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The U-box family genes in *Medicago truncatula*: Key elements in response to salt, cold, and drought stresses

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Abstract

The ubiquitination pathway regulates growth, development, and stress responses in plants, and the U-box protein family of ubiquitin ligases has important roles in this pathway. Here, 64 putative U-box proteins were identified in the *Medicago truncatula* genome. In addition to the conserved U-box motif, other functional domains, such as the ARM, kinase, KAP, and WD40 domains, were also detected. Phylogenetic analysis of the *M. truncatula* U-box proteins grouped them into six subfamilies, and chromosomal mapping and synteny analyses indicated that tandem and segmental duplications may have contributed to the expansion and evolution of the U-box gene family in this species. Using RNA-seq data from *M. truncatula* seedlings subjected to three different abiotic stresses, we identified 33 stress-inducible plant U-box genes (*MtPUBs*). Specifically, 25 salinity-, 15 drought-, and 16 cold-regulated *MtPUBs* were detected. Among them, *MtPUB10*, *MtPUB17*, *MtPUB18*, *MtPUB35*, *MtPUB42*, and *MtPUB44* responded to all three stress conditions. Expression profiling by qRT-PCR was consistent with the RNA-seq data, and stress-related elements were identified in the promoter regions. The present findings strongly indicate that U-box proteins play critical roles in abiotic stress response in *M. truncatula*.

Introduction

Ubiquitin-mediated proteolysis is required for most cellular processes, and the pathway is mediated by three sequential ubiquitination enzymes, E1, E2, and E3. E3 ubiquitin ligases are of particular importance as they confer substrate specificity that catalyzes the attachment of ubiquitin to protein targets [1,2]. The E3 ligases can be categorized into distinct families based on their protein domains (RING, HECT, or U-box domains) or mode of action [3,4]. The U-box E3 ligases, of which there are 64 members in *Arabidopsis*, were identified most recently and comprise the smallest E3 ligase family [5]. They have an approximately 70-amino-acid conserved U-box motif, which is present in U-box E3 ligases from yeast to humans [6]. A large

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expansion of the U-box gene family occurred in plants, which may be attributable to biological processes that are unique to the plant life cycle. It has been reported that plant U-box (PUB) proteins are largely involved in abiotic and biotic stress responses [7].

The *Arabidopsis* PUB protein AtCHIP plays an important role in temperature stress tolerance [8]. U-BOX17, another *Arabidopsis* PUB protein, and its tobacco homolog ACRE276 have been identified as positive regulators of cell death and defense [9], and subsequent studies yielded similar findings for the functions of these PUB proteins. AtPUB22 and AtPUB23 were found to have critical combinatory roles in response to drought stress [10], and they directly ubiquitinate RPN6, a 26S proteasome lid subunit, for subsequent degradation in *Arabidopsis* [7]. Similarly, AtPUB18 has a function linked to that of AtPUB19 in the negative regulation of ABA-mediated drought stress responses [11]. AtPUB13 acts as a node that connects flowering time regulation and salicylic acid (SA)-dependent defense signaling in *Arabidopsis* [12]. AtPUB30 acts in salt stress tolerance as a negative factor whose activity during germination is ABA independent [13]. The roles of PUBs in response to abiotic stresses have also been shown in other plants. For example, rice (*Oryza sativa*) *Spotted leaf11* (*Spl11*) encodes a U-box-containing E3 ligase and negatively regulates plant cell death and defense [14]. OsPUB15 helps reduce cellular oxidative stress during seedling establishment [15], and its ARM repeat domain is essential for its physical interaction with the kinase domain of PID2 (PID2K), an interaction observed both *in vitro* and *in vivo* [16]. *OsUPS*, another gene encoding a U-box-containing E3 ligase, responds to phosphate starvation in rice [17]. In hot pepper (*Capsicum annuum* L. cv. Pukang), CaPUB1 has been implicated in counteracting dehydration and high-salinity stress [18].

Efforts have been made to characterize these U-box genes in plant species as well as algae. Thus far, 30 full-length U-box genes have been identified in the *Chlamydomonas reinhardtii* genome sequence [19]. In *Arabidopsis* and rice, 64 and 77 U-box genes have been identified, respectively [20,21]. However, U-box genes have not been studied in the model legume *M. truncatula*. Here, we present a comprehensive analysis of the genes encoding U-box family proteins in *M. truncatula*.

Materials and methods

Identification of PUB proteins

Putative PUB proteins were identified in the *M. truncatula* genome database (http://www.medicagohapmap.org/tools/Blastform) using the BLAST program and the amino acid sequences of published U-box proteins as queries. The proteins identified by the BLAST program were used for domain searches from the Pfam (http://www.sanger.ac.uk/Software/Pfam/) and SMART (http://smart.embl-heidelberg.de/) databases with an E-value cut-off level of 1.0 or 10. These cut-off values were recommended for more reliable search results. Using the Pfam/SMART databases, the C-terminal domain of each PUB protein was analyzed with an E-value cut-off level of 1.0.

Alignments, phylogenetic analysis, intron/exon organization, and localization of PUB genes on chromosomes

The U-box domain (PF00646) was obtained from the Pfam database, and HMMER 3.0 (http://hmmer.janelia.org/) was used for U-box motif identification in each PUB protein. Clustal X (version 2.0; http://www.clustal.org/) was used for the multiple sequence alignment of all predicted U-box protein motifs. A neighbor-joining (NJ) tree was constructed by MEGA (version 5.1; Tamura et al. 2011), using the p-distance method with gaps treated by pairwise deletion

and a 1,000 bootstrap replicate. Intron/exon organization was determined using the *M. truncatula* genome database (http://www.medicagohapmap.org/home/view), and chromosomal maps were generated using the Genome Pixelizer Tcl/Tk script [www.atgc.org/GenomePixelizer (released 02/15/2002)]. Gene duplication was defined according to the following criteria: (1) The length of the sequence alignment covered ≥80% of the longest gene, and (2) the similarity of the aligned gene regions was ≥70% [22,23].

Promoter element analysis

To investigate *cis* elements in the promoter sequences of the U-box protein-encoding genes, the 1,500 bp DNA sequences upstream of the transcriptional start site were obtained from NCBI (http://www.ncbi.nlm.nih.gov/). The PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) was used to identify *cis* elements in the promoters and to collect data for the following: ABRE, ARE, AuxRR core, CGTCA motif, ERE, GARE motif, HSE, LTR, MBS, P Box, TC-rich repeat, TCA element, TGA element, and TGACG motif.

Plant materials and stress treatments

M. truncatula seeds were soaked with distilled water and placed on a plastic net floating on 1/4-strength Hoagland nutrient solution (1.0 mM Ca^{2+}, 1.5 mM K^{+}, 0.5 mM Mg^{2+}, 0.25 mM NH_4^{+}, 3.5 mM NO_3^{-}, 0.25 mM $H_2PO_4^{-}$, 0.523 mM SO_4^{2-}, 22 μM Fe^{2+}, 0.30 μM Cu^{2+}, 0.8 μM Zn^{2+}, 9 μM Mn^{2+}, 46 μM BO_3^{3-}, 0.1 μM MoO_4^{2-}). After germination, seedlings were grown under the following conditions for 4 weeks: 22–24˚C, 200 μmol $m^{-2}s^{-1}$ photosynthetic active radiation, and a photoperiod of 14/10 h (day/night).

Four weeks after germination, seedlings were subjected to various treatments. For drought treatment, the seedlings were transferred to dry Whatman 3MM paper in a sterile petri dish for 0, 2, 6, and 12 h. For cold treatment, the seedlings were transferred to 4˚C for 0, 2, 6, and 12 h. For salt treatment, the seedlings were transferred to solutions containing 300 mM NaCl for 0, 2, 6, and 12 h. After treatment, the seedlings were harvested, immediately frozen in liquid nitrogen, and stored at -80˚C for further analysis.

Statistical analysis

Experiments in the study were independently performed in triplicate. Each result in this study is the mean of at least three replicated treatments and each treatment contained at least 10 plants. The significant differences between treatments were statistically assessed by standard deviation and one-way analysis of variance (ANOVA). The data between differently treated groups were compared statistically by ANOVA, followed by the least significant difference (LSD) test if the ANOVA result was significant at $P<0.05$.

Library construction and sequencing

For RNA-seq analyses, RNA was extracted using Trizol. The 3'-tag digital gene expression libraries were prepared using the Illumina Gene Expression Sample Prep Kit based on the method described by Zhou *et al.* [24]. Deep sequencing were carried out using the Illumina HiSeq 3000 platform (Illumina, San Diego, CA, USA) following the manufacturer's instructions by Genergy Biotechnology Co. Ltd. (Shanghai, China). The raw data comprised 100-bp paired-end sequences, and the cleaned reads were then mapped to Arabidopsis thaliana genome (TAIR10) using default settings of TOPHATv2.0.8. The duplicated reads were removed and alignments with MAPQ score > 20 were used for further analysis. RNA-seq

alignments were processed using HTSeq-count, and differentially expressed genes were identified using DESeq with $|\log_2$ fold change$| > 3.5$.

