Genome-Wide Identification and Functional Analysis of U-Box E3 Ubiquitin Ligases Gene Family Related to Drought Stress Response in Chinese white pear (*Pyrus bretschneideri*)

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Abstract

Background

The plant U-box(PUB) proteins are a family of ubiquitin ligases (E3) enzymes that involved in diverse biological processes, as well as in responses to plant stress response. However, the characteristics and functional divergence of the *PUB* gene family has not yet been previously studied in the Chinese White Pear (*Pyrus bretschneideri*).

Results

In the present study, we identified 62 *PbrPUB*s in Chinese white pear genome. Based on the phylogenetic relationship, 62 *PUB* genes were clustered into five groups. The conserved motif and gene structure analysis provided further evidence to support the classification phylogenetic tree. The *PbrPUB* genes were unevenly distribution on 17 pear chromosomes, chromosome 15 housed most member of *PUB* family, with eight *PUB* genes. *Cis*-acting element analysis indicted *PUB* genes might participate in diverse biological processes, especially in the response to abiotic stresses. Based on RNA-data from “Dangshansuli” at seven tissues, we found that *PUB* genes exhibited diverse of expression level in seven tissues, and qRT-PCR experiment further support the reliable of RNA-Seq data. To identify candidate genes associated with resistance, we conducted qRT-PCR experiment the expression level of pear seed plant under four abiotic stresses, including: ABA, dehydration, salt and cold treatment. One candidate *PUB* gene associated with dehydration stress was selected to conduct further functional experiment. Subcellular localization revealed *PbrPUB18* gene was located on cell nucleus. Furthermore, heterologous over-expression of *PbrPUB18* in *Arabidopsis* indicated that the over-expression of *PbrPUB18* could enhance resistance in drought treatment. In conclusions, we systematically identified the *PUB* genes in pear, and provided valuable information for the molecular mechanism of *PUB* genes in pear.

Background

Plants are frequently exposed to various abiotic stresses such as drought, salt and low temperature during their life cycles. Several stresses often lead to oxidative damage and have adverse impacts on plant growth and development. To adapt to unfavorable environmental conditions, plants have evolved complex and efficient mechanisms [1]. Previous studies have identified four signal transduction pathways in response to abiotic stress, including transcriptional regulation, post-transcriptional modifications, epigenetic regulation, and post-translational modifications [2]. And ubiquitination is one of the most significant post-translational modifications. The ubiquitin/26S proteasome system (UPS) pathway is a pervasive and effective route for protein removal in eukaryotes [3, 4]. UPS include ubiquitin (Ub), ubiquitin-activating enzyme (E1), ubiquitin- conjugating enzyme (E2), ubiquitin ligase (E3), and the 26S proteasome. The central component of UPS is the highly conserved, 76 amino acid protein ubiquitin. Ubiquitin is bound to specific proteins and functions in the degradation of target proteins in an E1–E2–

E3 multienzyme cascade manner [5–8]. In the pathway, E3 enzymes are clearly the key factors that define substrate specificity. According to their reaction mechanism and subunit compositions, four main types were classified: Ubox, HECT (Homology to E6-Associated Carboxy-Terminus), RING (Really Interesting New Gene) and Cullin–RING ligases (CRLs)[4]. U-box ubiquitin ligases are characterized by a conserved U-box motif of about 70 amino acids. And U-box ubiquitin ligases were firstly discovered among E3 ubiquitin ligases, and was first clarified from ubiquitin fusion degradation protein-2 (UFD2) in yeast [9].

In comparison with the 2 and 21U-box (PUB) genes identified in *Saccharomyces cerevisiae* and *Homo sapiens* genomes, respectively, more U-box genes were widely distributed in plants. In *Arabidopsis thaliana*, about 61 plant U-box genes were predicted [9, 10], while 77 were found in *Oryza sativa* [11], 62 in *Solanum lycopersicum* [12], 93 in *Gossypium raimondii* [13], 91 in *Musa acuminata* [14], 61 in *Medicago truncatula* [15] 101 in *Brassica rapa* [16] and 125 in soybean [17]. Many previous studies have shown that PUB proteins are involved in biological processes such as plant hormone signaling regulations [6], self-incompatible or pseudo-self-compatibility regulations [18] as well as in biotic stress [19–21] and abiotic stress [5, 22, 23].

In a number of previous studies, U-box genes acted as regulators in diverse abiotic stress responses including drought, low temperature and salinity conditions. In *Arabidopsis thaliana*, *AtPUB18/AtPUB19* are negative regulators of ABA signaling by inducing ABA hypersensitivity, and *AtPUB22/AtPUB23* are negative regulators in drought stress responses in an ABA-independent pathway [24, 25]. *PUB25* and *PUB26*, two U-box typeE3 ubiquitin ligases, trigger cold signaling negative regulator *MYB15* to promote plant freezing tolerance[26]. *AtPUB44* ubiquitinates the AAO3 (abscisic aldehyde oxidase 3) via 26 proteasome and affects the ABA biosynthesis [27]. Furthermore, *AtPUB46* and *AtPUB48* were found to be more sensitive to drought [28]. In rice, *OsPUB15* has been implicated in positive regulating plant tolerance to salinity and drought stress [29]. In apple, *MdPUB29* may positively regulate salt tolerance [30].

The plant PUB family has been widely studied for abiotic stresses, mainly in model plants such as *Arabidopsis*, rice and tomato, and less on woody plants such as pear. Pear belongs to the *Pyrus* genus in the Rosaceae family, and is one of the most important fruit crops and widely distributed fruits in the world. However, the yield of pear frequently fell off on account of abiotic stress such as drought, cold and salinity. And these affect pear growth and development, furthermore limit pear crop productivity [31]. Therefore, it is significant to identify genetic determinants associated with drought, cold and salinity stresses tolerance in pear for agricultural development. The completion of Chinese White Pear (*Pyrus bretschneideri*) genome sequencing has provided a golden opportunity to study the function of the pear U-box proteins at the whole genome scale [32]. In this study, we conducted systematic identification of PUB genes in pear on whole-genome level, and further chromosome location, genomic structure, evolutionary analysis and experiment verification will establish a solid foundation for functional characterization of *PbrPUB* genes in the future.

