

Synergetic Modulation of Plant Cadmium Tolerance via MYB75-mediated ROS Homeostasis and Transcriptional Regulation

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Research Article

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Abstract

Cadmium (Cd) is a heavy metal with biological toxicity, which can be detoxified through chelation and compartmentation in plants. Transcriptional regulation mediates plant Cd tolerance by modulating these processes. However, the mechanism remains to be studied. Our results showed that a previously unknown function of MYB75 transcription factor in the regulation of Cd tolerance. Cd exposure stimulates anthocyanin accumulation by raising MYB75 expression. Enhanced Cd tolerance was observed in the MYB75-overexpressing plants, whereas increased Cd sensitivity was found in the MYB75 loss-of-function mutants. Under Cd stress conditions, lower reactive oxygen species (ROS) levels were detected in MYB75-overexpressing plants than in wild type plants. In contrast, higher ROS levels were found in MYB75 loss-of-function mutants. Overexpression of MYB75 were associated with increased glutathione (GSH) and phytochelatin (PC) content by anthocyanin-mediated ROS homeostasis. Furthermore, the expression of Cd stress-related gene including *ACBP2* and *ABCC2* was elevated in MYB75-overexpressing plants, and this upregulation was mediated through the mechanism by which MYB75 directly bind to the promoter of *ACBP2* and *ABCC2*. Our findings reveal an important role for MYB75 in regulation of plant Cd tolerance via anthocyanin-mediated ROS homeostasis and PC content, and through upregulation of Cd stress-related gene at transcriptional level.

Key Message

MYB75 enhances plant cadmium tolerance by mediating ROS homeostasis and cadmium tolerance-related genes expression.

Introduction

Human production processes such as industry and mining can increase concentrations of heavy metals in soil, air and water. The accumulation of heavy metals in soil can lead to the loss of soil fertility, the reduction of vegetation, and the pollution of water sources (Clemens et al., 2016, Zou et al., 2021). Cadmium (Cd) is a widespread heavy metal contamination, which has a serious toxic effect on plants, animals and human (Clemens, 2019). Cd is a nonessential and toxic element for plant growth and development. It is commonly known that Cd in water can rapidly enter plant via root, resulting in toxicological symptoms (Haider et al., 2021). Firstly, Cd can bind to the sulfhydryl, histidine, and carboxyl of structural protein, thus inhibiting protein function (Huybrechts et al., 2019). Cd also has similar chemical properties to other divalent cation, which hence can replace the metal ion in structural protein and cause impairment of protein function (Cuypers et al., 2010). Further, although Cd has non-redox property, it can indirectly induce the production of reactive oxygen species (ROS) (Wu et al., 2019, Zhang et al., 2020). ROS-mediated change of redox environment in plant cell can disrupt protein function and destroy cell structure, thus inhibiting physiological metabolic processes such as photosynthesis, respiration, photorespiration, and cell cycle (Waszczak et al., 2018). Consequently, improvement of Cd tolerance is critical for plant growth and development.

Cd in agricultural soil is mainly derived from atmospheric deposition and industrial effluent. Cd accumulation in plant is mostly due to the uptake of soil Cd via root. Previous researches have established that various plant transporters play an important role in uptake of Cd. Cd uptake and transport was implicated in several genes encoding transporters including *OsNramp5*, *HMA2*, *HMA4*, *IRT1*, and *PDR8* (Chang et al., 2020, Sheng et al., 2019, Wong et al., 2009a, Wong et al., 2009b, Wu et al., 2021). Cd is transported into and accumulated in plants, which is harmful to plant growth and development. But plants have evolved multiple defense mechanisms to resist and reduce the toxicity of Cd. Initially, Cd²⁺ chelated by Glutathione (GSH) and phytochelatin (PC) can sequester into vacuoles (González et al., 2021). Some transcription factors including ZAT6 and WRKY12, regulate Cd tolerance via modulation of GSH and PC biosynthesis at transcriptional level (Chen et al., 2016, Han et al., 2019). Next, plants may mediate the expression of metal uptake transporter genes and efflux transporter genes to compartment Cd distribution (Sheng et al., 2019). Finally, some membrane protein proteins such as ACBP2 and FP6 can bind Cd²⁺ via sulfhydryl groups on cysteine residues to form nontoxic or less toxic compound (Gao et al., 2010, Gao et al., 2009). PDF2.5 promotes Cd transfer from protoplast to cell wall, thus alleviating Cd toxicity (Luo et al., 2019). In addition, to decrease Cd-induced ROS accumulation, plants enhance ROS scavenging by activating antioxidant system. Plant Cd tolerance was enhanced by mediating these collective mechanisms at multiple levels, especially at transcriptional level.

MYB family transcription factors play a significant role in plant developmental processes as well as plant tolerance to diverse environmental stresses. Data from several studies suggest that MYB transcription factors participate in regulation of plant Cd tolerance, but details of the regulation mechanism need further research. MYB75, which is also defined as PRODUCTION OF ANTHOCYANIN PIGMENT 1 (PAP1), plays a key role in anthocyanin accumulation (Borevitz et al., 2000, He et al., 2021). However, the possible role of MYB75 in Cd tolerance and the details of the underlying mechanism has not been characterized.

To enrich the regulatory mechanisms in under Cd exposure, here we identify MYB75 as a new regulator of plant Cd tolerance. Under Cd stress, a *myb75-c* mutant shows increased sensitivity to Cd stress and accumulates more ROS but less PC, whereas the transgenic lines overexpressing MYB75 decrease ROS levels and lead to elevate PC content compared with wild type plants. Furthermore, MYB75 directly binds to the promoter of Cd stress-related gene including *ACBP2* and *ABCC2*, thus raising their transcription. Our study exposes that MYB75 acts as a positive regulator of Cd tolerance by anthocyanin-mediated ROS homeostasis and PC content, and through targeting *ACBP2* and *ABCC2* in *Arabidopsis*.

Materials And Methods

Plant materials and growth conditions

The *Arabidopsis thaliana pap1-D, 35S: MYB75* and *myb75-c* were described as previously (Zheng et al., 2020a, Zheng et al., 2019). The 1/2 MS with *Arabidopsis* seeds were placed at 4 °C for 2 d before moving to 22 °C under diverse light conditions. For Cd treatment, 100 mM CdCl₂ was added to the 1/2 MS medium (pH 5.8) to a final concentration of 50 μM CdCl₂, and 75 μM CdCl₂. Three-day-old wild type and

mutant or transgenic seedlings were transferred to 1/2 MS agar plates in the absence or presence of CdCl₂ for the indicated number of days. To reduce variation because of the precipitation of heavy metals, wild type and mutant or transgenic plants were grown adjacent to each other in the same plate and their growth was compared. After the indicated days of growth, the plants were sampled for root growth and fresh weight assays. *Nicotiana benthamiana* grown in soil at 22 °C under 16-h-light/8-h-dark conditions was used for the transient expression assays.

Chlorophyll measurement

The determination of chlorophyll contents was measured as described (Zhang et al., 2021). Briefly, a total of 100 mg of plant materials was pulverized with liquid nitrogen, incubated in a 1.0 mL of 80% acetone in the dark for 30 min and centrifuged for 5 min at 12000 rpm. Absorbance of the supernatant was measured at 645 and 663 nm and then total chlorophyll content was calculated.

Analysis of chlorophyll fluorescence

Chlorophyll fluorescence was determined with an pulse-modulated fluorometer (FMS-2, Hansatech, UK). For measurement of F_v/F_m , plants were dark adapted for 30 min. Minimal fluorescence (F_0) was measured during the weak measuring pulses, and maximal fluorescence (F_m) was measured by 0.8 s pulse of light at about 4,000 mmol m⁻² s⁻¹. An actinic light source was then applied to obtain steady-state fluorescence yield (F_s), after which a second saturation pulse was applied for 0.7 s to obtain light-adapted maximum fluorescence (F_m'). F_v/F_m , Φ_{PSII} were calculated as $(F_m - F_0)/F_m$, $(F_m' - F_s)/F_m'$, respectively (Baker, 2008).

Ion leakage assay

The determination of ion leakage was measured as described (Ding et al., 2018). Summarily, the injury seedlings were put into 15 ml tubes containing 5 ml deionized water, which were shaken at 22°C for 15 min, and the conductivity was measured as S1. After detecting S1, the tubes were put into boiled water at 100 °C for 15 min and shaken at 22 °C for 1 h, and then, S2 was measured. The formula (S1-S0)/(S2-S0) was used to calculate ion leakage (S0: conductivity of deionized water).

Oxidative damage estimation, in situ superoxide and H₂O₂ staining

Superoxide and H₂O₂ levels were visually detected with nitro blue tetrazolium (NBT) and 3, 3'-diaminobenzidine (DAB), respectively, as described previously (Zou et al., 2016). Seedlings were excised at the base with a razorblade and supplied through the cut ends with NBT (0.5 mg ml⁻¹) or DAB (2 mg ml⁻¹) solutions for 8 h. Leaves were then decolorized in boiling ethanol (95%) for 20 min. At least three seedlings were used for each treatment.

