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WRKY12 represses *GSH1* expression to negatively regulate cadmium tolerance in *Arabidopsis*

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Abstract

Key message **The WRKY transcription factor WRKY12 negatively regulates Cd tolerance in *Arabidopsis* via the glutathione-dependent phytochelatin synthesis pathway by directly targeting *GSH1* and indirectly repressing phytochelatin synthesis-related gene expression.**

Abstract Cadmium (Cd) is a widespread pollutant toxic to plants. The glutathione (GSH)-dependent phytochelatin (PC) synthesis pathway plays key roles in Cd detoxification. However, its regulatory mechanism remains largely unknown. Here, we showed a previously unknown function of the WRKY transcription factor *WRKY12* in the regulation of Cd tolerance by repressing the expression of PC synthesis-related genes. The expression of *WRKY12* was inhibited by Cd stress. Enhanced Cd tolerance was observed in the *WRKY12* loss-of-function mutants, whereas increased Cd sensitivity was found in the *WRKY12*-overexpressing plants. Overexpression and loss-of-function of *WRKY12* were associated respectively with increased and decreased Cd accumulation by repressing or releasing the expression of the genes involved in the PC synthesis pathway. Transient expression assay showed that WRKY12 repressed the expression of *GSH1, GSH2, PCS1*, and *PCS2*. Further analysis indicated that *WRKY12* could directly bind to the W-box of the promoter in *GSH1* but not in *GSH2, PCS1*, and *PCS2 in vivo*. Together, our results suggest that WRKY12 directly targets *GSH1* and indirectly represses PC synthesis-related gene expression to negatively regulate Cd accumulation and tolerance in *Arabidopsis*.

Keywords *Arabidopsis* · Cd tolerance · Glutathione · Phytochelatins · *WRKY12*

Introduction

Soil contamination with cadmium (Cd) is a global environmental problem because of its high toxicity and easy transmission through the food chain. Under excess Cd exposure, plants show limited plant growth, retarded photosynthesis, decreased respiration, leaf chlorosis, inhibited root growth, increased synthesis of reactive oxygen species (ROS), and even death (Smeets et al. 2008; Sharma and Dietz 2009; Wu et al. 2015).

Plants have employed various strategies to resist Cd stress and those include uptake, accumulation, translocation, and detoxification of the metal. In general, there are two major strategies for detoxifying excess Cd absorbed from soil, namely excluder strategy, which avoids Cd entering the plants or extrudes excess Cd (Mills et al. 2005; Moreno et al. 2008; Morel et al. 2009); and tolerance strategy, which chelates Cd in the cytosol and sequesters Cd in vacuoles (Verbruggen et al. 2009; Lin and Aarts 2012). Chelation, such as that with glutathione (GSH) and phytochelatins (PCs), is an important component involved in these mechanisms. GSH functions as a metal chelator, a cellular antioxidant, and a ROS signalling molecule, which is a γ-Glu-Cys-Gly tripeptide (Jozefczak et al. 2012; Seth et al. 2012). In *Arabidopsis thaliana*, there are two ATP-dependent enzymes, γ-glutamylcysteine synthetase (*GSH1*; EC.6.3.2.2) and GSH synthetase (*GSH2*; EC.6.3.2.3), involved in the GSH synthesis pathway (Shanmugam et al. 2012; Noctor et al. 2012).

Yangyang Han and Tingting Fan have contributed equally to this work.

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A GSH-deficient mutant, called cad2-1 mutant, is sensitive to Cd stress (Cobbett et al. 1998). Many studies have confirmed that PCs containing $(\gamma\text{-Glu-Cys})_n$-Gly ($n = 2$–11) are enzymatically synthesized directly from GSH by the enzyme PC synthase (PCS) (Rauser 1995). In *Arabidopsis thaliana*, PCSs are encoded by two genes, *PCS1* and *PCS2*, which are strongly induced by Cd stress (May et al. 1998; Noctor et al. 2002; Kühnlenz et al. 2014). Thus, GSH and PCs are known to have key roles in heavy metal detoxification.

It is commonly known that transcription factors (TFs) are critical components in signalling networks owing to their functions as regulators of the defence response by modulating the expression of the genes involved in defence response. WRKY TFs specifically recognize and bind the W-box (contains a TGAC core sequence) (Liu et al. 2014; Li et al. 2016). Many reports have demonstrated that WRKY TFs can be either positive or negative regulators of plant development and defence (Dong et al. 2003; Kalde et al. 2003; Luo et al. 2005; Devaiah et al. 2007; Eulgem and Somssich 2007; Chen et al. 2009, 2012; Wang et al. 2010; Ding et al. 2013; Kim et al. 2013; Liu et al. 2014; Li et al. 2016). *WRKY12*, which belongs to the subgroup IIc of the *WRKY* gene family, is involved in secondary cell wall formation by directly inhibiting *NST2* expression in pith cells (Wang et al. 2010) and modulates flowering time under short-day conditions (Li et al. 2016). A growing number of studies have found that TFs participate in the response to Cd stress by regulating the expression of downstream target genes such as *CaPF1, HsfA4a, BjCdR15/TGA3*, and *ZAT6* (Tang et al. 2005; Shim et al. 2009; Farinati et al. 2010; Chen et al. 2016). However, the possible role of *WRKY12* under Cd stress and the details of the underlying mechanism are yet to be revealed.