Results

Identification of *PbrPUB* gene family members

In our study, we used a strictly pipeline to identify PUB genes in pear genome. First, the hidden Markov model (HMM) of the U-box domain (PF04564) was downloaded from the Pfam30.0 database, and used as a query to identify the candidate PUB members in Chinese white pear genomic database (<http://peargenome.njau.edu.cn/>) using HMMER3.0. As a result, a total of 91 candidate PUB genes were identified in pear genome. Second, SMART tools were performed to verify the accuracy of 91 candidate PUB genes, and 29 PUB genes were removed because of the incomplete of PUB domain. At last, 62 PUB genes with complete U-box domain were obtained for further analysis (Table 1). We named these *PbrPUB* genes from *PbrPUB1* to *PbrPUB62* according to their location information on the chromosome. The ExPASy ProtParam (<https://www.expasy.org/>), an online proteomics and sequence analysis tool, was used to estimate molecular weight and isoelectric point (pI). The molecular weight for the *PbrPUB* gene family range from 39.33 kDa to 151.30 kDa (Kilodalton) and the pI range from 4.99 to 8.83, with an average of 6.78. Subcellular localization were also predicted by Cell-PLoc 2.0 (<http://www.csbio.sjtu.edu.cn/bioinf/Cell-PLoc-2/>), and we found that most PUB gene were located in nucleus, except six pub located in cytoplasm and three located in cell membrane.

Phylogenetic analysis of *PbrPUB* gene family members

To investigate the evolutionary relationships of *PbrPUB* genes family, we constructed a neighbor joining (NJ) phylogenetic tree using the Mega-X program based on the full-length PUB proteins of pear (62 members), tomato (62 members) and *Arabidopsis* (61 members) (Fig. 1a). The protein sequences of PUB genes of tomato and *Arabidopsis* were obtained from previous study [10, 12]. Based the result of phylogenetic tree, 185 members of PUB genes from these three species were clustered into five groups, including Group I, Group II, Group III, Group IV and Group V. The member number of Group III was biggest in five subgroups, and it harboured 64 PUB genes. However, Group IV harboured least PUB genes, with 10 PUB genes. In generally, the PUB genes of pear and tomato were clustered into one subclade, suggesting that pear and tomato exhibited relatively closer relationship compared to *Arabidopsis*.

It is interesting to note that the member number of PUB gene family in these three species is similar. This result indicated that the number of PUB genes in these three species is conserved. To explore which group of pears had occurred expansion or lost during evolution process, we measure the number of PUB genes of each species in each group. In pear, group I, II, III, IV and V contain 11, 21, 21, 3 and 6 *PbrPUB* gene family members, respectively. In tomato, groups I, II, III, IV and V contain 12, 21, 21, 3, and 5 *SlU-box* genes, respectively. In *Arabidopsis*, groups I, II, III, IV and V contain 20, 12, 22, 4 and 3 *AtPUB* genes, respectively (Fig. 1b). The member number of each group in pear and tomato is almost equal, suggesting that pear had not undergone expansion or lost compared to tomato. However, compared to pear and tomato, the group I of *Arabidopsis* had undergone rapid expansion, while the group II of *Arabidopsis* had undergone rapid lost.

Analysis of *PbrPUB* gene family conserved motifs and gene structures

To further verify the classification results of phylogenetic tree, we investigated the conserved motif and gene structure of *PbrPUB* genes in pear. The MultipleEm for Motif Elicitation (MEME) motif search tool were used to predict the conserved motif of *PbrPUB* genes. A total of 20 motifs were estimated in our study, and we named as motif 1–20 (Fig. 2a, 2b, Additional file 1: Fig. S1). Among them, motif 1, 3 and 5 were found in all groups, indicating that were highly conserved in all *PbrPUB* proteins. Based on the SMATR website, we determined that the U-box was comprised of Motif1, Motif3 and Motif5 (Additional file 2: Fig.S2). This result provided evidence to support the accuracy of PUB gene set identified in our study. Based on the SMATR website, we also found the other conserved domain: ARM and Pkinase domin. The ARM is comprised of motif 2, 4 and 7; the Pkinase is comprised of motif 11, 13 and 20. Generally, most *PbrPUB* members in the same groups had similar motifs. For example, most of the members of group II contained motif 6, 10 and 8. This result indicated that these three motifs might be key functional domain of group II PUB genes, suggesting that these proteins might have conservative functions.

To explore the gene structure of *PbrPUB* genes in pear, we extracted the exon-intron information of 62 *PbrPUB* genes from pear database using in-house scripts. Based on the information, TBtools software were preformed to show the gene structure of *PbrPUB* (Fig. 2c). The number of exon in PUB genes was greatly divergent, ranging from 1 to 20. Among 62 PUB genes in pear, *PbrPUB24* contained the greatest number of exon (20), while 16 PUB genes only contained one exon. Furthermore, the lengths of the exon and intron were differential. There are 30 PUB genes have been found contain UTR structure. Similarly, to the result of motif analysis, the PUB genes with similar gene structure were cluster into same subclade. For example, most members of class II only housed one exon. This result indicated that the members of same groups exhibited similar gene structure and conserved motifs. These results from conserved motifs and gene structure analysis provided strong evidence to support the accuracy of the classification result of phylogenetic tree.

Chromosomal localization and homologous gene analysis of PbrPUB genes

To further explore the distribution pattern of PUB gene in pear genome, the *PbrPUB* chromosomal distribution map was plotted using TBtools (Fig. 3a). The location information of PUB genes in pear were extracted by our in-house scripts. As a result, a total of 50 *PbrPUB* genes (82.26%) were unevenly mapped on the 17 pear chromosomes, and no member of *PbrPUB* gene family was mapped on chromosome 8. Therefore, we didn't shown chromosome 8 in our Fig. 3. In addition, 12 genes were located on scaffold contigs, and we also didn't show them in our Fig. 3. Chromosome 15 had the largest number of *PbrPUB* genes, with eight *PbrPUB* genes, followed by chromosome 5 with 6 genes. Chromosome 1, 2 and 12 each contained 4 *PbrPUB* genes. Two or three *PbrPUB* genes were mapped on chromosomes 3, 6, 7, 9, 10, 11, 13, 14, and 16. Chromosome 4 and chromosome 17 contained only one gene. We also identified the homologous genes of PUB gene family using MCscanX software. As result, 16 homologous gene pairs were identified in pear PUB gene family, which contained 26 homologous genes. Three homologous gene pairs were detected between chromosomes 5 and chromosomes 10 (Fig. 3b).

