Genome-Wide Identification and Expression Analysis of TALE Gene Family in Pomegranate (Punica granatum L.)

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Abstract: The three-amino-acid-loop-extension (TALE) gene family is a pivotal transcription factor that regulates the development of flower organs, flower meristem formation, organ morphogenesis and fruit development. A total of 17 genes of pomegranate TALE family were identified and analyzed in pomegranate via bioinformatics methods, which provided a theoretical basis for the functional research and utilization of pomegranate TALE family genes. The results showed that the PgTALE family genes were divided into eight subfamilies (KNOX-I, KNOX-II, KNOX-III, BELL-I, BELL-II, BELL-III, BELL-IV, and BELL-V). All PgTALEs had a KNOX domain or a BELL domain, and their structures were conservative. The 1500 bp promoter sequence had multiple cis-elements in response to hormones (auxin, gibberellin) and abiotic stress, indicating that most of PgTALE were involved in the growth and development of pomegranates and stress. Function prediction and protein-protein network analysis showed that PgTALE may participate in regulating the development of apical meristems, flowers, carpels, and ovules. Analysis of gene expression patterns showed that the pomegranate TALE gene family had a particular tissue expression specificity. In conclusion, the knowledge of the TALE gene gained in pomegranate may be applied to other fruit as well.

Keywords: pomegranate; TALE gene family; bioinformatics; expression analysis

1. Introduction

A homebox (HB) encodes the transcriptional regulatory factors with homeodomain, which play considerable roles in the development of plants and animals [1]. A typical homebox domain consists of 60 amino acids, to form three-helix regions, the first and second helices form a loop structure, and the second and third helices form a helix-corner-helical structure [2]. Plant homebox genes have been divided into distinct subgroups, Bharathan et al. [3] divided them into seven classes, including KNOTTED-like homeobox (KNOX/KNAT), BEL1-like homeobox (BEL/BLH), Zea mays homeobox (ZM-HOX), homebox from Arabidopsis thaliana 1 (HAT1), homebox from A. thaliana 2 (HAT2), A. thaliana homebox 8 (ATHB8) and GL2. Mukherjee et al. [4] classified them into 14 classes, containing homeodomain-leucine zipper I to IV (HD-ZIP I to IV), BELL, KNOX, plant zinc finger (PLINC), wuschel homebox (WOX), plant homeodomain (PHD), DDT, nodulin homeobox genes (NDX), luminidependens (LD), SAWADEE and PINTOX. While Burglin and Afsofter [5] classified them into 11 classes, including HD-ZIP I to IV, WOX, NDX, PHD, PLINC, LD, DDT, SAWADEE, PINTOX,
KNOX and BELL. In PlantTFDB, the homeobox genes consist of five families: HD-ZIP, TALE, WOX, HB-PHD, and HB-other [6].

Based on protein sequence and evolution, BELL and KNOX belong to the TALE gene family [7,8]. Except for some homeobox genes, TALE encodes an atypical structure forming two helices and three additional amino acid residues (P-Y-P) [9–11]. The TALE family plays a vital role in regulating plant growth and development [12–15], regulating the sporophyte program [16], the formation of plant meristems [7], and the maintenance of organ morphology [17], organ position [18], hormone regulation [19], signal transduction [20] and tuber formation [21]. Studies have shown that BELL and KNOX proteins specifically recognize and bind to form the BELL-KNOX heterodimer protein [22], which is essential for the nuclear localization of two transcription factor proteins and the activity of binding target gene [23,24]. TALE can form complexes to regulate ovule development [25]. After binding to the OVATE family protein (OPF), the BELL-KNOX dimer protein is reversely transferred from the nucleus to the cytoplasm to negatively regulate ovule development [26]. BELL proteins comprise two highly conserved domains: a POX domain (POX is composed of SKY and BEL) and homeodomain. The BELL plays essential roles in ovule development, frond development and fruit development [27,28]. BELL1 is expressed in the ovule and controlled the ovule integument identity. The dimer formed by the A. thaliana homeobox 1 (ATH1) protein and the shoot meristemless (STM) protein participates in the development of plant meristems [29], while the dimer formed by the ATH1 protein and the KNOTTED-like from A. thaliana 2 (KNOT2) protein regulates the development of plant inflorescence tissue [30]. The interaction of BLH6 and KNA17 affects the development of secondary cell walls [31]. The KNOX gene family contains KNOX1, KNOX2, ELK and homeodomain, except for a novel gene KNATM without the homeodomain [32,33]. In addition, KNOX1 and KNOX2 domains merge to form a MEINOX domain. KNOX1 is expressed in the meristem, which is necessary for meristem development and maintenance. Studies have shown that the KNOX2 gene is involved in regulating the secondary growth of plant cell walls and plays a crucial regulatory role in the development of roots, stems, seed coats and heartwood [30,34–36].

