PARAQUAT TOLERANCE3 is an E3 ligase and acts as a negative regulator of oxidative stress response

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Abstract

Oxidative damage could be caused in plant cells when biotic and abiotic stresses are imposed. While the response to oxidative stress is well studied, little is known about how the activated response is switched off when oxidative stress is diminished. By studying Arabidopsis mutant paraquat tolerance3, we identified the genetic locus PARAQUAT TOLERANCE3 (PQT3) as a major negative regulator of oxidative stress tolerance. PQT3, encoding an E3 ligase, is rapidly down-regulated by oxidative stress. PQT3 has E3 ubiquitin ligase activity in ubiquitination assay. Subsequently, we identified PRMT4b as a PQT3-interacting protein. By histone methylation, PRMT4b may regulate the expression of APX1 and GPX1, encoding two key enzymes against oxidative stress. Moreover, PQT3 is able to recognize PRMT4b for targeted degradation via 26S proteasome. Therefore, we have identified PQT3 as an E3 ligase that acts as a negative regulator of activated response to oxidative stress.
Sessile plants cannot avoid harsh living conditions such as drought, salinity, cold and hot temperature. These stresses induce damages to plants in different degrees. In most cases, they alter the normal cell homeostasis and increase the generation of reactive oxygen species (ROS) (Miller et al., 2010). Exposing plants to excessive light also causes ROS accumulation, serious damage to the photosynthetic system, and photo-inhibition (Mittler, 2002; Caverzan et al., 2012). Excessive ROS production results in oxidative stress which could destroy biological membranes and macromolecules, accelerate cell senescence, induce irreversible damages to cells and even lead to cell death (Gill and Tuteja, 2010). Under normal circumstances, ROS can be controlled at a low level in the organelles where ROS are produced, such as chloroplasts, mitochondria, and peroxisomes. However, the level of ROS is sharply increased under stress conditions (Moller, 2001; Mittler et al., 2004; del Rio et al., 2006; Leshem et al., 2006; Corpas et al., 2008; Miller et al., 2008; Van Breusegem et al., 2008; Miller et al., 2010). The production and elimination of ROS are dynamically balanced in plant cells (Apel and Hirt, 2004). Two protection systems, enzymatic and non-enzymatic, have evolved to scavenge ROS and protect plant cells from oxidative stress. Enzymatic system mainly includes the superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), glutathione peroxidase (GPX) and peroxiredoxin (PrxR), while non-enzymatic system mainly contains the ascorbic acid (AsA), alpha tocopherol (VE), and glutathione (GSH) (Takahashi and Asada, 1988; Apel and Hirt, 2004; Mittler et al., 2004). ROS are also important signal molecules that mediate the responses to pathogens, environmental stress, and stimuli in various developmental processes (Mittler et al., 2004; Torres and Dangl, 2005). The coordination mode of different networks of ROS elimination is very complex in Arabidopsis (Mittler et al., 2004).

The activation of oxidative response involves many layers of regulations (Mittler, 2002; Mittler et al., 2004). Little is known about the regulation by histone methylation of the genes involved in oxidative stress response. Histone methylation plays...
important roles in the plant development and growth as well as in some stress responses (Niu et al., 2007; Niu et al., 2008; Hong et al., 2010; Liu et al., 2010a; Yuan et al., 2013). The methylation marks are written on lysines or arginines respectively by histone lysine methyltransferases (HKMTs) and protein arginine methyltransferases (PRMTs). Nine PRMTs are found in the Arabidopsis genome (Niu et al., 2007). Two different types of PRMTs catalyze asymmetric di-methylation (ADMA) and symmetric di-methylation (SDMA) on the Arg residues, respectively (Scorilas et al., 2000; Liu et al., 2010a). A pair of PROTEIN ARGININE METHYLTRANSFERASE4 (PRMT4) homologs, AtPRMT4a and AtPRMT4b, is required for the asymmetrical di-methylation of Arg-2, Arg-17, and Arg-26 in histone H3 (Niu et al., 2007). Protein arginine methylation plays essential roles in diverse biological processes, such as RNA processing and transcriptional regulation (Scorilas et al., 2000).

Oxidative stress could be perceived by multiple mechanisms, including sensor or cellular receptor. The perception by receptors results in the activation of Ca\(^{2+}\)–calmodulin and mitogen-activated protein kinase (MAPK) cascade signaling transduction pathway. The activation or suppression of different transcription factors regulates a variety of defense pathway subsequently, such as ROS-scavenging, heat-shock proteins (HSPs), and photosynthesis (Mittler, 2002; Apel and Hirt, 2004; Mittler et al., 2004). While much attention has been paid to how plants respond to oxidative stress, we know little about how plants switch off the activated responses when stress is diminished. A common regulatory mechanism is to control the protein level of the stress responsive factors. The most studied mechanism of protein degradation is the ubiquitin/26S proteasome system (Santner and Estelle, 2010). The ubiquitin/26S proteasome pathway has been implicated in diverse aspects of eukaryotic cell regulation because of its ability to rapidly remove specific intracellular proteins (Moon et al., 2004; Dreher and Callis, 2007; Santner and Estelle, 2010). In this system, ubiquitin composed of 76 amino acids, is attached to the target protein under the action of three different enzymes (Dreher and Callis, 2007). The target proteins with ubiquitin have different fates. One kind of the ubiquitinated proteins can
be recognized and degraded by the 26S proteasome. Another type of labeled proteins, which are folded in wrong ways, can be recognized by their related enzymes to achieve the correct protein structures. The ubiquitin system may also directly affect protein activity and intracellular localization. It can also identify and modify many intracellular proteins, such as proteins involved in signal transduction, transcription factors, and receptors on cell surface, to participate in the regulation of physiological processes (Mukhopadhyay and Riezman, 2007).

In the ubiquitin degradation process, E3 played a crucial role. E3 is responsible for specific recognition of substrate protein and accurate positioning of the binding site between substrate protein and ubiquitin (Yee and Goring, 2009). In Arabidopsis thaliana, approximately 1400 genes encode components of the ubiquitin/26S proteasome pathway (Smalle and Vierstra, 2004). Approximately 90% of these genes encode subunits of the E3 ubiquitin ligases (Moon et al., 2004). Based on the domain interacted with E2 (ubiquitin conjugase) during the ubiquitin conjugation cascade, the large and diverse family of plant E3 ubiquitin ligases can be divided into HECT domain- and RING/U-box domain-containing E3 ligases (Santner and Estelle, 2010). The HECT family is relatively small compared with the RING domain-containing family that includes several hundreds of proteins and can be further divided into single subunit RING/U-box E3 ligases and multi subunit RING E3 ligases (Pickart, 2001; Moon et al., 2004). The large number of E3 ubiquitin ligases in higher plants indicates their important regulatory roles in diverse biological processes (Mazzucotelli et al., 2006).

Few E3 ligases have been identified to turn off the activated stress responses and the function of an enormous number of E3 ligases still remains to be identified. Here we report a novel negative regulator of oxidative stress response by PARAQUAT TOLERANCE3 (PQT3) in Arabidopsis. We isolated a paraquat tolerant mutant, paraquat tolerance3 (pqt3), and cloned the gene PQT3 that encodes an E3 ligase containing RING/U-box domain. The expression of APX1 and GPX1 was up-regulated in pqt3, while PQT3 was down regulated by oxidative stress. PQT3 was able to interact with PRMT4b. PRMT4b may catalyze histone methylation on APX1
and GPX1 chromatin and up-regulate their expressions, therefore protect plants from oxidative stress. When oxidative stress is diminished, PQT3 level increases and acts as E3 ubiquitin ligase to specifically target PRMT4b for degradation. Based on our results, PQT3 is a negative regulator that turns off the activated response of oxidative stress.

