Article

The WD40 Gene Family in Potato (*Solanum Tuberosum* L.): Genome-Wide Analysis and Identification of Anthocyanin and Drought-Related WD40s

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Received: 13 February 2020; Accepted: 12 March 2020; Published: 15 March 2020

Abstract: WD40 proteins, also known as WD40 domain proteins, constitute a large gene family in eukaryotes and play multiple roles in cellular processes. However, systematic identification and analysis of WD40 proteins have not yet been reported in potato (*Solanum tuberosum* L.). In the present study, 178 potato WD40 (*StWD40*) genes were identified and their distribution on chromosomes, gene structure, and conserved motifs were assessed. According to their structural and phylogenetic protein features, these 178 *StWD40* genes were classified into 14 clusters and 10 subfamilies. Collinearity analysis showed that segmental duplication events played a major role in the expansion of the *StWD40* gene family. Synteny analysis indicated that 45 and 23 pairs of *StWD40* genes were orthologous to *Arabidopsis* and wheat (*Triticum aestivum*), respectively, and that these gene pairs evolved under strong purifying selection. RNA-seq data from different tissues and abiotic stresses revealed tissue-specific expression and abiotic stress-responsive *StWD40* genes in doubled monoploid potato (DM). Furthermore, we further analyzed the WD40 genes might be involved in anthocyanin biosynthesis and drought stress in tetraploid potato cultivars based on RNA-seq data. In addition, a protein interaction network of two homologs of *Arabidopsis* *TTG1*, which is involved in anthocyanin biosynthesis, was constructed to identify proteins that might be related to anthocyanin biosynthesis. The result showed that there were 112 pairs of proteins interacting with *TTG1*, with 27 being differentially expressed in pigmented tissues. This study indicates that WD40 proteins in potato might be related to anthocyanin biosynthesis and abiotic stress responses.

Keywords: potato (*Solanum tuberosum*); WD40 protein; expression profiles; anthocyanin biosynthesis; drought stress

1. Introduction

WD40 proteins, also known as WD40 domain proteins, constitute a large gene family of eukaryotes [1]. The first WD40 protein was identified as a subunit of the bovine β -transduction

protein with a repeating 43 residue fragment containing highly a conserved glycine-histidine (GH) and tryptophan-aspartate (WD) motif [2]. The crystal structure of WD40 has a seven-leaf β -helical fold formed by repetition, with each fold containing a glycine histidine (GH) dipeptide of 11 to 24 residues at its N-terminus and the WD dipeptide at the C-terminus. [3,4], which forms a strong hydrogen bond network and a stable repeat fold [5].

The WD40 repeat proteins play multiple roles in cellular processes, including cell cycle regulation [6], cell apoptosis [7,8], autophagy [9,10], gene transcription [11], signal transduction [12,13], histone modification [14,15], DNA damage repair [16,17], RNA modification [18], cytoskeletal assembly, and chromatin assembly [19–21]. WD40 proteins function as scaffolds for protein–protein interactions or provide platforms to recruit diverse molecules that form functional complexes, indicating that WD40 has more interactions with other domains [1,21]. For instance, WD40 (TTG1) interacts with bHLH (basic-helix-loop-helix) and MYB (v-myb avian myeloblastosis viral oncogene homolog) to form the MBW (MYB-bHLH-WD40) complex, which not only regulates anthocyanin biosynthesis in *Arabidopsis* seedlings, but also positively or negatively regulates the extension phase of fiber development [22]. It was also shown that the MBW complex also directly regulates the activators and repressors, and the movement of repressors affects the trichome patterning on *Arabidopsis* leaves [23]. In addition, Derkacheva, et al. [24] found that WD40 (MSI1) links PRC2 to like heterochromatin protein 1 (LHP), which binds H3K27me3 in vitro and in vivo, which is required for the functional plant polycomb group (PcG) system. It was reported that CUL4, DDB1, and WD40, which is believed to act as a substrate receptor, form cullin-containing ubiquitin ligases (E3s) in *Arabidopsis* [25]. Lee et al. [26] found that 11 *Arabidopsis* WD40 proteins containing a DWD (DDB1-binding WD40 protein box) motif directly interact with DDB1, and may act as substrate receptors for the DDB1–CUL4 machinery.

To date, the WD40 protein family has been identified systematically in humans and multiple plant species [27–29], with 230 WD40s in *Arabidopsis thaliana*, 191 WD40s in cucumber (*Cucumis sativus*), 579 WD40s in cotton (*Gossypium hirsutum*), 200 WD40s in rice (*Oryza sativa*), and 743 WD40s in wheat (*Triticum aestivum*), respectively [30–33], but no report is available yet for potato (*Solanum tuberosum* L.).

Potato, which originated from the Andean regions of Peru and Bolivia [34], is the is the third most important food crop in the world after rice and wheat in terms of human consumption. In recent years, researchers have paid more attention to pigmented potato cultivars worldwide, which is a rich source of anthocyanins [35], which play important roles in plants. The color of anthocyanins attracts animals and insects, leading to seed transmission and pollination. Anthocyanins also protect plants from UV damage [36], and play a key role in tolerance to cold temperatures and drought [37,38]. Anthocyanins are natural colorants and have raised growing interest due to their extensive range of colors beneficial effects on human health [39]. Based upon many studies, such as cell line studies, animal models, and human clinical trials, it was suggested that anthocyanins possess anti-inflammatory and anticarcinogenic activity, and are useful for cardiovascular disease prevention, obesity control, and diabetes alleviation in humans [36].

The main factors that severely affect potato yield are various biotic and abiotic stresses, such as high temperature stress, cold stress, salinity, and drought stress, which badly restrict the growth and productivity of potato. Studies in other species showed that a large number of WD40 genes are involved in response to stress such as cold, heat, salinity, and drought [27,32,33,40]. Given the important role that WD40s play in plants, it is essential to identify and study the WD40 gene family in the potato genome.

In the current study, we identified a WD40 family with 178 members, and comprehensive analyses of the phylogenetic relationships, sequence features, gene duplications, and chromosome distribution were further performed. In addition, we analyzed the expression profile of *StWD40* genes under different tissue and abiotic stresses of double haploid (DM) potato. Additionally, RNA-seq data were used to analyze the expression profile of *StWD40* genes in white and pigment potato varieties, as well as in drought-sensitive and tolerant varieties of tetraploid potato. We also constructed a network of interactions with other potato genes for *TTG1* (*StAN11*), which is highly expressed in pigment potato

varieties. The results provide a basis for further study on functional characterization of the WD40 protein family.