Myrtales, the myrtle order of flowering plants, is placed in the Angiosperm Phylogeny Group IV (APG IV) botanical classification system [37]. Pomegranate (Punica granatum L.) is a considerable economic fruit tree of the Lythraceae family and widely cultivated worldwide. It was that pomegranate and the related species Eucalyptus grandis H., belonging to the order Myrtales, shared the paleotetraploidy event [38]. Studying the function and regulatory mechanism of TALE genes in pomegranate helps regulate pomegranate growth patterns, flower and fruit development. The completion of pomegranate genome data provided momentous data support for the study of pomegranate gene function [38–40]. In this study, the members of the TALE gene family were identified based on the genome sequence of 'Taishanhong', and their physical and chemical properties, protein structure, cis-elements, phylogenetic relationship, and gene tissue expression were analyzed. Through the systematic identification of PgTALE, the result lays a foundation for further study of the function of TALE genes in pomegranate.

2. Materials and Methods

2.1. Genome and Transcriptome Data Sources

Pomegranate genome sequences (ASM286412v1), protein sequences and transcriptome data were downloaded from NCBI (http://www.ncbi.nlm.nih.gov/), and the A. thaliana TALE protein sequence was downloaded from the A. thaliana database (http://www.arabidopsis.org), E. grandis, Populus trichocarpa, Malus domestica, Vitis vinifera and Solanum lycopersicum TALE protein sequence were downloaded from the PlantTFDB database (http://planttfdb.cbi.pku.edu.cn) [6] (Fasta file S1, Table S1).

2.2. Identification and Sequence Analysis of PgTALE Gene Family Members

The hidden Markov model file of the TALE family (E-value < 1e-5) was constructed by using TALE (PF00046) in the Pfam database [41] (https://pfam.xfam.org/) and hmmsearch program in
HMMPHMMER 3.0 software package (Virginia, USA) [42]. The candidate PgTALE protein conserved domains were searched, and the BELL domains (POX, homeodomain) or KNOX domains (KNOXI, KNOX2, ELK, homeodomain) were TALE gene family conserved. At the same time, using the published ‘Taishanhong’ protein sequences [38] and the TALE family protein sequences of 6 species (A. thaliana, E. grandis, P. trichocarpa, M. domestica, V. vinifera and S. lycopersicum) as baits to make a local BLASTP alignment (E-value < 1e⁻5, identity >50%), the repetition was removed, the candidate TALE protein sequences were screened. In addition, A. thaliana contains a member of the TALE gene family KNATM without homeodomain, we added a pomegranate gene homologous to KNATM [32], and then the target protein domains were detected by SMART and CDD [43,44]. The sequences without the TALE domain were removed. The online tool ExPASy Proteomics Server (https://web.expasy.org/protparam/) was used to predict the physical and chemical properties of PgTALE protein, such as amino acid sequence length, molecular weight, isoelectric point, grand average of hydropathicity [45]. Signal peptide of the PgTALE proteins was performed by SignalP 5.0 Sever (http://www.cbs.dtu.dk/services/SignalP). Subcellular localization of the PgTALE proteins was performed using CELLO (http://cello.life.nctu.edu.tw/) [46].

2.3. Construction of Phylogenetic tree of PgTALE Gene Family

Multiple sequence alignments of candidate proteins with A. thaliana, E. grandis, P. trichocarpa and V. vinifera TALE gene family proteins were performed using MAFFT [47]. The phylogenetic tree was constructed by using RA × ML -NC [48] with Bootstrap 1000 repeats and the best model of JTT + F + I + G4 selected by ModelFinder [49]. Then, the phylogenetic tree was beautified by using the online software tool EvolView (http://www.evolgenius.info/) [50].