Results

Identification and homology analysis of U-box proteins in *M. truncatula*

U-box domains (PF04564) were downloaded from the Pfam database and used as queries to identify U-box proteins in the *M. truncatula* genome database (http://www.medicagohapmap.org/tools/Blastform) using the BLAST program (HMMER 3.0, http://hmmer.janelia.org/). The identified proteins were used for a domain search of the Pfam (http://www.sanger.ac.uk/Software/Pfam/) and SMART (http://smart.embl-heidelberg.de/) databases with an E-value cut-off level of 1.0 or 10, which was recommended for more reliable search results. Using the Pfam/SMART databases, the C-terminal domains of each U-box protein with an E-value cut-off level of 1.0 were analyzed. We found 64 proteins containing at least one U-box motif in *M. truncatula* as annotated by the SMART/Pfam databases, and these proteins were designated as U-box proteins (MtPUB) (Table 1 and S1 Table). The isoelectric point (pI) bias of most of these U-box proteins was neutral. Only MtPUB10 and MtPUB11 had a pI greater than 10, and only MtPUB62 had a pI less than 5 (Table 1). Some of the genes encoding these U-box proteins had numerous introns; for example, *MtPUB9*, *MtPUB39*, *MtPUB47*, and *MtPUB64* all had more than 10 introns (Table 1).

Analysis of the functional domains of the *M. truncatula* U-box proteins

U-box proteins often contain several other functional domains at their N- or C-terminal regions. The SMART and Pfam database searches revealed that the U-box proteins contained several known or unknown conserved domains, which presumably participate in substrate recognition, and we designated these domains as functional domains (Fig 1). The types of functional domains in the U-box proteins are listed in Table 1. The 36 U-box proteins with one or more known functional domains were as follows, with the number in parentheses indicating the number of proteins: ARM(17), Armadillo/beta-catenin-like repeat; Kinase(8), protein tyrosine kinase; KAP(4), kinesin-associated protein; WD40(2), WD40 domain, G-beta repeat; USP-Kinase(1); Ufd2p(1), ubiquitin elongating factor core; TPR (1), TPR repeats; HEAT(1), HEAT repeats; and Pro isomerase(1), cyclophilin-type peptidyl-prolyl cis-trans isomerase/CLD. Some U-box proteins had no other obvious interaction domains or had a few rare or functionally uncertain domains; all of these were classified together as 'Unknown' (Fig 1).

Aside from the U-box motif, the ARM (Armadillo/beta-catenin-like repeat) domain, an approximately 40-amino-acid tandemly repeated sequence motif, was the most highly represented functional domain among the identified MtPUB proteins. In beta-catenin, these tandem repeats form a super-helix of helices that presumably mediates ligand interaction (Fig 1). U-box-ARM proteins have been reported in *Arabidopsis*. For example, *AtPUB18* and *AtPUB19* have related functions in negatively regulating ABA-mediated drought stress response [11]. The homologs of *AtPUB18* and *AtPUB19* in *M. truncatula* are *MtPUB35* and *MtPUB42* (S1 Fig). In *Medicago truncatula*, *MtPUB35* and *MtPUB42* have high sequence similarities with *AtPUB18* and *AtPUB19* (S1 Fig). *MtPUB32* also has high sequence similarity with *AtPUB13*, which encodes a U-box-ARM protein that links the flowering time and SA-dependent defense signaling pathways in Arabidopsis [12] (S1 Fig and S1 Table). U-box-ARM protein *AtPUB30* acts in salt stress tolerance as a negative factor independent of ABA during seed germination [13], and it is homologous to *MtPUB38* (S1 Fig). In rice, the U-box-ARM E3 ligase SPL11 negatively regulates plant cell death and defense[14]. OsPUB15, another U-box-ARM protein in rice, helps reduce cellular oxidative stress during seedling establishment [15]. *OsPUB15* is homologous to *MtPUB29* in *M. truncatula* (S1 Fig).

Table 1. Distribution of *MtPUB* genes in the *Medicago truncatula* genome.

S.No	Gene_ID	Accession number	Other domain	Predicted protein (aa)	Mol wt (kDa)	pI	Chromosome	No. of introns
1	*MtPUB1*	Medtr1g017770.1	Unknown	434	48.39	6.88	1	0
2	*MtPUB2*	Medtr1g056840.1	Unknown	411	46.09	8.47	1	1
3	*MtPUB3*	Medtr1g056870.1	Unknown	437	48.80	6.95	1	0
4	*MtPUB4*	Medtr1g056880.1	Unknown	437	48.90	7.91	1	0
5	*MtPUB5*	Medtr1g056910.1	Unknown	406	46.25	8.54	1	0
6	*MtPUB6*	Medtr1g069845.1	ARM	608	66.67	6.52	1	4
7	*MtPUB7*	Medtr1g076400.1	Unknown	1013	112.48	5.09	1	3
8	*MtPUB8*	Medtr1g079450.1	Unknown	446	49.65	8.05	1	1
9	*MtPUB9*	Medtr1g090320.1	WD40	1488	166.85	5.96	1	16
10	*MtPUB10*	Medtr1g093965.1	Unknown	200	21.84	10.05	1	3
11	*MtPUB11*	Medtr1g093995.1	Unknown	200	21.86	10.05	1	3
12	*MtPUB12*	Medtr1g094025.1	Unknown	296	33.24	8.14	1	3
13	*MtPUB13*	Medtr1g094215.1	ARM	447	48.10	6.13	1	3
14	*MtPUB14*	Medtr1g100820.1	Kinase	715	80.80	5.44	1	7
15	*MtPUB15*	Medtr2g007630.1	Unknown	259	28.73	9.58	2	3
16	*MtPUB16*	Medtr2g011140.1	Unknown	383	42.16	6.77	2	0
17	*MtPUB17*	Medtr2g018010.1	ARM	720	78.67	6.58	2	0
18	*MtPUB18*	Medtr2g087350.1	Unknown	403	45.33	8.61	2	0
19	*MtPUB19*	Medtr2g096850.1	Kinase	810	91.60	7.01	2	6
20	*MtPUB20*	Medtr3g008270.1	Kinase	797	88.60	6.48	3	9
21	*MtPUB21*	Medtr3g008280.1	Kinase	809	90.02	7.21	3	9
22	*MtPUB22*	Medtr3g065080.1	Unknown	439	49.13	8.08	3	0
23	*MtPUB23*	Medtr3g078160.1	Unknown	681	75.94	8.29	3	0
24	*MtPUB24*	Medtr3g078340.1	ARM	529	57.91	7.03	3	0
25	*MtPUB25*	Medtr3g085610.1	KAP	766	84.92	6.10	3	5
26	*MtPUB26*	Medtr3g095730.1	Unknown	419	46.77	8.92	3	0
27	*MtPUB27*	Medtr3g096370.1	Unknown	404	45.02	6.35	3	0
28	*MtPUB28*	Medtr3g115670.1	HEAT	814	89.47	5.06	3	3
29	*MtPUB29*	Medtr3g466220.1	ARM	836	90.68	5.45	3	3
30	*MtPUB30*	Medtr4g028960.1	ARM	701	76.42	6.83	4	0
31	*MtPUB31*	Medtr4g051515.1	Unknown	413	47.12	9.26	4	0
32	*MtPUB32*	Medtr4g063800.1	ARM	662	72.09	5.14	4	3
33	*MtPUB33*	Medtr4g085720.1	Unknown	410	45.35	7.81	4	0
34	*MtPUB34*	Medtr4g091880.1	Unknown	375	40.59	8.36	4	0
35	*MtPUB35*	Medtr4g107010.1	ARM	747	83.52	8.04	4	1
36	*MtPUB36*	Medtr4g125930.1	Kinase	764	85.49	6.00	4	8
37	*MtPUB37*	Medtr4g485520.1	ARM	652	70.78	7.01	4	3
38	*MtPUB38*	Medtr5g015210.1	Unknown	451	49.54	6.50	5	0
39	*MtPUB39*	Medtr5g015500.1	Pro isomerase	552	59.75	7.65	5	10
40	*MtPUB40*	Medtr5g020570.1	KAP	782	88.26	6.44	5	5
41	*MtPUB41*	Medtr5g032010.1	Kinase	808	92.93	7.98	5	8
42	*MtPUB42*	Medtr5g034440.1	ARM	689	76.82	7.19	5	0
43	*MtPUB43*	Medtr5g048050.1	Unknown	438	50.05	6.88	5	0
44	*MtPUB44*	Medtr5g077510.1	Unknown	442	49.45	8.53	5	0
45	*MtPUB45*	Medtr5g083030.1	ARM	694	76.93	6.78	5	0
46	*MtPUB46*	Medtr6g008170.1	KAP	554	61.48	8.22	6	0

(*Continued*)

Table 1. (*Continued*)

S.No	Gene_ID	Accession number	Other domain	Predicted protein (aa)	Mol wt (kDa)	pI	Chromosome	No. of introns
47	*MtPUB47*	Medtr6g013690.1	Ufd2p	1047	11.80	5.47	6	15
48	*MtPUB48*	Medtr6g071340.1	Unknown	418	47.61	5.56	6	0
49	*MtPUB49*	Medtr7g005940.1	Unknown	1073	12.18	7.21	7	8
50	*MtPUB50*	Medtr7g053260.1	Unknown	459	51.53	8.39	7	1
51	*MtPUB51*	Medtr7g059405.1	ARM	634	69.82	6.27	7	4
52	*MtPUB52*	Medtr7g077780.1	Kinase	896	100.38	6.02	7	8
53	*MtPUB53*	Medtr7g078330.1	ARM	646	72.78	5.05	7	3
54	*MtPUB54*	Medtr7g097020.1	ARM	767	84.39	7.44	7	5
55	*MtPUB55*	Medtr7g106340.1	Unknown	421	46.96	8.71	7	0
56	*MtPUB56*	Medtr7g116600.1	Unknown	460	51.32	8.35	7	1
57	*MtPUB57*	Medtr7g117890.1	ARM	1001	111.25	5.30	7	4
58	*MtPUB58*	Medtr8g011720.1	TPR	277	31.95	6.38	8	7
59	*MtPUB59*	Medtr8g027140.1	Unknown	1006	112.03	5.62	8	4
60	*MtPUB60*	Medtr8g068530.1	Kinase	769	88.97	5.81	8	7
61	*MtPUB61*	Medtr8g077205.1	KAP	760	85.19	6.66	8	4
62	*MtPUB62*	Medtr8g080280.1	Unknown	767	85.42	4.87	8	5
63	*MtPUB63*	Medtr8g092870.1	Unknown	418	46.35	7.48	8	0
64	*MtPUB64*	Medtr8g103227.1	WD40	1335	148.78	5.64	8	14

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Eight MtPUB proteins were found to have a kinase domain, indicating their potential involvement in signal transduction cascades via phosphorylation. The KAP (kinesin-associated protein) domain, found in four MtPUB proteins, is associated with motor function, consistent with the role of kinesins as intracellular multimeric transport motor proteins that move cellular cargo on microtubule tracks.

