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ORIGINAL ARTICLE

Genome-wide identification, sequence characterization, and protein–protein interaction properties of DDB1 (damaged DNA binding protein-1)-binding WD40-repeat family members in *Solanum lycopersicum*

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Abstract

Main conclusions **One hundred DDB1 (damaged DNA binding protein-1)-binding WD40-repeat domain (DWD) family genes were identified in the *S. lycopersicum* genome. The *DWD* genes encode proteins presumably functioning as the substrate recognition subunits of the cullin4-ring ubiquitin E3 ligase complex. These findings provide candidate genes and a research platform for further gene functionality and molecular breeding study.**

A subclass of DDB1 (damaged DNA binding protein-1)-binding WD40-repeat domain (DWD) family proteins has been demonstrated to function as the substrate recognition subunits of the cullin4-ring ubiquitin E3 ligase complex. However, little information is available about the cognate subfamily genes in tomato (*S. lycopersicum*). In this study, based on the recently released tomato genome sequences, 100 tomato genes encoding DWD proteins that potentially interact with DDB1 were identified and characterized, including analyses of the detailed annotations, chromosome locations and compositions of conserved amino acid domains. In addition, a phylogenetic tree, which comprises of three main groups, of the subfamily genes was constructed. The physical interaction between tomato DDB1 and 14 representative DWD proteins was determined by yeast two-hybrid and co-immunoprecipitation assays. The subcellular localization of these 14 representative DWD proteins was determined. Six of them were localized in both nucleus and cytoplasm, seven proteins exclusively in cytoplasm, and one protein either in nucleus and cytoplasm, or exclusively in cytoplasm. Comparative genomic analysis demonstrated that the expansion of these subfamily members in tomato predominantly resulted from two whole-genome triplication events in the evolution history.

Keywords WD40-repeat domain · Damaged DNA binding protein-1 · Phylogenetic analysis · Protein interaction · *Solanum lycopersicum*

Abbreviations

CDS	Coding sequence
CRL	Cullin-ring ubiquitin ligase
DDB1/2	Damaged DNA binding protein 1/2
DWD	Damaged DNA binding protein 1 (DDB1)-binding WD40-repeat domain family protein
HGNC	Gene nomenclature committee of human genome organization
HMM	Hidden markov model
PGDD	Plant genome duplication database
RBX1/2	Ring box 1/2
ROC1/2	Regulator of cullins 1/2
RPKM	Reads per kilobase of exon model per million mapped reads

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SGN	Sol genomics network
SKP1	S-phase kinase-associated protein 1
SlWDR	WD40-repeat domain family members in tomato
WD40	WD40-repeat domain

Introduction

The ubiquitin/26S proteasome system regulates a broad range of cellular processes in eukaryotic cells through protein ubiquitination and proteolytic degradation (Vierstra 2003, 2009). Three key enzymes involve in this system, including E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), and E3 (ubiquitin ligase). Unlike E1 and E2, there are large number of E3s, which are responsible for recruiting individual protein substrates and targeting them to 26S proteasome for degradation. The cullin-ring ubiquitin ligase (CRL) is the most abundant family of multi-subunit E3 ligases, with cullin proteins serving as scaffold for assembling. Two essential modules are assembled on cullins: a ring finger protein ROC1/2 (also called RBX1/2), which recruits E2, and the substrate recognition complex (Jackson and Xiong 2009; Zimmerman et al. 2010).

Six cullin genes have been identified in the human genome, *CUL1*, *CUL2*, *CUL3*, *CUL4A*, *CUL4B* and *CUL5*. For each cullin protein, eukaryotic cells have evolved distinct substrate recognition subunits for selective degradation (Zimmerman et al. 2010). For example, CUL1 utilizes the adaptor protein, S-phase kinase-associated protein 1 (SKP1), to bind to various F-box proteins, which specify substrates for ubiquitination; CUL2 and CUL5 bind to VHL-box or SOCS-box proteins through a heterodimeric linker complex containing elongins B and C, respectively. However, CUL3 relies on its N-terminal domain to bind to BTB domain proteins, which recognize different substrates (Zimmerman et al. 2010).

For the CUL4-based ROC1/RBX1-CUL4 E3 ligase complex (CRL4), the UV-damaged DNA binding protein-1 (DDB1) functions as the substrate adaptor (Jackson and Xiong 2009; Biedermann and Hellmann 2011). As the substrate recognition subunit, the WD40-repeat domain (WD40) proteins have been identified as the most abundant protein and associated with ROC1/RBX1–CUL4–DDB1 E3 complex via specifically binding to DDB1 (He et al. 2006; Fukumoto et al. 2008; Biedermann and Hellmann 2011). The WD40 protein contains a conserved WD40-repeat domain, usually including 6–8 WD40-repeats. A WD40-repeat is comprised of approximately 40–60 amino acids, including highly conserved dipeptide GH (Gly-His) and WD (Trp-Asp) at the N-terminus and C-terminus, respectively (Wu et al. 2012; Wang et al. 2013). Among the DDB1-binding WD40-repeat (DWD) proteins, a conserved motif, DWD (also known as WDXR) motif, exists within the WD40 repeat (Fukumoto et al. 2008; Jackson and Xiong 2009; Biedermann and Hellmann 2011). The DWD motif is required for the physical interaction between DWD proteins and DDB1 (Jackson and Xiong 2009). The DWD motif consists of 16 amino acids, of which four are highly conserved, including Asp (or Glu) at position 7, Trp (or Tyr) at position 13, Asp (or Glu) at position 14, and Arg (or Lys) at position 16 (Biedermann and Hellmann 2011). Among these residues, Arg was shown as the most important site for DWD–DDB1 linkage, evidenced by residue substitution experiments (Angers et al. 2006; Jin et al. 2006; Lee et al. 2008).

85 and 78 putative DWD proteins have been identified in Arabidopsis and rice, respectively (Lee et al. 2008). Several Arabidopsis DWD proteins have been characterized as the substrate receptors of CRL4 and demonstrated to be involved in stress response. AtDWA1, AtDWA2, AtDWA3 and Drs1 have been shown to participate in regulation of ABA signaling in response to environmental stress (Lee et al. 2010a, b, 2011). Three Arabidopsis DWD members, AtDWA1, AtDWA2 and AtDWA3 have been characterized to interact with DDB1A/B in vitro and in vivo. Among them, AtDWA1 and AtDWA2 were shown to directly interact with each other and form heterodimer (Lee et al. 2010b, 2011). An elevated protein level of ABA-responsive transcription factor gene *ABI5* was detected upon ABA treatment in the loss-of-function mutants of *AtDWA1*, *AtDWA2 and AtDWA3*. Moreover, *Drs1* (*drought sensitive 1*) gene was shown to regulate drought stress in an ABA-dependent manner (Lee et al. 2010a) and a recent study demonstrated ABD1 was an Arabidopsis WD40-repeat substrate receptor for CUL4-based E3 ligases that acts as a negative regulator of ABA signaling (Seo et al. 2014). Furthermore, Arabidopsis ATCSA-1, DDB2 and DHU1 are involved in the UV-response. Together with DDB2, ATCSA-1 is necessary for light-independent DNA repair processes after UV irradiation (Biedermann and Hellmann 2010). In contrast, DHU1 acts as a negative regulator as the *dhu* mutant exhibits enhanced UV-B tolerance (Kim et al. 2014).

