Genome-wide identification of candidate aquaporins involved in water accumulation of pomegranate outer seed coat

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ABSTRACT

Aquaporins (AQPs) are a class of highly conserved integral membrane proteins that facilitate the uptake and transport of water and other small molecules across cell membranes. However, little is known about AQP genes in pomegranate (*Punica granatum* L.) and their potential role in water accumulation of the outer seed coat. We identified 38 PgrAQP genes in the pomegranate genome and divided them into five subfamilies based on a comparative analysis. Purifying selection played a role in the evolution of PgrAQP genes and a whole-genome duplication event in Myrtales may have contributed to the expansion of PgrTIP, PgrSIP, and PgrXIP genes. Transcriptome data analysis revealed that the PgrAQP genes exhibited different tissue-specific expression patterns. Among them, the transcript abundance of *PgrPIPs* were significantly higher than that of other subfamilies. The mRNA transcription levels of *PgrPIP1.3*, *PgrPIP2.8*, and *PgrSIP1.2* showed a significant linear relationship with water accumulation in seed coats, indicating that PgrPIP1.3/PgrPIP2.8 located in the plasma membrane and PgrSIP1.2 proteins located on the tonoplast may be involved in water accumulation and contribute to the cell expansion of the outer seed coat, which then develops into juicy edible flesh. Overall, our results provided not only information on the characteristics and evolution of PgrAQPs, but also insights on the genetic improvement of outer seed coats.

Subjects Agricultural Science, Genomics, Molecular Biology, Plant Science
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INTRODUCTION

Pomegranate (*Punica granatum* L.) is an important economic fruit tree species due to its functional and nutraceutical properties, and it is widely consumed as a fruit, juice, wine, and medicine (*Johanningsmeier & Harris, 2011*; *Patel et al., 2008*). Pomegranate is native to Iran, the northern India side of the Himalayan Mountains, and is widely planted in Mediterranean-like climates around the world, including Tunisia, Turkey, Spain, Egypt,

Iran, Morocco, the USA, China, India, Argentina, Israel, and South Africa (*Qin et al., 2017*). The size of the juicy outer seed coat determines the edible quality of the fruit, so it has become an important fruit characteristic. Notably, the morphological characteristics of the pomegranate seed showed a compressed inner seed coat and an expanded outer seed coat, making it an appealing model for studying development of seed coats (*Luo et al., 2020*; *Niu et al., 2018*; *Qin et al., 2020*).

The development of the seed coat is accompanied by the import of organic and inorganic nutrients, including sugars, organic acids, cellulose, and water in developing seeds (*Qin et al., 2020*; *Uçar & Karagöz, 2009*; *Zarei et al., 2016*; *Zhou et al., 2007*). The symplastic pathway is one of the main pathways by which water moves, which is mediated by integral membrane proteins called aquaporins (AQPs), a major intrinsic protein family (*Adams & Wendel, 2005*; *Lian et al., 2004*; *Suga et al., 2002*). AQPs have been shown to transport small molecules such as carbon dioxide, glycerol, ammonia, urea, hydrogen peroxide, and selenite (*Ivanov et al., 2007*; *Yasui et al., 1999*; *Zwiazek et al., 2017*).

The general AQP structure is highly conserved in plants, and it is predicted to consist of six transmembrane (TM) α-helices (H1 to H6) and two half-helices (*Deshmukh et al., 2015*; *Lee et al., 2005*; *Tornroth-Horsefield et al., 2006*). These transmembrane α-helices are linked by five short loops (Loops A to E), Loops B and E contain the signature sequence Asparagine-Proline-Alanine (NPA) motif, which has the primary function of forming water-selective channels (*Chaumont et al., 2001*; *Gupta & Sankararamakrishnan, 2009*; *Wallace & Roberts, 2004*). Another important secondary structure known as an aromatic/arginine (ar/R) selectivity filter is formed by four residues contributed by transmembrane helices H2/H5 and the loop LE (*Azad et al., 2016*; *Deshmukh et al., 2015*; *Hove & Bhave, 2011*; *Tornroth-Horsefield et al., 2006*). These two structures predominantly determine the specificity of solute transport and transport rate (*Deshmukh et al., 2015*; *Lee et al., 2005*; *Tornroth-Horsefield et al., 2006*). According to sequence similarity and protein subcellular localization, AQPs in higher plant can be classified into five distinct subfamilies: plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), nodulin 26-like intrinsic proteins (NIPs) (*Pommerrenig, Diehn & Bienert, 2015*), small basic intrinsic proteins (SIPs), and unrecognized (or X) intrinsic proteins (XIPs) (*Chaumont et al., 2001*; *Danielson & Johanson, 2010*; *Kaldenhoff & Fischer, 2006*; *Khabudaev et al., 2014*; *Quigley et al., 2002*).

The AQP gene family has been widely studied in numerous plant species, such as *Arabidopsis thaliana* (*Quigley et al., 2002*), *Oryza sativa* (*Sakurai et al., 2005*), *Glycine max* (*Zhang et al., 2013*), cotton (*Li et al., 2019*), *Populus trichocarpa* (*Gupta & Sankararamakrishnan, 2009*), and grape (*Fouquet et al., 2008*), by blasting whole genome sequences. Previous studies have demonstrated that AQPs in higher plants play important roles in various physiological and development processes, such as cell and tissue expansion, fiber development, flower pollination, and seed development (*Azad et al., 2004*; *Eisenbarth & Weig, 2005*; *Gattolin, Sorieul & Frigerio, 2011*; *Soto et al., 2008*; *Soto et al., 2010*); (*Van der Willigen et al., 2006*; *Wudick et al., 2014*). For instance, in French bean (*Phaseolus vulgaris*), *PvPIPs* played a role in the import of water and nutrients by phloem-mediated transport and water recycling in the xylem in developing seeds (*Terashima & Ono, 2002*).

In rice, OsPIP1;1 and OsPIP1;3 functions as water channels. Over-expression of *OsPIP1;1* could increase rice yield and seed germination. Similarly, the overexpression of *OsPIP1;3* promoted the speed of seed germination under drought conditions (*Liu et al., 2013*; *Liu et al., 2007*). In *Pisum sativum* L., *PsNIP1* showed high expression level in developing pea seed coats, and the overexpression of *PsNIP1* increased the uptake of water and glycerol (*Schuurmans et al., 2003*; *Zhou et al., 2007*). Interestingly, the highly correlations between the expression of *AtTIP3/AtTIP1* and seed germination stages (a rapid imbibition of desiccated tissues and embryo growth, respectively) was observed, which also provided insight into the influence of AQPs on the seed developmental process (*Gattolin, Sorieul & Frigerio, 2011*). However, little is known about the role of AQPs on seed coat development.

In this study, we identified 38 *PgrAQP* genes in the pomegranate genome, then conducted gene structure, phylogenetics, and evolutionary divergence analyses. The expression patterns of *PgrAQP* genes in different tissues and seed developmental stages were analyzed, and the potential function of PgrPIP genes in response to drought stress was also identified. Furthermore, the candidate genes contributing to the water accumulation in the seed coat were selected based on a correlation analysis of gene expression and water accumulation in seed coats. Our findings provided fundamental information about the gene structure, phylogenetics, and evolutionary divergence of *PgrAQP*. In addition, this study also provided useful information for further investigation of the molecular mechanism by which PgrAQP functions in seed coat development.

MATERIALS AND METHODS

Identification of the *PgrAQP* genes in the pomegranate genome

The whole genome sequences of pomegranate were downloaded from NCBI Genome (https://www.ncbi.nlm.nih.gov/genome/?term=Punica+granatum+L). Predicted protein sequences were obtained using TBtools with the following sets: Sequence Toolkit: Batch translate CDS to protein (*Chen et al., 2020*). The amino acid sequences of the AQPs in *Arabidopsis* and *Populus trichocarpa* were employed as queries to blast searches against the whole-genome sequences in pomegranate using TBtools with a query over 50% and e-value less than 0.01 (*Quigley et al., 2002*). The candidate sequences were submitted to NCBI for EST blast searches. Finally, the AQP protein sequences of pomegranate were examined to verify the presence of the characteristic MIP and transmembrane helical domains using the SMART program (http://smart.embl-heidelberg.de/) and TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) (*Finn et al., 2014*; *Letunic, Khedkar & Bork, 2020*). The information of PgrAQP gene family (protein length, molecular weight, and isoelectric point) were determined using Expasy (https://web.expasy.org/compute_pi/) (*Artimo et al., 2012*). The subcellular localization of the PgrAQP proteins was predicted by WoLF PSORT (https://www.genscript.com/psort/wolf_psort.html) Cell-PLoc 2.0 (http://www.csbio.sjtu.edu.cn/bioinf/Cell-PLoc-2/)

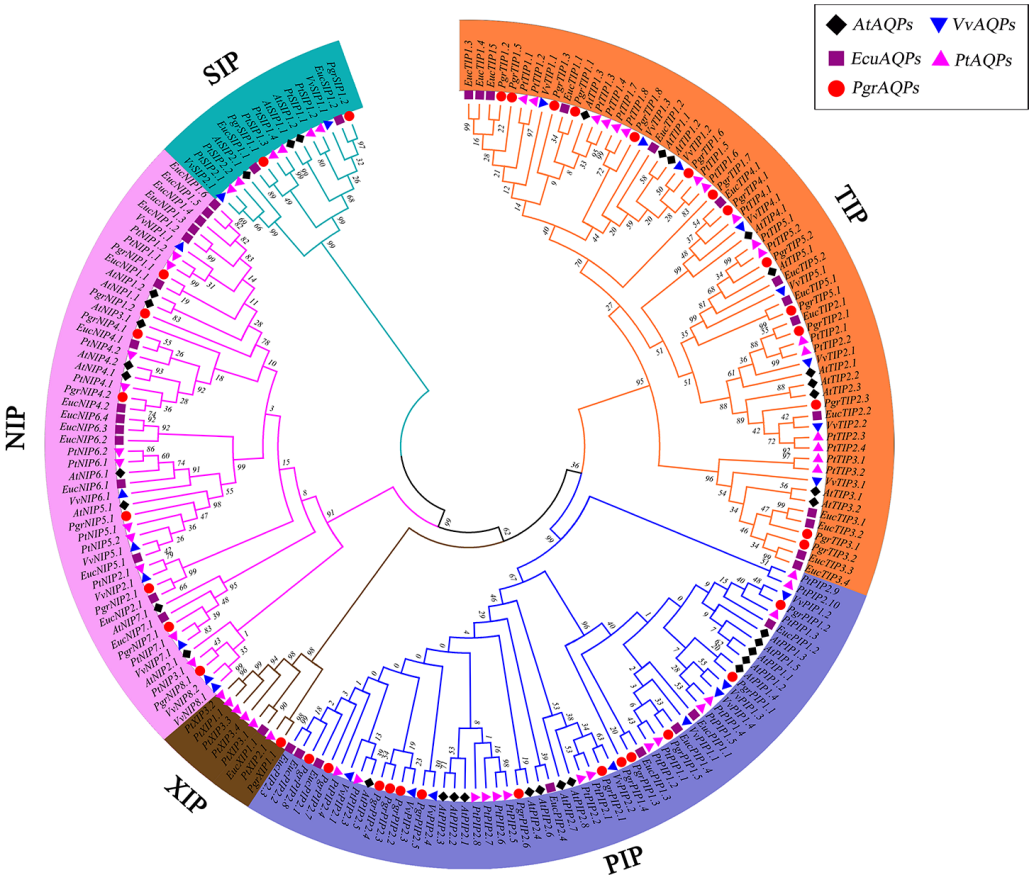

Figure 1 **Phylogenetic analysis of AQP proteins from pomegranate, grape, *Arabidopsis*, *Populus trichocarpa* and *Eucalyptus*.** The tree was generated by the neighbor-joining (NJ) method in MEGA 7. 0 with 1,000 bootstrap replicates. Different background colors indicate the different subfamilies of the AQP proteins.