O₂^{·-} and H₂O₂ content was measured as described (Lei et al., 2020). Briefly, the O₂^{·-} reacts with MSDS (hydroxylamine hydrochloride) to generate NO²⁻. Then a red azo compound is generated with the reaction

of p-aminobenzenesulfonic acid and α -naphthylamine, which possesses an absorption peak at 530 nm. The yellow titanium peroxide composite generated by the reaction of H_2O_2 which has an absorption peak at 415 nm is used to calculate the H_2O_2 content. Each sample has three biological repeats.

Determination of antioxidant enzymes

For the enzyme assays, 0.3 g of leaf were ground with 3 ml ice cold 25 mM Hepes buffer (pH 7.8) containing 0.2 mM EDTA, 2 mM ascorbate and 2% polyvinylpyrrolidone (PVP) (Zou et al., 2016). The homogenates were centrifuged at 4 °C for 20 min at 12,000 g and the resulting supernatants were used for the determination of enzymatic activity. Superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), peroxidase (POD) activities were assayed as described previously.

Real-time PCR analysis

RNA was extracted as described previously, and cDNA synthesis was performed by one microgram (Zhang et al., 2010). qRT-PCR analysis was conducted by using SYBR Green PCR Master Mix. Three separate experiments and technical triplicates of each experiment were implemented. Gene expression was standardized to the *ACTIN 8* transcript levels.

Cloning and transient expression assay

The 2000 bp promoters of *ACBP2* and *ABCC2* were PCR-amplified from Arabidopsis genomic DNA using specific primers (Supplementary Table S1). These promoters were cloned into the vector YY45 (YFP substitutes for β -glucuronidase [GUS] reporter), named *ProACBP2: GUS* and *ProABCC2: GUS*. These two constructs were co-transformed into epidermal cells of *Nicotiana benthamiana* with the 35S: MYB75-HA construct. Samples were then collected and labelled, and GUS staining was performed as described previously.

LUC reporter transactivation assays

The 2000 bp promoters of *ACBP2* and *ABCC2* were PCR-amplified from Arabidopsis genomic DNA using specific primers (Supplementary Table S1). These promoters were cloned into the vector pGreenII0800-LUC (Li et al., 2016), named *ProACBP2: LUC* and *ProABCC2: LUC*. These two constructs were co-transformed into wild type protoplasts with the 35S: MYB75-HA construct. For LUC assay, 100 μ l of protoplast lysis buffer was added to the frozen protoplasts and mixed with a pipette gun (Yoo et al., 2007). After 5 min incubation on ice, 20 ml of the lysate harvested by centrifugation at 1,000 g for 2 min and 100 ml LUC mix were used to measure LUC activity.

Electrophoresis Mobility Shift Assay

EMSA were performed as previously described (Zhang et al., 2021). Briefly, MBP-MYB75 was expressed in *E. coli* and purified using MBP Sepharose beads, respectively. The DNA fragment (Supplementary Table S1) was incubated with MBP-MYB75 in EMSA binding buffer (25 mM HEPES-KOH, pH 8.0, 50 mM KCl, 1 mM dithiothreitol [DTT] and 10% glycerol). An Electrophoretic Mobility Shift Assay (EMSA) Kit with SYBR Green and SYPRO Ruby EMSA stains (Thermo Fisher) were used to detect protein-DNA interactions.

ChIP assays

ChIP assays perform as described (Yu et al., 2008). The 4-week-old plants collected in 50 mL tubes, and 37 mL 1% formaldehyde solution was used for cross-linked under a vacuum for 20 min. The chromatin was collected and sheared by sonication to reduce the average DNA fragment size to around 500 bps, then the sonicated chromatin complex was immunoprecipitated by specific antibodies anti-HA. After reverse cross-linking, the immunoprecipitated DNA fragment was analysed by qPCR with specific primers shown in Supplementary Table S1.

Statistical analysis

Samples were analyzed in three individual biological replicates, and the data are indicated as the mean \pm SD. Two-way ANOVA (LSD's multiple-range test) or Student's t-test were performed at a significance level of $P < 0.05$.

Accession numbers

The Arabidopsis Genome Initiative identifiers for the genes described in this article are as follows: *MYB75* (AT1G56650), *MYB90* (AT1G66390), *TT8* (AT4G09820), *EGL3* (AT1G63650), *TTG1* (AT5G24520), *ACBP2* (AT4G27780), *ABCC2* (AT2G34660), *GSH1* (AT4G23100), *PDR8* (AT1G59870), *ATM3* (AT5G58270), *PDF2.5* (AT5G63660) and *ACTIN 8* (AT1G49240).

Results

MYB75 transcription factor participates in modulation of plant cadmium tolerance

Previous research has proved that plants exposed to Cd stress show transcriptional change of genes involved in phenylpropanoid metabolisms and anthocyanin accumulation (Dai et al., 2012, Herbette et al., 2006), thus we further investigated the mechanism underlying Cd stress-regulated anthocyanin accumulation. To test the impact of Cd stress on anthocyanin accumulation, seeds of the wild type were germinated on one-half-strength Murashige and Skoog (1/2 MS) agar plates containing either 0, 25, 50, 75, 100 μM CdCl_2 for 3 d. As shown in Fig. 1A, anthocyanin accumulation was elevated in wild type under Cd stress. Quantification of anthocyanin also verified that the anthocyanin level increased with the Cd concentration (Fig. 1B). Previous evidence demonstrated that anthocyanin biosynthesis derives from flavonoid biosynthetic pathway and three anthocyanin-specific genes encoding dihydroflavonol 4-reductasae (DFR), leucoanthocyanidin dioxygenase (LDOX), UDP-glucose: flavonoid 3-oglucosyl transferase (UF3GT) have been identified, and expression of these genes is regulated by MYB-bHLH-WD40 (MWB) protein complex (Xu et al., 2015). We hence determined the expression of regulatory gene in wild type without or with 25, 50, 75, 100 μM CdCl_2 . *MYB75* transcription was notably induced in response to Cd stress (Fig. 1B, Fig. S1A). However, Cd treatment would not alter the expression of other regulatory

gene (*MYB90*, *TT8* and *EGL3*) (Fig. S1B-D). These results suggest that MYB75 participates in Cd stress-induced anthocyanin accumulation.

Above findings lead us conclude that MYB75 transcription factor is involved in modulation of plant Cd tolerance. Consequently, *pap1-D* seedlings, the activation tag mutant constitutively overexpresses MYB75/PAP1 and *myb75-c* that MYB75 knockout mutant using the CRISPR-Cas9 system were used for determining the MYB75 function in plant Cd tolerance. *Arabidopsis* seedlings grown vertically in 1/2 MS agar plates without CdCl₂ for 3 d, then were moved to 1/2 MS agar plates without or with 50 or 75 μM CdCl₂ for 7 d. As can be seen from Fig. 1C, when grown on 1/2 MS media without CdCl₂, *pap1-D* and *myb75-c* exhibited no difference compared with wild type. Nevertheless, *pap1-D* showed higher tolerance in response to Cd stress compared with wild type (Fig. 1C). By comparison, we observed that *myb75-c* was more sensitive to Cd stress than wild type (Fig. 1C). These results were further verified by quantification of both the root length and fresh weight (Fig. 1D). Overall, these results suggest that MYB75 is involved in regulation of plant Cd tolerance.

It has previously been observed that Cd exposure weakens the photosystem, we next confirmed that whether MYB75 regulated Cd stress-induce photosystem damage. We maintained *Arabidopsis* seedlings grown in 1/2 MS media without or with 75 μM CdCl₂ for 21 d. From the Fig. 2A we can see that Cd exposure accelerated chlorophyll degradation. Quantification of chlorophyll verified that *pap1-D* showed more chlorophyll content compared with wild type (Fig. 2B). Meanwhile, the chlorophyll content of *myb75-c* mutant was significantly lower than that of wild type (Fig. 2B). Ion leakage can indicate the degree of damage in plants caused by environmental stresses, thus we analyzed the ion leakage under Cd exposure. As shown in Fig. 2C, ion leakage was much higher in *myb75-c* mutant than in wild type. Nonetheless, ion leakage of *pap1-D* was significantly lower than that of wild type (Fig. 2C).

We further determined the photosystem II (PSII) photochemistry by detecting Chlorophyll fluorescence including F_v/F_m and Φ_{PSII} . Compared with wild type, F_v/F_m of *pap1-D* showed no visible difference without CdCl₂ (Fig. 2D). When exposed to Cd stress, *pap1-D* exhibited higher levels of F_v/F_m than that of wild type (Fig. 2D). In contrast, levels of F_v/F_m were lower in *myb75-c* than in wild type (Fig. 2D). Similarly, levels of Φ_{PSII} were also higher in *pap1-D* and lower in *myb75-c* compared with wild type (Fig. 2E). These results reveal that MYB75 transcription factor alleviates Cd stress-induced photosystem damage. Together with above results, we conclude that MYB75 transcription factor participates in modulation of plant Cd tolerance.