2.4. Analysis of PgTALE Conserved Motifs and Gene Structure

The motif type and sequence of the PgTALE family were analyzed by MEME (http://meme-suite.org/tools/meme) [51], and the motif characteristics of PgTALE were obtained. According to the protein sequence and gene sequence of the PgTALE gene, the gene structure information of pomegranate TALE was obtained by Perl script (Perl file S1), including intron, exon and upstream and downstream sequence. In addition, a combined figure of phylogenetic tree, conserved motifs and gene structure was drawn by TBtools [52].

2.5. Analysis of PgTALE Protein Structure

Protein sequence similarity of more than 35% as a template, the tertiary structure and homologous modelling of PgTALE proteins were analyzed using the SWISS-MODEL (https://swissmodel.expasy.org/) [53], and Ramachandran Plots were used to display protein properties.

2.6. Analysis of Cis-elements and Protein-protein Interaction Network of PgTALE Gene Family

To analyze the cis-elements of the promoter region, the 1500 bp sequence upstream of the start codon was obtained from the pomegranate genome sequence by Perl script (Perl file S2), and the sequence was searched by PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) [54]. The protein–protein interaction network of the TALE family was analyzed by String (https://string-db.org/) [55].

2.7. Expression Analysis of PgTALE Gene Family

RNA-Seq data of tissues and organs closely related to pomegranate were downloaded from the NCBI database (Table 1). Subsequently, Kallisto version 0.44.0 software (California, USA) [56] was used to index the sequence with the ‘Taishanhong’ transcriptome file to calculate further and analyze gene expression. The corresponding expression levels (TPM values) of the TALE family members were obtained, and the obtained TPM values were converted by Log2 (TPM + 1). Finally, a heat map of the TALE gene was drawn by using the R package heatmap.
Table 1. RNA-Seq data of pomegranate.

| Accession No. | Cultivars | Sample Type | Library | Platform | Reference | Note |
|---------------|-----------|-------------|---------|----------|-----------|------|
| SRR5279396    | ‘Dabenzi’ | root        | Paired  | Illumina | HiSeq 4000 | [39] |
| SRR5279397    | ‘Dabenzi’ | leaf        | Paired  | Illumina | HiSeq 4000 | [39] |
| SRR5279395    | ‘Dabenzi’ | flower      | Paired  | Illumina | HiSeq 4000 | [39] |
| SRR5279391    | ‘Dabenzi’ | Inner seed coat (50 days after pollination) | Paired | Illumina | HiSeq 4000 | [39] |
| SRR5279388    | ‘Dabenzi’ | Outer seed coat (50 days after pollination) | Paired | Illumina | HiSeq 4000 | [39] |
| SRR5279394    | ‘Dabenzi’ | Pericarp (50 days after pollination) | Paired | Illumina | HiSeq 4000 | [39] |
| SRR5446598    | ‘Tunisia’ | flower (3.0–5.0 mm) | Paired | Illumina | HiSeq 2500 | [57] |
| SRR5446595    | ‘Tunisia’ | flower (5.1–13.0 mm) | Paired | Illumina | HiSeq 2500 | [57] |
| SRR5446592    | ‘Tunisia’ | flower (13.1–25.0 mm) | Paired | Illumina | HiSeq 2500 | [57] |
| SRR5446607    | ‘Tunisia’ | flower (3.0–5.0 mm) | Paired | Illumina | HiSeq 2500 | [57] |
| SRR5446604    | ‘Tunisia’ | flower (5.1–13.0 mm) | Paired | Illumina | HiSeq 2500 | [57] |
| SRR5446601    | ‘Tunisia’ | flower (13.1–25.0 mm) | Paired | Illumina | HiSeq 2500 | [57] |
| SRR5678820    | ‘Tunisia’ | Inner seed coat (50 days after pollination) | Paired | Illumina | HiSeq 4000 | [39] |
| SRR5678819    | ‘Baiyushizi’ | pericarp | Paired | Illumina | HiSeq 4000 | [58] |
| SRR1055290    | ‘nana’ | Mixed samples of leaves, flowers, fruit and roots | Single | 454 GS FLX Titanium | [40] |
| SRR1054190    | ‘Black127’ | Mixed samples of root, leaf, flower and fruit | Single | 454 GS FLX Titanium | [40] |