Two MtPUB proteins had WD40 domains. WD40 domain-containing proteins are made up of highly conserved repeating units approximately 40 amino acids long and usually ending with Trp-Asp (WD) [25]. They are found in all eukaryotes but not in prokaryotes, and they regulate numerous cellular functions, such as cell division, cell-fate determination, gene transcription, transmembrane signaling, mRNA modification, and vesicle fusion. The USP, Ufd2p, TPR, HEAT, and Pro isomerase domains were each present in only one MtPUB protein (Fig 1). WD40 and TPR domains are known to be involved in protein interactions [26,27]. Rice and *Arabidopsis* U-box proteins containing WD40 repeats are homologous to animal and human Prp19p proteins and are involved in preRNA splicing and other biological processes [7,28,29]. AtCHIP, the only TPR domain-containing U-box protein in *Arabidopsis*, is homologous to the mammalian CHIP (carboxyl terminus of Hsc70-interacting protein) and participates in abiotic stress response and the regulation of chloroplast protein turnover [30,31]. In humans and animals, CHIP interacts with molecular chaperones, such as Hsp70 and Hsp90, and acts as a partner in the cell to ensure protein stability. CHIP is involved in cell stress protection and several neurodegenerative diseases [32,33]. The homolog of *AtCHIP* in *M. truncatula* is *MtPUB58* (S1 Fig).

Phylogenetic and evolutionary analysis of U-box proteins in *M. truncatula*

For the phylogenetic analysis of the identified U-box proteins, we used HMMER 3.0 software (http://hmmer.janelia.org/) to analyze the motif sequences of each U-box protein. All of the U-

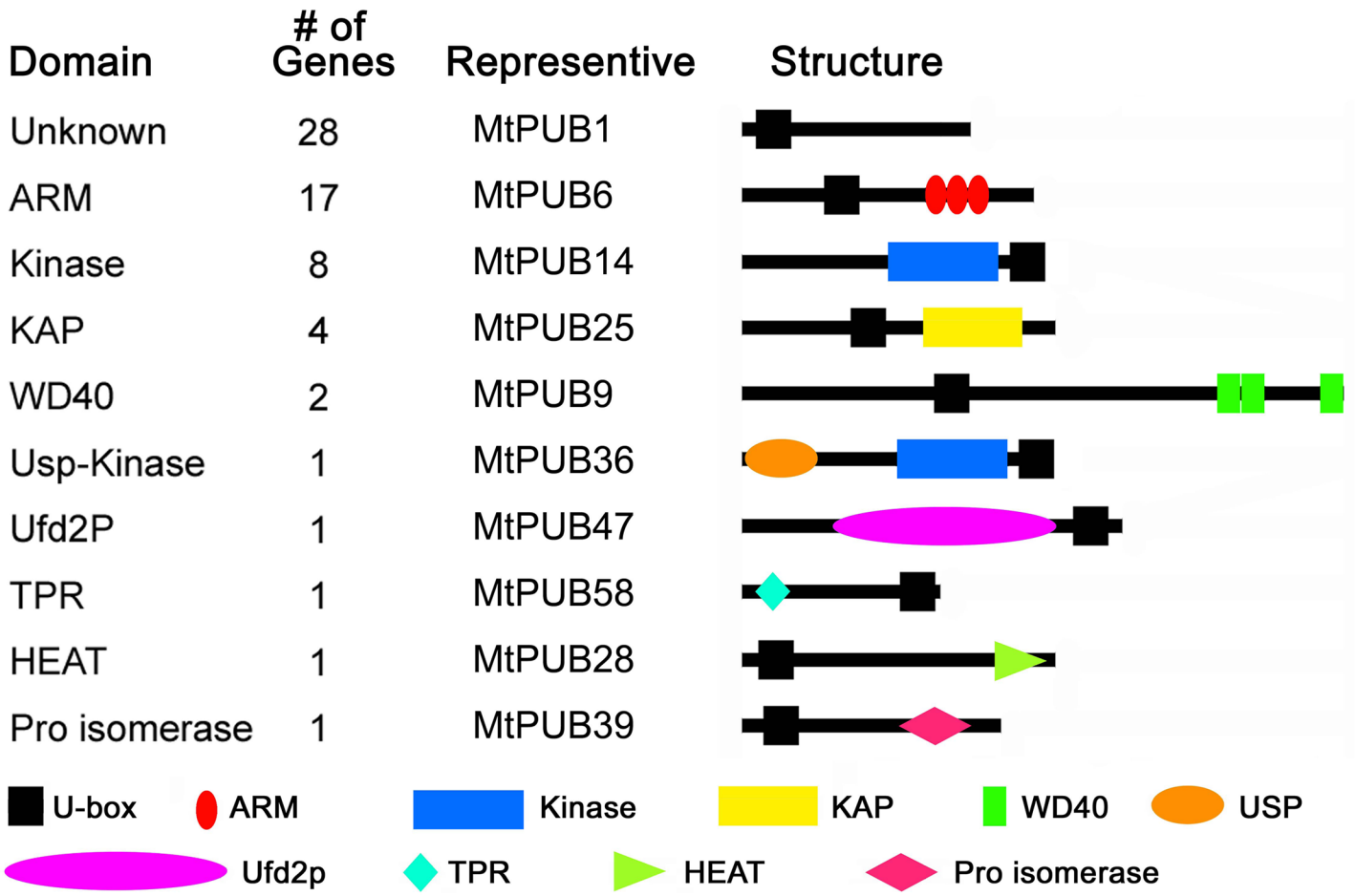

Fig 1. Number and domain structure of U-box proteins in *Medicago truncatula*. Shown on the left are the types of functional domains and the number of U-box proteins predicted to have those domains. The domain names are taken from the Pfam or SMART database. Domain abbreviations: Unknown, U-box proteins that have no obvious N- or C-terminal interaction domain or have rare or functionally uncertain domains; ARM, Armadillo/beta-catenin-like repeat; Kinase, protein tyrosine kinase; KAP, kinesin-associated protein; WD40, WD40 domain, G-beta repeat; USP-Kinase; Ufd2p, ubiquitin elongating factor core; TPR2, TPR repeats; HEAT, HEAT repeats; Pro isomerase, cyclophilin-type peptidyl-prolyl cis-trans isomerase/CLD.

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box proteins were found to contain only one U-box motif. Using the U-box motif sequence for the alignment, an unrooted phylogenetic tree of the entire dataset was created (Fig 2). The phylogenetic tree divided the 64 MtPUB proteins into six subfamilies according to the distribution of various branches, the length of each branch, and the phylogenetic relationship between MtPUB proteins.

The phylogenetic tree was color-coded according to the different functional domains (Fig 2). Most of the kinase domain-containing MtPUB proteins were in the G6 family. The ARM-containing MtPUB proteins generally localized in clades within the G1 family. This correlation further supports the phylogenetic relationships in the U-box tree and suggests a co-evolution of the U-box motif with other domains.

Locations of the U-box protein-encoding genes on *M. truncatula* chromosomes

The U-box protein-encoding genes were distributed randomly on all eight *M. truncatula* chromosomes. To determine whether the gene family in *M. truncatula* evolved through duplication

