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ORIGINAL PAPER

Genome-wide survey and expression analysis of the *PUB* family in Chinese cabbage (*Brassica rapa* ssp. *pekinesis*)

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Abstract U-box proteins are widely distributed among eukaryotic organisms and show a higher prevalence in plants than in other organisms. Plant U-box (PUB) proteins play crucial regulatory roles in various developmental and physiological processes. Previously, 64 and 77 *PUB* genes have been identified in Arabidopsis (*Arabidopsis thaliana*) and rice (*Oryza sativa*), respectively. In this study, 101 putative *PUB* genes were identified in the Chinese cabbage (*Brassica rapa* ssp. *pekinensis* line Chiifu-401-42) genome and compared with other 15 representative plants. By specific protein domains and a phylogenetic analysis, the *B. rapa PUB* (*BrPUB*) gene family was subdivided into 10 groups. Localization of *BrPUB* genes showed an uneven distribution on the ten chromosomes of *B. rapa*. The orthologous and co-orthologous *PUB* gene pairs were identified between *B. rapa* and *A. thaliana*. RNA-seq transcriptome data of different tissues revealed tissue-specific and differential expression profiles of the *BrPUBs*, and quantitative real-time PCR analysis showed inverse gene expression patterns of the *BrPUB-ARMs* in response to cold and heat stresses. Altogether, the identification, classification, phylogenetic analysis, chromosome distribution, conserved motifs, and expression patterns of BrPUBs were predicted and analysed. Importantly, this study of BrPUBs provides a rich resource that will aid in the determination of PUB functions in plant development.

Keywords Chinese cabbage · Plant U-box (PUB) proteins · Abiotic stress · RNA-seq · qRT-PCR

Introduction

The ubiquitin proteolytic pathway plays an important role in the degradation of target proteins. No part of the cell is beyond the reach of the ubiquitin–proteasome regulatory system (Glickman and Ciechanover 2002). Three enzymes are involved in the ubiquitination process: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin–protein ligase (E3). E1 transfers ubiquitin to E2, and E2 binds the ubiquitin. E3 removes the ubiquitin molecule from E2 and attaches it to the target substrate, forming a covalent bond between ubiquitin and the target. Finally, target proteins can be tagged by multiple ubiquitin molecules and then degraded by the 26S proteasome (Ciechanover and Schwartz 1998). The ubiquitin proteolytic pathway is involved in various cellular processes, such as cell cycle control, antigen processing, transcription, and receptor desensitization (Kirschner 1999; Hershko et al. 2000). Of the three enzymes involved in the ubiquitination process, E3 enzymes plays a central role in determining the specificity of the ubiquitination system and have been classified into two families (the HECT family and the RING

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finger family; Ciechanover 1998). Recently, the U-box protein family has been added as a third family of E3 enzymes. Moreover, the U-box protein–ubiquitin fusion degradation protein 2 (UFD2)—the first U-box protein identified in yeast—is thought to have E4 enzyme ability. E4 can mediate the assembly of polyubiquitin chains on proteins after the action of E3 and are commonly considered a specialized type of E3 enzyme (Koegl et al. 1999; Cyr et al. 2002). Previous studies of the *U-box* genes have included a systematic analysis of their E3 ligase activity and various functions in plant growth and development. However, there are still relatively few analyses of the response of these genes to stress conditions. *Brassica rapa* ssp. *pekinensis* (Chinese cabbage) is one of the most important *B. rapa* crops. This subspecies, which originated in China, has undergone thousands of years of cultivation and artificial selection. Moreover, Chinese cabbage is an increasingly important leaf vegetable worldwide due to its high yield and quality, and the growth, and development of this plant are significant for its yield. Recently, the Chinese cabbage (Chiifu-401-42) genome was sequenced and assembled, and this sequence provided the opportunity for analysis of *U-box* genes across the entire genome (Wang et al. 2011).

U-box proteins are characterized by the presence of a U-box domain of approximately 70 amino acids (Aravind and Koonin 2000; Ohi et al. 2003). The U-box domain is structurally similar to the RING domain but lacks the zinc-chelating residues of the RING finger and instead stabilizes its structure by forming salt-bridges (Ohi et al. 2003). In previous studies, the largest U-box protein family was identified in plants (Patterson 2002), and the U-box proteins found in plants were designated as Plant U-boxes (PUBs). In comparison with the two U-box genes identified in the yeast genome and the 21 U-box genes annotated in the human genome (Koegl et al. 1999; Hatakeyama et al. 2001; Ohi et al. 2003), 64 and 77 *PUB* genes have been predicted in the *Arabidopsis* genomes (Azevedo et al. 2001; Wiborg et al. 2008) and rice genomes (Zeng et al. 2008), respectively. The PUB proteins found in plants can be classified into various groups based on the presence of common domains (Azevedo et al. 2001; Andersen et al. 2004; Mudgil et al. 2004; Zeng et al. 2008; Yee and Goring 2009). ARM domain-containing PUB proteins and Kinase domain-containing PUB proteins are the two most prevalent groups found in plants. In addition to these common PUB groups, there are specific groups found only in certain species. For example, two PUB-MIF4G proteins and one PUB-TPR-Kinase protein were found to be specific to *Arabidopsis* and rice, respectively (Azevedo et al. 2001; Wiborg et al. 2008; Zeng et al. 2008).

Increasing lines of evidence suggest that the PUB proteins play crucial regulatory roles in various plant physiological and developmental processes, such as self-incompatibility (Stone et al. 1999, 2003; Indriolo et al. 2012, 2014), defence (Durrant et al. 2000; Kirsch et al. 2001; González-Lamothe et al. 2006; Yang et al. 2006; Yee and Goring 2009) and abiotic stress response (Yee and Goring 2009; Seo et al. 2012). Interestingly, the majority of PUB proteins with an elucidated biological function are from the PUB-ARM group (Yee and Goring 2009). However, the role of *PUB-ARM* genes in Chinese cabbage under abiotic stress is unknown. To assess how *PUB-ARM* genes in Chinese cabbage respond to different conditions, six abiotic stresses (GA, ABA, salinity, PEG, cold and heat stresses) were utilized. Plants are frequently subjected to salinity, osmotic, cold and heat stresses in their natural environments, and GA and ABA can regulate many aspects of plant growth and development (Monte et al. 2003; Seo et al. 2012).

B. rapa and *A. thaliana* both belong to the family Brassicaceae (Cruciferae). They have a close evolutionary relationship, providing a useful resource for comparative genomics analysis. During evolution, the PUB family underwent a large gene expansion, and the process of gene duplication and evolution of *PUB* genes is complex. More work should be done to elucidate the evolution and potential functions of *PUB* genes. To better understand the role of PUB proteins in Chinese cabbage, we took advantage of the available genome sequences to perform a genome-wide analysis of *PUB* genes in *B. rapa*. In total, we identified 101 *PUB* genes in the *B. rapa* genome and classified them using an improved method that differs from previous classifications. We further analysed the phylogenetic relationships, conserved motifs, chromosome distribution and gene duplication of these *BrPUB* genes. Moreover, we utilized publicly available gene expression data to analyse the expression patterns of these genes in different tissues. In addition, 41 *PUB-ARM* genes were selected for an examination of their comprehensive expressional profile under 6 different abiotic stresses. Our results provide useful information for in-depth studies of the PUB family in Brassica.

Materials and methods

Sequence retrieval in database

The genome sequences of *B. rapa* were downloaded from the *Brassica* database (BRAD, http://brassicadb.org/brad/) (Wang et al. 2011). To identify all of the members of the PUB family in *B. rapa*, we analysed the domains of all the *B. rapa* proteins using a Hidden Markov Model (HMM) profile (Finn et al. 2011) of the U-box domain (PF04564) retrieved from the Pfam 27.0 database (http://Pfam.sanger.ac.uk/) (Bateman et al. 2004) with an expected value (*e* value) cut-off of 1.0. Then, we verified these sequences

using the Pfam database (http://Pfam.sanger.ac.uk/), the SMART program (http://smart.embl-heidelberg.de/) (Letunic et al. 2012) and the NCBI database (http://www.ncbi.nlm.nih.gov/). The genome sequences of *Arabidopsis thaliana* were retrieved from the TAIR database (http://www.arabidopsis.org/). *A. thaliana* PUB (AtPUB) proteins were obtained using the same algorithms.

The rice PUB proteins were retrieved from previous analyses by Zeng et al. (Zeng et al. 2008). The Pfam 27.0 database (http://pfam.sanger.ac.uk/) was used to screen the genome assemblies of *Malus domestica*, *Cucumis sativus*, *Medicago truncatula*, *Populus trichocarpa*, *Carica papaya*, *Theobroma cacao*, *Vitis vinifera*, *Solanum lycopersicum*, *Sorghum bicolor*, *Selaginella moellendorffii*, *Physcomitrella patens*, *Chlamydomonas reinhardtii* and *Cyanidioschyzon merolae*. The genome data were downloaded from the Phytozome database (http://www.phytozome.net/), and the evolutionary relationships of these species were determined according to the PGDD database (http://chibba.agtec.uga.edu/duplication/) (Lee et al. 2013).

Phylogenetic analysis

Phylogenetic trees were produced individually using the full-length sequences of PUB proteins. The identified PUB proteins were aligned by the Muscle program (Edgar 2004). Phylogenetic analyses were conducted using MEGA6 (http://www.megasoftware.net/) with the neighbour-joining (NJ) method (Tamura et al. 2013). The bootstrap value was set at 1000, and the numbers of the clades mean bootstrap support values were expressed as percentages.

Physico-chemical characterization and conserved motifs analysis of PUB proteins

The physical and chemical characteristics of BrPUB proteins were predicted by Protparam (http://web.expasy.org/protparam) (Gasteiger et al. 2005). The conserved motifs of PUB proteins were detected by Multiple EM for motif elicitation (MEME, http://meme.sdsc.edu/meme/) (Bailey et al. 2009). The DNA sequences and coding domain sequences (CDS) of *BrPUB* genes were analysed by the tool GSDS (http://gsds.cbi.pku.edu.cn/) (Hu et al. 2014).

Physical locations of *BrPUB* genes and *BrPUB* gene duplications in the Chinese cabbage genome

To determine the physical locations of PUB genes in the Chinese cabbage genome, the starting and ending positions of all *BrPUB* genes on each chromosome were obtained from the BRAD database. Based on this information, a distribution map of the positions of *BrPUB* genes on 10 chromosomes was drawn using a Perl script. To identify gene duplications, all of the CDS sequences of the *BrPUB* genes were BLAST searched against each other (identity >85 %, *e* value <1e−10), and then, gene alignment coverage was obtained by pair-wise alignment using the date previously calculated by BLAST. Gene alignment coverage = (alignment length − mismatches)/length of the larger gene. The pairs that had a gene alignment coverage that was more than 0.75 were considered to be duplications. The *Ks* values were calculated by the method of Nei and Gojobori as implemented by the KaKs calculator (Zhang et al. 2006), and the divergence time was computed according to the synonymous substitution rate of 1.5×10^{-8} substitutions per site per year (Koch et al. 2000). Purple lines were used to link the duplicated genes between different chromosomes.

Identification of orthologous and paralogous genes

Homologous *PUB* genes between *B. rapa* and *A. thaliana* were identified by the OrthoMCL program (http://www.orthomcl.org/cgi-bin/OrthoMclWeb.cgi) (Li et al. 2003). BLASTP (*e* value ≤1e−10), and orthoMCL Pairs were used to find orthologs, in-paralogs and co-orthologs between the two species. Circos software (Krzywinski et al. 2009) was used to link these genes to their chromosomal positions. Cytoscape software was applied to build a network of their relationships (Shannon et al. 2003).