3. Results

3.1. Identification and Sequence Analysis of PgTALE Gene Family Members

In this study, 74 homebox gene family members were identified by using the hmmsearch method. The homebox family consists of five families (HD-ZIP, TALE, WOX, HB-PHD, and HB-other), and they share a PF number (PF00046). As TALE encodes an atypical structure forming two helices and three additional amino acid residues, 16 candidate members of the TALE gene family were identified, and all candidate proteins were identified to belong to the TALE protein family. 23 candidate members of the TALE gene family were identified by BLASTP. While Pg001623.1, Pg009439.1, Pg011532.1, Pg017964.1, Pg017965.1, Pg022249.1 and Pg027515.1 were removed because they did not contain TALE conserved domain. Our result showed that there were 17 members of the
TALE gene family in pomegranate (Table S2). *PgTALE* gene family was renamed, the results as shown in Table 2.

The physical and chemical properties of the *PgTALEs* was analyzed using the ExPaSy online tool. The results showed that the length of the 17 *PgTALE* gene coding regions ranged from 465 bp (*PgTALE17*) to 2400 bp (*PgTALE16*). The amino acid length of the TALE protein ranged from 154 aa (*PgTALE17*) to 799 aa (*PgTALE16*), and the protein molecular weight ranged from 17605.66 Da (*PgTALE17*) to 87381.25 Da (*PgTALE16*). The pI ranged from 5.13 (*PgTALE7*) to 8.78 (*PgTALE16*). Among them, the pl of three *PgTALE* proteins were higher than 7, suggesting that proteins were slightly alkaline; the other 14 *PgTALEs* were acidic proteins. The grand average of hydropathicity (GRAVY) was between −0.969 to −0.449, suggesting that *PgTALEs* are all hydrophilic proteins. The number of exons of the *PgTALEs* was 3–6. Besides, the signal peptide prediction showed that there were no signal peptides in all *PgTALE* proteins, which belonged to non-secreted proteins. Subcellular localization prediction suggested that all *PgTALE* proteins were distributed on the nucleus.

Table 2. The basic information of the TALE gene family in pomegranate.

| Gene Name | Gene ID | Location | ExonNo | CD S | AA | MW(Da) | pI | GRAVY | Subcellular Localization |
|-----------|---------|----------|--------|------|----|--------|----|-------|-------------------------|
| *PgTALE1* | Pg002952.1 | scaffold1:12366550:23692 | 4 | 2097 | 69 | 75,172.9 | 7.8 | −0.538 | Nuclear |
| *PgTALE2* | Pg005682.1 | scaffold13:4128457:41889 | 5 | 1035 | 34 | 38,810.1 | 5.2 | −0.795 | Nuclear |
| *PgTALE3* | Pg009001.1 | scaffold17:1307284:13128 | 4 | 1959 | 65 | 70,713.7 | 6.5 | −0.557 | Nuclear |
| *PgTALE4* | Pg011533.1 | scaffold2:5274898:527778 | 4 | 1932 | 64 | 70,086.6 | 6.3 | −0.566 | Nuclear |
| *PgTALE5* | Pg014946.1 | scaffold26:103332:10398 | 5 | 1065 | 35 | 40,403.9 | 6.1 | −0.969 | Nuclear |
| *PgTALE6* | Pg015766.1 | scaffold29:588196:590712 | 4 | 1905 | 63 | 70,031.4 | 5.8 | −0.717 | Nuclear |
| *PgTALE7* | Pg02248.1 | scaffold49:1377908:13861 | 5 | 1065 | 35 | 39,800.2 | 5.1 | −0.751 | Nuclear |
| *PgTALE8* | Pg024529.1 | scaffold6:3685906:368822 | 4 | 1563 | 52 | 58,385.6 | 6.1 | −0.449 | Nuclear |
| *PgTALE9* | Pg024817.1 | scaffold6:2019598:202237 | 5 | 1275 | 42 | 46,087.0 | 5.9 | −0.635 | Nuclear |
| *PgTALE10* | Pg026506.1 | scaffold7:1730705:173774 | 4 | 2103 | 70 | 76,325.2 | 7.8 | −0.647 | Nuclear |
| *PgTALE11* | Pg027513.1 | scaffold7:612910:617019 | 4 | 1023 | 34 | 37,775.8 | 6.5 | −0.534 | Nuclear |
| *PgTALE12* | Pg028434.1 | scaffold8:2877271:288087 | 5 | 909 | 30 | 33,829.1 | 6.2 | −0.635 | Nuclear |
| *PgTALE13* | Pg028770.1 | scaffold8:128287:132801 | 5 | 2136 | 71 | 78,483.7 | 6.5 | −0.513 | Nuclear |
| *PgTALE14* | Pg029909.1 | scaffold9:754612:75782 | 4 | 1851 | 61 | 68,481.0 | 6.2 | −0.707 | Nuclear |
| *PgTALE15* | Pg030082.1 | scaffold9:3730507:373380 | 6 | 1203 | 40 | 45,375.8 | 5.3 | −0.730 | Nuclear |
| *PgTALE16* | Pg030621.1 | scaffold9:200629:21887 | 6 | 2400 | 79 | 87,381.2 | 8.7 | −0.549 | Nuclear |
| *PgTALE17* | Pg005241.1 | scaffold13:2266264:22688 | 3 | 465 | 15 | 17,605.6 | 5.1 | −0.730 | Nuclear |
3.2. Phylogenetic Tree Analysis of PgTALE Gene Family

