1. Introduction

Domain-consistent proteins may belong to a family of proteins whose structure and function are similar. WD40 proteins constitute a diverse superfamily of proteins. Members of the family typically contain one or more conserved WD40 domains that include a glycine–histidine dimer (GH dipeptide) at the N-terminal end and a tryptophan–aspartic acid dimer (WD dipeptide) at the C-terminal end, which are named WD40 domains as they contain about 40 amino acid residues in total [1]. Since identification of the first WD40 protein structure [2,3], dozens of WD40 protein structures have been identified subsequently [4]. For example, the β subunit of a mammalian heterotrimer GTPase contains seven WD40 domains, with each domain forming a four-stranded antiparallel β-sheet and seven WD40 domains forming a highly stable β-propeller. A complete β-propeller requires at least four repeated WD40 domains. The β subunit of the G protein contains seven WD40 domains, of which the first and last participate in the formation of the same sheet, which may enhance the stability of the β-propeller structure [5,6]. In addition, the β-propeller structure determines the common mechanism of action of the WD40 protein family, which is
regulate the assembly of multiple protein complexes [6]. The repeated WD40 domain may be used as a scaffold and participates in the interaction of several proteins [4].

The WD40 protein family sequence is less conserved, and thus different members can participate in a variety of processes and show functional diversity [7,8]. Many WD40 proteins include domains with other functions, such as F-box, zinc finger, and bromodomain motifs [9]. Increasingly, WD40 proteins are shown to be involved in many important physiological and biochemical processes in plants, such as embryogenesis and gametogenesis [10], cell cycle and division [11–13], flower development and flowering [14–16], and secondary metabolic regulation [17]. WD40 proteins also participate in signal transduction and the response to diverse biological and abiotic stresses [18–23], and some members are involved in the response to hormones, such as auxin [24], brassinolide, and gibberellin [25,26]. In addition, genes involved in optical signal response have been identified. For example, CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1) has been isolated from Arabidopsis (Arabidopsis thaliana), rice (Oryza sativa), and apple (Malus × domestica) [27]. Many experiments have shown that WD40 proteins play a role in the dark to inhibit the light morphological pathways of plants. Among the most studied WD40 proteins in plants is a protein associated with anthocyanin synthesis, such as petunia (Petunia hybrid) ANT-HOCYANIN 11 (AN11) [28], Arabidopsis TRANSPARENT TESTA GLABRA 1 (TTG1) [29], corn (Zea mays) PALL ALEURONE COLOR 1 (PAC1), and pomegranate (Punica granatum) TTG1 [30,31], which form the TTG1/bHLH/MYB transcriptional complex together with MYB and bHLH proteins to regulate anthocyanin expression in plants [32,33].

With the advent of whole genome sequencing, the WD40 protein family has been systematically identified in various species [9,34–36]. Amongst plant species specifically, genes encoding WD40-family proteins have been identified in thale cress (Arabidopsis thaliana) [9], foxtail millet (Setaria italica) [37], cucumber (Cucumis sativus) [38], rice (Oryza sativa) [7], peach (Prunus persica) [39] and cotton (Gossypium hirsutum) [40]. However, although the WD40 family has been studied in many plant species, it has yet to be investigated in apple.

Apple is among the four most widely cultivated fruit crops in the world. During growth, apple trees often experience drought, low temperature, and other abiotic stresses. Such stresses affect the yield and quality of the fruit. Therefore, it is important to identify the genes associated with drought and low temperature responses to elucidate the stress resistance mechanism of apple. A growing body of evidence suggests that WD40 genes are involved in plant defence and response to a variety of biotic and abiotic stresses [18,20,22,41,42]. Considering their role in molecular network interactions, stress response and their abundance, a comprehensive analysis and identification of the WD40 protein family would be valuable. High-quality de novo assembled apple genome [43] has allowed us to carry out significant identification of all apple WD40 genes.