RNA-Seq data analysis

Previously generated and analysed Illumina RNA-seq data were used for this study (Tong et al. 2013). Four tissues (root, stem, leaf and flower) of *B. rapa* accession Chiifu-401-42 were analysed. The abundance of transcriptional data is expressed as fragments per kilobase of exon per million fragments mapped (FPKM). The expression cluster of *BrPUB* genes from the aforementioned tissues was analysed by Cluster 3.0 (http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm). The heat maps were constructed by Tree View (http://jtreeview.sourceforge.net/).

Plant materials, growth conditions and abiotic stress treatments

Chinese cabbage (Chiifu-401–42) grown in plastic pots in a 3:1 soil–vermiculite mixture in a controlled-environment growth chamber was used as starting material. The artificial growth conditions were set at 24/16 °C, with a photoperiod of 16/8 h for day/night and a relative humidity of 65–70 %. Five-leafed plants were used for different abiotic stress treatments. The plants were irrigated with 100 μM gibberellic acid (GA), 100 μM abscisic acid (ABA), 15 %

(w/v) polyethylene glycol (PEG) 6000 and 250 mM NaCl. The above plants were watered thoroughly and deeply. In addition, some five-leaf stage plants were transferred from the normal growth condition to 4 and 38 °C for cold and heat treatments, respectively. All treatments were performed over a continuous time course (0, 1, 6, 12 h). Five-leaf stage plants exposed to normal artificial growth conditions were used as controls. Young leaves from control and stress-treated plants were collected as samples in three biological replicates for RNA preparation, and the samples were quickly frozen in liquid nitrogen and stored at −70 °C until use.

RNA isolation, reverse transcription and quantitative real-time PCR (qRT-PCR) analysis

Total RNA was extracted from plant leaves with an RNA kit (TaKaRa, Dalian, China). RNA was reverse transcribed into cDNA using a Prime Script RT reagent kit (TaKaRa, Dalian, China). Then, the cDNA was diluted 1:20 with ddH_2O to be used as a template for qRT-PCR. Quantitative real-time PCR was performed using the SYBR Premix Ex Taq kit® (TaKaRa, Dalian, China) on an ABI Prism 7500 detection system (Applied Biosystems, Foster City, CA, USA) with the following cycling profile: 95 °C for 30 s, 95 °C for 5 s (40 cycles), 60 °C for 30 s, and melting curve analysis at 65 °C for 10 s with 61 cycles. The reaction mixture volume for qRT-PCR is 20 μL. Experimental repeat runs for three biological and three technical replicates were included in the analysis. The actin gene *Bra028615* was used as an internal control to normalize the expression level of the target genes. Specific primers used for qRT-PCR were designed by Beacon Designer 7 and are shown in Table S10. An analysis of the relative gene expression using the comparative Ct value method was performed (Heid et al. 1996). The results were calculated by the $2^{-\Delta\Delta Ct}$ according to previous reports (Livak and Schmittgen 2001).

Results

Identification, classification and phylogenetic analysis of BrPUB proteins in Chinese cabbage and comparative analyses

To identify PUB proteins in Chinese cabbage, we searched the entire *B. rapa* genome sequence for genes containing a U-box domain using an HMM profile of the U-box domain PF04564 with an *e* value cut-off of 1e−1. A total of 101 putative *PUB* genes in *B. rapa* were identified and were designated as *BrPUB 1–101* according to the generic system (Table 1). Using the same algorithms, 62 *PUB* genes in the Arabidopsis database were also identified. Compared with the recent report by Wiborg et al. (2008), 2 AtPUB proteins were absent from this study because At3g49065 was removed by TAIR and At5g05230 lacked a clear U-box domain.

PUB proteins can be further classified on the basis of other domains present. The amino acid sequences of the U-box proteins in *B. rapa* and *A. thaliana* were used to search against the SMART program and the NCBI protein database to identify other domains. In addition to the U-box, a variety of other protein domains are present in these proteins (Table 2). Based on the presence of these other domains, PUB proteins in *B. rapa* and *A. thaliana* were divided into 10 and 8 groups, respectively. Notably, in addition to the U-box and the other domains used for classification, proteins within a group sometimes contained other domains (Zeng et al. 2008).

Due to the structure of PUB protein domains and the low support values for informative characters, we used phylogenetic analysis to support our group designations (Fig. S1, Fig. 1). First, a phylogenetic tree was drawn by means of MEGA 6 software (using the NJ method and bootstrapping 1000 times) to identify the phylogenetic relationships among all AtPUB proteins (Fig. S1). The topology of the phylogenetic tree allowed us to divide the AtPUBs into eight subfamilies (Groups III–X). The classifications based on protein domains and phylogenetic analyses were completely identical, indicating that the two methods were in strong agreement. In our subfamily classification of AtPUB proteins, we also referred to the classification model constructed by Azevedo et al. and Heise et al. (http://www.arabidopsis.org/browse/genefamily/pub.jsp) (Azevedo et al. 2001; Heise et al. 2002). In a previous study, AtPUBs were divided into seven subfamilies (Class I–VII), so we labelled the previously defined clades in the trees shown in Fig. S1 to facilitate a comparison. Some of the groups (e.g. Group III, VI, IX and X) were supported by previous studies, while others (e.g. Group IV, V, VII and VIII) changed. The differences were as follows: the former Class V was further divided into two parts, one defined as a new group (Group IX) and the other combined with Class IV to form a new group (Group VII); the former Class VI was further divided into two groups (Groups IV and V).

Next, 101 BrPUBs and 62 AtPUBs were chosen for a combined phylogenetic analysis (Fig. 1). Based on the phylogenetic relationship, 101 BrPUBs and 62 AtPUBs were clustered into 10 groups, which approach was identical with the above classification results. Every group contained BrPUBs, but no AtPUBs were found in Groups I and II. Group V, with 41 BrPUBs and 28 AtPUBs, was found to be the largest group and consisted of proteins containing the ARM domain. The ARM repeat block is an approximately 40-amino acid motif that was first identified in

Table 1 The information of 101 *PUB* genes in *B. rapa*

Group	*B. rapa* gene	BrPUB ID	Chr	Gene start	Gene end	AtPUB ID	*A. thaliana* gene	Identity (%)	*e* value
I	Bra006452	BrPUB22	A03	3550082	3558713				
II	Bra006450	BrPUB20	A03	3541084	3546714				
III	Bra024442	BrPUB60	A06	16402178	16404865	AtPUB49	AT5G67530.1	91.23	0
IV	Bra005504	BrPUB46	A05	5854472	5859143	AtPUB60	AT2G33340.2	90.94	0
	Bra030575	BrPUB81	A08	20488427	20494491	AtPUB59	AT1G04510.1	91.01	0
V	Bra004351	BrPUB71	A07	21443605	21446909	AtPUB42	AT1G68940.1	89.32	0
	Bra015754	BrPUB73	A07	24299428	24302268	AtPUB43	AT1G76390.2	84.22	0
	Bra012249	BrPUB67	A07	11314048	11317364	AtPUB44	AT1G20780.1	84.16	0
	Bra016453	BrPUB80	A08	17769701	17773164	AtPUB44	AT1G20780.1	82.15	0
	Bra018212	BrPUB56	A06	10808458	10810619	AtPUB13	AT3G46510.1	88.46	0
	Bra033820	BrPUB4	A01	13317744	13320019	AtPUB13	AT3G46510.1	87.31	0
	Bra035665	BrPUB40	A04	13064190	13066948	AtPUB12	AT2G28830.1	85.49	0
	Bra007105	BrPUB93	A09	28863652	28866326	AtPUB14	AT3G54850.1	85.79	0
	Bra016165	BrPUB72	A07	22380457	22383313	AtPUB10	AT1G71020.1	82.86	2e−137
	Bra012355	BrPUB66	A07	10494043	10496207	AtPUB11, AtCMPG6	AT1G23030.1	85.06	0
	Bra024559	BrPUB90	A09	25636306	25638524	AtPUB11, AtCMPG6	AT1G23030.1	86.8	0
	Bra027977	BrPUB87	A09	10713152	10715646	AtPUB15	AT5G42340.1	84.54	0
	Bra031431	BrPUB7	A01	17355509	17366323	AtPUB37	AT5G46210.1	95.45	0.67
	Bra002153	BrPUB100	A10	11279455	11287038	AtPUB46	AT5G18320.1	78.35	2e−80
	Bra006451	BrPUB21	A03	3547700	3549530	AtPUB46	AT5G18320.1	78.19	6e−20
	Bra040374	BrPUB11	A01	25488516	25490097	AtPUB9	AT3G07360.3	86.7	0
	Bra001247	BrPUB26	A03	15535292	15537053	AtPUB9	AT3G07360.3	87.36	0
	Bra029642	BrPUB53	A05	23573551	23575305	AtPUB9	AT3G07360.1	84.87	0
	Bra013521	BrPUB2	A01	6112142	6113263	AtPUB8, AtCMPG3	AT4G21350.1	85.7	0
	Bra019924	BrPUB54	A06	3704177	3706264	AtPUB18	AT1G10560.1	83.63	0
	Bra031521	BrPUB6	A01	16601769	16603802	AtPUB19	AT1G60190.1	82.81	5e−160
	Bra001998	BrPUB62	A07	2748977	2750770	AtPUB16	AT5G01830.1	81.21	0
	Bra019022	BrPUB33	A03	26746302	26748326	AtPUB16	AT5G01830.1	81.26	0
	Bra021899	BrPUB41	A04	15206158	15208143				
	Bra010834	BrPUB77	A08	15922711	15924870	AtPUB17	AT1G29340.1	84	0
	Bra032309	BrPUB88	A09	22708966	22711080	AtPUB17	AT1G29340.1	80.85	9e−162
	Bra032180	BrPUB39	A04	10637352	10639522	AtPUB4	AT2G23140.2	86.47	2e−109
	Bra029250	BrPUB18	A02	25769338	25770996	AtPUB41	AT5G62560.1	84.14	5e−172
	Bra035890	BrPUB83	A09	3665376	3667161	AtPUB41	AT5G62560.1	81.69	0
	Bra031875	BrPUB19	A02	26452785	26454425	AtPUB38	AT5G65200.1	82.43	2e−32
	Bra025605	BrPUB37	A04	7889532	7891220	AtPUB40	AT5G40140.1	82.11	0
	Bra007097	BrPUB92	A09	28809870	28812278	AtPUB3	AT3G54790.2	87.18	0
	Bra014787	BrPUB35	A04	3159586	3161959	AtPUB3	AT3G54790.2	89.56	0
	Bra039185	BrPUB95	A09	33162268	33168145	AtPUB4	AT2G23140.2	85.17	0
	Bra012155	BrPUB69	A07	11903745	11906654	AtPUB2	AT5G67340.1	84.49	8e−88
	Bra037117	BrPUB86	A09	4530505	4533138	AtPUB2	AT5G67340.1	84.37	0
	Bra010960	BrPUB79	A08	16510327	16513362	AtPUB6	AT1G24330.1	86.14	0
	Bra030028	BrPUB65	A07	9291552	9294465	AtPUB6	AT1G24330.1	88.08	0
	Bra033994	BrPUB15	A02	9363884	9367129	AtPUB7	AT1G67530.2	86.57	0
	Bra010914	BrPUB78	A08	16303027	16306346	AtPUB45	AT1G27910.1	86.52	0
	Bra032852	BrPUB89	A09	23568001	23571646	AtPUB45	AT1G27910.1	87.43	0