2. Materials and Methods

2.1. Identification of *StWD40* Proteins in Potato

In order to identify the *StWD40* genes in potato and *Arabidopsis thaliana*, the whole protein sequence of potato was downloaded from the Potato Genome Sequencing Consortium (PGSC, http://solanaceae.plantbiology.msu.edu/pgsc_download.shtml, version 4.03) and the whole protein sequence of *Arabidopsis thaliana* was downloaded from Ensembl (<http://plants.ensembl.org/index.html>, version TAIR 10.41). The hidden Markov model (HMM) profile of the WD40 domain (PF00400) was downloaded from Pfam (<http://pfam.xfam.org/family/PF00400>). All WD40 proteins were identified by HMMER3.1 software (<http://hmmmer.org/download.html>) and the cutoff value was set to 1.0. All *StWD40* genes were confirmed by SMART (<http://smart.embl-heidelberg.de>) and Pfam (<http://pfam.xfam.org/search>).

2.2. Sequence Analysis and Structural Characterization in Potato

The coding sequence (CDS), protein sequence, and genomic sequence of the identified *StWD40* genes were downloaded from PGSC. All *StWD40* sequences were submitted to EXPASY (<http://web.expasy.org/protparam/>) to analyze the number of amino acids, molecular weight (MW), and theoretical isoelectric point (pI) [41,42]. The MEME program (version 5.0.4, <http://alternate.meme-suite.org/tools/meme>) was used to identify motifs in the *StWD40* sequence with the following parameters: 10 motifs with an optimal motif width of 6 to 50 amino acid residues and any number of repeats [43]. The exon–intron structures of the *StWD40* genes were graphically displayed by the Gene Structure Display Server (GSDS2.0, <http://gsds.cbi.pku.edu.cn/>) [44].

2.3. Chromosomal Localization and Gene Duplication in Potato

The chromosome distribution information of each *StWD40* gene was acquired from PGSC. MapChart software was used to draw the chromosomal positions map of *StWD40* genes and the relative distances. The events of the *StWD40* duplication was confirmed based on two criteria: (a) The length of the shorter aligned sequence covered >70% of the longer sequence, and (b) the similarity of the two aligned sequences was >70% [45,46]. Two genes located in the same chromosomal fragment of less than 100 kb and separated by five or fewer genes were identified as tandem duplicated genes [47]. The segmental duplications were confirmed by searching the Plant Genome Duplication Database (<http://chibba.pgml.uga.edu/duplication/>) [48]. MCScanX was used to analyze the synteny of the WD40 genes between potato and other plants [49], and figures were drawn by Circos v0.69 [50]. To further estimate the duplication events of WD40s, K_a (non-synonymous)/ K_s (synonymous) was calculated using KaKs Calculator 2.0 [51].

2.4. Phylogenetic Analysis and Classification of *StWD40* Genes

The full-length amino acid sequences of 232 AtWD40s and the newly identified 178 *StWD40*s were used for phylogenetic analysis. Multiple sequence alignments were performed using ClustalW progress. An unrooted maximum likelihood phylogenetic tree was constructed using MEGA (Molecular Evolutionary Genetics Analysis) 7.0 software [52] with a bootstrap test including 1000 iterations using the Poisson method.

2.5. Plant Materials and Treatments

The research materials were three potato varieties of different colors, including “Heimeiren” (HM), a purple potato cultivar with purple skin and purple flesh, “Xindaping” (XD), a white potato cultivar with white skin and white flesh, and “Lingtianhongmei” (LT), a red potato cultivar with red skin and red flesh, and were grown in a greenhouse at Gansu Agricultural University in Lanzhou, Gansu

Province, China. Six fresh tubers of each variety were used (diameter = 4–5 cm) to harvest the skin tissues and flesh tissues. Skin tissues were carefully obtained from cortex tissue. Flesh tissues were isolated with at least 5 mm distance from the skin. The samples were immediately frozen in liquid nitrogen and stored in a refrigerator at -80°C for use.

For the drought stress experiment, “Atlantic” (A, a drought-sensitive cultivar) and “Qingshu No. 9” (Q, a drought-tolerant cultivar) were grown in the field under a rain exclusion shelter at the Dingxi Academy of Agricultural Science in Dingxi, Gansu Province, China. The treatments included drought stress and a well-watered control (watering provided by a drip irrigation system). The experimental design was a randomized block with three replications of each treatment, with 10 plants in each experimental unit. Before seedling emergence, both treatments were irrigated suitably and equally. After emergence, plants under the drought stress treatment did not receive water for the remainder of the growing season. At the early flowering stage, full-blooming stage, and flower-falling stages, the foliage of three plants in each experimental unit were harvested and pooled as a single sample. Samples were immediately frozen in liquid nitrogen and stored at -80°C until later use.

2.6. RNA Extraction, qPCR, and Statistical Analysis

For quantitative real-time PCR (qPCR) analysis, total RNA extractions of different tissues (including skin and flesh obtained from three color potato cultivars and leaves obtained from two potato cultivars under droughts stress) were carried out using an RNA extraction kit (Tiangen DP419, Beijing, China). The integrity of RNA was monitored by agarose gel electrophoresis and the concentration was detected using a Nanodrop ND-2000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). A total of 2 μg RNA per sample was used for first-strand cDNA synthesis. Elimination of genomic DNA contamination and first-strand cDNA synthesis were carried out using FastKing RT kit with gDNase (Tiangen KR116, Beijing, China). The qPCR was conducted with a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using SuperReal PreMix Plus (SYBRGreen FP205; Tiangen, Beijing, China) with three biological replicates. The qPCR reaction conditions were as follows: 30 s at 95°C , followed by 40 cycles of 5 s at 95°C , 30 s at 60°C , followed by 65– 95°C melting curve detection. The qPCR efficiency of each gene was obtained by analyzing the standard curve of a cDNA gradient dilution. The housekeeping gene was *StEF-1 α* (AB061263) [53]. Relative expression was calculated using the comparative C_t ($2^{-\Delta\Delta C_t}$) method [54]. The oligodT primers were used for qPCR and were synthesized by Sangon Biotech (Shanghai, China). The primers and melt curves are listed in Table S1 and Figure S1.

Data quantified from the qPCR of three biological replicates were performed with a one-way ANOVA analysis of variance using the statistical software package SPSS 22.0. Differences in gene expression levels were processed by Duncan’s multiple range tests and the least significant difference (LSD). Significance was defined at a level of $p < 0.05$.