In addition, DWD proteins, including MSI1, MSI4, PRL1, TRIP-1 and VIP3, have been characterized by their distinct physiological roles in regulating growth and development of Arabidopsis (Jiang and Clouse 2001; Zhang et al. 2003; Lee et al. 2008; Dumbliauskas et al. 2011; Pazhouhandeh et al. 2011). MSI1 appears to be involved in regulating *MEDEA* parental imprinting during seed development through its association with CUL4–DDB1 (Dumbliauskas et al. 2011). MSI4 represses *FLOWERING LOCUS C (FLC)* expression via association with both CUL4–DDB1 and CLF–Polycomb Repressive Complex 2

(PRC2) to regulate the flower timing (Pazhouhandeh et al. 2011), whereas VIP3 controls the flower timing by promoting *FLC* expression (Zhang et al. 2003).

Distinct functions have been assigned to *S. lycopersicum* DDB1, including regulation of chloroplast division and secondary metabolism (Lieberman et al. 2004; Liu et al. 2004; Wang et al. 2008; Azari et al. 2010; Rohrmann et al. 2011), epigenetic regulation and cell proliferation (Liu et al. 2012a; Tang et al. 2012), as well as basal defense against biotic stress (Liu et al. 2012b). Recently, we characterized a tomato DWD protein (DDI1) as a substrate receptor that is involved in response to multiple abiotic stresses, including UV radiation, high salinity and osmotic stress (Miao et al. 2014). However, little is known for majority of tomato DWD gene family members. In this study, based on the released tomato genome sequences (The Tomato Genome Consortium 2012), we carried out a genome-wide identification of DWD family members in *S. lycopersicum*. Detailed analyses, including chromosome distribution, conserved domains composition, gene phylogeny and duplication, expression pattern, subcellular localization and interaction with DDB1, were performed. Our results can provide information for further genetic manipulation of these *DWD* genes for improvement of agronomic traits and/or stress tolerance in tomato and probably other Solanaceae plants.

Materials and methods

Data sets

The annotated genome sequences of *S. lycopersicum* were obtained from SGN (ITAG Release 2.3, http://solgenomics.net). The collinear genomic blocks in the tomato genome and between tomato and grapevine genome were obtained from PGDD (http://chibba.agtec.uga.edu/duplication/; Lee et al. 2013).

Plant materials

The *Nicotiana benthamiana* plants were grown in an artificial climate incubator, under standard conditions (26 °C day, 22 °C dark; 16 h day, 8 h dark). The 4-week-old *N. benthamiana* plants were chosen for *Agrobacterium tumefaciens* GV2260-mediated transient infiltration. The harvested tobacco leaves were immediately frozen in liquid nitrogen and stored at −80 °C.

Identification of tomato *DWD* genes in *S. lycopersicum*

HMM profile of WD40-repeat domain (PF00400) downloaded from Pfam database (http://pfam.sanger.ac.uk/; Finn et al. 2014) was exploited for the identification of *WD40* genes in the *S. lycopersicum* genome using HMMER 3.0 (Finn et al. 2011). The default parameters were employed. Subsequently, the candidate WD40s' sequences were determined by analyzing the presence of WD40-repeat domain using the InterProScan (http://www.ebi.ac.uk/Tools/pfa/iprscan/; Jones et al. 2014) and SMART database (http://smart.embl-heidelberg.de/; Letunic et al. 2012). Among the verified WD40 members, a conserved 16 amino acid sequence was used to manually identify the DWD motifs in WD40-repeats. This 16 amino acid sequence is: [IFVL]-[IFVL]-[AGST]-[AGST]-[AGST]-x-[DE]-x(2)-[IFVL]-x-[IFVL]-[WY]-[DE]-[IFVL]-[RK]. Finally, the tomato WD40s containing the DWD motifs were designated as DWD (DDB1-binding WD40-repeat) members in *S. lycopersicum*.

Chromosome location and duplication events of tomato *DWD* genes

The locations of tomato *DWD* genes were assigned on 12 chromosomes according to the GFF3 files in ITAG Release 2.3 from SGN (http://solgenomics.net). The syntenic genomic blocks including the *DWD* genes in tomato and between tomato and grape genomes were described using Circos (Krzywinski et al. 2009). The tandem duplicated tomato *DWD* pairs were defined according to Hanada et al. (2008).

Identification of conserved domains in tomato DWD proteins

The domains search program from Pfam (http://pfam.sanger.ac.uk/; Finn et al. 2014) was used for identification of conserved domains in the tomato DWDs' protein sequences. The threshold used was E value $\leq 1E-5$ by searching the PfamA database.

Phylogenetic analyses of tomato *DWD* genes

The DWD motif sequences in WD40-repeat domains from tomato and Arabidopsis were retrieved. For the tree construction, the sequence alignments were performed using Clustal X 2.1 with default settings (Larkin et al. 2007). The unrooted phylogenetic tree was constructed based on the alignments, using MEGA 6.0 with the neighbor-joining (NJ) method (Tamura et al. 2013). The parameters used in the tree construction were Dayhoff model plus gamma-distributed rates and 1,000 bootstraps. The trees were visualized and optimized in ITOL (Letunic and Bork 2007).

Yeast two-hybrid assay

The yeast two-hybrid interaction assay was performed as described previously (Serino et al. 1999). The activation

domain fusion constructs were co-transformed into yeast strain EGY48 containing reporter plasmid pSH18–34. The full-length cDNA sequences of tomato *DDB1* (Genbank Accession: NM_001247346) and fourteen tomato *DWD* genes (*SlWDR13*, *SlWDR25*, *SlWDR28*, *SlWDR31*, *SlWDR43*, *SlWDR45*, *SlWDR141*, *SlWDR163*, *SlWDR167*, *SlWDR171*, *SlWDR186*, *SlWDR221*, *SlWDR237* and *SlWDR247*) were subcloned into pEG202 and pJG4–5, respectively.

Yeast transformants were verified by growth on Glu/CM, -Ura, -His, -Trp dropout plates. Interactions between tomato DDB1 and DWD proteins were monitored by blue coloration of yeast colonies grown on medium containing X-gal and confirmed by their growth on medium without leucine.

Co-immunoprecipitation assay

Fourteen selected tomato *DWD* genes were subcloned into pBTEX and transiently expressed in 4-week-old *N. benthamiana* leaves mediated by *A. tumefaciens* GV2260. Two days after agrobacterial infiltration, the infected leaf tissues ($\approx$3 cm^2) were ground to fine powder with liquid nitrogen and homogenized in 1.0 mL protein extraction buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 10 % glycerol, 1 % PVPP, 1 mM phenylmethylsulfonyl fluoride, and complete cocktail of protease inhibitors). Subsequently, the lysate was centrifuged at 12,000 rpm/4 °C for 10 min. The supernatant was incubated with 10 µL anti-HA affinity matrix (Sigma, USA) at 4 °C for 2 h. The affinity matrix was then washed five times with the wash buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 10 % glycerol, 1 mM phenylmethylsulfonyl fluoride) and resuspended with 2× loading buffer (Tris–HCl pH 6.8, 100 mM bromophenol blue 0.2 %, SDS 4 %, glycerol 20 %, β-mercaptoethanol 200 mM). The immunoprecipitated protein complex was separated by SDS-PAGE electrophoresis, followed by Western blotting using anti-HA or anti-FLAG antibody (Roche, USA).