To clarify the evolutionary relationship and possible biological functions of members of the PgTALE gene family, the phylogenetic tree of the TALE gene was constructed based on the amino acid sequences of the pomegranate, *A. thaliana*, *E. grandis*, *P. trichocarpa* and *V. vinifera* (Figure 1). Based on the classification of *A. thaliana* TALE gene family (BELL and KNOX family), the pomegranate BELL proteins were classified into five subfamilies: BELL-I (one member), BELL-II (two), BELL-III (one), BELL-IV (two) and BELL-V (three), and KNOX proteins were classified into 3 subfamilies: KNOX-I (five), KNOX-II (two), KNOX-III (five). In each clade, there are branches from the same species, which may be caused by gene duplications [59].

![Phylogenetic Tree](image)

*Figure 1.* The phylogenetic tree of the three-amino-acid-loop-extension (TALE) gene family in pomegranate, *A. thaliana*, *E. grandis*, *P. trichocarpa* and *V. vinifera*. The phylogenetic tree was constructed by RA × ML - NC with Bootstrap 1000 repeats and the best model of JTT + F + I + G4. Pomegranate, *A. thaliana*, *E. grandis*, *P. trichocarpa* and *V. vinifera* TALE proteins are marked with a solid red star, solid green star, red check, blue triangle and blue circle, respectively.

3.3. Analysis of Conserved Motifs and Gene Structures of PgTALE Gene Family

The conserved motifs of PgTALE were identified. 10 conserved motifs (Figure 2), in which Motif 1 represents the homeodomain (homeobox domain, HOX), Motif 4 represents the ELK domain, and Motif 7 represents SKY domain. The location information of the PgTALE protein domain was analyzed. The results showed that the six members of KNOX subfamily contained KNOX1, KNOX2, ELK and HOX domains, and only *PgTALE17* did not contain HOX domains. BELL subfamily all contained POX and HOX domains.
Structural analysis showed that the gene structure of *PgTALE* was a similarity, and there were little differences in the number of exons and introns among *PgTALE* genes. The number of exons and introns of the *PgTALE* genes were 3–6 and 2–5, respectively (Figure 2). Members of the same subfamily of *PgTALE* showed similar gene structure and protein conserved motif distribution. For example, five members of KNOX group (*PgTALE2, PgTALE5, PgTALE7, PgTALE9* and *PgTALE12*) contained five exons and four introns, and only one member (*PgTALE17*) contained 3 exons and 2 introns. Seven members of BELL group (*PgTALE1, PgTALE3, PgTALE4, PgTALE6, PgTALE8, PgTALE10* and *PgTALE14*) contained four exons and three introns. The above results indicated that the *PgTALE* gene family had a certain degree of conservation regardless of its genetic structure or protein conserved motifs.