2.7. RNA-Seq Data Analysis

Total RNA of the aforementioned samples was chosen for further RNA-seq library construction. Enrichment of mRNA, fragmentation, addition of adapters, size selection, PCR amplification, and Next-Generation Illumina sequencing were performed by Biomarker Technologies Corporation (Beijing, China). After RNA sequencing, the raw data were uploaded to NCBI (Project ID PRJNA541919 and PRJNA541096). High-quality, clean reads were obtained by trimming the raw reads and filtering out contaminants, adapters, phred scores of less than 20, and uncertain bases. The cleaned data were aligned to PGSC_DM_v3.4 gene models downloaded from Solanaceae Genomics Resource at Michigan State University (http://solanaceae.plantbiology.msu.edu/pgsc_download.shtml) by Bowtie2 (v2.2.9). The number of mapped clean reads were counted (by fragment) and subjected to differential expression analysis using the edge R package (<http://www.r-project.org/>). Genes with the absolute value of $\log_2\text{FC}$ ($\log_2$ fold change) of ≥ 1 and a false discovery rate (FDR) of < 0.05 were considered to be significant differentially expressed genes (DEGs).

The DEGs were annotated against nonredundant database (Nr), SwissProt/UniProt Plant Proteins, Kyoto Encyclopedia of Genes and Genomes (KEGG), Cluster of Orthologous Groups of proteins (COG/KOG) and the potato protein database (<http://ftp.jgi-psf.org/pub/compngen/phytozome/v9.0/Stuberosum>) by BLASTX, with a cut-off E-value of 1.0e-5. Then, DEGs were subjected to enrichment analysis of GO (Gene Ontology) functions and KEGG pathways.

To study the expression patterns of *WD40* genes in various tissues (carpels, petals, sepals, stamens, whole mature flowers, immature fruits, mature fruits, internal to the fruit, stolons, tubers, stems, leaves, petioles, callus, roots, and shoots) and under abiotic stress (salt treatment: 150 mM NaCl; mannitol-induced drought stress: 260 μ M mannitol; heat treatment: 35 °C), the same methods as mentioned above were used, Illumina RNA-seq data were downloaded from the PGSC [55], and TBtools software was used to generate the heatmap [56].

2.8. The Interaction Network of *StAN11*(TTG1) Proteins

STRING is a database of known and predicted protein–protein interactions. The interactions include direct (physical) and indirect (functional) associations and each protein–protein interaction is annotated with one or more “scores”. These scores are indicators of confidence to show how likely STRING judges an interaction to be true, and to provide the available evidence. The threshold of combined score >400 indicate a confidence of medium or better.

Based on the RNA-seq dataset of pigmented cultivars, we selected PG0026477 and PG0000561 (annotated as *StAN11*) to predict the protein interaction network. Specific protein interactions were constructed using online STRING v11.0 software (Search Tool for the Retrieval of Interacting Genes/Proteins, <http://string-db.org/>) with a combined score >400 (medium confidence) [57]. As active interaction sources, text mining, experiments, databases, co-expression, neighborhood, gene fusion, and co-occurrence were selected. Genes with FPKM (fragments per kilobase of exon per million fragments mapped) > 5 were retained to construct the protein interaction network. The interaction network was visualized by Cytoscape v3.7.1 [58].

3. Results

3.1. Identification of *StWD40* Proteins

The Hidden Markov Model (HMM) search was performed against the potato and *Arabidopsis* genomes, excluding sequences without a typical WD40 domain via the Pfam (pfam00400) and SMART (SM00320) websites. Ultimately, a total of 178 *StWD40* genes in potato and 232 *AtWD40* genes in *Arabidopsis* were obtained, of which 230 *AtWD40* genes were consistent with those identified by Li et al. (2014) [31] and two more *AtWD40* genes were identified in our work. The *StWD40* protein varied largely in length and physicochemical properties; the length of amino acid residues of *StWD40* proteins ranged from 61 to 1552, the molecular weights were between 6.43 and 173.70 kDa, and the isoelectric point (pI) values ranged from 4.26 to 9.72 (Table S2). The transcript IDs were converted to their corresponding gene IDs, and the “SC0003DMG40” in the gene ID was omitted for convenience. The gene IDs are listed in Table S2.

3.2. Chromosomal Distribution, Gene Structure, and Subfamily Classification of *StWD40* Proteins

The position of each *StWD40* gene was determined by mapping their sequences to the chromosomes of the potato genome, showing that 177 *StWD40* genes (PG0019022 was not located) were distributed extensively and unevenly on 12 chromosomes (Figure 1A). A further 26 *StWD40* genes were located on chromosome 3, which had the largest number of *StWD40* genes, whereas chromosomes 5 and 10 had the least number of *StWD40* genes (Figure 1B). Most *StWD40* genes were located at the proximal or distal ends of these chromosomes, and high densities of *StWD40* genes were distributed at the bottoms of chromosomes 1, 3, 7, and 12. In contrast, relatively high densities of *StWD40* genes were detected at the tops of chromosomes 5 and 11.

Figure 1. Genomic distributions of 177 *StWD40* genes (except for unlocated *PG001922*) on 12 potato chromosomes. (A) “*StWD40* gene distribution map” on 12 potato chromosomes. Tandemly duplicated genes are marked by purple boxes. (B) Numbers of *StWD40* genes on each potato chromosome.

The full-length amino acid sequences of 178 newly identified *StWD40*s were used to construct an unrooted phylogenetic tree by MEGA 7.0 software, as shown in Figure 2A. In order to gain insight into the structures of the *StWD40* genes, we analyzed their exon and intron organization. The results showed that the numbers of exons and introns in the *StWD40* gene family differed greatly (Figure 2B). There were 23 *StWD40* genes (19.92%), which only had one exon and no intron. More than half (101) of the *StWD40* genes contained 1–9 introns, and the rest of them (54) contained 10 or more introns. The details are listed in Table S2.

Figure 2. Phylogenetic relationships, gene structure, conserved motifs, and domain composition analysis of the WD40 genes. Phylogenetic tree and subfamily classification of StWD40 proteins. Boxes of different colors represent 10 subfamilies of StWD40 proteins; the light green box represents subfamily

A, the light blue box represents subfamily B, the purple box represents subfamily C, the black box represents subfamily D, the orange box represents subfamily E, the red box represents subfamily F, the dark green box represents subfamily G, the yellow box represents subfamily H, the blue box represents subfamily I, and the grey box represents subfamily J (A). Exon/intron organization of the *StWD40* genes. The blue box indicates the exon and the black line of the same length indicates the intron. The upstream/downstream area is indicated by a red box. The numbers 0, 1, and 2 represent the splicing phase of the intron (B). Distribution of conserved motifs in the *StWD40* genes. Boxes of different colors represent 10 putative motifs (C). The 10 classification domains of *StWD40* genes are represented by boxes of different colors (D).