MYB75 positively regulates plant Cd tolerance

To further examine the impact of MYB75 on plant Cd tolerance, we generated transgenic plants expressing *MYB75* driven by the constitutive 35S promoter in wild type background. The *35S: MYB75 #7* and *35S: MYB75 #10* transgenic plants exhibited increased tolerance to Cd stress compared with wild type when grown on 1/2 MS media with 50 or 75 μM CdCl₂ (Fig. 3A). Quantitative analysis of root length

and fresh weight further confirmed these results (Fig. 3B, C), indicating that MYB75 positively regulates plant Cd stress.

Next, the *35S: MYB75 #7* and *35S: MYB75 #10* transgenic plants grown on 1/2 MS media without or with 75 μM CdCl_2 were examined. The chlorophyll content of *35S: MYB75 #7* and *35S: MYB75 #10* was significantly higher than that of wild type (Fig. 4A, B). As can be seen from Fig. 4C, ion leakage of *35S: MYB75 #7* and *35S: MYB75 #10* transgenic plants was significantly lower than that of wild type. When exposed to Cd stress, *35S: MYB75 #7* and *35S: MYB75 #10* transgenic plants exhibited higher levels of F_v/F_m and ΦPSII than that of wild type (Fig. 4D, E). Taken together, MYB75 transcription factor enhances plant Cd tolerance under Cd exposure.

MYB75 transcription factor-elevated Cd tolerance is involved in antioxidant system

Much work so far has focused on the key function of MYB75 transcription factor in regulation of anthocyanin accumulation. Given that anthocyanin is critical for scavenge ROS via their antioxidant capability (Li et al., 2017, Nakabayashi et al., 2014), we further examined the ROS levels in MYB75-overexpressing lines and *myb75-c* mutant under normal or Cd stress conditions. Nitroblue tetrazolium (NBT) staining indicated that content of superoxide ($\text{O}_2^{\cdot-}$) was much lower in *pap1-D*, *35S: MYB75 #7* and *35S: MYB75 #10* plants compared with wild type under Cd stress (Fig. 5A). On the contrary, $\text{O}_2^{\cdot-}$ content of *myb75-c* was much higher than that of wild type under Cd stress (Fig. 5A). These results were further ascertained by quantification of $\text{O}_2^{\cdot-}$ content (Fig. 5B). Moreover, we observed that *pap1-D*, *35S: MYB75 #7* and *35S: MYB75 #10* plants accumulated less hydrogen peroxide (H_2O_2) content than wild type under Cd stress (Fig. 5C). In contrast, H_2O_2 content of *myb75-c* was much higher than that of wild type under Cd stress (Fig. 5C). Quantification of H_2O_2 content also confirmed these results (Fig. 5D). Collectively, these results demonstrated that MYB75 transcription factor plays positive role in protection of plants from Cd exposure by alleviating oxidative damage.

Extensive research has shown that plants have highly effective antioxidant mechanisms involving superoxide dismutase (SOD) and catalase (CAT) to scavenge ROS. Cd exposure led to decreases in antioxidant enzyme activities (Fig. 5E, F). It is mostly likely that Cd^{2+} inhibits the function of antioxidant enzyme. Intriguingly, diminution of SOD and CAT activities in *myb75-c* mutant were significantly aggravated under Cd exposure (Fig. 5E, F). Oppositely, SOD and CAT activities of *pap1-D*, *35S: MYB75 #7* and *35S: MYB75 #10* plants were significantly higher than that of wild type (Fig. 5E, F). In summary, MYB75 transcription factor positively regulates Cd tolerance through activating antioxidant system and alleviating oxidative damage.

MYB75 transcription factor stimulates GSH-dependent PC synthesis pathway and Cd accumulation

Reduced glutathione (GSH)-oxidized glutathione (GSSG) conversion by ROS homeostasis in plant cell has been intensively investigated (Noctor et al., 2012). To clarify whether ROS homeostasis affects GSH content, we examined catalase-overexpressing plants (35S: *CAT2* and 35S: *CAT3*) under Cd exposure. When compared with wild type, 35S: *CAT2* and 35S: *CAT3* plants exhibited less H₂O₂ content, but more GSH and PC content under Cd stress (Fig. S2). Given that MYB75 transcription factor declined the ROS levels via anthocyanin and antioxidant enzyme, we next determined the GSH levels without or with Cd treatment. As shown in Fig. 6A, no significant difference was detected in total glutathione (GSH plus 2GSSG) between the wild type, the *pap1-D*, 35S: *MYB75 #7*, 35S: *MYB75 #10* plants, and the *myb75-c* mutants without Cd treatment. Cd exposure significantly depressed GSH concentrations in these plants (Fig. 6A). Nevertheless, compared with wild type, GSH levels was higher in *pap1-D*, 35S: *MYB75 #7*, 35S: *MYB75 #10* plants and lower in *myb75-c* mutants (Fig. 6A). Previous research has established that phytochelatin (PC), the important component involved in chelating Cd²⁺, derived directly from GSH via the PC synthase (PCS). The PC content was elevated significantly in *pap1-D*, 35S: *MYB75 #7*, 35S: *MYB75 #10* plants and diminished in *myb75-c* mutants compared with wild type under Cd exposure, suggesting MYB75 transcription factor positively regulates PC levels. Taken together, these results verified that MYB75 positively regulates Cd tolerance through stimulating GSH-dependent PC synthesis pathway.

Given the observed change of PC, we further test whether MYB75 affects Cd content through measuring Cd content under Cd stress. As can be seen from Fig. 6C, *pap1-D*, 35S: *MYB75 #7*, 35S: *MYB75 #10* plants showed higher Cd content in roots and shoots than wild type. On the contrary, Cd content was reduced in *myb75-c* under Cd stress compared with that in the wild type. These results suggest that MYB75 mediates Cd accumulation in roots and shoots under Cd stress.

MYB75 transcription factor directly regulates the Cd tolerance-related gene expression

Transcription factor found to be influencing Cd tolerance have been explored in several studies (Agarwal et al., 2020, Zhang et al., 2019). Therefore, we further investigated whether MYB75 regulates Cd tolerance at transcriptional level. We determined Cd tolerance-related gene expression such as *ACBP2*, *ABCC2*, *GSH1*, *PDR8*, *ATM3*, and *PDF2.5*. Intriguingly, the transcription levels of *GSH1*, *PDR8*, *ATM3*, and *PDF2.5* were induced by Cd stress, but these gene expression in *pap1-D*, 35S: *MYB75 #7*, 35S: *MYB75 #10* and *myb75-c* exhibited no difference compared with wild type (Fig. S3). However, under Cd stress, the transcription levels of *ACBP2*, which binds Cd²⁺, were significantly higher in *pap1-D*, 35S: *MYB75 #7*, 35S: *MYB75 #10* plants than in wild type, while its expression levels in *myb75-c* mutant were significantly lower than that in the wild type (Fig. 7A). We also noticed that expression levels of *ABCC2*, an ABCC-type phytochelatin transporter, were elevated in *pap1-D*, 35S: *MYB75 #7*, 35S: *MYB75 #10* plants, but reduced in *myb75-c* mutant without or with Cd stress (Fig. 7B).

On the basis of positive impact of MYB75 on *ACBP2* and *ABCC2* expression, we further surveyed the GUS activity by transient expression analysis in *Nicotiana benthamiana*. From the Fig. 7C-E and Fig. S4, we can see that MYB75 prompted the expression levels of *ProACBP2: GUS* and *ProABCC2: GUS*, indicating

that MYB75 has the capacity of prompting reporter activity driven by the promoters of *ACBP2* and *ABCC2*. Moreover, we performed luciferase (LUC) reporter transactivation assays in *Arabidopsis* protoplasts. Promoters of *ACBP2* and *ABCC2* were fused with LUC gene to generate promoter-LUC reporter constructs (Fig. 7F). These reporter constructs were co-expressed with empty vector or MYB75-HA in wild type protoplasts treated with MG132, and the reporter gene expression was used to evaluate MYB75 transcriptional activity. We noticed that MYB75 induced *ProACBP2-LUC* expression compared with empty vector (Fig. 7G). Consistently, MYB75 also induced *ProABCC2-LUC* expression (Fig. 7H). Based on these findings, we conclude that MYB75 positively regulates *ACBP2* and *ABCC2* expression.

MYB75 transcription factor directly binds to the promoter of *ACBP2* and *ABCC2*

Taking the above observations into account, we further examined the binding of MYB75 to the promoter regions of *ACBP2* and *ABCC2* *in vitro* and *in vivo*. MYB-recognizing element (MRE) has been identified as the core binding motif of MYB75. We found two MREs within the promoters of *ACBP2* and *ABCC2* (Fig. 8A, B). To test whether MYB can directly bind to the promoter regions of these target genes, we firstly performed electrophoretic mobility shift assay (EMSA). The results revealed that MYB75 protein tagged with maltose binding protein (MBP) (MBP-MYB75) bound to the P1 probe of *ACBP2* and C1 probe *ABCC2*, while the binding was abolished by mutation of MYB75 binding sites in the probes (Fig. 8A, B). Interestingly, MBP-MYB75 weakly bound to the P2 probe in the promoter of *ACBP2*, but did not bind to the C2 in the promoter of *ABCC2* (Fig. 8A, B). We next employed chromatin immunoprecipitation (ChIP) to further test the affinity of MYB75 for promoters of *ACBP2* and *ABCC2*. We immunoprecipitated HA-MYB75 protein from *35S: MYB75 #7* transgenic plants treated with MG132 by using anti-GFP antibody. TA3, a retrotransposable element, was used as the internal control. The ChIP-qPCR results revealed that MYB75 significantly enriched the fragments containing P1 and P2 of *ProACBP2*, C1 of *ProABCC2* (Fig. 8C, D). These results indicate that MYB75 directly regulates the transcription of *ACBP2* and *ABCC2* by binding to their promoters.