Determination of subcellular localization of tomato DWD proteins

Fourteen selected full-length cDNAs of tomato *DWD* genes were PCR-amplified and sequenced. These cDNA sequences were cloned into the pART27 vector to express tomato DWD–GFP fusion protein driven by the CaMV 35S promoter. The resulting DWD–GFP constructs and free GFP control vector were transiently expressed in 4-week-old *N. Benthamiana* leaves via *A. tumefaciens* GV2260-mediated infiltration at the inoculum of $OD_{600} = 0.6$. Two days after agrobacterial infiltration, the infected leaf tissues were examined under the OLYMPUS FV1000-IX81 microscope.

Analysis of gene expression patterns of tomato *DWD* genes

Two sets of transcriptome sequencing data of tomato fruit were downloaded, including assembled cDNA sequences and corresponding expression values (Matas et al. 2011; Tang et al. 2013). The assembled cDNA sequences were aligned to CDS sequences of tomato *DWD* genes using local BlastN program (Altschul et al. 1990). For tomato *DWD* genes with more than one corresponding cDNAs, the median expression values were calculated. The expression data of log2 scale was hierarchically clustered using Euclidean distance with average linkage in MeV 4.8.1 (Saeed et al. 2003).

Results

Identification of tomato *DWD* genes in *S. lycopersicum*

A systematic analysis was performed to identify the WD40 family genes in the tomato genome, using WD40-repeat domain's HMM profile (PF00400). As a result, a total of 276 non-redundant putative WD40 members were identified. These candidates were further verified by searching for the presence of WD40 domain in the database of InterProScan and SMART. Eventually, 273 WD40 genes were identified in the tomato genome (Supplementary Table S1).

According to the nomenclature of gene families and groups in HGNC (http://www.genenames.org/genefamilies/a-z), the tomato WD40 genes were named "SlWDR". Consequently, the 273 members were numbered based on their physical positions on chromosomes, resembling the situations in cucumber and Chinese cabbage (Ling et al. 2011; Tang et al. 2014).

Next, we manually searched the DWD motif within the WD40-repeats in each SlWDR protein sequence according to the studies in Arabidopsis and rice (Lee et al. 2008). Consequently, 100 putative DWD proteins were identified in the tomato genome and 125 DWD motifs were identified in these 100 DWD proteins (Table 1). SlWDR14 with 2515 amino acids (aa) is the largest member in this family, whereas the smallest member is SlWDR238 containing 139 aa. The average length of tomato DWD proteins is 569 aa. The detailed information of individual tomato *DWD* genes is listed in Table 1, including the SGN accession number, encoded-protein length, chromosome location and DWD motif number.

Table 1 The detailed information of verified tomato DWD members list

Gene name	Accession in SGN	Protein length	Chromosome location	DWD motif number
SlWDR1	Solyc01g021640.2.1	496	1	1
SlWDR2	Solyc01g060050.1.1	608	1	1
SlWDR3	Solyc01g066740.2.1	1,321	1	1
SlWDR6	Solyc01g079510.2.1	1,667	1	1
SlWDR7	Solyc01g080690.2.1	469	1	2
SlWDR13	Solyc01g094480.2.1	482	1	2
SlWDR14	Solyc01g096110.2.1	2,515	1	1
SlWDR16	Solyc01g098090.2.1	709	1	1
SlWDR23	Solyc01g104510.2.1	424	1	1
SlWDR24	Solyc01g107160.2.1	340	1	1
SlWDR25	Solyc01g107360.2.1	806	1	1
SlWDR28	Solyc01g109560.2.1	377	1	1
SlWDR30	Solyc01g111030.1.1	470	1	2
SlWDR31	Solyc01g111590.2.1	403	1	1
SlWDR33	Solyc02g014460.2.1	474	2	1
SlWDR34	Solyc02g021360.2.1	798	2	1
SlWDR36	Solyc02g038750.2.1	354	2	1
SlWDR37	Solyc02g064800.2.1	774	2	1
SlWDR38	Solyc02g069770.2.1	516	2	1
SlWDR41	Solyc02g078800.2.1	1,029	2	1
SlWDR42	Solyc02g078830.2.1	337	2	1
SlWDR43	Solyc02g078970.2.1	570	2	1
SlWDR45	Solyc02g079110.2.1	442	2	1
SlWDR47	Solyc02g083940.2.1	332	2	1
SlWDR48	Solyc02g086470.2.1	514	2	2
SlWDR51	Solyc02g088780.2.1	423	2	2
SlWDR54	Solyc02g091790.2.1	314	2	1
SlWDR57	Solyc03g059100.1.1	326	3	3
SlWDR58	Solyc03g059310.2.1	234	3	1
SlWDR59	Solyc03g062940.2.1	316	3	2
SlWDR72	Solyc03g112530.2.1	481	3	1
SlWDR73	Solyc03g113850.1.1	474	3	1
SlWDR83	Solyc03g119090.2.1	609	3	1
SlWDR84	Solyc03g119650.2.1	1,215	3	2
SlWDR91	Solyc03g121580.2.1	460	3	1
SlWDR94	Solyc04g008720.2.1	810	4	1
SlWDR97	Solyc04g012170.2.1	1,516	4	1
SlWDR98	Solyc04g016510.2.1	488	4	2
SlWDR99	Solyc04g045290.1.1	143	4	1
SlWDR101	Solyc04g072890.2.1	439	4	1
SlWDR102	Solyc04g076020.2.1	761	4	2
SlWDR106	Solyc04g078320.2.1	592	4	1
SlWDR110	Solyc04g082300.2.1	350	4	2
SlWDR114	Solyc05g008190.2.1	527	5	1
SlWDR116	Solyc05g012300.1.1	219	5	2
SlWDR117	Solyc05g012320.1.1	266	5	2
SlWDR118	Solyc05g012720.2.1	278	5	2
SlWDR120	Solyc05g014090.1.1	397	5	1