To further study the diversification of these *StWD40* genes in potato, we searched for conserved motifs of these proteins using the online MEME program. In total, 10 conserved motifs were identified and designated as motifs 1 to 10 (Figure 2C). All 178 *StWD40* proteins had motif 1 and motif 2, which contained 11 and 15 amino acids, respectively. There were 171 *StWD40* proteins that contained motif 3. Motif 8 and motif 9 were the least distributed, of which nine *StWD40* proteins contained motif 8 and six *StWD40* proteins contained motif 9. We found that the WD40 domain was composed of a variety of motif combinations, such as motif 2 and motif 3, motif 3 and motif 6, motif 2 and motif 4, and motif 4 and motif 6. Motif 1, motif 2, and motif 6 predominantly occurred at the C-terminus of the WD40 domain, and motif 3 and motif 4 were predominantly present at the N-terminus of the WD40 domain. The details of the 10 motifs are in Table S3.

To analyze whether these motifs were conserved in orthologs of *Arabidopsis* and wheat, the special conserved motif in wheat and *Arabidopsis*, which were orthologous to *StWD40*, were identified using the MEME website (Table S4, Figure S2). We found that motif 1, motif 2, motif 4, motif 5, and motif 6 identified in *StWD40* were relatively conserved in potato, wheat, and *Arabidopsis*, and that motif 1, motif 2, and the sequence “AFSPDGX” in motif 5 were more conserved than others. These motifs make up the largest part of the WD40 domain, but not entirely. The results indicated that only the WD40 domain was conserved during evolution in different species.

According to their domain compositions, the 178 *StWD40*s were grouped into 10 subfamilies, of which 129 *StWD40*s were classified into subfamily A, containing only WD40 domains, whereas the other 49 *StWD40*s containing the same domains were grouped into subfamilies B–J (Figure 2A,D, Table S5). Four *StWD40*s were classified into subfamily B, which contained the WD40 domain and CAF1 domains. Seven *StWD40*s, containing the WD40 domain and the LisH domain, were classified into subfamily C. Six *StWD40*s were grouped into subfamily D, which contained the WD40 domain and UTP (U3 small nucleolar RNA-associated protein) (12, 13, 15 or 21). Four *StWD40*s contained the WD40 domain and the zinc finger domain and were assigned to subfamily E. Five *StWD40*s were classified into subfamily F, containing the WD40 domain and the NLE (Notchless) (NUC139, NUC141) domain. Two *StWD40*s were classified into subfamily G, containing the WD40 domain, the Coatomer_WDAD (Coatomer WD associated region) domain and the COPI_C (Coatomer alpha subunit C-terminus) domain. Only one *StWD40* contained the WD40 domain and the protein kinase domain and was grouped into subfamily F. Five *StWD40*s were classified into subfamily I, containing the WD40 domain and the F-BOX/U-BOX contained. Fifteen *StWD40*s were classified into subfamily J, which contained the WD40 domain and other domains.

3.3. Gene Duplication and Genome Synteny

Tandem duplication and segmental duplication contribute to expansion of new gene family members and new functions in plant genome evolution [59]. The segmental and tandem duplication events in the *StWD40* family were investigated to elucidate the *StWD40* gene duplication events in potato. In our study, three pairs of genes (3.37%) were identified as tandemly duplicated genes, of which two pairs were located on chromosome 3 and one pair was located on chromosome 7 (Figure 1A, Table

S6). In addition, 14 pairs of genes (13.48%) might be related to segmental duplication events (Figure 3, Table S6).

Figure 3. Segmental duplications of *StWD40* in potato. The red line indicates the syntenicity of *StWD40* and the gray line indicates the syntenicity within the genome.

The substitution rate of non-synonymous (K_a) and synonymous (K_s) is the basis for evaluating the positive selection pressure of duplication events. $K_a/K_s = 1$ indicates neutral selection, $K_a/K_s < 1$ denotes purifying selection, and $K_a/K_s > 1$ signifies positive selection. The K_a/K_s of duplication *StWD40* genes were calculated using KaKs Calculator 2.0, showing that the K_a/K_s of tandem duplication ranged from 0.2307 to 0.6398 with a mean value of 0.4317 and that the K_a/K_s of segmental duplication varied from 0.0818 and 0.4012 with a mean value of 0.2463. The K_a/K_s values for all duplication events were less than 1, implying that most of these genes evolved under the effect of purifying selection.

To further investigate the potential evolution processes of the *StWD40* gene family, we analyzed the syntenic relationship of *WD40* genes among potato, *Arabidopsis*, and wheat. A total of 45 pairs of orthologs were identified between potato and *Arabidopsis* (Figure 4, Table S7), and the K_a/K_s ranged from 0.0142 and 0.9421 with a mean value of 0.1797. Another 23 pairs of orthologs between potato and wheat were identified (Figure 4, Table S8), and the K_a/K_s ranged from 0.0406 to 0.8655 with a mean value of 0.2763. These results imply that duplication events played an important role in evolution and

functional divergence, and that segmental duplication played a predominant role in the expansion of the *StWD40* family.

Figure 4. Comparative physical mapping showing the orthologous relationships of *StWD40* genes with *Arabidopsis* (A) and wheat (B). Average values of K_a , K_s , and K_a/K_s (C–E), respectively, of duplicated genes. There were 45 and 23 WD40 genes identified to be orthologous to *Arabidopsis* (A) and wheat (B), respectively. The horizontal axes in (C–E) stand for tandem duplication (Tandem), segmental duplication (Segmental), and the duplication between potato and *Arabidopsis* (St–Ar) and wheat (St–Ta).

3.4. Phylogenetic Analysis of WD40 Genes

To analyze the evolutionary relationship of the WD40 family between potato and *Arabidopsis*, the protein sequences of 178 identified *StWD40*s and 232 identified *AtWD40*s were used to generate an unrooted phylogenetic tree using the Maximum Likelihood (M-L) method via MEGA 7.0 software. The WD40s were divided into 14 major distinct clusters (Clusters a–n): 13 *AtWD40*s and eight *StWD40*s belonged to Cluster a, 10 *AtWD40*s and nine *StWD40*s were assigned to Cluster b, seven *AtWD40*s and three *StWD40*s were classified into Cluster c, 10 *AtWD40*s and 11 *StWD40*s were grouped into Cluster d, and 10 *AtWD40*s and four *StWD40*s belonged to Cluster k (Figure 5). The *StWD40*s of these clusters (a, b, c, d, and k) originated from subfamily A. The *StWD40*s belonging to subfamily B were assigned to Cluster n, which had four *AtWD40*s and 19 *StWD40*s. Noticeably, the genes in a set of orthologous pairs between potato and *Arabidopsis* were divided in the same cluster (Figure 5, Table S7), indicating the conservation of synteny between potato and *Arabidopsis* proteins. We found that members of one subfamily were not assigned to one cluster; however, members of one subfamily and in one cluster had similar numbers and locations of WD40 domains.