Table 1 continued

Group	*B. rapa* gene	BrPUB ID	Chr	Gene start	Gene end	AtPUB ID	*A. thaliana* gene	Identity (%)	*e* value
VI	Bra010551	BrPUB75	A08	14343058	14350562				
	Bra010559	BrPUB76	A08	14389946	14392391	AtPUB5	AT4G36550.1	80.35	6e−163
	Bra011690	BrPUB1	A01	1038663	1041058	AtPUB5	AT4G36550.1	80.15	2e−110
	Bra001717	BrPUB28	A03	18039246	18040710	AtPUB29	AT3G18710.1	82.25	3e−77
	Bra022347	BrPUB50	A05	18997726	18999000	AtPUB29	AT3G18710.1	82	5e−113
	Bra037568	BrPUB9	A01	21162921	21164162	AtPUB29	AT3G18710.1	83.46	1e−88
	Bra024318	BrPUB58	A06	15524576	15525835	AtPUB27, AtCMPG2	AT5G64660.1	86.86	0
	Bra029975	BrPUB5	A01	14896391	14897722	AtPUB30	AT3G49810.1	86.42	0
	Bra037833	BrPUB85	A09	4439104	4440435	AtPUB31, AtCMPG4	AT5G65920.1	82.32	0
	Bra022415	BrPUB49	A05	18571449	18572717	AtPUB25	AT3G19380.1	81.66	0
	Bra022417	BrPUB48	A05	18561390	18562658	AtPUB25	AT3G19380.1	81.66	0
	Bra038205	BrPUB8	A01	20824385	20825638	AtPUB25	AT3G19380.1	84.03	3e−126
	Bra014206	BrPUB74	A08	2540087	2541358	AtPUB26	AT1G49780.1	86.32	1e−175
	Bra025442	BrPUB38	A04	9075313	9076629	AtPUB21, AtCMPG5	AT5G37490.1	83.24	0
	Bra039752	BrPUB14	A02	8739507	8740775	AtPUB20, AtCMPG1	AT1G66160.1	86.52	9e−93
	Bra001424	BrPUB27	A03	16327497	16329687	AtPUB24	AT3G11840.1	82.68	2e−134
	Bra034806	BrPUB51	A05	22236223	22238008	AtPUB24	AT3G11840.1	82.3	0
	Bra038683	BrPUB10	A01	24501764	24503458	AtPUB24	AT3G11840.1	84.92	6e−162
	Bra005309	BrPUB45	A05	4719051	4720214	AtPUB23	AT2G35930.1	88.38	0
	Bra017278	BrPUB42	A04	15688937	15690175	AtPUB23	AT2G35930.1	88.98	0
	Bra023044	BrPUB24	A03	8375076	8376305	AtPUB23	AT2G35930.1	88.57	0
	Bra033430	BrPUB36	A04	4501980	4503254	AtPUB22	AT3G52450.1	88.85	0
	Bra006925	BrPUB91	A09	27731071	27732378	AtPUB22	AT3G52450.1	89.37	0
	Bra012803	BrPUB31	A03	22171466	22172737	AtPUB22	AT3G52450.1	88.21	0
VII	Bra007600	BrPUB94	A09	31480022	31481881	AtPUB36	AT3G61390.2	81.78	0
	Bra014439	BrPUB34	A04	657815	659273	AtPUB36	AT3G61390.2	84.78	4e−79
	Bra003448	BrPUB70	A07	16526311	16530887	AtPUB36	AT3G61390.2	83.94	3e−111
	Bra033243	BrPUB97	A10	3363397	3366437	AtPUB56	AT1G01670.1	80.85	3e−145
	Bra033244	BrPUB96	A10	3360431	3363061	AtPUB55	AT1G01660.1	91.3	0
	Bra000412	BrPUB25	A03	11010262	11012094	AtPUB54	AT1G01680.1	86.56	9e−126
	Bra033242	BrPUB98	A10	3366774	3368219	AtPUB54	AT1G01680.1	86.65	6e−108
	Bra024393	BrPUB59	A06	16078745	16081693	AtPUB50	AT5G65500.1	85.53	0
	Bra037819	BrPUB84	A09	4353628	4356571	AtPUB50	AT5G65500.1	86.07	0
	Bra004947	BrPUB44	A05	2603473	2606441	AtPUB33	AT2G45910.1	85.44	0
	Bra039305	BrPUB43	A04	18796043	18799636	AtPUB33	AT2G45910.1	81.69	0
	Bra018013	BrPUB55	A06	9230936	9234960	AtPUB32	AT3G49060.1	87.44	0
	Bra019576	BrPUB57	A06	13409187	13413538	AtPUB32	AT3G49060.1	87.44	0
	Bra002768	BrPUB99	A10	7784024	7787506	AtPUB61	AT5G57035.1	87.52	0
	Bra006836	BrPUB23	A03	5273791	5277047	AtPUB61	AT5G57035.1	87.99	0
	Bra012181	BrPUB68	A07	11733267	11741634	AtPUB34	AT2G19410.1	89.26	0
	Bra038999	BrPUB61	A07	759287	762879	AtPUB34	AT2G19410.1	86.85	0
	Bra012943	BrPUB30	A03	21424804	21427718	AtPUB51	AT5G61560.2	90.01	0
	Bra029318	BrPUB16	A02	25338390	25341936	AtPUB51	AT5G61560.1	89	0
	Bra015196	BrPUB64	A07	3977312	3980780	AtPUB53	AT5G51270.1	81.99	1e−89
	Bra015198	BrPUB63	A07	3917933	3921025	AtPUB53	AT5G51270.1	86.1	0
	Bra022543	BrPUB13	A02	8122684	8125824	AtPUB53	AT5G51270.1	86.1	0
	Bra013868	BrPUB3	A01	8144520	8148023	AtPUB35	AT4G25160.1	89.6	0
	Bra019185	BrPUB32	A03	25845779	25849564	AtPUB35	AT4G25160.1	86.32	0
	Bra035920	BrPUB82	A09	3502632	3506123	AtPUB52	AT5G61550.2	86.13	0
	Bra012944	BrPUB29	A03	21412661	21416343	AtPUB52	AT5G61550.2	87.94	0
	Bra029317	BrPUB17	A02	25345668	25349410	AtPUB52	AT5G61550.2	85.39	0
VIII	Bra037092	BrPUB47	A05	9382343	9384317	AtPUB58	AT1G56040.1	81.97	2e−60

Table 1 continued

Group	*B. rapa* gene	BrPUB ID	Chr	Gene start	Gene end	AtPUB ID	*A. thaliana* gene	Identity (%)	*e* value
IX	Bra029643	BrPUB52	A05	23570258	23572006	AtCHIP	AT3G07370.1	88.87	0
X	Bra008700	BrPUB101	A10	12257412	12262539	AtUFD2	AT5G15400.1	90.65	0
	Bra023516	BrPUB12	A02	2699148	2704059	AtUFD2	AT5G15400.1	89.02	0

Table 2 Domain organizations of *B. rapa* and *Arabidopsis* PUB proteins

Group	Domain organization (N-terminus → C-terminus)	Number of proteins included	
		Brassica rapa	*Arabidopsis*
I	U-box + TIR + NB-ARC + LRR3 + RPT	1	0
II	U-box + GDA1_CD39	1	0
III	U-box +Pro_isomerase	1	1
IV	U-box + WD40	2	2
V	U-box +ARM	41	28
VI	U-box only	24	13
VII	Kinase +U-box	27	14
VIII	MIF4G + U-Box	1	2
IX	UFD2 + U-box	2	1
X	TPR + U-Box	1	1

Drosophila (Riggleman et al. 1989). It was found to take part in protein–protein interactions and in the regulation of cell death and defence (Huber and Weis 2001; Zeng et al. 2004). Group VII, with 27 BrPUBs and 13 AtPUBs, was the second largest group, consisting of proteins containing one or more kinase domain(s) in the N-terminal region. Group VI, with 24 BrPUBs and 14 AtPUBs, was the third largest group and only contained the U-box domain. In addition to these three classes, there are other smaller subfamilies characterized by specific domains such as WD40 repeats, TPR motifs, and MIF4G motifs. Both WD40 and TPR domains have been shown to function in protein–protein interactions (Das et al. 1998; Blatch and Lässle 1999; Espejo et al. 2002), and MIF4G has been shown to function in RNA metabolism (Ponting 2000; Kraft et al. 2013).

For comparative genomic analyses, we searched for PUB protein-coding sequences in the genomes of 16 representative species (Fig. 2). Our findings showed that the number of *PUB* genes in Algae, Bryophyta or Lycophytes is less than that in Angiospermae which occurred during the whole-genome duplication events (Fig. 2). Surprisingly, we found that the number of *PUB* genes in *C. reinhardtii* was much more than that in *C. merolae,* and the number of *PUB* genes in higher plants was relatively stable, which indicated that the PUB proteins may have expanded before divergence of higher plants and lower plant species. The density of PUB proteins in the whole Chinese cabbage genome (0.356) was less than that in *A. thaliana* (0.459), while it was greater than that of other 14 species, suggesting that they may have important roles in Brassicaceae plants. Although there were more PUB proteins in *M. domestica* than in Chinese cabbage, its PUB protein densities (0.136) were lower than that in Chinese cabbage because of its large genome sizes.

Characterization of PUB proteins and conserved motif identification in Chinese cabbage

The physical and chemical characteristics of all BrPUB proteins were analysed (Table S1). The number of amino acid residues encoded by the BrPUB proteins ranged from 238 to 1661. The molecular weights (MWs) of BrPUB proteins varied from 26.8 to 223.9 kD. The theoretical isoelectric points (pI) of the 101 family members varied from 5.01 to 9.24. The protein characteristics of three biggest groups (Group V, VI and VII) were compared using the MWs and pI of their BrPUB members (Fig. 3). Comparative analysis of different groups indicated that most of the proteins from Group V (PUB-ARM, 73.2 %) and Group VII (PUB-Kinase, 70.4 %) were acidic, with a wide fluctuation in MWs. A majority of Group VI (U-box only, 87.5 %) proteins showed a wider fluctuation in pI values and lower MWs than the proteins in Groups V and VII.

The instability index is used to evaluate the stability of protein structure. The average instability index of the 87 BrPUB proteins was less than 40.0, indicating that most BrPUBs were unstable proteins. Only 14 proteins were determined to be stable, with instability indices varying from 29.75 to 39.99. Most BrPUBs contained numerous aliphatic amino acids, and the aliphatic index reached an average of 93.78. The hydropathicity value of + of the proteins was less than zero, suggesting that most BrPUBs are hydrophilic.

