenetic Analysis

To explore the phylogenetic relationship of the AOMT genes among six species, including 10 pomegranate OMT genes, 16 OMT *D. zibethinus* genes, 7 *C. sinensis* OMT genes, 6 *A. thaliana* OMT genes, 9 *M. domestica* OMT genes and 10 *V. vinifera* OMT genes, these identified AOMT gene sequences were utilized to perform multiple sequence alignment analysis with MAFFT (a multiple sequence alignment program) software [32]. Then, a maximum likelihood tree was established using PhyML [33] with the JTT model, SPR topology search, 1000 bootstrap replicates and aLRT statistics. The tree was visualized using Figtree v1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/).

2.4. Exon-Intron Structure

Gene Structure Displayer server (GSDS) website [34] (http://gsds.cbi.pku.edu.cn) was used to analyze the AOMT gene structure. Each plant species GFF annotation was downloaded corresponding to the genome. The required annotation contents were filtered out using perl script and were uploaded to the GSDS website for the AOMT exon-intron structure.

2.5. Motif Analysis

In order to further identified and analyzed the protein conserved motifs of the PgAOMT family members, The online tool Multiple Em for Motif Elicitation (MEME: http://meme.nbcr.net/meme/intro.html) [35] was employed with the following parameters: the motif width was 20~100 aa, and the number of motifs was 6.

2.6. RNA Extraction, cDNA Synthesis and RT-PCR Analysis

Total RNA of pomegranate young leaves was extracted using RNAprep Pure total RNA extraction kit. RNA was purified using DNase I digestion kit (Vazyme-innovation in enzyme technology, Nanjing, China). The obtained RNA was reverse transcribed into cDNA by HiScript®II 1st Strand cDNA Synthesis Kit (Vazyme-innovation in enzyme technology, Nanjing, China). The pomegranate target genes were screened according to the verified *A. thaliana*, *M. domestica*, *D. zibethinus*, *C. sinensis* and *V. vinifera* AOMT homologous gene. Then, the primers were designed depending on the candidate genes with Primer5.0 for PCR amplification based on the predicted genes through ePCR and were listed in Supplementary S3. Finally, the GBclonart seamless cloning kit (GBI, Suzhou,

China) was used to recombine gene as Zhang et al. [36] described and the cloned genes were sequenced.

2.7. Expression Analyses of PgAOMT Gene Family Members

The public transcriptomes pomegranate peel (SRR5279392, SRR5279393, SRR5279394) and aril (SRR5279386, SRR5279387, SRR5279388) during development were downloaded from NCBI. Quality controls for these transcriptomes were examined by the NGS QC Toolkit v2.3.3. Then, the gene expression was quantified by the kallisto v0.43.1. The data was calculated by reads per kilobase per million mapped reads (RPKM) as transcript abundance and the RPKM values were transformed in log2 fold change. The differently expressed PgAOMT genes in peel and aril across development periods were analyzed with DESeq2 software [37]. According to the threshold *p*-value adjust <0.01 and log2(fold-change) $\geq$2, differentially expressed genes (DEGs) were screened out. Up-regulated DEGs and down-regulated DEGs were classified. The volcano map generation was exhibited by R software. To validate the result of the RNA-seq data, three samples for each tissue (peel and aril) were collected for the expression levels of PgAOMT genes.

RNA samples of aril and peel during development (July, August, September and October) were used for qRT-PCR experiments. The operation method of RNA extraction and cDNA reserve transcription is the same as 2.7 mentioned. qRT amplification was performed in triplicate using the Luna® Universal qPCR Master Mix (NEB, Ipswich, MA, USA). The qRT-PCR primers of AOMTs listed in Supplementary S3. The expression levels of these genes were assessed using the method described by Liu et al. [38]. The correlative expression data was calculated according to the $2^{-(\Delta\Delta CT)}$ method [39]. Finally, the expression spectrum was draw out using R software.

3. Results

3.1. Identification of the AOMT Genes in Pomegranate

To identify the AOMT genes of pomegranate, we used SelectHMM to (e-value $\leq$ 10) screen and CDD and SMART to identify all possible AOMT members which contain complete domains. Altogether 10 OMT candidate genes were identified from pomegranate genome.

