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Genome-Wide Identification and Expression Analysis of the *fw2.2-like* Gene Family in Pear

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Abstract: Fruit size is a major factor determining yield, quality, and consumer acceptability. *fw2.2* (*fruit weight-2.2*) is a primary quantitative trait locus that was the first to be cloned, accounting for 30% of the variation in tomato fruit size. The various homologs of *fw2.2* (*fw2.2-like*) have been identified in many plants and belong to a large family. To date, there has been no report that has carried out a comprehensive identification of *fw2.2-like* members in pear. In this study, a total of 14 *fw2.2-like* genes were identified in the pear (*Pyrus bretschneideri* Rehd) genome and designated as *PbFWL1-14*. All of the *PbFWL* genes were unevenly distributed on nine chromosomes, and each chromosome contained between one and four genes. All PbFWL proteins contained more than two conserved motifs, and *PbFWL* genes contained more than one intron, and the genes of the same subfamily seemed to have a similar intron gene structure. According to the neighbor-joining phylogenetic tree, a total of 78 fw2.2/CNR (cell number regulator) from five plant species, including pear, maize, tomato, peach, rice, and physalis, could be divided into seven subgroups, and PbFWL proteins were mainly distributed in subgroups 1, 3, 4, 5, and 6. The Ka/Ks analysis also revealed that the *fw2.2-like* gene family of pear may have been subjected to strong purifying selection pressure during its evolution. A cis-element analysis found that many cis-elements responsive to hormones and stress were discovered in promotion regions for all *PbFWLs*. When combining real-time quantitative PCR analysis detection results, *PbFWL1/2/5* were found to be the most likely candidate genes for regulating pear fruit size.

Keywords: fruit size; *fw2.2-like* gene family; gene expression

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1. Introduction

Fruit size is one major factor determining yield, quality, and consumer acceptability. Genes participate in fruit weight or size gain under suitable growth conditions by regulating cell number and size [1,2]. At present, some genes are known to affect plant and organ size by regulating cell numbers or size [3–7]. Tomato *fruit-weight2.2* (*fw2.2*) was the first major quantitative trait locus (QTLs) to be cloned, and it regulates tomato fruit size and weight by controlling the carpel cell number, accounting for 30% of fruit size variation, with negative regulation of fruit size [8]. Alterations in fruit size, imparted by *fw2.2* alleles, are due to a variation in gene expression and not due to a different sequence and structure of the encoded protein [3].

Various homologs of fw2.2 proteins can be found throughout the plant kingdom as well as in mammals (fw2.2-like, also referred to as CNR or FWL (fw2.2-like) in the literature) [9]. Thibivilliers et al. used fw2.2 for sequence homology comparison and found 134 *FWL* genes in 13 plant species. Most plant FWL proteins consist of 100 to 250 amino acids, some being greater than 400 amino acids, and amino acid sequence comparisons between fw2.2 protein homologs revealed that they all have cysteine-rich PLAC8 structural domains [10]. They have functions in regulating organ size, maintaining metal ion homeostasis in vivo, and controlling calcium ion uptake [11]. Among them, the function of regulating organ size is universal in plants. For example, the maize gene cell number regulator1 (*Zea mays*;

ZmCNR1) negatively controls the size of the whole plant [9]. The biological function of other *fw2.2-like* genes that negatively regulate fruit development has also been described in husk tomato (*Physalis floridana*; *PfCNR1*) [12], sweet cherry (*Prunus avium*; *PavCNR12* and *PavCNR20*) [13], avocado (*Persea americana*; *Pafw2.2-like*) [14], and loquat (*Eriobotrya japonica*; *EjFWL1* and *EjFWL2*) [15]. The homologs of fw2.2 seem to belong to a large family of the given organisms. The *fw2.2-like* gene family has been studied in a variety of plants, including arabidopsis [9], oilseed rape [16], rice [17], maize [9], and pomegranate [18].

Pear, belonging to the genus Pyrus in the Rosaceae family, is one of the most important fresh fruits in the world; at least 22 different species are maintained worldwide [19]. These accessions have abundant fruit size variability, and the weight of a single fruit ranges from a few grams to several hundred grams [20]. In the production and cultivation of fruit trees, the size of the fruit is the main determinant of fruit grading and the economic efficiency of the orchard. For example, the Korla fragrant pear (*Pyrus sinkiangensis* Yü) is a well-received variety growing in the Bayingolin Mongol Autonomous Prefecture and the Aksu region in China. The Korla fragrant pear cultivate with a single fruit weight of 120 g is 3~4 times more expensive than the Korla fragrant pear cultivar with a single fruit weight of 80 g [21]. Therefore, breeding new large-fruited cultivars is a very major breeding objective. However, the long juvenile period of pears, which can last 5 years or even longer, greatly hampers the rapid phenotypic evaluation of fruit quality traits. Therefore, understanding the markers and genes associated with pear fruit size will greatly improve the efficiency of breeding large-fruited cultivars, as it would allow for the early elimination of seedlings that are likely to produce fruit that does not meet the breeding target. Pear fruit weight is a quantitative trait, like weight in tomatoes. Many QTL loci associated with fruit size have been detected in pear, and these loci are mostly concentrated in the LG7 and LG11 linkage groups [22,23]. The *fw2.2-like* gene family provides a good source of candidate genes for studying the molecular mechanism of pear fruit size. However, the *fw2.2-like* gene family genome-wide identification has not been reported in pear. In this study, we carried out a genome-wide analysis of Chinese white pear (*Pyrus bretschneideri*) *fw2.2-like* genes and selected 'Duli' pear (small-fruited), Korla fragrant pear (medium-fruited), 'Zaomeixiang' pear (medium-fruited, which is the large-fruited bud varietal of Korla fragrant pear), and 'Yali' pear (large-fruited) as research materials, focusing on the two most vigorous cell division periods, those of flowering and young fruit expansion, to explore the pear *fw2.2-like* gene family and investigated their potential functions in controlling fruit size.

2. Materials and Methods

2.1. Identification of the fw2.2-like Family in Pear

The protein sequence data of Chinese white pear (*Pyrus bretschneideri*) are available in the pear genome project (http://peargenome.njau.edu.cn/ accessed on 4 January 2022) [20]. We used a combination of two methods to search for members of the *fw2.2-like* family in *Pyrus bretschneideri*: an HMM search with the PLAC8 domain HMM profile (PF04749) and BLASTp searches, each using fw2.2 (Solyc02G090730.2.1) protein sequences from tomato as queries. First, the HMM profiles of PLAC8 were retained from the Pfam database (http://pfam.xfam.org/ accessed on 4 January 2022) and were used to identify the putative fw2.2-like proteins with the best domain E-value cutoffs of $\leq 1 \times 10^{-4}$ [18]. The tomato fw2.2 sequences were used as the query with which to perform a BLAST search in *Pyrus bretschneideri*, with a cutoff E-value of $\leq 1 \times 10^{-5}$. To validate the HMM and BLAST search, these potential sequences were submitted to NCBI Batch CD-Search tools (CDD) [24], Pfam [25], and SMART (http://smart.embl-heidelberg.de/ accessed on 7 March 2022) in order to confirm the conserved PLAC8 domain. The predicted protein sequences lacking the PLAC8 domain or containing other structures were excluded. All reliable fw2.2-like sequences were uploaded to ExPASy [26] in order to calculate the number of amino acids, isoelectric points (pI), and molecular weights. The subcellular localization of the identified PLAC8 proteins was determined using Plant-mPLoc [27]. Multiple sequence alignment was performed using ClustalX (http://www.ebi.ac.uk accessed on 20 March 2022).