The aim of the present study was to investigate the functions and the underlying mechanisms of *WRKY12* in plant response to Cd stress.

Results

WRKY12 is repressed by Cd stress

Considering that *WRKY13* is involved in the regulation of Cd stress response (Sheng et al. 2018), *WRKY12* and *WRKY13* belong to the same subgroup in the *WRKY* gene family, and their amino acid sequences are highly similar (Li et al. 2016), we speculated that *WRKY12* may also respond to Cd stress. To verify this hypothesis, we analysed the transcription level of *WRKY12* under Cd stress. The results showed that the *WRKY12* transcription was inhibited by Cd stress (Fig. 1A), thus providing evidence that *WRKY12* gene may be involved in regulating plant Cd tolerance.

We also examined the expression pattern of *WRKY12* in different tissues and detected the expression of *WRKY12* in all tissues examined, but the highest level was found in the stem (Fig. 1B).

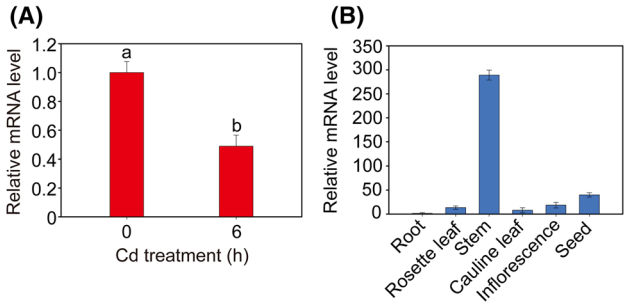

Fig. 1 Expression patterns of *WRKY12*. **A** The expression of *WRKY12* was induced by Cd stress. Two-week-old WT seedlings were treated with $CdCl_2$ (50 μM) for 0 and 6 h for qRT-PCR analysis. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal control. Data present means ± SE ($n = 3$). Bars with different lowercase letters are significantly different at $P < 0.05$ (Tukey's test). **B** The expression pattern of *WRKY12* was analysed by qRT-PCR in different tissues of wild-type (WT) plants. GAPDH was used as internal control. The samples were isolated from the root, rosette leaves, stem, cauline leaves, inflorescences, and seeds of the WT plants

Loss-of-function mutations of the WRKY12 gene lead to enhanced Cd tolerance

To further verify the potential regulatory role of *WRKY12* in response to Cd stress, two T-DNA insertion mutants were obtained from the SALK *Arabidopsis* T-DNA mutant collection, named *wrky12-1* (CS 435919) and *wrky12-2* (CS 374453) (Alonso et al. 2003). The two insertion sites of the *wrky12-1* and *wrky12-2* were located upstream of the gene coding region (Fig. 2A). qRT-PCR results detected no transcripts of *WRKY12* in the two mutants (Fig. 2B). There were no significant differences in growth between the WT and mutants in the control 1/2 MS medium. However, under Cd stress, the mutants exhibited enhanced Cd tolerance compared with the WT (Fig. 2C). Compared with WT plants, the root length and FW of the mutants were significantly increased in a Cd concentration-dependent manner ($P < 0.05$; Fig. 2D, E). Together, these results indicated that the *WRKY12* loss-of-function results in increased Cd tolerance.

To test whether *wkry12* mutants are also involved in the regulation of other heavy metal stresses, the *wkry12* mutants were grown on 1/2 MS medium with or without $Pb(NO_3)_2$, Na_3AsO_4, and $ZnSO_4$. When Na_3AsO_4 or $ZnSO_4$ was added, no significant differences between WT and *wrky12* mutant plants were observed. However, the *wrky12* mutants showed enhanced tolerance to $Pb(NO_3)_2$ (Supplementary Fig. S1).