A phylogenetic tree of all BrPUBs was drawn with MEGA 6 (using the NJ method and bootstrapping 1,000 times) to identify phylogenetic relationships among all of the BrPUB proteins (Fig. 4a). The conserved motifs were searched by the MEME program (Fig. 4b), and the LOGOs of the protein motifs were also obtained by MEME (Fig. S2). The exon and intron distribution map showing the gene structure of BrPUBs was analysed using the GSDS2.0 program (Fig. 4c). In general, 101 BrPUBs were clustered into ten large groups identical with our classification results. Most members of a given group shared a similar motif composition and similar exon and intron

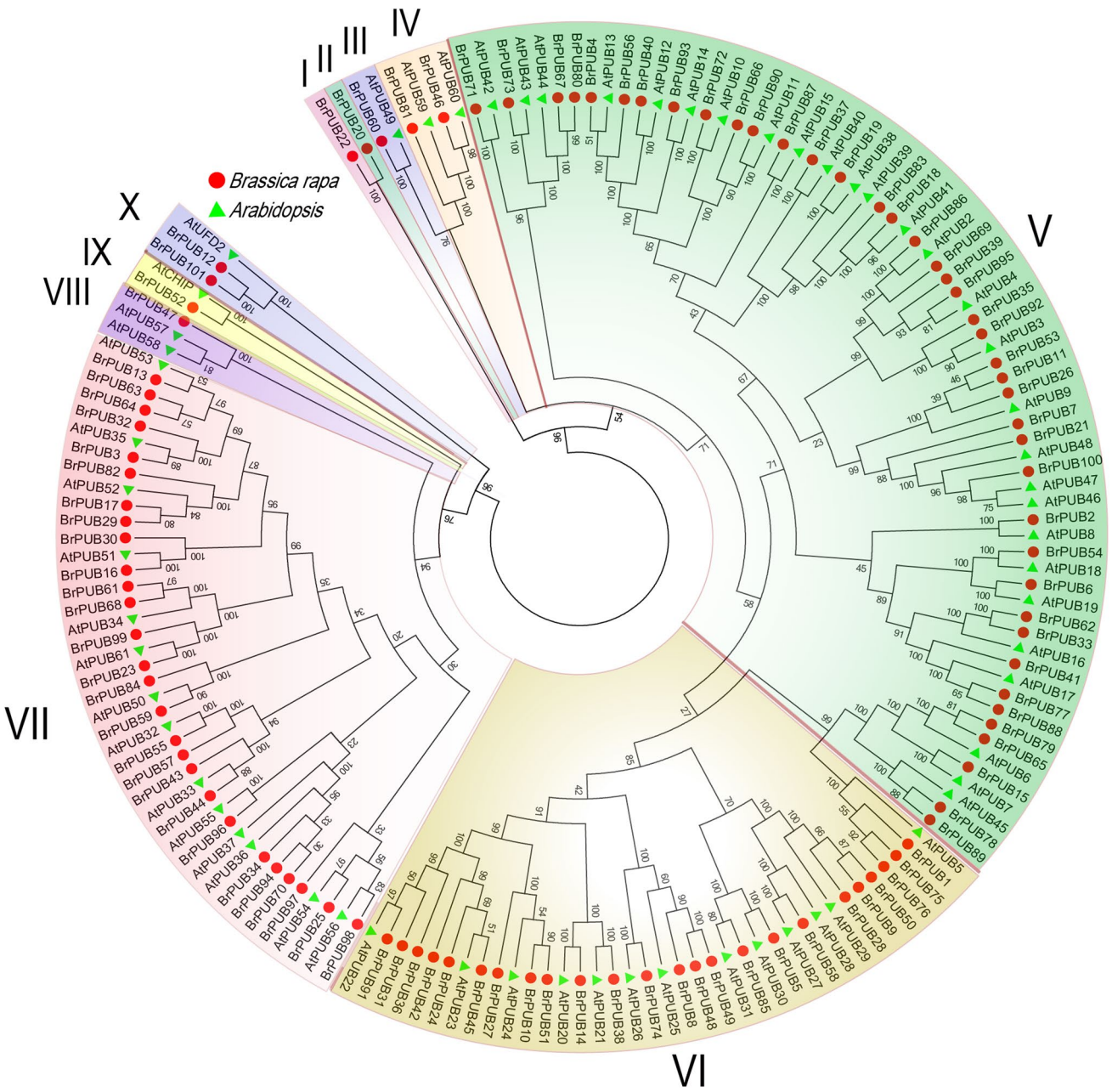
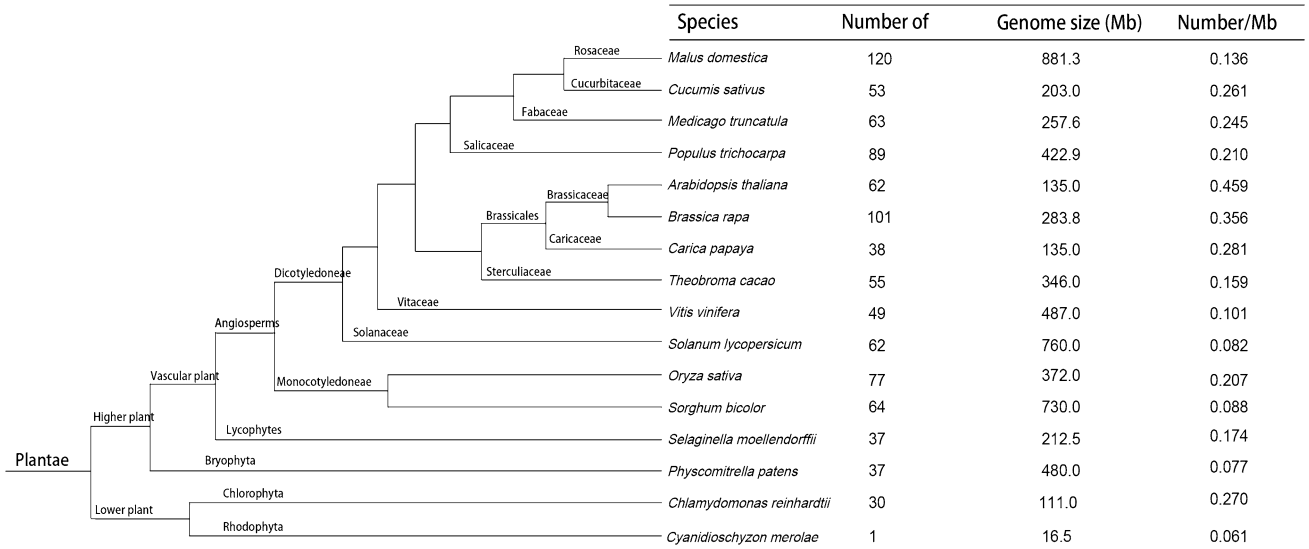


Fig. 1 Phylogenetic tree of PUB proteins of Chinese cabbage and *Arabidopsis*. Multiple sequence alignment of the full-length sequences of the PUB proteins was done using MUSCLE, and the phylogenetic tree was constructed using MEGA 6 by the NJ method with 1000 bootstrap replicates. The tree was divided into ten phylogenetic subgroups, designated as Group I–X.10 clades with *different colours* are marked. Members of Chinese cabbage and *Arabidopsis* are denoted by *circles* and *triangles*, respectively (color figure online)

distributions. This strong concordance with phylogenetic relationship may indicate functional similarities within a subgroup.

Furthermore, with the exception of the U-box domain, different groups showed different motif profiles, but only some of the motifs could be annotated by the tool SMART. All of the BrPUBs contained motifs 1 and 3, which were parts of U-box domain. The majority of BrPUBs contained the highly conserved motifs 4, 8, 12, 13 and 14, which may be essential for the functions of BrPUBs. In addition, an inspection of motif distribution indicated that some motifs were only present in specific classes of the BrPUB family. For example, motifs 22 and 24 were characteristic of Group V and corresponded to the ARM domain. Motif 5 corresponding to the Kinase domain, and motif 29, was specific to Group VII. Moreover, motif 28 existed mainly in Groups



Species	Number of	Genome size (Mb)	Number/Mb
Malus domestica	120	881.3	0.136
Cucumis sativus	53	203.0	0.261
Medicago truncatula	63	257.6	0.245
Populus trichocarpa	89	422.9	0.210
Arabidopsis thaliana	62	135.0	0.459
Brassica rapa	101	283.8	0.356
Carica papaya	38	135.0	0.281
Theobroma cacao	55	346.0	0.159
Vitis vinifera	49	487.0	0.101
Solanum lycopersicum	62	760.0	0.082
Oryza sativa	77	372.0	0.207
Sorghum bicolor	64	730.0	0.088
Selaginella moellendorffii	37	212.5	0.174
Physcomitrella patens	37	480.0	0.077
Chlamydomonas reinhardtii	30	111.0	0.270
Cyanidioschyzon merolae	1	16.5	0.061

Fig. 2 Summary of PUB genes family among 16 genomes of different plant species

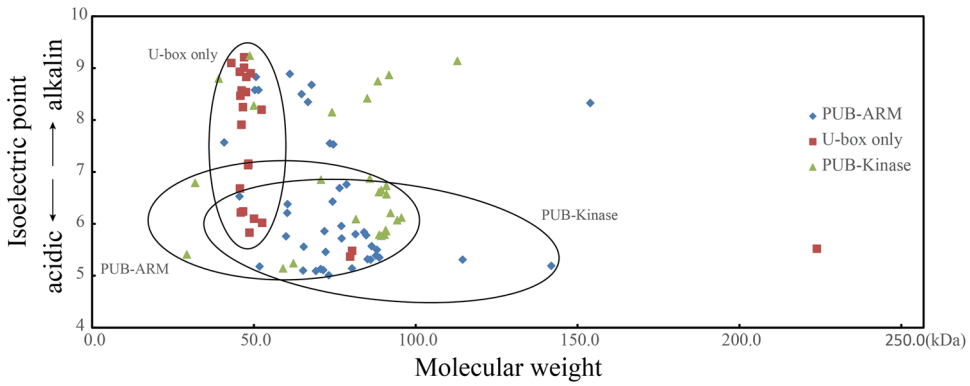


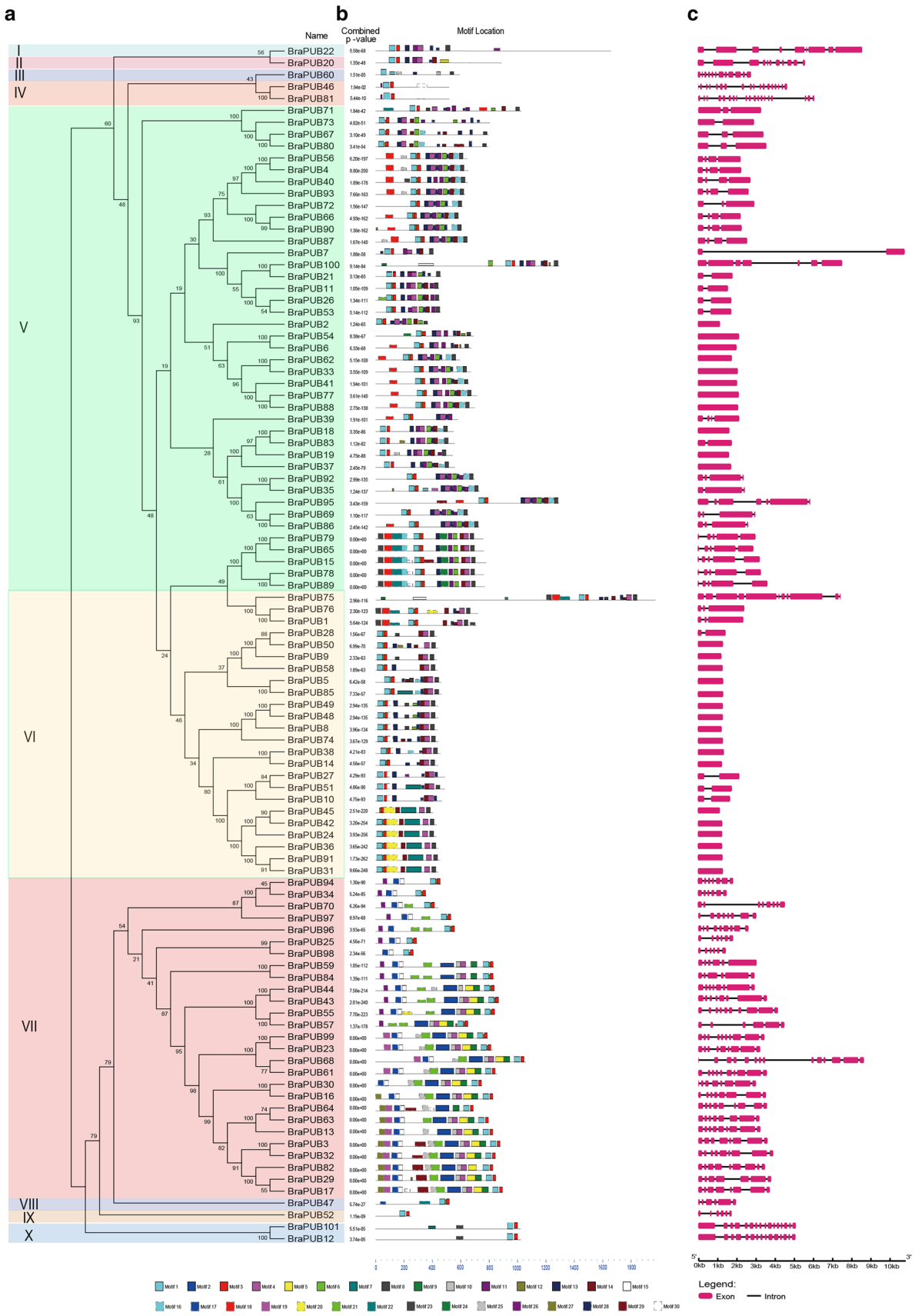
Fig. 3 Putative isoelectric points and molecular weights of PUB-ARM proteins, U-box-only proteins and PUB-Kinase proteins in *B. rapa*