2.2. Analysis of *fw2.2-like* Gene Structure and Conserved Motifs

Sequences of pear genome-wide *fw2.2-like* gene family members were analyzed and structures were mapped using the online program GSDS (http://gsds.cbi.pku.edu.cn/ accessed on 22 March 2022). Conserved motifs in the pear PbFWLs protein were identified using the online software MEME 5.5.1 (http://meme-suite.org/ accessed on 23 March 2022) with the following optimized parameters: number of repeats, arbitrary; maximum number of motifs, 6; and optimal width of each motif, between 6 and 100 residues.

2.3. Analysis of Cis-Acting Elements in *fw2.2-like* Gene Promoters

The upstream sequence (1.5 kb) of the coding sequence of the *fw2.2-like* gene was retrieved from the Chinese white pear genomic data and then submitted to PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/ accessed on 26 March 2022) for analysis of promoter sequences. The cis-acting element distribution was mapped using GSDS2.0 (http://gsds.cbi.pku.edu.cn accessed on 26 March 2022) [28].

2.4. Chromosomal Localization and Gene Duplication

The localization data of *fw2.2-like* pears were also obtained from genome annotation files (https://www.rosaceae.org/species/pyrus_betulifolia/genome_v1.0 accessed on 2 April 2022) [20]. Chromosome localization was mapped using MapChart 2.2 software [29]. In addition, gene duplication maps were obtained using MCScanX [30]. Gene duplications were confirmed using two criteria: (i) if shorter paired sequences covered more than 70% of longer sequences; (ii) if there was a similarity of paired sequences >70% [31,32]. Two genes located in the same chromosomal segment at less than 100 kb and separated by five or fewer genes were identified as tandem duplicates [33]. The differentiation time was calculated using the formula $T = Ks/2r$, where Ks is the synonymous mutation at each locus and r is the rate of differentiation of the plant nuclear genes. For dicotyledons, r was considered to be 1.5×10^{-8} synonymous mutations per locus per year [34].

2.5. Phylogenetic Analysis of *fw2.2-like* Proteins

PbFWLs were aligned with fw2.2/CNR proteins from other plants, such as those of *Zea mays* (12 sequences) [9], tomato (19 sequences and 1 sequence from wild tomato) [13,35], peach (23 sequences) [13], rice (8 sequences) [17], and physalis (1 sequence) [12], and multiple sequence comparisons were performed using ClustalW. Phylogenetic trees were constructed using comparisons from MEGA 7.0 [36] with the maximum likelihood method and a bootstrap set to 1000. The parameters were referenced in Guo et al. [9].

2.6. Plant Material

The three pear cultivars (Korla fragrant pear, 'Zaomeixiang' pear, and 'Yali' pear) and one wild pear ('Duli' pear) which were used in this study are listed in Supplementary Table S1. The 25-year-old trees were selected from the pear germplasm collection at the Research Center of Xinjiang Korla Fragrant Pear (Korla, China). These cultivated pear trees, grafted onto 'Duli' pear rootstocks, were spaced 2.5 × 4 m apart. They received annual routine horticultural care, were hand-pollinated with 'Dangshan Su' pollen at the anthesis on branches in the middle periphery of the canopy, and tagged.

2.7. Measuring the Characteristics of Fruit at Early Stages of Pear Fruit Development

Material at the anthesis (−4 and 0 days after full bloom, −4 and 0 DAFB) was collected randomly at the periphery of the tree in the middle of the canopy on untagged branches. A total of 10 flowers were collected at a time; all parts above the ovary (including stigma, stamens, and petals) and the stalk were removed to obtain the receptacle. Fruiting samples were collected starting with 10 DAFB, and fruit marked by pre-tagging was collected every 10 days until 50 DAFB (fruit cell division was expected to end around 50 DAFB), at a rate of 10 fruits at a time, with sepals and stalk parts removed. The longitudinal diameter of the receptacle (fruit) was measured using vernier calipers, the fresh weight

of the receptacle (fruit) was weighed on a thousandth of a scale, and the receptacle (fruit) volume was calculated according to the longitudinal diameter method V = 4/3πd3 (V is the receptacle or fruit volume and d is the average of 1/2 receptacle or the fruit's longitudinal diameter) [37]. This was repeated 3 times.

2.8. Measurements of Receptacle/Fruit Cell Size and Number

Pear fruits developed from receptacles, and a previous study showed that the receptacle develops into the pulp of pear, and the size difference between cultivars is mainly due to the number of mesocarp cells [38,39]. Thus, 10 tissues (receptacle equatorial axes and mesocarp tissue) per cultivar were preserved in an FAA solution (70% ethanol: acetic acid: formalin = 90:5:5) for paraffin sectioning. The tissues fixed in an FAA fixative solution were dehydrated, cleared, and embedded. Fruit sections were produced with a rotary microtome (ERM 3100, Heston, Jiangsu, China), and the sections were stained with the safranin and fast green counterstain method. The stained surfaces were observed under a Nikon Eclipse 80i microscope (Nikon, Tokyo, Japan) and photographed. The measurement of cell size and cell number in the receptacle/mesocarp was conducted according to the specifications of Tian et al. [40]. We used the following formula to calculate the cell volume: $V = 4/3\pi r^3$ (V represents the cell volume, r represents the average value of the vertical and horizontal diameter of 1/2 cells); cell number = fruit volume/cell volume [37]. Relative cell proliferation rate = (cell number 2—cell number 1)/(time 2—time 1), −4 to 0 DAFB for the first period P1 (period 1), and so on, 0 to 10 DAFB, and 10 to 20 DAFB for P2 and P3, respectively.

2.9. RNA Isolation and qRT-PCR

The receptacle and mesocarp tissue RNA was extracted from Korla fragrant pear, 'Zaomeixiang' pear, 'Yali' pear, and 'Duli' pear on −4 DAFB, 0 DAFB, 10 DAFB, 20 DAFB, 30 DAFB, and 40 DAFB. The receptacle (fruit) was cut crosswise along the equatorial side of the receptacle (fruit) with a scalpel, and the ovary (mesocarp) tissue was taken in the middle of the crosswise section and placed in an ultra-low-temperature refrigerator at −80 °C to freeze and for preservation. Total RNA was extracted using a polysaccharide polyphenol plant total RNA extraction kit (TIANGEND, Beijing, China). The cDNA was synthesized by reverse transcription according to the instructions in the FastKing RT Kit (with gDNase) (TIANGEND, Beijing, China). Based on the obtained cDNA sequence of the pear fw2.2-like gene, qRT-PCR primers were designed and examined using Primer 5.0 software, using the pear *TUB2* gene as an internal reference; all primer information is listed in Supplementary Table S2. The expression of each internal reference gene was detected using the BIO-RAD CFX Connect™ Fluorescent Quantitative PCR Assay System. The reagent used was Power SYBR® Green PCR Master Mix (Applied Biosystems Inc., MA, USA, Cat. No. 4367659), and the PCR programs used were 95 °C, 3 min pre-denaturation; 95 °C, 10 s denaturation; 55 °C, 20 s annealing; 72 °C, 20 s extension, with 40 cycles in total. Each sample was repeated 3 times. The relative expression of genes was calculated using $2^{-\Delta\Delta Ct}$.