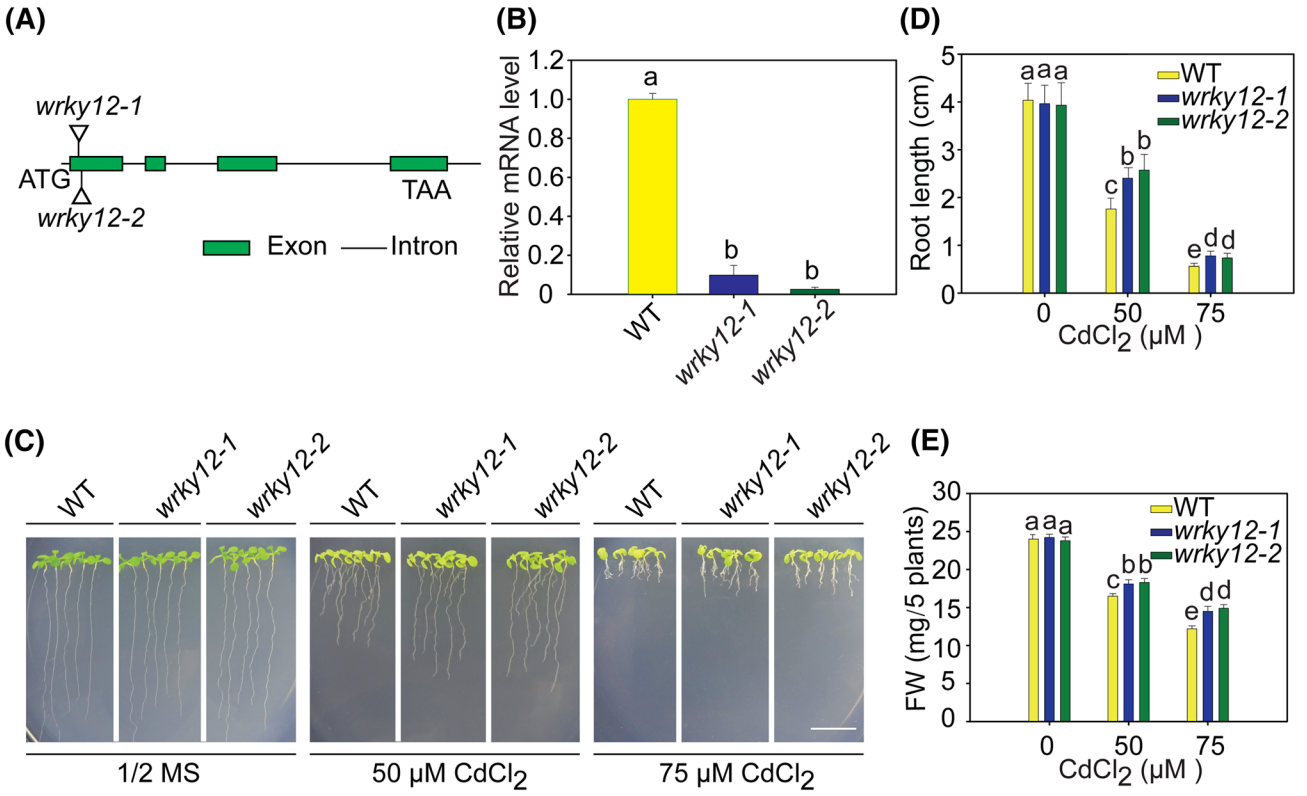

Fig. 2 The *wrky12* mutants are tolerant to Cd stress. **A** Genetic map of the *WRKY12* gene. Black boxes and black lines indicate exons and introns, respectively, in *WRKY12*. The positions of T-DNA insertion are indicated by triangles. **B** The expression of *WRKY12* in the *wrky12* mutants and wild-type (WT) plants by qRT-PCR using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as internal control. Data are presented as the means ± SE ($n = 3$). Bars with different lowercase letters are significantly different at $P < 0.05$ (Tukey's test). **C** The *WRKY12* loss-of-function mutation produced phenotypes resistant to Cd stress. Seedlings were grown on half-strength Murashige and Skoog (1/2 MS) medium for 3 days then treated with 0, 50, or 7 5 μM $CdCl_2$ for 2 weeks. Bar = 1 cm. **D** and **E** Root length (**D**) and fresh weight (**E**) of plants described in **C**. Data present the means ± SE ($n = 3$). Bars with different lowercase letters are significantly different at $P < 0.05$ (Tukey's test)

Overexpression of the WRKY12 gene results in enhanced Cd sensitivity

To further examine whether *WRKY12* regulates Cd tolerance, we generated a *35S:WRKY12* construct and transformed it into the WT plants. At least five transgenic lines were obtained, of which two lines *OE5* and *OE11* (Fig. 3A) were chosen for further study. There were no differences in growth and development among WT, *wrky12* mutant, and *WRKY12*-OE plants under normal growth conditions (Supplementary Fig. S2).