I, V and VI, while motif 20 was specific to Group VI. An analysis of the primary gene structure showed that members in one group usually shared a similar genetic structure. Interestingly, with the exception of Groups V and VI, all of the other groups had at least six exons.

Chromosomal distribution, copy number variation and differential retention of PUB genes in Chinese cabbage

Genome chromosomal distribution analysis revealed that Chinese cabbage PUBs were distributed across all 10 chromosomes and all 3 subgenomes (Fig. 5; Table S3). In total, 101 *BrPUB* genes were mapped separately onto chromosomes A01–A10. On average, one *PUB* gene was present every 0.9 Mb. These *BrPUB* genes were distributed unevenly on the 10 chromosomes (A1–A10) of Chinese cabbage with the largest number—14 PUB genes each—detected on chromosomes 3 and 9 (Fig. 4). Every chromosome had at least six PUB genes. Furthermore, the 101 *BrPUB* genes were mapped onto the chromosomes in relation to the three subgenomes (LF, MF1, and MF2): 44 in LF, 33 in MF1, and 24 in MF2 (Table S3).

A comparison of homologous *PUB* genes in Arabidopsis and the three *B. rapa* subgenomes (LF, MF1 and MF2) revealed that most *BrPUB* genes in the collinear blocks were conserved throughout the divergent evolution of *Arabidopsis* and *B. rapa* (Table S3). The gene dosage hypothesis predicts that genes whose products interact with other gene products in a dose-sensitive manner, creating a web of dependency, are more likely to be retained (Thomas et al. 2006; Birchler and Veitia 2007). Interestingly, genes of Groups VI and VII were retained following triplication and fractionation in *B. rapa* at a higher rate than Group V genes (Fig. S3). At 46.2 %, Group VI and Group VII genes were retained at two or three copies, which is greater than the retention of Group V genes (38.7 %), while more of the Group V genes (17.9 %) were completely lost.


a
b
c
Name
Combined p -value
Motif Location
I
II
III
IV
V
VI
VII
VIII
IX
X
BraPUB22
BraPUB20
BraPUB60
BraPUB46
BraPUB81
BraPUB71
BraPUB73
BraPUB67
BraPUB80
BraPUB56
BraPUB4
BraPUB40
BraPUB93
BraPUB72
BraPUB66
BraPUB90
BraPUB87
BraPUB7
BraPUB100
BraPUB21
BraPUB11
BraPUB26
BraPUB53
BraPUB2
BraPUB54
BraPUB6
BraPUB62
BraPUB33
BraPUB41
BraPUB77
BraPUB88
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BraPUB78
BraPUB89
BraPUB75
BraPUB76
BraPUB1
BraPUB28
BraPUB50
BraPUB9
BraPUB58
BraPUB5
BraPUB85
BraPUB49
BraPUB48
BraPUB8
BraPUB74
BraPUB38
BraPUB14
BraPUB27
BraPUB51
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0 200 400 600 800 1000 1200 1400 1600 1800
Motif 1 Motif 2 Motif 3 Motif 4 Motif 5 Motif 6 Motif 7 Motif 8 Motif 9 Motif 10 Motif 11 Motif 12 Motif 13 Motif 14 Motif 15
Motif 16 Motif 17 Motif 18 Motif 19 Motif 20 Motif 21 Motif 22 Motif 23 Motif 24 Motif 25 Motif 26 Motif 27 Motif 28 Motif 29 Motif 30
5' 3'
0kb 1kb 2kb 3kb 4kb 5kb 6kb 7kb 8kb 9kb 10kb
Legend:
Exon
Intron

◀ **Fig. 4** Phylogenetic relationships, conserved motif compositions and gene structures of BrPUB proteins. The NJ tree of BrPUB proteins, their motif locations and gene structure. **a** The multiple sequence alignment of 101 full-length BrPUB proteins was done using MUSCLE, and the phylogenetic tree was constructed using MEGA 6 by the NJ method with 1000 bootstrap replicates. The tree was divided into ten phylogenetic subgroups designated as Group I–X marked with *different colour* backgrounds. **b** The schematic representation of the conserved motifs in the BrPUB proteins detected by MEME analysis. Each motif is represented by a *colour box* numbered at the *bottom*. **c** The gene structures of *BrPUBs* were analysed by the tool GSDS. Introns and exons are represented by *black lines* and *coloured boxes*, respectively (color figure online)

Orthologous groups and the duplication of PUB genes

Angiosperms have a propensity for chromosomal duplication and occasionally triplication (Lee et al. 2013). Extensive genome replication, especially gene-level triplication after divergence from Arabidopsis, could increase the flexibility of *B. rapa* (Wang et al. 2011). In fact, the number of *BrPUB* genes was notably lower than the triplicate number observed for *AtPUBs*, indicating that gene loss occurred during polyploid speciation. In this study, we analysed ortholog PUB gene groups between Chinese cabbage and *Arabidopsis* using the OrthoMCL program. A total of 166 orthologous gene pairs and 17 co-orthologous gene pairs were identified in the PUB proteins of these two species (Table S4). In addition, 117 and 24 paralogous PUB gene pairs were identified in Chinese cabbage and *Arabidopsis*, respectively (Table S4). The relationships among the orthologous, co-orthologous and paralogous PUB genes between *B. rapa* and Arabidopsis were assessed by Circos (Fig. 6). The numbers of homologous *BrPUB* genes on Arabidopsis chromosomes 5 and 1 were higher than those on other chromosomes among the orthologous gene pairs of *B. rapa* and Arabidopsis. Only four Arabidopsis U-box genes had no orthologous *BrPUB* gene; these genes have been duplicated in Arabidopsis after the split. Thirteen *AtPUB* genes were found to have only one ortholog in *B. rapa*; these genes were present before the divergence, but two of the three copies were lost after *B. rapa* genome triplication (Fig. S4a). Forty-five *AtPUB* genes had at least two orthologs in *B. rapa*, and these genes were preferentially retained after triplication (Fig. S4b, c).

Gene duplication events are important for the study of the evolutionary mechanisms utilized by plant genomes; both tandem and segmental gene duplications have had a significant influence on the expansion and evolution of gene families in plant genomes (De Bodt et al. 2005). Typically, one (sometimes two) gene duplicates in tandem as the original event, which is defined as tandem duplication and often produces gene clusters or hotspots. Segmental duplication occurs when genes are duplicated as a segment, resulting from massive genome-scale events, such as polyploidy or duplications of large chromosome-level regions. Segmental duplications often result in many homologs on different chromosomes (Cannon et al. 2004; Freeling 2009). The large size of the *BrPUB* gene family suggests that this family may have undergone frequent duplication events during evolution. The duplicated genes were identified based on amino acid identity and gene alignment coverage (Tables S5, S6). As shown in Fig. 5, a total of 36 duplicated genes were identified in *B. rapa* (amino acid identity >85 %, gene alignment coverage >0.75), including 34 segmental duplication events between chromosomes as well as two tandem duplication events on the same chromosome (*BrPUB48* and *BrPUB49*). The divergence time of duplicated *BrPUB* gene pairs ranged from 8.43 to 19.83 million years ago (MYA) and averaged 13.13 MYA, which indicates that the divergence of duplicated *PUB* genes in *B. rapa* occurred largely before the triplication events (5–9 MYA) (Table S6).

Expressional pattern analysis of *BrPUBs* in different tissues

We analysed Illumina RNA-seq transcriptomic data in four different tissues (roots, stems, leaves and flowers). The FPKM values were taken to represent the transcriptional level. More than 29,000 Chinese cabbage genes were extracted from the four tissues. The transcriptional levels of 93.0 % of the *BrPUBs* gene were obtained from at least one tissue, with an overall coverage of 78.2 % among the four tissues. However, 7 *BrPUBs (BrPUB32, 34, 39, 48, 49, 62* and *82*) were not detected (Fig. 7; Table S7). These seven *BrPUBs* may not be expressed or may have spatially or temporally restricted expressional patterns (Table S7). Some *BrPUBs* exhibited tissue-specific expression. For instance, 3 *PUB-ARM* genes (*BrPUB41*, *69*, and *87*) were expressed specifically in flowers, while *BrPUB94* was expressed specifically in roots. *BrPUB24, 31* and *42* were not expressed in leaves, and *BrPUB16* had a low expression level in roots. Interestingly, the FPKM values of four *BrPUBs* (*BrPUB11, 42, 53* and *91)* in root were greater than 100, demonstrating that these genes may be important in *B. rapa* root development. In addition, *BrPUB6,* mostly expressed in the root, was homologous with *AtPUB19*, a negative regulator of drought response mediated by abscisic acid (Seo et al. 2012).