3. Results

3.1. Identification and Sequence Analysis of fw2.2-likes in Pear

BLASTp and HMM searches were conducted to extensively identify pear fw2.2-likes using tomato fw2.2 protein sequences as queries. Thirty-six *fw2.2-like* genes were obtained in pear; then, the fw2.2-like protein sequences encoded by nonrepresentative transcripts were excluded. The remaining sequences were checked using CDD, Pfam, and SMART to determine if the remaining sequences had the full PLAC8 structural domain. In total, 14 sequences were confirmed as pear fw2.2-like proteins and were thus named PbFWL1 to PbFWL14. Gene names, gene IDs, chromosomal locations, gene location, amino acid numbers, molecular weights, isoelectric point (pI), instability index, and grand average of hydropathicity (GRAVY) are listed in Table 1. The predicted subcellular localization was the cell membrane and nucleus for PbFWL8 and PbFWL9 and the cell membrane for the other fw2.2-likes. The protein length of the pear *fw2.2-like* family gene members ranged from

151 (PbFWL1) to 415 amino acids (PbFWL9). The molecular weight of the pear *fw2.2-like* family genes ranged between 16.99 kDa (PbFWL1) and 47.51 kDa (PbFWL9). The *fw2.2-like* family genes of pear were distributed on nine chromosomes. The predicted pI values of PbFWL proteins ranged from 4.79 (PbFWL5) to 7.79 (PbFWL6). Their instability index was greater than 40. The hydrophilic index was between −0.484 and 0.239, and the cysteine residue content was between 15 and 20, which was higher than the average protein.

A comparison of the protein sequences of PbFWLs, ZmCNR1, OsFWL1, OsFWL3, OsFWL4, PfCNR1, and fw2.2 (Figure 1) showed that they all have PLAC8 structural domains and belong to the PLAC8 supergene family. In the PLAC8 domain, all homologs and some amino acids in between seem to be highly conserved. These amino acids are composed of two common protein motifs (CXXXXXCPC and QEYRELK), but the CXXXXXCPC motif was not observed in PbFWL8.

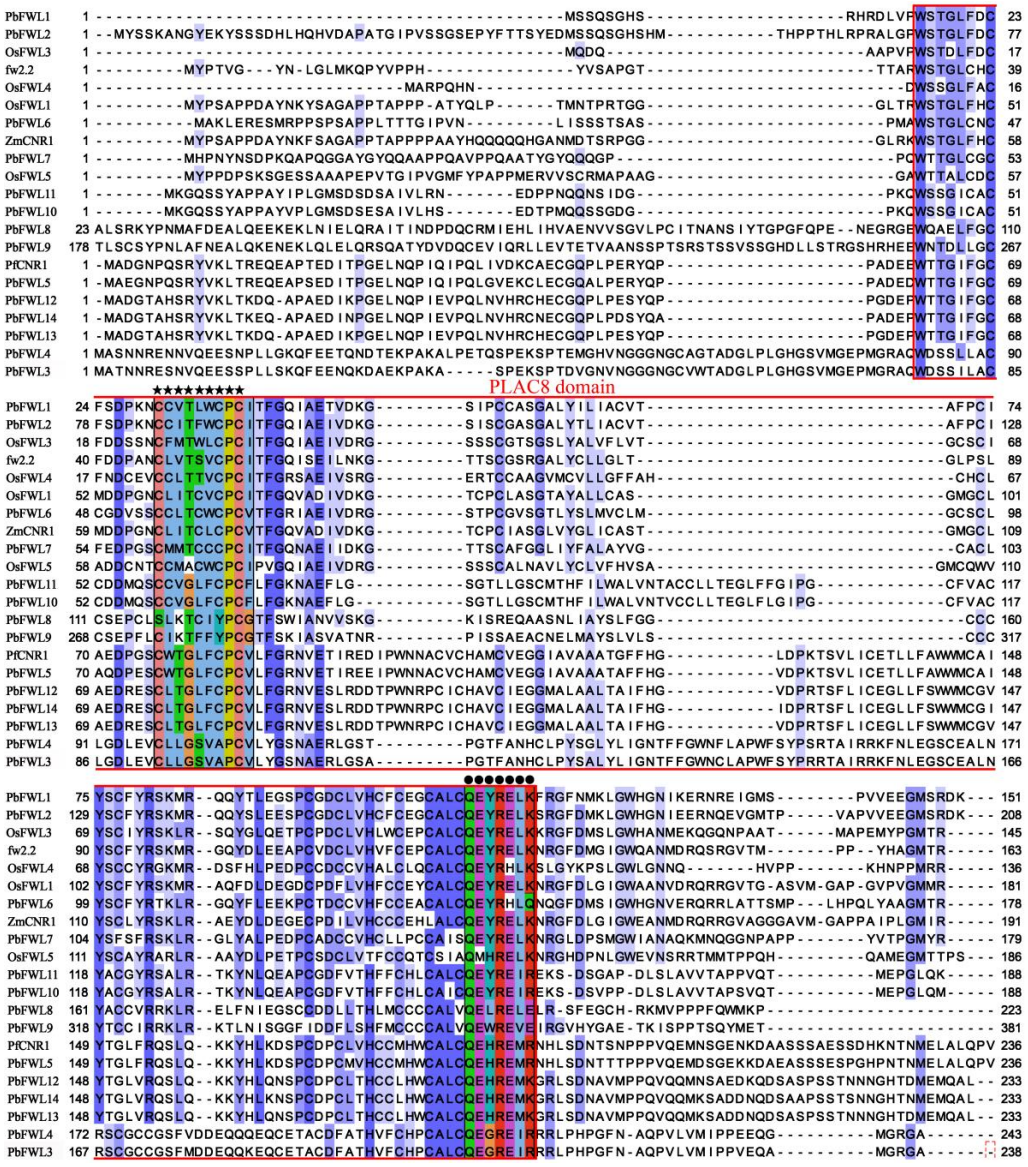

Figure 1. Multiple sequence alignment of PbFWLs, ZmCNR1, OsFWL1, OsFWL3, OsFWL4, OsFWL5, PfCNR1, and fw2.2. Identical and similar amino acids are shown in light blue and blue, respectively. The PLAC8 structure domain is indicated by the red box. The conserved motifs CXXXXXCPC and QEYRELK are indicated by black five-pointed stars and filled circles.

Table 1. Sequence characteristics of 14 genes identified in pear.