In this study, apple WD40 genes were identified, classified, and collinearity and evolutionary relationships were analysed. We also examined the tissue-specific expression and expression patterns of MdWD40 genes under abiotic stresses. Our results suggest that some MdWD40 genes may have an important role in response to abiotic stress. This study provides a basis for further exploration of the role of WD40 genes in apple.

2. Materials and Methods

2.1. Identification of Apple MdWD40 Genes and Their Chromosomal Location

The WD40 domain hidden Markov model PF0040 was downloaded from the Pfam database (http://pfam.xfam.org/ accessed on 28 January 2022). Apple WD40 genes were identified using the PF0040 file followed by a search of the Apple Genome Database (https://www.rosaceae.org/species/malus/malus_x_domestica/genome_GDDH13_v1.1 accessed on 28 January 2022) [44], and only genes with e-values < 0.01 were retained for further analysis [45]. In addition, Arabidopsis WD40 protein sequences [38] were used as queries for a BLASTP search of the Apple Genome Database, and only sequences with e-values < $1 \times 10^{-4}$ were selected as candidate proteins [46]. The presence of the WD40 conserved domain in the candidate genes was verified by means of conserved domain prediction using
the online Pfam software (http://pfam.xfam.org/ accessed on 28 January 2022). Specific data, such as molecular weight and theoretical pl of the candidate MdWD40 proteins were obtained from the ExPASy online portal (https://www.expasy.org/ accessed on 28 January 2022). The chromosomal location was downloaded from the Apple Genome Database and the genome location of MdWD40 genes was depicted using Mapdraw [47].

2.2. Classification and Phylogenetic Analysis of Apple WD40 Proteins

Other domains in the apple WD40 protein sequences were predicted using the online Pfam software [48]. A phylogenetic tree of 325 identified apple WD40 protein sequences (21 sequences were excluded on account of being short) and 229 identified Arabidopsis WD40 protein sequences (one sequence was excluded because it was short) was reconstructed using the neighbour-joining method (execution parameter: Bootstrap, “1000”; mode, “p-distance”; Gaps/Missing Date Treatment, “Pairwise deletion”) implemented in the phylogenetic software MEGA 5.1 [49]. The Arabidopsis WD40 protein sequences were downloaded from The Arabidopsis Information Resource (TAIR) (https://www.arabidopsis.org/ accessed on 28 January 2022).

2.3. Exon/Intron Structure of Apple WD40 Genes

The gene annotation file was downloaded from the Apple Genome Database to obtain annotations associated with the apple WD40 genes. The apple WD40 gene family exon/intron map was generated using the online tool Gene Structure Display Server 2.0 (http://gsds.cbi.pku.edu.cn/ accessed on 28 January 2022).

2.4. Synteny Analysis of Apple WD40 Genes

The WD40 protein sequences for apple and the reference species Arabidopsis were subjected to a BLAST analysis (parameters: -evalue, “1 × 10^{-5}”; -m, “8”), and then gff files annotated with the apple and Arabidopsis databases were integrated and analysed using MCScanX software [50]. The Circos version 0.63 software was used to display syntenic relationships between the proteins [51].

2.5. Plant Material and Treatments

Fuji apple (Malus × domestica ‘Fuji’) was used for the stress treatments. The trees were planted in Baishui, Shanxi province, China. Apple tissues and organs were sampled as follows: root, new lateral roots of 1–3 cm diameter; young leaves, leaves that were just unfolded on new shoots; old leaves, leaves from the middle portion of growing branches in the lower part of the tree body; flower buds, unopened buds; flowers, flowers at anthesis; young fruit, fruit sampled about 60 days after anthesis; mature fruit, fruit sampled 160 days after flowering; seeds (young and mature) were sampled coincident with the fruit samples [52].