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Sequence alignment of *PgrAQP* genes, phylogenetic analysis, and classification

The predicted plant AQP protein sequences were aligned using the ClustalW tool in MEGA7.0 (*Kumar, Stecher & Tamura, 2016*). *Arabidopsis thaliana,* grape (*Vitis vinifera*), *Populus trichocarpa* and eucalyptus (*Eucalyptus grandis*) AQP protein sequences were collected from NCBI (https://www.ncbi.nlm.nih.gov/). A phylogenic tree was constructed with MEGA7.0 using the neighbor-joining method and bootstrap parameter with 1,000 replicates. For this study, Pomegranate AQPs were named according to the sequence homology and phylogenetic relationships with *Populus trichocarpa* . According to the name of the best hit gene in *Populus trichocarpa*, the subfamily classification (PIP, NIP, SIP, TIP, and XIP) and corresponding names of AQPs are shown in Fig. 1. TBtools software was used to determine the localization of *PgrAQP* genes on pomegranate chromosomes.

Gene structure and conserved motif analysis of *PgrAQPs*

The conserved motifs in the proteins were identified using Multiple Expectation Maximization for Motif Elucidation (MEME v4.12.0, http://meme-suite.org/tools/meme) with the following parameters: maximum number of motifs, 10; width of optimum motif, ≥15 and ≤50 (*Bailey et al., 2009*). The gene exon–intron structures of AQPs were confirmed using the GSDS database by aligning the open reading frame (ORF) with their corresponding genomic sequences (*Hu et al., 2015a*). Sequences representing NPA motifs, ar/R filter, and Froger's residue positions were manually identified based on multiple sequence alignments of pomegranate AQPs with heterologous AQPs of *Arabidopsis* (*Kumar, Stecher & Tamura, 2016*). TBtools software was used to construct a chromosome localization map of AQP family genes.

Gene duplication and synteny analysis of the *PgrAQP* gene family

Gene duplication events of *PgrAQP* genes were analyzed using TBtools with the following parameters: Blast Compare Two Seqs, Outfml: Table, NumofThreads: 2, E-value:1e−5, NumofHits: 5, NumofAligns: 5; File Merger For MCScanX, Merge Mode: GtfGff2 SimGxf (*Chen et al., 2020*). Duplication types were divided into whole-genome (WGD), segmental, and tandem duplications (*Qiao et al., 2015*). Tandem duplicated genes were defined as two homologous genes within a range of 100-kb and sequence alignment coverage over 75% (*Gu et al., 2002*; *Wang et al., 2010*; *Yang et al., 2008*). The nonsynonymous substitution ratios (*Ka*), synonymous substitution ratios (*Ks*), and *Ka/Ks* ratios of the *PgrAQP* family genes were calculated using the TBtools simple *Ka/Ks* calculator program, with the following parameters: Simple Ka/Ks Calculator (NG). The *Ks* value was used to calculate the divergence time of duplication events (T =*Ks*/2R Mya, Millions of years), where R is the rate of synonymous substitutions, $R = 1.5 \times 10^{-8}$ substitutions per synonymous site per year for dicotyledonous plants and T refers to divergence time (*Edlund, Swanson & Preuss, 2004*). The microsyntenic relationship of AQP genes between pomegranate, grape, *Arabidopsis*, and eucalyptus was determined using TBtools Dual Systeny Plot for MCscanX program (*Chen et al., 2020*).

Plant materials and analysis of transcript profiles of *PgrAQP* genes

Two major pomegranate cultivars, 'Dabenzi' (a hard-seeded cultivar) and 'Tunisia' (a soft-seeded cultivar), were planted in Anhui Province (Hefei, 31°51′9.05″N, 117°06′34.33″E), China and grown under the same fertilization and irrigation conditions. Seeds from five fruits of the pomegranate cultivar 'Dabenzi' and 'Tunisia' were collected at 50, 95, and 140 days after pollination (DAP). For outer seed coats, the seeds from fruits collected 95 DAP and 140 DAP was used because it was difficult to visually distinguish the inner and outer seed coats of seeds were collected at 50 DAP. Three biological replicates were taken for RNA sequencing. For each treatment, the fresh weights of the total inner and outer seed coats were recorded and immediately frozen in liquid nitrogen. Part of the inner and outer seed coats were stored at −80 °C until they were used for transcriptome sequencing. RNA extraction was performed as described previously (*Qin et al., 2020*). Briefly, the total RNA was isolated using a Plant RNeasy Mini Elute Cleanup kit (Qiagen, Beijing,

China) and the sequencing libraries were prepared using the NEBNext Ultra^{TM} RNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA) following the manufacturer's protocols. Transcriptome sequencing was conducted using an Illumina HiSeq 2000 platform.

The remaining samples were used for the measurement of water accumulation. The water accumulation (%) in the different seed coats was determined by the fresh weight (FW) and drought weight (DW) using the formula (FW−DW)/FW ×100. The freeze-drying analysis was performed using GOLD-SIM FD83 (SIM International group co. ltd, USA). The experiments were repeated three times.

The abundances of pomegranate AQP transcripts, in the root, flower, leaf, and three developmental stages of the peel and seed coat (inner and outer seed coat), were collected from the microarray data previously published by *Qin et al. (2017)*. Transcriptional abundances of AQP genes were estimated using the fragments per kilobase of exon per million mapped reads (FRKM) method, and a heat map was generated based on the $\log_2$ FRKM transformation value using TBtools (*Hu et al., 2018*), with the following protocol: starting from the "Graphics", click "Heatmap lllustrator", select the Heatmap button, then set input files in each filed, click "Start" and graph will be generated.

Verification of PgrAQP genes function responding to water deficit in pomegranate root

For the analysis of PgrAQP genes expression patterns in response to water deficit, the PEG treatment experiment was performed. For the hydroponic culture experiment, the full Hoagland nutrient solution contained 20% (v/v) polyethylene glycol 6000 (PEG6000). The solution pH was adjusted to 5.5. The roots samples were collected after 0 h, 1 h, 6 h, 12 h and 24 h of treatment, and were quickly frozen in liquid nitrogen and stored at -80 for subsequent RNA isolation.

For performing qRT-PCR analysis, approximately 2 ug of NDA-eliminated total RNA from pomegranate roots were used to synthesize cDNA using a reverse transcription kit (TaKaRa). qRT-PCR was performed on the Applied Biosystems (ABI) StepOne Plus PCR system (Applied Biosystems) using the LightCylcer 96 SYB GREEN I Master (Roche, Indianapolis, IN, USA) in a 20 ul reaction solution. The PgrAQP genes that showed higher expressed in the roots from the RNA-Seq data were selected for qRT-PCR analysis. The relative transcript abundance of each gene was normalized to the pomegranate actin (OWM91407) with the cycle threshold (Ct) $2(^{-\Delta\Delta Ct})$ method. Three biological replicates and technical replicates were used for each gene. All the specific primers used for each target gene are listed in Table S1.

Statistical analysis

The data were analyzed by ANOVA (SPSS 16.0; SPSS Inc., Chicago, IL, USA), followed by Turkey's test ($P < 0.05$) to determine differences of inner and outer seed coats. The data represent the mean ± SE of three independent biological replicates.

RESULTS

Genome-wide identification of PgrAQP genes in pomegranate reveal each Aquaporin subfamily presents particular physicochemical characteristics

A total of 38 *PgrAQP* members were identified in the pomegranate genome via a genome-wide search using the AQP protein sequences in *Arabidopsis* and *Populus trichocarpa* as queries (*Quigley et al., 2002*). A subsequent conserved domain analysis also confirmed all of the predicted AQPs (Fig. S1). The characteristics of *PgrAQP* family genes are shown in Table S2, including the gene ID, protein length, relative molecular weight, transmembrane domains (TMDs), subcellular localization, and calculated isoelectric point (pI). The 38 predicted PgrAQP genes encoded proteins that varied in length from 245 to 359 amino acid residues, with a relative molecular weight of 22.86 to 35.18 kDa, and a calculated pI ranging from 5.06 to 10.16. The average PI value of PgrTIPs was less than other AQPs due to the loss of basic residues in the C-terminal domain. The grand average of hydrophobicity index (GRAVY) was used to evaluated protein hydrophobicity and hydrophilicity. The results showed the GRAVY of PgrAQP proteins were all positive, ranging from 0.203 to 0.967, which indicated that all of PgrAQP proteins were hydrophobic. Furthermore, the lowest average of GRAVY value (0.44) was found in the PgrPIP subfamily, suggesting that this subfamily has better interaction with water molecules.

The predicted transmembrane domains (TMDs) showed that most *PgrAQP* genes (28 of 38, 73.7%) contained six TMDs, 2.7%, 15.8%, and 7.8% of PgrAQP genes contained four, five, and seven TMDs, respectively (Fig. S2). Based on subcellular localization predicted by WoLF PSORT, most PgrPIP and PgrNIP proteins were predicted to localize in plasma membranes, while only PgrPIP2.2 was found in the chloroplast. All PgrTIP proteins were predicted to localized to vacuoles. For PgrSIP proteins, PgrSIP1.1 and PgrSIP1.2 were found in the chloroplast and vacuole, respectively. PgrXIP proteins were predicted to localized in plasma membranes.

Phylogenetic characterization of the pomegranate *PgrAQP* gene family

To investigate the evolutionary relationship of pomegranate AQP family genes, a total of 193 AQP protein sequences from the four species studied (38 in pomegranate, 35 in *Arabidopsis*, 33 in grape, 55 in *Populus trichocarpa* and 40 in eucalyptus) were identified. An unrooted phylogenetic tree was constructed based on the alignments of their amino acid sequences in MEGA 7 using the neighbor-joining method (*Kumar, Stecher & Tamura, 2016*). By comparing amino acid sequences of PgrAQPs with APQs from three other plant species, 38 PgrAQPs were divided into five different subfamilies, that is, 14 PgrTIPs, 13 *PgrPIPs*, eight *PgrNIPs*, two *PgrSIPs*, and one *PgrXIP* (Fig. 1). The *PgrPIPs* divided into two major subgroups, *PgrPIP1s* and *PgrPIP2s*, which comprised five and eight members, respectively. Furthermore, PgrNIPs formed six subgroups (*PgrNIP1, PgrNIP1, PgrNIP4, PgrNIP5, PgrNIP7,* and *PgrNIP8*) in pomegranate. As the largest sub-family, the TIPs members were classified into five subgroups, consisting of seven PgTIP1s, two PgTIP2s, two PgTIP3s, one PgTIP4, and two PgTIP5s. SIPs and XIPs formed one group, containing

two and one members, respectively. In the phylogenetic tree, *PgrAQP* genes were more closely related to AQPs in eucalyptus than that in *Arabidopsis* and grape, which is in accordance with the evolutionary relationships among these species (*Qin et al., 2017*).

Gene structure and conserved motif analysis of PgrAQP genes confirm the phylogenetic classification

Gene structure and conserved motifs can provide information for exploring the evolutionary relationships among a gene family. The number of introns of *PgrAQP* genes ranged from zero to four, while the length of exons was highly similar for each subfamily (Fig. S3). Among them, most of the *PgrTIPs* genes had two introns, except for *PgrTIP1.6* and *PgrTIP1.8*, which had only one intron. For PgrPIP genes, most of members had three introns, whereas *PgPIP2.8* contained two introns. The numbers of introns in *PgrNIPs* ranged from one to four introns. Four out of 10 members had four introns (*PgrNIP2.1*, *PgrNIP4.1*, *PgrNIP4.2*, and *PgrNIP3.1*), three members had three introns (*PgrNIP1.2*, *PgrNIP5.1*, and *PgrNIP7.1*), and two members had two introns (*PgrNIP1.1* and *PgrNIP1.3*). *PgrXIP2.1* had three introns. The SIP family formed a small subfamily, among which, two members had two introns and one member had no introns.