Gene Name	Gene ID	Chr	Gene Location (bp)	AA	MW (kDa)	pI	Instability Index(II)	GRAVY	cys	S.L.
PbFWL1	rna5458	16	312,566(-)314,118	151	16.99	7.41	54.00	−0.124	16	Cell membrane
PbFWL2	rna5456	16	310,397(-)314,118	208	23.08	5.67	54.46	−0.333	15	Cell membrane
PbFWL3	rna12156	4	11,872,621(-)11,875,857	246	26.72	5.29	56.18	−0.363	16	Cell membrane
PbFWL4	rna4422	12	12,008,588(-)12,011,010	251	27.19	4.93	56.13	−0.445	15	Cell membrane
PbFWL5	rna27717	7	6,529,212(-)6,531,603	242	26.91	4.79	56.34	−0.314	17	Cell membrane
PbFWL6	rna1124	3	23,944,140(-)23,945,131	178	19.54	7.79	53.62	0.022	20	Cell membrane
PbFWL7	rna4877	6	18,544,328(-)18,545,220	179	19.33	6.04	50.31	−0.232	16	Cell membrane
PbFWL8	rna23574	7	17,632,755(-)17,634,097	242	27.16	5.81	45.29	−0.135	19	Cell membrane Nucleus
PbFWL9	rna17541	15	14,880,874(-)14,886,180	415	47.51	6.58	49.00	−0.484	18	Cell membrane Nucleus
PbFWL10	rna19452	12	1,210,378(-)1,213,360	180	19.42	4.87	60.41	0.239	17	Cell membrane
PbFWL11	rna6937	14	433,409(-)436,373	188	20.36	4.9	59.62	0.134	17	Cell membrane
PbFWL12	rna24884	7	19,812,175(-)19,824,369	239	26.55	5.28	47.81	−0.365	17	Cell membrane
PbFWL13	rna27387	7	18,336,597(-)18,354,923	239	26.53	5.27	45.69	−0.365	17	Cell membrane
PbFWL14	rna41755	1	5,027,259(-)5,029,618	239	26.47	5.28	41.77	−0.318	17	Cell membrane

3.2. Phylogenetic Analyses of PbFWL Gene Family Members

To clarify the affinities and evolutionary distances between the pear fw2.2-like protein family and fw2.2-like proteins of other species, the full-length amino acid sequences of reported maize (12 members) [9], tomato (20 members) [13,35], peach (23 members) [13], rice (8 members) [8], physalis (1 member) [12] and pear *fw2.2-like* gene family members (14 members) were used in MEGA7.0 software to construct a phylogenetic tree (Figure 2). The protein sequences of *fw2.2-like* genes of tomato, *Zea mays*, peach, rice, and physalis were obtained from a previous study. Phylogenetic analysis showed that the 78 fw2.2/CNRs clustered into 7 distinct groups. In the clockwise direction, each group number is shown from 1~7. fw2.2/CNR members from the same species tended to cluster together in different phylogenetic tree groups. Group 1 contained 61.5% fw2.2/CNR proteins, labeled clade 1 in group 1 containing fw2.2, ZmCNR1, OsFWL1, and OsFWL3; this clade contained just two pear proteins, PbFWL1 and PbFWL2. PbFWL6 is a member of an adjacent subclade, labeled as 2 in group 1. A global pairwise amino acid comparison between fw2.2 and PbFWL1 showed 50.9% identity, and PbFWL2 showed 44.23% identity. fw2.2 was slightly more similar to PbFWL6 at 46.63%, with PbFWL2 and PbFWL6 showing 43.33% similar identity. However, the maximum likelihood test results indicate that PbFWL2 is closer to fw2.2 than PbFWL6. This closer proximity of PbFWL2 to fw2.2 was also revealed by three other family relationship algorithms, maximum parsimony, minimum evolution, and UPGMA cluster analysis, each completed using bootstrap tests. Group 6 contains 15.4% fw2.2/CNR proteins, labeled clade 3 in group 6, containing PfCNR1 and PbFWL5; a global pairwise amino acid comparison of PfCNR1 and PbFWL5 showed 85.1% identity.

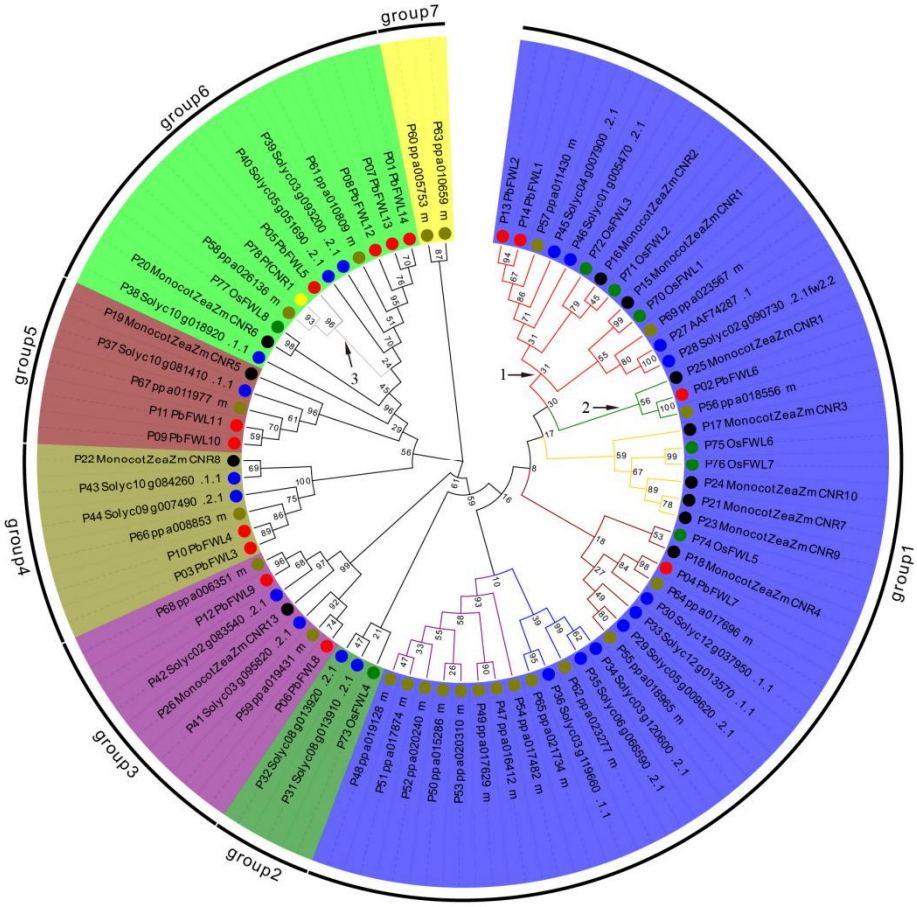

Figure 2. Phylogenetic tree analysis of fw2.2/CNR proteins in pear and other plants. The solid red circles represent pears, the solid black circles represent corn, the solid blue circles represent tomatoes, the solid olive circles represent peaches, and the solid green circles represent rice.

3.3. Gene Structure and Motif Analysis

To understand the structural components of the *PbFWL* genes, we extracted the exon–intron information of 14 *PbFWL* genes from the pear database using in-house scripts. The examined genes between CDSs contained 3 to 7 exons and 2 to 6 introns (Figure 3B). PbFWL6 had 3 exons and 2 introns, and the examined genes had the smallest exon/intron number among all identified PbFWL genes. *PbFWL9* had 7 exons and 6 introns, which represented the largest exon/intron number among the examined *PbFWL* genes. Exon/intron numbers varied among the homoeologous genes of the same family member but were essentially the same within the same subfamily (cluster I: exon: 4 to 5/intron: 3 to 4, cluster II: exon: 3/intron 2, cluster III: exon: 3/intron: 2, cluster IV: exon: 6 to 7/intron: 5 to 6, cluster V: exon: 3 to 4/intron: 2 to 3). Furthermore, all genes except *PbFWL6* and *PbFWL7* contained untranslated regions (UTR).