In 1/2 MS media, no significant differences were observed between the WT and the two overexpressing plants; however, under Cd stress, the *WRKY12*-OE plants displayed enhanced Cd sensitivity compared with the WT plants (Fig. 3B). This was further confirmed by quantitative analysis of the root length ($P < 0.05$; Fig. 3C) and the fresh weight ($P < 0.05$; Fig. 3D). These results suggest that *WRKY12* negatively regulates Cd tolerance.

Alteration of WRKY12 gene expression affects Cd accumulation

To test whether the expression change of *WRKY12* affects Cd content, we measured Cd content in WT, *wrky12* mutant, and *WRKY12*-OE plants under Cd stress. A higher Cd content was observed in roots and shoots of the *wrky12* mutant plants than in the WT ($P < 0.05$; Fig. 4). In contrast, Cd content was reduced in the *WRKY12*-OE lines under Cd stress compared with that in the WT ($P < 0.05$; Fig. 4). These results suggest that the sequestration mechanism may be involved in *WRKY12*-mediated Cd tolerance.

WRKY12 gene negatively regulates Cd tolerance via the GSH-dependent PC synthesis pathway

The GSH-dependent PC synthesis pathway is an important pathway for heavy metal detoxification in plants. Accordingly, we tested whether *WRKY12* regulates Cd tolerance

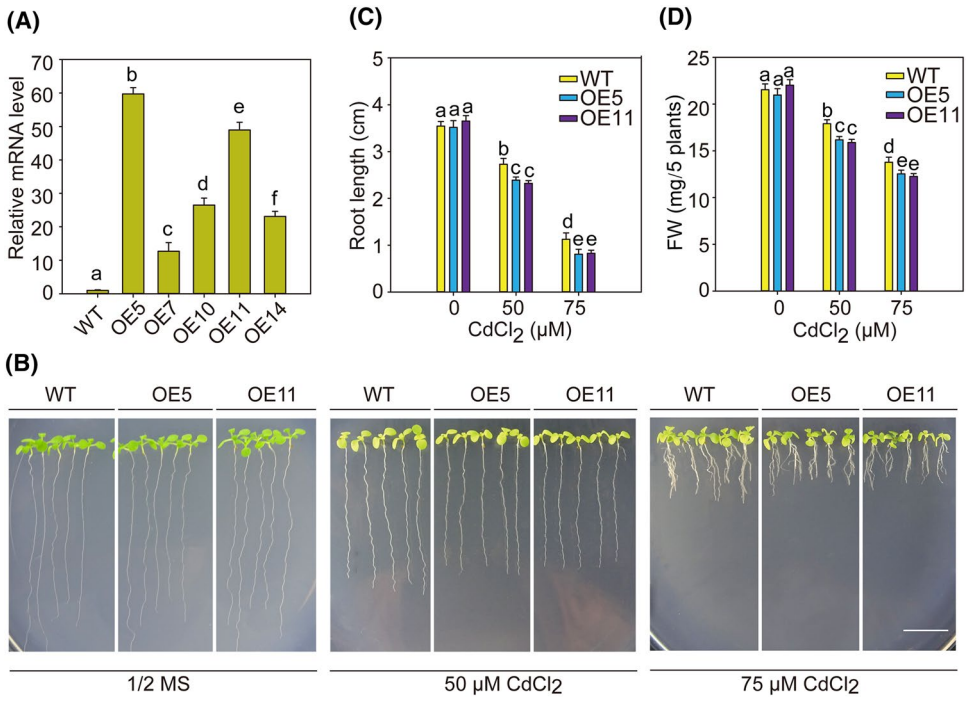

Fig. 3 Overexpression of *WRKY12* leads to sensitivity to Cd stress. **A** qRT-PCR test of *WRKY12* expression in wild-type (WT) and *WRKY12*-overexpressing (OE) lines. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as internal control. Data present the means ± SE ($n = 3$). Bars with different lowercase letters are significantly different at $P < 0.05$ (Tukey's test). **B** Growth of WT and *WRKY12*-OE lines (OE5 and OE9) under Cd stress. Seedlings were grown on half-strength Murashige and Skoog (1/2 MS) medium with 0, 50, or 75 μM $CdCl_2$ for 2 weeks. Bar = 1 cm. **C** and **D** Root length (**C**) and fresh weight (**D**) of plants described in **B**. Data present the means ± SE ($n = 3$). Bars with different lowercase letters are significantly different at $P < 0.05$ (Tukey's test)