Discussion

As a large number of heavy metal pollutants, Cd pollution has become a global crisis with industrial development, causes serious environmental damage and human disease. High concentration of Cd in the soil also inhibits plant growth and crop yield. In recent years, there has been an increasing interest in studying the mechanism by which plants enrich and detoxify Cd. In this study, we established that MYB75 transcription factor positively regulates plant Cd stress via multiple pathways. Our research revealed that MYB75 stimulates GSH and PC content and activates of Cd tolerance-related gene at transcriptional level, such as *ACBP2* and *ABCC2*, thus inspiring a new transcription factor function in elevating plant Cd tolerance.

MYB75 positively regulates plant Cd tolerance via anthocyanin

It is well established that anthocyanin accumulation is induced by extreme environmental factors, such as high light, drought, cold, thus protecting plants against ROS damage (Allan et al., 2018). Several studies suggested that Cd stress stimulated anthocyanin accumulation. Anthocyanin biosynthetic genes transcription were induced under Cd exposure (Dobrikova et al., 2021, Mwamba et al., 2020). As we all know, anthocyanin biosynthetic genes were regulated by MBW protein complex. It seems that Cd stress affected anthocyanin regulatory genes expression. Our results illuminated that Cd exposure primarily induce MYB75 expression, thus prompting MBW protein complex and stimulating anthocyanin accumulation (Fig. 1A, B). These findings reveal that Cd exposure aggravated anthocyanin accumulation via transcriptional regulation of MBW protein complex.

Several researches demonstrated that there was a negative correlation between the chlorophyll and anthocyanin content under Cd stress (Szopinski et al., 2020, Vazquez et al., 2020). We found that *pap1-D, 35S: MYB75 #7* and *35S: MYB75 #10*, which accumulated abundant anthocyanin, exhibited higher chlorophyll level and fluorescence than wild type (Fig. 2 and 3). These results imply that anthocyanin can protect photosystem from ROS damage under Cd exposure. It has previously been observed that exogenous anthocyanin increased intracellular GSH levels (Norris et al., 2016). We verified that endogenous anthocyanin elevated GSH and PC levels through modulating ROS homeostasis (Fig. 6A, B). Our results are also in accord with previous studies indicating that anthocyanin is beneficial to Cd uptake and hyperaccumulate Cd under Cd exposure (Mwamba et al., 2020, Szopinski et al., 2020) (Fig. 6C). Previous studies showed that anthocyanins and organic acids can facilitate localization of Cd into vacuole (Glinska et al., 2013, Sebastian et al., 2018, Verbruggen et al., 2009). We speculated that endogenous anthocyanins can benefit Cd²⁺ sequestration to reduce Cd toxicity. These observations may support the hypothesis that plants adaptively prompt anthocyanin accumulation during suffering Cd stress thus scavenging ROS and expediting Cd²⁺ sequestration to alleviate Cd toxicity.

MYB75 enhances plant Cd tolerance at transcriptional level

Under Cd exposure, plants evolve an adaptive mechanism by which transcription factors regulate expression of numerous Cd tolerance-related genes at transcriptional level. ZAT6 regulates Cd tolerance by upregulating PC synthesis-related genes (Chen et al., 2016). MYB49, the R2R3-MYB transcription factor, results Cd accumulation through inducing *HIPP22* and *HIPP44* transcription (Zhang et al., 2019). Cd-induced WRKY13 activates *DCD* expression to increase the production of H₂S, brings about higher Cd tolerance (Zhang et al., 2020). In our study, LUC and GUS staining assays suggested that MYB75 reinforced the activities of the promoters of *ACBP2* and *ABCC2* (Fig. 7C-H). EMSA assays demonstrated that MYB75 directly bound to the MYB-recognizing element in the promoter of *ACBP2* and *ABCC2* (Fig. 8A, B). ChIP-qPCR assays proved that Cd stress strengthened binding of MYB75 to *ACBP2* and *ABCC2* (Fig. 8C, D). These results imply that MYB75 directly regulates the *ACBP2* and *ABCC2* expression.

Previous studies confirmed that *ACBP2* alleviates Cd toxicity through binding Cd^{2+} (Gao et al., 2010). Additionally, ABA and drought induces *ACBP2* transcription thus enhancing drought tolerance (Du et al., 2013). Intriguingly, anthocyanin accumulation is raised by ABA and drought. It seems that MYB75 was activated upon drought stress and then elevated *ACBP2* expression to improve drought tolerance. These findings suggested that MYB75 perhaps participated in diversified stress response. Prior reports have shown that *ABCC2* exhibited PC transport activity in plant vacuole (Song et al., 2010). Subsequent research observed that anthocyanin is transported to vacuole via *ABCC2* (Behrens et al., 2019). It can thus be suggested that vacuole played an important role in detoxifying Cd via *ABCC2*-modulated Cd^{2+} sequestration. According to these data, we infer that regulation of MYB75 on Cd^{2+} sequestration and compartmentation at transcriptional and metabolic level enhances plant Cd tolerance.

MYB75 balance growth and the stress response

Recent studies suggested that reciprocal regulation between stress-response and growth-control pathways occurs at multiple levels. Plants need to strictly limit the allocation of carbon sources to balance development and extreme environmental response. Extensive research has shown that MYB75 functions in regulation of secondary cell wall formation during plant development (Bhargava et al., 2010). MYB75 transcription facilitates carbon flow into the anthocyanin pathway rather than lignin pathway under various environmental stresses. Our previous studies demonstrated plant fine tune anthocyanin accumulation via post-translational regulation of MYB75 through HAT1 transcription factor and SUMO E3 ligase SIZ1 during growth (Zheng et al., 2020b, Zheng et al., 2019). In general, therefore, it seems that MYB75 primarily regulates secondary cell wall formation under normal condition. Once plant suffers stress including Cd and drought, the activities of MYB75 is enhanced via multiple levels of regulation, thus promoting anthocyanin accumulation to adapt to the extreme environment.

In conclusion, a working model was proposed based on our study (Fig. 9). When the plants are confronted with Cd stress, expression of MYB75 transcription factor could be quickly induced. Cd-induced MYB75 stimulates GSH-dependent PC synthesis via anthocyanin-mediated ROS homeostasis. On the other side, MYB75 activates *ACBP2* and *ABCC2* expression by directly binding to the promoters of *ACBP2* and *ABCC2*, followed by Cd sequestration and compartmentation, thus enhancing plant Cd tolerance.

Declarations

Author contributions

D.W.Z conceived and supervised the study. T.Z and F.Y designed experiments and analyzed data. T.Z performed experiments. T.Z and D.W.Z wrote the manuscript.

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Declaration of Competing Interest

The authors have declared that no competing interests exist.