The MEME website identified the conserved motifs of PbFWL proteins, revealing six in total. The length of these conserved motifs ranged from 15 to 50 amino acids (Supplementary Table S3). Using Pfam, CDD, and SMART analysis, motifs 1, 2, 3, 4, and 6 were found to correspond exactly to the regions of the PLAC8 domain (Figure 3C). We also identified motifs unique to each cluster. These included motifs 3, 5, and 6, which were only present in cluster I; motif 1 was missing in cluster III. The only exceptions were that motif 4 was missing in PbFWL8 (cluster IV). According to the analyses of conserved motifs and gene structures, although the number of conserved motifs and the length of exons and introns varied to some extent, the conserved motifs and gene structures of the same subfamily members were highly consistent.

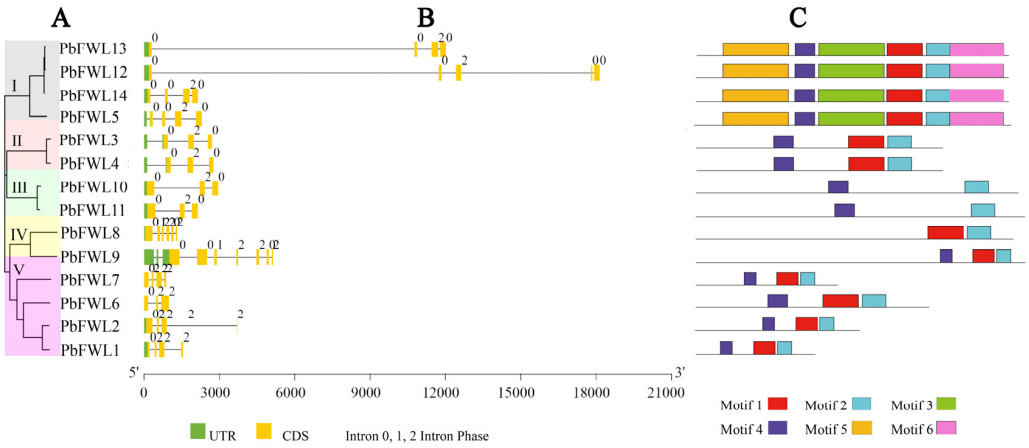

Figure 3. Gene structures of *PbFWL* genes, phylogenetic relationships, and conserved motifs of PbFWL proteins. (**A**) Phylogenetic tree of 14 PbFWL proteins in pear. The full-length amino acid sequences of 14 PbFWL proteins were imported into MEGA 7.0 software to construct an unrooted neighbor-joining phylogenetic tree. (**B**) Gene structure analysis of pear *fw2.2-like* members. The yellow boxes represent exons. The black lines represent introns. The green boxes represent UTR. The numbers 0, 1, and 2 represent the intron phases. (**C**) The conserved motifs in PbFWL proteins. The six predicted conserved structural domains are indicated by differently colored boxes. The motif information is shown in Supplementary Table S3.

3.4. Chromosomal Distribution and Synteny Analysis of PbFWLs in Pyrus Bretschneideri

To further study the relationship between the genetic evolution within the *fw2.2-like* gene family in pear, we analyzed the chromosomal location and synteny of the *PbFWL* genes. The physical mapping of *PbFWLs* on the chromosomes of *Pyrus bretschneideri* showed that the distribution of *PbFWLs* was unequal; these genes were distributed on nine chromosomes, with one or more genes per chromosome (Figure 4A). One pair of genes (*PbFWL1*/*PbFWL2*) was repeated in tandem, and five pairs (*PbFWL10*/*PbFWL11*, *PbFWL13*/*PbFWL14*, *PbFWL12*/*PbFWL14*, *PbFWL12*/*PbFWL13*, *PbFWL3*/*PbFWL4*) were segmentally duplicated (Figure 4B). To better understand the drivers of pear *fw2.2-like* gene evolution, *Ka*/*Ks* values of gene duplication pairs were calculated. The results showed that the *Ka*/*Ks* values of all gene duplication pairs were <1, indicating that the pear *fw2.2-like* gene family may have undergone purifying selection during evolution. The *Ks* values ranged from 0.02 to 0.231, indicating that the earliest duplication occurred 7.7 million years ago and that the latest duplication occurred 0.667 million years ago (Supplementary Table S4).

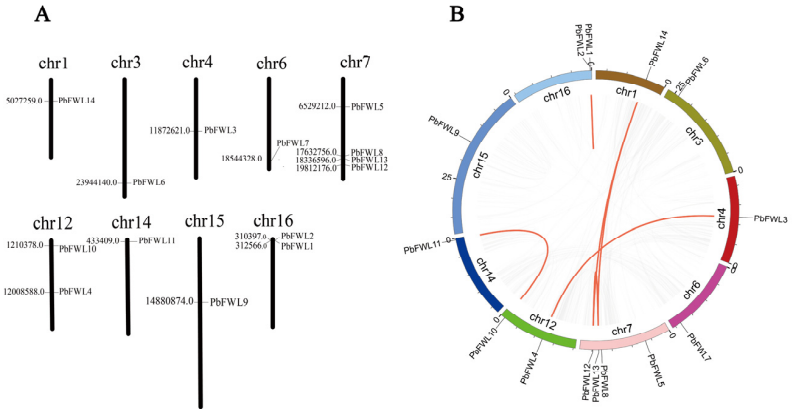

Figure 4. Schematic diagram of chromosome distribution and interchromosomal relationships of *PbFWLs* in pears. (**A**) The distribution pattern of *PbFWLs* in 9 pear chromosomes, where the chromosome number is indicated at the top of each chromosome. (**B**) The distribution pattern synteny analysis of *fw2.2-like* gene family of pear; the gray lines indicate all synteny blocks in the pear genome, and the red lines indicate duplicated *PbFWL* gene pairs.

3.5. Analysis of the fw2.2-like Gene Family Promoter Elements in Pear

Cis-acting elements are important cues for predicting gene function. Thus, in order to understand the potential role of the *fw2.2-like* gene family in pear growth and development, we analyzed the promoter cis-acting elements of the pear *fw2.2-like* gene family. As a result, we found many cis-acting elements in the initiation of pear *fw2.2-like* gene family genes, and 15 of them were selected as interesting cis-acting elements for further analysis, specifically those related to stress, hormones, plant growth, and development (Figure 5). Additionally, we found that all *PbFWL* genes contain cis-acting elements related to hormone regulation, such as gibberellin (GA), methyl jasmonate (MeJA), abscisic acid (ABA), auxin, and salicylic-acid-responsive elements.