Two-year-old Fuji seedlings from tissue-cultured seedlings were grown in pots in a greenhouse prior to stress treatment. For treatment with exogenous ABA, ABA solution (300 µm) was sprayed on the leaves and distilled water was sprayed as the control. For low-temperature treatment, seedlings were treated at 4 °C in a light incubator (continuous white light of 500 µmol m^{-2} s^{-1}). The control seedlings were cultured in a light incubator maintained at 25 °C. Samples in the ABA and low-temperature treatments were collected at 0, 0.5, 1, 4, 8, and 12 h after treatment [53,54]. The drought treatment was applied by irrigating each pot with 500 mL PEG6000 solution (0.2 kg L^{-1}). The same volume of distilled water was applied to the controls. Samples were collected at 0, 0.5, 1, 12, 24, 48, and 72 h after PEG6000 treatment [55]. Nine seedlings were used for each treatment with three seedlings considered as one biological replicate.

2.6. Expression Analysis of WD40 Genes in Apple

Total RNA was extracted from the apple samples using the Plant RNA Kit (Omega Bio-Tek, Norcross, GA, USA). A sample (1 µg) of total RNA was used for first-strand cDNA
synthesis using the PrimeScript RT Reagent kit with gDNA Eraser (TaKaRa Biotechnology, Dalian, China), which was stored at −80 °C for subsequent quantitative real-time PCR (qRT-PCR) analyses.

The qRT-PCR analyses were performed on an ABI StepOne Plus system (ABI, Foster City, CA, USA) using SYBR Green fluorescent dye (TaKaRa Biotechnology). The gene MdActin (XM_008393049) was used as an internal reference gene. All primers were gene-specific and designed with Primer 5.0 (Supplementary File 1: Table S6). Each PCR was conducted in a 20 µL reaction volume, which contained 10 µL SYBR Premix ExTaq II (TaKaRa Biotechnology), 0.8 µL of each forward primer and reverse primer (1.0 µM), 0.4 µL ROX Reference Dye, 1.0 µL cDNA template, and 7.0 µL sterile water. The PCR cycle parameters were 40 cycles of 95 °C for 30 s, 95 °C for 5 s, and 60 °C for 30 s.

The 2−ΔΔCt method was used to calculate the relative gene expression [56]. Thermal mapping was conducted using the Morpheus online tool (https://software.broadinstitute.org/morpheus/ accessed on 28 January 2022). The IBM SPSS Statistics 19.0 software package (IBM Corporation, Armonk, NY, USA) was used to process the expression data and assess statistical significance using Student’s t-test. Origin 7.5 software (Microcal Software Inc., Northampton, MA, USA) was used for plotting the expression data.

3. Results

3.1. Genome-Wide Identification of WD40 Genes in Apple

In total, 346 MdWD40 genes (Supplementary File 1: Table S1) were identified. With the exception of the common conserved WD40 domain of the 346 MdWD40 proteins, the physical and chemical properties of the proteins encoded by the MdWD40 genes varied greatly. The open reading frame of the members ranged from 231 to 10,803 bp, the length of the polypeptide varied from 76 to 3600 amino acids, the molecular weight ranged from 8.24 to 401.37 kDa, and the pI ranged from 4.33 to 11.8, which indicated that different MdWD40 proteins showed different acid–base properties.

The 346 MdWD40 genes were distributed on the 17 chromosomes of apple, but were not evenly distributed (Figure 1). The highest number of MdWD40 genes (39) was located on chromosome 15, whereas chromosome 4 carried the lowest number (11). The distribution on each chromosome revealed that some regions of the chromosomes showed a relatively high number of MdWD40 genes. For example, MdWD40 genes on chromosome 6 were mainly concentrated on the lower arm, whereas MdWD40 genes on chromosome 13 were mainly concentrated on the upper arm.