Figure 5. Phylogenetic classification of WD40 proteins from potato and *Arabidopsis*. The 14 clusters are marked with different colors. The blue circles represent potato WD40 proteins and the red circles represent *Arabidopsis* WD40 proteins. The orthologous genes of potato and *Arabidopsis* are represented by stars of different colors. The numbers represent branch lengths.

3.5. Expression Profiles of *St*WD40 Genes in Different Tissues

To understand the tissue-specific expression patterns of the *St*WD40 genes, the transcript abundance in different tissues, including flowers (carpels, petals, sepals, stamens and whole mature flowers), fruits (immature, mature and internal to the fruit), stolons, tubers, stems, leaves (leaves and petioles), and other tissues (callus, roots and shoots) of DM potato were analyzed based on transcriptome data downloaded from PGSC [55]. The results showed that 33.71% of *St*WD40 genes (60/178) were highly expressed in all tissues with FPKM > 5, 8.43% (15/178) of *St*WD40 genes showed little expression with FPKM less than 2, and one gene (*PG0037204*) was not expressed in any of the 16 tissues (FPKM = 0). Some of these displayed a tissue-specific expression pattern, such as *PG0006520*, *PG1015451*, and *PG0010081*, which were highly expressed only in stamens and mature flowers (FPKM > 14) and were expressed at low levels in the other tissues (FPKM < 2). Compared with other tissues, four genes were highly expressed in specific tissues (FPKM > 5 and log2FC > 1): *PG0044039* was in

fruit, *PG0002566* was in tubers and calluses, and *PG0026477* and *PG0016973* were in tubers and stolons (Figure S3A, Table S9).

3.6. Expression Profiles of *StWD40* Genes in Pigmented Potato Cultivars

To investigate whether the *StWD40* genes are involved in anthocyanin biosynthesis, expression profile analysis of the *StWD40* genes in different tuber tissues (skin and flesh) of tetraploid pigmented potato cultivars was performed based on RNA-seq data. The upregulated and downregulated *StWD40* genes in pigmented tuber skins of three potato varieties were analyzed. Six *StWD40* genes (*PG0002566*, *PG0013161*, *PG0015613*, *PG0016973*, *PG0028735*, and *PG0029342*) were upregulated in pigmented skin compared with the white skin of XD. Nine *StWD40* genes (*PG0006156*, *PG0009000*, *PG0016078*, *PG0019207*, *PG0021607*, *PG0022734*, *PG1001493*, *PG0025006*, and *PG2012456*) were downregulated in pigmented skin. Among them, *PG0013161* belonged to subfamily D, *PG0029342* belonged to subfamily J, and the other genes belonged to subfamily A. In pigmented skin, *PG0002566* was upregulated both in the skin of HM and LT, and *PG0013161*, *PG0015613*, *PG0016973*, *PG0028735*, and *PG0029342* were only upregulated in the skin of HM. Additionally, *PG0019207* was downregulated in the skin of HM and LT. *PG0006156*, *PG0016078*, *PG0021607*, *PG0022734*, and *PG1001493* were downregulated in the skin of HM, and *PG0009000*, *PG0025006*, and *PG2012456* were downregulated in the skin of LT (FPKM > 1 and $|\log_2\text{FC}| > 1$) (Figure 6, Table S10). These downregulated genes all belonged to subfamily A. Seventeen *StWD40* genes were not expressed (FPKM = 0) and 26 *StWD40* genes showed little expression with an FPKM value less than 1.

Previous studies showed that *TTG1* participates in the pigmentation of seed coat and the development of trichomes in *Arabidopsis* [60]. In the PGSC database, the best blast matches for *TTG1*

(*StAN11*) were *PG0000561*, *PG0026477*, and *PG0044039*, and their expression was analyzed in this RNA-seq dataset. *PG0000561* showed higher expression in pigmented skin than in white skin (FPKM = 45.02 in HM and FPKM = 54.68 in LT with increases of 0.36 log₂FC and 0.64 log₂FC, respectively). *PG0026477* was highly expressed in white and pigmented skin, with FPKM ranging from 42.17 to 54.11. In the flesh, *PG0000561* and *PG0026477* were upregulated in HM (log₂FC > 1, FPKM > 1) and highly expressed in LT, with log₂FC ranging from 0.94 to 0.97. However, *PG0044039* showed little expression in all tissues, with FPKM < 1.

3.7. Expression Profiles of *StWD40* Genes under Abiotic Stresses

The RNA-seq data were downloaded from PGSC to analyze differential expression of *StWD40* genes under various abiotic stresses. The results showed that 10, 15, and 19 *StWD40* genes were differentially expressed (FPKM > 1 and |log₂FC| > 1) under salt, mannitol, and heat treatment, respectively (Figure S3B and Table S11). Among them, 6, 7, and 11 *StWD40* genes were upregulated under salt, mannitol, and heat treatment, and 3, 8, and 8 *StWD40* genes were downregulated under these abiotic stresses, respectively. Only one gene (*PG0015970*) was differentially expressed under three stress treatments (FPKM > 1 and |log₂FC| > 1). Three *StWD40* genes were differentially expressed under two stress treatments, with FPKM > 1 and |log₂FC| > 1. A total of 35 *StWD40* genes only responded to a single stress (FPKM > 1 and |log₂FC| > 1) (Figure S3B, Table S11).

To further investigate how the *StWD40* genes might respond to drought stress, the tetraploid drought-sensitive cultivar “Atlantic” (A) and the tetraploid drought-tolerant cultivar “Qingshu No. 9” (Q) were subjected to drought stress. The RNA-seq data showed that 15 *StWD40* genes were not expressed (FPKM = 0), and 12 genes were upregulated or downregulated in response to drought stress, of which six *StWD40* genes responded to drought stress at the early flowering stage, eight genes were expressed at the full-blooming stage, and five genes were expressed at the full-flowering stage (FPKM > 1 and |log₂FC| > 1). *PG0005946* showed no difference in A and Q at the early flowering stage and full-blooming stage but was downregulated in Q at the flower-falling stage under drought stress. *PG0003339* was upregulated in Q at the flowering and full-blooming stages. *PG0002566* was downregulated in Q from the early flowering stage until the flower-falling stage, and the other genes were only upregulated or downregulated at one stage (Figure 6, Table S12). Combined with the data analysis of PGSC mannitol stress, we found that the upregulated genes (*PG0011337*, *PG0006033m* and *PG0007963*) in DM under mannitol stress were more highly expressed in drought-sensitive cultivar A than in drought-tolerant cultivar Q. The down-regulated genes (*PG0009000*, *PG0010747*, *PG0015829*, *PG0017881*, and *PG0022734*) in DM under mannitol stress were more highly expressed in drought-tolerant cultivar Q than in drought-sensitive cultivar A. These genes may be involved in drought stress and need further study.