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Figures

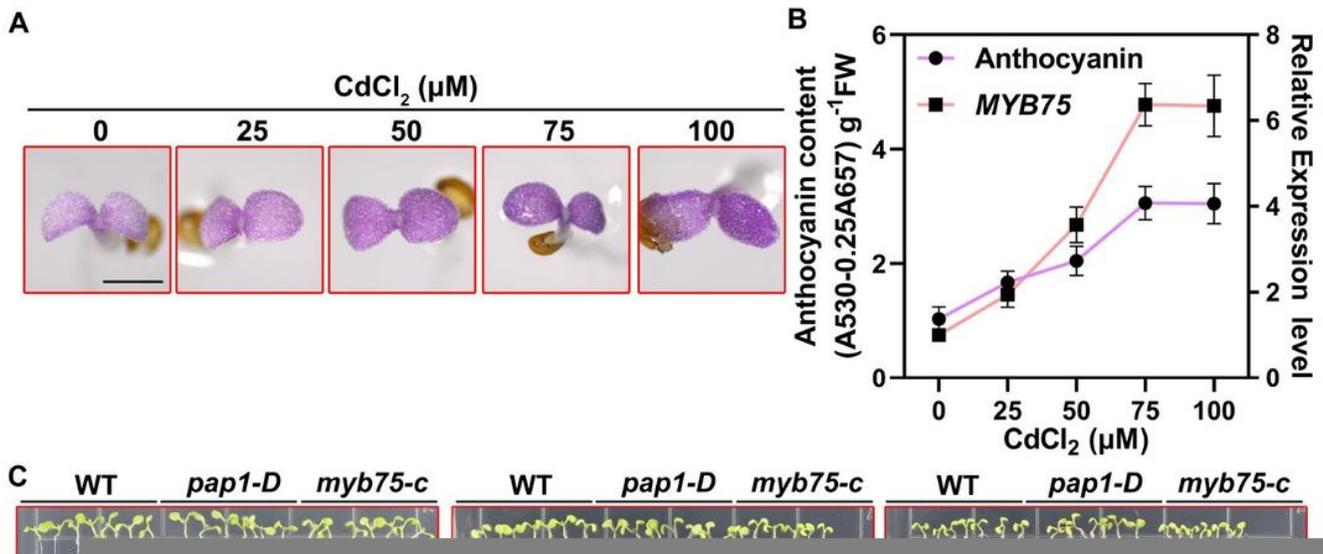


Figure 1

MYB75 transcription factor is involved in plant Cd tolerance. (A) 3-day-old WT Arabidopsis seedlings grown on plates under different Cd stress. Bars = 0.25 cm. (B) Anthocyanin levels and MYB75 transcripts in extracts from seedlings in (A). The experiments were performed in biological triplicate (representing anthocyanin content measured from 0.2 g WT plants and treatment were pooled for one replicate). FW, fresh weight. Error bars denote \pm SD (n=3). (C) Phenotypes of WT, *pap1-D* and *myb75-c* mutants under Cd stress. 3-day-old seedlings grown on 1/2 MS medium were transferred to 1/2 MS medium without or with 50 or 75 μ M CdCl₂. Photographs were taken 7 d after transfer. Bar = 1cm. (D) Root length of seedlings described in (C). Data are means \pm SD; n = 26 biologically independent roots. Different letters represented

statistically significant differences (two-way ANOVA, $p < 0.05$). (E) Fresh weight of seedlings described in C. Five plants per genotype from one plate were measured for each repeat. Data are means \pm SD ($n = 3$). Different letters represented statistically significant differences (two-way ANOVA, $p < 0.05$).

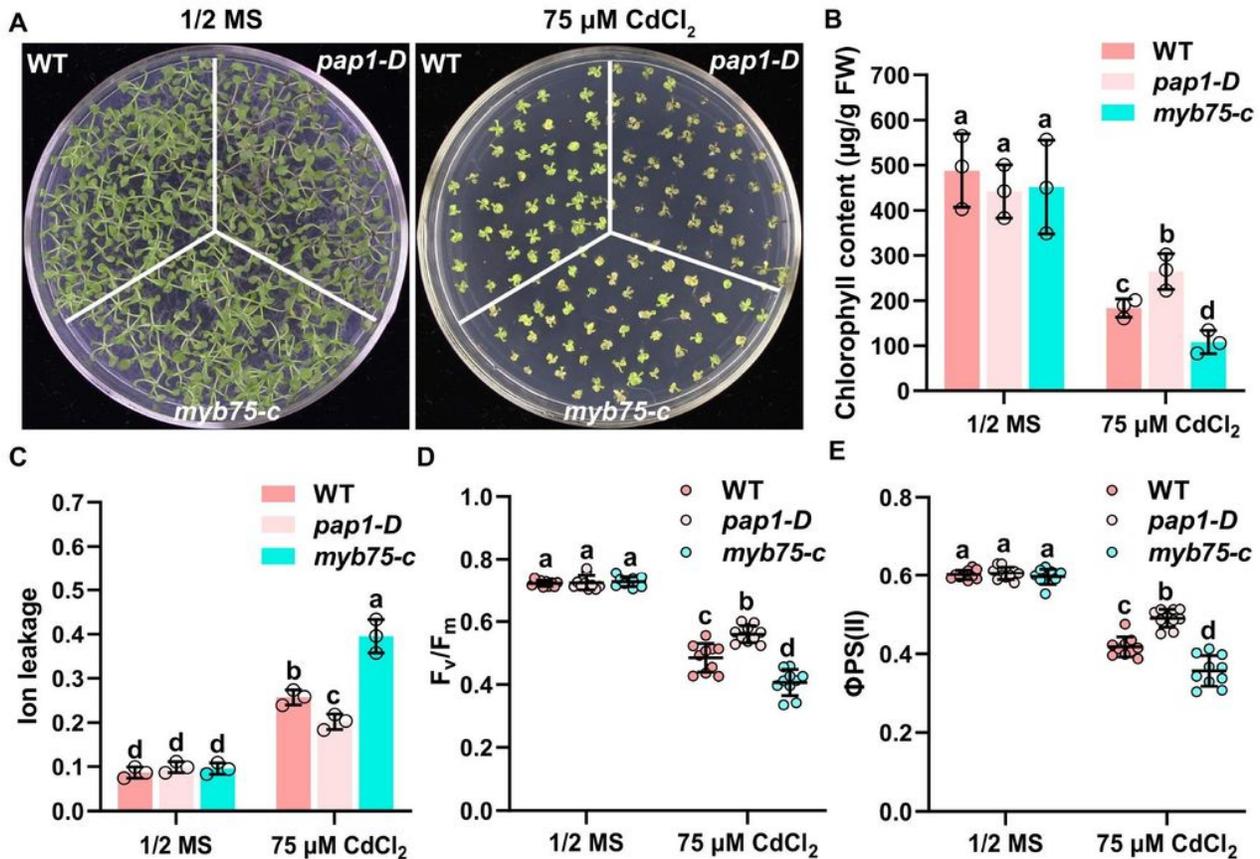


Figure 2

MYB75 enhances plant Cd tolerance. (A) 21-day-old Arabidopsis seedlings of WT, *pap1-D*, *myb75-c* grown on plates under different Cd stress. (B) Chlorophyll levels in extracts from seedlings in (A). The experiments were performed in biological triplicate (representing chlorophyll content measured from 15 plants of each genotype and treatment were pooled for one replicate). FW, fresh weight. Error bars denote \pm SD ($n = 3$). Different letters represented statistically significant differences (two-way ANOVA, $p < 0.05$). (C) Ion leakage of the plants in (A). Error bars denote \pm SD ($n = 3$). (D) F_v/F_m of seedlings described in (A). Data are means \pm SD; $n = 10$ biologically independent seedlings. Different letters represented statistically significant differences (two-way ANOVA, $p < 0.05$). (E) $\Phi\text{PS(II)}$ of seedlings described in (A). Data are means \pm SD; $n = 10$ biologically independent seedlings. Different letters represented statistically significant differences (two-way ANOVA, $p < 0.05$).

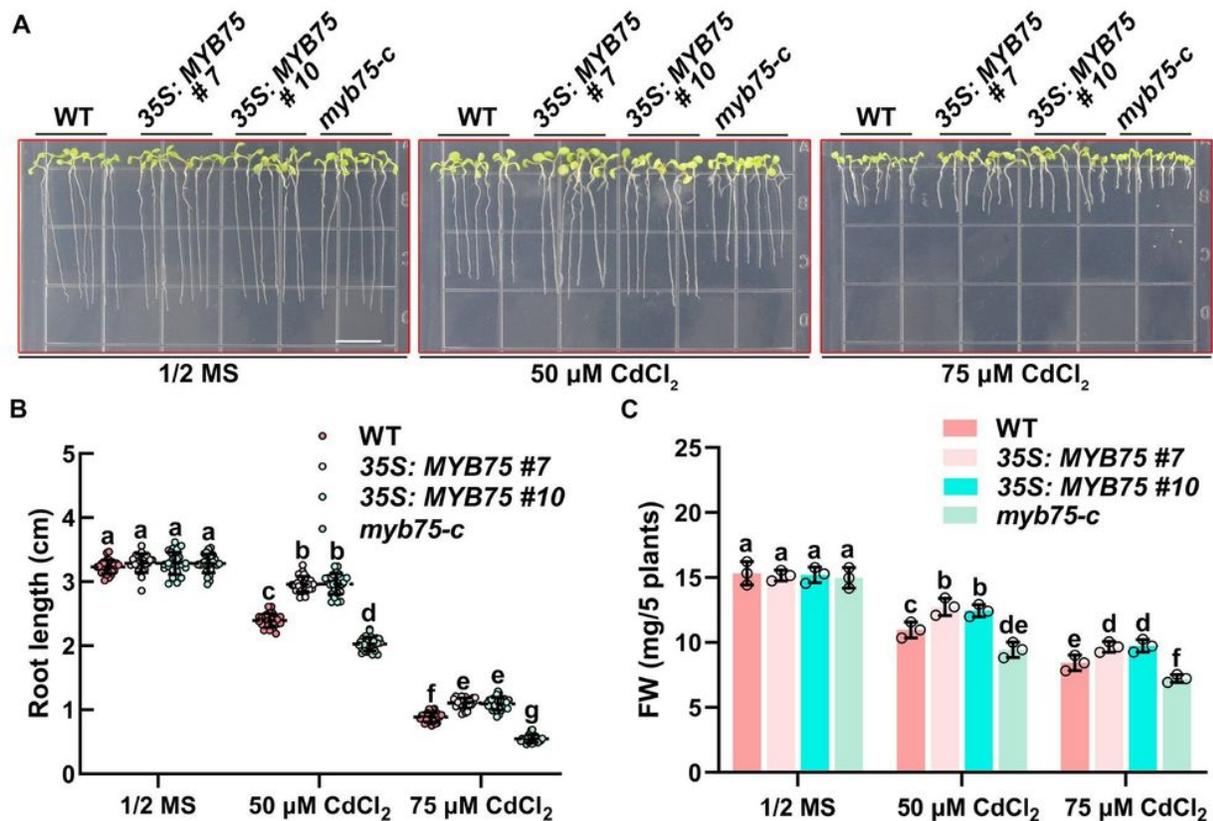


Figure 3

Cd tolerance in the MYB75 overexpression lines. (A) Phenotypes of WT, 35S: MYB75 #7, 35S: MYB75 #10 and *myb75-c* mutants under Cd stress. Three-day-old seedlings grown on 1/2 MS medium were transferred to 1/2 MS medium without or with 50 or 75 μM CdCl₂. Photographs were taken 7 d after transfer. Bar = 1 cm. (B) Root length of seedlings described in (A). Data are means \pm SD; n = 26 biologically independent roots. Different letters represented statistically significant differences (two-way ANOVA, $p < 0.05$). (C) Fresh weight of seedlings described in A. Five plants per genotype from one plate were measured for each repeat. Data are means \pm SD (n=3). Different letters represented statistically significant differences (two-way ANOVA, $p < 0.05$).