By analyzing the intron–exon structure of *PgrAQP* genes, we found that the number of introns of each AQPs subfamily (PIP, NIP, TIP, SIP, and XIP) are highly conserved and similar when compared with plant species, such as banana, watermelon, chickpea, and sweet orange, suggesting similar intron loss or gain events were experienced in AQP subfamily over the course of evolution (*Deokar & Tar'an, 2016*; *Hu et al., 2015b*; *MartinsCde et al., 2015*; *Zhou et al., 2019*). Multiple sequence alignments showed that PgrNIPs and PgrPIPs were the most diverse (38.5%) and conserved (72.7%) subfamily at the amino acid level, respectively (Table S3). This finding is consistent with the AQPs from *Nicotiana tabacum*, suggesting that the function and regulatory mechanism of *PgrPIP* subfamily genes showed more conserved than *PgrNIP* subfamily genes in pomegranate (*De Rosa et al., 2020*; *Deokar & Tar'an, 2016*). Furthermore, the relative conservation of protein sequences and ar/R selectivity filter among the *PgrPIP* and *PgrTIP* subfamily genes suggested that these proteins may share a conserved function in transporting water and other small neutral solutes (Tables S3 and S4) (*Zhu et al., 2019*).

To detect the structural diversity and provide further support of the grouping of PgrAQPs, a total of 10 conserved motifs were identified (Fig. S3). Generally, motif compositions were conserved within each subfamily. For PgrPIP, eight motifs were found in all family members. Motifs 1, 2, 3, 6, 8, and 10 were common for TIP and NIP subfamily members, except for PgrNIP7.1. Interestingly, motifs 4 and 5 were only identified in the PIP subfamily and similar motifs were found in bread wheat, indicating the PIP subfamily may have unique functions (*Madrid-Espinoza et al., 2018*).

Comparison of substrate-specific residues in *PgrAQP* proteins

The NPA motifs, ar/R selectively filter, and Froger's positions were identified by multiple sequence alignment between the *PgrAQPs* and *AtAQPs* using MEGA 7.0. These highly conserved motifs and positions were critical for the substrate selectively of AQPs (*Tornroth-Horsefield et al., 2006*). Conserved domain searches using CDD tool from NCBI confirmed

all the predicted AQP genes in Pomegranate encoded MIP domains (Fig. S3). As shown in Table S4, all of PgrTIPs, PIPs, and XIP proteins harbored two conserved NPA domains in both loop B (LB) and loop E (LE). For the PgrNIPs subfamily, except for PgrNIP5.1, the rest of numbers showed the third residue of the first NPA motifs was serine rather than alanine. In addition, PgrSIP1.2 showed the substitution of alanine by threonine in their first NPA motif.

The residues of the ar/R selectivity filter and Froger's position displayed conserved regions within each subfamily, but regions were more variable across different subfamilies (*Kayum et al., 2017*). For example, all of the members of the PgrPIPs showed conservative residues at the ar/R selectively filter with phenylalanine-histidine-threonine-arginine, which is typical of aquaporin protein structure. The residues of Froger's position were conserved in PgrPIPs, including S at P2, A at P3, Y at P4, and W at P5, while the P1 position was variable with the Q/M residues. Different residues were observed at the ar/R selectively filter and Froger's position in PgrNIPs, which had W/G/A/V-V/S/I-A/G-R and F/L/Y-S/T-A-Y/F-I/V/LM residue compositions, respectively. For PgrTIPs members, the residues of P3, P4, and P5 positions were highly conserved, while the P1 and P2 positions were variable residues. The ar/R selectivity filters were variable with H/S/N-I/V-A/P-V/R/C residues. The residues of Froger's position were conserved in PgrSIP1.1 and PgrSIP1.2, but the ar/R filter showed distinct difference.

The pore diameter and hydrophobicity of AQP proteins determines their substrate specificity (*Almasalmeh et al., 2014*; *Hove & Bhave, 2011*). The highly conserved amino acid features of AQPs included six transmembrane domains, the NPA domain, and the ar/R selectivity filter (*Froger et al., 1998*). For example, all of the PgrPIP subfamily members showed a highly conserved ar/R filter structure (T-H-R-T) (Table S4), which was observed in PIP family genes from other plant species, such as watermelon, *Arabidopsis*, *Brassica rapa,* soybean, and chickpea, indicating that the substrate specificity of this subfamily may be more specific than others subfamilies (*Deokar & Tar'an, 2016*; *Kayum et al., 2017*; *Quigley et al., 2002*; *Zhang et al., 2013*). Among the different TIP subgroups, the highly conserved NPA motif, ar/R H2, H5, and Forger's P3 to P5 were observed. Furthermore, the highly conserved ar/R filter (H-I-A-V) and Froger's positions (T-S-A-Y-W) of the *PgrTIP1* subfamily were reported to function as urea and H_2O_2 transporter (*Hove & Bhave, 2011*). In addition, the conserved ar/R filter G-S-G-R residues were found in *PgrNIP2.1*, and this characteristic was identified as the indicator of Si transporters, indicating that *PgrNIP2.1* may be involved in the transport of Si (*Deshmukh et al., 2013*; *Deshmukh et al., 2015*; *Zhou et al., 2019*). In pomegranate, valine was present at position H5 in the ar/R selectivity filters of *PgrXIP2.1*, suggesting that the hydrophobicity of *PgrXIP2.1* is greater than other PgrAQPs subfamily members (*Danielson & Johanson, 2008*; *Gupta & Sankararamakrishnan, 2009*).

Segmental duplication events have contributed to the expansion of the *PgrAQP* family genes

To investigate the localization of *PgrAQP* genes and duplication events in pomegranate, we anchored the *PgrAQPs* on chromosomes and conducted a duplication analysis. The physical position of the *PgrAQP* genes were found to be unevenly distributed across all

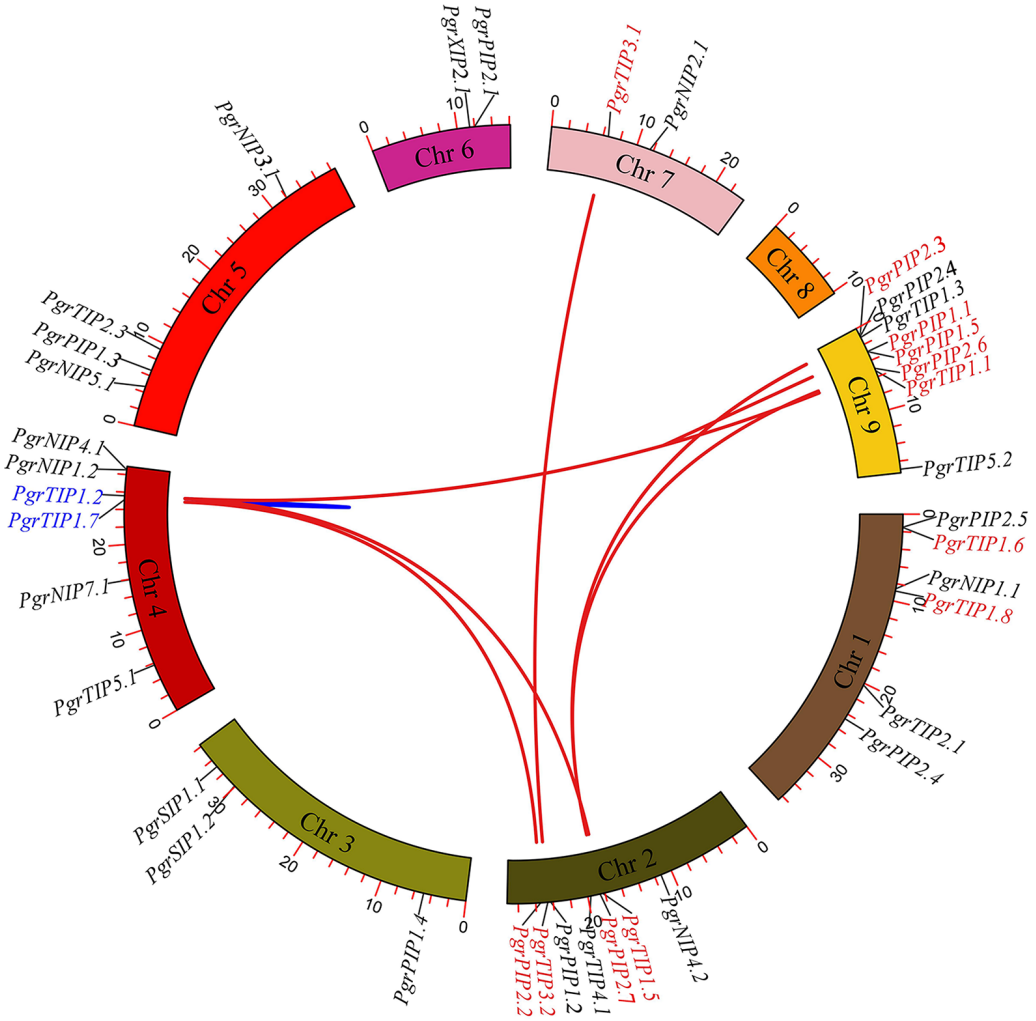

Figure 2 **Analysis of chromosomal locations and syntenic relationships of *PgrAQP* genes.** The AQP genes in pomegranate were mapped to different chromosomes using TBtools, and AQP genes in red and blue represent genes with segmental and tandem duplications, respectively.

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pomegranate chromosomes (Fig. 2). The PIP subfamily genes were randomly anchored on chromosomes, except for Chromosome 7, and TIP subfamily genes were found in all chromosomes except Chromosome 4. Aside from Chromosomes 2 and 8, NIP group genes were located in each chromosome. Genes in the SIP subfamily were present only on Chromosome 4.

We further analyzed the gene duplication modes of *PgrAQP* genes in pomegranate. As shown in Fig. 2, 47% of the *PgrAQP* genes had been duplicated by tandem/segmental duplication events. We found one tandem duplication event in Chromosome 3 (*PgrTIP1.2*/*PgrTIP1.7*). There were eight pairs of segmental duplications detected among six chromosomes. As shown in Table 1, the proportion of segmental *PgrAQP* gene duplications was 88%, indicating that segmental duplication events have played a key role in the expansion of the *PgrAQP* gene family. To access the selection pressure and the date

Table 1 The *Ka* and *Ks* values of duplicated *PgrAQP* gene pairs.

Duplicated gene pairs	Duplicate type	*Ka*	*Ks*	*Ka/Ks*	Time (Mya)	Purify selection
PgrTIP1.2 vs PgrTIP1.5	Segmental	0.074	1.54	0.048	5.12	Yes
PgrTIP3.1 vs PgrTIP3.2	Segmental	0.254	1.19	0.213	3.97	Yes
PgrPIP1.5 vs PgrPIP1.1	Segmental	0.078	0.86	0.090	2.88	Yes
PgrPIP2.5 vs PgrPIP2.2	Segmental	0.123	1.26	0.098	4.21	Yes
PgrPIP2.7 vs PgrPIP2.3	Segmental	0.102	1.38	0.074	4.59	Yes
PgrPIP2.6 vs PgrPIP2.7	Segmental	0.073	1.30	0.057	4.32	Yes
PgrTIP1.2 vs PgrTIP1.1	Segmental	0.096	2.09	0.046	6.97	Yes
PgrTIP1.2 vs PgrTIP1.7	Tandem	0.101	0.52	0.194	1.74	Yes

Notes.
Ka, non-synonymous substitution rate; *Ks*, synonymous substitution rate; Mya, Million years ago.

at which such duplication events occurred, estimation of the *Ka* and *Ks* substitution rates of these duplication *PgrAQP* gene pairs were calculated. A *Ka/Ks* ratio >1 and <1 indicate positive Darwinian selection or purifying selection, whereas a value of 1 indicates neutral selection. The *Ka/Ks* ratios of *PgrAQ* P duplication gene pairs showed a *Ka/Ks* ratio of <1, indicating that these *PgrAQP* genes have experienced purifying selection during the course of evolution. According to the mathematical formula ($T = Ks/2\lambda$) used to calculate the evolutionary date, we assessed the divergence time of these duplication events and found that the gene duplication events occurred approximately 1.74–6.97 million years ago.