According to the on-line analysis software ExPASY [40], we provided characteristics of PgOMT genes including protein length (PL), molecular weight (MW), isoelectric point (PI), instability index and mean value of hydrophilicity (GRAVY) (Table 1). The 10 predicted PgOMT proteins ranged from 93 (*PgOMT08*) to 414 amino acids (aa) (*PgOMT10*), with an average of 232 aa. The proteins MW ranged from 10,153.52 (*PgOMT08*) to 45,306.63 (*PgOMT10*) Ku, with an average of 25,669.56 Ku. The PI varied from 4.79 (*PgOMT07*) to 9.26 (*PgOMT10*), with an average of 5.83. The instability index varied from 23.63 (*PgOMT06*) to 56.67 (*PgOMT05*). All proteins were hydrophilic except PgOMT10 and PgOMT07 based on the GRAVY.

Table 1. The basic information of PgOMT gene family.

Species	Gene ID	Gene Name	Protein Length/aa	Molecular Weight/ku	PI	Instability Index	GRAVY
Pomegranate	*Pg002344.1*	*PgOMT01*	250	27,320.50	5.45	46.31	−0.088
	Pg002346.1	*PgOMT02*	250	27,519.67	5.05	45.46	−0.052
	Pg002348.1	*PgOMT03*	239	26,378.53	5.35	31.20	−0.079
	Pg002351.1	*PgOMT04*	242	27,172.27	5.44	36.49	−0.160
	Pg002849.1	*PgOMT05*	183	20,421.47	6.21	56.67	−0.152
	Pg003086.1	*PgOMT06*	109	11,927.73	5.89	23.63	−0.110
	Pg006183.1	*PgOMT07*	286	31,785.38	4.79	39.20	0.009
	Pg017894.1	*PgOMT08*	93	10,153.52	5.18	45.87	−0.200
	Pg021629.1	*PgOMT09*	258	28,709.93	5.72	37.09	−0.246
	Pg026019.1	*PgOMT10*	414	45,306.63	9.26	34.14	0.093

3.2. Phylogenetic Analysis of PgAOMT Gene Family

A comprehensive and robust phylogenetic tree is the basis of studying phylogenetic genomics. To study the AOMT gene family evolutionary relationship, a total of 58 candidate OMT genes were identified from pomegranate (10 OMT genes), *D. zibethinus* (16 OMT genes), *C. sinensis* (7 OMT genes), *A. thaliana* (6 OMT genes), *M. domestica* (9 OMT genes) and *V. vinifera* (10 OMT genes) and constructed a maximum likelihood (ML) tree (Figure 1) using PhyML [33]. This ML tree has robust bootstrap-supported values in general.