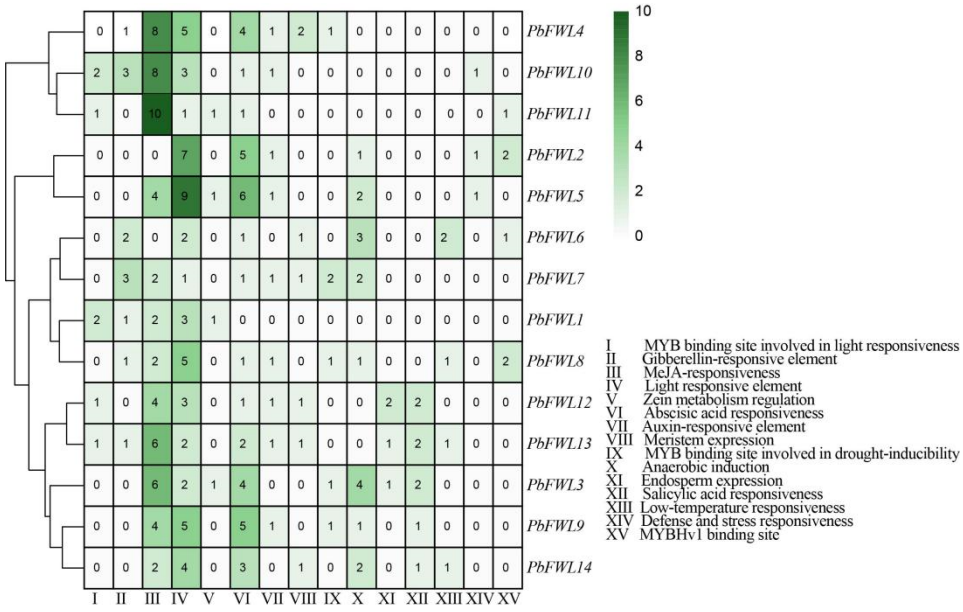

Figure 5. Predicted cis-elements in the promoter of the *PbFWL* genes. The color scale at the top right indicates the number of cis-acting elements. Green color indicates the number of cis-acting elements on *fw2.2-like* member. There were a total of 15 cis-acting elements, including (I) MYB binding site involved in light responsiveness; (II) Gibberellin-responsive element; (III) MeJA responsiveness; (IV) light-responsive element; (V) Zein metabolism regulation; (VI) abscisic acid responsiveness; (VII) auxin-responsive element; (VIII) Meristem expression; (IX) MYB binding site involved in drought inducibility; (X) anaerobic induction; (XI) endosperm expression; (XII) salicylic acid responsiveness; (XIII) low-temperature responsiveness; (XIV) defense and stress responsiveness; and (XV) MYBHv1 binding site.

3.6. Differential Expression of the PbFWL Genes during Early Fruit Development among Pear Varieties with Different Fruit Sizes

To determine whether *PbFWLs* are involved in the regulation of receptacle and fruit development early in fruit development, we analyzed the correlation of *PbFWL* gene expression with cell division related to the receptacle and flesh using large-fruited ('Yali' pear), medium-fruited (Korla fragrant pear, 'Zaomeixiang' pear), and small-fruited wild cultivars ('Duli' pear) during early fruit development stages. There were apparent differences in the receptacle and fruit size between the 'Duli' pear and the other three pear cultivars (Supplementary Figure S1). The observation of cells in the receptacle and early fruit showed that cells in the preliminary blooming stage (−4 and 0 DAFB) were of similar size, and that the cell size of the small-fruited wild cultivar was greater than that of the other cultivars at 10 to 40 DAFB (Figure 6A,B). In general, cell numbers in the same periods were higher for the 'Yali' pear than the 'Zaomeixiang' pear, followed by the Korla fragrant pear and the 'Duli' pear (Figure 6C). Analysis of the relative cell proliferation rates during the adjacent time periods revealed that the receptacle cells had two cell proliferation phases: a first

phase before 0 DAFB (P1), when the cell proliferation occurs slowly, and a second phase from 10 DAFB to 50 DAFB (P3 to P5), when cells proliferated rapidly (Figure 6D).

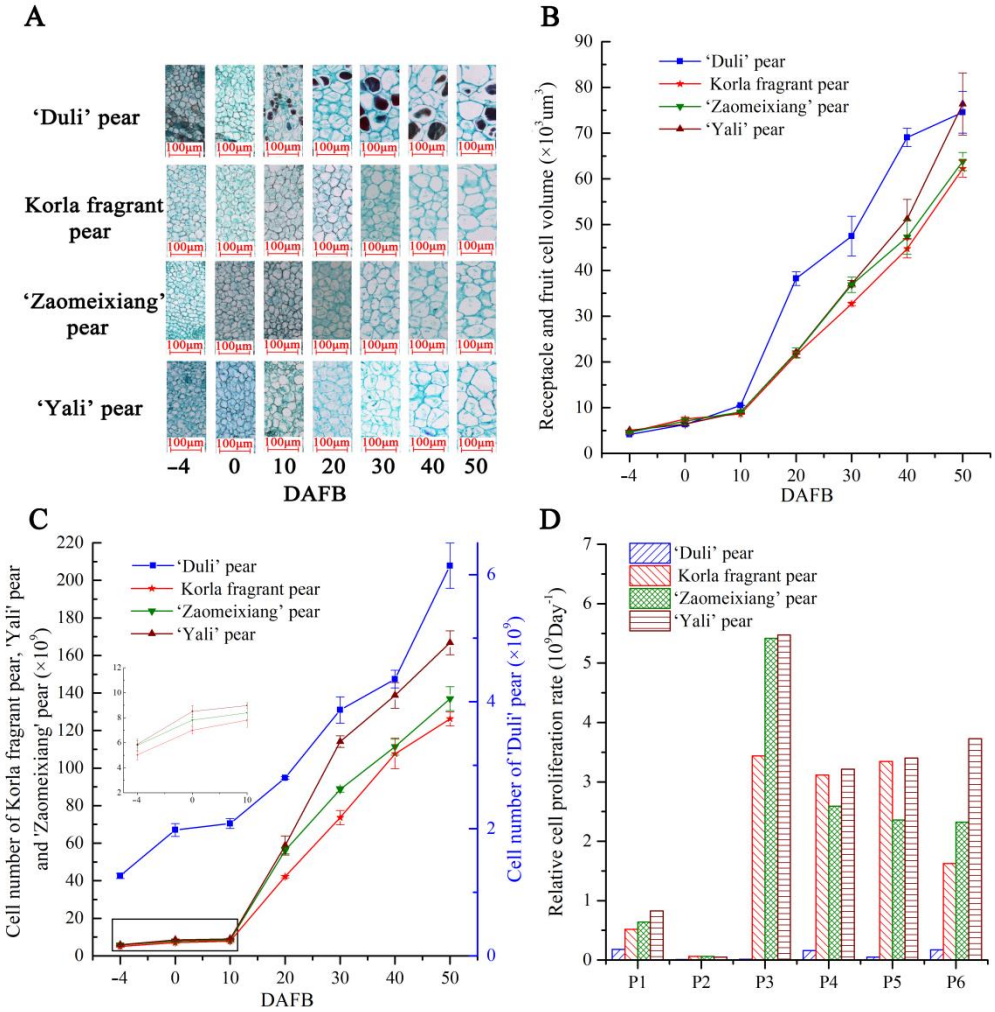

Figure 6. Cell proliferation and cell expansion during early pear fruit morphogenesis with different fruit sizes. (**A**) Longitudinal sections of material tissues in different stages are shown; the bar stands for 100 μm. (**B**) Cell size. (**C**) Cell number. (**D**) The relative cell proliferation rate. The vertical bars represent the standard error of triplicate experiments.