Further, we constructed the comparative synteny maps of three plants species (pomegranate *vs*. *Arabidopsis*, pomegranate vs. grape, and pomegranate vs. *Eucalyptus*) to explore the evolutionary process of *PgrAQP* genes (Fig. 3, Table S5) and found that 24, 28, and 41 orthologous AQP gene pairs were identified, respectively. Remarkably, the numbers of orthologous TIP gene pairs between pomegranate and grape/*Eucalyptus* were significantly higher than that in *Arabidopsis*. Nevertheless, the PIP genes were highly conserved within the species. The syntenic relationship detected in TIP genes indicates that the expansion of *PgrTIPs* and *VvTIPs*/*EucTIPs* genes may have occurred after that of *Arabidopsis*, while the PIP genes have been evolutionarily conserved.

Analysis of *PgrAQP* gene expression profiles of different pomegranate tissues and water deficit

Identifying tissue-specific genes is a basic strategy to select candidate genes involved in biological processes. To explore the possible functions of *PgrAQP* genes in various developmental stages of different organs of pomegranate, a heat map of *Pgr* AQP expression profiles was conducted. The heat map showed various expression patterns of the 38 *PgrAQP* genes analyzed (Fig. 4A, Table S6). Most of TIPs, such as *PgrTIP1.1*, *PgrTIP1.4*, *PgrTIP1.6*, *PgrTIP1.8*, and *PgrTIP2.1*, showed higher expression in the roots, leaves, and flowers, whereas the transcripts of *PgrTIP2.3*, *PgrTIP3.1,* and *PgrTIP3.2* showed extremely low expression levels. For the NIPs subfamily, *PgrNIP4.1* and *PgrNIP5.1* were highly expressed in the leaves and roots, respectively. Interestingly, the transcripts of *PgrNIP1.3* could only be detected in flowers, indicating that *PgrNIP1* might be involved in the development of

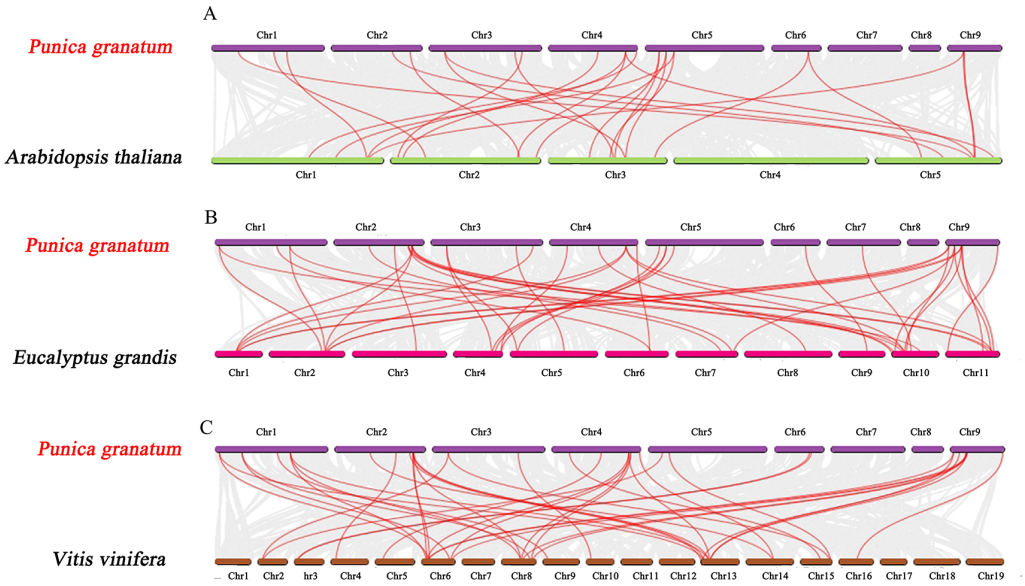

Figure 3 Synteny analysis of *PgrAQP* genes between pomegranate and three plant species. (A) Pomegranate and *A. thaliana*, (B) pomegranate and *Vitis vinifera*, (C) pomegranate and *Eucalyptus grandis*. The gray lines indicated collinearity between pomegranate and other species. The red lines highlight the syntenic AQP gene pairs. The chromosome name is indicated at the top of every chromosome.

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pomegranate flowers, while other members were transcribed at extremely low levels. The accumulation of transcripts of two *PgrSIPs* was detected in all tissues analyzed, whereas *PgrSIP1.2* had higher relative expression levels than that of *PgrSIP1.1*. In the PIPs subfamily, *PgrPIP3*, *PgrPIP2.3*, and *PgrPIP2.4* had a low level of transcripts in all tissues and stages analyzed, whereas the remaining members had higher expression levels in all tissues analyzed. Notably, *PgrPIP1.3, PgrPIP1.5, PgrPIP2.1,* and *PgrPIP2.8* had higher expression levels in the peel and seed coats at all three experimental stages, indicating that these genes may play important roles in seed development.

To reveal the potential function of PgrAQPs in response to water deficit in pomegranate root, the transcript levels of seven selected PgrAQPs from PIP subfamily for seedling under 20% PEG supply condition were tested using qRT-PCR. According to the qRT-PCR results, except the *PgrPIP1.5*, of which the transcript was enhanced in the roots after the 24 h of 20% PEG supply conditions, the expression of the other five paralogues, *PgrPIP1.1, PgrPIP1.2, PgrPIP1.3, PgrPIP2.1* and *PgrPIP1.4,* were significantly repressed under the 20% PEG supply condition (Fig. 5). It should be noted that the expression levels of the *PgrPIP1.2* was strongly and rapidly decreased under the 20% PEG treatment. In contrast, the expression pattern of *PgrPIP2.4* showed barely changed until 12 h after 20% PEG treatment, the down-regulation was observed at 24 h.

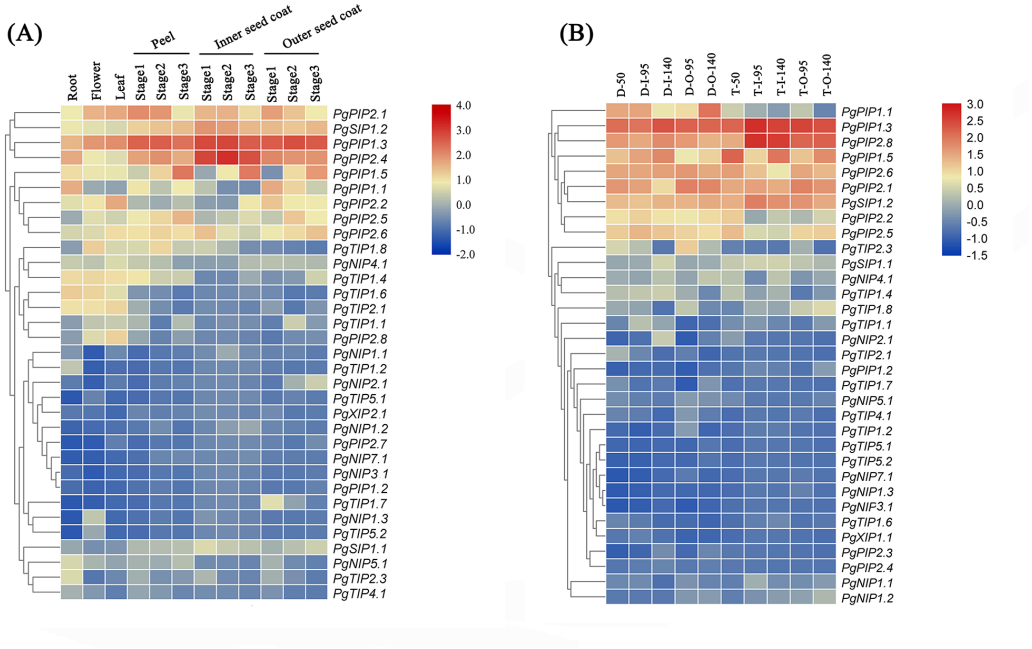

Figure 4 **Expression analysis of the *PgrAQP* genes in pomegranate.** (A) Expression profile of *PgrAQP* genes in the cultivated pomegranate cultivar 'Dabenzi,' including roots, flowers, leaves, and three stages of the peel, inner, and outer seed coats (50, 95, and 140 days after pollination). (B) Expression profiling of *PgrAQP* genes at different developmental stages of the seed coats in pomegranate. The abbreviations are as follows: D: *P. granatum* 'Dabenzi,' T: *P. granatum* 'Tunisia,' O: Outer seed coat, I: Inner seed coat. The number represents the number of days after pollination (DAP). The heat map was generated using TBtools. Expression data were calculated with log2 normalization based on FPKM values.

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Identification of candidate *PgrAQPs* involved in water accumulation in the outer seed coat of pomegranate

To understand water transport and accumulation in pomegranate seed coats, especially of the juicy outer seed coat, we detected water accumulation in the inner and outer seed coats at different developmental stages, as well as the relative transcript levels of *PgrAQP* genes in corresponding samples. We found that the water content was significantly increased in outer seed coats during seed development in 'Dabenzi' and 'Tunisia,' and the water that accumulated in the outer seed coats was higher than that in the inner seed coats (Fig. 6). Genes, including *PgrPIP1.3, PgrPIP2.8, PgrPIP1.5, PgrPIP2.6, PgrPIP2.1, PgrPIP2.2, PgrPIP2.5*, and *PgSIP1.2*, had relatively high levels of transcript accumulation in inner and outer seed coats. Among the abovementioned genes, *PgrPIP1.3, PgrPIP2.8,* and *PgSIP1.2*, had high levels of accumulation of transcripts only in the outer seed coat at the later developmental stages (Fig. 5), which indicated that these genes may be involved in the accumulation of water in outer seed coats at the later developmental stages.

In Arabidopsis, a plasma membrane aquaporin, AtPIP1;2 is involved in aquaporin-mediated leaf water transport, lateral root formation, and water uptake in root. To further understand the function of *PgrPIP1.3*, *PgrPIP2.8*, and *PgrSIP1.2* in the water accumulation in the seed coat, the linear relationship between the water content in the seed coat and

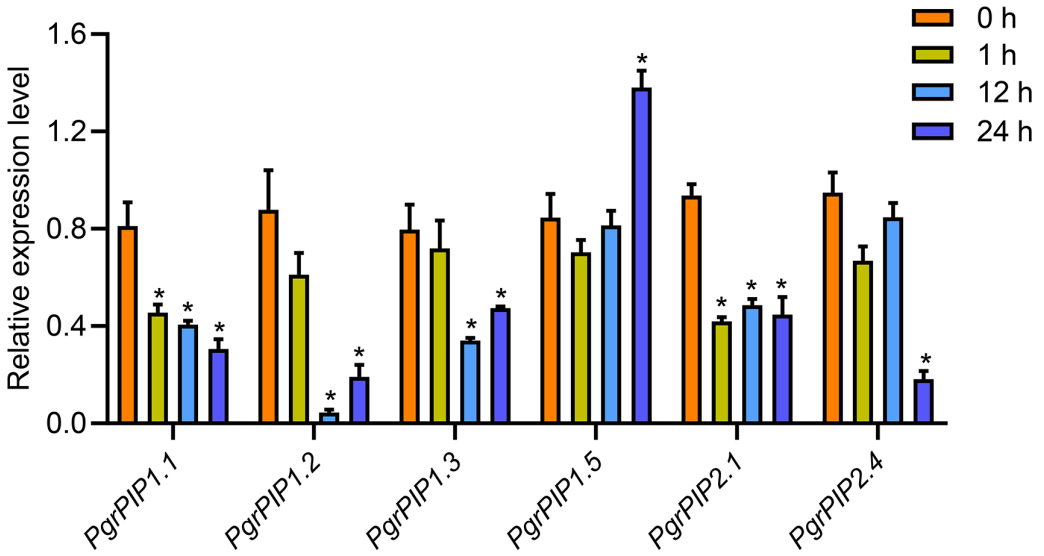

Figure 5 **Expression profiles of six Pomegranate PIPs roots in response to water deficit.** qRT-PCR was performed to determined the relative transcript level for the six *PgrPIP* genes. Relative expression level was normalized relative to untreated control group (0 h PEG treatment). Error bar represents SE of three independent biological replicated. Asterisk indicate significant differences, $^{*}P < 0.05$.