Cis-acting elements analysis in the putative promoter of *PbrPUB* genes

Cis-acting elements were important clues for the prediction of gene functions. To further investigate the function of *PbrPUB* genes, we predicted the *cis*-acting element of the putative promoter region of *PbrPUB* genes using PlantCARE database. In this study, the 2000 bp upstream region of transcription initiation site of *PbrPUB* genes was defined as the putative promoter region. As a result, a total of 41 *cis*-acting elements were identified (Fig. 4), and we selected 15 interesting *cis*-acting elements for further analysis. These 15 *cis*-acting were associated with stress, hormone, plant growth and development. As shown in Fig. 4a, some diverse distribution patterns of *cis*-acting elements were observed in the promoter region of *PbrPUB* genes, indicating that the *PUB* gene family of pear particular in various different biology process. Meanwhile, we found that all *PbrPUB* genes contained the *cis*-acting related to hormone regulation, such as, abscisic acid (ABA), methyl jasmonate (MeJA), gibberellin (GA), salicylic acid, and auxin responsiveness elements. Previous study had reported that *DSG1*, which encodes a U-box domain, could regulate cell division and elongation by responding to multiple hormones, such as auxin, salicylic acid and ethylene [33]. In our study, 55 genes were identified as the responsiveness elements of ABA, suggesting that *PUB* gene family might particular in resistance under ABA treatment (Fig. 4b). In *Arabidopsis*, *AtPUB9*, *AtPUB18*, *AtPUB19*, and *AtPUB44* were identified to involve in ABA response [34]. It is notable that the element related to MYB binding site involved in drought was predicted in 44 *PbrPUB* genes, suggesting that these 44 *PbrPUB* genes might mediated by MYB genes response to drought stress. Moreover, there were 30 *PbrPUB* genes have *cis*-acting elements related to cold, suggesting that these 30 *PbrPUB* genes might particular in resistance under low temp treatment. As we all know, flavonoid biosynthesis is one of important phenomenon during the process of response to stress in plant. In the study, we found *PbrPUB10*, *PbrPUB24* and *PbrPUB5* contained MYB binding site involved in flavonoid biosynthetic.

Expression profile of *PbrPUB* genes in different tissues of pear

To further explore the tissues-specific expression of *PbrPUB* genes, we conducted RNA-seq analysis using transcriptomic data from seven different tissues of 'Dangshansuli' cultivar pear, including stem, ovary, petal, sepal, bud, fruit and leaf [35]. We used RPKM (reads per kilobase per million) values to estimate the expression level of *PbrPUB* genes. The RPKM value equal to 0 means no expression in one library, and the RPKM value greater than 10 means highly expression in one library. Then, we investigated the expression level of 62 *PUB* genes. Pheatmap, an R package, was used to show the expression patterns of 62 *PbrPUB* genes (Fig. 5a). Based on the expression pattern of 62 *PUB* genes, they were clustered into four main classes. Gene in Class IV exhibited highly expression level in all of seven examined tissues, while gene in Class II exhibited almost no expression in all of seven tissues. Class I was specifically expressed in pear leaf, and a diversity of expression pattern were detected in Class III (Fig. 5a). Among the 62 *PbrPUB* genes, 52 genes (83.87%) were at least expressed in one tissue, even though the transcript abundance of several genes was relatively lower for certain tissues. Approximately 10 non-expressed *PUB* genes were identified in all of seven tissues, and they may be lost the function during the evolution process of *PUB* genes family in pear. 29 *PbrPUB* genes were expressed in all seven different tissues, indicating that they

have various roles in the development of different tissues. Interesting, we found 28 PUB genes exhibited highest expression in leaf, suggesting that these 28 genes might involve the development of leaf. Due to leaf is an important plant organ involved resistance, we referred that these 28 PUB genes might particular in resistance in the process of pear growth and development.

To verify the transcriptome sequences analysis was reliable, 15 *PbrPUB* genes were randomly selected to conduct a quantitative real-time PCR (qRT-PCR) experiment to investigate the expression levels in seven different tissues of the 'Dangshansuli' pear, including stem, leaf, petal, fruit, sepal, bud, and ovary (Fig. 5b). We found that all of 15 *PbrPUB* genes exhibited a diversity of expression patterns in the seven different tissues, suggesting that *PbrPUB* genes family may function in different tissues and participate in diverse metabolic processes. Seven genes (*PbrPUB1*, *PbrPUB3*, *PbrPUB7*, *PbrPUB9*, *PbrPUB18*, *PbrPUB36* and *PbrPUB38*) exhibited a similar expression pattern with a high expression level in leaf tissues, suggesting that *PbrPUB* genes play critical functions during leaf development. All of these seven genes exhibited highly expression level in leaf in transcriptome data. These result provided further evidence to support our transcriptome sequences analysis was reliable. Interestingly, most of 15 PUB genes were highly expressed in reproductive organ, suggesting that *PbrPUB* genes might associate with the development of reproductive organ.

The expression pattern of PbrPUB genes under abiotic stresses

Previous study had extensively reported *PbrPUB* gene family involved in various abiotic stresses [36]. To explore the function of PUB gene family in pear, *Pyrus betulaefolia* from grown 45-day-old seedlings were subjected to four different stress treatments including dehydration, low temperature, ABA and salt. To identify whether PUB genes in pear could respond to abiotic stresses, 11 *PbrPUB* genes were randomly selected from each group to conduct a qRT-PCR experiment to detect their expression level. 11 genes are comprise of 2 from group I (*PbrPUB1* and *PbrPUB14*), 4 from group II (*PbrPUB12*, *PbrPUB18*, *PbrPUB36* and *PbrPUB38*), 2 from group III (*PbrPUB3* and *PbrPUB25*), 2 from group IV (*PbrPUB7* and *PbrPUB48*) and 1 from group V (*PbrPUB34*).

To identify candidate *PbrPUB* genes related to dehydration treatment, the shoots of seedlings of pear were placed on dry filter papers for 0, 1, 6, 9, 12 and 24 h at ambient environment (Fig. 6a). Among the eleven PUB genes, 9 PUB genes were up-regulated expressed and one PUB gene (*PbrPUB7*) was down-regulated expressed under dehydration stress. However, *PbrPUB14* was not significantly differential expressed under dehydration stress. Among the 9 up-regulated genes, *PbrPUB18* exhibited highly increased expression level during the process of dehydration treatment, while *PbrPUB12*, *PbrPUB3* and *PbrPUB36* were up-regulated expressed during 12 h dehydration treatment and recovered to normal levels at 24 h. *PbrPUB1*, *PbrPUB38* and *PbrPUB25*, exhibited highest expression level at 1 h, where *PbrPUB12*, *PbrPUB14*, *PbrPUB3* and *PbrPUB36* exhibited highest expression level at 12 h under dehydration treatment. These results suggested that *PbrPUB1*, *PbrPUB38* and *PbrPUB25* respond to dehydration treatment faster than that of *PbrPUB12*, *PbrPUB14*, *PbrPUB3* and *PbrPUB36*. Therefore, PUB gene family in pear play vital role in the process of low temperature stress response.