Results

Loss of At4g17410 confers the paraquat tolerance of pqt3

The mutant pqt3 was isolated from an activation-tagging library as described (Xi et al., 2012). This library containing approximately 55,000 independent lines was screened for mutants with enhanced tolerance to different stresses (Yu et al., 2008; Wu et al., 2010). To isolate tolerant mutant to oxidative stress, we germinate seeds on MS medium with 2 µM paraquat, green seedlings that could continued to grow were rescued as putative mutants with improved paraquat tolerance and named paraquat tolerance (pqt) mutants. The pqt3, as one mutant of them, was further characterized and marked as pqt3-1. The enhanced oxidative tolerance of pqt3-1 mutant was confirmed by germinating seeds on MS medium containing 0 or 2 µM of paraquat. In presence of paraquat, more than 60% pqt3-1 seeds germinated with green cotyledons but only 2% wild type seeds germinated, while all seeds of both wild type and pqt3-1 germinated on MS medium without paraquat (Figures 1A and 1B). Genetic analysis showed that the mutation was recessive. All F1 backcross offsprings (pqt3-1 x wild type) were paraquat sensitive and F2 selfing population showed typical 3:1 segregation ratio (sensitive: resistant; 85:27, $\chi^2 = 0.0476$). The result suggested that pqt3-1 mutant may have a more efficient mechanism of ROS scavenge, which was caused by loss-of-function mutation in a single nuclear gene PQT3 (At4g17410).

In pqt3-1 mutant, a single T-DNA insertion was located in the fourth intron of At4g17410 (Supplemental Figure 1A). The exact integration site of the T-DNA right border was 803bp downstream of the ATG initiation codon of At4g17410. As a result,
the expression of *At4g17410* was completely disrupted as confirmed by RT-PCR analysis (Supplemental Figures 1B and 1D). The expressions of its neighboring genes, *At4g17390* and *At4g17420*, were not affected (Supplemental Figure 1B).

The *At4g17410* locus consists of 13 exons and 12 introns. The open reading frame is predicted to encode a polypeptide composed of 827 amino acids with an estimated molecular mass of 91kD. Based on the conserved RING/U-box domain, this protein is predicted as an E3 ligase.

To further determine whether the loss of *At4g17410* resulted in the enhanced oxidative tolerance of *pqt3-1* mutant, we used another allele of *pqt3*, the T-DNA insertion mutant Salk_065409, which was ordered from Arabidopsis Biological Resource Center (ABRC) and its T-DNA insertion was confirmed by RT-PCR (Supplemental Figures 1A, 1C and 1D). As the first identified *pqt3* mutant was named as *pqt3-1*, the Salk_065409 was marked as *pqt3-2*. The *pqt3-2* mutant showed similar enhanced oxidative tolerance to paraquat and had high survival ratio under different concentrations of paraquat treatment as *pqt3-1* did (Figure 1C). The survival ratio of *pqt3-1* and *pqt3-2* were 50% and 20%, respectively, under 2 μM paraquat treatment, while none of the wild type seedlings survived under the same condition. In addition, both *pqt3-1* and *pqt3-2* showed a late-flowering phenotype (Figure 1D). To confirm further, we generated functional complementation (FC) lines and 35Spro:*PQT3* overexpression lines (Supplemental Figures 1E and 1F). FC lines and 35Spro:*PQT3* lines showed similar if not higher paraquat sensitivity to wild type under 2 μM paraquat treatment while the *pqt3-1* and the *pqt3-2* mutants displayed enhanced paraquat tolerance (Figures 1E and 1F). These results indicate that *PQT3* is a negative regulator of oxidative stress tolerance and is responsible for the phenotype of *pqt3* mutants.

After 6 μM paraquat treatment for 12 or 24 hours, the result of DAB staining showed that the brown precipitate on the leaves of the wild type was more than that of *pqt3* mutants (Figures 1G to 1R). As several stresses could cause oxidative damage to plants, the sensitivity of *pqt3* mutants to other environmental stresses was analyzed subsequently. The result indicated that *pqt3* mutants have enhanced tolerance to CdCl₂,
mannitol, NaCl, and drought stress (Supplemental Figure 2).

**The expression pattern and protein localization of PQT3**

To investigate the spatiotemporal pattern of PQT3 expression, we generated PQT3pro:GUS reporter lines. GUS staining results showed that PQT3 was expressed in both shoot and root tissues under normal condition (Figures 2A to 2G). GUS expression was detected in the root tissues at all developmental stages we analyzed (Figures 2A to 2C). For the 1-week-old seedlings, strong GUS staining was observed in the cotyledons, hypocotyls and root tissues (Figure 2A). For the 3-week-old seedlings, strong GUS staining was also detected in cotyledons, young leaves, and root tissues, but weakly stained in older leaves (Figure 2B). In 7-week-old adult plants, GUS expression was detected in rosette leaves, cauline leaves, the tip and basal junction of siliques, and was significantly higher in the flower petals, stamens and stigma of pistil (Figures 2D to 2G).

PQT3 has two predicted nuclear localization signals (NLSs) in the carboxyl terminus (Supplemental Figure 3), implicating its nuclear localization. To confirm this, the 35Spro:PQT3-GFP construct was made and transiently expressed in onion epidermal cells. The 35Spro:GFP construct was used as control (Figures 2H to 2J). PQT3-GFP signal was indeed detected in the nucleus (Figures 2K to 2M).

For further confirmation, we transformed the PQT3pro: PQT3-GFP fusion construct into the Arabidopsis and obtained transgenic plants. Fluorescent microscopy results showed that GFP signal was accumulated in the nucleus of root cells (Figures 2N to 2P), which is in agreement with the presence of two NLSs of the PQT3.

**PQT3 is rapidly down regulated by paraquat treatment and other stress conditions**

Interestingly, the expression of PQT3 was quickly down-regulated by 6 μM paraquat treatment and maintained at a low level as long as the paraquat treatment was applied (Figure 3A). This result is supportive for our previous opinion that PQT3 is a negative regulator of plant oxidative tolerance. By extrapolation, the expression
of PQT3 could be down-regulated by other stress conditions. Indeed, our results showed that the expression of PQT3 was down-regulated by H2O2 (Figure 3B), mannitol (Figure 3C), drought (Figure 3D), CdCl2 (Figure 3E), and NaCl treatment (Figure 3F) at the indicated time points. Among these stresses, CdCl2 treatment led to the most significant reduction of PQT3 expression (Figure 3E). The PQT3pro:GUS pattern was observed under different stresses subsequently (Figures 3G to 3DD).

Compared with transgenic seedlings under normal conditions (Figures 3G, 3M, 3S and 3Y), GUS staining was weaker in the seedlings under paraquat (Figures 3H, 3N, 3T and 3Z), H2O2 (Figures 3I, 3O, 3U and 3AA), mannitol (Figures 3J, 3P, 3V and 3BB), CdCl2 (Figures 3K, 3Q, 3W and 3CC), and NaCl treatment (Figures 3L, 3R, 3X and 3DD). The changed PQT3pro:GUS pattern was consistent with the altered expression of PQT3 detected by quantitative RT-PCR under different stresses.

APX1 and GPX1 were up-regulated in pqt3 mutant

Enzymatic protection systems were very important for ROS elimination. The transcript levels of ascorbate peroxidase (APX), catalase (CAT), glutathione peroxidase (GPX), cytosolic Cu/Zn SOD (CSD1), plastidic Cu/Zn SOD (CSD2), FeSOD (FSD), atypical Cys-His rich thioredoxin (ACHT), glutaredoxin C (GRXC), 2-Cys peroxiredoxin B (2CPB), peroxiredoxin Q (PRXQ), and mitochondrial MnSOD (MSD) were analyzed by quantitative RT-PCR in pqt3 and wild type. The results show that transcript levels of APX1 and GPX1 were up-regulated in pqt3 under normal conditions compared with that in the wild type (Figures 4A to 4J). The elevated transcript levels of APX1 and GPX1 may contribute to the enhanced oxidative stress tolerance. The enzyme activity of APX and GPX in wild type and pqt3 mutant was also detected. The pqt3 mutant had higher enzyme activity of APX and GPX compared with wild type (Figures 4K and 4L).