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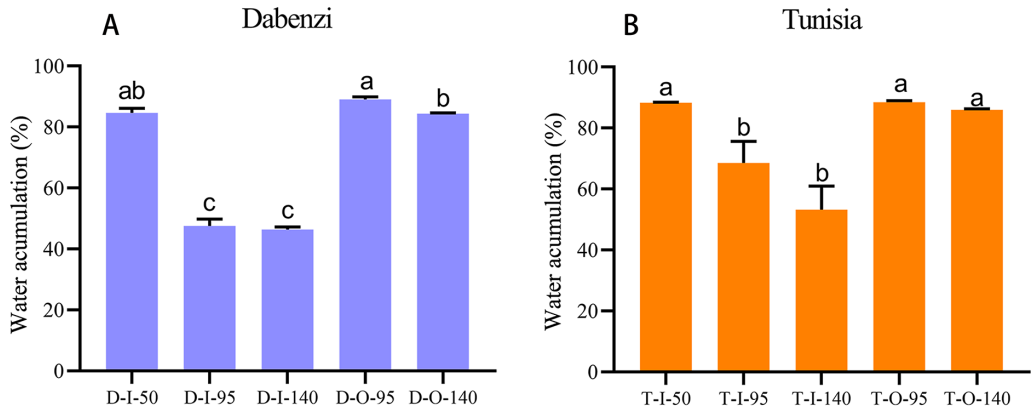

Figure 6 **Water accumulation in seed coats of pomegranate.** The abbreviations in the sample designations represent the cultivar names: D for*P. granatum* 'Dabenzi' and T for*P. granatum* '*Tunisia*.' The second letter represents tissue: O and I stands for the outer seed coats and the inner seed coat, respectively. The numbers represent the three stages of seed coat development at different days after flowering (DAF). Error bar represents SE of three independent biological replicated. Difference letters indicate a significant difference ($P < 0.05$).

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differences in transcript levels for *PrgPIP1.3* and *PgrSIP1.2* in seed coat development was determined. Significant positive correlations in both *PgrPIP1.3* and *PgrSIP1.2* and water content in outer seed coats were observed (Fig. 7). Therefore, it was concluded that *PgrPIP1.3* and *PgrSIP1.2* might be involved in the accumulation of water in pomegranate seed coats.

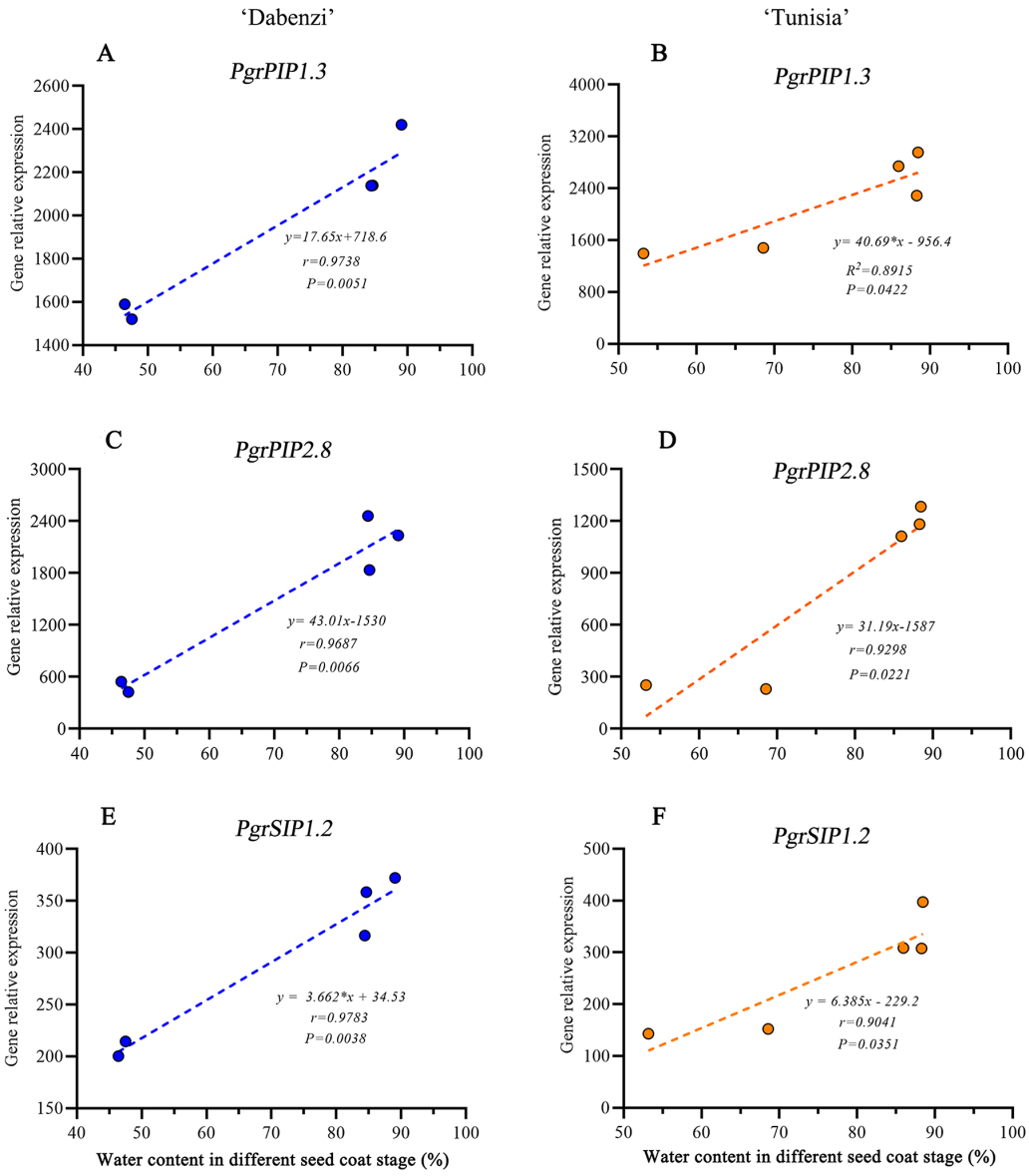

Figure 7 Linear regression between gene expression of PgrAQPs and water accumulation. Correlation analysis between gene expression of *PgrPIP1.3*, *PgrPIP2.8* and *PgrSIP1.2* and water accumulation in seed coats from *P. granatum* 'Dabenzi' (A, C, E) and *P. granatum* 'Tunisia' (B, D, F).

Full-size DOI: 10.7717/peerj.11810/fig-7

DISCUSSION

PgrAQP identification and structure analysis

AQP proteins play an important role in various physiological and developmental processes of different organs and tissues, and function as a transporter for water and/or small neutral solutes in plants. Numerous studies have been conducted on AQPs in plants, such as in *Arabidopsis*, grape, soybean, rice, and *Populus trichocarpa* (*Fouquet et al., 2008*; *Gupta & Sankararamakrishnan, 2009*; *Quigley et al., 2002*; *Sakurai et al., 2005*; *Zhang et al., 2013*).

However, the genome-wide identification of the AQP gene family in pomegranate was absent due to the limitations of an available genome sequence (*Luo et al., 2020*; *Qin et al., 2017*). In this study, 38 *PgrAQP* genes were identified and characterized in pomegranate. The characteristics of *PgrAQP* were comparable with other plants species. For instance, the numbers of exons and structures of intron/exons in the *PgrAQP* genes were highly conserved in different species, such as *Arabidopsis*, olive, chickpea, *Arachis hypogea*, and banana (*Deokar & Tar'an, 2016*; *Faize et al., 2020*; *Hu et al., 2015b*; *Quigley et al., 2002*; *Shivaraj et al., 2019*). Similar to the organizations of intron/exons, *PgrAQP* proteins of each subfamily possess the same conserved motifs (Fig. 2). These results suggest that the gene structures of PgrAQPs are closely related to homologous genes.

Furthermore, the TMDs showed that some of the PgrAQPs were lack one (PgrSIP1.1) or two (PgrNIP5.1, PgrPIP2.8) TMDs (Fig. S3). Variation in the number of TMDs to different plant species has been reported (*Ayadi et al., 2011*; *Zhu et al., 2019*). For instance, a truncated form of wheat TdPIP2;1 aquaporin, showed no water transport activity. Interestingly, the truncated tdpip2;1 could reach the plasma membrane by interact with the functional TdPIP2;1, and then may affect the functional form and reduce the water transport activity of aquaporin (*Ayadi et al., 2011*). Therefore, the absence of TMDs may affect the PgrAQPs subcellular localization and water transport activity.

The evolutionary relationships among the *PgrAQP* gene family

Gene duplication is considered as a major driving force for the evolution of gene families, and several duplication events have been identified over the course of evolution of some plant species. At approximately 117 million years ago (Mya), all core eudicots experienced a genome triplication event (the γ event), including Arabidopsis, *Eucalyptus*, grape, and pomegranate (*Jiao et al., 2012*; *Jiao et al., 2014*). Then, Arabidopsis experienced two recent WGDs (α and β), whereas pomegranate and *Eucalyptus* underwent a Myrtales lineage-specific WGD event (109.9Mya, M), but grape did not undergo any additional WGDs (*Myburg et al., 2014*; *Qin et al., 2017*). In *Arabidopsis*, a total of 35 AQP genes were identified and further evolution analysis revealed that *AtAQPs* result from different types of gene duplication, such as γ WGD (1), β WGD (2), α WGD (8), tandem (2), and transposed (4) (*Bowers et al., 2003*; *Zwiazek et al., 2017*). For poplar, tandem duplication (4) and the recent WGD (20) were the major driving forces for 55 AQP genes (*Zou & Yang, 2019*).

In this study, the 38-member PgrAQP family is comparable to *Arabidopsis* (33) and *Eucalyptus* (40), but had a greater number of genes than grape (28). Furthermore, all the PgrNIP, PgrSIP, and PgrXIP subfamilies were found to have a close relationship with the corresponding genes in *Eucalyptus* AQPs, which is consistent with the evolutionary relationships among the species, suggesting that these AQP subfamilies might have functional conservation in Myrtales (*Qin et al., 2017*). It is reasonable to deduce that WGD events may function as a trigger of *PgrAQP* family genes expansion. In additional, we identified eight gene duplication events in the *PgrAQPs* subfamily, including seven segmental duplications and one tandem duplication event (Fig. 2, Table 1). Interestingly, the *Ka/Ks* ratio of the eight duplications was <1, indicating that the evolution of the *PgrAQP* genes is mediated by large-scale purifying selection, similar to the AQP family

in *B. rapa* and wheat (*Kayum et al., 2017*; *Madrid-Espinoza et al., 2018*). The synonymous substitution rate was also used to estimate the evolutionary timescale, and the divergence time of duplicated *PgrAQP* genes occurred 1.77 to 6.97 million years ago, which is in accordance with the divergence time of *BrAQPs*. This indicates that duplicated divergence of the PgrAQP genes occurred after the triplication events and Myrtales WGD duplication events (*Kayum et al., 2017*; *Qin et al., 2017*).

According to a genome-wide analysis of different organisms, it was hypothesized that the frequency of gene duplication events was important to the evolution of a species (*Flagel & Wendel, 2009*; *Lynch & Conery, 2000*). In cotton and sesame, tandem duplicated genes showed functional differentiation, although they shared structural conservation (*Li et al., 2019*; *Wu et al, 2016*). In our study, *PgrTIP1.2* and *PgrTIP1.7* were identified as tandem duplicated genes, and they were predominantly expressed in roots and outer seed coats in pomegranate, respectively. Our results provided more information for understanding the evolution of plant AQPs.