To verify whether PUB gene in pear involve in low temperature stress, the seedlings were placed in the chamber set at 4°C for 0, 1, 6, 9, 12, 24, 48 and 96 h (Fig. 6b). We detected the expression level of those 11 *PbrPUB* genes by qRT-PCR experiment. 4 genes (*PbrPUB12*, *PbrPUB3*, *PbrPUB36* and *PbrPUB48*) were up-regulated expressed under cold stress, suggesting that those *PbrPUB* genes might respond to low temperature. *PbrPUB12*, *PbrPUB48* and *PbrPUB36* were highly increased during the 48 h low temperature exposure. The expression level of *PbrPUB3* was reached to double peak at 1 h and 48 h.

To explore the functions of *PbrPUB* genes under the salt stress, the seedlings were placed in solution containing 200 mM NaCl solution for 0, 2, 4, 6, 8, 12 and 36 h (Fig. 6c). We detected the expression level of those 11 *PbrPUB* genes by qRT-PCR experiment. On the whole, all of the selected 11 *PbrPUB* genes were significantly up-regulated expressed under the 200 mM salt stress treatment. The expression level of *PbrPUB14*, *PbrPUB25*, *PbrPUB3*, *PbrPUB48* and *PbrPUB7* were highly increased during the 12 h salt exposure. Moreover, *PbrPUB1*, *PbrPUB12*, *PbrPUB18*, *PbrPUB34*, *PbrPUB36* and *PbrPUB38* were highest expressed at 4 h under salt stress, suggesting that these 6 *PbrPUB* genes respond to salt treatment actively. We focus on the expression level of *PbrPUB18*. In the 4–8 h, the expression level of *PbrPUB18* was significantly increased, and then it was down-regulated at 12 h, finally recovered normal level at 36 h.

Previous study had reported that PUB gene involved in ABA-mediated drought stress responses. To investigate the roles of PUB gene family in ABA stress, the seedlings were dipped in solution containing 100 µM ABA for 0, 1, 3, 6, 9, 12 and 36 h (Fig. 6d). As results, all of 11 PUB genes were respond to the ABA stress, and these gene were unregulated expressed at first, and then were down-regulated at 36 h after ABA treatment. These results indicated that PUB genes play important roles in ABA-regulated pathway. The expression levels of three genes (*PbrPUB1*, *PbrPUB25*, and *PbrPUB36*) were reached to peak at 1 h, suggesting that these three genes were actively responded to ABA stress. Interestingly, we found that *PbrPUB18* was expressed in 6 h and 12 h after ABA treatment.

Subcellular localization of PbrPUB18

To further explore the function of PUB gene in pear, we constructed subcellular localization experiment to identify where the PUB genes act function. To further verify the biologic function of *PbrPUB* genes in pear under drought stress, *PbrPUB18* was selected from 9 up-regulated expressed genes for further study. The full-length ORF of *PbrPUB18* was fused to the N-terminal of GFP (Green Fluorescent Protein), under the control of *CaMV35S* promoter to form a fusion construct 35S-*PbrPUB18*-GFP. Then the 35S-*PbrPUB18*-GFP fusion protein and 35S-GFP (as a control) was transient transformed into tobacco leaves and the fluorescence signal was observed with a confocal laser scanning microscope. The green fluorescence of GFP control was found in the membrane and the nucleus (Fig. 7a). In contrast, 35S-*PbrPUB18*-GFP fused GFP protein was only existed in the nucleus and integrated perfectly with DAPI (4', 6-diamidino-2-phenylindole) regime (Fig. 7b), suggesting that *PbrPUB18* was located in the nucleus, which was consistent with our prediction in Table 1.

Assessment of drought tolerance in transgenic lines of PbrPUB18

To further confirm the biologic function of *PbrPUB18* gene under drought stress, *Arabidopsis* Col-0 plants (WT) were transformed by the floral dip method [37]. Two overexpression lines OE-4 and OE-5 were screened out by PCR identification at DNA level in T0 generation plants. The selected robust *Arabidopsis thaliana* seedlings were transplanted into the soil as T1 generation plants, and the T1 generation positive seedlings were identified again by semi-quantitative PCR at mRNA level. QRT-PCR also verified the expression of *PbrPUB18* in OE-4 and OE-5 far above in WT (Additional file 3 Fig S3). To assess the function of overexpression *PbrPUB18* in *Arabidopsis* on drought tolerance, 15-day-old WT and transgenic lines were used to expose to drought stress by withholding water for 12 days. There was no morphological difference between WT and the transgenic lines in the normal condition. After 12 days without water, the two transgenic lines showed more tolerance to the drought stress, as manifested by lesser leaf-wilting symptoms compared with the WT plants (Fig. 8a). In addition, chlorophyll fluorescence measurements were recorded to further verify drought tolerance of WT and the transgenic lines (Fig. 8b). The maximum quantum efficiency of the photochemistry (Fv/Fm) values was not affected by species and growth conditions, but under stress conditions, this parameter decreased significantly. After 12 days drought treatment, the Fv/Fm values of WT were significantly lower than those of the two transgenic lines, suggesting WT showed more sensitivity to the drought stress (Fig. 8e). Electrolyte leakage (EL), an important indicator of cell injury, was measured after drought stress. The EL of two transgenic lines was only approximately 15 %–20 % compared to WT (37.3 %), suggesting that WT suffered more severe membrane damage than transgenic lines of *Arabidopsis* by overexpressing *PbrPUB18* (Fig. 8c). The transgenic plants displayed significantly lower malondialdehyde (MDA) contents than WT exposure to drought condition (Fig. 8d).