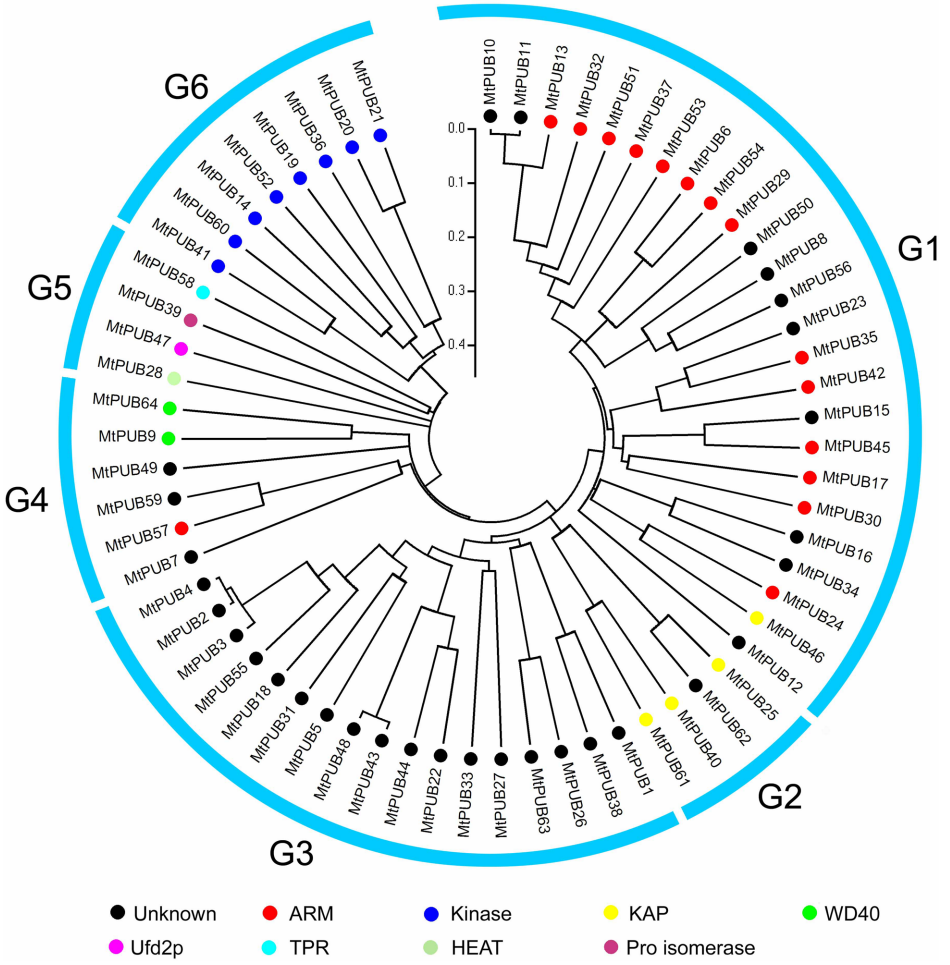

Fig 2. Phylogenetic tree of the U-box protein family from *Medicago truncatula*. The 70-amino-acid U-box motifs from the 64 putative U-box proteins were aligned by CLUSTAL X 2.0, and the unrooted NJ phylogenetic tree was constructed by MEGA 5.1, using the p-distance method and a bootstrap value of 1,000. The six groups identified from the phylogenetic analysis are marked on the outside. The bar represents the branch length equivalent to 0.05 amino acid changes per residue. Table 1 provides additional information for the corresponding genes.

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events, we obtained the chromosomal locations of the U-box protein-encoding genes from the *M. truncatula* genomic database and mapped the loci on the chromosomes (Fig 3). With 14 U-box genes, chromosome 1 had the largest number, whereas chromosome 6 had only three U-box genes. Some U-box genes were arranged in tandem repeats either in the same or inverse orientation, representing local gene duplications. As shown in Fig 3, there were four segmental duplication events between chromosomes, suggesting that tandem duplications of chromosomal regions played a major role in the expansion of this gene family.

Expression analysis of U-box protein-encoding genes in various tissues

Using an existing database (http://mtgea.noble.org/v2/), we were able to survey the expression of many *MtPUB* genes in different tissues. A few *MtPUBs* were expressed only in certain tissues. For example, *MtPUB18* and *MtPUB49* were mainly expressed in roots; *MtPUB40* expression was largely restricted to leaves and roots; *MtPUB27* was expressed in flowers and pods;

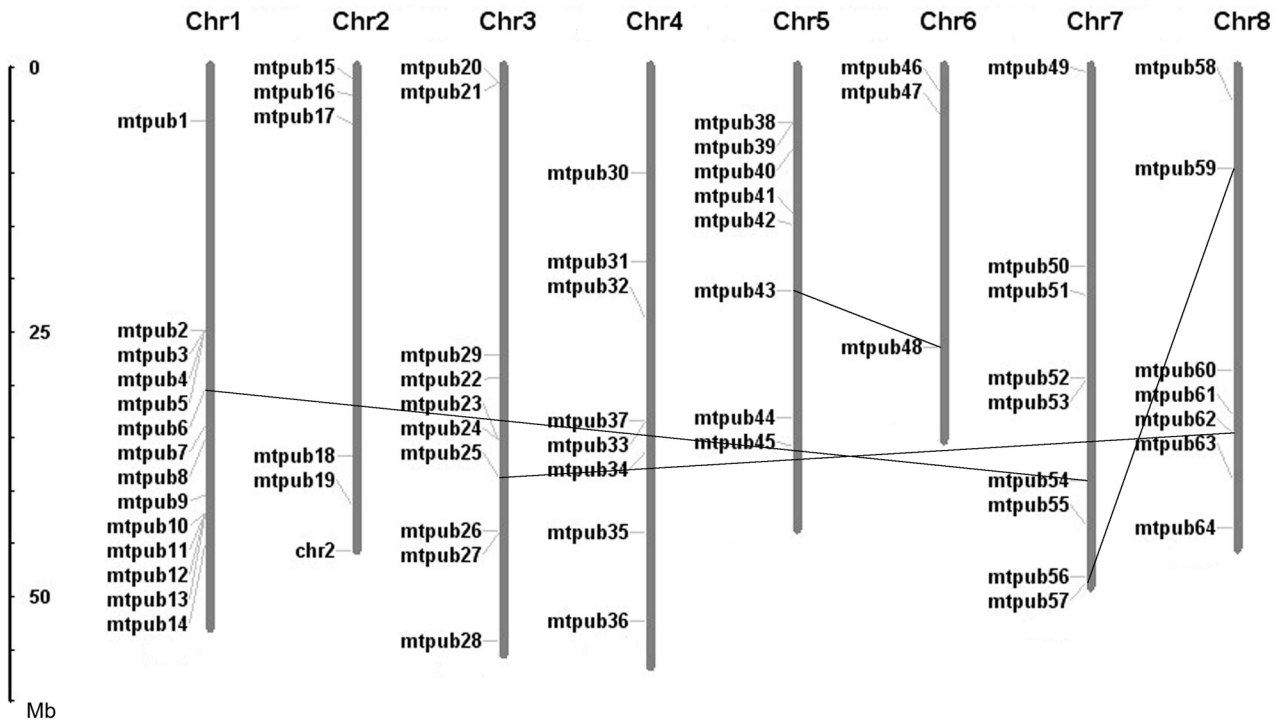

Fig 3. Locations and duplications of *Medicago truncatula* U-box genes on chromosomes 1–8. Genes lying on duplicated segments of genome have been joined by lines. The scale represents megabases (Mb). The chromosome numbers are indicated at the top of each bar.

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and *MtPUB44* was expressed in roots and mature seeds (Fig 4A). Because legume root nodules plays an important role in symbiotic nitrogen fixation, we also identified *MtPUBs* that were differentially expressed in the nodule. Strong expression of *MtPUB42* and *MtPUB47* could be seen in root nodules, while the expression of *MtPUB18*, *MtPUB40*, and *MtPUB49* in root nodules was low (Fig 4B).

Identification of stress-responsive *MtPUBs*

To study the expression of the U-box family genes under abiotic stress, 4-week-old *M. truncatula* seedlings were collected and treated with drought, salt, or cold stress for 0, 2, 6, and 12 h. Total RNA was extracted, and libraries were constructed for RNA-seq. In general, under drought, salt, and cold stress, there were more up-regulated genes than down-regulated genes, and the difference was most obvious at 2 h (S2 Fig). Salt stress had the strongest correlation with drought stress, and the R value was more than 0.95 at 2, 6, and 12 h (S3 Fig). The analysis showed that some of the 64 U-box family genes could be induced by salt, drought, or cold stress, but a few genes were down-regulated (Fig 5, Tables 2–4). After drought treatment, *MtPUB1*, *MtPUB7*, *MtPUB10*, *MtPUB13*, *MtPUB17*, *MtPUB18*, *MtPUB22*, *MtPUB31*, *MtPUB35*, *MtPUB42*, *MtPUB43*, *MtPUB44*, *MtPUB52*, *MtPUB57*, and *MtPUB59* were up-regulated (Table 2). (A gene was considered up-regulated if its expression was increased at 2, 6, and 12 h and if the $\log_2$ fold change > 1 for at least one of these time points.) Using the same criteria, we found that *MtPUB1*, *MtPUB8*, *MtPUB10*, *MtPUB15*, *MtPUB17*, *MtPUB18*, *MtPUB23*, *MtPUB25*, *MtPUB26*, *MtPUB31*, *MtPUB33*, *MtPUB34*, *MtPUB35*, *MtPUB42*, *MtPUB43*, *MtPUB44*, *MtPUB48*, *MtPUB51*, *MtPUB52*, *MtPUB55*, *MtPUB57*, *MtPUB59*, *MtPUB60*, *MtPUB61*, and *MtPUB64* were up-regulated under salt stress (Table 3). After cold treatment, *MtPUB7*, *MtPUB10*, *MtPUB11*, *MtPUB12*,