PQT3 possesses E3 ligase activity

Not all the proteins with the predicted RING domain function as an ubiquitin ligase (Deshaies and Joazeiro, 2009). The E3 activity of PQT3 was determined via
self-ubiquitination system. The full-length PQT3 protein (GST-PQT3) showed the E3 ubiquitin ligase activity (Figure 5). The ubiquitinated bands of PQT3 were detected by western blotting in the presence of E1 (from wheat), E2 (UBCh5b, from human), and 6xHis-tagged ubiquitin (UBQ14, from Arabidopsis). Self-ubiquitination of PQT3 was not detected in the absence of any of the essential reaction components (Figure 5). The result demonstrates that PQT3 has the E3 ubiquitin ligase activity.

**PQT3 interacts with PRMT4b**

To study the molecular mechanisms that underlie the enhanced stress tolerance of *pqt3*, we screened cDNA library for potential candidate target proteins of PQT3 using yeast-two-hybrid (Y2H). Several proteins were isolated from the screen. Among these candidate interactors, PRMT4b, a member of arginine methyl transferase family, was frequently presented. To reveal the domain of PQT3 responsible for the interaction with PRMT4b, the PQT3 protein was divided into four parts: N-terminal DWNN, zfCCHC, U-box (RING finger), and C-terminal section containing the NLS1 and NLS2 domains, based on the predicted domains of PQT3 protein (Supplemental Figure 3). Full-length PQT3 and four protein sections were used for Y2H assay as baits. Full-length protein and the C-terminus containing the NLS1 and NLS2 domains of PQT3 were able to interact with PRMT4b in Y2H assays (Figures 6A to 6C). The interaction between PQT3 and PRMT4b was confirmed by colonies that grew on the SC-Leu-Trp-His plate with 50 mM 3-amino-1,2,4-triazole (3-AT) and displayed the blue color in X-gal assay (Figures 6B and 6C). PRMT4a is not a potential target of PQT3 in previous Y2H library screening. The Y2H assay was also performed to further study the potential interaction between PQT3 and PRMT4a, since *PRMT4a* is a close related gene of *PRMT4b*. The result showed that the PQT3 can not interact with PRMT4a (Supplemental Figure 4).

Protein pull-down assay was performed to confirm the interaction between PQT3 and PRMT4b in vitro. MBP-PQT3-C66 protein containing the NLS domain (Supplemental Figure 3) and His-PRMT4b protein were expressed in *E. coli* and purified subsequently. His-PRMT4b was incubated with amylose resin bound with
recombinant MBP-PQT3-C66 protein. Pulled-down protein complex was detected by SDS-PAGE (Figure 6D) and western blotting using anti-His antibody (Figure 6E). The pull-down result clearly shows that PQT3 interacts with PRMT4b in vitro.

To determine whether the interaction also occurs in vivo, we used the bimolecular fluorescence complementation (BiFC) system. Full-length PQT3 cDNA was fused to the N-terminal region of the yellow fluorescent protein (YFP), while full-length PRMT4b cDNA was fused to the C-terminal region of YFP. The empty plasmids were used as negative controls. Different plasmid combinations were co-infiltrated into epidermal cell of N. benthamiana leaves. The yellow fluorescence was observed in epidermal cell containing both NE-PQT3 (the N-terminal part of YFP fused with PQT3) and CE-PRMT4b (the C-terminal part of YFP fused with PRMT4b) (Figure 6F). No fluorescence was observed from the negative controls (NE-PQT3/CE, NE/CE-PRMT4b and NE/CE) (Figures 6G, 6H and 6I). The nuclei were stained by Hoechst and detected by confocal subsequently. These results indicate that PQT3 can interact with PRMT4b in the nucleus of plant cell.

**PRMT4b is an ubiquitination substrate of PQT3**

To determine whether PRMT4b is a substrate recognized by PQT3 being as an E3-ligase, we resorted to the in planta ubiquitination assay (Liu et al., 2010b). Leaf infiltration was conducted via Agrobacterium tumefaciens strains containing different combination of constructs. The infiltrated parts of N. benthamiana leaves were harvested. Total protein was extracted and detected via western blotting with anti-HA antibody. A smear of bands, which were larger than the size of HA-PRMT4b and showed the features of ubiquitinated form of the PRMT4b proteins, could be examined by anti-HA antibody in the samples co-infiltrated with PQT3 and HA-PRMT4b (Figure 7A). The cell lysates were immunoprecipitated with anti-HA antibody subsequently. Immunoprecipitated samples were detected via western blotting with anti-ubiquitin antibody. In the PQT3-PRMT4b co-infiltration sample, these high molecular size bands could also be detected by anti-ubiquitin antibody (Figure 7B). These results indicate that these high molecular size bands were
ubiquitinated forms of PRMT4b. PQT3 protein could ubiquitinate the PRMT4b protein in tobacco. The decline of PRMT4b protein was also found in the samples co-infiltrated with PQT3 and HA-PRMT4b (Figure 7A).

The transcript level of PRMT4b could be induced in both wild type and pqt3 seedlings by paraquat treatment (Figure 7C). The mRNA level of PRMT4b was similar between wild type and pqt3 seedlings without or with paraquat treatment for 0.5h. The transcript level of PRMT4b in pqt3 seedlings was lower than that of the wild type under paraquat treatment for 1h (Figure 7C). The PRMT4b protein levels were also detected using the seedlings treated without or with paraquat. As shown in the Figure 7D, the protein level of PRMT4b in pqt3-1 was higher than that of the wild type under normal conditions. The protein level of PRMT4b in wild type remains lower than that of pqt3-1 under paraquat treatment at the same time point, although protein level of PRMT4b increased gradually in wild type and pqt3-1 with the prolonged paraquat treatment (Figure 7D). Under MG132 treatment, the protein level of PRMT4b was increased in both wild type and pqt3-1 mutant. When the seedlings were co-treated with paraquat and MG132 for 12 h, PRMT4b was accumulated in both wild type and pqt3-1 mutant, and no significant difference of PRMT4b protein level was found between the wild type and pqt3-1 mutant (Figure 7D) because the degradation of the PRMT4b protein through ubiquitination-26S proteasome pathway was inhibited by MG132.

In order to further demonstrate the PQT3-dependent ubiquitination of PRMT4b, the phenotype of wild type and pqt3-1 mutant treated without or with paraquat in presence or absence of proteasome inhibitor MG132 was studied. Survival ratio of pqt3-1 seedlings was higher than that of wild type under paraquat treatment. No significant difference of survival could be observed when the wild type and pqt3-1 seedlings were co-treated with paraquat and MG132 (Figure 7E). The wild type gained enhanced paraquat tolerance as pqt3-1 mutant under MG132 treatment.

PRMT4b is involved in the oxidative stress tolerance

To reveal whether PQT3-enhanced the oxidative tolerance of plants is via
PRMT4b in Arabidopsis, we obtained the prmt4b mutant and 35Spro:PRMT4b lines (Supplemental Figures 1G, 1H and 1L) and observed the phenotypes of prmt4b and 35Spro:PRMT4b under different concentrations of paraquat treatment firstly. Survival ratio of wild type, pqt3-1, pqt3-2, prmt4b and 35Spro:PRMT4b were counted (Figure 8A). 35Spro:PRMT4b had similar phenotype as pqt3-1 and pqt3-2, while the prmt4b mutant was more sensitive to paraquat treatment than wild type (Figure 8A). Under CdCl2 treatment, primary root elongation of prmt4b mutant was also slower than that of wild type (Figure 8B). Furthermore, the overexpression lines of PRMT4b were analyzed under other stress conditions. 35Spro:PRMT4b increased tolerance to CdCl2 and NaCl stresses, as compared with wild type. The 35Spro:PRMT4b showed the opposite of the 35Spro:PQT3 which was more sensitive to CdCl2 and NaCl stresses as compared with wild type (Supplemental Figure 5). These results show that PRMT4b is a positive regulator for plant oxidative tolerance, which is also consistent with the function of PQT3. The prmt4a mutant and prmt4aprmt4b double mutants were subsequently obtained and examined (Supplemental Figures 1I to 1K). The prmt4aprmt4b double mutants had the similar phenotype with prmt4b under paraquat treatment, and the knockout of PRMT4a did not affect the oxidative tolerance (Figures 8C to 8E).