Potential functions of *PgrAQP* genes

As the largest AQP subfamily in most plant species, PIPs play a crucial role in water absorption of roots and leaves. Furthermore, PIPs can affect photosynthesis by enhancing the diffusion of CO_2 in mesophyll tissue of rice and *N. tabacum* (*Flexas et al., 2006*; *Xu et al., 2019*). In this study, *PgrPIP* subfamily genes had higher expression levels in all analyzed samples compared with other analyzed AQPs. Interestingly, among the *RhPIPs*, *RhPIP1s* and *RhPIP2s* were involved in the expansion of rose petals via an ethylene-dependent pathway (*Chen et al., 2013*; *Ma et al., 2008*). The expression analysis showed that *PgrPIP1.3* and *PgrPIP2.1* had similar expression patterns in pomegranate flowers, suggesting a similar role of PIPs during pomegranate flower development (Fig. 4). In the higher plants, TIPs are widely used as markers for vacuolar compartments and function as transporter for small solutes in various tissue (*Bienert et al., 2007*; *Holm et al., 2005*; *Liu et al., 2003*; *Porcel et al., 2018*). In *Arabidopsis*, at least six TIP subfamily numbers, including *TIP1;1*, *TIP1;2*, *TIP2;1*, *TIP2;2*, *TIP2;3*, and *TIP4;1*, showed specific expression patterns in roots (*Gattolin, Sorieul & Frigerio, 2011*). In addition, the rice *OsTIP2;1* was only detected in roots (*Nguyen, Moon & Jung, 2013*). In pomegranate, *PgrTIP1.2* and *PgrTIP2.3* show higher expression patterns in the roots than other organs, indicated that these gene may specifically participate in the absorption and transport of small solutes, such as NH_4^+, H_2O_2, and urea, in the roots of pomegranate (*Bienert et al., 2007*; *Holm et al., 2005*; *Liu et al., 2003*). In our study, the XIP and majority of NIPs showed lower expression levels than PIPs and TIPs. Interestingly, we found that *PgrNIP5.1* was highly expressed in roots. Such specific root expression of NIPs was reported for *AtNIP5;1* and *HvNIP2;1* (*Schnurbusch et al., 2010*; *Takano et al., 2006*), which were involved in boron (B) homeostasis, indicating that *PgNIP5;1* may participate in B absorption and translocation in pomegranate roots.

Drought causes tissue dehydration due to an imbalance between plant water uptake and transpiration. Evidence shows that AQPs play an important role in drought tolerance in plants. Ectopically expression of *MpPIP2;1* in *Arabidopsis* has been shown to enhance drought and salinity tolerance. The decrease or increase of the *AQPs* transcript levels could

prevent water losses or helps plants to direct water flow to specific organ under drought stress. In this study, we found most members of the *PgrPIP* genes were suppressed by drought stress (Fig. 5), including *PgrPIP1.1, PgrPIP1.2, PgrPIP1.3, PgrPIP2.1* and *PgrPIP2.4*, suggested their involvement in reduced water losses in Pomegranate plants. Furthermore, the upregulation of the transcript levels of *PgrPIP1.5* was observed under 20% PEG supply condition, indicated that PgrPIP1.5 might play a critical for tolerance to drought in pomegranate.

Identification of candidate *PgrAQP* genes involved in pomegranate seed coat development

Plant cell expansion is primarily driven by turgor and requires steady water intake, the rate of tissue growth is primarily restricted due to decreasing of turgor (*Peret et al., 2012*; *Picaud et al., 2003*; *Reuscher et al., 2013*). For example, in higher plants, the development of a seed coat was primarily initiated by fertilization and driven by cell expansion and growth (*Figueiredo et al., 2016*). Accordingly, most of the AQPs were reported to be strongly expressed in tissues that can be hydraulically limited during growth. In *Arabidopsis*, the specific expression pattern and regulatory mechanism showed that *AtTIP1;1* plays a critical role in cell expansion (*Beebo et al., 2009*; *Ludevid et al., 1992*). Overexpression of ginseng TIP in *Arabidopsis* resulted in a significant increase of leaf cell sizes compared with the wild type plants (*Lin et al., 2007*). In rice, *OsPIP1;1* is highly expressed in leaves and roots, and overexpression of *OsPIP1;1* exhibited a higher germination rate than the control plants (*Liu et al., 2013*).

In pomegranate, the expanded outer seed coats had higher water accumulation than the rigid inner seed coats in both the hard-seeded cultivar 'Dabenzi' and the soft-seeded cultivar 'Tunisia' (Fig. 6). Accordingly, significant positive correlations were found between the expression level of *PgrSIP1.2, PgrPIP1.3*, and *PgrPIP2.8* and water content in the seed coats (Fig. 7). PgrSIP1.2 was predicted to be localized in the vacuole. Considering that promotion of cell expansion and maintenance of turgor requires the transfer of substantial amounts of water to cells, it is reasonable to proposed that *PgrPIP1.3*, *PgrPIP2.8*, and *PgrSIP1.2* might be involved in mediating the water accumulation in the inner and outer seed coats of pomegranate.

CONCLUSIONS

In this study, a total of 38 AQP genes were identified and their characteristics, including protein physicochemical properties, gene structure, phyletic evolutionary, and expression patterns were studied. These PgrAQP genes are distributed across nine pomegranate chromosomes and divided into five subfamilies. Purifying selection were undergone during the evolution of *PgrAQP* family genes basing on the syntenic relationships and duplication events analysis, and a whole-genome duplication event in Myrtales may contribute to the expansion of *PgrTIP*, *PgrSIP*, and *PgrXIP* genes. Furthermore, the high expression of *PgrPIP1.3, PgrPIP2.8,* and *PgrSIP1.2* in seed coats and the positive correlation between transcript levels of *PgrAQP* genes and the water content revealed these three genes may be the potential candidate genes involving in outer seed coat development. Hence, further

studies on functions of this three AQP genes are needed for genetic improvement of outer seed coats in pomegranate.

ADDITIONAL INFORMATION AND DECLARATIONS

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Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Jianjian Liu conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Gaihua Qin and Jianrong Zhao conceived and designed the experiments, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Chunyan Liu, Jie Zhou and Bingxin Lu performed the experiments, authored or reviewed drafts of the paper, and approved the final draft.
- Xiuli Liu and Jiyu Li analyzed the data, prepared figures and/or tables, and approved the final draft.

Data Availability

The following information was supplied regarding data availability:

The raw measurements of water content in both inner and outer seed coats, and RNA-Seq data are available in the

The raw RNA-seq data of different pomegranate tissue (SRP100581) and development stages of seed coats (PRJNA548841) are available in the Sequence Read Archive (SRA): SRX2914315.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.11810#supplemental-information.

REFERENCES

Adams KL, Wendel JF. 2005. Polyploidy and genome evolution in plants. *Current Opinion in Plant Biology* **8**:135–141 DOI 10.1016/j.pbi.2005.01.001.

Almasalmeh A, Krenc D, Wu B, Beitz E. 2014. Structural determinants of the hydrogen peroxide permeability of aquaporins. *FEBS Journal* **281**:647–656 DOI 10.1111/febs.12653.

Artimo P, Jonnalagedda M, Arnold K, Baratin D, Csardi G, De Castro E, Duvaud S, Flegel V, Fortier A, Gasteiger E, Grosdidier A, Hernandez C, Ioannidis V, Kuznetsov D, Liechti R, Moretti S, Mostaguir K, Redaschi N, Rossier G, Xenarios I, Stockinger H. 2012. ExPASy: SIB bioinformatics resource portal. *Nucleic Acids Research* **40**:W597–603 DOI 10.1093/nar/gks400.

Ayadi M, Cavez D, Miled N, Chaumont F, Masmoudi K. 2011. Identification and characterization of two plasma membrane aquaporins in durum wheat (Triticum turgidum L. subsp. durum) and their role in abiotic stress tolerance. *Plant Physiology and Biochemistry* **49(9)**:1029–1039 DOI 10.1016/j.plaphy.2011.06.002.

Azad AK, Ahmed J, Alum MA, Hasan MM, Ishikawa T, Sawa Y, Katsuhara M. 2016. Genome-wide characterization of major intrinsic proteins in four grass plants and their non-aqua transport selectivity profiles with comparative perspective. *PLOS ONE* **11**:e0157735 DOI 10.1371/journal.pone.0157735.

Azad AK, Sawa Y, Ishikawa T, Shibata H. 2004. Phosphorylation of plasma membrane aquaporin regulates temperature-dependent opening of tulip petals. *Plant and Cell Physiology* **45**:608–617 DOI 10.1093/pcp/pch069.

Bailey TL, Boden M, Buske FA, Frith M, Grant CE, Clementi L, Ren J, Li WW, Noble WS. 2009. MEME SUITE: tools for motif discovery and searching. *Nucleic Acids Research* **37**:W202–W208 DOI 10.1093/nar/gkp335.

Beebo A, Thomas D, Der C, Sanchez L, Leborgne-Castel N, Marty F, Schoefs B, Bouhidel K. 2009. Life with and without AtTIP1;1, an Arabidopsis aquaporin preferentially localized in the apposing tonoplasts of adjacent vacuoles. *Plant Moleculat Biology* **70**:193–209 DOI 10.1007/s11103-009-9465-2.

Bienert GP, Moller AL, Kristiansen KA, Schulz A, Moller IM, Schjoerring JK, Jahn TP. 2007. Specific aquaporins facilitate the diffusion of hydrogen peroxide across membranes. *Journal of Biological Chemistry* **282**:1183–1192 DOI 10.1074/jbc.M603761200.

Bowers JE, Chapman BA, Rong J, Paterson AH. 2003. Unravelling angiosperm genome evolution by phylogenetic analysis of chromosomal duplication events. *Nature* **422**:433–438 DOI 10.1038/nature01521.

Chaumont F, Barrieu F, Wojcik E, Chrispeels MJ, Jung R. 2001. Aquaporins constitute a large and highly divergent protein family in maize. *Plant Physiology* **125**:1206–1215 DOI 10.1104/pp.125.3.1206.

Chen C, Chen H, Zhang Y, Thomas HR, Frank MH, He Y, Xia R. 2020. TBtools: an integrative toolkit developed for interactive analyses of big biological data. *Molecular Plant* **13**:1194–1202 DOI 10.1016/j.molp.2020.06.009.

Chen W, Yin X, Wang L, Tian J, Yang R, Liu D, Yu Z, Ma N, Gao J. 2013. Involvement of rose aquaporin RhPIP1;1 in ethylene-regulated petal expansion through interaction with RhPIP2;1. *Plant Molecular Biology* **83**:219–233 DOI 10.1007/s11103-013-0084-6.

Danielson JA, Johanson U. 2008. Unexpected complexity of the aquaporin gene family in the moss Physcomitrella patens. *BMC Plant Biology* **8**:45 DOI 10.1186/1471-2229-8-45.

Danielson JA, Johanson U. 2010. Phylogeny of major intrinsic proteins. *Advances in Experimental Medicine and Biology* **679**:19–31 DOI 10.1007/978-1-4419-6315-4_2.

De Rosa A, Watson-Lazowski A, Evans JR, Groszmann M. 2020. Genome-wide identification and characterisation of Aquaporins in Nicotiana tabacum and their relationships with other Solanaceae species. *BMC Plant Biology* **20**:266 DOI 10.1186/s12870-020-02412-5.

Deokar AA, Tar'an B. 2016. Genome-wide analysis of the aquaporin gene family in Chickpea (Cicer arietinum L.). *Frontiers in Plant Science* **7**:1802 DOI 10.3389/fpls.2016.01802.

Deshmukh RK, Vivancos J, Guérin V, Sonah H, Labbé C, Belzile F, Bélanger RR. 2013. Identification and functional characterization of silicon transporters in soybean using comparative genomics of major intrinsic proteins in Arabidopsis and rice. *Plant Molecular Biology* **83**:303–315 DOI 10.1007/s11103-013-0087-3.

Deshmukh RK, Vivancos J, Ramakrishnan G, Guérin V, Carpentier G, Sonah H, Labbé C, Isenring P, Belzile FJ, Bélanger RR. 2015. A precise spacing between the NPA domains of aquaporins is essential for silicon permeability in plants. *The Plant Journal* **83**:489–500 DOI 10.1111/tpj.12904.

Edlund AF, Swanson R, Preuss D. 2004. Pollen and stigma structure and function: the role of diversity in pollination. *The Plant Cell* **16**(**Suppl**):S84–S97 DOI 10.1105/tpc.015800.