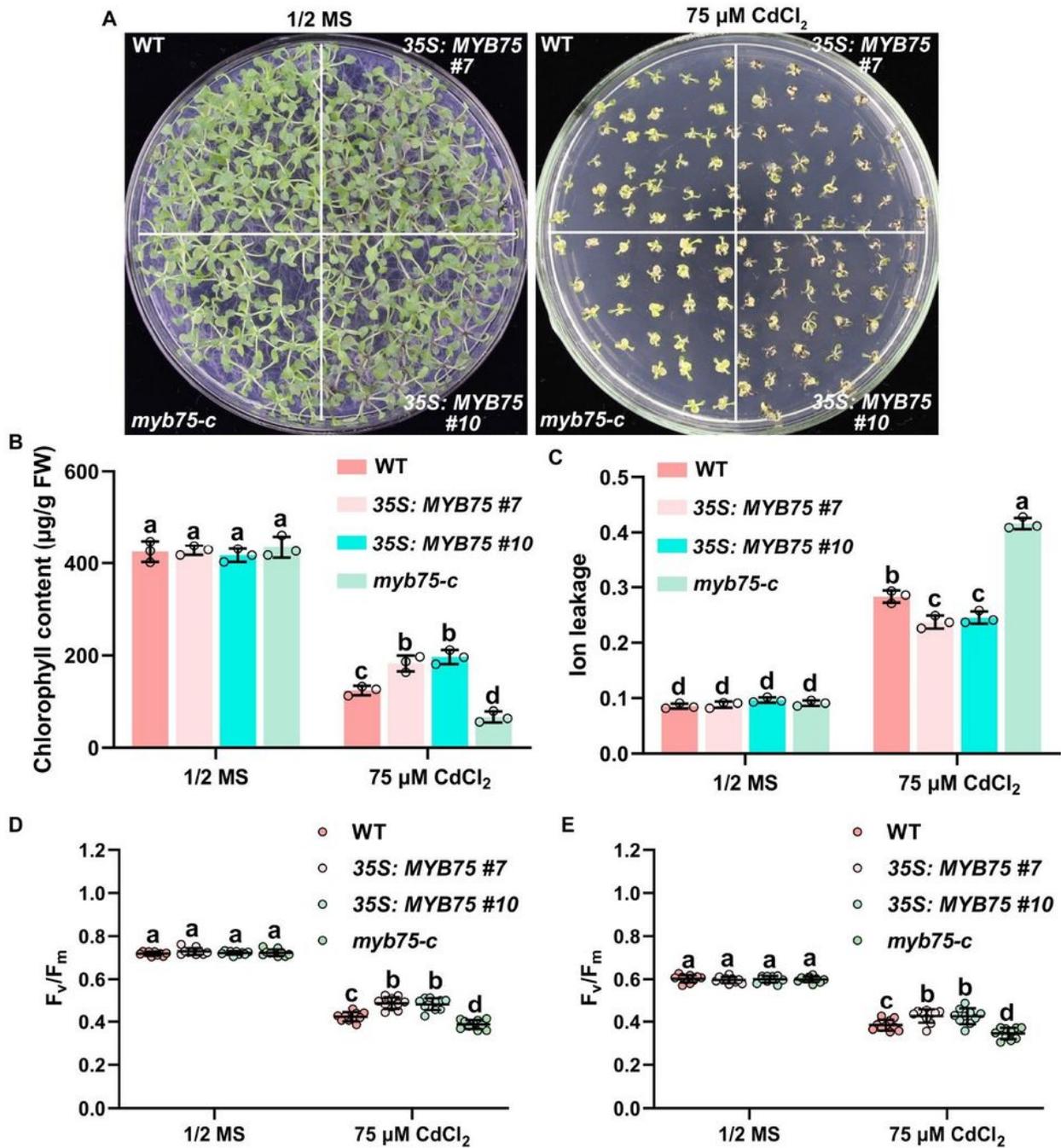


Figure 4

MYB75 overexpression enhances plant Cd tolerance (A) 21-day-old Arabidopsis seedlings of WT, *35S: MYB75 #7*, *35S: MYB75 #10* and *myb75-c* grown on plates under different Cd stress. (B) Chlorophyll levels in extracts from seedlings in (A). The experiments were performed in biological triplicate (representing chlorophyll content measured from 15 plants of each genotype and treatment were pooled for one replicate). FW, fresh weight. Error bars denote \pm SD ($n=3$). Different letters represented statistically

significant differences (two-way ANOVA, $p < 0.05$). (C) Ion leakage of the plants in (A). Error bars denote \pm SD ($n = 3$). (D) F_v/F_m of seedlings described in (A). Data are means \pm SD; $n = 10$ biologically independent seedlings. Different letters represented statistically significant differences (two-way ANOVA, $p < 0.05$). (E) $\Phi PS(II)$ of seedlings described in (A). Data are means \pm SD; $n = 10$ biologically independent seedlings. Different letters represented statistically significant differences (two-way ANOVA, $p < 0.05$).