Table 1 continued

Gene name	Accession in SGN	Protein length	Chromosome location	DWD motif number
SlWDR124	Solyc05g018780.1.1	379	5	1
SlWDR125	Solyc05g025510.2.1	580	5	1
SlWDR126	Solyc05g025630.1.1	463	5	1
SlWDR130	Solyc05g053130.2.1	728	5	1
SlWDR134	Solyc06g008880.2.1	474	6	2
SlWDR141	Solyc06g064830.2.1	514	6	1
SlWDR156	Solyc07g008860.2.1	397	7	2
SlWDR160	Solyc07g039200.2.1	462	7	1
SlWDR161	Solyc07g039330.2.1	377	7	1
SlWDR162	Solyc07g040790.2.1	435	7	1
SlWDR163	Solyc07g041080.2.1	323	7	1
SlWDR164	Solyc07g044850.2.1	580	7	1
SlWDR167	Solyc07g053660.2.1	452	7	2
SlWDR168	Solyc07g063120.2.1	830	7	1
SlWDR171	Solyc07g064090.2.1	372	7	1
SlWDR172	Solyc07g065950.2.1	1,407	7	1
SlWDR173	Solyc07g066060.2.1	313	7	1
SlWDR174	Solyc07g066130.1.1	645	7	1
SlWDR175	Solyc07g066140.1.1	470	7	1
SlWDR180	Solyc08g008160.2.1	415	8	1
SlWDR183	Solyc08g023590.2.1	386	8	1
SlWDR186	Solyc08g067040.2.1	764	8	2
SlWDR191	Solyc08g081990.2.1	1,052	8	1
SlWDR197	Solyc09g009710.2.1	905	9	1
SlWDR198	Solyc09g010620.1.1	163	9	2
SlWDR204	Solyc09g031610.2.1	587	9	1
SlWDR209	Solyc09g065290.2.1	315	9	2
SlWDR212	Solyc09g075000.2.1	385	9	1
SlWDR214	Solyc09g091020.2.1	776	9	1
SlWDR215	Solyc09g098330.2.1	440	9	1
SlWDR216	Solyc10g005730.2.1	446	10	1
SlWDR219	Solyc10g011690.2.1	1,019	10	2
SlWDR221	Solyc10g047000.1.1	350	10	1
SlWDR223	Solyc10g080180.1.1	499	10	1
SlWDR224	Solyc10g085580.1.1	701	10	1
SlWDR228	Solyc11g005190.1.1	360	11	2
SlWDR229	Solyc11g005550.1.1	299	11	1
SlWDR232	Solyc11g007640.1.1	175	11	1
SlWDR236	Solyc11g011980.1.1	672	11	1
SlWDR237	Solyc11g017070.1.1	326	11	1
SlWDR238	Solyc11g020290.1.1	139	11	1
SlWDR244	Solyc11g072540.1.1	473	11	2
SlWDR246	Solyc12g005950.1.1	677	12	2
SlWDR247	Solyc12g009030.1.1	323	12	1
SlWDR248	Solyc12g013840.1.1	862	12	1
SlWDR252	Solyc12g035360.1.1	333	12	1
SlWDR263	Solyc12g088290.1.1	820	12	1
SlWDR264	Solyc12g088340.1.1	453	12	1

Table 1 continued

Gene name	Accession in SGN	Protein length	Chromosome location	DWD motif number
SlWDR265	Solyc12g088540.1.1	499	12	1
SlWDR270	Solyc12g098690.1.1	429	12	1
SlWDR271	Solyc12g099010.1.1	1,468	12	1
SlWDR273	Solyc00g059100.2.1	325	Unanchored	1

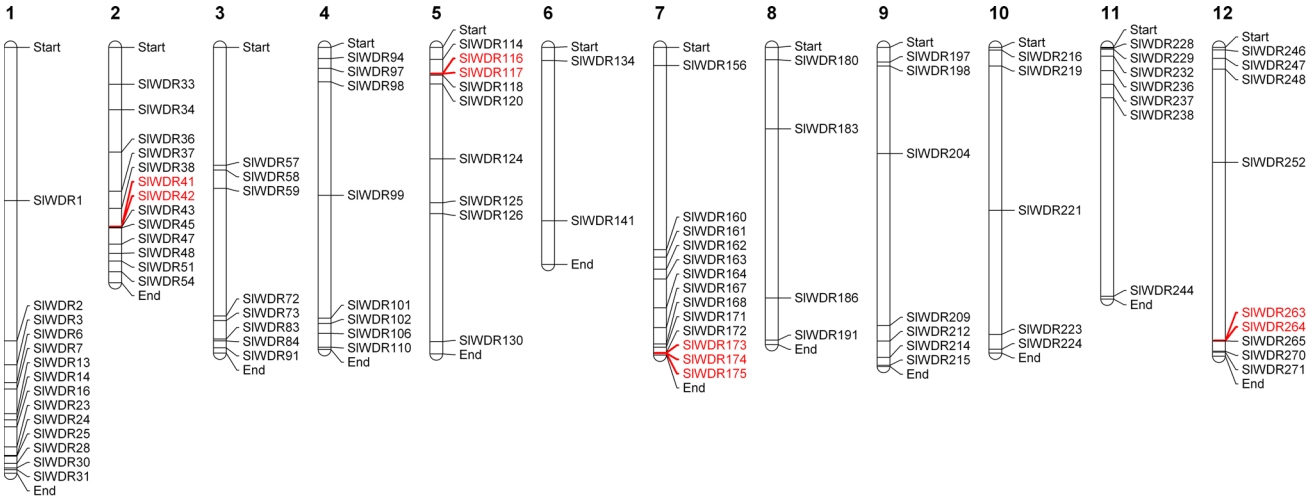


Fig. 1 Chromosome distribution of tomato *DWD* genes. Tandemly duplicated genes are indicated in *red*. The chromosome location is referred to the gff3 data from SGN (http://solgenomics.net). The 'start' and 'end' indicate the end of each chromosome, respectively

Chromosomal distribution of tomato *DWD* genes

The 100 tomato *DWD* genes are distributed across 12 tomato chromosomes, with an exception of *SlWDR273* (SGN accession: Solyc00g059100.2.1) located in the unanchored scaffolds, chromosome 00:14071311–14075040. Chromosomes 1, 2 and 7 possess 14, 13, and 13 *DWD* genes, respectively, accounting for 40 % tomato *DWD* members (Fig. 1). Interestingly, most of tomato *DWD* genes are located at the chromosome ends (Fig. 1).

Based on their physical locations, we further determined the tandem duplication of tomato *DWD* genes, which was defined as an array of two or more homologous genes within a distance less than 100 kb (Hanada et al. 2008). We identified, on chromosomes 2, 5, 7 and 12, four tomato *DWD* gene clusters with nine members as tandem duplications (genes labeled in red in Fig. 1).

The conserved domains in tomato DWD proteins

Based on the online Blast analysis in Pfam database, the conserved domain compositions and structures in tomato DWD protein sequences were elucidated (Supplementary Table S2). Among the 100 members, seventy-six have one, twenty-three have two and one has three DWD motifs (Table 1), consistent with previous reports that DWD proteins usually possess one and sometimes two, but rarely three DWD motifs (He et al. 2006; Lee et al. 2008).

In addition to the DWD motif, there are 35 distinct conserved domains present in 29 tomato DWD proteins, estimated by an E value $\leq 1E-5$. Detailed analyses demonstrated these 35 domains belonged to 19 different categories, of which CAF1C_H4-bd (6 locations) and Pkinase (5 locations) are the most common. CAF1C_H4-bd appears in CAF1 complex which is involved DNA replication and facilitates replication-dependent nucleosome assembly with histones (Murzina et al. 2008). Pkinase is a structurally conserved protein domain containing the catalytic function of protein kinases, which are involved in many cellular processes, including immune response (Hanks and Quinn 1991). Our findings support a hypothesis that DWD motif specifically interacts with DDB1, while a variety of additional modules might target and bind to different substrates (Lee et al. 2008).

The conserved domains' structures of the 100 tomato DWDs represent four distinct architectures: type I, only DWD motif exists (1 DWD motif in 53 DWD members, 2 DWD motifs in 17 DWD members and 3 motifs in one

DWD member); type II, 1–2 DWD motifs exist in the N-terminus with other domains in the C-terminus (in 5 DWD members); type III, 1–2 DWD motifs reside in the C-terminus with other domains in the N-terminus (in 19 DWD members); type IV, the DWD motifs are located between the conserved domains of the N-terminus and the C-terminus (in 4 DWD members). An exception is that all the conserved modules are located in the N-terminus in SlWDR23 (Supplementary Table S2).