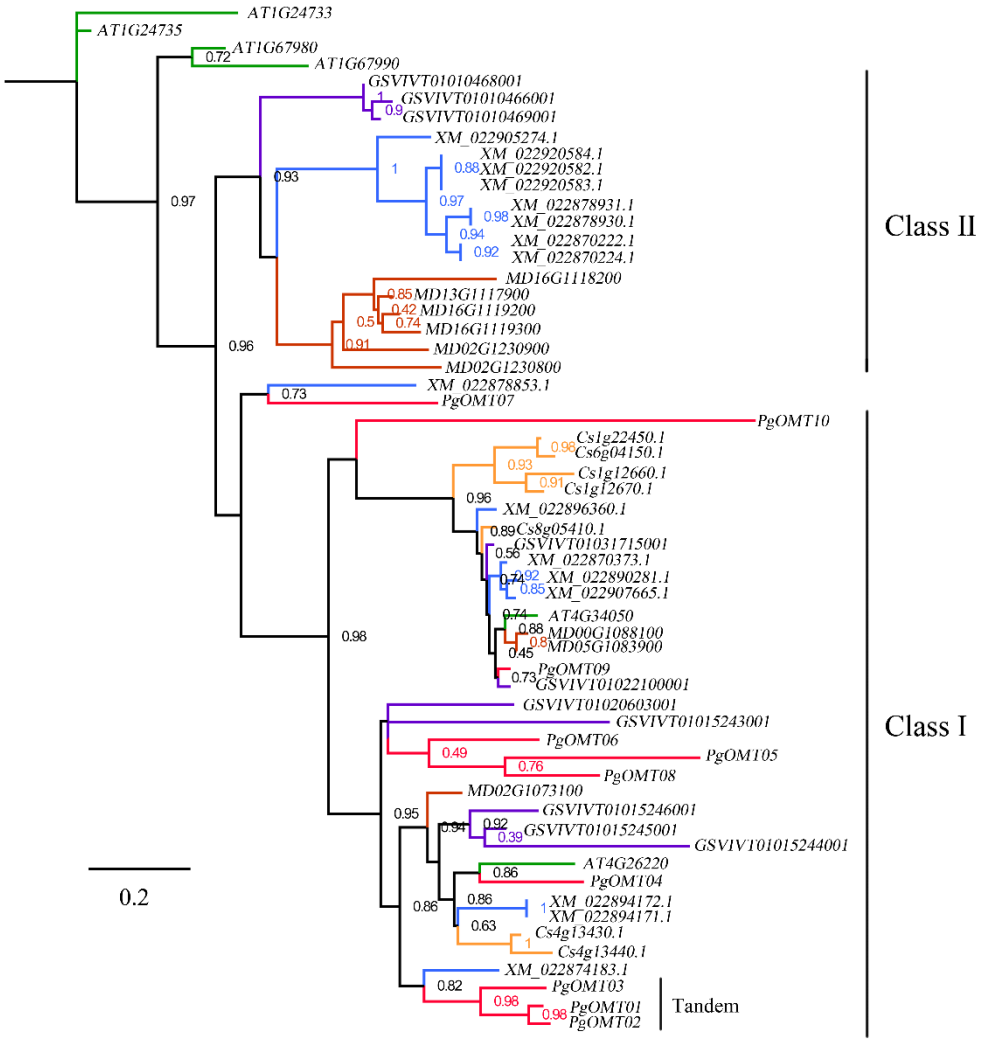

Figure 1. OMT (*O*-methyltransferases) gene family phylogenetic tree of 6 species including pomegranate, *A. thaliana*, *V. vinifera*, *M. domestica*, *C. sinensis* and *D. zibethinus* presented by various branch colors. A total of 58 sequences were aligned with MAFFT (a multiple sequence alignment program) and the phylogenetic tree was contrasted using JTT (Jones-Taylor-Thornton) model. According to the phylogenetic relationship, 58 OMTs were divided into Class I (AOMT (anthocyanin *O*-methyltransferases) group) and Class II (OMT group). Node values beside branches were bootstrap-supported values generated from 1000 replicates. Pg: pomegranate; AT: *A. thaliana*; Cs: *C. sinensis*; GSVIVT: *V. vinifera*; MD: *M. domestica*; XM: *D. zibethinus*.

Based on the phylogenetic tree branches and bootstrap-supported values, the OMT genes can be divided into Class I and Class II. In Class I, six species OMT genes evenly distributed, while in Class II, just *D. zibethinus*, *M. domestica* and *V. vinifera* were the main species. We defined Class I and Class II as AOMT group and OMT group, respectively (Figure 1). Meanwhile, it was speculated that the AOMT group evolved from the OMT group.

It is apparent that each of the four species (*A. thaliana*, *V. vinifera*, *M. domestica* and *D. zibethinus*) constituted the gene cluster respectively in the basal part of the ML tree (Figure 1), indicating that OMT gene family has undergone species-specific expansion during evolution. In Class I, the branches were made up of *GSVIVT01020603001*, *GSVIVT01015243001*, *GSVIVT01015246001*, *GSVIVT01015245001*, *GSVIVT01015244001*, *PgOMT06*, *PgOMT05*, *PgOMT08*, *PgOMT04*, *PgOMT03*, *PgOMT01* and *PgOMT02*, which represented a ratio of 5:7 of grape to pomegranate (Figure 1). It was indicated that pomegranate experienced one WGD event after paleohexaploidy shared with *V. vinifera* and considerable fraction duplicates were lost during the subsequent gene loss event [20,41–43].