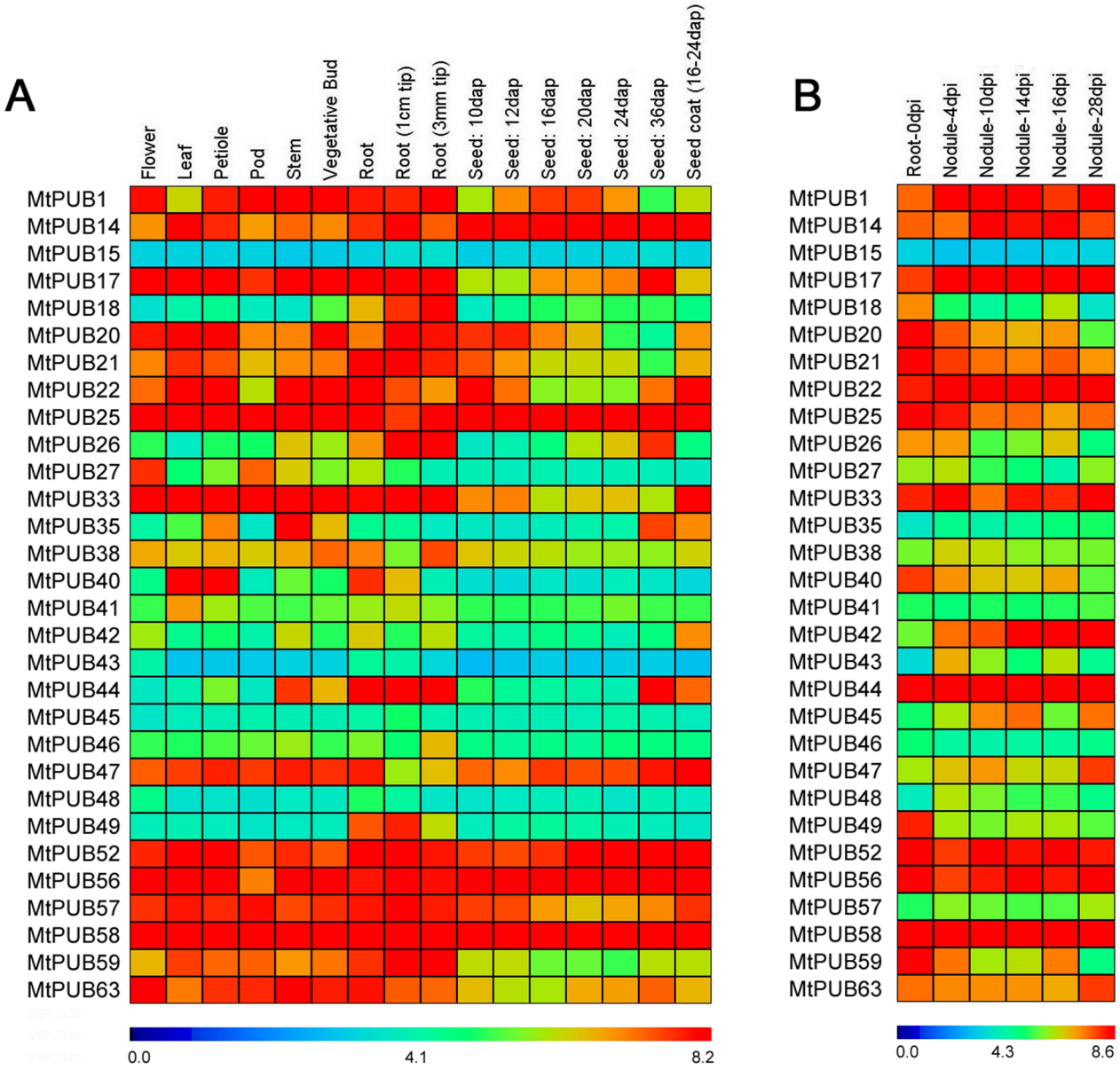

Fig 4. Expression profiles of *Medicago truncatula* U-box protein-encoding genes during panicle development (A) and root development (B). The average log signal values of U-box protein-encoding genes in various tissues/organs and developmental stages (mentioned at the top of each lane) are presented. The data comes from this site (http://mtgea.noble.org/v2/annotation_search_form.php#gid). The color scale (representing log signal values) is shown at the bottom. dap: days after pollination.

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MtPUB17, *MtPUB18*, *MtPUB22*, *MtPUB25*, *MtPUB29*, *MtPUB33*, *MtPUB35*, *MtPUB42*, *MtPUB44*, *MtPUB45*, *MtPUB56*, and *MtPUB61* were up-regulated (Table 4).

As indicated above, fewer *MtPUB* genes were down-regulated under the analyzed stress conditions. Under drought treatment, *MtPUB2*, *MtPUB3*, *MtPUB4*, *MtPUB6*, *MtPUB9*, *MtPUB11*, *MtPUB20*, *MtPUB27*, *MtPUB40*, *MtPUB46*, and *MtPUB63* were down-regulated (Fig 5B, Table 2). (A gene was considered down-regulated if its expression was decreased at 2, 6, and 12 h and if the log2 fold change < -1 for at least one of these time points). Using the same criteria, *MtPUB2*, *MtPUB3*, *MtPUB4*, *MtPUB40*, *MtPUB46*, and *MtPUB49* were down-regulated under

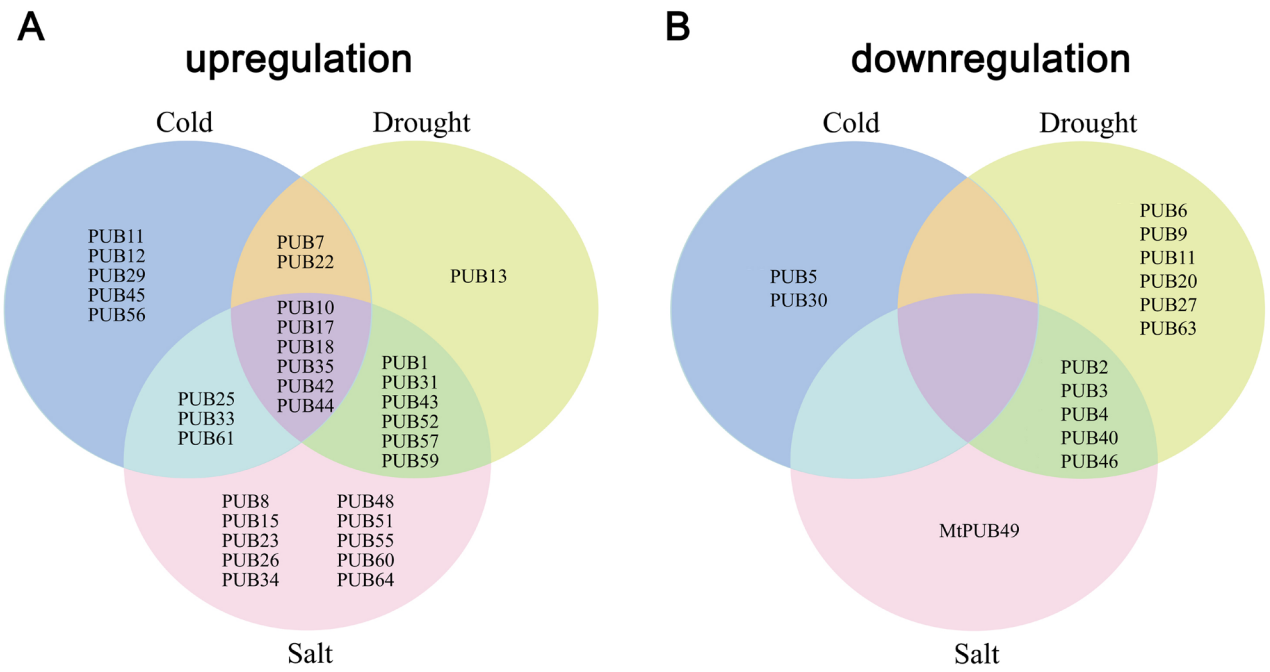

Fig 5. Venn diagram showing common and unique differential *MtPUB* gene expression under three treatment conditions. Among them, 25 high-salinity-, 15 drought-, and 16 cold- up regulated U-box genes were detected and 6 U-box genes were observed to respond remarkably to all three stresses. in contrast, 6 high-salinity-, 11 drought-, and 2 cold- down regulated U-box genes were detected.

https://doi.org/10.1371/journal.pone.0182402.g005

salt stress (Table 3). After cold treatment, only *MtPUB5* and *MtPUB30* were down-regulated. We also identified *MtPUB* genes that were induced by more than one stress condition (Fig 5). For example, *MtPUB10*, *MtPUB17*, *MtPUB18*, *MtPUB35*, *MtPUB42*, and *MtPUB44* were induced by salt, drought, and cold treatment. In addition, *MtPUB2*, *MtPUB3*, *MtPUB4*, *MtPUB40*, and *MtPUB46* were down-regulated under salt stress and under drought stress (Fig 5).

To verify the above data, we conducted qRT-PCR to examine the expression patterns of 17 *MtPUB* genes under the different stress conditions (Fig 6 and S3 Table). Under drought stress, the transcript levels of the following U-box protein-encoding genes increased: *MtPUB7*, *MtPUB11*, *MtPUB18*, *MtPUB22*, *MtPUB31*, *MtPUB35*, *MtPUB42*, *MtPUB43*, *MtPUB44*, *MtPUB45*, and *MtPUB64*. Among these, *MtPUB31*, *MtPUB35*, *MtPUB42*, *MtPUB43*, *MtPUB44*, *MtPUB45*, and *MtPUB64* were strongly induced. *MtPUB35*, *MtPUB42*, *MtPUB43*, *MtPUB44*, and *MtPUB45* were also strongly induced by salt stress treatment. Under cold stress, the transcript levels of *MtPUB3*, *MtPUB4*, *MtPUB22*, *MtPUB35*, *MtPUB42*, *MtPUB43*, *MtPUB44*, *MtPUB45*, and *MtPUB64* increased, and among these, *MtPUB18*, *MtPUB22*, *MtPUB35*, *MtPUB42*, *MtPUB43*, and *MtPUB44* were strongly induced. It is worth noting that the domain analysis identified MtPUB35 and MtPUB42 as U-box-ARM proteins and that U-box-ARM proteins in *Arabidopsis* and rice are known to have important roles in plant stress response [15].

A few *MtPUBs* were down-regulated under stress, including *MtPUB40*, which was down-regulated under all three abiotic stress conditions. *MtPUB3* and *MtPUB4* were down-regulated under drought and salt stress, and *MtPUB2* was down-regulated under drought and cold stress. These data illustrate the consistency between the qRT-PCR and high-throughput sequencing analyses (Fig 5 and Fig 6, Tables 2–4). Some U-box protein-encoding genes were induced by all three stress conditions and may therefore have important roles in response to abiotic stress; however, further study is required to characterize the functions of these and other *MtPUB* genes.