**Figure 2.** Phylogenetic tree, conserved motifs and gene structures of the *PgTALE* gene family. (A) The phylogenetic tree of 17 *PgTALE* proteins. (B) Conserved motifs in the TALE proteins. The motifs were identified by the MEME Suite. Different conserved motifs, numbers 1–10, are displayed in different colored boxes. (C) Gene structures of the TALE genes. The intron/exon structure was mapped by TBtools. The black dotted line represents introns. (D) Sequence logos of 10 conserved motifs were identified.
3.4. Protein Structure Analysis and Protein Interaction Networks of Pomegranate TALE Gene Family

The spatial structure of proteins plays a role in the biological function of proteins. The tertiary structure of the protein was analyzed, which found that the structure of the PgTALE family members was similar (Figure 3), except for PgTALE17 without a template (protein sequence similarity of less than 35%) that we cannot predict protein tertiary structure. The protein is a multi-chain folded protein, mainly α-helix. The calculation test showed that the Ramachandran Favoured value of the PgTALE family was above 90%, and PgTALE2 and PgTALE15 reached 100%, except PgTALE9 was only 87.27%. The results showed that the PgTALE protein had a stable spatial structure.

![Figure 3. Tertiary structure and Ramachandran plot of PgTALE protein. The tertiary structure of the protein was analyzed through the online SWISS-MODEL.](image)

Protein function prediction suggested that PgTALE2, PgTALE7 and PgTALE15 played roles in meristem function (Figure 4), contributing to the shoot apical meristem (SAM) maintenance and organ separation. They may also be involved in maintaining cells in a meristematic state. In addition, PgTALE14 might involve in the regular pattern of organ initiation. PgTALE11 may be required for SAM formation in embryogenesis. PgTALE12 may be involved in secondary cell wall biosynthesis. PgTALE13 might be required for the SAM to respond appropriately to floral inductive signals.

The protein-protein interaction of PgTALE was analyzed for predicting its potential function, signal transduction and metabolic pathways. It was predicted that there were interactions between PgTALE14 and AG, SEP3, KNAT1, INO and other proteins to regulate ovule development. In addition, BEL1 can form heterodimers with KNAT1, it predicted that PgTALE14 (BELL family) may interact with PgTALE5 (KNOX family) to form heterodimers. PgTALE8 might interact with STM and KNAT6 and enhance the apical meristem of these genes.

From the figure of gene co-expression (Figure 4), we can see the level of co-expression of KNAT1/KNAT3/KNAT6/STM/BEL1/BLH6/ATH1 and other genes. Among them, KNAT1 and KNAT6, KNAT1 and STM were higher than that of other genes. They may participate in or respond to a biological or abiotic stress process, and it may be inferred that PgTALE2/PgTALE5/PgTALE11 may also have similar functions.
3.5. Analysis of Cis-elements of PgTALE Gene Family

In this study, the upstream 1500 bp sequence of PgTALE gene was extracted, the possible cis-elements in the promoter region were found (Table S3). 13 cis-elements related to abiotic stress were found, which were ABRE, ARE, AuxRR-core, CAAT-box, CGTCA-motif, GARE-motif, LTR, MBS, P-box, TATC-box, TCA-element, TGA-element and TGACG-motif (Figure 5; Table S4). AuxRR-core and TGA-element are auxin-responsive elements. CGTCA-motif and TGACG-motif are MeJA-responsiveness elements, while GARE-motif, P-box and TATC-box are gibberellin response elements. The PgTALE genes contain the enhancer response element CAAT-box. 64.7% of the PgTALE genes contain ABA response element ABRE and the cold stress response element LTR. 70.6% of the PgTALE genes contain the antioxidant response element ARE. 41.2% of the PgTALE gene contains MeJA-responsiveness response elements CGTCA-motif and TGACG-motif, the salicylic acid response element TCA-element, 29.4%, 23.5%, 35.3% of the PgTALE genes contain gibberellin response elements GARE-motif, P-box, TATC-box, 29.4% of the PgTALE genes contain the drought stress response element MBS. Besides, only the PgTALE8 gene contains the auxin response element AuxRR-core, and PgTALE12 and PgTALE13 contain the auxin response element TGA-element.
3.6. Expression Analysis of PgTALE Gene Family

To further analyze the characteristics and function of the PgTALE genes, the tissue-specific expression of the TALE gene was analyzed (Figure 6; Table S5). The results showed that the vast majority of PgTALE genes were expressed in different tissues, but PgBLH8 was expressed in trace or no expression in all tissues.