In the drought tolerance stress assay, we found that two transgenic lines had lower values of EL and MDA, implying that they might be subjected to lighter extent oxidative stress than the wild type. Histochemical staining with 3, 3'-diaminobenzidine (DAB) and nitro-blue tetrazolium chloride (NBT) was used to analyze the *in situ* accumulation of H_2O_2 and O_2^- , two main reactive oxygen species (ROS), respectively. After drought stress the leaves of WT lines (Fig. 8f) were stained to a more serious extent compared with transgenic *Arabidopsis*, implying that more ROS was produced in the WT under the drought conditions. Similar to staining results, quantitative measurements further demonstrated that H_2O_2 contents in the two transgenic lines were remarkably lower than those of WT (Fig. 8g). And anti- O_2^- contents in the two transgenic lines were remarkably more than those of WT (Fig. 8h), implying that less ROS was produced in the two transgenic lines under the drought conditions.

Discussion

Genome-wide and phylogenetic analysis of PbrPUB genes in pear

As a family of ubiquitin ligases, U-box genes encode a conserved U-box motif of about 70 amino acids and regulated the ubiquitination of the substrates [23]. U-box genes were widely distributed in the plants and reported to participate in many biological processes including plant hormone signaling regulations [6], self-incompatible or pseudo-self-compatibility regulations [18] as well as in biotic stress [19–21] and

abiotic stress [5, 22, 23]. Due to PUB gene play important role during plant development, PUB genes have been identified in different plant species, such as *Arabidopsis thaliana* (61) [9, 10], rice (77) [11], tomato (62) [12], cotton (93) [13], and banana (91) [14]. Pear, one of Rosaceae fruit trees, is widely cultivated all over the world. However, the analysis related to PUB genes in pear was poor until now. In the present study, 62 genes were identified as PUB gene family in pear using bioinformatics analysis, and the number of PUB gene in pear is similar to that of *Arabidopsis thaliana* (61) and tomato (62). We therefore speculate that the number of PUB genes in plant kingdom is relatively conserved.

Phylogenetic tree analysis indicated that a total of 185 PUB protein members in these three species (containing 62 pear, 62 tomato, and 61 *Arabidopsis*) were categorized into five subgroups (I-V). Although there are some differences, this phylogenetic tree was largely consistent with the results from a previous study [12, 14]. For example, 125 *GmPUB* genes in soybean proteins were classified into six groups using phylogenetic tree analysis [17]. Through the phylogenetic relationship analysis, it was showed that *PbrPUBs* exhibited closer relations with *SIU-boxs* compared with *AtPUBs*. This result was consistent with the fact that pear and tomato exhibited closely relationship than *Arabidopsis*. Although the number of PUB genes was similar in three species, we found that the genes of Group I in had undergone rapid expansion and Group II had undergone rapid lost. In addition to the U-box domain, 62 *PbrPUB* proteins are found to bind to different domains including armadillo (ARM) repeats, the tetratricopeptide (TPR) domain and WD40 repeats. The majority of PUB proteins that have been elucidated for biological functions are from the U-box proteins with ARM repeats [18]. The ARM repeats have been shown primarily mediating the interaction with substrates, suggesting that interaction make the substrates available for ubiquitination [23]. 25 member of PUB genes in pear only housed U-box domain, and 25 members housed both U-box and ARM domain. Moreover, TPR domain was found in *PbrPUB14* gene and WD40 repeats was found in *PbrPUB40* gene.

The function predication of PUB gene family in pear based on cis-acting and specific-tissues expression analysis

The *cis*-acting analysis of putative promoter indicated the U-box gene family was involved in stress-related mechanisms, hormonal regulation, growth and development. Previous study had reported that PUB proteins were responded with ABA. For instance, *AtPUB44* could regulated the biosynthesis of ABA through ubiquitinating the AAO3 (abscisic aldehyde oxidase 3) via 26 proteasomes [38]. In additional, one transcription factor of ABI3 was regulated by *AtPUB9* and increased the ABA sensitivity of *Arabidopsis* during seedling germination [39]. *AtPUB18*, *AtPUB19* and *AtPUB44* were found to directly interrupt the biosynthesis of ABA directly. In our study, 55 genes contained the ABA responsiveness elements on the putative promoter region. Especially, we found that eight ABA responsiveness elements were identified in the promoter region of *PbrPUB43*. This result indicted that PUB gene might play important role during ABA signal transduction in pear. In *Arabidopsis* and *Nicotiana*, the expression levels of PUB genes were regulated by abiotic and biotic stress [40]. In here, we found MYB binding site involved in drought induction responsive element, abscisic acid responsive element, defense and stress responsive element, low temperature responsive element, wound responsive element in the promoter region of PUB genes in

pear. The present of these elements indicated that most of PUB genes in pear were involved in stress response.

Based on our previous RNA-Seq data in seven different tissues of ‘Dangshansuli’ cultivar pear, transcriptome sequencing analysis and qRT-PCR expression profiling were conducted to investigate PUB gene expression patterns in seven different tissues. The members of the PUB gene family exhibited a variety of expression patterns in seven different tissues. These results indicated that PUB might participate in a diversity of functions during plant growth and development. Among the 62 members of the PUB gene family in pear, 29 *PbrPUB* genes were expressed in all seven different tissues. Additionally, 72.58 % of *PbrPUBs* were detected in transcriptional abundance in pear sepal. Whereas of *PbrPUBs* expressed in all tissues, 45.16 % were highest in leaves, suggesting these genes may have important roles in the development of pear leaves. Further, the expression levels of 15 randomly selected members of the *PbrPUB* gene family were validated using qRT-PCR. Based on qRT-PCR expression profiles, we found that the 15 *PbrPUB* genes were highly expressed in petal, sepal, ovary and leaf, suggesting that *PbrPUB* genes may function in the development of petal, sepal, ovary and leaf.

Roles of *PbrPUB* genes in response to different abiotic stresses

Previous studies have reported that PUB genes in plants involved in the process of stress responses [24, 26, 41, 42]. A large number of PUB genes were induced and expressed during abiotic stress conditions [40]. In this study, the differential expression levels of 11 *PbrPUB* genes under various abiotic stresses were investigated by using qRT-PCR, including drought, low temperature and salt stress. From the result, *PbrPUB12*, *PbrPUB3*, *PbrPUB36* and *PbrPUB48* were significantly up-regulated and expressed under four treatments, suggesting these three genes could respond to dehydration, ABA, cold and salt stress. *PbrPUB7* was down-regulated and expressed under dehydration stress, suggesting that *PbrPUB7* might negatively regulate the response process of dehydration. Drought is one of the most critical stresses and could significantly affect the growth of a plant. In our study, we found that the expression level of *PbrPUB18* was significantly up-regulated after dehydration treatment, suggesting that *PbrPUB18* genes might respond to drought stress. Therefore, *PbrPUB18* gene was selected for further functional identification. Subcellular localization experiment suggested that *PbrPUB18* was located at the cell nucleus. This result indicated that *PbrPUB18* might act biologically at the cell nucleus. Furthermore, different from previous studies, overexpression of *PbrPUB18* gene in *Arabidopsis thaliana* resulted in resistance to drought tolerance. Summary, we systematically identified the PUB gene family in pear, and further functional identification laid a foundation for the functional study of PUB genes in pear in the future.