Eisenbarth DA, Weig AR. 2005. Dynamics of aquaporins and water relations during hypocotyl elongation in Ricinus communis L. seedlings. *Journal of Experimental Botany* **56**:1831–1842 DOI 10.1093/jxb/eri173.

Faize M, Fumanal B, Luque F, Ramirez-Tejero JA, Zou Z, Qiao X, Faize L, Gousset-Dupont A, Roeckel-Drevet P, Label P, Venisse JS. 2020. Genome wild analysis and molecular understanding of the aquaporin diversity in olive trees (Olea Europaea L.). *International Journal of Molecular Sciences* **21**(**11**):4183 DOI 10.3390/ijms21114183.

Figueiredo DD, Batista RA, Roszak PJ, Hennig L, Kohler C. 2016. Auxin production in the endosperm drives seed coat development in Arabidopsis. *Elife* **5** DOI 10.7554/eLife.20542.

Finn RD, Bateman A, Clements J, Coggill P, Eberhardt RY, Eddy SR, Heger A, Hetherington K, Holm L, Mistry J, Sonnhammer EL, Tate J, Punta M. 2014. Pfam: the protein families database. *Nucleic Acids Research* **42**:D222–D230 DOI 10.1093/nar/gkt1223.

Flagel LE, Wendel JF. 2009. Gene duplication and evolutionary novelty in plants. *New Phytologist* **183**:557–564 DOI 10.1111/j.1469-8137.2009.02923.x.

Flexas J, Ribas-Carbo M, Hanson DT, Bota J, Otto B, Cifre J, McDowell N, Medrano H, Kaldenhoff R. 2006. Tobacco aquaporin NtAQP1 is involved in mesophyll conductance to CO2 in vivo. *The Plant Journal* **48**:427–439 DOI 10.1111/j.1365-313X.2006.02879.

Fouquet R, Léon C, Ollat N, Barrieu F. 2008. Identification of grapevine aquaporins and expression analysis in developing berries. *Plant Cell Reports* **27**:1541–1550 DOI 10.1007/s00299-008-0566-1.

Froger A, Tallur B, Thomas D, Delamarche C. 1998. Prediction of functional residues in water channels and related proteins. *Protein Science* **7**:1458–1468 DOI 10.1002/pro.5560070623.

Gattolin S, Sorieul M, Frigerio L. 2011. Mapping of tonoplast intrinsic proteins in maturing and germinating Arabidopsis seeds reveals dual localization of embryonic TIPs to the tonoplast and plasma membrane. *Molecular Plant* **4**:180–189 DOI 10.1093/mp/ssq051.

Gu Z, Cavalcanti A, Chen FC, Bouman P, Li WH. 2002. Extent of gene duplication in the genomes of Drosophila, nematode, and yeast. *Molecular Biology and Evolution* **19**:256–262 DOI 10.1093/oxfordjournals.molbev.a004079.

Gupta AB, Sankararamakrishnan R. 2009. Genome-wide analysis of major intrinsic proteins in the tree plant Populus trichocarpa: characterization of XIP subfamily of aquaporins from evolutionary perspective. *BMC Plant Biology* **9**:134 DOI 10.1186/1471-2229-9-134.

Holm LM, Jahn TP, Moller AL, Schjoerring JK, Ferri D, Klaerke DA, Zeuthen T. 2005. NH3 and NH4+ permeability in aquaporin-expressing Xenopus oocytes, Pflugers Archiv. *European Journal of Physiology* **450**:415–428 DOI 10.1007/s00424-005-1399-1.

Hove RM, Bhave M. 2011. Plant aquaporins with non-aqua functions: deciphering the signature sequences. *Plant Molecular Biology* **75**:413–430 DOI 10.1007/s11103-011-9737-5.

Hu W, Hou X, Huang C, Yan Y, Tie W, Ding Z, Wei Y, Liu J, Miao H, Lu Z, Li M, Xu B, Jin Z. 2015b. Genome-wide identification and expression analyses of aquaporin gene family during development and abiotic stress in banana. *International Journal of Molecular Sciences* **16**:19728–19751 DOI 10.3390/ijms160819728.

Hu W, Hua X, Zhang Q, Wang J, Shen Q, Zhang X, Wang K, Yu Q, Lin YR, Ming R, Zhang J. 2018. New insights into the evolution and functional divergence of the SWEET family in Saccharum based on comparative genomics. *BMC Plant Biology* **18**:270 DOI 10.1186/s12870-018-1495-y.

Hu B, Jin J, Guo AY, Zhang H, Luo J, Gao G. 2015a. GSDS 2.0: an upgraded gene feature visualization server. *Bioinformatics* **31**:1296–1297 DOI 10.1093/bioinformatics/btu817.

Ivanov II, Loktyushkin AV, Gus'kova RA, Vasil'ev NS, Fedorov GE, Rubin AB. 2007. Oxygen channels of erythrocyte membrane. *Dokl Biochem Biophys* **414**:137–140 DOI 10.1134/s160767290703012.x.

Jiao Y, Leebens-Mack J, Ayyampalayam S, Bowers JE, McKain MR, McNeal J, Rolf M, Ruzicka DR, Wafula E, Wickett NJ, Wu X, Zhang Y, Wang J, Zhang Y, Carpenter EJ, Deyholos MK, Kutchan TM, Chanderbali AS, Soltis PS, Stevenson DW, McCombie R, Pires JC, Wong GK, Soltis DE, Depamphilis CW. 2012. A genome triplication associated with early diversification of the core eudicots. *Genome Biology* **13**:R3 DOI 10.1186/gb-2012-13-1-r3.

Jiao Y, Li J, Tang H, Paterson AH. 2014. Integrated syntenic and phylogenomic analyses reveal an ancient genome duplication in monocots. *The Plant Cell* **26**:2792–2802 DOI 10.1105/tpc.114.127597.

Johanningsmeier SD, Harris GK. 2011. Pomegranate as a functional food and nutraceutical source. *Annual Review of Food Science and Technology* **2**:181–201 DOI 10.1146/annurev-food-030810-153709.

Kaldenhoff R, Fischer M. 2006. Functional aquaporin diversity in plants. *Biochimica Et Biophysica Acta/General Subjects* **1758**:1134–1141 DOI 10.1016/j.bbamem.2006.03.012.

Kayum MA, Park JI, Nath UK, Biswas MK, Kim HT, Nou IS. 2017. Genome-wide expression profiling of aquaporin genes confer responses to abiotic and biotic stresses in Brassica rapa. *BMC Plant Biology* **17**:23 DOI 10.1186/s12870-017-0979-5.

Khabudaev KV, Petrova DP, Grachev MA, Likhoshway YV. 2014. A new subfamily LIP of the major intrinsic proteins. *BMC Genomics* **15**:173 DOI 10.1186/1471-2164-15-173.

Kumar S, Stecher G, Tamura K. 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution* **33**:1870–1874 DOI 10.1093/molbev/msw054.

Lee JK, Kozono D, Remis J, Kitagawa Y, Agre P, Stroud RM. 2005. Structural basis for conductance by the archaeal aquaporin AqpM at 1.68 A. *Proceedings of the National Academy of Sciences of the United States of America* **102**:18932–18937 DOI 10.1073/pnas.0509469102.

Letunic I, Khedkar S, Bork P. 2020. SMART: recent updates, new developments and status in 2020. *Nucleic Acids Research* **49(D1)**:D458–D460 DOI 10.1093/nar/gkaa937.

Li W, Zhang D, Zhu G, Mi X, Guo W. 2019. Combining genome-wide and transcriptome-wide analyses reveal the evolutionary conservation and functional diversity of aquaporins in cotton. *BMC Genomics* **20**:538 DOI 10.1186/s12864-019-5928-2.

Lian HL, Yu X, Ye Q, Ding X, Kitagawa Y, Kwak SS, Su WA, Tang ZC. 2004. The role of aquaporin RWC3 in drought avoidance in rice. *Plant and Cell Physiology* **45**:481–489 DOI 10.1093/pcp/pch058.

Lin W, Peng Y, Li G, Arora R, Tang Z, Su W, Cai W. 2007. Isolation and functional characterization of PgTIP1, a hormone-autotrophic cells-specific tonoplast aquaporin in ginseng. *Journal of Experimental Botany* **58**:947–956 DOI 10.1093/jxb/erl255.

Liu C, Fukumoto T, Matsumoto T, Gena P, Frascaria D, Kaneko T, Katsuhara M, Zhong S, Sun X, Zhu Y, Iwasaki I, Ding X, Calamita G, Kitagawa Y. 2013. Aquaporin OsPIP1;1 promotes rice salt resistance and seed germination. *Plant Physiology and Biochemistry* **63**:151–158 DOI 10.1016/j.plaphy.2012.11.018.

Liu LH, Ludewig U, Gassert B, Frommer WB, von Wiren N. 2003. Urea transport by nitrogen-regulated tonoplast intrinsic proteins in Arabidopsis. *Plant Physiology* **133**:1220–1228 DOI 10.1104/pp.103.027409.

Liu HY, Yu X, Cui DY, Sun MH, Sun WN, Tang ZC, Kwak SS, Su WA. 2007. The role of water channel proteins and nitric oxide signaling in rice seed germination. *Cell Research* **17**:638–649 DOI 10.1038/cr.2007.34.

Ludevid D, Hofte H, Himelblau E, Chrispeels MJ. 1992. The expression pattern of the tonoplast intrinsic protein gamma-TIP in arabidopsis thaliana is correlated with cell enlargement. *Plant Physiology* **100**:1633–1639 DOI 10.1104/pp.100.4.1633.

Luo X, Li H, Wu Z, Yao W, Zhao P, Cao D, Yu H, Li K, Poudel K, Zhao D, Zhang F, Xia X, Chen L, Wang Q, Jing D, Cao S. 2020. The pomegranate (Punica granatum L.) draft genome dissects genetic divergence between soft- and hard-seeded cultivars. *Plant Biotechnology Journal* **18**:955–968 DOI 10.1111/pbi.13260.

Lynch M, Conery JS. 2000. The evolutionary fate and consequences of duplicate genes. *Science* **290**:1151–1155 DOI 10.1126/science.290.5494.1151.

Ma N, Xue J, Li Y, Liu X, Dai F, Jia W, Luo Y, Gao J. 2008. Rh-PIP2;1, a rose aquaporin gene, is involved in ethylene-regulated petal expansion. *Plant Physiology* **148**:894–907 DOI 10.1104/pp.108.120154.

Madrid-Espinoza J, Brunel-Saldias N, Guerra FP, Gutierrez A, Del Pozo A. 2018. Genome-wide identification and transcriptional regulation of aquaporin genes in bread wheat (Triticum aestivum L.) under water stress. *Genes* **9(10)**:497 DOI 10.3390/genes9100497.

Martins Cde P, Pedrosa AM, Du D, Goncalves LP, Yu Q, Gmitter Jr FG, Costa MG. 2015. Genome-wide characterization and expression analysis of major intrinsic proteins during abiotic and biotic stresses in sweet orange (Citrus sinensis L. Osb.). *PLOS ONE* **10**:e0138786 DOI 10.1371/journal.pone.0138786.

Myburg AA, Grattapaglia D, Tuskan GA, Hellsten U, Hayes RD, Grimwood J, Jenkins J, Lindquist E, Tice H, Bauer D, Goodstein DM, Dubchak I, Poliakov A, Mizrachi E, Kullan AR, Hussey SG, Pinard D, Merwe Kvander, Singh P, Van Jaarsveld I, Silva-Junior OB, Togawa RC, Pappas MR, Faria DA, Sansaloni CP, Petroli CD, Yang X, Ranjan P, Tschaplinski TJ, Ye CY, Li T, Sterck L, Vanneste K, Murat F, Soler M, Clemente HS, Saidi N, Cassan-Wang H, Dunand C, Hefer CA, Bornberg-Bauer E, Kersting AR, Vining K, Amarasinghe V, Ranik M, Naithani S, Elser J, Boyd AE, Liston A, Spatafora JW, Dharmwardhana P, Raja R, Sullivan C, Romanel E, Alves-Ferreira M, Kulheim C, Foley W, Carocha V, Paiva J, Kudrna D, Brommonschenkel SH, Pasquali G, Byrne M, Rigault P, Tibbits J, Spokevicius A, Jones RC, Steane DA, Vaillancourt RE, Potts BM, Joubert F, Barry K, Pappas GJ, Strauss SH, Jaiswal P, Grima-Pettenati J, Salse J, Vande Peer Y, Rokhsar

DS, Schmutz J. 2014. The genome of Eucalyptus grandis. *Nature* **510**:356–362 DOI 10.1038/nature13308.