Table 2. Read abundance of *MtPUB* genes in the drought-0, drought-2, drought-6, and drought-12 libraries.

Gene_ID	Drought-0	Drought-2	Drought-6	Drought-12	log_2 (Drought-2/ Drought-0)	log_2 (Drought-6/ Drought-0)	log_2 (Drought-12/ Drought-0)
MtPUB1	79	146	178	111	0.89	1.17*	0.49
MtPUB2	59	27	13	4	-1.11*	-2.17*	-3.91*
MtPUB3	69	41	14	13	-0.76	-2.29*	-2.45*
MtPUB4	158	76	41	14	-1.05*	-1.97*	-3.49*
MtPUB5	62	176	53	55	1.51*	-0.23	-0.16
MtPUB6	299	200	91	131	-0.58	-1.71*	-1.19*
MtPUB7	348	841	784	717	1.27*	1.17*	1.04*
MtPUB8	3	6	1	4	1.07*	-1.53*	0.44
MtPUB9	941	633	624	343	-0.57	-0.59	-1.46*
MtPUB10	10	11	23	35	0.09	1.13*	1.72*
MtPUB11	4	4	1	2	-0.12	-1.94*	-0.64
MtPUB12	5	9	9	7	0.85	0.86	0.51
MtPUB13	463	911	1106	901	0.98	1.26*	0.96
MtPUB14	393	446	539	442	0.18	0.46	0.17
MtPUB15	2	3	2	2	0.47	0.11	0.34
MtPUB16	1	1	1	1	0	0	0
MtPUB17	520	730	1218	905	0.49	1.23*	0.80
MtPUB18	51	155	122	67	1.59*	1.25*	0.38
MtPUB19	526	661	798	914	0.33	0.60	0.80
MtPUB20	449	393	191	232	-0.19	-1.23*	-0.95
MtPUB21	448	478	281	325	0.09	-0.67	-0.46
MtPUB22	119	336	237	176	1.50*	1.00*	0.57
MtPUB23	174	96	452	560	-0.85	1.38*	1.69*
MtPUB24	444	449	484	468	0.02	0.13	0.08
MtPUB25	274	304	286	354	0.15	0.06	0.37
MtPUB26	219	340	233	222	0.63	0.09	0.02
MtPUB27	143	143	69	46	0.00	-1.05*	-1.63*
MtPUB28	1009	1266	1309	1224	0.33	0.38	0.28
MtPUB29	768	883	1182	953	0.20	0.62	0.31
MtPUB30	361	308	313	283	-0.23	-0.21	-0.35
MtPUB31	26	362	60	36	3.82*	1.23*	0.49
MtPUB32	1348	1690	1839	1330	0.33	0.45	-0.02
MtPUB33	159	328	150	165	1.04*	-0.08	0.05
MtPUB34	238	209	254	251	-0.19	0.09	0.07
MtPUB35	47	1924	3780	3587	5.34*	6.32*	6.24*
MtPUB36	461	771	700	702	0.74	0.60	0.61
MtPUB37	561	394	355	321	-0.51	-0.66	-0.81
MtPUB38	169	233	97	81	0.47	-0.81	-1.05*
MtPUB39	415	462	571	540	0.15	0.46	0.38
MtPUB40	688	662	176	184	-0.06	-1.97*	-1.91*
MtPUB41	405	361	681	712	-0.16	0.75	0.81
MtPUB42	36	439	1544	912	3.61*	5.42*	4.66*
MtPUB43	10	70	10	27	2.87*	0.04	1.52*
MtPUB44	226	1168	693	341	2.37*	1.62*	0.60
MtPUB45	6	21	9	5	1.90*	0.60	-0.09
MtPUB46	208	95	109	96	-1.13*	-0.94	-1.11*

(*Continued*)

Table 2. (*Continued*)

Gene_ID	Drought-0	Drought-2	Drought-6	Drought-12	log_2 (Drought-2/ Drought-0)	log_2 (Drought-6/ Drought-0)	log_2 (Drought-12/ Drought-0)
MtPUB47	1422	1781	2647	2632	0.32	0.90	0.89
MtPUB48	32	99	32	45	1.62*	-0.02	0.47
MtPUB49	279	153	160	143	-0.87	-0.80	-0.97
MtPUB50	178	126	136	154	-0.50	-0.39	-0.21
MtPUB51	119	110	113	127	-0.10	-0.07	0.09
MtPUB52	806	1059	1823	2081	0.39	1.18*	1.37*
MtPUB53	1	1	1	1	0	0	0
MtPUB54	222	156	237	157	-0.51	0.10	-0.50
MtPUB55	96	436	89	29	2.19*	-0.11	-1.74*
MtPUB56	261	284	156	220	0.12	-0.74	-0.24
MtPUB57	832	1663	1887	1736	1.00*	1.18*	1.06*
MtPUB58	230	300	244	241	0.38	0.09	0.07
MtPUB59	328	584	609	728	0.83	0.89	1.15*
MtPUB60	1	1	6	1	0	2.70*	0
MtPUB61	262	456	431	470	0.80	0.72	0.84
MtPUB62	1125	1514	1961	1846	0.43	0.80	0.71
MtPUB63	149	135	76	67	-0.14	-0.98	-1.16*
MtPUB64	2	10	16	1	2.41*	3.07*	-0.96

Values indicate number of reads.

* indicates a significant difference in expression compared to the 0 h time point ($P < 0.01$ and $|log_2N| \geq 1$). Drought-0, Drought-2, Drought-6, and Drought-12 indicate 0, 2, 6, and 12 h drought treatment, respectively.

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Stress-associated *cis*-acting elements in *MtPUB* promoters

Cis-regulatory elements and *trans*-acting factors involved in stress-induced gene expression have been extensively analyzed [7]. To identify promoter elements at *MtPUB* loci, we analyzed the 1500 bp upstream promoter sequences of the 64 *MtPUBs* using the PlantCARE database (http://intra.psb.ugent.be:8080/PlantCARE) [34]. The elements listed in S2 Table include several known stress-related elements, including the MYB binding site involved in drought inducibility (MBS), anaerobic induction elements (AREs), heat-stress-responsive elements (HSEs), low-temperature-responsive elements (LTRs), ABA-responsive elements (ABREs), and stress-responsive elements (TC-rich repeats) and so on [35,36]. Among the 64 *MtPUBs*, 27 had ABREs, suggesting they might be involved in ABA-mediated stress response processes. Forty-five *MtPUBs* had AREs, elements involved in the response to hypoxic, low-temperature, and dehydration stresses [37]. The presence of ABREs and AREs in some *MtPUBs* suggests that they might be regulated by stress conditions. For example, we found more than two AREs and ABREs in the promoters of *MtPUB13*, *MtPUB17*, *MtPUB42*, *MtPUB48*, and *MtPUB57*. These findings from the analysis of stress-responsive *cis* elements provide auxiliary evidence that some *MtPUBs* are likely to be involved in the response to abiotic stresses.

Discussion

2.1 U-box family genes structure and evolution

The global identification of U-box genes should help improve the understanding of gene expression and regulatory mechanisms that underlie plant tolerance to abiotic stresses such as salinity, drought, and cold. This study identified 64 U-box genes from *M. truncatula*, which is

Table 3. Read abundance of *MtPUB* genes in the salt-0, salt-2, salt-6, and salt-12 libraries.

Gene_ID	Salt-0	Salt-2	Salt-6	Salt-12	log_2 (Salt-2/Salt-0)	log_2 (Salt-6/Salt-0)	log_2 (Salt-12/Salt-0)
MtPUB1	109	142	158	223	0.38	0.54	1.03*
MtPUB2	44	18	13	24	-1.31*	-1.81*	-0.91
MtPUB3	44	42	13	13	-0.09	-1.81*	-1.78*
MtPUB4	159	81	54	42	-0.97	-1.56*	-1.91*
MtPUB5	61	43	70	147	-0.52	0.20	1.27*
MtPUB6	249	183	242	179	-0.45	-0.04	-0.48
MtPUB7	375	518	578	621	0.46	0.62	0.73
MtPUB8	1	3	1	10	1.73*	0	3.37*
MtPUB9	975	654	837	944	-0.58	-0.22	-0.05
MtPUB10	13	13	37	25	0.01	1.47*	0.91
MtPUB11	1	3	1	1	1.35*	0	0
MtPUB12	1	2	2	1	0.82	0.83	0
MtPUB13	484	809	775	927	0.74	0.68	0.94
MtPUB14	375	509	615	377	0.44	0.71	0.01
MtPUB15	1	9	7	4	3.25*	2.85*	1.87*
MtPUB16	1	1	3	1	0	1.74*	0
MtPUB17	527	752	854	1116	0.51	0.70	1.08*
MtPUB18	34	117	103	171	1.76*	1.58*	2.32*
MtPUB19	587	525	601	526	-0.16	0.04	-0.16
MtPUB20	534	351	428	483	-0.61	-0.32	-0.15
MtPUB21	464	383	408	667	-0.28	-0.18	0.52
MtPUB22	131	242	223	240	0.88	0.76	0.87
MtPUB23	191	217	348	428	0.18	0.86	1.16*
MtPUB24	393	458	450	585	0.22	0.19	0.57
MtPUB25	220	411	397	526	0.90	0.85	1.26*
MtPUB26	237	281	395	797	0.24	0.74	1.75*
MtPUB27	110	63	104	58	-0.80	-0.08	-0.92
MtPUB28	929	1282	1146	1239	0.47	0.30	0.42
MtPUB29	745	963	1003	1122	0.37	0.43	0.59
MtPUB30	419	340	396	989	-0.30	-0.08	1.24*
MtPUB31	28	92	108	367	1.73*	1.97*	3.73*
MtPUB32	1387	1959	2172	2297	0.50	0.65	0.73
MtPUB33	123	264	169	302	1.10*	0.45	1.29*
MtPUB34	177	249	254	388	0.49	0.52	1.13*
MtPUB35	80	1755	1351	1129	4.45*	4.08*	3.82*
MtPUB36	513	703	683	589	0.45	0.41	0.20
MtPUB37	483	491	490	550	0.02	0.02	0.19
MtPUB38	158	106	127	151	-0.58	-0.32	-0.06
MtPUB39	458	508	509	674	0.15	0.16	0.56
MtPUB40	807	220	240	153	-1.88*	-1.75*	-2.40*
MtPUB41	421	314	520	514	-0.42	0.31	0.29
MtPUB42	25	1145	1127	1003	5.49*	5.47*	5.30*
MtPUB43	13	32	34	72	1.27*	1.34*	2.43*
MtPUB44	248	528	507	1083	1.09*	1.03*	2.12*
MtPUB45	5	17	4	1	1.66*	-0.41	-2.45*
MtPUB46	274	104	142	119	-1.40*	-0.94	-1.20*