**Increased H3R17 methylation on APX1 and GPX1 chromatin in pqt3**

The transcript level of APX1 and GPX1 was higher in pqt3 than that in wild type under normal conditions (Figures. 4A and 4B). PQT3 may regulate the transcription of APX1 and GPX1 via PRMT4b. Therefore, chromatin immunoprecipitation (ChIP) assays were carried out to compare the modification status of H3R17me2a in the chromatin of APX1 and GPX1 between wild type and pqt3 mutant. ChIP assays were performed with wild type and pqt3 plants using antibody against H3R17me2a. As shown in the Figures 9A and 9B, the APX1 and GPX1 chromatin was divided into different regions and the enriched chromosome fragments were detected by quantitative RT-PCR. The results showed that histone H3R17me2a modification of APX1 and GPX1 chromatin was increased in pqt3 mutant (Figures 9C and 9D). These
results suggest that PRMT4b may target \textit{APX1} and \textit{GPX1} to enhance the oxidative tolerance by increasing asymmetric dimethylation of H3 at R-17 in \textit{APX1} and \textit{GPX1} chromatin.

**Genetic analysis of PRMT4b acting as a target of PQT3 in oxidative tolerance**

To confirm further that PRMT4b is the target of PQT3, the \textit{pqt3prmt4b} double mutants were obtained (Supplemental Figures 1M and 1N). The survival ratio of \textit{pqt3prmt4b} under paraquat treatment was intermediate between \textit{pqt3} and \textit{prmt4b}, which demonstrates that the PRMT4b protein is one of the targets of PQT3 and suggests that PQT3 may also target other proteins that contribute to the tolerance to oxidative stress (Figures 10A to 10C).

We also obtained the \textit{pqt3prmt4a} double mutants (Supplemental Figures 1O and 1P) and found that the survival ratio of \textit{pqt3prmt4a} under paraquat treatment was not significantly different from that of \textit{pqt3} mutant (Figures 10D to 10F), again indicating that PRMT4a is not involved in oxidative stress response.

**Discussion**

PQT3 is a member of Arabidopsis RING-finger/U-box E3 ligase family. Secondary structure prediction using InterProScan protein sequence analysis software revealed four conserved domains including DWNN (domain with no name), zinc finger domain, RING-finger domain, and U-box domain in N-terminus of PQT3 protein (Supplemental Figures. 6A and 6B). DWNN is a novel ubiquitin-like domain, which is a highly-conserved domain in eukaryotic animals and plants (Pugh et al., 2006). The DWNN domain of PQT3 contains 76 amine acids. DWNN domain is only found in the N-terminus of the members in the splicing-associated RBBP6 (Retinoblastoma Binding Protein 6) protein family (Pugh et al., 2006). The RING-finger domain was also found in RBBP6 protein family. The existence of the RING-finger domain suggests that DWNN domain may act as ubiquitin-like
regulatory factors (Pugh et al., 2006). CCHC-type Zinc finger domain is also known as zinc knuckle, which can be found in a large number of RNA binding proteins (Lopato et al., 1999; Krishna et al., 2003). As mentioned above, RING-finger domain can combine with the E2 in the cascade reaction of ubiquitination system, while U-box is a modified RING-finger (Freemont, 2000). In addition, two predicted NLS sequences (471-477 and 696-711 amino acids) were also found in the C-terminus of PQT3, which is consistent with nuclear localization of the protein (Figures 2H to 2P; Supplemental Figures 6A and 6B). Phylogenetic tree analysis further revealed the high homology proteins of PQT3 in other species (Supplemental Figure 6C). DWNN domain and RING-finger/U-box domain in N-terminus of PQT3 were highly conserved in homologous proteins in different plant species (Supplemental Figure 7). The PQT3 may play its role as an ubiquitin ligase in different species and its function may be conserved throughout the plant kingdom.

In vitro ubiquitination assay shows that PQT3 has the E3 ligase activity (Figure 5). For in planta ubiquitination assay, as compared with the control sample, the enhanced high molecular size bands in the PQT3-PRMT4b co-infiltration sample indicate that PRMT4b protein could be ubiquitinated by expressed PQT3 protein in N. benthamiana (Figures 7A and 7B). The transcript level of PRMT4b was not significantly different between wild type and pqt3 mutant without or with paraquat treatment for 0.5 h (Figure 7C). The transcript level of PRMT4b was lower in pqt3 mutant than that in wild type under paraquat treatment for 1 h (Figure 7C). One possible reason is that pqt3 has enhanced oxidative tolerance to paraquat which could induce the transcript level of PRMT4b (Figures 1 and 7C). In addition, the reduced transcript level of PRMT4b in pqt3 may be due to a negative feedback regulation that is activated by the accumulation of PRMT4b protein under paraquat treatment (Figure 7D). In Arabidopsis, pqt3-1 and pqt3-2 had higher protein level of PRMT4b than the wild type, as the PRMT4b protein was degraded by PQT3 in wild type under normal conditions (Figure 7D). The transcript level of PRMT4b could be induced in wild type under paraquat treatment (Figure 7C), while the transcript level of PQT3 was decreased by paraquat treatment (Figure 3A). PQT3-mediated PRMT4b degradation
was weakened in wild type. Consequently, the level of PRMT4b protein in wild type was elevated, but it was lower than that of pqt3 (Figure 7D). Proteasome inhibitor MG132 could block the degradation of the PRMT4b protein in wild type and enhance paraquat tolerance of wild type (Figures 7D and 7E). Above all, PQT3, as an E3 ligase, play its role in oxidative tolerance through the ubiquitination-degradation of PRMT4b.

A series of environmental stress could lead to oxidative damage to plants (Zhu, 2000; Mittler, 2002; Apel and Hirt, 2004). Both biotic stress and abiotic stress result in the production of ROS which in excess cause oxidative stress. Besides paraquat tolerance, pqt3 mutants have enhanced tolerance to various environment stresses (Figure 1; Supplemental Figure 2). Further studies may reveal new mechanisms of PQT3 in multiple stress tolerance. Analysis of PQT3-interacting proteins may be a good start point to further understand the function of PQT3. In the screen for PQT3-interacting proteins with Y2H, we isolated 47 colonies that were positive for the expressions of the His3 and LacZ reporter genes. Among these proteins, PRMT4b, as previously mentioned, may be responsible for the increased degree of Arg-17 methylation which may further regulate APX1 and GPX1 genes to enhance oxidative tolerance of plants (Figures 4A, 4B and 9). It has been reported that arginine methylation was involved in transcriptional regulation, signal transduction, DNA repair, RNA processing, and nuclear transport (Bedford and Richard, 2005; Pahlich et al., 2006; Bedford and Clarke, 2009; Liu et al., 2010a; Yu, 2011; Ahmad and Cao, 2012; Blackwell and Ceman, 2012). PRMTs have been extensively analyzed, and the functions of PRMTs involved in flowering time, circadian cycle, vegetative growth, and the plant response to salinity and ABA have been reported (Niu et al., 2008; Ahmad and Cao, 2012). Here, the prmt4b mutant was found to be more sensitive to paraquat and CdCl2 treatment than wild type (Figure 8). Thus, PRMT4b plays a role in plant response to oxidative stress. It is known that PRMT4-mediated methylation at Arg-17 of histone H3 is linked to transcription activation (Bauer et al., 2002). The Arg-17 of histone H3 is the major site of PRMT4-mediated methylation, although it was reported that other protein site could also be methylated by PRMT4.
We suggest that the increased Arg methylation degree of specific regions in APX1 and GPX1 chromatin was caused by PRMT4b (Figure 9).

The increased transcript level of APX1 and GPX1 may be caused by PRMT4b-mediated histone Arg methylation (Figures 4A, 4B and 9). APX1 is a cytosolic enzyme involved in H₂O₂ degradation using ascorbate as the electron donor (Correa-Aragunde et al., 2013). The response of APX1 to oxidative stress has been studies in Arabidopsis. APX1 plays an important role in the response to stress combination (Koussevitzky et al., 2008). It could be activated by multiple stresses to protect plants against oxidative stress (Davletova et al., 2005; Vandrauwera et al., 2011; Maruta et al., 2012). The tobacco could be more tolerant against UV-C-induced oxidative damage through the overexpression of APX1 (Saxena et al., 2011). GPXs also have important functions in oxidative signaling, which can protect plants from harmful effects of excessive oxidation (Passaia et al., 2014). It has also been reported that overexpression of a tobacco glutathione S-transferase with glutathione peroxidase activity (GST/GPX) in transgenic tobacco seedlings lead to enhanced peroxide scavenging and reduced oxidative damage (Roxas et al., 2000).