3.3. Gene Structure and Protein Conserved Motifs of PgAOMT Genes Family

To study the AOMT gene structure, we analyzed the distribution and amount of the exons and introns among six species OMT genes on the basis of phylogenetic similarities (Figure 2A,B). The results showed that the majority of OMT genes contained introns and the number of introns varied from 0 to 8 (Figure 2B), only *AT1G24733*, *MD02G1230800* and *PgOMT08* had no intron. In the same subclade, OMTs seem to have similar intron/exons distribution, while there were some special cases such as *GSVIVT01010469001*, *MD16G1118200* and *PgOMT10*, that were obviously different from the genes of the same subclade in length and number of intron (Figure 2A,B). In addition, the introns of early copy genes tended to be longer (Figure 2A,B). These supported the assumption that the length and number of introns facilitate gene family differentiation. Combined with previous phylogenetic analysis, it was speculated that gene duplication events with gene loss events influenced PgOMTs gene structure.

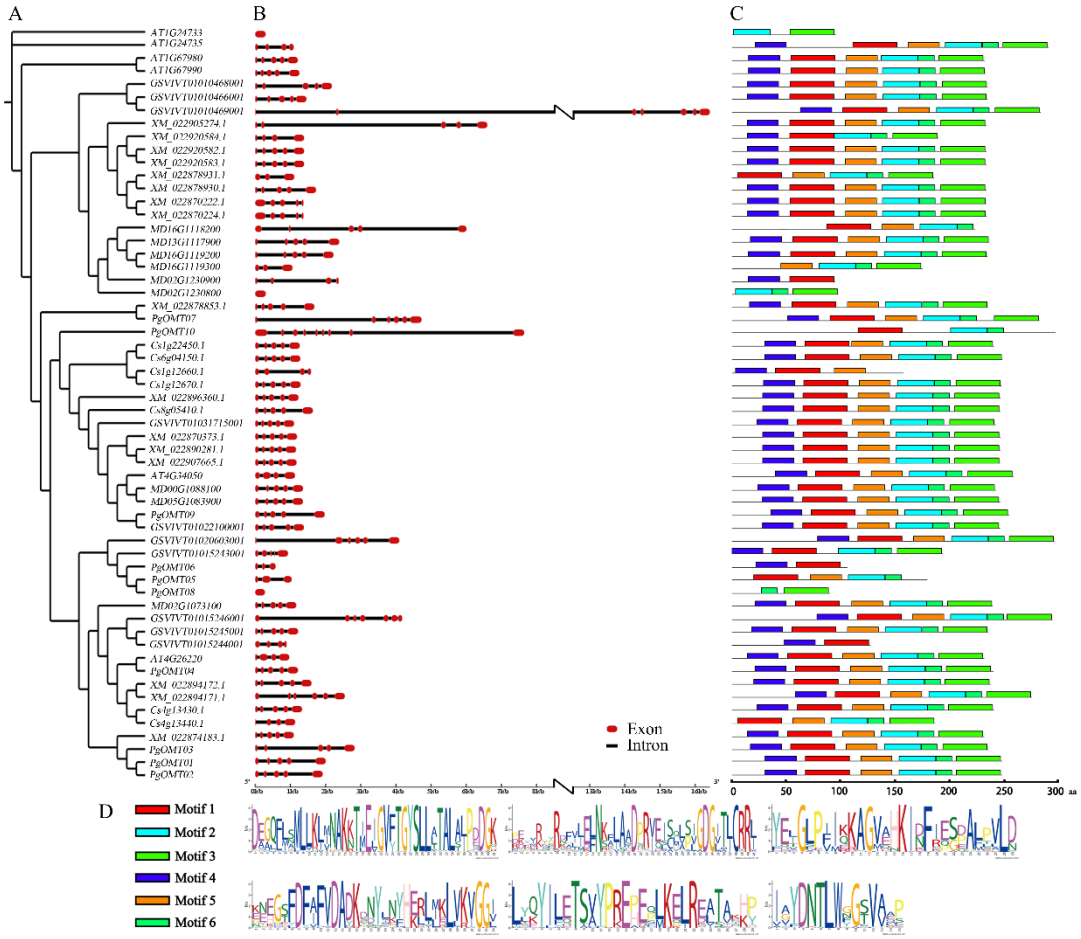

Figure 2. Phylogenetic tree (**A**), gene structures (**B**), conserved motif distribution (**C**) and 6 conserved motifs (**D**) of OMT gene from 6 species. Gene structures and protein conserved motifs were visualized by online software GSDS and MEME, respectively.