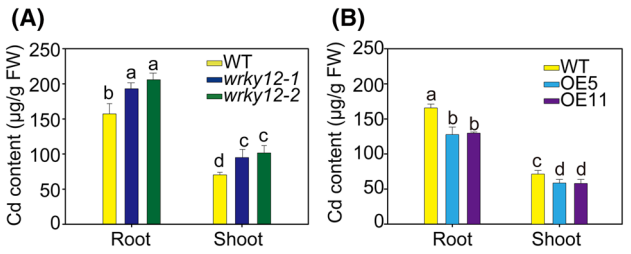

Fig. 4 Cd contents in shoots and roots of wild-type (WT), *wrky12* mutants (**A**), and *WRKY12*-overexpressing (OE) lines (**B**) treated with $CdCl_2$. These plants were grown on half-strength Murashige and Skoog medium containing 50 μM $CdCl_2$ for 2 weeks, and roots and shoots of these samples were collected. Data present the means ± SE ($n = 3$). Bars with different lowercase letters are significantly different at $P < 0.05$ (Tukey's test)

through the GSH-dependent PC synthesis pathway by treating plants with BSO, a GSH synthesis inhibitor. The WT and *wrky12* mutants exhibited a similar growth when grown in 1/2 MS media with or without BSO alone (Fig. 5A, B). When both BSO and Cd were applied to the medium, the tolerant phenotypes of the *wrky12* mutants disappeared (Fig. 5A, B). These results suggest that the accumulation of Cd caused by the knockout of *WRKY12* is GSH-dependent.

Next, we further analysed the levels of GSH and PCs in WT, *wrky12*, and *WRKY12*-OE plants subjected to Cd treatment. No significant difference was detected in total glutathione (GSH plus 2 glutathione disulphide [GSSG]) between the wild type, the *wrky12* mutants, and the *WRKY12*-OE lines without Cd treatment (Fig. 5C). Under Cd stress, *GSH* concentrations decreased significantly in the wild type, *wrky12* mutants, and *WRKY12*-OE plants; however, compared with WT plants, its content was higher in *wrky12* mutants and lower in *WRKY12*-OE lines (Fig. 5C). In addition, the PC content was increased significantly in the *wrky12* mutants and decreased in the *WRKY12*-OE lines compared with the WT (Fig. 5D). These results suggest that *WRKY12* negatively regulates Cd tolerance via the GSH-dependent PC synthesis pathway.

WRKY12 protein represses the expression of the PCs synthesis-related genes

On the basis of the above results, we hypothesized that *WRKY12* regulates the expressions of PC synthesis-related genes. Therefore, the genes *GSH1, GSH2, PCS1*, and *PCS2*, which are involved in this pathway, were analysed (Zhu et al. 1999a, b; Brunetti et al. 2011; Kühnlenz et al. 2014).