Phylogenetic analyses of tomato *DWD* genes

To investigate the phylogenetic relationship among tomato *DWD* genes, a phylogenetic tree was constructed using amino acid sequences of 125 DWD motifs derived from 100 tomato DWD proteins. The DWD motif sequences used for tree construction are listed in Supplementary Table S3. In the phylogenetic tree, there are four main distinct clusters, Group Ia, Group Ib, Group II and Group III (Fig. 2). In the phylogenetic tree, even in the DWD motifs derived from the same protein sequence, the divergence of DWD motifs is evident (Fig. 2). For instance, *SlWDR57* (squares labeled in Fig. 2) contained three DWD motifs, which were clustered into distinct clades. In addition, an alternative phylogenetic tree was constructed using Arabidopsis (101) and tomato (125) DWD motifs' sequences (Supplementary Table S3). The interspersed distribution of the DWD motifs from different species implied the sequence conservation of DWD motifs between tomato and Arabidopsis during the evolutionary process (Supplementary Fig. 1). The two phylogenetic trees could provide a potential support for functional similarities and divergences between Arabidopsis and tomato DWDs as well.

Based on the tomato DWD phylogenetic tree, 14 representative tomato DWD members (circles labeled in Fig. 2) were selected to test protein–protein interaction with DDB1 and to determine their subcellular localization. The results are described below.

The interactions between tomato DWD proteins and DDB1

To investigate whether the identified tomato DWD proteins can act as the substrate receptors for the CUL4–DDB1 E3

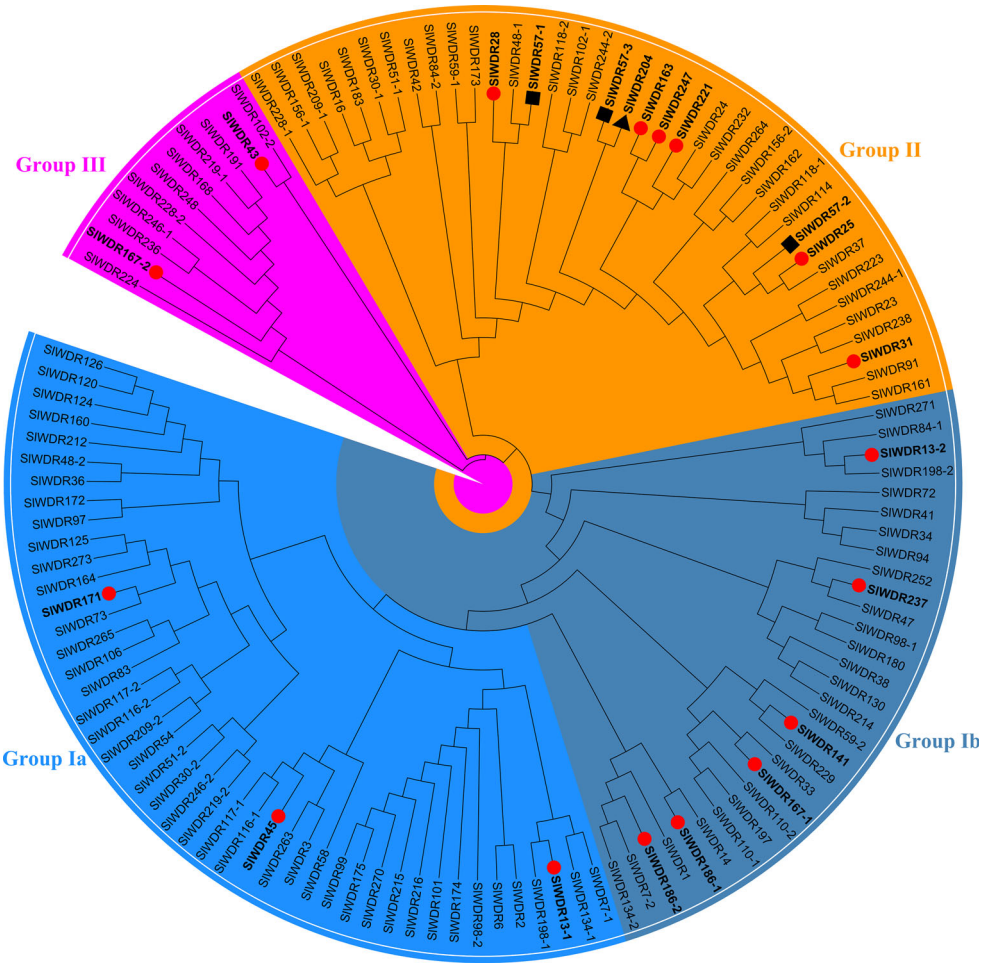


Fig. 2 Phylogenetic tree of tomato *DWD* genes. The 14 representative genes selected for further DDB1–DWD interaction test and subcellular localization are labeled with *circles*. The three divergent DWD motifs, which were derived from SlWDR57, are labeled with *squares*. The characterized tomato DWD protein, tomato DDI1 (SlWDR204, SGN accession: Solyc09g031610.2.1), is labeled with *triangles*

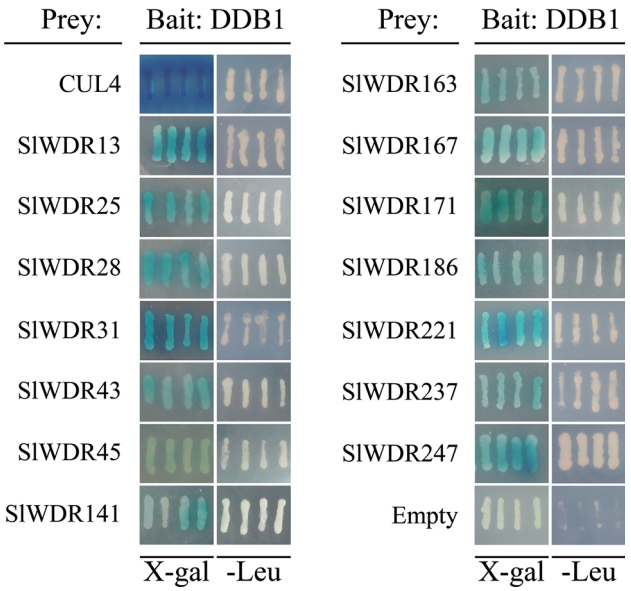


Fig. 3 Interactions between tomato DWD proteins and DDB1 verified by yeast two-hybrid assay. Yeast strains harboring the distinct bait and prey constructs are indicated. The interactions between tomato DWD proteins and DDB1 were monitored by *coloration* of colonies grown on the medium with X-gal and growth of colonies on the medium lacking leucine. The DDB1-binding protein CUL4 served as a positive control, whereas the empty vector was included as a negative control

ligase complex, we selected 14 tomato representative DWDs to test whether they could bind to DDB1 using yeast two-hybrid and co-immunoprecipitation assays. The tested DWDs include SlWDR13, SlWDR25, SlWDR28, SlWDR31, SlWDR43, SlWDR45, SlWDR141, SlWDR163, SlWDR167, SlWDR171, SlWDR186, SlWDR221, SlWDR237, and SlWDR247. The primes for vector construction are listed in Supplementary Table S4.