Conclusions

In our study, a total of 62 *PbrPUB* members were identified in the Chinese white pear genome, and were unevenly distributed on 17 pear chromosomes. According to phylogenetic tree analysis, the pear PUB gene family was divided into five groups. The conserved motif and gene structure analysis provided strong evidence to support the result of the classification phylogenetic tree. *Cis*-acting element analysis

indicted *PUB* genes might participate in diverse biological processes, especially in the response to abiotic stresses and phytohormones. Transcription sequencing data from different seven tissues exhibited diverse of expression level of *PbrPUB* genes. Further qRT-PCR was used to identify candidate genes associated with abiotic stresses. In addition, *PbrPUB18* was cloned and functionally identified. Subcellular localization revealed *PbrPUB18* genes were located on cell nucleus. Heterologous over-expression of *PbrPUB18* in *Arabidopsis* indicated that the over-expression of *PbrPUB18* could enhance resistance in drought treatment. Our data and analysis lay a solid foundation for the future research on molecular mechanism of *PbrPUB* genes in responding to abiotic stresses.

Methods

Genome identification of *PUB* gene family members in Chinese white pear

To identify the potential members of the *PUB* protein family, the complete genome sequence of pear (*Pyrus bretschneideri*) was downloaded from the pear genome project (<http://peargenome.njau.edu.cn/>) [32]. The hidden Markov Model (HMM) profile of the U-box domain (PF04564) was obtained from Pfam30.0 (<http://pfam.xfam.org/>), and was used as a query to identify the candidate *PUB*s from the pear genome protein database using HMMER3.0.

All candidate *PUB* genes were further verified by using SMART conserved domain search tools (<http://smart.embl-heidelberg.de/>) to ensure that each candidate protein contained a U-box domain. In addition, the ExPASy ProtParam (<https://www.expasy.org/>), an online proteomics and sequence analysis tool, was performed to predict the physical and chemical properties of *PbrPUB* proteins, such as the number of amino acids (AA), molecular weights, isoelectric points, and instability index [43]. The subcellular localization prediction was carried out in Cell-PLoc 2.0 (<http://www.csbio.sjtu.edu.cn/bioinf/Cell-PLoc-2/>) [44].

PbrPUB protein phylogenetic analysis

Arabidopsis *PUB* protein sequences were downloaded from the *Arabidopsis* Information Resource (TAIR) (<https://www.Arabidopsis.org/browse/genefamily/pub.jsp>) [10] Tomato *PUB* protein sequences were obtained from previous study [12]. All *PUB* full-length protein sequences in *Pyrus bretschneideri*, *Arabidopsis thaliana* and *Solanum lycopersicum* were aligned using ClustalW with default parameters. Then, MEGA-X was used to construct an unrooted phylogenetic tree based on the result of multiple sequence alignment using the NJ (Neighbor Joining) method with 1000 bootstrap replicates [45]. Evolview (<https://evolgenius.info/evolview-v2/#login>) [46] was used to visualize the phylogenetic tree.

Gene structure, motif analysis and cis-acting elements analysis

To identify and visualize the structural organization (introns, exons, and untranslated regions) of the pear *PUB* gene family, the information of gene structure was extracted from whole genome database of pear

using in-house scripts. The novel conserved motifs of *PbrPUB* genes were identified by MEME suite (<http://meme-suite.org/tools/meme>). A total of 20 motifs and a width limit of 200 amino acids were used for the analysis with other default parameters. TBtools were used to visualize the results of gene structure and conserved motif analysis.

Bedtools software was used to extract the 2000 bp upstream (putative promoter region) sequences of the transcription start site of all *PbrPUB* genes [47]. Then, PlantCare (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>) was used to predict the *cis*-acting elements in the putative promoter region of *PbrPUB* genes [48]. TBtools were used to visualize the distribution of putative *cis*-acting elements.

Synteny analysis and chromosomal localization

Synteny analysis was performed by the method described in the Plant Duplicate Gene Database (PlantDGD) (<http://pdgd.njau.edu.cn:8080/>). The collinear block was identified by *PbrPUB* duplication events in the MCScanX [49]. The data were integrated and plotted by using Circos [50]. Based on the annotation information of PUB genes in pear, the corresponding location distributions of *PbrPUB* genes in chromosomes were displayed by TBtools [51].

Gene expression analysis of *PbrPUB* on the RNA-Seq. Data

The RNA-seq data of the cultivar ‘Dangshansuli’ in seven different tissues were acquired from our previous study, including bud, stem, leaf, sepal, petal, fruit and ovary [52]. We downloaded them from the National Center for Biotechnology Information database (NCBI, <https://www.ncbi.nlm.nih.gov/>). Then, Heatmap.2, afunction in R, was used to plot heatmaps based on the $\log_2$ (RPKM + 1) value of each *PbrPUB* gene.

Plant materials and stress treatments

The test material *Pyrus betulaefolia* were collected from the pear germplasm orchard of the Center of Pear Engineering Technology Research situated at Hushu in Nanjing, which were cultivated in the experimental base of the National Center of Pear Engineering Technology Research, Nanjing Agricultural University. To explore the expression levels of *PbrPUB* genes under abiotic Stresses, uniform and healthy shoots of *Pyrus betulaefolia* from grown 45-day-old seedlings were subjected to various stress treatments. The shoots were washed and cultured for 1 day in a growth chamber to minimize the mechanical stress on the tissues, followed by exposure to corresponding stress treatments, which were carried out as follows. For dehydration treatment, the shoots were placed on dry filter papers for 0, 1, 6, 9, 12 and 24 h at ambient environment. For cold stress, the seedlings were placed in the chamber set at 4°C for 0, 1, 6, 9, 12, 24, 48 and 96 h. For salt stress, the seedlings were placed in solution containing 200 mM NaCl solution for 0, 2, 4, 6, 8, 12 and 36 h. In addition, ABA treatment was carried out as follows. The seedlings were dipped in solution containing 100 μ M ABA for 0, 1, 3, 6, 9, 12 and 36 h. For each treatment, at least 30 seedlings were used, and the leaves were sampled from three randomly collected seedlings at

the designated time points, immediately frozen in liquid nitrogen and stored at -80°C for the purpose of extracting RNA for expression analysis.