The pqt3 mutants also have late-flowering phenotype (Figure 1D). It has been reported that prmt4aprmt4b double mutants display late-flowering phenotype (Niu et al., 2008). The Y2H result showed that the PQT3 can not interact with PRMT4a (Supplemental Figure 4). As compared with wild type, the prmt4a mutant also has no significant difference in the oxidative tolerance (Figures 8C to 8E). The phenotype of pqt3prmt4a mutant demonstrates that PRMT4a protein was not involved in the regulation of oxidative stress response by PQT3 (Figures 10D to 10F). The late-flowering phenotype of pqt3 may be regulated by other mechanisms, rather than PRMT4a and PRMT4b. The flowering-related transcription factor AGAMOUS (AG) was found to be a potential target interacted with PQT3 in Y2H library screening. AG is involved in carpel development, leaf development, identification of floral organs, and stamen development (Urbanus et al., 2010). Targeted removal of AG by PQT3 may be related to the late-flowering phenotype of pqt3. By interacting
with different partners, PQT3 may mediate multiple functions in diverse biological processes.

In conclusion, oxidative stress activates the expression of PRMT4b, represses the expression of PQT3, and weakens PQT3-mediated the ubiquitinated degradation of PRMT4b, synergistically resulting in increased accumulation of PRMT4b. Consequently, the increased level of PRMT4b protein may lead to higher degree of histone methylation on the APX1 and GPX1 chromatin. As a result, the transcription of APX1 and GPX1 is activated, leading to more APX1 and GPX1, which enhance oxidative tolerance of plants. When the stress disappears, transcription repression of PQT3 by oxidative stress is removed. The function of PQT3, as a negative regulator of oxidative stress response, is restored. The PRMT4b is then degraded by PQT3 in ubiquitination pathway. The activated response to oxidative stress is switched off. We propose a working model for PQT3 as a negative regulator of oxidative stress response (Figure 11).

Methods

Mutant screen from the activation-tagging library

The paraquat tolerant mutant PQT3 was isolated from an activation-tagging library including approximately 55,000 individual lines generated using pSKI015 in the Columbia ecotype as described (Xi et al., 2012).

Plant material and growth conditions

The wild type used in the study is Arabidopsis thaliana ecotype Columbia that is the genetic background for all the mutants and transgenic plants. Salk_065409 (pqt3-2), Salk_097442C (prmt4b) and Salk_033423 (prmt4a) were ordered from ABRC (Arabidopsis Biological Resource Center). The seeds were sterilized in 10% bleach for 10 min. Then the seeds were washed for 5 times at least with sterile water. For vernalization, the seeds were kept in the dark with water at 4°C for 3 days to
ensure the synchronous germination. Sterile seeds were germinated on half-strength MS medium. The seedlings were grown at 22°C under 16-h-light (100 μE m⁻² s⁻¹) /8-h-dark cycle.

**Transformation of Arabidopsis**

The constructs were electroporated into competent cell of *Agrobacterium tumefaciens* C58C1. The floral-dip method was used to transfer these constructs into Arabidopsis as described (Clough and Bent, 1998; Bent, 2000).

**PCR analysis**

Total RNA was extracted from various tissues of plants using TRIzol reagent. Then RNA reverse reaction was carried out by TransScript Kit (TransGen Biotech). For RT-PCR analysis, PCR was performed using specific primers and the products were detected by agarose gel electrophoresis. For quantitative RT-PCR analysis, the transcript level was detected on an Applied Biosystem Step One real-time PCR system using SYBR Premix Ex Taq II (TaKaRa) and specific primers were listed in the Supplemental Table 1. *UBQ5* was used as the internal control.

**Identification of pqt3 mutant, FC line, 35Spro:PQT3, and 35Spro:PRMT4b.**

Homozygous T-DNA insertion mutants of Salk_150614 were identified using genomic PCR as described (Alonso et al., 2003). RT-PCR was carried out as previously described to confirm the results of genomic PCR. The 35Spro:PQT3 and 35Spro:PRMT4b plasmids were transformed into Col-0 to obtain the overexpression lines of PQT3 and PRMT4b. For the FC line, the 35Spro: PQT3 was transformed into pqt3 mutant, and the line with the same expression level of PQT3 as wild type was chosen and used as FC line. 35Spro: PQT3, 35Spro: PRMT4b and FC line were identified by glufosinate screening and RT-PCR.

**Stress tolerance assay**
The seeds were germinated on half-strength MS media containing different concentrations of paraquat, mannitol, CdCl₂ and NaCl, respectively. The phenotype was observed and survival ratio was scored at the indicated time points.

For drought tolerance assay, the pqt3-1 mutant, pqt3-2 mutant and wild type seeds were germinated in one pot at same density. When seedlings were 15 day old, watering was withheld for 15 day before re-watering. The photos were taken before re-watering and after re-watering for 1 day and 7 days. The survival ratio was scored after re-watering for 1 day and 7 days.

**DAB staining**

DAB staining was performed as described (Daudi and O’Brien, 2012). DAB staining solution (pH 6.0) was prepared by adding 0.05% (v/v) Tween-20 and 10 mM Na₂HPO₄ to the DAB solution (1 mg/ml DAB, pH3.0). For each treatment condition, at least 3 leaves per plant were obtained from 3 independent plants for each line (Col-0, pqt3-1 and pqt3-2). Arabidopsis leaves from different lines were treated using MS liquid medium without or with 6 μM paraquat for 3 h. These leaves were stained in 6-well culture plates with DAB staining solution subsequently and 10 mM Na₂HPO₄ (pH 6.0) was used as the negative control. The 6-well plates were covered with aluminum foil and placed on a shaker for 4 - 5 h. Follow the incubation, replace the DAB staining solution with bleaching solution (ethanol: acetic acid: glycerol = 3:1:1). Place the 6-well plates into a boiling water bath (90 - 95°C) for 15 - 20 mins. Replace the bleaching solution with fresh bleaching solution. The brown precipitate formed by the DAB reaction with H₂O₂ could be observed on the leaves. Photos were taken using a camera. Special attention should be paid to light avoidance through the whole operation. The experiment was repeated for three times.

**The detection of GUS activity**

The promoter of PQT3 was cloned into pCB308R (Xiang et al., 1999; Lei et al., 2007). The transgenic lines containing PQT3pro: GUS were isolated by glufosinate
screening. The T2 population was used for GUS staining. Histochemical staining for GUS activity in Arabidopsis was performed as described previously (Jefferson et al., 1987) and GUS staining solution was prepared as described before (Xi et al., 2012). The experimental materials were incubated in staining solution at 37°C. Then Arabidopsis tissues were destained and stored in 70% ethanol. The GUS activities of individual parts were observed via a light microscope (ZEISS Axio skop2 plus) with a video camera.

**Subcellular localization assay**

The full length CDS of *PQT3* was cloned into the binary vector pCB2008E to construct the PQT3-GFP fusion vector (Lei et al., 2007). The inserted sequence was confirmed by sequencing. The construct was delivered into the onion epidermal cells via microprojectile bombardment of particle gun for transient expression assay. It was also transferred into Col-0 via floral-dip method to create transgenic plant for the analysis of PQT3-GFP fusion protein localization. The green fluorescence in onion epidermal cells and the root tissues of transgenic lines were observed using a fluorescence microscope (ZEISS Axio skop2 plus) with a video camera.