The *fw2.2* gene negatively regulates tomato fruit size and weight by affecting carpel cell division. Based on the results of pear receptacle (fruit) tissue slices in this experiment (Figure 6), we speculated that the accumulation level of *PbFWLs* transcripts in small-fruited wild cultivar ('Duli' pear) is larger than that in medium-fruited cultivars (Korla fragrant pear and 'Zaomeixiang' pear) followed by large-fruited cultivar ('Yali' pear). To verify this conclusion, we assessed the gene expression levels of 14 selected *PbFWL* genes using qRT-PCR of the receptacle or pulp tissues at different developmental stages (−4, 0, 10, 20, 30, and 40 DAFB) in the 'Duli' pear, Korla fragrant pear, 'Zaomeixiang' pear, and 'Yali' pear. Since the sequences of *PbFWL12* and *PbFWL13* are highly similar, it is impossible to design primers to separate the two sequences. Finally, the expression levels of 13 *PbFWL* genes were monitored (Figure 7). Gene expression data showed that 13 *PbFWL* genes did not exhibit regular expression patterns in the receptacle. Except for *PbFWL1/2/5*, the remaining *PbFWL* genes also did not exhibit regular expression patterns in the pulp. Lower *PbFWL1/2/5* transcription levels were found in the fruits at the fruit cell division phase (10 to 40 DADB) in the large-fruited cultivar ('Yali' pear) compared to those levels in the same phase in medium-fruited cultivars (Korla fragrant pear and 'Zaomeixiang' pear), and medium-fruited cultivars levels were lower than small-fruited wild cultivar levels

('Duli' pear). The transcript levels of *PbFWL1/2/5* were the highest at the 40 DAFB stage of small-fruited wild cultivars and the 10 DAFB stage of medium and large fruits. The above description of correlation was an artificial judgment and lacks statistical support, and so we quantified the division of pulp cells with gene expressions falling between 10 to 40 DAFB for correlation analysis (Table 2). The results showed that between 10 to 40 DAFB, the expression of *PbFWL1/2/5* correlated with the division of pulp cells during this period, that of *PbFWL1* was significantly negatively correlated, while that of *PbFWL2* and *PbFWL5* were highly significantly negatively correlated.

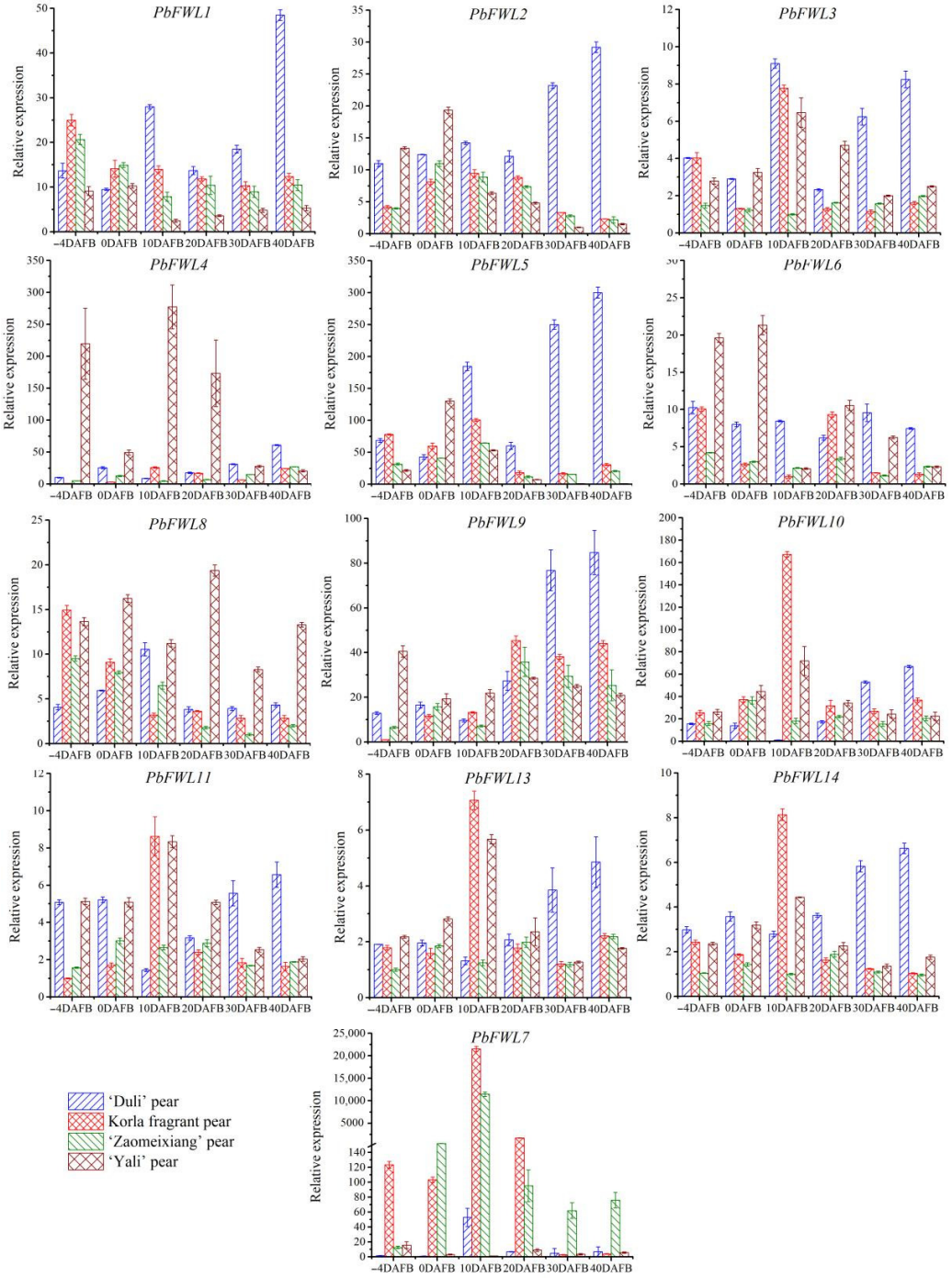

Figure 7. Relative expression levels of *PbFWL* genes in 'Duli' pear, Korla fragrant pear, 'Zaomeixiang' pear, and 'Yali' pear flower bud and fruit development. The vertical bars represent the standard error of triplicate experiments.

Table 2. Correlation between *PbFWL1/2/5* expression and pulp cell devision in 10 to 40 DAFB.

	Cell Division (C)	Expression		
		PbFWL1	***PbFWL2***	***PbFWL3***
'Duli' pear	0.036	27.179	19.683	198.781
Korla fragrant pear	0.126	12.127	5.962	41.572
'Zaomeixiang' pear	0.124	9.432	5.305	28.070
'Yali' pear	0.132	4.067	3.410	15.589
Correlation		$r = -0.958$ $P = 0.042$	$r = -0.996$ $P = 0.004$	$r = -0.996$ $P = 0.004$

Not: C indicates average cell cycle of pulp cells in 10 to 40 DAFB, C = log2 [NUM (40 DAFB)/NUM (10 DAFB)]; Expression (E) indicates average expression of gene in 10 to 40 DAFB, Expression = [E (10 DAFB) + E (20 DAFB) + E (30 DAFB) + E (40 DAFB)]/4.