3.2. Classification and Phylogenetic Analysis of MdWD40 Proteins

In the present study, 346 MdWD40 proteins were divided into 12 subfamilies (A–L) based on the domain composition (Figure 2, Supplementary File 1: Table S2). Of the proteins, 251 that contained only the WD40 domain were classified into subfamily A. Proteins that contained other domains, in addition to the WD40 domain, were divided into the following subfamilies: five proteins containing a zinc finger domain were classified into subfamily B; nine proteins of subfamily C contained a UTP domain; six proteins containing the Beige/BEACH domain were classified into subfamily D; subfamily E contained six proteins with a NLE (NUC) domain; eight proteins that included a LisH domain were classified into subfamily F; five proteins with F-BOX and U-BOX domains were classified in subfamily G; eight proteins with histone-binding protein RBBP4 or subunit C of CAF1 complex domains were classified in subfamily H; eight proteins with Coatomer (COPI) alpha subunit C-terminus or Coatomer WD associated region (WDAD) were classified in subfamily I; subfamily J comprised two members that contained a protein kinase domain; two proteins containing breast carcinoma amplified sequence 3 (BCAS3) were classified in subfamily K; and subfamily L consisted of 36 members that contained other domains, including DENN, DUP, Cyclophilin, and domains with an unknown function.
Figure 1. Positions of MdWD40 genes on the 17 chromosomes of apple.

To explore the evolutionary relationships of WD40 proteins between apple and Arabidopsis, a phylogenetic tree was constructed for Arabidopsis and apple WD40 proteins. In the unrooted phylogenetic tree, the proteins were divided into 14 groups (Figure 3a, Supplementary File 1: Table S3). Group 14 contained the highest number of MdWD40 proteins (53), and Group 1 contained the fewest MdWD40 proteins (1). Except for Groups 1 and 2, apple sequences in the other groups outnumbered those of Arabidopsis. Given that the exon/intron structure may also explain the evolutionary relationship, we selected two groups randomly (Groups 3 and 13) to construct an exon/intron structure map of apple WD40 genes (Figure 3b). In general, the exon/intron structure of the genes within the same group were similar, whereas the exon/intron structures of the two subfamilies differed markedly.

Figure 2. Classification and Phylogenetic Analysis of MdWD40 Proteins. In the present study, 346 MdWD40 proteins were divided into 12 subfamilies (A–L) based on the domain composition. Of the proteins, 251 that contained only the WD40 domain were classified into subfamily A. Proteins that contained other domains, in addition to the WD40 domain, were divided into the following subfamilies: five proteins containing a zinc finger domain were classified into subfamily B; nine proteins of subfamily C contained a UTP domain; six proteins containing the Beige/BEACH domain were classified into subfamily D; subfamily E contained six proteins with a NLE (NUC) domain; eight proteins that included a LisH domain were classified into subfamily F; five proteins with F-BOX and U-BOX domains were classified in subfamily G; eight proteins with histone-binding protein RBBP4 or subunit C of CAF1 complex domains were classified in subfamily H; eight proteins with Coatomer (COPI) alpha subunit C-terminus or Coatomer WD associated region (WDAD) were classified in subfamily I; subfamily J comprised two members that contained a protein kinase domain; two proteins containing breast carcinoma amplified sequence 3 (BCAS3) were classified in subfamily K; and subfamily L consisted of 36 members that contained other domains, including DENN, DUP, Cyclophilin, and domains with an unknown function.
Figure 2. Structure of representative MdWD40 proteins from each subfamily. The protein structure is based on the presence of WD40 and other additional domains as identified using Pfam. The letter denoting the subfamily to which each corresponding protein belongs, and the gene ID are given on the left.