PgTALE5, PgTALE12, and PgTALE15 are expressed during functional male flower development, indicating that these genes may be involved in the female and male organ differentiation; PgTALE1 and PgTALE9 are higher expressed in leaves, bisexual and functional male flower, indicating that they may be related to the differentiation of male and female organs of pomegranate flowers and regulating leaf development. There are also differences in the expression of different PgTALE genes in different tissue, such as PgTALE2 is not expressed in the inner seed coat, outer seed coat and pericarp. The expression of PgTALE9 is the highest in the functional male flower (5.1 m–13.0 mm), and the expression of PgTALE10 is the highest in the pericarp. However, there are some differences in the expression of different PgTALE genes in different pomegranate varieties, such as PgTALE7 and PgTALE14 in the varieties of ‘Dabenzi’, ‘Tunisia’ and ‘Baiyushizi’. In the same pomegranate variety ‘Dabenzi’, there are also significant differences in tissue expression between leaves and outer seed coat. For example, the expression of PgTALE16 is higher in leaves, but the lowest in the outer seed coat.

![Heatmap of PgTALE gene expression in different tissues](image)

**Figure 6.** Heatmap of PgTALE gene expression in different tissues. Note: S1: Root; S2: Flesh leaf; S3: Flower; S4: Inner seed coat of ‘Dabenzi’ (50 days after pollination); S5: Outer seed coat (50 days after pollination); S6: Pericarp (50 days after pollination); S7: Functional male flowers (1.3–25.0 mm); S8: Functional male flowers (5.1–13.0 mm); S9: Functional male flowers (3.0–5.0 mm); S10: Female fertility (4.1–5.0 mm); S11: Female fertility (5.1–13.0 mm); S12: Female fertility (3.0–5.0 mm); S13: Inner seed coat of ‘Tunisia’ (50 days after pollination); S14: Inner seed coat of ‘Baiyushizi’ (50 days after pollination); S15: Pericarp of ‘Wonderful’; S16: Mix of leaves, flowers, fruit and roots of ‘nana’; S17: Mix of leaves, flowers, fruit and roots of ‘Black127’ (cultivars S1–S6 are ‘Dabenzi’, cultivars S7–S13 are ‘Tunisia’).

4. Discussion

The TALE gene family is found in plant meristems and is related to the differentiation and signal transduction of meristems, for example, it can inhibit the expression of the critical enzyme gene ga20ox1 in the GA pathway [60]. In other important fruits belonging to the Rosaceae family, TALE are involved in the rootstock responding to apple cold stress [61], the cherry anthesis [62]. In addition,
it regulated tomato fruit development [28]. Currently, the TALE gene family has been found in many plants: 33 AtTALE genes in *A. thaliana*, 40 LjTALE genes in *Lotus japonicas* K. [63], 46 GaTALE genes in *Gossypium* *arboretum* L., 47 GrTALE genes in *G. raimondii* L., 88 GkTALE genes in *G. barbadense* L., 94 GhTALE genes *G. hirsutum* L. [8], and 35 PiTALE genes in poplar [64], 7 VsTALE genes in *Vandenboschia speciose* G. [16]. Therefore, the copy number of the TALE gene family in different species is different. At present, the genomic data of three pomegranate varieties have been released in China, but there are no reports on the identification and analysis of pomegranate TALE family genes. In this study, for the first time, 17 TALE genes were identified in the pomegranate. Through the analysis of the physicochemical properties of the protein (Table 1), it was found that pomegranate TALE proteins are all hydrophilic proteins that are consistent with studies in *Populus* and *L. japonicas* [63,64]. Domain differences may represent the regulatory effects of promoting or inhibiting. In addition, the *PgTALE* genes are divided into eight subfamilies (Figure 1), which is consistent with the *A. thaliana* and cotton TALE gene subfamily classification [8].