**Enzyme activity assay**

For APX and GPX enzyme activity assay, Arabidopsis seedlings were ground in liquid nitrogen and resuspended in precooling Enzyme extraction buffer (50 mM phosphate buffer (Na2HPO4-NaH2PO4), PH=7.0; 1 mM EDTA; 0.05% (v/v) TritonX-100; 2% (w/v) PVPP and 1 mM ascorbic acid) on ice. Extraction solution was centrifuged for 20 min (16000 g/min, 4°C). The protein concentration of supernatant was detected by One-drop micro-ultraviolet spectrophotometer and SDS-PAGE before the supernatant was used for enzyme activity analysis. The detection of APX activity was performed as described with modifications (Nakano and Asada, 1981). For APX activity, 50 μl enzyme and 2950 μl reaction mixture (50 mM Tris-HCl, pH7.0; 0.1 mM EDTA; 0.1 mM H2O2 and 0.5 mM ascorbic acid) was
mixed. The decreased OD_{290} was recorded per 10 s. The amount of enzyme oxidized 1 mM AsA in one minute set as one activity unit (U) of APX. The activity of APX was expressed as U \cdot g^{-1} protein. GPX activity was measured indirectly through the detection of glutathione reductase (GR) activity using GPX Activity Measurement Kit (Beyotime Biotech, China). GR activity was detected as described with modifications (Halliwell and Foyer, 1978). The OD_{340} was recorded per 30 s. The amount of enzyme consumed 1 mM NADPH in one minute set as one activity unit (U) of GPX. The activity of GPX was also expressed as U \cdot g^{-1} protein.

**Western blot**

For western blot analysis, proteins were separated by SDS-PAGE in a 12% acrylamide gel and electroblotted to nitrocellulose membrane (Immobilion-P, MILLIPORE Corporation, USA). Antibodies used in western blot were as follows: anti-HA antibody (HA-Tag, 26D11, Mouse mAb, M20003, Abmart, Shanghai, China), 1:1000 for western blot; anti-PRMT4b antibody, 1:500 for western blot; anti-Ubiquitin antibody (ab7254, abcam, USA), 1:1000 for western blot; anti-His antibody (His-Tag, 2A8, Mouse mAb, M30111, Abmart, Shanghai, China), 1:1000 for western blot and goat anti-mouse IgG-HRP (Santa Cruz Biotechnology, USA), 1:5000 for western blot. The results were examined with a CCD camera system (Image Quant LAS 4000) using Super Signal West Femto Trial Kit (Thermo, USA).

**In vitro E3 ubiquitin ligase activity assay**

The *in vitro* E3 ligase activity assay was performed as described previously (Zhang et al., 2007). GST-PQT3 fusion protein was obtained from *E. coli* and purified subsequently. His-tagged Arabidopsis ubiquitin (UBQ14) was also expressed using bacterial expression system and purified. In addition, the wheat (*Triticum aestivum*) E1 (GI: 136632) and human E2 (UBCh5b) were also used in the reaction. Reactions were performed for 1.5 h at 30°C. For the immunoblot, Nickel-HRP (nickel–nitrilotriacetic acid agarose conjugated to horseradish peroxidase)
(Kirkegaard & Perry Laboratories, http://www.kpl.com/) was used for the detection of His-tagged ubiquitin.

**Y2H screening and confirmation**

The full-length *PQT3* cDNA was cloned into the bait vector pDEST32 and transformed into the yeast strain Mav203 subsequently. Yeast two-hybrid screening was performed using two-hybrid cDNA library of Arabidopsis. Yeast cells harboring the bait were transformed with a cDNA library containing inserts for prey proteins fused to GAL4-AD. Positive clones were screened using SD/-Leu-Trp-His and X-gal assay. Then it was identified by nucleotide sequencing with corresponding primers. Two-hybrid screening was performed via the protocol described in Two-Hybrid System Manual (Invitrogen, USA). The yeast two-hybrid was carried out to confirm the result of screening. The full-length CDS of *PQT3* and the four segments of *PQT3* were cloned into the bait vector pDEST32 and *PRMT4b* was cloned into the prey vector pDEST22. The primers used for yeast two-hybrid were listed in Supplemental Table 1.

**Pull-down assay**

MBP-PQT3-C66 and His-AtPRMT4b fusion protein were expressed using prokaryotic expression system and purified. MBP-PQT3-C66 fusion protein was incubated with MBP beads (amylose resin) at 4°C for 2 h, and the MBP tag was used as a negative control. The beads were cleaned with washing buffer for 4 times. Then the beads were incubated with His-AtPRMT4b at 4°C for 2 h respectively. The beads were cleaned with washing buffer for 4 times. Pulled-down mixtures were separated by SDS-PAGE and further transferred to nitrocellulose membrane. The result was detected using western blot with anti-His antibody.

**Agroinfiltration procedure**

*Agrobacterium tumefaciens* strain C58C1 was used in the experiments.
Agroinfiltration procedure was performed as described previously (Liu et al., 2010b). At first, these strains were grown on LB medium containing Kanamycin. Single colony was transferred into 5 ml LB liquid medium with Kanamycin and grown for 48 h in a 28°C shaker. The bacteria solution was inoculated into new LB liquid medium containing 10 mM 2-(N-morpholine)-ethanesulfonic acid (MES; pH 5.6) and 40 μM acetosyringone (1:100 ratio, v/v). Bacteria were developed in a 28°C shaker until OD₆₀₀ reached 3.0 approximately. The bacteria were collected gently by means of 10 min centrifugation (3200 g/min), and the pellets were resuspended with 10 mM MgCl₂ until OD₆₀₀ reached 1.5 approximately. The bacteria solution was kept at room temperature with a final concentration of 200 μM acetosyringone for at least 3 h without shaking. The different plastid combinations were transformed into epidermal cells of *N. benthamiana* leaves by disposable syringe.

**BiFC analysis**

NE-PQT3 (the N-terminal part of YFP fused with PQT3) and CE-PRMT4b (the C-terminal part of YFP fused with PRMT4b) were constructed. These constructs were transferred to Agrobacterium strains C58C1 respectively. As mentioned above, the different plastid combinations were transformed into epidermal cells of *N. benthamiana* leaves by agroinfiltration. YFP was observed 1-2 days after leaf infiltration using confocal. The nuclei were stained by Hoechst subsequently and fluorescence detection by confocal was performed. The primers used for BiFC were listed in Supplemental Table 1.

**Protein extraction**

Native extraction buffer 1 [ NB1; 50 mM TRIS-MES pH 8.0, 0.5 M sucrose, 1 mM MgCl₂, 10 mM EDTA, 5 mM DTT, protease inhibitor cocktail for plant cell and tissue extracts (Sigma, USA) ] was chosen for protein extraction buffer. Other steps of protein extraction were carried out as described previously (Liu et al., 2010b).
In planta ubiquitination assay

Agrobacterium strains containing different constructs were mixed and infiltrated into the leaves of *N. benthamiana*. Total proteins were extracted from plant leaves 1 day after infiltration. The total proteins were separated by SDS-PAGE and analyzed via western blot using anti-HA antibody. Ponceau S staining of the Rubisco protein was used as loading control. In the presence of MG132, total proteins were immunoprecipitated with anti-HA antibody (Abmart) and protein A agarose beads (Millipore, USA) subsequently. Detailed steps of immunoprecipitation were performed as described previously (Liu et al., 2010b). Immunoprecipitated samples were analyzed using western blot with anti-ubiquitin antibody (Abcam). The results were examined with a CCD camera system (Image Quant LAS 4000).