(*Continued*)

Table 3. (*Continued*)

Gene_ID	Salt-0	Salt-2	Salt-6	Salt-12	log_2 (Salt-2/Salt-0)	log_2 (Salt-6/Salt-0)	log_2 (Salt-12/Salt-0)
MtPUB47	1416	1755	2165	2306	0.31	0.61	0.70
MtPUB48	47	86	83	131	0.88	0.84	1.49*
MtPUB49	272	176	187	94	-0.63	-0.54	-1.53*
MtPUB50	207	208	240	137	0.01	0.21	-0.60
MtPUB51	110	127	168	251	0.20	0.61	1.19*
MtPUB52	908	1296	1477	1886	0.51	0.70	1.05*
MtPUB53	1	1	1	1	0	0	0
MtPUB54	209	191	351	307	-0.13	0.75	0.55
MtPUB55	83	289	93	259	1.79*	0.15	1.64*
MtPUB56	295	268	190	218	-0.14	-0.64	-0.44
MtPUB57	797	1701	1589	2026	1.09*	1.00*	1.35*
MtPUB58	236	298	291	302	0.34	0.30	0.35
MtPUB59	286	585	647	694	1.03*	1.18*	1.28*
MtPUB60	1	9	5	2	3.12*	2.29*	1.22*
MtPUB61	204	319	465	638	0.65	1.19*	1.65*
MtPUB62	1122	1812	1784	1790	0.69	0.67	0.67
MtPUB63	134	84	90	98	-0.68	-0.57	-0.44
MtPUB64	1	10	12	18	3.36*	3.57*	4.19*

Values indicate number of reads.

* indicates a significant difference in expression compared to the 0 h time point ($P < 0.01$ and $|log_2N| \geq 1$). Salt-0, Salt-2, Salt-6, and Salt-12 indicate 0, 2, 6, and 12 h salt treatment, respectively.

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similar to the number identified in *Arabidopsis* (61) (S1 Table) [38] and rice (77) (S1 Table) [5]. Compared to higher plants, there are far fewer U-box proteins in yeast (3) and human (20) [39], indicating an uneven distribution of U-box proteins among species of different kingdoms. Considering the percentage of U-box genes among total genes in the genome, the percentage in *M. truncatula* (0.134%) was lower than that in *Arabidopsis* (0.249%). Through the phylogenetic tree analysis, we found that multiple members in each class of U-box proteins raised the possibility of functional redundancy among the members, such as *MtPUB10* and *MtPUB11* (Fig 2). Such functional redundancy may represent a daunting challenge for the functional characterization of *PUB* genes.

In addition to the U-box domain, other important domains, including the ARM, kinase, KAP, and WD40 domains, were present in the identified proteins. The most highly represented was the ARM domain, an approximately 40-amino-acid long tandemly repeated sequence motif (Fig 1). This domain was first identified in the *Drosophila melanogaster* segment polarity protein Armadillo, which is involved in Wingless signal transduction [40]. Structural characteristics of the ARM motif suggest its involvement in protein-protein interaction, which has been demonstrated in several cases [41]. In a few cases, HEAT repeats were detected in proximity to the ARM repeats. In animals, the functions of ARM-repeat proteins are significant, including cytoskeletal regulation and intracellular signaling transduction.

We analyzed the chromosomal locations of the U-box protein-encoding genes on the *M. truncatula* genome (Fig 3). Profiling of the gene distribution on the eight *M. truncatula* chromosomes indicated that the gene family evolved in this species through a large number of duplication events. Gene duplication was defined according to the following criteria: (1) The length of the sequence alignment covered ≥80% of the longest gene, and (2) the similarity of

Table 4. Read abundance of *MtPUB* genes in the cold-0, cold-2, cold-6, and cold-12 libraries.

Gene_ID	Cold-0	Cold-2	Cold-6	Cold-12	log_2 (Cold-2/Cold-0)	log_2 (Cold-6/Cold-0)	log_2 (Cold-12/Cold-0)
MtPUB1	83	154	43	50	0.90	-0.94	-0.72
MtPUB2	57	146	63	30	1.37*	0.14	-0.90
MtPUB3	68	149	71	54	1.13*	0.05	-0.33
MtPUB4	162	380	205	121	1.23*	0.34	-0.42
MtPUB5	60	48	28	21	-0.31	-1.11*	-1.54*
MtPUB6	283	290	400	446	0.04	0.50	0.66
MtPUB7	340	1346	1024	771	1.99*	1.59*	1.18*
MtPUB8	1	1	2	1		0.74	
MtPUB9	969	743	1315	1567	-0.38	0.44	0.69
MtPUB10	7	17	20	15	1.26*	1.50*	1.06*
MtPUB11	3	13	12	9	2.13*	2.01*	1.62*
MtPUB12	4	5	11	8	0.25	1.43*	0.96
MtPUB13	459	558	555	627	0.28	0.27	0.45
MtPUB14	370	401	396	446	0.12	0.10	0.27
MtPUB15	1	1	2	1	0	0.74	0
MtPUB16	1	1	1	1	0	0	0
MtPUB17	505	1059	686	750	1.07*	0.44	0.57
MtPUB18	56	76	271	152	0.45	2.28*	1.45*
MtPUB19	509	570	867	719	0.16	0.77	0.50
MtPUB20	448	487	483	508	0.12	0.11	0.18
MtPUB21	372	520	322	404	0.48	-0.21	0.12
MtPUB22	193	2724	268	295	3.82*	0.47	0.61
MtPUB23	188	72	450	278	-1.38*	1.26*	0.57
MtPUB24	420	438	355	439	0.06	-0.24	0.07
MtPUB25	296	330	619	797	0.16	1.07*	1.43*
MtPUB26	254	168	163	229	-0.60	-0.64	-0.15
MtPUB27	125	89	123	102	-0.49	-0.02	-0.30
MtPUB28	960	963	1088	1170	0.00	0.18	0.29
MtPUB29	689	1078	1388	1378	0.65	1.01*	1.00*
MtPUB30	364	232	164	240	-0.65	-1.15*	-0.60
MtPUB31	26	23	16	49	-0.14	-0.71	0.91
MtPUB32	1314	1815	2248	1965	0.47	0.77	0.58
MtPUB33	176	898	367	276	2.35*	1.06*	0.65
MtPUB34	247	232	148	191	-0.09	-0.74	-0.37
MtPUB35	33	392	158	162	3.57*	2.26*	2.29*
MtPUB36	407	413	544	654	0.02	0.42	0.68
MtPUB37	526	480	270	270	-0.13	-0.96	-0.96
MtPUB38	166	169	138	170	0.02	-0.27	0.04
MtPUB39	389	453	432	579	0.22	0.15	0.58
MtPUB40	713	720	760	728	0.01	0.09	0.03
MtPUB41	413	348	273	399	-0.25	-0.60	-0.05
MtPUB42	37	222	684	449	2.58*	4.20*	3.60*
MtPUB43	17	68	23	8	2.05*	0.49	-1.04*
MtPUB44	274	2081	506	532	2.93*	0.89	0.96
MtPUB45	3	17	7	5	2.49*	1.20*	0.76
MtPUB46	212	215	218	229	0.02	0.04	0.11

(*Continued*)

Table 4. (*Continued*)

Gene_ID	Cold-0	Cold-2	Cold-6	Cold-12	log_2 (Cold-2/Cold-0)	log_2 (Cold-6/Cold-0)	log_2 (Cold-12/Cold-0)
MtPUB47	1392	1537	1539	1790	0.14	0.14	0.36
MtPUB48	62	148	50	25	1.26*	-0.31	-1.32*
MtPUB49	350	269	237	525	-0.38	-0.56	0.58
MtPUB50	174	194	174	148	0.16	0.00	-0.23
MtPUB51	121	136	143	152	0.17	0.24	0.33
MtPUB52	792	937	775	958	0.24	-0.03	0.27
MtPUB53	1	1	1	1	0	0	0
MtPUB54	205	226	195	219	0.14	-0.07	0.10
MtPUB55	95	949	227	77	3.32*	1.25*	-0.31
MtPUB56	298	626	925	467	1.07*	1.64*	0.65
MtPUB57	874	1148	1370	1357	0.39	0.65	0.63
MtPUB58	255	250	261	297	-0.03	0.03	0.22
MtPUB59	278	358	353	385	0.37	0.34	0.47
MtPUB60	1	1	1	1	0	0	0
MtPUB61	198	432	337	378	1.12*	0.77	0.93
MtPUB62	1146	891	2446	2168	-0.36	1.09*	0.92
MtPUB63	160	188	170	100	0.23	0.08	-0.67
MtPUB64	4	4	2	8	0.00	-0.81	0.96

Values indicate number of reads.