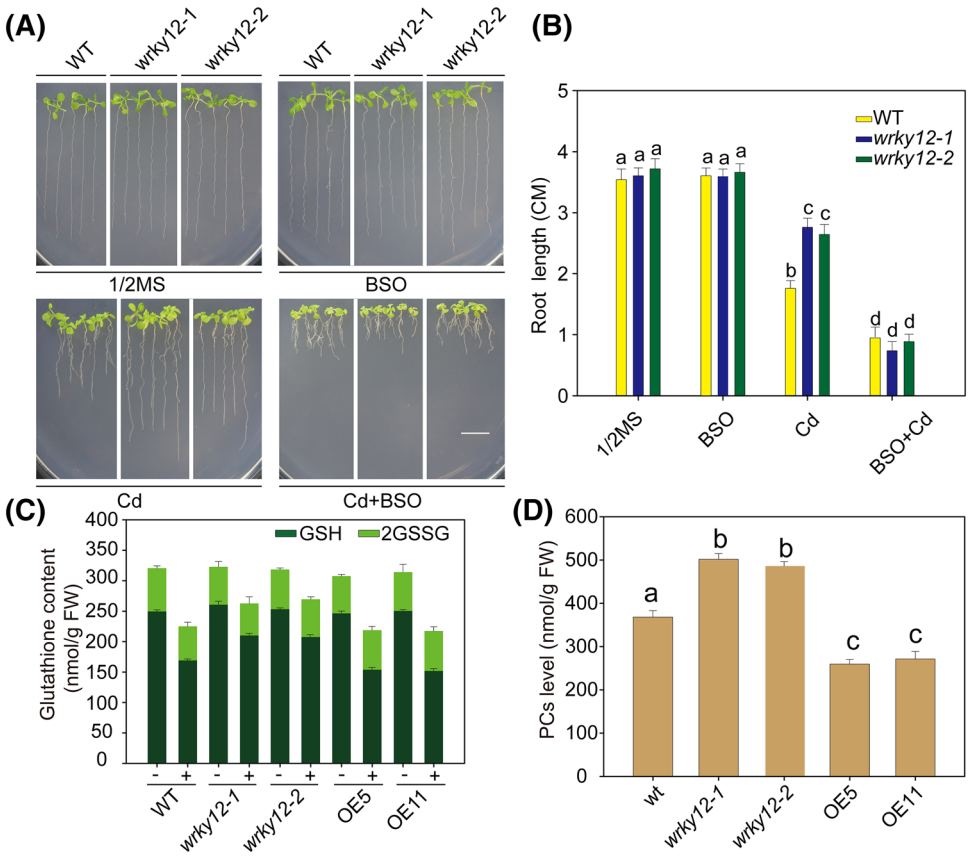

Fig. 5 *WRKY12* mediates Cd tolerance through the glutathione-dependent pathway. **A** Analysis of buthionine sulfoximine (BSO) effect on the growth of wild-type (WT) and *wrky12* mutant plants. Seedlings were grown on half-strength Murashige and Skoog (1/2 MS) medium with no $CdCl_2$, 50 μM $CdCl_2$, or 0.1 mM BSO for 2 weeks. **B** Root length of plants described in **A**. Data are presented as means ± SE. Bars with different lowercase letters are significantly different at $P < 0.05$ (Tukey's test). **C** and **D** Measurements of total glutathione (GSH plus 2 glutathione disulphide [GSSG]) (**C**) and phytochelatin (PC) (**D**) contents in the WT, *wrky12* mutants, and the *WRKY12*-overexpressing (OE) lines. Seedlings were grown on 1/2 MS medium for 2 weeks, and after treating them with 0 or 50 μM $CdCl_2$ for 24 h, their GSH/GSSG (**C**) and PC (**D**) contents were quantified. Data present the means ± SE ($n = 3$). Bars with different lowercase letters are significantly different at $P < 0.05$ (Tukey's test)

Compared with the expressions in the WT, the transcription levels of these genes induced by $CdCl_2$ were significantly increased in the *wrky12* mutants but decreased in the *WRKY12*-OE seedlings (Fig. 6A). These results imply that *WKY12* represses the expressions of the PCs synthesis-related genes. In addition, we also detected the expression pattern of *PDR8*, a gene encoding a Cd extrusion pump conferring Cd tolerance. Our data showed no significant differences in the transcription of *PDR8* between the WT, *wrky12* mutants, and *WRKY12*-OE seedlings (Supplementary Fig. S3).

To further test whether WRKY12 suppresses the transcriptions of *GSH1, GSH2, PCS1*, and *PCS2*, we observed the GUS activity by transient expression analysis in *Nicotiana benthamiana*. As shown in Fig. 6B, C, WRKY12 inhibited the expressions of *ProGSH1:GUS, ProGSH2:GUS, ProPCS1:GUS*, and *ProPCS2:GUS*. These results suggest that WRKY12 has the capacity of inhibiting reporter activity driven by the promoters of *GSH1, GSH2, PCS1*, and *PCS2*.

The WRKY12 gene directly binds to the promoter of GSH1

It is known that WRKY proteins can bind to W-box motifs present in the promoters of target genes (Ulker and Somssich 2004; Jiang et al. 2014). Using promoter sequence analysis, we detected many W-boxes in the promoter regions of *GSH1, GSH2, PCS1*, and *PCS2* (Fig. 7A) and speculated that WRKY12 may directly bind to these boxes and affect their expressions. To test this speculation, we performed ChIP-qPCR assay using seedlings of a transgenic line *WRKY12-GFP*. As shown in Fig. 7B, *WRKY12* could directly bind to the W-box of the promoter in *GSH1* but not in *GSH2, PCS1*, and *PCS2*. These results indicate that *WRKY12* directly regulates the transcription of *GSH1* by