3.3. Amplification Pattern of WD40 Genes in Apple

To investigate the possible relationship between number of WD40 genes and potential WD40 gene duplication in apple, we performed a collinearity analysis, which included tandem repeats and fragment repeats (Figure 4, Supplementary File 1: Table S4). On the basis of Holub’s definition, a tandem repeat is when two or more genes are present in a chromosome within a 200 kb fragment [57]. The present study identified four pairs of MdWD40 genes with tandem repeats: MdWD40-104/MdWD40-105, MdWD40-260/MdWD40-261, MdWD40-270/MdWD40-271, and MdWD40-319/MdWD40-320. A total of 118 pairs of WD40 genes were predicted to have arisen by fragment replication, which accounted for about 68% of the total MdWD40 genes. Only one pair of replicated genomic regions were located on the same chromosome, i.e., chromosome 15. MdWD40-267 and MdWD40-283 were corresponding collinear genes. The remainder of the predicted collinear genes were distributed on different chromosomes. Further analysis revealed that only one pair of MdWD40 genes on chr1 and chr15, chr1 and chr16, chr2 and chr9, chr3 and chr13, chr4 and chr11, chr9 and chr15, and chr11 and chr12 were fragment repeats. In addition, chr1 and chr7, chr2 and chr7, chr2 and chr15, chr3 and chr11, chr4 and chr12, chr5 and chr10, chr6 and chr14, chr8 and chr15, chr9 and chr17, chr12 and chr14, and chr13 and chr16 contained many pairs of genes that are collinear.
Figure 3. (a) Phylogenetic relationships of WD40 proteins from apple and Arabidopsis. The sequences were aligned using CLUSTALW implemented in MEGA 5.1 and the unrooted phylogenetic tree was reconstructed using the neighbour-joining method. The proteins were classified into 14 distinct subfamilies. Each subfamily was assigned a different colour. (b) Gene structure of MdWD40 genes in Group 3 and Group 13. Green boxes represent a UTR, yellow boxes represent the CDS, black lines represent an intron.
3.4. Synteny Analysis of WD40 Genes in Apple and Arabidopsis

To further explore the origin of the apple WD40 gene family and its evolutionary relationship with Arabidopsis, the apple and Arabidopsis WD40 gene families were co-linearly analysed and mapped. The results showed that 67 pairs of genes showed collinearity, thus these MdWD40 genes and the corresponding AtWD40 genes shared a common ancestor (Figure 5, Supplementary File 1: Table S4). In these linear relationships, the majority were an apple MdWD40 gene corresponding to an Arabidopsis WD40 gene, such as AT3G26640–MdWD40-150 and AT1G03380–MdWD40-202. In addition, an apple WD40 gene may correspond to multiple Arabidopsis WD40 genes, or multiple apple WD40 genes may correspond to an Arabidopsis gene, such as AT1G18080/AT3G18130–MdWD40-256 and AT5G15550–MdWD40-155/MdWD40-321.
3.5. Gene Ontology Annotation

A GO-Slim analysis performed using Blast2GO predicted the putative participation of MdWD40 proteins in diverse biological processes. Many MdWD40 proteins were localized in the nucleus and participated in the formation of the Cul4-RING ubiquitin ligase complex and the heterotrimeric G-protein complex in the cell component category. Regarding molecular functions, 42% of MdWD40 genes were classified as molecule binding, followed by myosin heavy chain kinase activity, and histone acetyltransferase activity. In total, 134 MdWD40 proteins were involved in biological processes, including response to stress, cell division, tissue development, histone acetate, signal transfer, and rRNA processing (Figure 6, Supplementary File 1: Table S5).
Figure 6. Gene ontology (GO) annotation of MdWD40 proteins. The Blast2Go program was used to define GO within three categories: (a) biological processes, (b) molecular functions, and (c) cellular component.