Quantitative real-time PCR (qRT-PCR) analysis

Total RNA was isolated using a Plant Total RNA Isolation Kit Plus (FOREGENE Co. Ltd., Chengdu, China) and reverse transcribed into cDNA using the PrimeScript™ RT reagent kit (Takara, Dalian, China) according to manufacturer's the instructions. QRT-PCR was performed on a BioRad CFX96 real-time system using an SYBR®Green PCR Master Mix kit (Takara) using 20 µL of reaction mixture consisting 10 µL of 2×SYBR-PreMix EX Taq, 0.25 µM of each primer (Additional file 4: Table S1) and 50 ng of cDNA template. Fifteen pairs of the most specific primers were designed by Primer 5.0 software (PREMIER Biosoft International, Palo Alto, CA, USA) and checked by using NCBI online software (<https://www.ncbi.nlm.nih.gov/>). The protocol for real-time PCR was as follows: initiation with a 10 min denaturation at 95°C, followed by 55 cycles of amplification with 15 s of denaturation at 95°C, 15 s of annealing at 58°C and 20 s of extension at 72°C. Reads for fluorescence data collection occurred at 60°C. A melting curve was performed from 60 to 95°C to check the specificity of the amplified product.

The $2^{-\Delta\Delta CT}$ method was applied to calculate the relative expression level of each gene [53]. Relative expression levels were calculated by normalizing to expression of the pear *Tubulin* gene (AB239681), which was used as an internal control for *Pyrus betulaefolia*. Expression of *AtActin* was used as an internal reference for *Arabidopsis*. The expression analysis at each time point was repeated at least three times, and the data are shown as the mean values ± SE.

Subcellular localization

The full-length cDNA of *PbrPUB18* without a stop codon was amplified by RT-PCR with primer pair (GSP16, Additional file 4: Table S1) containing restriction sites of *Xba*I and *Bam*HI, then inserted into the pCAMBIA1302 vector and fused in-frame to the N-terminal of GFP (Green Fluorescent Protein), under the control of *CaMV*35S promoter to form a fusion construct 35S pro: *PbrPUB18*-GFP. After validation by sequencing, the fusion constructs 35S pro: *PbrPUB18*-GFP and 35S pro: GFP (as a control) were mobilized into *Agrobacterium tumefaciens* strain GV3101 by heat shock. Transient transformation of *Nicotiana benthamiana* was done as described earlier [54]. The fluorescence signal was observed with a confocal laser scanning microscope (LSM410; Carl Zeiss) after 72 h post infiltration and the position of nucleus was revealed by staining with 4', 6-diamidino-2-phenylindole (DAPI).

Arabidopsis transformation and characterization of transgenic plants

Arabidopsis thaliana ecotype Columbia Col-0 plants were transformed for heterologous over-expression *PbrPUB18* by using the floral dip method [37]. And *Agrobacterium tumefaciens* suspension containing the vector 35S-*PbrPUB18*-GFP (OD₆₀₀ = 0.80) was used for transformation. T0 seeds were identified by Murashige and Skoog (MS) solid medium with 20 mg·L⁻¹ hygromycin and then verified by PCR analysis using specific primers pair (GSP17, Additional file 4: Table S1). Semi-quantitative RT-PCR and qRT-PCR

was used to further analyze the transcript levels of *PbrPUB18* in T1 plants with primers pair (GSP18 and GSP5, Table S1). Two overexpressing lines (OE-4 and OE-5) of *PbrPUB18* were selected to generate T3 homozygous plants for the subsequent stress tolerance assay.

Assessment of drought tolerance in transgenic lines

To test the drought tolerance, The WT and transgenic lines were subjected to drought. For drought treatments, 15-day-old soil-grown *Arabidopsis* seedlings were subjected to withholding water for 12 days. At the end of treatment, the leaves were collected for measurement of electrolyte leakage, MDA content and ROS level. Electrolyte leakage was measured as described [55]. The MDA content, H_2O_2 and O_2^- content were measured using specific analytical kits (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China) following the manufacturer's instructions. Histochemical staining with 3, 3'-diaminobenzidine (DAB) and nitro-blue tetrazolium chloride (NBT) was used to analyse the *in situ* accumulation of H_2O_2 and O_2^- respectively, according to [56]. In addition, chlorophyll fluorescence measurements were recorded using an IMAGING-PAM chlorophyll fluorometer and ImagingWin software (Walz; Effeltrich, Germany) according to [57]. For chlorophyll fluorescence imaging, the 20 mins-dark-adapted plants were illuminated under an initial saturating pulse of $> 1800 \mu\text{mol photons m}^{-2}\text{s}^{-1}$. Then the maximum quantum efficiency of the photochemistry (F_v/F_m) values was obtained.

Statistical analysis

Three independent technical replicates were used for each sample, shown as mean $\pm$ standard error (SE). Statistical analyses were carried out with SPSS (IBM SPSS 17) statistical software package. Analysis of variance (ANOVA) was used to compare the statistical difference based on Duncan's multiple range test, at the significance levels of $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***).

Abbreviations

PUB: Plant U-box gene; UPS: Ubiquitin/26S proteasome system; Ub: Ubiquitin; E1: Ubiquitin-activating enzyme; E2: Ubiquitin- conjugating enzyme; E3: Ubiquitin ligase; HECT: Homology to E6-Associated Carboxy-Terminus; RING: Really Interesting New Gene; CRLs: Cullin–RING ligases; UFD2: Ubiquitin fusion degradation protein-2; AAO3: Absciscic aldehyde oxidase 3; ABA: Absciscic acid; MeJA: Methyl jasmonate; GAs: Gibberellin; RPKM: Reads per kilobase per million; qRT-PCR: Quantitative real-time PCR; GFP: Green fluorescent protein; DAPI: 4', 6-diamidino-2-phenylindole; F_v/F_m : The maximum quantum efficiency of the photochemistry; WT: Wide type; MDA: Malondialdehyde; EL: Electrolyte leakage; DAB: 3, 3'-diaminobenzidine; NBT: Nitro-blue tetrazolium chloride; ROS: Reactive oxygen species; TPR: Tetratricopeptide; ARM: Armadillo

Declarations

Ethics approval and consent to participate

Not Applicable.