* indicates a significant difference in expression compared to the 0 h time point ($P < 0.01$ and $|log_2N| \geq 1$). Cold-0, Cold-2, Cold-6, and Cold-12 indicate 0, 2, 6, and 12 h cold treatment, respectively.

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the aligned gene regions was ≥70% [22,23]. The 64 U-box genes in *M*. *truncatula* were distributed on all eight chromosomes, but in some cases, the genes were concentrated in certain chromosomal regions, such as the bottom half of chromosome 1. In addition, we found some U-box genes were arranged in tandem repeats of two genes, representative of local gene duplications. This finding suggests that tandem duplications of chromosomal regions may have played an important role in the expansion of this gene family. On the other hand, we also found tandem U-box genes harboring different functional domains, indicative of diversification by domain shuffling after tandem duplication, which would promote functional diversity of the U-box genes.

2.2 U-box family genes tissue-differentially expression and function

The functions of U-box genes in *M*. *truncatula* remain poorly understood. Some of them were constitutively expressed, such as *MtPUB25*, *MtPUB52*, *MtPUB56*, and *MtPUB58*, whose high expression levels in tissues suggest they may be essential for *M*. *truncatula* growth and development (Fig 4). Other U-box genes, such as *MtPUB42*, had low expression levels in all tissues but were clearly induced by stress according to the RNA-seq data, indicating a potential role in abiotic stress. Finally, tissue-specific expression was also observed, such as the root-specific expression of *MtPUB49*, indicating that some U-box genes may have tissue-specific or organ-specific functions (Fig 4).

2.3 U-box family genes in response to various abiotic stresses

It remains unclear why plants have more U-box proteins than other organisms. One possibility is that U-box proteins significantly contribute to the ability of plants to respond to diverse

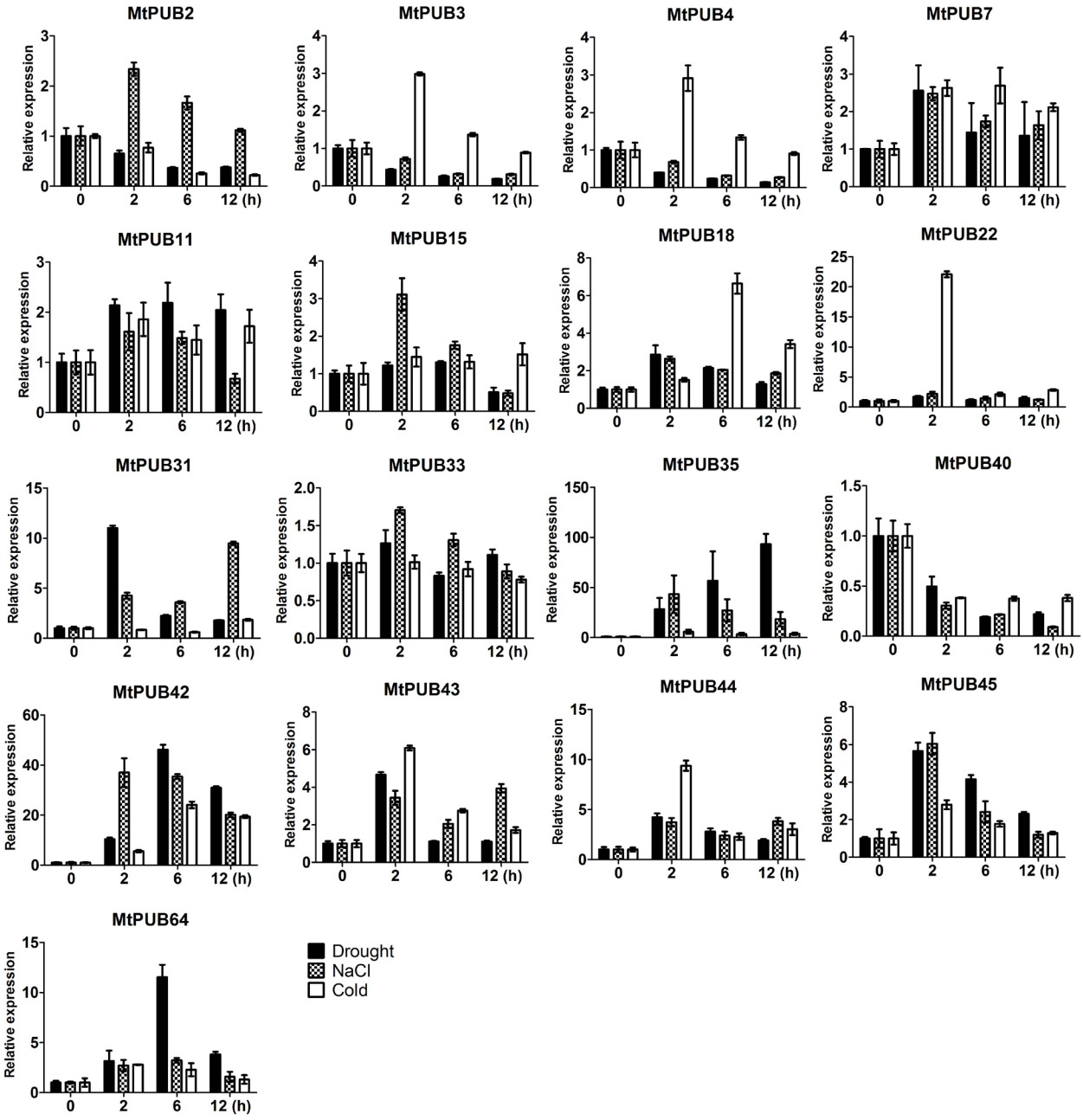

Fig 6. The expression of U-box protein-encoding genes induced by drought, salt, and cold stress as determined by qRT-PCR. Four-week-old seedlings were treated with drought (by transferring them to dry Whatman 3MM paper in a sterile petri dish), NaCl (300 mM), or cold (4˚C) for 0, 2, 6, and 12 h.

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environmental stresses, due to plant immobility and the lack of an animal-like immune system [39]. There has been increasing evidence supporting this hypothesis in recent years, which prompted us to investigate whether *M. truncatula* PUB proteins are induced by abiotic stress. The number of up-regulated U-box genes was 15, 25, and 16 under drought, salt, and cold stress, respectively. In contrast, the number of down-regulated U-box genes was 11, 6, and 3, respectively (Fig 5, Tables 2–4). Thus, abiotic stress mainly induces U-box gene expression. Many genes were induced by two or three stress conditions and may therefore play a role

under various environmental stresses. Our results showed that, as in other species, the expression of many *MtPUB* genes, such as *MtPUB10*, *MtPUB17*, *MtPUB18*, *MtPUB35*, *MtPUB42*, and *MtPUB44*, could be induced by drought, salt, and cold stress (Fig 5, Tables 2–4).

In higher plants, U-box-ARM proteins have been implicated in the regulation of cell death and defense [9] and in reducing cellular oxidative stress during seedling establishment in rice [15]. *MtPUB35* and *MtPUB42* were found to encode ARM domain-containing proteins and were up-regulated more than 10-fold at different time points under all three stresses (Fig 5, Tables 2–4). In addition to their classification as U-box-ARM protein-encoding genes with markedly induced expression under abiotic stresses, the proteins encoded by *MtPUB35* and *MtPUB42* were grouped together in the G1 subfamily in the phylogenetic analysis. Analysis of *cis* sequences revealed 4 and 3 ABRE elements in *MtPUB35* and *MtPUB42*, respectively, as well as 5 ARE elements in *MtPUB42* (S2 Table), further indicating that the two U-Box-ARM genes are important for stress response. Further study of these genes is therefore warranted. In short, these results are consistent with the findings in other plants that U-box-ARM proteins have the potential to regulate plant responses to abiotic stresses. *M. truncatula* homologs of other characterized *PUB* genes were also identified in the present study. For example, the *Arabidopsis* genes *AtPUB22* and *AtPUB23* play a key role in drought stress response [10], so *MtPUB18*, the homologous gene in *M. truncatula*, may also be associated with drought stress. Similarly, *MtPUB44* may be involved in disease resistance, as it is homologous to tobacco *NtCMPG1*, which has been shown to be essential for disease resistance [42]. Taken together, the present findings suggest that PUB proteins likely play critical roles in stress response in *M. truncatula*.

Supporting information

S1 Fig. A phylogenetic tree of U-Box protein (Pub) family from 3 species (Mt,At,Os).
(PDF)

S2 Fig. Abundance of transcriptions in stress treatment vs. non-stress treatment samples.
(PDF)

S3 Fig. Abundance of transcriptions between two samples.
(PDF)

S1 Table. U-box protein-encoding genes in Medicago truncatula, Arabidopsis thaliana, and Oryza sativa. Detailed genomic information, including the gene name, gene ID, and protein sequence, is provided for each U-box gene.
(XLS)

S2 Table. 15 types of cis-acting elements and the number of times they occurred in each U-box protein-encoding gene.
(XLS)

S3 Table. Primer sequences used for this study.
(XLS)

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