As shown in Fig. 3, blue colorization of yeast colonies grown on the medium with X-gal indicates the interaction between tomato DDB1 and DWDs. Their interactions were further verified by growth of these yeast colonies on Leu-deficient medium (Fig. 3). Thus, our data suggest that the selected tomato DWD proteins are able to bind to DDB1.

To further test the interactions between tomato DWD proteins and DDB1 *in planta*, co-immunoprecipitation assay was performed. The epitope-tagged DWD-Flag and DDB1-HA were transiently co-expressed in the leaves of *N. benthamiana*. As shown in Fig. 4, tomato DWD proteins could be efficiently retrieved from the DDB1 immuno-complex, whereas no interaction was observed in the negative control. These results suggest that tomato DWD proteins identified by our bioinformatics analyses could directly bind to DDB1 and probably bring together CUL4–DDB1 complex and its substrates for degradation.

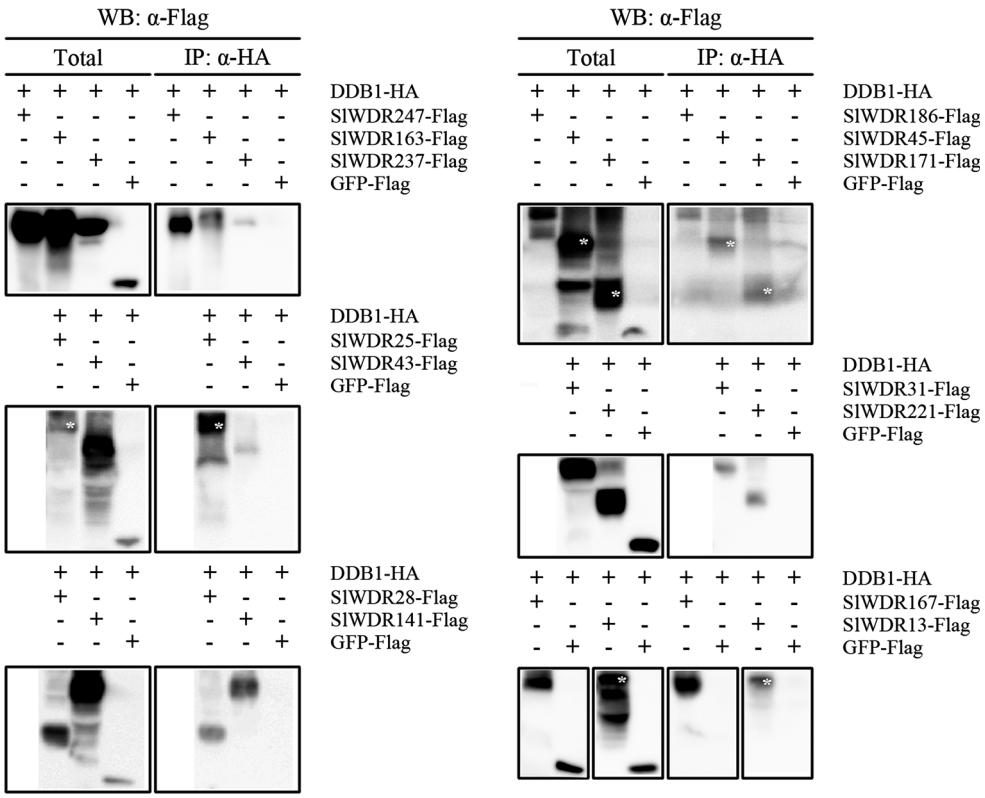


Fig. 4 In vivo interactions between tomato DWD proteins and DDB1 detected by the co-immunoprecipitation assay. The crude protein extracts were isolated from transiently infiltrated *N. benthamiana* leaves expressing the distinct combinations of DDB1-HA and DWDs-FLAG, or DDB1-HA and GFP-FLAG. The total protein extracts (total) and the immunoprecipitates (IP) were subjected to immunoblot analysis with antibodies against FLAG. The asterisk indicates the positions of DWD-FLAG

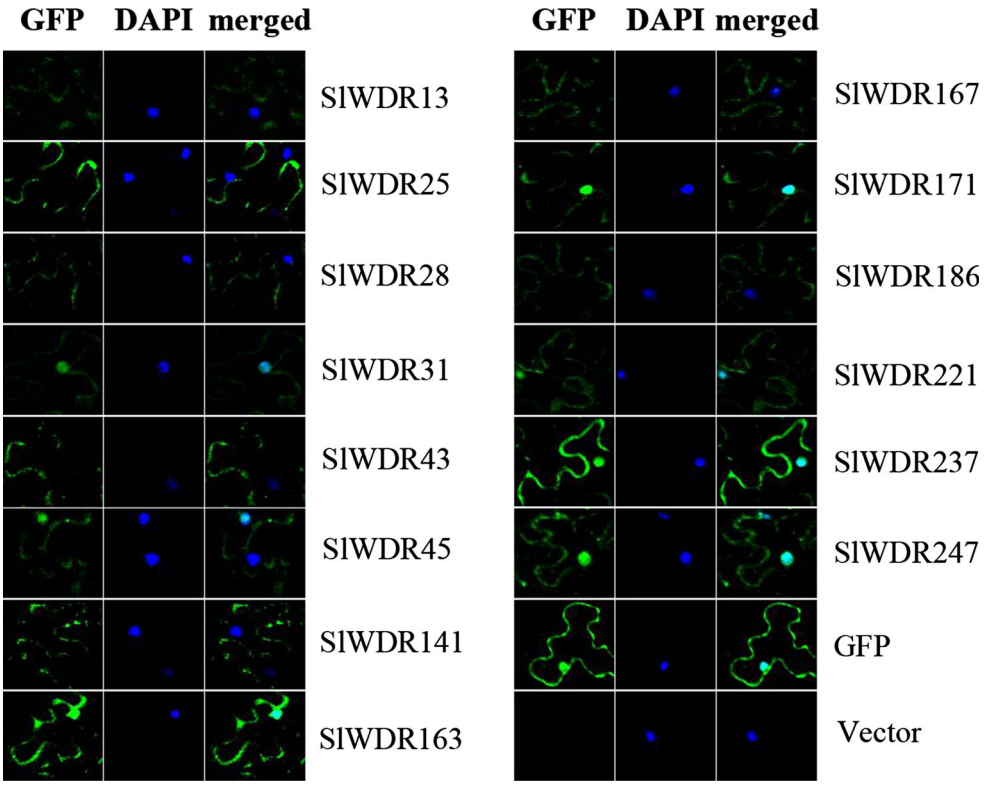


Fig. 5 Subcellular localization of tomato DWD proteins. *Agrobacteria* harboring appropriate GFP-tagged DWD constructs were infiltrated into *N. benthamiana* leaves. Two days after agro-infiltration, the infected leaf tissues were subjected to confocal laser scanning microscopy to examine the fluorescence signal (*left panel*). DAPI (4, 6-diamidino-2-phenylindole) staining indicates the localization of nucleus. Free GFP and empty vector were included positive control and negative control, respectively