3.6. Organ Expression Patterns of WD40 Genes in Apple at Different Growth Stages

Increasing evidence shows that many WD40 proteins play an important role in response to biotic and abiotic stresses. Eight Arabidopsis WD40 genes (AT1G80710, AT2G19430, AT5G58230, AT5G13480, AT1G03380, AT1G61210, AT1G18080, and AT5G67320) have been reported to be involved in ABA, drought, and low temperature responses [18, 20, 42, 58–63]. Therefore, we located the homologous genes MdWD40-17, MdWD40-24, MdWD40-32, MdWD40-37, MdWD40-55, MdWD40-70/MdWD40-219 (their protein similarity was 91.75%), MdWD40-256, and MdWD40-283 in apple. At the same time, MdWD40-340, MdWD40-235, MdWD40-259, MdWD40-266, MdWD40-308, MdWD40-7, MdWD40-125, MdWD40-307, MdWD40-74, and MdWD40-172 are 10 candidate genes associated with abiotic stresses, such as drought and low temperature, that were identified on the basis of the results of GO annotation. The 19 candidate MdWD40 genes were expressed in all organs. However, they showed significant spatiotemporal differences in expression (Figure 7). The 19 MdWD40 genes were roughly divided into two categories: 12 genes (MdWD40-283, MdWD40-24, MdWD40-256, MdWD40-32, MdWD40-37, MdWD40-266, MdWD40-340, MdWD40-55, MdWD40-259, MdWD40-235, MdWD40-70, and MdWD40-7) were more highly expressed in stems, leaves, and seeds than in roots or fruit; and seven genes (MdWD40-17, MdWD40-308, MdWD40-219, MdWD40-74, MdWD40-172, MdWD40-125, and MdWD40-307) were more highly expressed in roots and fruit than the other organs.
3.7. Response of WD40 Genes to ABA, Drought, and Low Temperature in Apple

To explore the response mode of MdWD40 genes to ABA, drought, and low-temperature stress, we analysed the expression level of the 19 candidate stress-related MdWD40 genes in leaves of apple seedlings treated with ABA, polyethylene glycol (PEG) 6000, and 4 °C. Eight genes showed responses to ABA, drought, and low-temperature stress (Figure 8 and Figures S1–S3). MdWD40-17, MdWD40-70, MdWD40-74, and MdWD40-219 were upregulated to different degrees in response to ABA treatment, and the timing of the peak in up-regulation also differed; MdWD40-74 expression peaked at 1 h of treatment, whereas MdWD40-17, MdWD40-70, and MdWD40-219 each peaked at 8 h. However, MdWD40-24, MdWD40-256, and MdWD40-307 were downregulated in response to ABA treatment. With regard to the PEG6000 treatment, six genes showed significant changes in expression, of which MdWD40-17, MdWD40-70, MdWD40-74, and MdWD40-283 were upregulated. The timing of their expression peak was delayed, and the earliest expression detected was at 12 h. The expression patterns of MdWD40-74 and MdWD40-283 were similar and increased at 72 h. MdWD40-24 and MdWD40-256 showed a downward trend in relative expression in response to drought treatment. Only three genes showed a significant change in expression after low-temperature treatment, in which MdWD40-17 and MdWD40-283 were upregulated while MdWD40-24 were downregulated. Thus, we observed that the latter three genes underwent significant changes in expression in response to ABA, drought, and low-temperature treatment.
Figure 8. Expression patterns of apple WD40 genes under abiotic stresses. Quantitative reverse transcription PCR (qRT-PCR) analysis of apple WD40 genes following treatment with (a) ABA at 0, 0.5, 1, 4, 8, and 12 h, (b) PEG6000 at 0, 0.5, 1, 12, 24, 48, and 72 h, and (c) 4 °C at 0, 0.5, 1, 4, 8, and 12 h. Bars and error bars are the mean ± SD of three replicate reactions. Asterisks indicate significantly up- or down-regulated expression of a gene under the differential treatment (Student’s t-test; * p < 0.05, ** p < 0.01).

4. Discussion

4.1. Identification, Classification, and Evolutionary Relationships of the WD40 Gene Family in Apple

The WD40 superfamily of proteins is widely distributed in animals and plants [35,64,65]. Its members perform a variety of functions and are involved in diverse processes, including signal transduction, cell division, cytoskeleton formation, and stress response [12,13,19,66].
Identification of members of the WD40 gene family provides the foundation for a comprehensive study of WD40 genes and their functions in apple. The current results show that many MdWD40 proteins contain not only the WD40 domain, but also additional domains, which is consistent with the WD40 proteins of Arabidopsis, rice, and foxtail millet [7,9,37]. This finding suggests that MdWD40 proteins serve a variety of functions.