The cis-elements exist at the gene promoter site and specifically binds transcription factors to regulate gene transcription. This study found that the *PgTALE* promoter sequence contained multiple cis-elements related to hormonal response and abiotic stress, which are rich in methyl jasmonate response element, abscisic acid response element and gibberellin response element, which is similar to antecedent studies [8]. It indicated that the promoter of the TALE gene has a certain conservative. Previous studies have found that ABRE is associated with plant drought, ABA induction, and high salt stress in plants [31,65]. In addition, there are a series of elements related to stress, such as ARE, MBS and LTR. The results indicate that *PgTALE* plays a role in pomegranate abiotic stress. Gene function prediction and protein-protein network analysis also show that the *PgTALE* family plays a significant role in regulating ovule and inflorescence development. Gene functional prediction and protein-protein network analysis also showed that there are some interactions between *PgTALE14*, AG and KNAT1 in floral organs; the results were consistent with the previous study [7,25].

The tissue expression analysis of the *PgTALE* found that most of them were expressed in diverse tissues and varieties, but diverse *PgTALE* genes were expressed in different tissue varieties and showed specific differences. It was similar to the results of TALE genes in *A. thaliana* [66]. It can be speculated that the TALE family of pomegranate has similar functions to this family in other plants. According to the function of *BEL1* in *A. thaliana*, we speculated that its homologous gene *PgTALE14* has important regulatory significance in the development of pomegranate ovules [67,68]; *PgTALE8*, as the homologous gene of *ATH1*, controls inflorescence development [69,70]. The specificity of tissue and variety expression is speculated to be closely related to its gene function. For example, *PgTALE1*, *PgTALE6*, *PgTALE9*, *PgTALE10*, *PgTALE12* and *PgTALE14* had high expression levels in the functional male flowers, bisexual flowers, and fruit tissues. It is predicted that *PgTALE* may have roles in maintaining flower organ and fruit development. However, due to the inconsistency of some sequencing platforms (Illumina and 454) in RNA-seq data, to a certain extent, it may lead to the uneven sequencing depth among tissue samples and the gap in reading length, which has a certain impact on the analysis results, while the difference in pomegranate varieties also has a certain error on the expression analysis results. After the normalization of RNA-seq data, the error may be reduced.

5. Conclusions

In this study, 17 *PgTALE* members were identified in pomegranate and explored their phylogenetic relationships. The *PgTALE* gene structure of all members of the subfamily is very similar. *PgTALE* may participate in the apical meristems, flower organ and fruit development, and the subfamily genes may have the same expression pattern. These conclusions are the foundation for the function research of the *PgTALE* gene and provide a reference for exploring its evolutionary process.
Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1. Fasta file S1: TALE sequences of 6 species as baits, Perl file S1: Gene structure, Perl file S2: Promoter, Table S1: IDs of TALE sequences of 6 species, Table S2: Domains of 17 PgTALE, Table S3: All cis-elements of 17 PgTALE, Table S4: 13 cis-elements related to abiotic stress of 17 PgTALE, Table S5: RNA-seq data of 17 PgTALE.

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Abbreviations:

| Abbreviations | Full Name                                |
|---------------|------------------------------------------|
| TALE          | three-amino-acid-loop-extension          |
| KNOX/KNAT     | KNOTTED-like homebox                     |
| BELL/BLH      | BEL1-like                                |
| HB            | homeobox                                 |
| ZM-HOX        | Zea mays homebox                         |
| HAT1          | homeobox from *Arabidopsis thaliana* 1   |
| HAT2          | homeobox from *A. thaliana* 2            |
| ATHB8         | *A. thaliana* homeobox 8                 |
| HD-ZIP        | homeodomain-leucine zipper               |
| PLINC         | plant zinc finger                        |
| WOX           | wuschel homeobox                         |
| PHD           | plant homeodomain                        |
| NDX           | nodulin homeobox genes                   |
| LD            | luminiidependens                         |
| OFP           | OVATE family protein                     |
| ATH1          | *A. thaliana* homeobox 1                 |
| STM           | shoot meristemless                       |
| KNAT2         | KNOTTED-like from *A. thaliana* 2        |
| APG IV        | Angiosperm Phylogeny Group IV            |
| HOX           | homeobox domain                          |
| SAM           | shoot apical meristem                    |

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