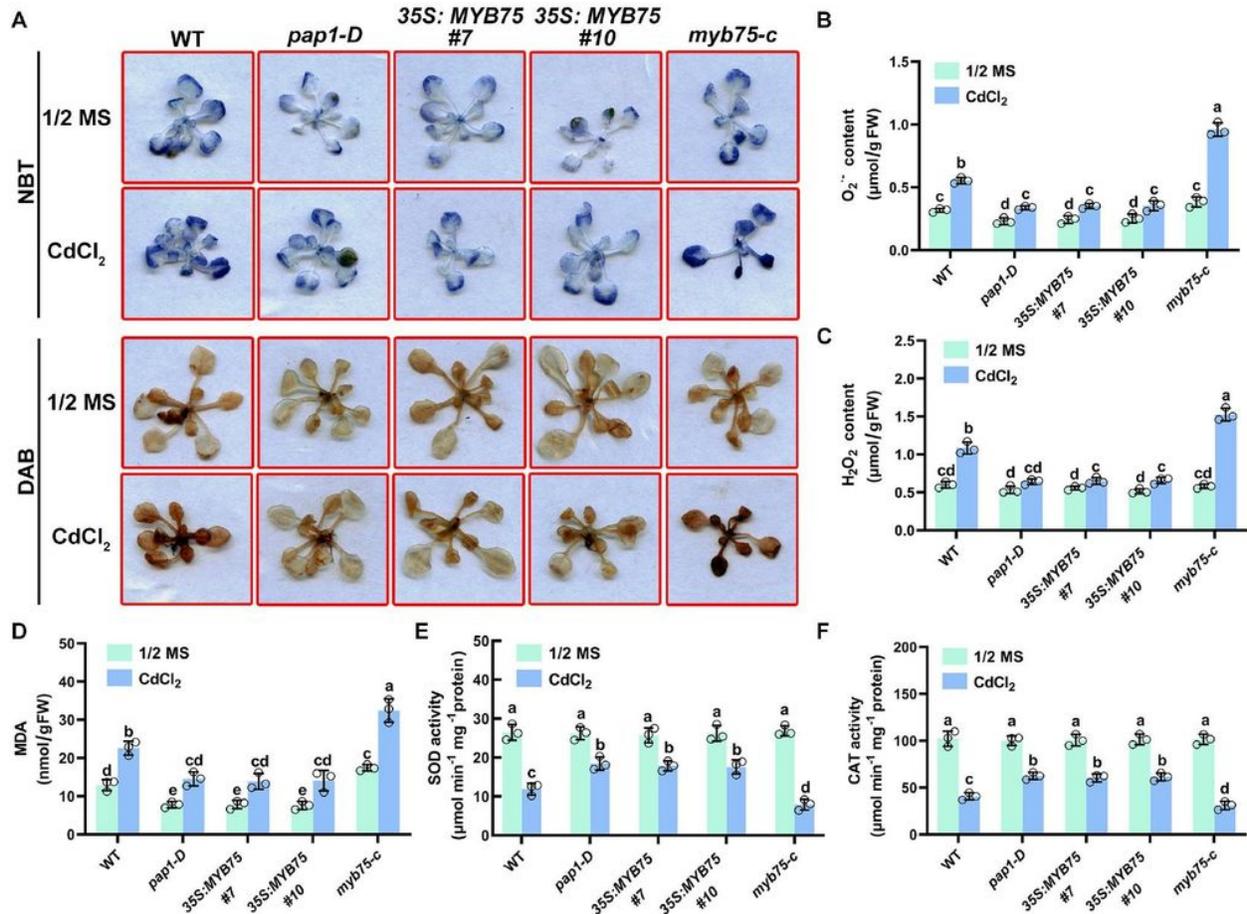


Figure 5

MYB75 modulates ROS homeostasis under Cd stress. (A) Representative images of NBT-stained and DAB-stained leaves from WT, *pap1-D*, *35S: MYB75 #7*, *35S: MYB75 #10* and *myb75-c* seedlings grown on medium for 2 weeks and then treated with (+) or without (-) 75 μM CdCl_2 for 24 h. (B) $O_2^{\cdot-}$ content in extracts from seedlings in (A). The experiments were performed in biological triplicate (representing $O_2^{\cdot-}$ content measured from 10 plants of each genotype and treatment were pooled for one replicate). FW, fresh weight. Error bars denote \pm SD ($n = 3$). Different letters represented statistically significant differences (two-way ANOVA, $p < 0.05$). (C) H_2O_2 content in extracts from seedlings in (A). The experiments were performed in biological triplicate (representing H_2O_2 content measured from 10 plants of each

genotype and treatment were pooled for one replicate). FW, fresh weight. Error bars denote \pm SD (n=3). Different letters represented statistically significant differences (two-way ANOVA, $p < 0.05$). (D) MDA contents in extracts from seedlings in (A). The experiments were performed in biological triplicate (representing MDA content measured from 10 plants of each genotype and treatment were pooled for one replicate). FW, fresh weight. Error bars denote \pm SD (n=3). Different letters represented statistically significant differences (two-way ANOVA, $p < 0.05$). (E) SOD activity in extracts from seedlings in (A). Error bars denote \pm SD (n=3). Different letters represented statistically significant differences (two-way ANOVA, $p < 0.05$). (F) CAT activity in extracts from seedlings in (A). Error bars denote \pm SD (n=3). Different letters represented statistically significant differences (two-way ANOVA, $p < 0.05$).

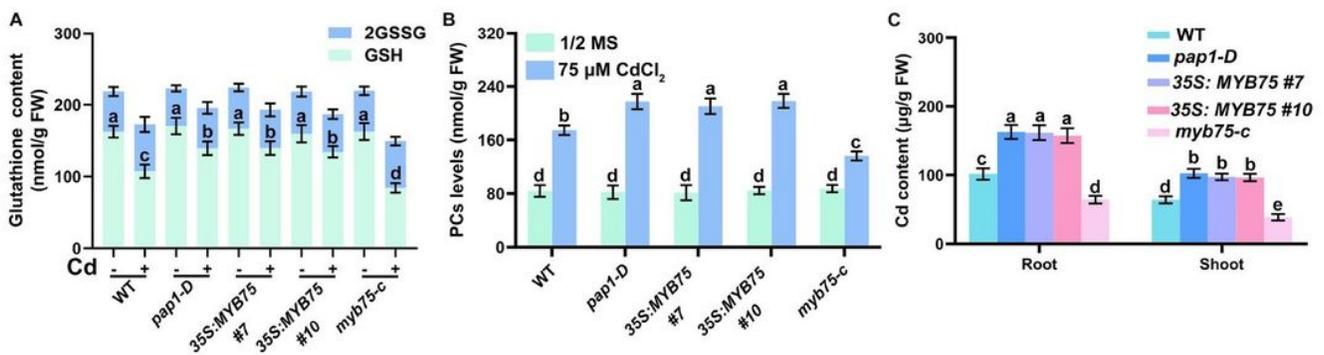


Figure 6

MYB75 mediates GSH, PCs levels and Cd distribution. (A) Glutathione contents in WT, *pap1-D*, 35S:MYB75 #7, 35S:MYB75 #10 and *myb75-c* seedlings grown on medium for 2 weeks and then treated with (+) or without (-) 75 μ M CdCl₂ for 24 h. The experiments were performed in biological triplicate (representing glutathione content measured from 10 plants of each genotype and treatment were pooled for one replicate). FW, fresh weight. Error bars denote \pm SD (n=3). Different letters represented statistically significant differences (two-way ANOVA, $p < 0.05$). (B) PC contents in WT, *pap1-D*, 35S:MYB75 #7, 35S:MYB75 #10 and *myb75-c* seedlings grown on medium for 2 weeks and then treated with or without 75 μ M CdCl₂ for 24 h. The experiments were performed in biological triplicate (representing PC content measured from 10 plants of each genotype and treatment were pooled for one replicate). FW, fresh weight. Error bars denote \pm SD (n=3). Different letters represented statistically significant differences (two-way ANOVA, $p < 0.05$). (C) Cd contents in WT, *pap1-D*, 35S:MYB75 #7, 35S:MYB75 #10 and *myb75-c* seedlings grown on medium for 2 weeks. The experiments were performed in biological triplicate (representing Cd content measured from 10 plants of each genotype and treatment were pooled for one replicate). FW, fresh weight. Error bars denote \pm SD (n=3). Different letters represented statistically significant differences (two-way ANOVA, $p < 0.05$).

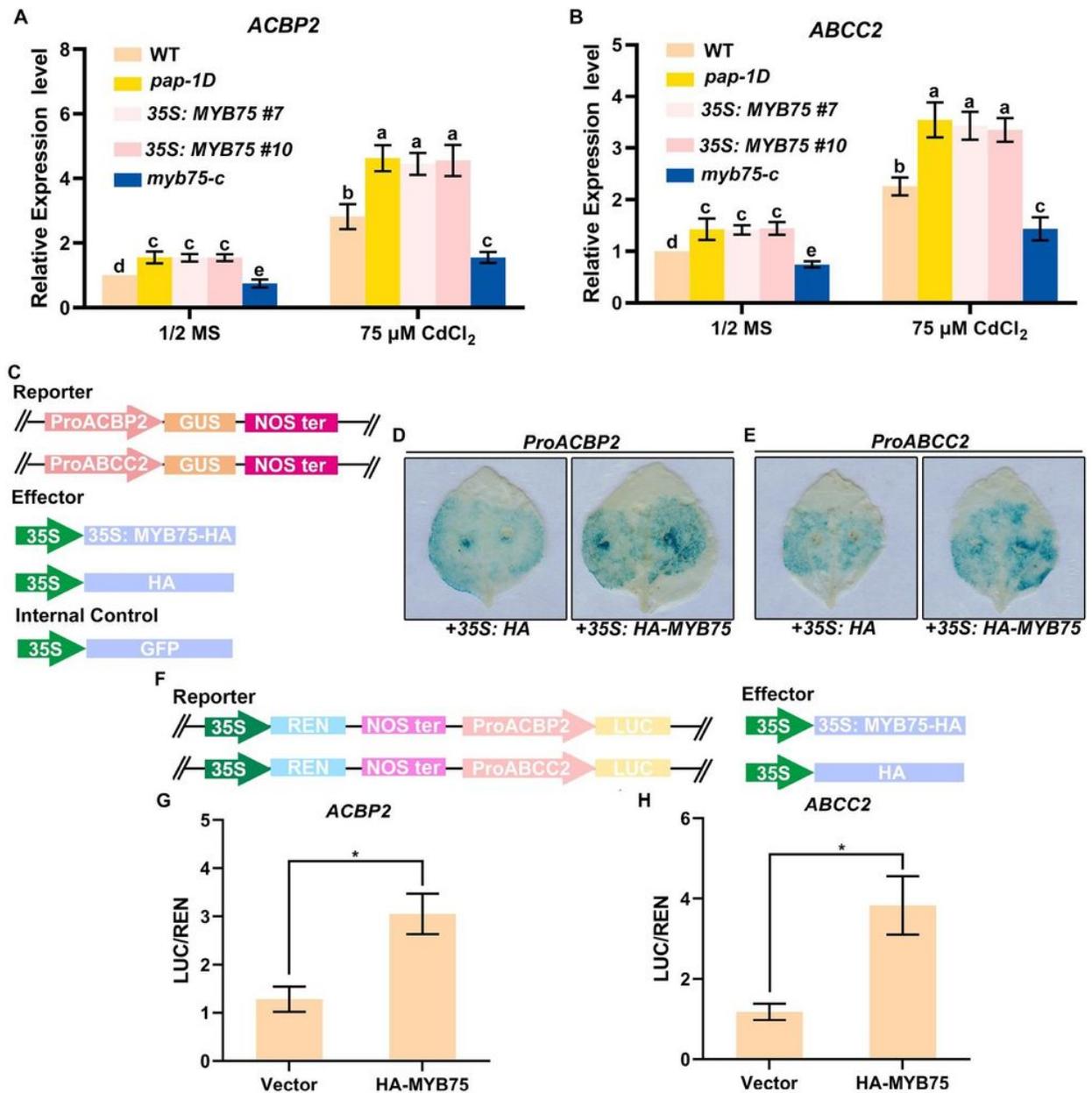


Figure 7

MYB75 regulates *ACBP2* and *ABCC2* expression. (A, B) qPCR analysis of *ACBP2* and *ABCC2* expression levels. Seedlings grown on plates for 2 weeks respectively, then treated with 75 μ M CdCl₂ for 4 h, and then their mRNAs were isolated for qPCR analysis. Expression levels were standardized to *ACTIN 8*, the results of WT under control conditions were set at 1. Error bars denote \pm SD (n= 3). Different letters represented statistically significant differences (two-way ANOVA, p<0.05). (C) A schematic of the *ACBP2*-promoter and *ABCC2*-promoter reporter construct, the effector plasmid, and the transfection control plasmid. (D, E)