Nguyen MX, Moon S, Jung KH. 2013. Genome-wide expression analysis of rice aquaporin genes and development of a functional gene network mediated by aquaporin expression in roots. *Planta* **238**:669–681 DOI 10.1007/s00425-013-1918-9.

Niu J, Cao D, Li H, Xue H, Chen L, Liu B, Cao S. 2018. Quantitative proteomics of pomegranate varieties with contrasting seed hardness during seed development stages. *Tree Genetics & Genomes* **14**:14 DOI 10.1007/s11295-018-1229-1.

Patel C, Dadhaniya P, Hingorani L, Soni MG. 2008. Safety assessment of pomegranate fruit extract: acute and subchronic toxicity studies. *Food and Chemical Toxicology* **46**:2728–2735 DOI 10.1016/j.fct.2008.04.035.

Peret B, Li G, Zhao J, Band LR, Voss U, Postaire O, Luu DT, Da Ines O, Casimiro I, Lucas M, Wells DM, Lazzerini L, Nacry P, King JR, Jensen OE, Schaffner AR, Maurel C, Bennett MJ. 2012. Auxin regulates aquaporin function to facilitate lateral root emergence. *Nature Cell Biology* **14**:991–998 DOI 10.1038/ncb2573.

Picaud S, Becq F, Dedaldechamp F, Ageorges A, Delrot S. 2003. Cloning and expression of two plasma membrane aquaporins expressed during the ripening of grape berry. *Functional Plant Biology* **30**:621–630 DOI 10.1071/FP02116.

Pommerrenig B, Diehn TA, Bienert GP. 2015. Metalloido-porins: essentiality of Nodulin 26-like intrinsic proteins in metalloid transport. *Plant Science* **238**:212–227 DOI 10.1016/j.plantsci.2015.06.002.

Porcel R, Bustamante A, Ros R, Serrano R, Mulet-Salort JM. 2018. BvCOLD1: a novel aquaporin from sugar beet (Beta vulgaris L.) involved in boron homeostasis and abiotic stress. *Plant, Cell and Environment* **41**:2844–2857 DOI 10.1111/pce.13416.

Qiao X, Li M, Li L, Yin H, Wu J, Zhang S. 2015. Genome-wide identification and comparative analysis of the heat shock transcription factor family in Chinese white pear (Pyrus bretschneideri) and five other Rosaceae species. *BMC Plant Biology* **15**:12 DOI 10.1186/s12870-014-0401-5.

Qin G, Liu C, Li J, Qi Y, Gao Z, Zhang X, Yi X, Pan H, Ming R, Xu Y. 2020. Diversity of metabolite accumulation patterns in inner and outer seed coats of pomegranate: exploring their relationship with genetic mechanisms of seed coat development. *Horticulture Research* **7**:10 DOI 10.1038/s41438-019-0233-4.

Qin G, Xu C, Ming R, Tang H, Guyot R, Kramer EM, Hu Y, Yi X, Qi Y, Xu X, Gao Z, Pan H, Jian J, Tian Y, Yue Z, Xu Y. 2017. The pomegranate (Punica granatum L.) genome and the genomics of punicalagin biosynthesis. *The Plant Journal* **91**:1108–1128 DOI 10.1111/tpj.13625.

Quigley F, Rosenberg JM, Shachar-Hill Y, Bohnert HJ. 2002. From genome to function: the Arabidopsis aquaporins. *Genome Biology* **3**:RESEARCH0001 DOI 10.1186/gb-2001-3-1-research0001.

Reuscher S, Akiyama M, Mori C, Aoki K, Shibata D, Shiratake K. 2013. Genome-wide identification and expression analysis of aquaporins in tomato. *PLOS ONE* **8**:e79052 DOI 10.1371/journal.pone.0079052.

Sakurai J, Ishikawa F, Yamaguchi T, Uemura M, Maeshima M. 2005. Identification of 33 rice aquaporin genes and analysis of their expression and function. *Plant and Cell Physiology* **46**:1568–1577 DOI 10.1093/pcp/pci172.

Schnurbusch T, Hayes J, Hrmova M, Baumann U, Ramesh SA, Tyerman SD, Langridge P, Sutton T. 2010. Boron toxicity tolerance in barley through reduced expression of the multifunctional aquaporin HvNIP2;1. *Journal of Plant Physiology* **153**:1706–1715 DOI 10.1104/pp.110.158832.

Schuurmans JA, van Dongen JT, Rutjens BP, Boonman A, Pieterse CM, Borstlap AC. 2003. Members of the aquaporin family in the developing pea seed coat include representatives of the PIP, TIP, and NIP subfamilies. *Plant Molecular Biology* **53(5)**:633–645 DOI 10.1023/B:PLAN.0000019070.60954.77.

Shivaraj SM, Deshmukh R, Sonah H, Belanger RR. 2019. Identification and characterization of aquaporin genes in Arachis duranensis and Arachis ipaensis genomes, the diploid progenitors of peanut. *BMC Genomics* **20**:222 DOI 10.1186/s12864-019-5606-4.

Soto G, Alleva K, Mazzella MA, Amodeo G, Muschietti JP. 2008. AtTIP1;3 and AtTIP5;1, the only highly expressed Arabidopsis pollen-specific aquaporins, transport water and urea. *FEBS Letters* **582**:4077–4082 DOI 10.1016/j.febslet.2008.11.002.

Soto G, Fox R, Ayub N, Alleva K, Guaimas F, Erijman EJ, Mazzella A, Amodeo G, Muschietti J. 2010. TIP5;1 is an aquaporin specifically targeted to pollen mitochondria and is probably involved in nitrogen remobilization in Arabidopsis thaliana. *The Plant Journal* **64**:1038–1047 DOI 10.1111/j.1365-313X.2010.04395.

Suga N, Takada H, Nomura A, Ohga S, Ishii E, Ihara K, Ohshima K, Hara T. 2002. Perforin defects of primary haemophagocytic lymphohistiocytosis in Japan. *British Journal of Haematology* **116**:346–349 DOI 10.1046/j.1365-2141.2002.03266.x.

Takano J, Wada M, Ludewig U, Schaaf G, von Wiren N, Fujiwara T. 2006. The Arabidopsis major intrinsic protein NIP5;1 is essential for efficient boron uptake and plant development under boron limitation. *The Plant Cell* **18**:1498–1509 DOI 10.1105/tpc.106.041640.

Terashima I, Ono K. 2002. Effects of HgCl(2) on CO(2) dependence of leaf photosynthesis: evidence indicating involvement of aquaporins in CO(2) diffusion across the plasma membrane. *Plant and Cell Physiology* **43**:70–78 DOI 10.1093/pcp/pcf001.

Tornroth-Horsefield S, Wang Y, Hedfalk K, Johanson U, Karlsson M, Tajkhorshid E, Neutze R, Kjellbom P. 2006. Structural mechanism of plant aquaporin gating. *Nature* **439**:688–694 DOI 10.1038/nature04316.

Uçar S, Karagöz S. 2009. The slow pyrolysis of pomegranate seeds: the effect of temperature on the product yields and bio-oil properties. *Journal of Analytical and Applied Pyrolysis* **84**:151–156 DOI 10.1016/j.jaap.2009.01.005.

Van der Willigen C, Postaire O, Tournaire-Roux C, Boursiac Y, Maurel C. 2006. Expression and inhibition of aquaporins in germinating Arabidopsis seeds. *Plant and Cell Physiology* **47**:1241–1250 DOI 10.1093/pcp/pcj094.

Wallace IS, Roberts DM. 2004. Homology modeling of representative subfamilies of Arabidopsis major intrinsic proteins, Classification based on the aromatic/arginine selectivity filter. *Plant Physiology* **135**:1059–1068 DOI 10.1104/pp.103.033415.

Wang L, Guo K, Li Y, Tu Y, Hu H, Wang B, Cui X, Peng L. 2010. Expression profiling and integrative analysis of the CESA/CSL superfamily in rice. *BMC Plant Biology* **10**:282 DOI 10.1186/1471-2229-10-282.

Wu X-Y CC-z, Lü G-Q, Wang X-Y. 2016. Identification and Characterization of the AQP Gene Family in Sesame. *Science China Chemistry* **49**:1844–1858 DOI 10.3864/j.issn.0578-1752.2016.10.002.

Wudick MM, Luu DT, Tournaire-Roux C, Sakamoto W, Maurel C. 2014. Vegetative and sperm cell-specific aquaporins of Arabidopsis highlight the vacuolar equipment of pollen and contribute to plant reproduction. *Plant Physiology* **164**:1697–1706 DOI 10.1104/pp.113.228700.

Xu F, Wang K, Yuan W, Xu W, Liu S, Kronzucker HJ, Chen G, Miao R, Zhang M, Ding M, Xiao L, Kai L, Zhang J, Zhu Y. 2019. Overexpression of rice aquaporin OsPIP1;2 improves yield by enhancing mesophyll CO2 conductance and phloem sucrose transport. *Journal of Experimental Botany* **70**:671–681 DOI 10.1093/jxb/ery386.

Yang S, Zhang X, Yue JX, Tian D, Chen JQ. 2008. Recent duplications dominate NBS-encoding gene expansion in two woody species. *Molecular Genetics and Genomics* **280**:187–198 DOI 10.1007/s00438-008-0355-0.

Yasui M, Hazama A, Kwon TH, Nielsen S, Guggino WB, Agre P. 1999. Rapid gating and anion permeability of an intracellular aquaporin. *Nature* **402**:184–187 DOI 10.1038/46045.

Zarei A, Zamani Z, Fatahi R, Mousavi A, Salami SA, Avila C, Cánovas FM. 2016. Differential expression of cell wall related genes in the seeds of soft- and hard-seeded pomegranate genotypes. *Scientia Horticulturae* **205**:7–16 DOI 10.1016/j.scienta.2016.03.043.

Zhang DY, Ali Z, Wang CB, Xu L, Yi JX, Xu ZL, Liu XQ, He XL, Huang YH, Khan IA, Trethowan RM, Ma HX. 2013. Genome-wide sequence characterization and expression analysis of major intrinsic proteins in soybean (Glycine max L.). *PLOS ONE* **8**:e56312 DOI 10.1371/journal.pone.0056312.

Zhou Y, Setz N, Niemietz C, Qu H, Offler CE, Tyerman SD, Patrick JW. 2007. Aquaporins and unloading of phloem-imported water in coats of developing bean seeds. *Plant, Cell and Environment* **30**:1566–1577 DOI 10.1111/j.1365-3040.2007.01732.x.

Zhou Y, Tao J, Ahammed GJ, Li J, Yang Y. 2019. Genome-wide identification and expression analysis of aquaporin gene family related to abiotic stress in watermelon. *Genome* **62**:643–656 DOI 10.1139/gen-2019-0061.

Zhu YX, Yang L, Liu N, Yang J, Zhou XK, Xia YC, He Y, He YQ, Gong HJ, Ma DF, Yin JY. 2019. Genome-wide identification, structure characterization, and expression pattern profiling of aquaporin gene family in cucumber. *BMC Plant Biology* **19**:345 DOI 10.1186/s12870-019-1953-1.

Zou Z, Yang J. 2019. Genome-wide comparison reveals divergence of cassava and rubber aquaporin family genes after the recent whole-genome duplication. *BMC Genomics* **20**:380 DOI 10.1186/s12864-019-5780-4.

Zwiazek J, Xu H, Tan X, Navarro-Ródenas A, Morte A. 2017. Significance of oxygen transport through aquaporins. *Scientific Reports* **7**:40411 DOI 10.1038/srep40411.