To research the distribution of AOMT proteins conserved motifs, six species protein sequences were analyzed using MEME. Most OMT proteins have six conserved motifs (Figure 2C,D). However, some proteins such as *AT1G24733* only had motif 1 and 2. Combined with the phylogenetic tree, it explained why *AT1G24733* and *AT1G24735* that formed an individual branch (Figure 2A,C). It was observed that four AOMTs including *PgOMT01*, *PgOMT02*, *PgOMT03* and *PgOMT04* belonging to the same subclade contained all 6 motifs. While motif distribution of three other AOMTs from the same subclade were different, as follows, *PgOMT10* possesses motif 1, 2 and 6; *PgOMT06* possesses motif 1 and 4; *PgOMT05* contains motif 1, 2, 5 and 6 and *PgOMT08* contains motif 3 and 6 (Figure 2A,C). These illustrated that there were obvious differences among PgOMT proteins after the WGD event. The loss and retention of motifs may be related to the evolution of subclade.

3.4. RT-PCR Analysis of PgAOMT Candidate Gene Sequences

The RT-PCR technique was used to detect the reliability of five OMT genes. Five target sequences were obtained after reverse transcription. The sequencing reads of five OMT genes were aligned with predicted PgAOMTs downloaded from the database using MEGA-X software [44] for screening (Supplementary S4). Through alignment, it was found that although there were differences among several base pairs in partial regions, the structure of the five sequencing OMT genes was consistent with the predicted sequences on the whole.

The inconsistent regions (Supplementary S4: the red box areas) were randomly sampled. As can be seen from the enlarged partial drawing, the alignment between two sequences in the first group were identical 100%. The alignment areas of the sequences of the remaining four groups were basically the same, although there were some differences. It was concluded that the five OMT genes are all AOMT genes and their quality was high, which can meet the requirements of the subsequent analysis.

3.5. Expression Patterns of PgAOMT in Peel and Aril during Different Development

Some research has revealed that AOMT genes play roles in anthocyanin methylation that influence the plant coloration and improve self-protection from environmental stress [8,18]. To gain insight into the functions of the PgAOMT genes in the development of aril and peel, the expression patterns of PgOMT genes in aril and peel were analyzed using public RNA-Seq data from NCBI (Figure 3A). The volcano plot analysis showed that *PgOMT09* in aril was significantly down-regulated (Figure 3A). The DEG analyses demonstrated that *PgOMT09* played pivotal roles in aril development.

To further understand the physiological role of the PgAOMTs, we determined the PgAOMT gene family members expression in peel and aril during different developmental stages (July, August, September and October) (Figure 3B). The histograms revealed that the transcript abundance of seven PgAOMT genes was different significantly in aril and peel, suggesting that there were differences in their functions of PgAOMT in aril and peel development. Notably, *PgOMT04* exhibited high expression both in peel and aril development. *PgOMT09* expression was down-regulated significantly at the early stage of aril development. *PgOMT01*, *PgOMT02* and *PgOMT03* belonging to the same subclade exhibited low or no expression. These phenomena indicated that during the genome duplication events, some genes (such as *PgOMT09*) evolved new functions and some genes (such as *PgOMT01*, *PgOMT02* and *PgOMT03*) became nonfunctional pseudogenes due to segmental large-scale duplication events arose from tandem duplication [2]. Additionally, PgAOMT genes had aril/peel-specific expression patterns in pomegranate (Figure 3B). For instance, *PgOMT01* and *PgOMT03* only expressed in aril. *PgOMT05*, *PgOMT07* and *PgOMT10* were more highly expressed in aril than in peel.