We selected 19 *StWD40* genes which had relatively higher expression levels in pigmented tissues or differential expression levels in Q and A under drought stress as targets to validate the reliability of the RNA-seq dataset via qPCR. The results confirmed that the qPCR expression patterns were in agreement with the RNA-Seq dataset (Figures 7 and 8), showing a high correlation ($R^2 = 0.853$) described by a simple linear regression equation ($y = 1.0149x - 0.1912$) between the RNA-seq dataset of pigmented potato cultivars and qPCR, and a high correlation ($R^2 = 0.830$) described by a simple linear regression equation ($y = 0.8221x + 0.1007$) between the RNA-seq dataset of two potato cultivars under drought stress and qPCR, indicating consistency between the two analytical methods.

Figure 7. The qPCR expression analyses of 19 *StWD40* genes in white and pigmented skin and flesh. XDS, HMS, and LTS represent the white skin of the white potato cultivar (XD), the purple skin of the purple potato cultivar (HM), and the red skin of the red potato cultivar (LT), respectively. XDF, HMF, and LTF represent the white flesh of XD, the purple flesh of HM, and the red flesh of LT, respectively. Data represent the mean of three biological replicates $\pm$ standard error of the mean. Standard errors are shown as bars above the columns. Different letters above bars denote significant difference at $p < 0.05$.

Figure 8. The qPCR expression analyses of 19 *StWD40* genes in a drought-sensitive cultivar (Atlantic, A) and a drought-tolerant cultivar (Qingshu No. 9, Q) cultivar under drought stress at the early flowering stage (S1), full-blooming stage (S2), and flower-falling stage (S3). Data represent the mean of three biological replicates $\pm$ standard error of the mean. Standard errors are shown as bars above the columns. Different letters above bars denote significant difference at $p < 0.05$.

3.8. The Interaction Network of *StAN11* (TTG1) Proteins

Studies showed that TTG1 is involved in anthocyanin synthesis [23,61]. We further investigated proteins that interact with *StAN11* (TTG1) and may be involved in the synthesis of anthocyanins. Based on the RNA-seq dataset of pigmented cultivars, we selected PG0026477 and PG0000561 (annotated as *StAN11*) to predict the protein interaction network. Genes with FPKM > 5 were retained to construct a protein interaction network with a median confidence (combination score >400). The results indicated that a total of 112 pairs of interacting proteins were predicted, of which 39 proteins interacted with PG0026477 and PG0000561, 27 proteins interacted with PG0026477, and seven proteins interacted with PG0000561. Among them, PG0026477 interacted with all MYB transcription factors in the network and with bHLH1-1. In addition, we found that PG0026477 directly interacted with structural genes such as leucoanthocyanidin oxygenase (*ANS*), flavonoid 3',5'-hydroxylase (*F3'5'H*), dihydroflavonol 4-reductase (*DFR*), flavanone 3 beta-hydroxylase (*F3H*) and flavanol synthase (*FLS*). Unlike PG0026477,

PG0000561 did not interact with MYB and bHLH1-1, but with DFR, F3H, and KCS10 (3-ketoacyl-CoA synthase 10), which were upregulated in pigmented tissues. (Figure 9, Tables S13 and S14).

Figure 9. Protein interaction network predicted for StAN11 (TTG1) with interacting potato proteins (combined score of >400, medium confidence). The edge color was shown from green to red in accordance with the combined score. (A) Interacting potato proteins with PG0026477. (B) Interacting potato proteins with PG0000561. MYB (v-myb avian myeloblastosis viral oncogene homolog) transcript factors are highlighted in yellow and bHLH1-1 is highlighted in red. Proteins that interacted with PG0026477 and PG0000561 are highlighted in orange.

4. Discussion

4.1. Expansions of WD40 Gene Family in Potato

The series and segment repeat modes play a major role in expansion of the StWD40 family. In the present study, the proportion of tandem duplications was 3.37% of all *StWD40* genes (6 of 178), and segmental duplications accounted for 13.48% of the total (24 of 178). All duplication events of *StWD40s* were from subfamily A, with no duplication events in other subfamilies, which was different from wheat [30]. Thus, duplication was the major driving force of the evolution of *StWD40* subfamily A. This may be because *StWD40* genes have multiple mutations at the C-terminus and the N-terminus and cannot form other conserved domains. Subfamily A contains 131 *StWD40* genes with abundant functions. For example, *AtJGB* (AT2G26490) is a negative regulator of pollen germination [62] and *PG0010081*, *PG0006520*, and *PG1015451* were clustered with *AtJGB* in one cluster, which was specifically expressed in mature flowers and stamens. *ATG18A* (AT3G62770) is involved in autophagy [63] and *PG0024704* was clustered with *ATG18A* in one cluster. *AtBUB31* (AT3G19590) is involved in cell division [64] and *PG0025750* was orthologous to *AtBUB31*. *PG2021193* was clustered with *AtYAO* (AT4G21130) in one cluster, which plays a role in embryo sac development [65]. *AtTTG1* (AT5G24520) is involved in anthocyanin synthesis [61]. Based on the PGSC dataset, *PG0026477*, *PG0000561*, and *PG0044039* were annotated as *TTG1* (*StAN11*) in potato, but all members of this subfamily only contained the WD40 domain. In summary, gene duplication provided a large genome with numerous functional genes for adaptation to various environments in potato.

4.2. Phylogenetic Analysis and Evolution of StWD40s

Numerous phylogenetic analyses of WD40 proteins were conducted in *Arabidopsis* [31]. In order to obtain an overview of the 178 *StWD40* proteins and their relationships with *Arabidopsis*, a phylogenetic tree combining potato and *Arabidopsis* WD40 proteins was constructed. The results showed that 410 WD40 members (178 *StWD40* proteins and 232 *AtWD40* proteins) were divided into 14 clades (Figure 5). Comparing the full-length cDNA sequences with the corresponding genomic DNA sequences, we

observed that 154 (86.52%) *StWD40* coding sequences were interrupted by introns and 24 (13.48%) *StWD40* sequences had no introns. Among them, *PG0010959* had 30 introns, which was the most introns among the *StWD40* genes. Furthermore, the *StWD40* genes in the same branch not only had high sequence similarity, but also had similar intron–exon structures (Figure 2B). We further analyzed the structure of 45 *StWD40* genes that were orthologous to *Arabidopsis*. Genes in orthologous pairs usually exhibited the same genetic structure (Figure S4). It is noteworthy that the exon length of the *StWD40* genes, which were orthologous to *Arabidopsis*, were similar to that of *Arabidopsis*, but differed in intron length. The results suggested that introns in *WD40* played a minor role in the evolution of the *Arabidopsis* and potato *WD40* gene family through some unknown mechanisms. This is consistent with previous studies in cucumber [31].