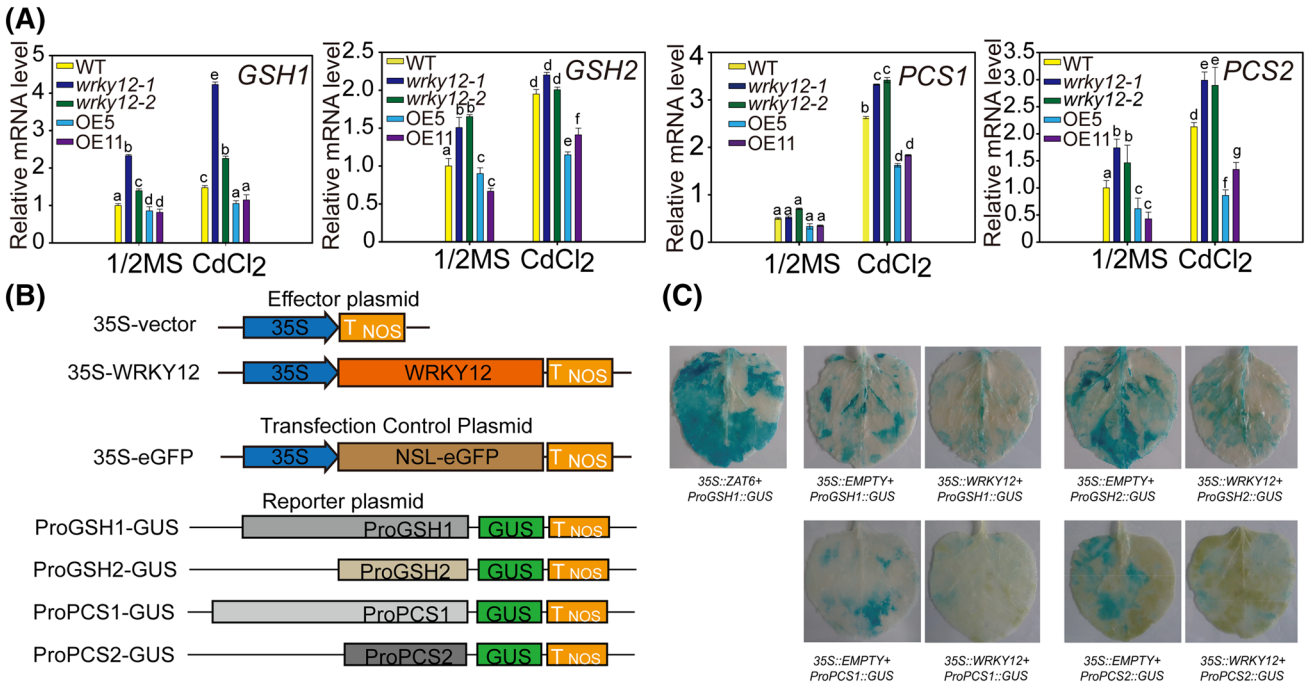

Fig. 6 The expressions of genes in glutathione (GSH)-dependent phytochelatin (PC) synthesis pathway were suppressed by *WRKY12*. **A** The transcriptions of GSH/PC synthetic genes *GSH1, GSH2, PCS1*, and *PCS2* in the wild type (WT), *wrky12* mutant, and *WRKY12*-overexpressing (OE) lines by quantitative analysis. Two-week-old seedlings were treated with 0 or 50 μM $CdCl_2$ for 6 h. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as internal control. All the data shown here are presented as the means ± SE ($n = 3$). Bars with different lowercase letters are significantly different at $P < 0.05$ (Tukey's test). **B** Schematics of all constructs used for transient expression assays in *Nicotiana benthamiana* leaves. The *GSH1, GSH2, PCS1*, or *PCS2* promoter was fused to the *β*-glucuronidase (*GUS*) reporter gene. *35S:WRKY12* acted as an effector. The *35S* promoter was fused to the green fluorescent protein (*GFP*) gene as an internal control. **C** GUS staining showed expressions of *ProGSH1, ProGSH2, ProPCS1*, or *ProPCS2* after co-expression with *WRKY12*. *ProGSH1:GUS, ProGSH2:GUS, ProPCS1:GUS*, or *ProPCS2:GUS* was co-transformed with *35S:WRKY12* into *N. benthamiana*, and the *35S*-empty vector was used as effector plasmid control

binding to its promoter, and indirectly represses the expression of *GSH2, PCS1*, and *PCS2*.

Discussion

The WRKY TF family has been reported to be involved in a variety of heavy metal stresses. For example, *WRKY46* responds to Al stress by negatively regulating *ALMT1* expression, and loss of function of *WRKY46* leads to increased malate secretion and reduced Al accumulation (Ding et al. 2013). The activities of *Zea mays ZmWRKY4* and *Thellungiella salsuginea EsWRKY33* were induced by $CdCl_2$ and $AgNO_3$, respectively (Mucha et al. 2015; Hong et al. 2017). *WRKY53* in *Thlaspi caerulescens* has also been reported to play a potential role in Cd stress response (Wei et al. 2008). *ThWRKY7* can specifically bind to the promoter of *ThVHAc1* and improve Cd stress tolerance by regulating ROS homeostasis in *Tamarix hispida* (Yang et al. 2016). We also reported that *WRKY13* positively regulates Cd tolerance in *Arabidopsis* (Sheng et al. 2018). In the present study, we found that *WRKY12* plays a negative role in regulating Cd stress tolerance and its expression is inhibited by Cd stress. Moreover, the *wrky12* mutants displayed enhanced Cd tolerance; conversely, the *WRKY12*-overexpressing lines showed a phenotype sensitive to Cd stress. These data suggested that *WRKY12* was able to mediate the Cd stress response in *Arabidopsis*. Additionally, no differences were observed between the mutants and the WT under As and Zn stresses, but the tolerance phenotype was observed under Pb stress. One possible explanation is that plant response to Pb stress may also require phytochelatins (Clemens 2006; Verbruggen et al. 2009; Chen et al. 2015).