Expression patterns of *BrPUB-ARMs* under different abiotic conditions

All 41 *PUB-ARM* genes were selected for an examination of their comprehensive expressional profiles under

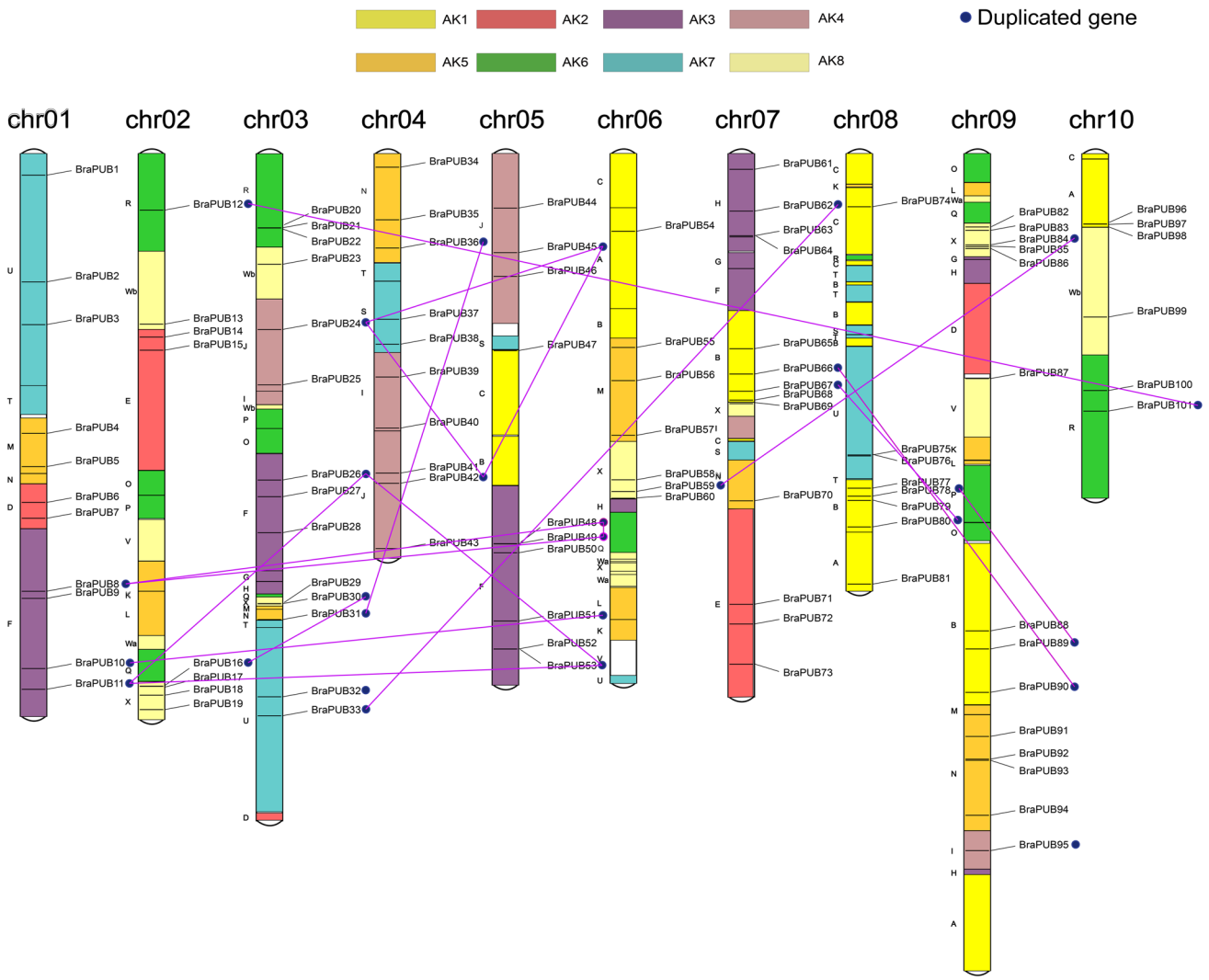

Fig. 5 Distribution of the *BrPUB* genes on 10 Chinese cabbage chromosomes. The 101 *BrPUB* genes unevenly located on each conserved collinear blocks of the chromosomes. The chromosome numbers are marked above each chromosome. The *PUB* genes present on duplicated chromosomal segments are connected by *purple lines* between the two relevant chromosomes. The conserved collinear blocks on each chromosome are labeled A to X and are *colour coded* according to inferred ancestral chromosomes following an established convention (color figure online)

cold, heat, GA, ABA, PEG, and salt treatments by qRT-PCR (Table S8). The results are shown using the program TreeView (Figs. 8, 9).

The results showed that 41*BrPUB-ARM* genes responded to the treatments differently. Interestingly, some *BrPUB-ARM* genes displayed inverse gene expression patterns in response to cold and heat treatments. For instance, *BrPUB41*, *54*, *69*, and *71* were up-regulated under heat treatment, but were down-regulated under cold treatment. In contrast, *BraPUB18*, *19*, *21*, *33*, *62*, *67*, *79*, *86*, and *100* were up-regulated under cold treatment, but were down-regulated under heat treatment (Fig. 8). Moreover, the expressions of *BrPUB19*, *21*, *53*, *56*, *66*, *83* and *90* were down-regulated or barely altered under all the treatments, except under cold treatment, while *BraPUB26* did not respond to treatments other than cold and heat (Fig. S5). Under GA stress, the expressions of *BrPUB72* and *95* were up-regulated at 4, 6 and 12 h (Fig. 8a). Moreover, the expression of *BrPUB72* was 20 times grater than that of the control at 6 and 12 h (Fig. 9b). Under ABA stress, the expressions of two *BrPUB* genes (*BrPUB71* and *100*) were 20 times greater than that of the control at 6 and 12 h (Fig. 9c). *BrPUB6*, *54*, *67*, and *86* were up-regulated at 1 h and then decreased rapidly in later stages (Fig. 9a). Under NaCl stress, the expressions of six *BrPUB* genes (*BrPUB2*, *7*, *19*, *41*, *65*, and *87*) were up-regulated at 1 h and then decreased in later stages (Fig. 9a). Under PEG stress, most *BrPUBs* were down-regulated or showed no

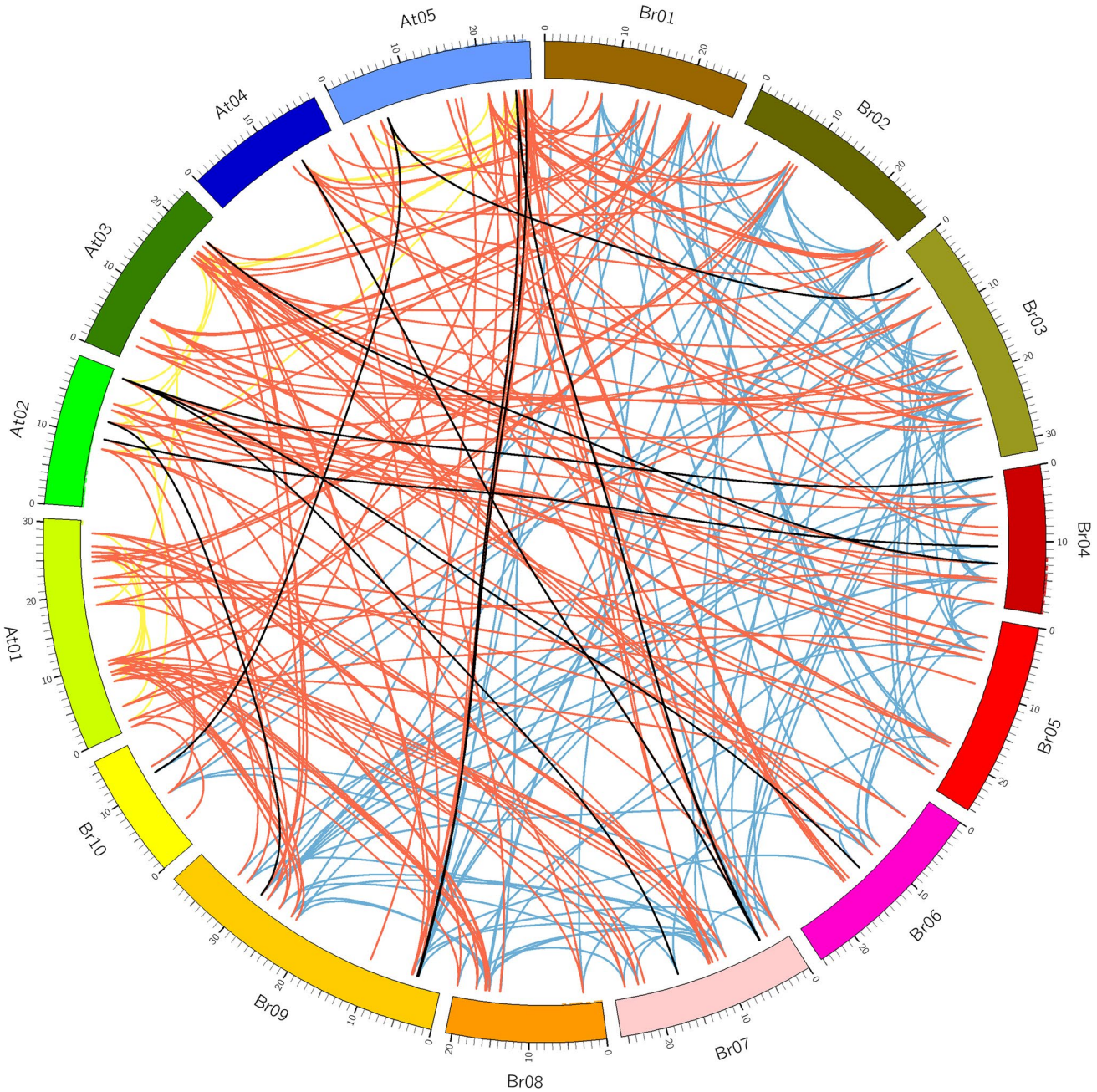

Fig. 6 Ortholog groups of *PUB* genes in *B. rapa* and *Arabidopsis* Genome. Ten Chinese cabbage chromosomes and five *Arabidopsis* chromosomes are marked with *different colors* with their names on the periphery. The *lines* in the figure represent four pairs. The *lines* of orthologous gene pairs are coloured in *red*; the *lines* of co-orthologous gene pairs are coloured in *black*; the *lines* of paralogous gene pairs in Chinese cabbage are coloured in *blue* and the *lines* of paralogous gene pairs in *Arabidopsis* are coloured in *yellow*. The figure was created by the software Circos (color figure online)

change, while only *BrPUB72* was obviously up-regulated at 6 and 12 h (Fig. 9a, e). With the *BrPUB-ARM* genes showing increased expression in response to a range of abiotic stresses (Figs. 8, 9, Fig. S5), it seems likely that these proteins play a role in mediating ubiquitin-directed protein degradation in adaptation-related responses to various environmental stresses.

Eleven duplicated *BrPUB-ARM* genes were investigated under different stresses in *B. rapa*, and a line chart was used to show the trends among them (Fig. 10; Table S9). Similar expression patterns were found in two gene pairs, including *BrPUB* (*78*: *89*; *33*: *62*) and *BrPUB* (*66*: *90*; *11*: *26*: *53*). Overall, the most duplicated gene pairs showed similar expression, except for the *BrPUB67* and *80* gene pair.