GUS staining of the *ProACBP2: GUS* and *ProABCC2: GUS* reporter after coexpression of *35S: MYB75-HA* in *Nicotiana benthamiana*. Coexpression of the *ProACBP2: GUS* and *ProABCC2: GUS* reporter and 35S empty vector was used as the effector plasmid control. (F) A schematic of the *ACBP2-promoter* and *ABCC2-promoter* reporter construct, the effector plasmid for LUC assays. (G, H) LUC assays of the *ProACBP2: LUC* and *ProABCC2: LUC* reporter after coexpression of *35S: MYB75-HA* in *Nicotiana benthamiana*. Coexpression of the *ProACBP2: LUC* and *ProABCC2: LUC* reporter and 35S empty vector was used as the effector plasmid control. Error bars denote \pm SD (n= 3). Asterisk represented statistically significant differences (t-test, p<0.05).

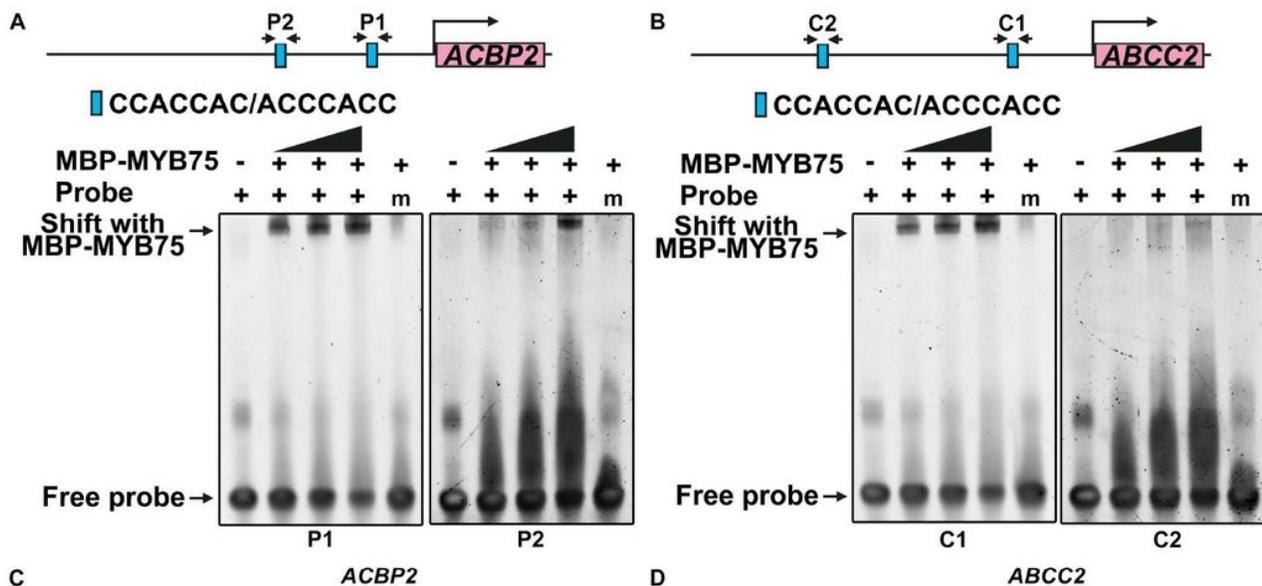


Figure 8

MYB75 directly binds to the promoter of *ACBP2* and *ABCC2*. (A, B) EMSA assays of MYB75 binding to *ACBP2* and *ABCC2* promoter. Wild-type and mutant probes were incubated with MBP-MYB75, and free and bound DNAs were separated on an acrylamide gel. (C, D) ChIP assays of MYB75 binding to *ACBP2* and *ABCC2* promoter. The 14-day-old seedlings were treated with or without 75 μ M CdCl₂ for 4h

respectively, then harvested for CHIP-qPCR assay using anti-HA antibody. TA3 was used as the internal control. Different letters represented statistically significant differences (two-way ANOVA, $p < 0.05$).

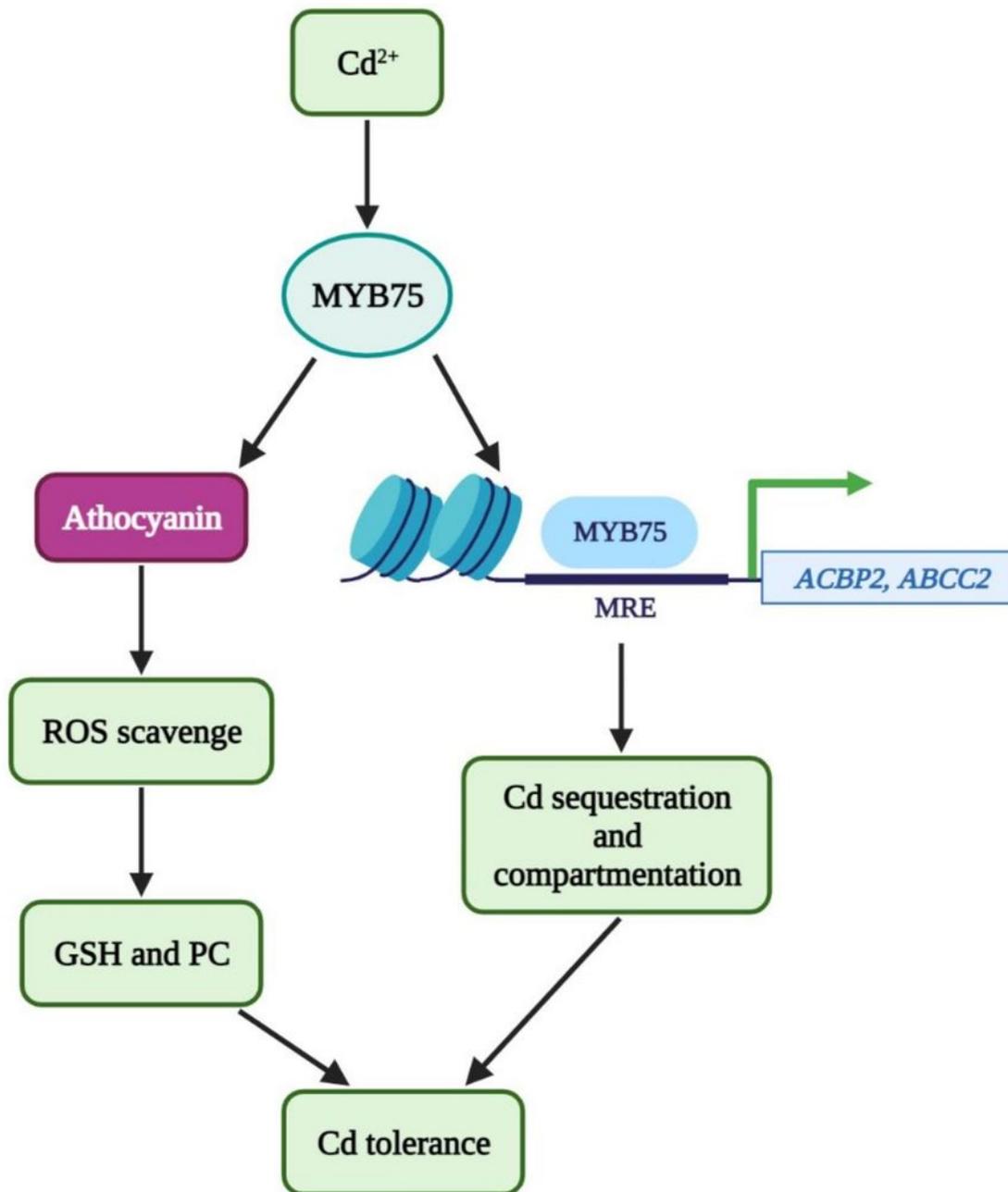


Figure 9

A working model for MYB75-regulated plant Cd tolerance. When plants were confronted with Cd stress, MYB75 transcription factor could be induced quickly. On the one hand, MYB75 activated anthocyanin

accumulation, followed by the ROS scavenge, which resulted in the elevation of GSH and PC content. On the other hand, MYB75 directly bind to the promoter of Cd tolerance-related gene, such as *ACBP2* and *ABCC2*, thus activating their expression, which brought about Cd sequestration and compartmentation. The synergetic modulation via MYB75 enhanced plant tolerance in response to Cd stress.

Supplementary Files

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