4.3. Expression Profile Analysis of *StWD40* Genes

Tissue expression profiling and analysis of published datasets revealed that many *StWD40* genes were expressed in various tissues of potato, and some of them were tissue-specific. The *JINGUBANG* gene is a novel negative regulator of pollen germination in *Arabidopsis*, and *JGB* forms a regulatory complex with *TCP4* to control pollen Jasmonic acid synthesis and ensure pollination in moist environments [61]. In potato, *PG0010081*, *PG0006520*, and *PG1015451* were specifically expressed in mature flowers and stamens, which were annotated as *JGB*. *PG0026477* and *PG0016973* were differentially expressed in tubers and stolons of potato. *PG0026477* was annotated as *AN11* in the PGSC database, homologous with *TTG1* in *Arabidopsis*, and *PG0016973* was annotated as a transducing family protein. *PG0002566* was annotated as Coatomer subunit beta'-2 in the PGSC database, and was specifically expressed in tuber and callus tissues. It is speculated that these may be involved in the formation of potato tubers but this has not yet been reported; their functions need to be further studied. *PG0044039* and *PG0000561* were a pair of tandem duplication genes, with *PG0044039* being specifically expressed in fruit and *PG0000561* being highly expressed in tubers. According to the results of previous studies, some evolving new members may lose their original functions or acquire new functions in gene duplication events [66], which may be a reason for differences in expression patterns between *PG0044039* and *PG0000561*.

We also analyzed the expression profiles of *StWD40* genes in different tuber tissues (skin and flesh) of tetraploid pigmented potato cultivars. Studies showed that *TTG1* is involved in regulating anthocyanin biosynthesis [60,61]. *PG0026477*, *PG0000561*, and *PG0044039* were annotated as *StAN11* genes, namely *TRANSPARENT TESTA GLABRA 1 (TTG1)*, with *PG0044039* and *PG0000561* being a pair of tandem duplication genes. *PG0026477* has already been cloned [67]. Previous studies found that *PG0026477* is involved in anthocyanin biosynthesis [67,68], but *PG0000561* and *PG0044039* have not been reported. *PG0026477* was only upregulated in purple and red flesh and less expressed in pigmented skin than in white skin. *PG0000561* was upregulated in pigmented flesh and also was more highly expressed in pigmented skin. Based on the protein interaction network, *PG0026477* interacted with *HLH1-1 (PG0012891)*, which was upregulated in pigmented tissues but not expressed in white skin and flesh. Moreover, all of the MYB transcription factors in the interaction network interacted with *PG0026477*, further suggesting that *PG0026477* synthesizes the MBW complex with *HLH1-1* and MYB transcription factors and that this complex is involved in the synthesis of anthocyanins. In addition, *PG0002566* was upregulated in pigmented skin ($\log_2\text{FC} > 7$) and HM flesh ($\log_2\text{FC} = 3.48$). *PG0000235* and *PG0004584* were upregulated in pigmented flesh, with $\log_2\text{FC} > 2$. These genes may be involved in the synthesis of anthocyanins, but they have not yet been reported in *Arabidopsis*. Therefore, their role in anthocyanin synthesis remains to be further studied.

Numerous *WD40* proteins have been characterized by genetic analysis and found in response to various abiotic stresses [40,69,70]. There is no uniform gene expression pattern for *StWD40* genes. A total of 178 *StWD40* genes were differentially expressed under salt, mannitol, and heat stress, indicating that they may be involved in crosstalk between different signal transduction pathways in response to abiotic stresses [27,28,32]. Some *StWD40* genes were induced under various stresses,

however, some just responded to one stress stimulus. In addition, some *StWD40* genes had different expression patterns, as some of these genes were highly expressed in one stress, but rarely expressed or did not change in response to other stresses.

In general, members within one cluster may have common evolutionary origins and conserved functions, therefore, the functions of certain members can be confirmed by the known functions of members in the same cluster [71]. *PG0015970* was downregulated under salt, mannitol, and heat treatments. *PG0015970* was clustered with three *AtRACK1* homologous genes in one cluster, namely *RACK1A* (*AT1G18080*), *RACK1B* (*AT1G48630*), and *RACK1C* (*AT3G18130*), respectively. Previous studies showed that *RACK1* can interact with many signal molecules and affect different signal transduction pathways [72–74]. Liu, et al. [72] found that overexpression of the *MaRACK1* gene in *Arabidopsis* decreased tolerance to drought and salt stresses and *MaRACK1* overexpression changed expression levels of genes in response to stress and stimuli. Guo et al. [73] found that the *RACK1* gene may integrate ethylene signaling to regulate plant growth and development in *Arabidopsis*. *PG1005658* was homologous to *AT1G18080*, which was downregulated under salt and mannitol treatments. The above results indicated that *PG0015970* and *PG1005658* may have similar functions to *RACK1* under abiotic stress. Autophagy is a vital means of recovering damaged cellular components or degrading various proteins when plants are starved or oxidatively stressed [10,75]. The autophagy-related gene (*ATG18*), a phosphoinositide-binding protein that acts through the WD40 domain, is required for vesicle formation in autophagy [63]. In the present study, we found that *PG0024704*, which was in the same cluster as *ATG18A*, *C*, *D*, and *E*, was significantly downregulated under heat stress, but expression was increased under mannitol stress and did not respond to salt stress. These results were inconsistent with those from research in apple (*Malus*) [63]. *PG0002566* and *PG2005959* were differentially expressed in three periods (early flowering stage, full-blooming stage, and flower-falling stage) under drought stress in a drought-sensitive cultivar (A) and a drought-tolerant cultivar (Q) (Figure 8), and *PG0002566* was also upregulated in pigmented skins. However, the function of *PG0002566* has not yet been reported. In summary, *StWD40* genes may play a role in response to abiotic stress in potato and possibly participate in the communication pathway during different signal transduction pathways.