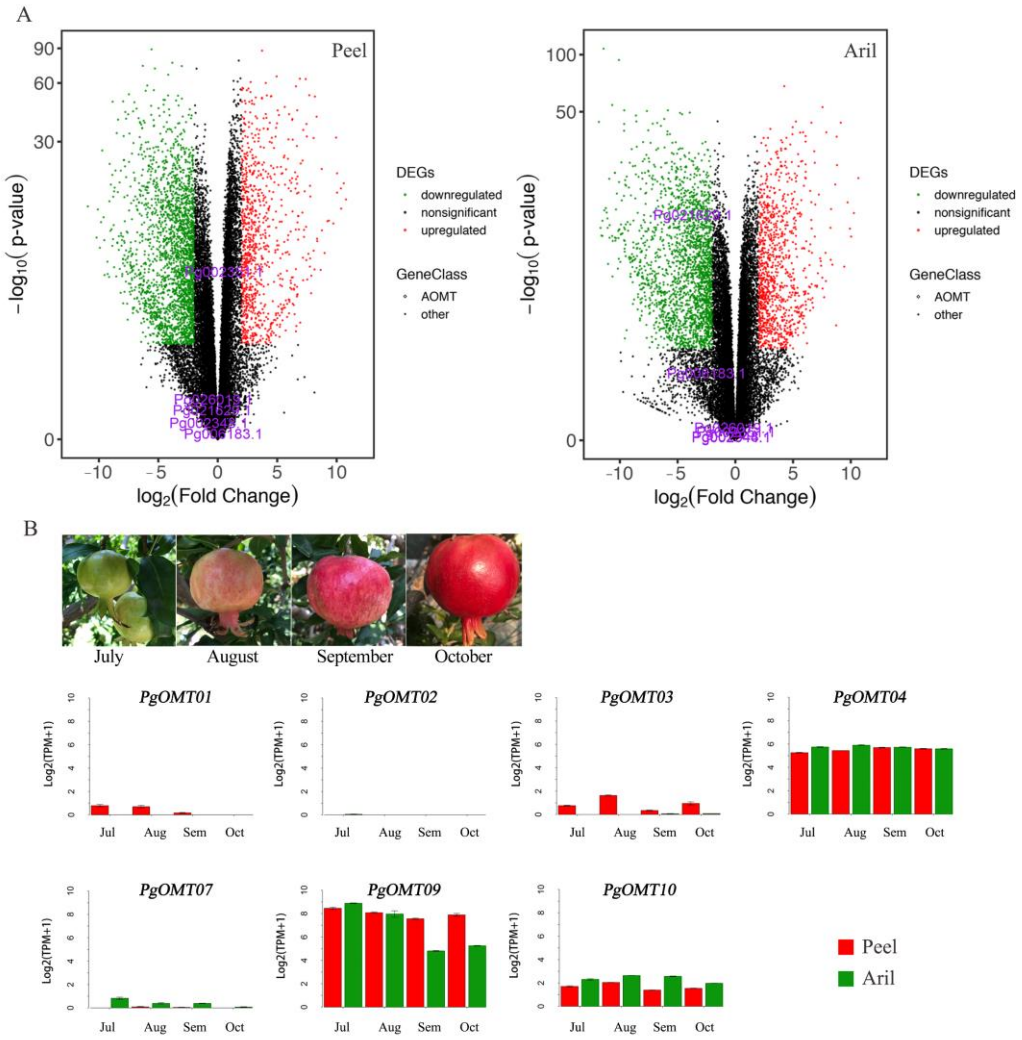

Figure 3. Expression pattern of PgAOMTs. (**A**) Up-regulated (red point) and down-regulated (blue point) differently expressed AOMTs during peel and aril development. (**B**) Expression of PgAOMTs in peel and aril during development.