Chromatin immunoprecipitation-PCR assay

The *pqt3* mutant and wild type were used for ChIP assay. *UBQ5* was used as an internal control. ChIP was performed as previously described (Gendrel et al., 2005; Pei et al., 2007). The regions with enriched Arg-17 methylation were precipitated from input DNA with anti-H3R17 antibodies (anti-Histone H3 asymmetric dimethyl R17 antibody-ChIP grade, ab8284, Abcam, USA). The enrichments of DNA fragments were detected by quantitative RT-PCR using specific primers corresponding to different regions of *APX1* and *GPX1* chromatins as described previously (Bastow et al., 2004; Liu et al., 2007). The primers used in ChIP assay were listed in Supplemental Table 1.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *TUB8*, At5g23860; *UBQ5*, At3g62250; *PQT3*, At4g17410; *PRMT4b*, At3g06930; *PRMT4a*, At5g49020; *APX1*, At1g07890; *APX2*, At3g09640; *APX3*, At4g35000; *APX4*, At4g09010; *APX5*, At4g35970; *APX6*, At4g32320; *sAPX*, At4g08390; *tAPX*, At1g77490; *CSD1*, At1g08830; *CSD2*, At2g28190; *CAT1*, At1g20630; *CAT2*,
At4g35090; CAT3, At1g20620; FSD1, At4g25100; FSD2, At5g51100; FSD3, At5g23310; GPX1, At2g25080; GPX2, At2g31570; GPX3, At2g43350; GPX4, At2g48150; GPX5, At3g63080; GPX6, At4g11600; GPX7, At4g31870; GPX8, At1g63460; ACHT1, At4g26160; ACHT2, At4g29670; ACHT3, At2g33270; ACHT4, At1g08570; ACHT5, At5g61440; 2CPB, At5g06290; PRXQ, At3g26060; GRXC1, At5g63030; GRXC2, At5g40370; GRXC5, At4g28730; MSD, At3g10920; AG, At4g18960.

Supplemental Data

Supplemental Figure 1. Identification of mutants, homozygous Salk line and transgenic lines.

Supplemental Figure 2. Phenotype of pqt3 mutants under other environmental stresses leading to oxidative damage.

Supplemental Figure 3. Sections of PQT3 protein used in Y2H and pull-down assays.

Supplemental Figure 4. Y2H assay for PQT3 and PRMT4a.

Supplemental Figure 5. Phenotype of PQT3 and PRMT4b overexpression lines under other environmental stresses lead to oxidative damage.

Supplemental Figure 6. Functional domains and phylogenetic tree of PQT3 protein.

Supplemental Figure 7. Homologous sequences alignment of PQT3 protein.

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AUTHOR CONTRIBUTIONS

C.L. and J.D. designed the study, performed the experiments and interpreted the results. XT.C. designed the study, performed the experiments, interpreted the results, and wrote the manuscript. P.Z. and R.L. contributed to part of the experiments. T.Z. and XF.C. contributed prmt4b mutants, PRMT4b overexpression lines, and PRMT4b antibody. P.W. and Q.X. contributed to the verification of E3 ligase activity of PQT3. C.X. designed the experiments, discussed the results, edited the manuscript and supervised the project.

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Figure Legends

Figure 1. Phenotype of *pqt3*-1 and *pqt3*-2 mutants.

(A) Confirmation of paraquat (PQ) tolerant phenotype. 7-day-old seedlings of wild type and *pqt3* mutant were grown on MS medium supplemented with 0 or 2 μM paraquat.

(B) Survival ratio of wild type and *pqt3* mutant grown in (A). Values are mean ±SD (n= 30 plants, ***P < 0.001). Asterisks indicate Student’s t-test significant differences.

(C) Multiple mutant alleles analysis of paraquat tolerance for the *At4g17410* locus. Survival ratio of wild type, *pqt3*-1 and *pqt3*-2 (Salk_065409) grown in the 0, 0.5, 1, and 2 μM paraquat medium was counted. Values are mean ±SD (n= 30 plants, ***P < 0.001). Asterisks indicate Student’s t-test significant differences.

(D) Late-flowering phenotype of *pqt3*-1 and *pqt3*-2 compared with wild type. Plants were grown under long day photoperiod (16 h light and 8 h dark).

(E) The phenotype of 7-day-old *pqt3*-1, wild type, function complementation (FC), *pqt3*-2, and 35Spro:PQT3 seedlings under 0 μM (the top picture) or 2 μM (the bottom picture) paraquat treatment. Bar= 0.5 cm.

(F) The survival ratio of *pqt3*-1, wild type, FC, *pqt3*-2, and 35Spro:PQT3 seeds germinated and grown on MS medium containing 0 μM or 2 μM paraquat for 7 days was counted. Values are mean ±SD (n= 30 plants, ***P < 0.001). Asterisks indicate Student’s t-test significant differences.

(G to R) DAB staining. The leaves of wild type, *pqt3*-1, and *pqt3*-2 were treated without (G to J) or with 6 μM paraquat for 12 (K to N) or 24 h (O to R). 10 mM Na₂HPO₄ was used as the negative control to stain the leaves of wild type treated without (G) or with 6 μM paraquat for 12 (K) or 24 h (O). Without PQ treatment, the leaves of wild type (H), *pqt3*-1 (I), and *pqt3*-2 (J) were stained using DAB staining solution. After 12 h paraquat treatment, DAB staining solution was used to stain the leaves of wild type (L), *pqt3*-1 (M), and *pqt3*-2 (N). The leaves of wild type (P), *pqt3*-1 (Q), and *pqt3*-2 (R) were stained by DAB staining solution after 24 h paraquat treatment. Bar= 0.5 cm.
Figure 2. Expression pattern and subcellular localization of PQT3.

(A) GUS expression in 1-week-old seedling. Transgenic Arabidopsis plants expressing \( PQT3pro:GUS \) were generated and analyzed for GUS expression. Bar= 0.5 cm.
(B) GUS expression in 3-week-old seedling. Bar= 0.5 cm.
(C) GUS expression in root tissue of 7-week-old plant.
(D) GUS expression in flower tissue.
(E and F) GUS expression in silique tip (E) and junction (F).
(G) GUS expression in 7-week-old adult Arabidopsis.

(H to J) \( 35Spro:GFP \) was transiently expressed in onion epidermal cells as control. The GFP can be observed in both plasma membrane and nucleus.
(K to M) Nucleus localization of the PQT3-GFP fusion protein in onion epidermal cell was observed. Bar= 20 \( \mu \)m.
(N to P) The nucleus localization of PQT3-GFP fusion protein in the root tissue of stable transgenic seedlings expressing \( PQT3pro:PQT3-GFP \). Bar= 20 \( \mu \)m.

Figure 3. The expression of PQT3 was down-regulated by various oxidative stress conditions.

(A) The transcript level of \( PQT3 \) was down-regulated by paraquat treatment. 1-week-old seedlings were treated with 6 \( \mu \)M paraquat for the indicated times before RNA extraction for quantitative RT-PCR analysis. Values are mean ±SD (n=3 experiments, \( ***P < 0.001 \)). Asterisks indicate Student’s t-test significant differences.

(B to F) The expression of \( PQT3 \) was down-regulated by other stress conditions. 1-week-old seedlings were treated by 10 mM \( \text{H}_2\text{O}_2 \) (B), 200 mM mannitol (C), drought (D), 200 mM CdCl\(_2\) (E), and 150 mM NaCl (F) for the indicated times before RNA extraction for quantitative RT-PCR analysis. Values are mean ±SD (n=3 experiments, \( *P < 0.05, **P < 0.01, ***P < 0.001 \)). Asterisks indicate Student’s t-test significant differences.

(G to DD) GUS staining of 7-day-old transgenic lines contained \( PQT3pro:GUS \)
without or with 6 μM parquat, 10 mM H₂O₂, 200 mM mannitol, 200 mM CdCl₂, 
150 mM NaCl treatment for 3 h. GUS expressions were significantly reduced in 
primary root tip (G to L), root elongation zone (M to R), root maturation zone (S to 
X), root zone with LRP (Y to DD) under stress conditions. Bar= 50 μm.

**Figure 4. The analysis of antioxidant enzyme in wild type and pqt3 mutant.**

(A to J) Quantitative RT-PCR analysis of transcript levels of antioxidant enzyme 
genes. RNA samples were isolated from 7-day-old wild type and pqt3 seedlings for 
quantitative RT-PCR analysis. The transcript levels of APX (A), GPX (B), ACHT (C), 
FSD (D), CAT (E), GRXC (F), CSD (G), PRXQ (H), 2CPB (I) and MSD (J) were 
analyzed. Values are mean ±SD (n=3 experiments, ***P < 0.001). Asterisks indicate 
Student’s t-test significant differences.

(K and L) Enzyme activity of APX (K) and GPX (L) in wild type and pqt3 mutant. 
The pqt3 mutant has higher enzyme activity of APX and GPX than wild type. Values 
are mean ±SD (n=3 experiments, ***P < 0.001). Asterisks indicate Student’s t-test 
significant differences.