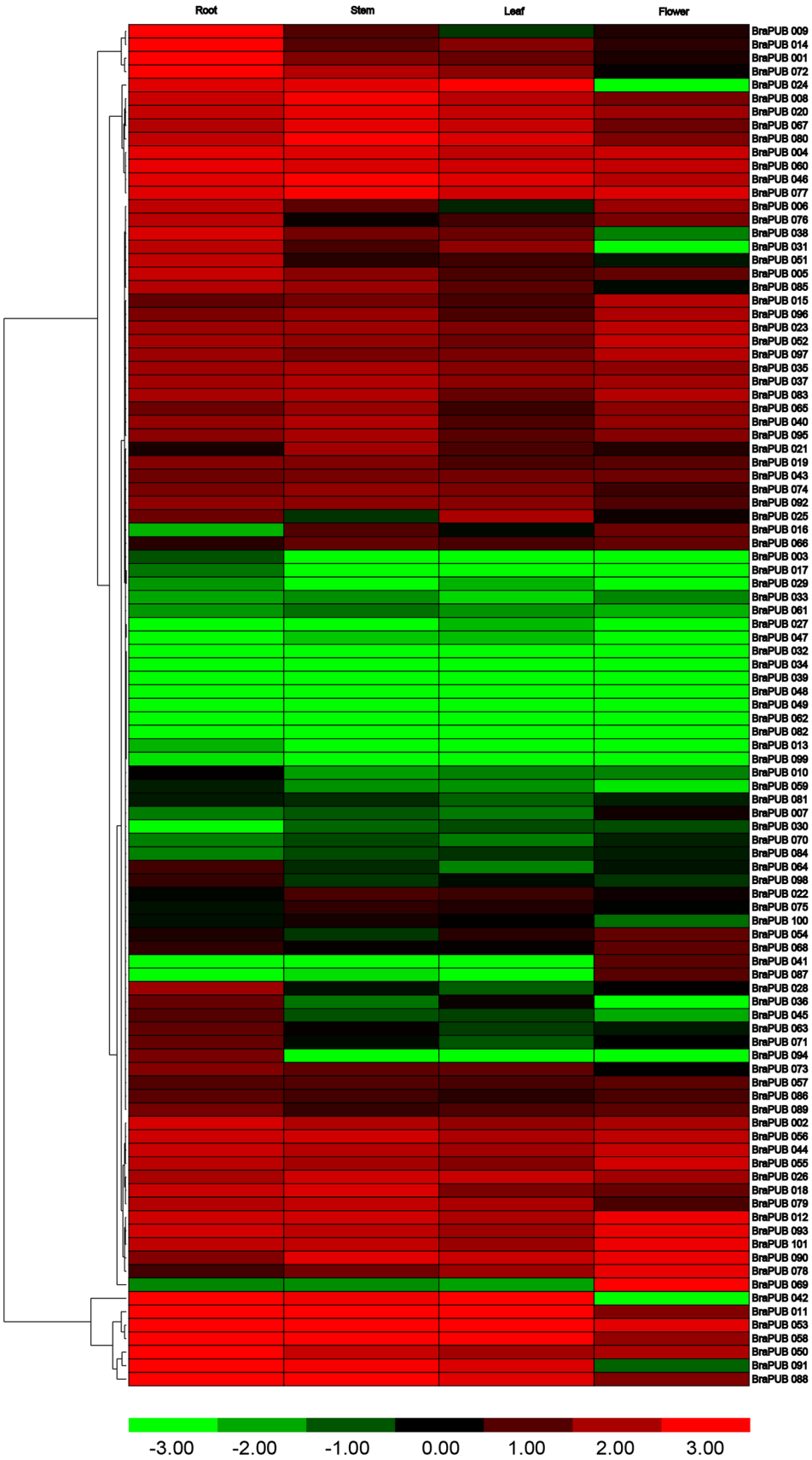

Fig. 7 Heat map of *BrPUB* genes in root, stem, leaf and flower. The *bar* at the *bottom* of the heat map represents relative expression values

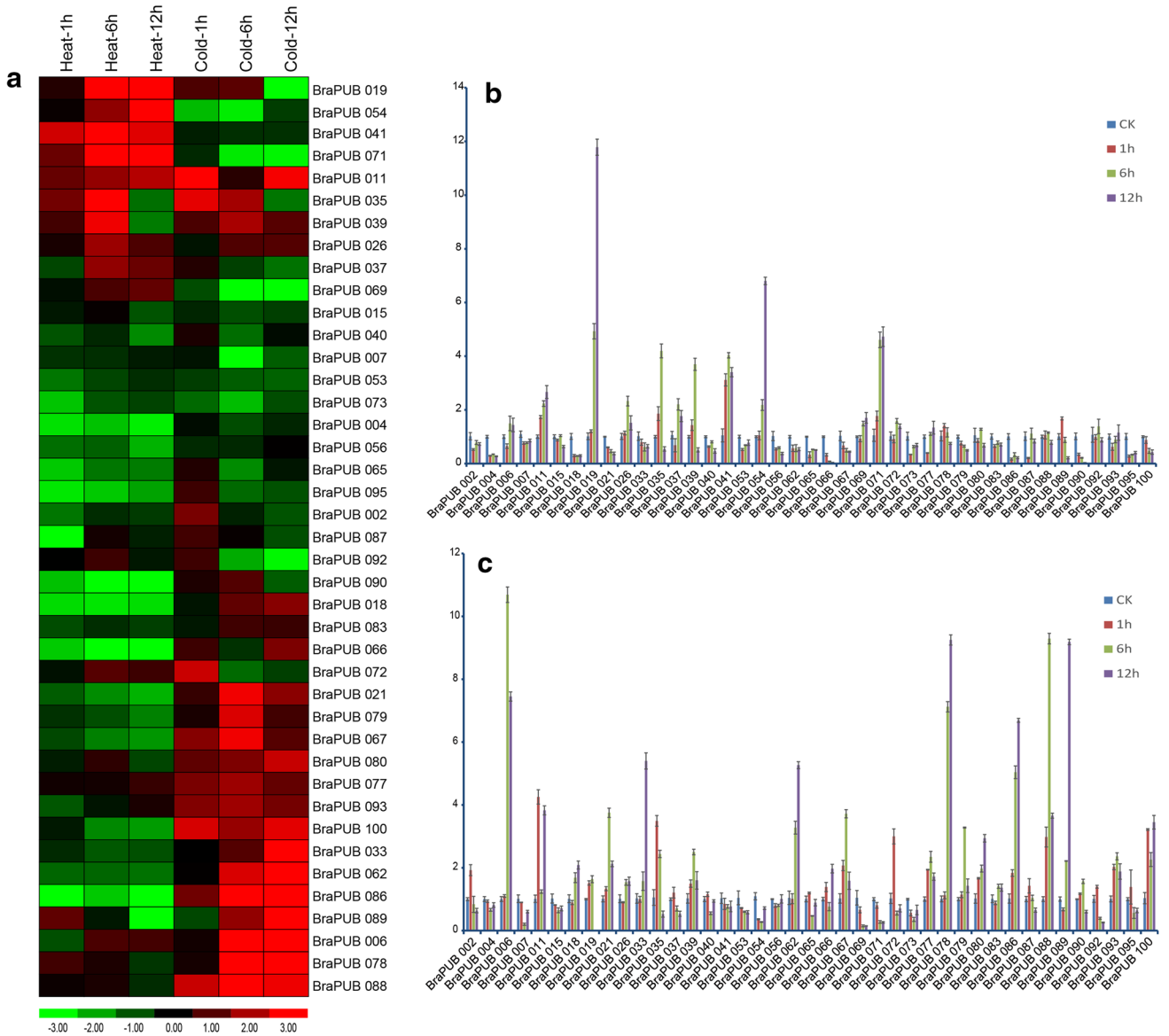

Fig. 8 Expression analysis of *B. rapa PUB-ARM* genes under heat and cold abiotic stresses. **a** Heat map representation and hierarchical clustering of *B. rapa PUB-ARM* genes under heat and cold stresses. **b** The relative expression ratios of *B. rapa PUB-ARM* genes under heat stresses. **c** The relative expression ratios of *B. rapa PUB-ARM* genes under cold stresses. The relative expression levels of *BrPUB* in leaves under these stresses were quantified against the control transcript levels. The *bar* at the *bottom* of each heat map represents relative expression values

Discussion

Chinese cabbage is an important vegetable that is cultivated worldwide. The PUB family proteins, which have E3 ligase activity, play crucial regulatory roles in various plant developmental and physiological processes. With the rapid development of high-speed sequencing technologies and the implementation of many whole-genome-sequencing projects, PUB family diversity is likely to expand, and species-specific functions of *PUB* genes, including conserved members of this family, are likely to emerge (Yee and Goring 2009). In this study, we analyse the *PUB* gene family in Chinese cabbage and in 15 other species, including 13 higher plants and 2 lower plants. A total of 938 *PUB* genes are identified and analysed in our research. After careful comparison, we found that the *PUB* genes in *C. reinhardtii* have already expanded compared with *C. merolae* and its PUB protein density (0.270) was even greater than that of most of the high plants. Thus, the important of PUB proteins in various physiological processes, particularly basic function, can be shown through the evolution of the *PUB* gene family in lower plant. The density of PUB proteins in

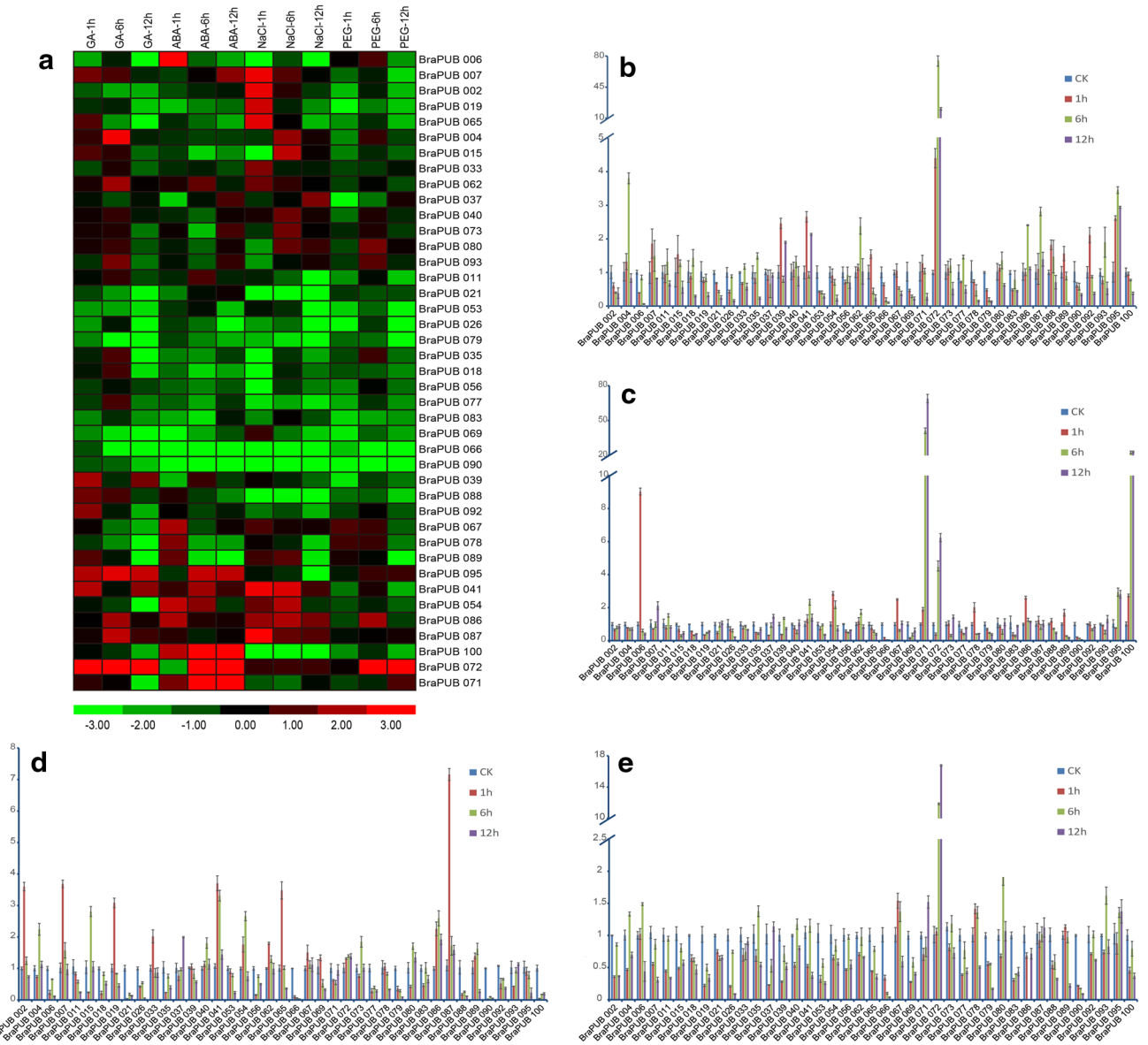

Fig. 9 Expression analyses of *B. rapa PUB-ARM* genes under four abiotic stresses. **a** Heat map representation and hierarchical clustering of *BrPUB* genes during GA, ABA, NaCl and PEG stresses. **b** The relative expression ratios of *B. rapa PUB-ARM* genes under GA stresses. **c** The relative expression ratios of *B. rapa PUB-ARM* genes under ABA stresses. **d** The relative expression ratios of *B. rapa PUB-ARM* genes under NaCl stresses. **e** The relative expression ratios of *B. rapa PUB-ARM* genes under PEG stresses. Every stress contains three durations: 1, 4 and 12 h. The relative expression levels of *BrPUB* in leaves under these stresses were quantified against the control transcript levels. The *bar* at the *bottom* of each heat map represents relative expression values

Brassicales was greater than that of other species used in our analyses, which indicated that the PUB proteins may have very important roles in Brassicales species.