4. Discussion

AOMT enzymes are known to play roles in anthocyanin methylation. It can increase diversity and improve the stability of anthocyanin [8,18]. Few AOMTs were identified and characterized such as *VvAOMT* in grape, *CkmOMT2* in cyclamen, *AnthOMT* in tomato, *DIFe1*, *DIFe2a* and *DIFe2b* in *Petunia* spp. and *PsAOMT* and *PtAOMT* in purple-flowered and red-flower *Paeonia* plants, respectively [8,17,18,45,46]. So far, few articles have described the evolution of the OMT gene family. Three published pomegranate genomes have been sequenced and these provide valuable resources to explore functional genes in genome-wide data [20,47,48]. In addition, pomegranate possesses diverse anthocyanins [49,50] and AOMTs expansion occurred after duplication events [20]. Here, we identified and analyzed the expression patterns of AOMTs based on phylogenomic analyses to speculate on the evolution of PgAOMT gene family.

It has been reported that pomegranate shared the paleohexaploidy event with all eudicots and the paleotetraploidy event with *E. grandis* [20]. Gene duplication has been deemed as a predominant mechanism to increase functional diversification and the enhanced expression divergence of duplicated genes can substantially contribute to physiological diversification [51]. Hence, exploring the duplication events to illuminate gene functional diversification is significant. A total of 58 OMTs were identified from the selected six species. They were divided into Class I and Class II by constructing an ML tree that analyzed through bootstrap-supported values (Figure 1). Of the 10 candidate PgOMT

genes, nine PgOMTs belong to Class I and only one belonged to Class II (Figure 1). We defined Class I as AOMT group and Class II as OMT group. It was speculated that AOMT group evolved from OMT group. In the subclade of Class I, there was a ratio of five grape to seven pomegranate, suggesting that there might be pomegranate genome duplication and loss events [20,52]. In contrast with the number of AOMTs in other species, PgAOMTs copy number was more than that of *E. grandis* (6) and *C. sinensis* (2) [53,54]. In our study, the number of AOMTs from pomegranate was more than that from other species (Figure 1), suggesting a lineage-specific expansion event occurred in pomegranate. High copy number of PgAOMTs may contribute to the diverse anthocyanins forming distinct colors of pomegranate [20].

By analyzing AOMT genes structure combined with the phylogenetic tree, it was obvious that the gene structure of AOMTs in Class I have some differences, but members in the same subclade seem to be similar (Figure 2A,B). Notably, in the clade of *PgOMT06*, *PgOMT05* and *PgOMT08*, the three PgAOMTs have fewer introns than others (Figure 2B). The absence of introns of the three PgAOMTs proved the presumption that there might be gene loss events in PgAOMT family due to neo-, sub- or non-functionalization [55,56]. Furthermore, we found that the motifs encoding PgAOMTs in earlier evolved clades were absent to varying degrees in Class I. For example, PgOMT10 only contained motif 1, 2 and 6; PgOMT06 only contained motif 1 and 4; PgOMT05 possessed motif 1, 2 and 5; PgOMT08 possessed motif 3 and 6, whereas the PgAOMTs that later evolved contained all 6 motifs (Figure 2A,C). In general, the AOMTs phylogenetic analysis is consistent with the gene structure.

Some gene families evolved and expanded through tandem duplication or segmental duplication along with high rates of birth and death. Gene family's expansion in these ways is of important significance to diversify their functions [57]. Vice versa, gene function may feedback on copy number and genome organization and thus give rise to the widely varying patterns of tandem or segmental duplication [57]. In brief, genes exist in the form of clusters after gene tandem duplication [58]. Deciphering gene clusters evolution is vital to offer new insight into gene family evolutionary scenario. By analyzing the gene mapping in pomegranate, it presented the existence of tandem duplication (Figure 1, *PgOMT01*, *PgOMT02* and *PgOMT03*), which are identical to those confirmed by Yuan et al. [20]. Pomegranate genome large-scale duplication gave rise to AOMT tandem duplication [20]. Gene tandem duplication events drove the evolution of PgAOMT family to some extent.

Pseudogenes tend to have significantly lower expression compared with annotated genes [59]. They are considered as nonfunctional genes that have similar structure to functional genes in a domain family. Hence, numerous pseudogenes were misidentified as functional genes during the genome annotation process [59]. Three tandem duplicated genes of *PgOMT01*, *PgOMT02* and *PgOMT03* have low or no gene expression levels in peel or aril development (Figure 3B), suggesting they were pseudogenes and genes may experience rapid birth and death during AOMT family evolution [60]. In addition, the expression of *PgOMT04* and *PgOMT10* demonstrated significantly higher than others both in peel and aril development (Figure 3B). The high expression supported the assumption that the PgAOMTs evolved new functions after genome duplication [36].