Consent for publication

Not Applicable.

Availability of data and material

All needed genome sequences and genome annotation files of Chinese white pear were obtained from the Nanjing Agricultural University pear genome project website (<http://peargenome.njau.edu.cn>). All data generated or analysed during this study are included in this published article and its supplementary information files

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

CMW and XSH designed and carried out the experiments, and CMW carried out all bioinformatics analysis and wrote the manuscript. YQD and BBS contributed to genes expression analysis. BBS and XSH directed and revised the manuscript. All authors read, reviewed and approved the final manuscript.

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Tables

Due to technical limitations, table 1 is only available as a download in the Supplemental Files section.

Figures

Figure 1

Phylogenetic tree analysis of PUB gene family. (a) A Neighbor-Joining (NJ) tree of PUB proteins from three species, including pear, tomato and Arabidopsis. The phylogenetic tree was constructed by Mega-X software with 1000 boot strap. The red star, green triangle and blue tick represents the PUB proteins in Arabidopsis, pear and tomato, respectively. All of 185 PUB genes from three species were clustered into five subgroups, named Group I, II, III, IV and V; (b) Five pie plots represented the percentage of PUB genes

of three species in five groups. The orange part represented the percentage of PUB genes in pear, and the blue part represented the percentage of PUB genes in tomato, and the purple part presented the percentage of PUB genes in Arabidopsis.

Figure 2

The conserved motifs and gene structure analysis of PUB gene family in pear. (a) A Neighbor-Joining (NJ) phylogenetic tree of 62 pear PUB genes. The phylogenetic tree was constructed by Mega-X with 1000 bootstrap. The red branches indicated group I; the blue branches indicated group II; the orange branches indicated group III; the green branches indicated group IV; the purple branches indicated group V; (b) The conserved motifs analysis of PbrPUB genes in pear. A total of 20 motifs were predicated by

MEME tool, named Motif 1-20. The scale bar indicates 200 aa; (c) The gene structure analysis of PUB genes in pear, including UTR, intron and exon. The green rectangles represented UTR; the yellow rectangles represented Exon; the grey lines presented Intron. The scale bar indicates 2 kb.

Figure 3

The location distribution and synteny analysis of PUB genes in pear genome. (a) The distribution pattern of PUB genes family in 17 pear chromosomes. Due to no PUB genes were mapped on Chromosomes 8, we didn't show it in the Fig.; (b) The distribution pattern synteny analysis of PUB genes family. The red lines indicated the syntenic gene pairs of PUB gene family.

Figure 4

The cis-acting elements analysis of putative promoter of 62 PbrPUB genes. (a) The distribution pattern of 15 cis-acting elements of putative promoter of PUB gene family in pear. The phylogenetic tree was constructed by Mega-X using NJ method with 1000 bootstrap; (b) The number of 15 cis-acting elements of putative promoter of PbrPUB genes. The color scale at the top right indicated the number of cis-acting elements. Green color indicated the number of cis-acting elements on PUB member. 15 cis-acting elements including: (I) Absciscic acid responsiveness; (II) MeJA-responsiveness; (III) Gibberellin-responsive element; (IV) Light responsive element; (V) MYB binding site involved in drought-inducibility; (VI) Salicylic acid responsiveness; (VII) Anaerobic induction; (VIII) Auxin-responsive element; (IX) Zein metabolism regulation; (X) Defense and stress responsive element; (XI) Low-temperature responsiveness; (XII) MYB binding site involved in light responsiveness; (XIII) Endosperm expression; (XIV) Wound-responsive element; (XV) MYB binding site involved in flavonoid biosynthetic genes regulation.

Figure 5

The expression pattern analysis of PUB gene family in seven different tissues. (a) The heatmap of expression level of PUB gene family in seven different tissues, including stem, ovary, petal, sepal, bud, fruit and leaf. Pheatmap, an R package, were used to generate the heatmap. The color scale represented the RPKM (reads per kilobase per million) values normalized by log2. Red color represented high expression, while blue represented low expression; (b) The expression levels of 15 randomly selected genes in seven different tissues were detected by qRT-PCR experiment. Seven tissues are comprised of bud, stem, ovary, petal, sepal, fruit and leaf. The x-axes represented seven different tissues; the y-axes represented the relatively expression of PUB genes.

Figure 6

The expression level of 11 randomly selected PUB genes in four stresses were detected by qRT-PCR experiment. (a) For dehydration treatment, the shoots were placed on dry filter papers for 0, 1, 6, 9, 12 and 24 h; (b) For cold stress, the seedlings were placed in the chamber set at 4°C for 0, 1, 6, 9, 12, 24, 48 and 96 h; (c) For salt stress, the seedlings were placed in solution containing 200 mM NaCl solution for 0, 2, 4, 6, 8, 12 and 36 h; (d) For ABA stress, The seedlings were dipped in solution containing 100 μ M ABA for 0,

1, 3, 6, 9, 12 and 36 h. The x-axes represented time after treatment; the y-axes represented the relatively expression of PUB genes.

Figure 7

The Subcellular localization of PbrPUB18. (a) Tobacco leaf epidermal cells were transiently transformed with constructs containing 35S:GFP vector alone as control; (b) Transient expression of fusion plasmid (35S:PbrPUB18-GFP) in tobacco leaf epidermal cells. The nucleus was identified by DAPI staining. Green fluorescence images, DAPI staining mages, blight field images and the merged images are shown from left to right. Scale bars =20μm.

Figure 8

Drought tolerance assay of transgenic *Arabidopsis* plants overexpressing PbrPUB18. (a) Phenotypes of 15-day-old transgenic plants and WT before and after 12 days drought stress; (b) Images of (Fv/Fm). The false color code depicted on the right of the image ranges from 0 (black) to 1.0 (purple); Electrolyte leakage (c), MDA contents (d) in the WT, OE-4 and OE-5 after drought treatment; (e) Fv/Fm of WT, OE-4 and OE-5 before and after drought stress; (f) Histochemical staining with DAB and NBT for detection of

H₂O₂ and O₂⁻, respectively, in WT, OE-4 and OE-5 after drought stress for 12 days; Levels of H₂O₂ (g) and anti-O₂⁻ (h) in Arabidopsis WT, OE-4 and OE-5 after drought treatment. Asterisks indicate that the value is significantly different from that of the WT at the same time point (**P< 0.01; ***P< 0.001).

Supplementary Files

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