Subcellular localization of tomato DWD proteins

To determine the subcellular localization of the 14 selected DWD proteins, 35S::DWD–GFP constructs were generated using primers listed in Supplementary Table S4. The fused DWD–GFP constructs were transiently expressed in *N. benthamiana* via *A. tumefaciens* GV2260-mediated infiltration. Two days after agro-infiltration, the epidermal cells were collected from infected leaf tissues for examination of the green fluorescence, as well as DAPI staining of the nucleus. In comparison with the universally distributed fluorescence signal of free GFP, the fluorescence signal of six fusion proteins (SlWDR31-GFP, SlWDR163-GFP, SlWDR171-GFP, SlWDR221-GFP, SlWDR237-GFP, SlWDR247-GFP) was detected in both nucleus and cytoplasm, while in seven fusion proteins (SlWDR13-GFP, SlWDR25-GFP, SlWDR28-GFP, SlWDR43-GFP, SlWDR141-GFP, SlWDR167-GFP, SlWDR186-GFP) the florescence signal was visualized exclusively in cytoplasm (Fig. 5). Interestingly, the fluorescence signal derived from SlWDR45-GFP could be observed either in nucleus and cytoplasm, or exclusively in cytoplasm (Fig. 5). The DWD localization results were consistent with those of tomato CUL4 and DDB1, whose YFP-derived proteins were visualized in both nucleus and cytoplasm (Wang et al. 2008).

Discussion

In this study, we have identified one hundred *DWD* genes in the *S. lycopersicum* genome. The structures of their encoded proteins, phylogenetic relationships, physical interactions with tomato DDB1 and subcellular localizations were determined. In addition to the DWD motif, a variety of additional conserved domains were found in tomato DWD proteins. Compared to those in Arabidopsis (85) and rice (78), the tomato DWD family members (100) underwent distinct expansion, possibly resulting from whole-genome triplications as well as small-scale tandem duplications.

Comparative genomic analysis has demonstrated that the *S. lycopersicum* genome possessed two whole-genome triplication events, including an ancient triplication shared by core eudicots, and a recent event affecting the Solanaceae lineage, which caused large expansion and evolution of speciation-related gene families (The Tomato Genome Consortium 2012). We postulated the two whole-genome triplications might be involved in expansion of tomato *DWD* members. To test this hypothesis, we examined the relationship of orthologous *DWD* genes in the syntenic regions between grape and tomato genomes in PGDD database (Lee et al. 2013). As a result, 49 collinear regions were found, including 46 tomato *DWD* genes and their putative grape orthologs (Fig. 6; Supplementary Table S5).

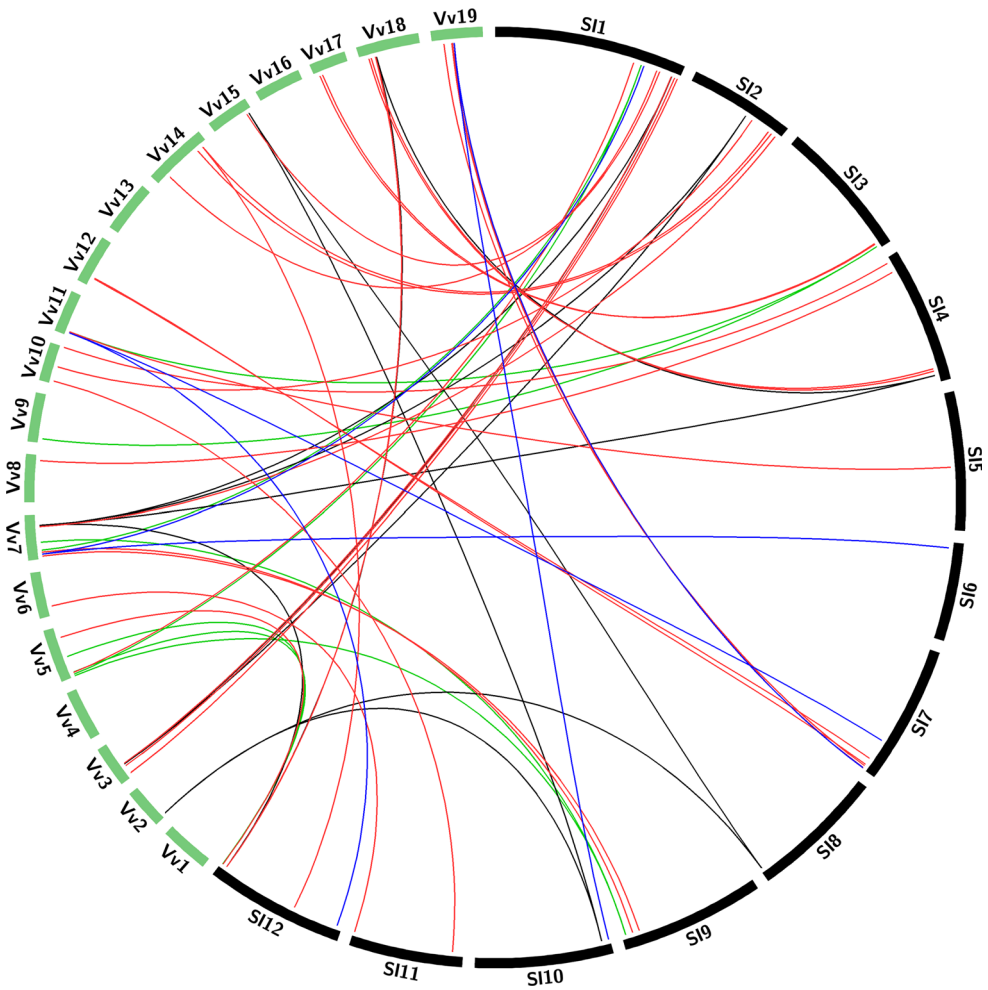
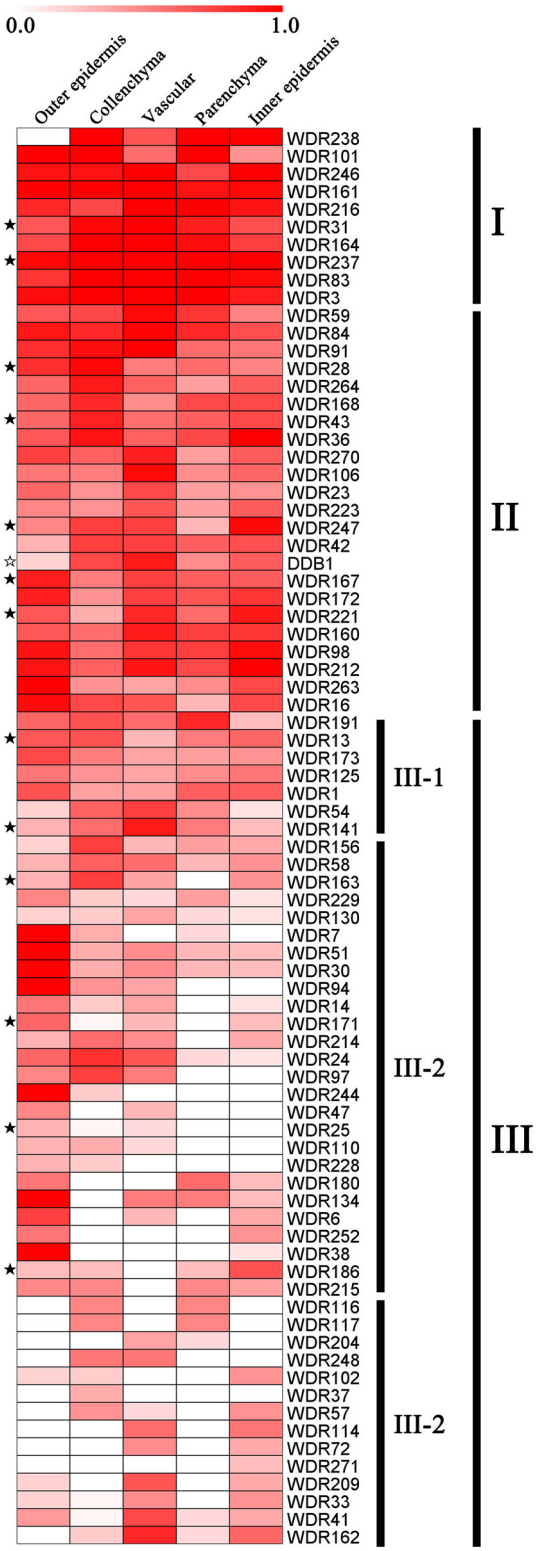