4. Discussion

The *fw2.2-like* gene family and the broader family of proteins containing the PLAC8 structural domain are key components in the regulation of cell division and organ growth [41]. *fw2.2-like* gene family members have been isolated and identified in multiple species, but the number of *fw2.2-like* gene family members differs across different species [42]. In this study, we described the identification of 14 homologous proteins to *fw2.2* in pear, which were named the *PbFWL* genes. The number of *fw2.2-like* family gene members in maize was similar (12) [9] and less than that in tomato (19) [43] and peach (23) [13]. Although the number of *fw2.2-like* gene members in pear is the same as that in maize, the genome size of pear is 527 Mb [9], which is much smaller than that of maize at 2300 Mb [44]. Thus, the number of fw2.2-like genes in the genome does not depend entirely on the genome size. In plants, the *fw2.2-like/CNR* gene family members display short gene sequences. The length of amino acids in pear *PbFWL* genes ranged from 151 aa to 415 aa, which is very similar to that of maize (157 aa to 429 aa) [9]. An analysis of the isolated sequences revealed that the Cys-rich amino acids constitute the characteristic PLAC8 domain (PF04749) [45]. In the PLAC8 domain, there are two highly conserved motifs, CCXXXXCPC and QEYRELK. The function of QEYRELK is unknown [43,46].

In tomato, *fw2.2* gene family members are located on the cell membrane [43]. However, in this paper, the pear fw2.2-like gene family members are not entirely located on the cell membrane. This result is consistent with that of rice [17]. PbFWL8 and PbFWL9 are predicted to be both membrane proteins and nuclear proteins, and this double-localization of proteins is common [47]. However, software predictions can be inaccurate in studies of the exact location of family proteins in cells. Further experiments, such as transient gene expression assays, are needed. The PbFWL proteins' instability index was greater than 40, and the hydrophilicity index was between −0.484 and 0.134, indicating that putative pear PbFWLs are unstable hydraulic proteins. This result is consistent with that of FW in pomegranate [18]. The evolutionary tree shows that the members of tomato *fw2.2* gene families are mainly located in group 1, which reflects that the evolution direction of tomato *fw2.2* family genes may have a clear bias—that is, they evolve in the order of group 1. The pear *fw2.2-like* gene family members have four members in group 1 and group 6, indicating that the members of the pear *fw2.2-like* family are mainly evolved in the direction of group 1 and group 6. Members in groups 1 and 6 may play a more important role in the pear. Guo et al. studied the maize *fw2.2-like* gene family to investigate how the *fw2.2-like* gene affects maize plant or organ size, and phylogenetic and protein analyses revealed that ZmCNR1 is closest to fw2.2 and that they are structurally similar; for these reasons, ZmCNR1 was considered to be the best possible candidate maize direct line for the tomato fw2.2 homologs. Therefore, ZmCNR1 was selected for functional validation of the transgene [9]. In our study, the fw2.2 and ZmCNR1 specific subclades to which they belong, labeled clade 1 in Figure 3, contain just two pear proteins, PbFWL1 and PbFWL2. In addition, PfCNR1 and PbFWL5 specific subclades were labeled as clade 3. We speculate that PbFWL1, PbFWL2, and PbFWL5 may be involved in regulating cell division in organs such as pear fruits.

In *PbFWLs*, we found 6 tandem repeat genes, accounting for 42.8% of the total family members. Among them, three tandem repeat genes were distributed in group 6, which

indicated that tandem repeats played essential roles in amplifying members in this group. Paralogous pairs with a *Ka*/*Ks* ratio <1 in pear, *PbFWL* genes indicate mutation restriction with purifying selection, similar to what occurs in pomegranate *FWs* [18]. Additionally, cis-elements have an important influence on the expression of downstream genes. In our study, the promoter regions of multiple family members had cis-elements that respond to ABA, IAA, and MeJA signals. The vital role of ABA in response to drought and other abiotic stresses has been confirmed [48]. MeJA is an essential signaling molecule that regulates plant resistance to biotic stress [49]. Therefore, it is speculated that *PbFWL* genes may be involved in the stress response of pears to biotic or abiotic stresses.

Tomato *fw2.2* was the first cloned quantitative trait gene for of control fruit size in plants, accounting for 30% of the fruit weight variation. The transcript levels of small-fruited wild tomatoes are higher than those of large-fruited cultivated tomatoes, regulating the number of carpel cells and affecting fruit size [50,51]. The function of *FWL* in controlling cell proliferation is universal in plants [42]. For example, the expression of the avocado *PaFWL* in small-fruit pulp tissue is higher than that in normal pulp tissue [14]; *PfCNR1* affects the cell cycle and thus controls the size of berries and seeds in physalis [12]. Previous studies have shown that, during the cell division of pear fruit, *PbFWL1* and *PbFWL2* are more highly expressed in small fruits than in large fruits, with expression being negatively correlated with cell division in pear fruit [40]. This study found that the expression levels of 13 member genes of the pear *fw2.2-like* gene family do not correlate with the division of torus cells. Only the expression levels of *PbFWL1*, *PbFWL2*, and *PbFWL5* from 10 to 40 DAFB were higher in small fruit than in medium fruit, followed by large fruit, with expression negatively correlated with pear fruit cell division. The expression levels of *PbFWL1*, *PbFWL2*, and *PbFWL5* in the pulp of the large and medium fruits reached their peaks during the 10 DAFB period. In summary, we speculate that the functions of *PbFWL1*/*2*/*5* genes are similar to those of *fw2.2* and *PfCNR1*; the difference is that the pear *fw2.2-like* gene regulates the division of pulp cells in the young fruit stage of pear, affecting the size of pear fruits. *PbFWL1/2/5*, as membrane proteins, may interact with other genes to affect pear fruit size directly or indirectly. In addition, Arabidopsis *AtPCR1* and rice *OsFWL4* endow plants with cadmium resistance [17,52]. Therefore, *PbFWLs* may have other functions, which need to be studied in the future.

5. Conclusions

Fw2.2 is a primary quantitative trait locus that controls fruit size. The various homologs of *fw2.2* (*fw2.2-like*) have been identified in many plants and constitute a large family found in their plant inner organisms. In this study, we identified 14 *PbFWLs* from the genome of *Pyrus bretschneideri*, and the biological information about *fw2.2-like* family members was analyzed. The RT-qPCR results showed that *PbFW1*/*2*/*5* regulated fruit size at the young fruit stage and was the transcription factor regulating pear fruit size variation, while the expression of other *PbFWLs* was poorly correlated with fruit size. This study provides a good foundation for our next study on the molecular mechanism of how the pear *fw2.2-like* gene regulates fruit size.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae9040429/s1, Figure S1: Fruit size variation in pears; Table S1: Plant materials used in this study and their characteristic fruit size and maturation period; Table S2: Sequence of primers used in the experiment; Table S3: List of the putative motifs of PbFWL proteins; Table S4: Ka/Ks value of homologous *PbFWL* gene pairs.

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