The phylogenetic analysis resolved MdWD40 and AtWD40 proteins into 14 groups. Apple WD40 proteins were more numerous than Arabidopsis AtWD40 proteins in Groups 14, 12, 9, and 7, which indicated that in apple WD40 genes had undergone amplification specifically in these four groups. Many subgroups in the evolutionary tree shared one or more domains in addition to the WD40 domain. This finding was consistent with the subfamily definition mentioned above.

4.2. Amplification of WD40 Genes in Apple

Gene replication is an important driving force of plant evolution and many angiosperms have experienced whole-genome replication events (α, β, and γ) [67,68]. A previous study showed that a relatively recent (at least 50 million years ago) genome-wide replication transformed the chromosomes of apple from the original nine to the current 17 chromosomes. Four pairs of chromosomes (5 and 10, 3 and 11, 9 and 17, and 13 and 16) were derived from four ancestral chromosomes I, II, III, and IV, respectively. Chromosomes 4, 12, 6, and 14 are derived from ancestral chromosomes V and VI, and chromosomes 1, 2, 7, 8, and 15 are derived from chromosomes VII, VIII, and IX [69]. The results of colinear analysis of WD40 genes in apple showed that 110 pairs (93%) of colinear relationships were observed on chromosomes 1 and 7, 2 and 7, 5 and 10, 3 and 11, 4 and 12, 6 and 14, 2 and 15, 8 and 15, 13 and 16, and 9 and 17. These results are consistent with the modern whole-genome replication model for apple. Fragment replication includes small fragments replicated between chromosomes and the above-mentioned modern whole-genome replication [67]. Small fragment replication of apple WD40 genes involved eight pairs, whereas only four pairs of tandem repeats were observed among the 346 MdWD40 genes. It is suggested that MdWD40 gene amplification was mainly caused by the modern whole-genome replication events.

4.3. Collinearity Analysis of Apple WD40 Genes and Arabidopsis WD40 Genes

Genomic comparison is a relatively fast and efficient means of transferal of genomic knowledge of a taxonomic unit, whose genomic structure, function, and evolution have been thoroughly studied, to a less studied taxonomic unit [70]. Sixty-seven pairs of apple and Arabidopsis WD40 genes showed a collinear relationship (Table S4). This result indicated that these gene pairs show an orthologous relationship, share common ancestors, have been conserved during the evolutionary process, and may perform similar functions. Many WD40 genes in Arabidopsis have been identified and will provide a reference for future studies of apple WD40 genes.

4.4. GO Analysis of Apple WD40 Genes

Currently, WD40 motifs have been observed in many regulatory proteins involved in processes such as cell division, mRNA modification, transmembrane signal transduction, and transport of substances [6,7,9]. The GO annotation of apple WD40 genes showed that the genes may be involved in these processes as well. The GO annotation also predicted possible involvement of apple WD40 genes in defence against diverse biological and abiotic stresses. With regard to molecular function, many MdWD40 proteins were indicated to be involved in molecular binding, which is consistent with the molecular function of WD40 proteins to mediate protein–protein interactions. The annotations for the cell component category reflect that many MdWD40 proteins are important components of the nucleus, Cul4-RING ubiquitin ligase complex, and heterotrimeric G-protein complex, which are consistent with the function of WD40 proteins in cell division, chromosome modification,
and signal transduction [7,37]. These observations further illustrate the versatility of WD40 proteins in apple.