Fig. 6 Duplicated tomato *DWD* genes in synteny genomic blocks. The lines link tomato *DWD* genes and their putative grape homologs, which are located in the synteny blocks between tomato and grape genome. The *red*, *green* and *blue lines* represent the one–one, one–two and two–one homologous relationships between tomato *DWD* genes and their putative grapevine homologs. The *black lines* represent more complicated orthologous relationships

Of the 46 tomato *DWD* genes, 30 possess one–one orthologous relationship with grape orthologs. In addition, four and six tomato *DWD* members have one–two and two–one orthologous relationships with grape orthologs, which presumably resulted from the ancient and recent triplications, respectively. The rest of six tomato *DWD* genes seemingly have a more complicated relationship with grape orthologs. In the collinear blocks of the tomato genome, we additionally analyzed the collinear regions containing *DWD* genes. Eight syntenic regions were found, with eight *DWD* gene pairs having one–one paralogous relationship (Supplementary Fig. 2 and Table S5). These data demonstrated that both the ancient and recent whole-genome triplication events contributed to *DWD* genes' expansion in tomato. In addition, it might be noteworthy that there is a small portion (nine genes in five clusters, 9 %) of expanded *DWD* members ascribed to tandem duplication events in tomato (Fig. 1). Combing these results, the driving force of expansion of tomato *DWD* members was mainly attributed to the two whole-genome triplications, rather than tandem duplication.

Although the 100 tomato DWD members were determined by presence of DWD motif sequence, their genuine existence and ability to bind to DDB1 were evidenced by yeast two-hybrid assay and co-immunoprecipitation analysis (Figs. 3, 4). The phylogeny-dependent selection of tomato DWD members for these experimental assays could fully represent the whole gene family with interspersed distribution in Group I, II and III (Fig. 2), and positive results have been achieved for all 14 representative DWD members without exception.

In addition to the DWD motif, we found a number of conserved domains present in tomato DWD proteins, comprising of four main composition patterns (Supplementary Table S2), which are probably required for recognition specificity of substrates targeted by the RBX1–CUL4–DDB1 E3 ligases. Based on the full-length sequences of encoded proteins, we constructed a phylogenetic tree reflecting the orthologous relationship between tomato and Arabidopsis *DWD* genes, revealing that similar domain composition patterns were present in tomato DWDs and their Arabidopsis counterparts (Supplementary



◀**Fig. 7** Expression level of tomato *DWD* genes in five tomato fruit tissues. The expression pattern of 79 tomato *DWD* genes in different tissues of ten DAP tomato fruits includes out epidermis, collenchyma, vascular, parenchyma and inner epidermis. The tomato DWD members, which physically interact with tomato DDB1 in the protein interaction assays, are indicated with *filled stars*. The *DDB1* gene is labeled with *open stars*. The tomato *DWD* genes are classified into three groups, I, II, and III, based on the clustering of expression value

Fig. 3). The characterized Arabidopsis *DWD* genes should provide valuable clues for further functional elucidation of tomato *DWD* homologs (Supplementary Table S6).

Intriguingly, although the Arabidopsis *DDB2* gene (At5g58760) and its tomato ortholog *DDI1* (*SlWDR204*, SGN accession: Solyc09g031610.2.1) (triangles labeled in Fig. 2) encode proteins containing a single DWD motif without any additional conserved domain, both of them play significant physiological roles in response to UV radiation, are localized in the nucleus, and interact with their corresponding CUL4 and DDB1 orthologs (Biedermann and Hellmann 2010; Miao et al. 2014).

Since DWD proteins have been defined as substrate receptor that is able to physically interact with DDB1 and form CRL4 E3 ligase complexes, it should be interesting to compare the expression patterns of *DDB1* and *DWD* genes. To this end, the spatial gene expression of tomato *DDB1* and 79 *DWD* genes representing five tomato fruit tissues was determined (Matas et al. 2011) (Fig. 7; Supplementary Table S7). Depending on their expression patterns, 79 *DWD* genes were classified into three groups, 22 genes in Class II showed a tendency of co-expression with *DDB1* (Fig. 7; Supplementary Fig. 4). Our another independent study showed that up to 86 tomato *DWD* genes were expressed at early fruit developmental stage in both wild-type and the *DDB1*-defective *hp1* mutant (Tang et al. 2013) (Supplementary Table S8). Significantly, the decreased transcription level of the defective *DDB1* was detected in the *hp1* mutant fruit, accompanied by reduced expression level of 26 *DWD* genes (26/87, 30 %), of which 17 had 1.2–1.5 fold and 9 with more than 1.5 fold change at the transcript level. Out of these co-expressed tomato DWD members, SlWDR28, SlWDR43, SlWDR167, SlWDR221, and SlWDR247 were included in the 14 representative DWD members selected for the protein interaction and subcellular localization assays (★ labeled in class II in Fig. 7). The subcellular localization of SlWDR221 and SlWDR247 was shown to target to both nucleus and cytoplasm, resembling that of tomato CUL4 and DDB1 (Wang et al. 2008). Nevertheless, a large number of *DWD* genes displayed distinct expression patterns from *DDB1*, suggesting the assembly of various cellular CRL4 E3 ligase complexes could be restrained strictly by regulation of the abundance of individual components spatially and temporally.

We also found six tested DWD proteins were localized in both nucleus and cytoplasm when overexpressed from the CaMV 35S promoter. This prompted us to use cNLS

Mapper (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi; Kosugi et al. 2009) to determine if these DWD proteins possessed nuclear localization signals (NLS). As a result, the NLS sequences were identified in SlWDR31, SlWDR171 and SlWDR237 with cutoff score close to or exceeding 5, while no significant NLS sequence was found in the other three DWDs (Supplementary Fig. S5). Given the fact that tomato DDB1 is localized in both nucleus and cytoplasm and physically interacts with DWD proteins (Wang et al. 2008), the nuclear localization of the tomato DWD members without NLS might be attributed to their physical association with the nucleus-localized DDB1.

Author contribution YZ, MM, XT and WW carried out the experiments; YZ, SH and JY contributed to the data analyses; YZ, SH and YL designed experiments, wrote and revised the manuscript; SH and YL conceived strategies and managed projects. All authors read and approved the manuscript.

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