4.4. The Interaction Network of *StAN11* Proteins

In *Arabidopsis*, *TTG1* interacts with *MYB* and *bHLH1* to form MBW complexes that are involved in anthocyanin synthesis [23]. Based on the PGSC database, *PG0026477* and *PG0000561* were annotated as *StAN11*, which was homologous to *Arabidopsis* *TTG1*. *PG0026477b* not only interacted with the *MYB* transcription factor and *bHLH1* (*PG0012891*), but also directly interacted with multiple genes that were involved in the flavonoid biosynthesis pathway, such as *ANS* (*PG0003090*, *PG0003091*, *PG0003880*, *PG0027333*, *PG0027396*, and *PG0033567*), *F3'5'H* (*PG0000425*), *DFR* (*PG0003605*), *F3H* (*PG0003563*), and *FLS* (*PG1024988*) [76–78]. *PG0026477*, annotated as *StAN11*, plays the same role as the *TTG1* gene in anthocyanin synthesis, which was reported to form a MBW complex with the *MYB* transcription factor and *bHLH1*. In addition, *PG0000516* was also annotated as *StAN11*, but did not interact with *MYB* and *bHLH1*. *PG0000561* was highly expressed in pigmented tissues and directly interacted with *DFR* and *F3H*. *KCS10* (*PG0010819*) interacted with *PG0000561* and was upregulated in pigmented skin. Studies showed that *KCS10* encodes a protein that is probably involved in the synthesis of long-chain lipids in the cuticle [79], but whether it is involved in the synthesis of anthocyanins has not been reported. Studies of *StAN11* and the proteins interacting with it could provide further understanding regarding the complex regulatory system in potato anthocyanin synthesis.

5. Conclusions

In this study, 178 *StWD40* genes were identified and analyzed at the genome-wide level. All these genes were distributed unevenly on 12 chromosomes. A total of 178 *StWD40*s were phylogenetically divided into 14 distinct clusters based on highly conserved gene structures and motifs. According to their domain compositions, the 178 *StWD40*s were grouped into 10 subfamilies. Collinearity analysis

showed that segmental duplication events played a crucial role in the expansion of the *StWD40* gene family. Synteny analysis indicated that 45 and 23 *StWD40* genes were orthologous to *Arabidopsis* and wheat, respectively. According to Ka/Ks ratios, these gene pairs evolved under strong purifying selection. Based on RNA-seq, we performed a comprehensive expression analysis of *StWD40* genes in different tissues, multiple abiotic stresses, colorful potato cultivars (XDP, HM, and LT), and different drought tolerance cultivars (Q and A) in order to identify associations of *StWD40* genes with spatial distribution, anthocyanin biosynthesis, and multiple abiotic stresses. Furthermore, we analyzed two StAN11 protein interaction networks that were highly expressed in anthocyanin biosynthesis. The results of this study increase our understanding of the characterization of the *StWD40* superfamily and provide valuable information regarding further functional elucidation of these genes in potato.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2073-4395/10/3/401/s1>. Table S1: Primers used for qPCR; Table S2: Characteristic features of 178 *StWD40* genes; Table S3: Specific conserved motifs identified by MEME among potato WD40 proteins; Table S4: Specific conserved motifs identified by MEME among WD40 proteins in potato, wheat, and *Arabidopsis*; Table S5: Domain composition and number of members of 10 subfamilies of 178 *StWD40*s; Table S6: Ka/Ks ratios of tandemly and segmentally duplicated *StWD40*s; Table S7: Ka/Ks ratios of the syntenic relationships for WD40 between potato and *Arabidopsis*; Table S8: Ka/Ks ratios of the syntenic relationships for WD40 between potato and wheat; Table S9: The FPKM value of *StWD40* genes in different tissues; Table S10: The FPKM value of *StWD40* genes in white and pigmented tissues; Table S11: The FPKM value of *StWD40* genes under salt, mannitol, and heat stresses; Table S12: RNA-seq data of *StWD40* genes under drought stress; Table S13: Protein interaction network of StAN11 (TTG1); Table S14: The FPKM value of genes in protein interaction networks; Figure S1: The melt curves of 19 *StWD40* genes and the internal standard gene; Figure S2: Distribution of conserved motifs of the WD40 proteins in potato, wheat, and *Arabidopsis*. Boxes of different colors represent 10 putative motifs. Figure S3: Expression profiles of *StWD40* genes in different tissues and under heat, salt, and mannitol, treatments based on transcriptome data. Heat map representation and hierarchical clustering of *StWD40* genes in (A) different tissues and (B) under abiotic stress. (A) Heat map representation and hierarchical clustering of *StWD40* genes across different tissues and developmental stages. (B) Heat map representation and hierarchical clustering of *StWD40* genes under abiotic stress (heat, salt, and mannitol) in doubled monoploid potato. CK represents the control. Heat treatment was 35 °C for 24 h, salt treatment was 150 mM NaCl for 24 h, mannitol treatment was 260 uM for 24 h. The color in the heat map represents the FPKM value using logarithm with a base of 2. Figure S4: Comparison of 45 pairs of orthologous gene structures between potato and *Arabidopsis*. The blue box indicates the exon and the black line of the same length indicates the intron. The upstream/downstream area is indicated by a red box. The numbers 0, 1, and 2 represent the splicing phase of the intron.

Author Contributions: Conceptualization, Yuhui Liu and J.Z.; data curation, Z.L., B.S., Yuanming Li, C.L., and Z.C.; formal analysis, Z.L.; funding acquisition, Yuhui Liu and J.Z.; software, Z.L.; Writing—original draft, Z.L.; Writing—review and editing, Yuhui Liu and J.A.C. All authors have read and agreed to the published version of the manuscript.

Funding: The Research Program Sponsored by the Gansu Science Foundation for Distinguished Young Scholars (17JR5RA138), the National Natural Science Foundation of China (31601356 and 31860398), China Agriculture Potato Research System (CARS-09-P14), Special Funds for Discipline Construction of Gansu Agricultural University (GSAU-XKJS-2018-218), Gansu Provincial Key Laboratory of Aridland Crop Science, Gansu Agricultural University (No. GSCS-2017-2), the Program for Longyuan Innovation Teams of Gansu Province and Fuxi Talent Project of Gansu Agricultural University (Gaufx-02Y04).

Acknowledgments: We wish to thank Yuanyuan Pu, Lijuan Niu and Wenhao Li of Gansu Agricultural University for their help in the improvement of the manuscript. We also thank Shujuan Jiao, Yunchun Wei and Tiqian Han of Gansu Agricultural University for their help in the experiments.

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

Abbreviations

DM	doubled monoploid potato
HMM	hidden Markov model
Ka	non-synonymous substitution rate
Ks	synonymous substitution rate
PG	PGSC0003DMG40
qPCR	quantitative real-time PCR
RNA-seq	RNA sequencing

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