4.5. Apple WD40 Gene-Specific Expression and Response to Exogenous ABA, Drought, and Low Temperature

The 19 candidate stress-related MdWD40 genes showed differential expression in six organs of apple at different developmental stages. MdWD40-24, 25, 32, 37, 266, 340, 55, 259, 235, 70, and 7 showed higher expression levels in stems, leaves, and seeds, which indicated that the genes might perform important functions in the development of stems, leaves, and seeds. MdWD40-24 and AT2G19430 showed a close phylogenetic relationship. In Arabidopsis, AT2G19430 has been reported to be closely associated with seed germination [58], thus based on homology MdWD40-24 might play an important role in the germination of apple seeds. MdWD40-17, 308, 219, 74, 172, 125, and 307 showed a higher expression level in roots and fruit, which indicated that these genes may play an important role in development of apple roots and fruit. In addition, MdWD40-17 showed a higher expression level in flowers at anthesis compared with that of flower buds, and thus might be involved in the flowering process. The homologous gene ATIG80710 also shows higher abundance in flowers, which suggests the possibility that the homologs serve similar biological functions in different species [42].

Many WD40 proteins participate in the responses to a variety of abiotic stresses [20,21,23,60,63,71]. However, evidence that apple WD40 family genes are responsive to abiotic stresses is still lacking. Abscisic acid is generally considered to be a stress hormone involved in responses to environmental stress [72–74]. Following ABA treatment, a distinct change in the expression of eight MdWD40 genes was observed, which suggested involvement of the genes in the ABA signalling pathway. Treatment with PEG6000 reduces the osmotic potential and thus simulates drought stress [75]. After irrigation with PEG6000, the expression of six MdWD40 genes was altered in which MdWD40-17, MdWD40-70, MdWD40-74, and MdWD40-283 were upregulated while MdWD40-24 and MdWD40-256 were downregulated. We observed that these six genes coincidently responded to exogenous ABA and drought stress. Based on the relationship with ABA, the regulatory pathways of drought stress-responsive genes can be divided into two signal transduction pathways: ABA-dependent and ABA-independent types [76]. The six ABA- and drought-responsive genes may participate in the drought stress response via ABA-dependent pathways. Low temperature can cause ABA accumulation. The accumulation of ABA can play an extremely important role in resistance to low-temperature stress in plants, thereby improving the cold resistance of plants [77]. Thus, it is envisaged that the three genes MdWD40-17, MdWD40-24, and MdWD40-283 would likely respond to ABA, drought, and low temperature simultaneously. Further research is required to elucidate the mechanism by which MdWD40 genes respond to ABA, drought, and low-temperature stress.

In conclusion, 346 MdWD40 genes were identified and analysed on a genome-wide scale. Collinearity analysis indicated that the recent whole-genome replication events were the major driving force of MdWD40 family evolution. The expression patterns of MdWD40 genes under different stresses indicated that some MdWD40 genes were involved in responses to stresses of ABA, drought, and low temperature in apple. Our results provide a strong contribution to a better understanding of the evolutionary history of the WD40 genes and also provide a reference for the further functional investigation of these selected candidate WD40 proteins in apple.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/10.3390/horticulturae8020141/s1, Figure S1: qRT-PCR analysis of apple MdWD40 genes following treatments with ABA; Figure S2: qRT-PCR analysis of apple WD40 genes following treatments with PEG6000. Figure S3: qRT-PCR analysis of apple WD40 genes following treatments with low temperature. Table S1: information of apple WD40 proteins. Table S2: domain classification of apple WD40 proteins. Table S3: phylogenetic classification of MdWD40 proteins and
AtWD40 proteins. Table S4: synteny information of apple WD40 proteins. Table S5: gene ontology annotation of apple WD40 proteins. Table S6: list of primers for qRT-PCR.

**Author Contributions:** B.Z., D.Q., Y.Y., Z.Z. (Zhenzhen Zhu) and Z.Z. (Zhengyang Zhao) designed the experiments. B.Z., X.L. and Z.Z. (Zhenzhen Zhu) performed the experiments. D.Q. and H.Y. analysed the data. B.Z. and D.Q. wrote the manuscript. B.Z., D.Q., Y.Y. and Z.Z. (Zhengyang Zhao) revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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