Anthocyanin accumulation is known to be induced for resisting abiotic stresses [61]. AOMTs have been identified that are capable of anthocyanin methylation, particularly endows plant with an enhanced protection, have experienced genome duplication, presumably due to selection imposed by rapid changes in abiotic environments [18,20]. As we all know, methylation modification plays roles in increasing anthocyanin diversity and stability. In addition, the anthocyanin methylation level is significantly correlated with the hue towards red or purple of plant tissues [8,45,62]. Zhao et al. [63] proposed that AOMT could be responsible for pomegranate peel and aril color transition from white to red. It has been proposed that the methylation of B-ring hydroxyl groups generates the shift towards red [64]. In "Taishanhong" pomegranate, Cyanidin-3Glucoside and Delphinidin-

3-glucoside were detected the major anthocyanins, but methylated anthocyanin contents have not been reported [65].

Subsequently, qRT-PCR (Figure 3) results showed that AOMT genes were expressed in peel and aril of pomegranate fruit. *PgOMT04* and *PgOMT09* in peel and *PgOMT04* in aril with higher expression throughout the development period, indicating that these O-methylations of anthocyanin and others may be closely related with fruit development and the ripening process. *PgOMT09* was down-regulated continuously in aril during the early development process, suggesting that it played roles in early development. *PgOMT03* and *PgOMT10* in peel and aril and *PgOMT10* in aril showed fluctuating expression pattern, suggesting that they may play roles in specific stages of fruit development. In contrast, *PgOMT09* expressed both in peel and aril, with distinct expression pattern, indicating that there were different accumulation of O-methylation anthocyanin between them. Interestingly, *PgOMT01* was specifically expressed in pomegranate peel, suggesting that its specific formation of O-methylated anthocyanin or others would occur in fruit peel.

5. Conclusions

In this study, we identified 10 OMTs from pomegranate genome and they were divided into two groups (AOMT and OMT group) by phylogenetic analysis. Pomegranate have undergone WGD, tandem duplication and gene loss events, presumably due to selection for improving their behavioral response to rapid changes of environment. The expression patterns of PgAOMTs indicated that some members function in anthocyanin methylation which enhances diversity and stability of anthocyanins and improves protection against stresses such as ultraviolet. This study also provides fundamental information on AOMTs for the coloration of pomegranate.

Supplementary Materials: The following are available online at https://www.mdpi.com/2073-4395/11/2/318/s1. Supplementary S1: A summary of species for phylogenetic analysis and their genomic source. Supplementary S2: Simplified species tree of 6 species. Supplementary S3: Oligo nucleotide primers information. Supplementary S4: Sequence analyses of the AOMT genes. (Note: 1~10 are represented respectively in the figure: ①Pg002351.1, ②OMT1, ③Pg002348.1, ④OMT2, ⑤Pg002346.1, ⑥OMT3, ⑦Pg026019.1, ⑧OMT4, ⑨Pg006183.1, ⑩OMT5).

Author Contributions: Conceptualization, X.Z. (Xinhui Zhang) and W.Y.; Writing—original draft preparation, X.Z. (Xinhui Zhang) and W.Y.; Writing—review and editing, X.Z. (Xinhui Zhang), W.Y.; Y.Z., Y.R., X.Z. (Xueqing Zhao) and Z.Y.; Visualization, X.Z. (Xinhui Zhang); Project administration, Z.Y.; All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by the Research Fund for Postgraduate Innovation Project of Jiangsu Province (KYLX16_0857), the Doctorate Fellowship Foundation of Nanjing Forestry University, the Initiative Project for Talents of Nanjing Forestry University (GXL2014070) and the Priority Academic Program Development of Jiangsu High Education Institutions (PAPD).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author and the public pomegranate transcriptomes presented in this study are available in insert article.

Conflicts of Interest: The authors declare no conflict of interest.

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