**Figure 5. PQT3 has E3 ubiquitin ligase activity.**

GST-PQT3 was expressed in E. coli and purified. Nickel-HRP was used to detect 
His-tagged ubiquitin. The ubiquitination activity of GST-PQT3 was observed only in 
the presence of E1 (from wheat), E2 (UBCh5b, from human), and 6×His-tagged 
ubiquitin (Ub). GST was used as negative control. The numbers on the right indicate 
the molecular masses of marker proteins.

**Figure 6. PQT3 interacts with PRMT4b.**

(A to C) Y2H assay. PQT3 and its four protein sections were used as the bait.
PRMT4b was used as the prey. Krev1/RalGDS-wt act as strong positive control and 
Krev1/RalGDS-m1 act as week positive control. Krev1/RalGDS-m2 was used for 
negative control. The yeast harboring various constructs was grown on SC-Leu-Trp
medium (A). The yeast was transferred to SC-Leu-Trp-His medium with 50 mM 3-AT (B) or used for X-gal staining (C).

(D and E) The pull-down assay between PQT3 and PRMT4b. His-PRMT4b was incubated with amylose resin bound with recombinant MBP-PQT3-C66 protein. Pulled-down protein complex was detected by SDS-PAGE (D) and western blot using anti-His antibody (E). MBP protein was used as a negative control.

(F to I) BiFC assay. Different plasmid combinations were expressed in epidermal cells of *N. benthamiana* leaves. Yellow fluorescence (YFP) was observed in epidermal cell expressing both NE-PQT3 (the N-terminal part of YFP fused with PQT3) and CE-PRMT4b (the C-terminal part of YFP fused with PRMT4b) (F). No fluorescence was observed from the negative controls: NE-PQT3 / CE (G), NE / CE-PRMT4 (H), and NE / CE (I). The nuclei were stained by Hoechst and the fluorescence was detected by confocal. NE indicates pSPYNE vector and CE indicates pSPYCE vector.

**Figure 7. PRMT4b is a substrate recognized by PQT3.**

(A and B) *In planta* ubiquitination assay. Total protein samples were isolated from the infiltrated parts of *N. benthamiana* leaves 1 day after agroinfiltration. The total protein was analyzed via western blot using anti-HA antibody (A). The total proteins were separated via a SDS-PAGE and Ponceau S staining of the Rubisco protein was shown as a loading control. Immunoprecipitated samples were analyzed using western blot with anti-ubiquitin antibody subsequently (B). The numbers on the right indicate the molecular masses of marker proteins.

(C) The mRNA level of *PRMT4b* in wild type and *pqt3* mutant under paraquat treatment. Total RNA was isolated from 14-day-old wild type and *pqt3* seedlings without or with 6 μM paraquat treatment for quantitative RT-PCR analysis. Values are mean ±SD (n=3 experiments).

(D) The effect of proteasome inhibitor MG132 on the protein level of PRMT4b in wild type and *pqt3-1* mutant with paraquat treatment in time-course. Wild type and *pqt3-1* mutant were grown for 14 days, then the seedlings were treated without or
with 6 µM paraquat for 6 or 12 h in presence or absence of 50 µM proteasome inhibitor MG132. The total proteins were extracted. Western blot was performed using anti-PRMT4b antibody. Ponceau S staining of the Rubisco protein serves as a loading control.

(E) The survival ratio of wild type and *pqt3* mutant germinated and grown on MS medium containing 0 µM or 1 µM paraquat without or with 15 µM MG132 for 7 days were counted. DMSO was used as control. Values are mean ±SD (n= 30 plants, *P < 0.05). Asterisk indicates Student’s t-test significant difference.

Figure 8. PRMT4b was involved in oxidative tolerance of Arabidopsis.

(A) Paraquat tolerance assay. Survival ratio of wild type, *pqt3-1* mutant, *pqt3-2* mutant (Salk_065409), *prmt4b* mutant and 35Spro:PRMT4b grown on 0, 2, 3, or 5 µM paraquat medium were counted. The assay was repeated for three times. Values are mean ±SD (n= 50 plants, *P < 0.05, **P < 0.01, ***P < 0.001). Asterisks indicate Student’s t-test significant differences.

(B) Primary root elongation of wild type and *prmt4b* mutant seedlings grown on MS without or with 150 mM CdCl2 was measured. Values are mean ±SD (n= 30 plants, **P < 0.01). Asterisks indicate Student’s t-test significant differences.

(C to E) The phenotype of wild type, *prmt4a*, *prmt4b*, and *prmt4aprmt4b* grown on MS without (C) or with 1 µM paraquat (D). The survival ratio of wild type, *prmt4a*, *prmt4b*, and *prmt4aprmt4b* germinated and grown on MS medium containing 0 µM or 1 µM paraquat for 5 days were counted (E). Values are mean ±SD (n= 36 plants, *P < 0.05, **P < 0.01). Asterisks indicate Student’s t-test significant differences.

Figure 9. Arg-17 methylation in specific regions of *APX1* and *GPX1* chromatin was increased in *pqt3* mutant.

(A and B) The illustration of *APX1* and *GPX1* chromatin. A to F represent different regions of *APX1* chromatin (A). A to I represent different regions of *GPX1* chromatin (B). Promoters (black lines), exons (white boxes), and introns (black lines) of *APX1* and *GPX1* were shown.
(C and D) ChIP-PCR assay. Quantitative PCR was performed to verify each chromatin region of APX1 and GPX1 using specific primers. Fragments C, D and E in APX1 chromatin were enriched by anti-H3R17 antibodies (C). Fragments C, D and I in GPX1 chromatin were enriched by anti-H3R17 antibodies (D). UBQ5 was used as an internal control. Values are mean ±SD (n=3 experiments, **P < 0.01, ***P < 0.001). Asterisks indicate Student’s t-test significant differences.

Figure 10. The phenotypes of pqt3prmt4b and pqt3prmt4a double mutants under paraquat treatment.

(A and B) The phenotypes of pqt3, pqt3prmt4b, wild type and prmt4b seedlings grown on MS without (A) or with 2 μM paraquat (B).

(C) The survival ratio of pqt3, pqt3prmt4b, wild type and prmt4b under 2 μM paraquat treatment for 7 days were counted. Values are mean ±SD (n= 30 plants, **P < 0.01, ***P < 0.001). Asterisks indicate Student’s t-test significant differences.

(D) The survival ratio of pqt3, pqt3prmt4a, wild type and prmt4a under 1 μM paraquat treatment for 7 days were counted. Values are mean ±SD (n= 30 plants, **P < 0.01).

(E and F) The phenotypes of pqt3, pqt3prmt4a, wild type and prmt4a seedlings grown on MS without (E) or with 1 μM paraquat (F).

Figure 11. A working model for PQT3 acting as a negative regulator of oxidative stress response.

Many environmental stresses cause oxidative stress in plants. Under oxidative stress, stress signaling up-regulates PRMT4b expression and down-regulates PQT3 expression, leading to higher PRMT4b activity that will activate APX1 and GPX1 and enhance antioxidation capacity of APX1 and GPX1. When oxidative stress is diminished, PRMT4b expression is decreased and PQT3 expression is increased. As a result, PQT3 activity is increased, leading to faster removal of PRMT4b via 26S proteasome. Together with decreased PRMT4b expression, PRMT4b activity will
rapidly drop, leading to decreased expression of \( APX1 \) and \( GPX1 \). The activated response of oxidative stress is then switched off.
|           | GST | GST-PQT3 |
|-----------|-----|----------|
| His-Ub    | +   | + + + +  |
| E1        | +   | - + + +  |
| E2        | +   | + - + +  |

Ubiquitinated

- 72 kD
- 26 kD E2
- Free Ub
A

Survival (%) vs PQ concentration (μM)

Col-0
pq3-1
pq3-2
prmt4b
35S:PRMT4b

B

Primary root length (cm)

Col-0
prmt4b

C

Col-0
prmt4a4b
prmt4a
prmt4b

D

E

Survival (%) vs PQ concentration (μM)

Col-0
prmt4a
prmt4b
prmt4a4b