A total of 101 genes were identified as members of the Chinese cabbage PUB family in our research. These genes were classified into ten groups based on both structural and phylogenetic analyses. It is noteworthy that one PUB protein (AtPUB5) in Arabidopsis and three PUB proteins (BrPUB1, 75 and 76) in *B. rapa* were classified as U-box-only proteins based on a domain analysis, but they appeared more like Group V (PUB-ARM) proteins based on the phylogenetic analysis. This could be due to deletion of the ARM domain during evolutionary development. This classification is further supported by protein and gene structural analyses; the protein and gene structures of these four proteins more closely resemble PUB-ARM proteins (Fig. 5); the result of AtPUB5 was not shown. Compared with Arabidopsis and rice, two new types of PUB proteins were found in *B. rapa*, including proteins in Groups I (U-box + TIR + NB-ARC + LRR3 + RPT) and

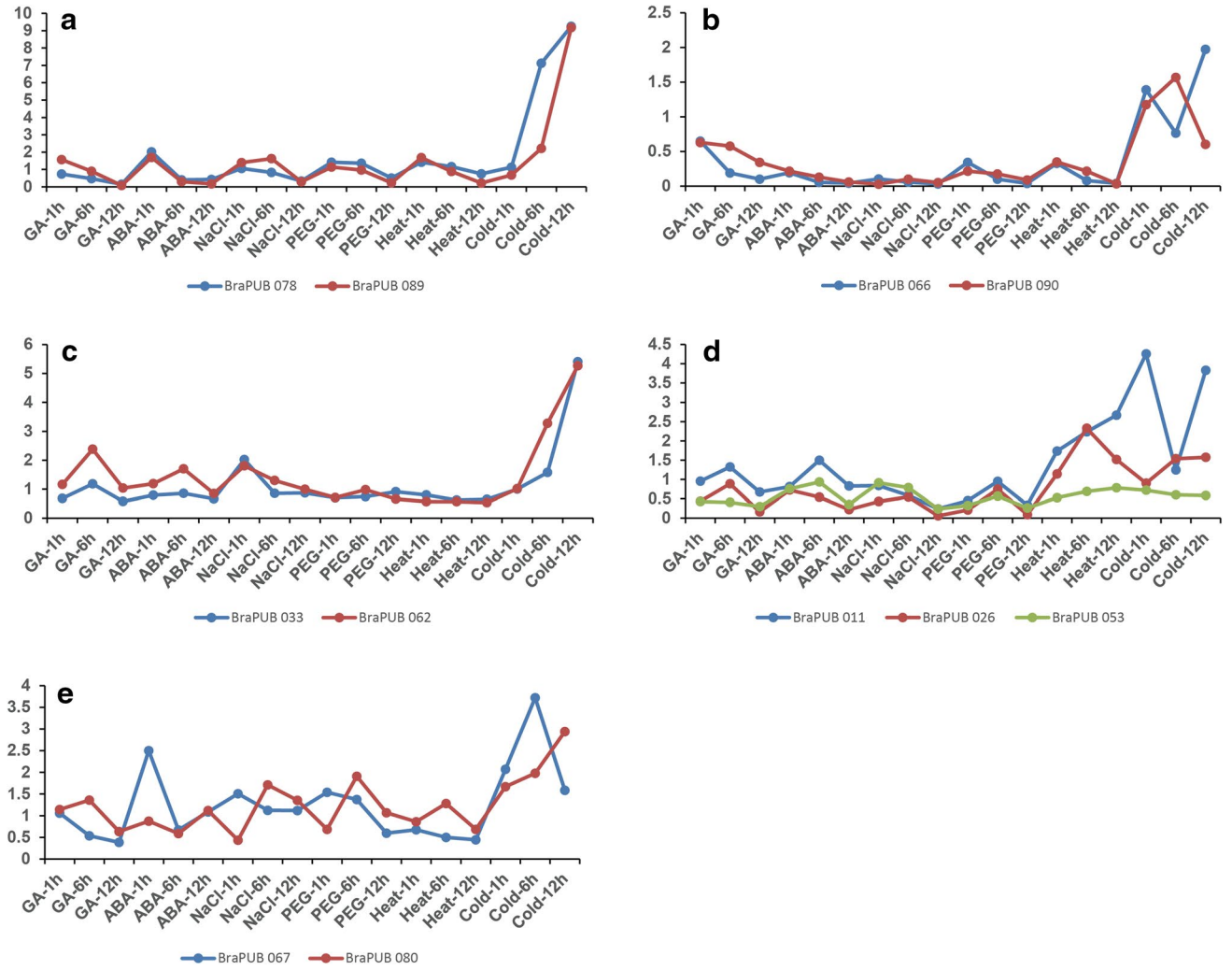

Fig. 10 *PUB-ARM* genes lying on duplicated segments of *B. rapa* genome and their expression pattern. Expression patterns of duplicated genes have been compared. *X*-axis represents the abiotic stresses. *Y*-axis represents the raw expression value

II (U-box + GDA1_CD39), which arose during the triplication that occurred after the divergence from *Arabidopsis*. Moreover, the numbers of members in each group in *B. rapa* are different compared with *Arabidopsis* and rice. Thus, the *PUB* genes in these species are present in different groups with different gene numbers.

In some taxa—especially in higher plant lineages—all chromosomes double on the genomic level, which initiates the process of fractionation among the new homologs and is known as whole-genome duplication (WGD) (Freeling 2009). The Brassicaceae genome experienced two WGD events—*Arabidopsis* beta and *Arabidopsis* alpha—during the phylogeny of the species (Bowers et al. 2003). Later, the Chinese cabbage genome experienced one WGT event against *Arabidopsis* (Wang et al. 2011). WGD results in gene duplication and is typically followed by substantial gene loss (Lee et al. 2013). The gene balance hypothesis predicts that genes that have products that participate in macromolecular complexes or in transcriptional or signalling networks are more likely to be retained (Birchler and Veitia 2007). The PUB-ARM proteins have been shown to function in large complexes during flower development (Liu et al. 2012), salt stress (Ni et al. 2010; Salt et al. 2011) and drought stress (Drechsel et al. 2011), and the majority of PUB proteins with an elucidated biological functions belong to this group. In this study, more Group VI (U-box only) and VII (Kinase + U-box) *PUB* genes were found to be retained in duplicate or triplicate than Group V (U-box + ARM) genes, indicating that Group VI and VII *PUB* genes may have important roles during plant growth and development. We speculate that the *PUB* genes in Group V were preferentially lost compared with other groups, possibly due to the functional divergence of these genes between *A. thaliana* and *B. rapa*. The genome of

B. rapa is almost a complete triplication of the genome of *A. thaliana*, and both segmental and partial tandem gene duplications play important roles in the expansion and evolution of gene families in plant genomes. In this study, each *PUB* gene in Arabidopsis had one–ten orthologous genes derived from Chinese cabbage, indicating that genomic duplications and partial tandem duplications were accompanied by the triplication. Further analysis identified 36 duplicated genes in the *B. rapa* genome, of which only two had undergone tandem duplication, while 34 *BrPUB* genes had undergone segmental duplication.

The largest PUB group in *B. rapa* is the ARM domain-containing group of proteins, and all members in this group were found to have E3 ligase activity (Mudgil et al. 2004; Samuel et al. 2006). The *PUB-ARM* genes may be affected by various abiotic stresses. In previous studies, the *PUB-ARM* genes in *Capsicum annuum* were shown to be affected by salinity stress (Cho et al. 2006), and other plants were affected by the application of hormones, such as GA (Monte et al. 2003) and ABA (Samuel et al. 2008). However, how *PUB-ARM* genes in Chinese cabbage respond to abiotic stresses had not been studied previously. In this study, all 41 *B. rapa PUB-ARM* genes were identified, and their expression patterns under different stress treatments were analysed. Many *BrPUB-ARM* genes responded to temperature stress. In particular, some had inverse expression profiles under cold and heat treatments. *BrPUB41*, *54*, *69*, and *71* are strongly up-regulated under heat treatment, but are down-regulated under cold treatment. Conversely, *BrPUB18*, *19*, *21*, *33*, *62*, *67*, *79*, *86* and *100* are strongly up-regulated under cold treatment, but are down-regulated under heat treatment. Two *PUB-ARM* genes, *AtPUB18* and *19* in Arabidopsis, were found to be expressed transiently under ABA treatment (Yee and Goring 2009). In this study, several *BrPUB-ARM* genes were up-regulated under ABA treatment (Fig. 2b, c), including *BrPUB6* and *54*, which are most closely related to *AtPUB19* and *18*, respectively. Interestingly, other *BrPUB* genes were strongly up-regulated under ABA treatment, including *BrPUB71* and *100* (Fig. 2c). *BrPUB72* was expressed at relatively high levels under GA and PEG treatments (6 and 12 h). These results further support the hypothesis that *PUB* genes play important roles in the face of various environmental stresses.

In summary, a total of 101 *PUB* genes were identified in the Chinese cabbage genome. We used an improved method which was based on protein domain and phylogenetic analyses to classify the PUB proteins from Chinese cabbage and *A. thaliana*—a method that is of value in the study of other species. This classification is likely to assist in clarifying a molecular genetic basis for determining the functions of PUB proteins in the ubiquitin-mediated proteolysis pathway and to provide functional gene resources for transgenic research. Our results suggest that Group VI and VII *PUB* genes, which have received little attention, are preferentially retained compared with Group V genes based on the gene-dosage hypothesis. In addition, the expansion of the *PUB* genes in *B. rapa* was attributed to segmental duplications rather than tandem duplications. Tissue expression pattern analysis of the *PUB* genes, together with the expression patterns of *BrPUB* genes under different abiotic stresses, provided a basic resource for the examination of the molecular regulation of Chinese cabbage development and stress resistance. Our study is the first systematic and comprehensive analysis of PUB genes in Chinese cabbage, but the functional mechanisms of *BrPUBs* need to be further explored. Nevertheless, this study provides a preliminary exploration of *BrPUBs* and lays the foundation for the study of PUB genes in other species.

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Conflict of interest All authors declare that they have no conflict of interest.

Ethical standard This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent Informed consent was obtained from all individual participants included in the study.

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