The GSH-dependent PC synthesis pathway is an important mechanism for chelating and detoxifying Cd in higher plants (Cobbett and Goldsbrough 2002; Lin and Aarts 2012). Under Cd stress, GSH and PC could chelate Cd ions to form Cd-GSH and Cd-PCs complexes, which are then sequestered to the vacuoles (Cobbett and Goldsbrough 2002). Therefore, PCs play a vital role in Cd accumulation and detoxification though PC-conjugated vacuolar sequestration (Clemens 2006). In the present study, when Cd and

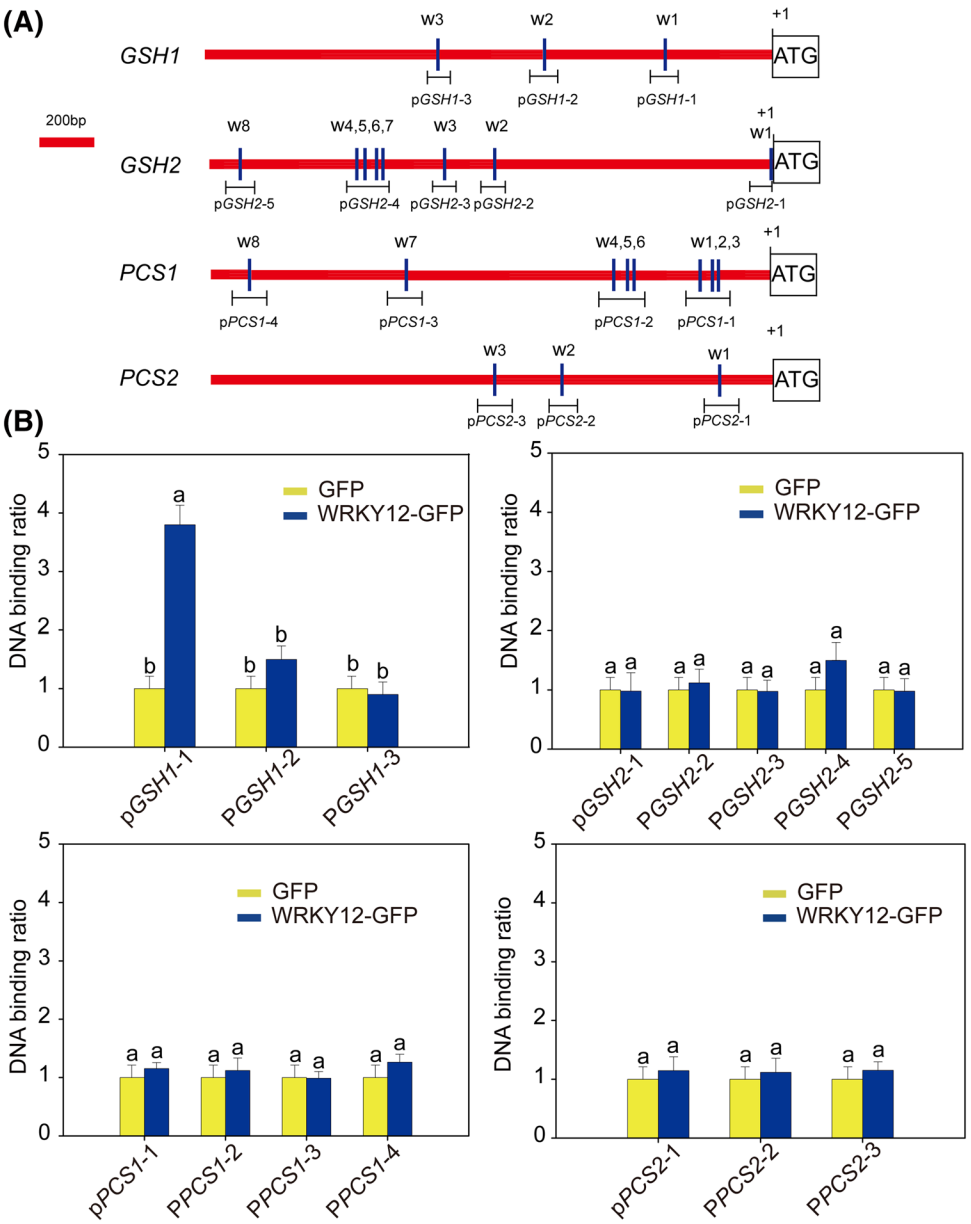

Fig. 7 WRKY12 directly binds to the promoter of *GSH1* in vivo. **A** Schematic diagram of the *GSH1, GSH2, PCS1*, and *PCS2* promoters showing W-box presence in different regions. Bars indicate W-box (TGAC); lines beneath the bars represent the sequences for chromatin immunoprecipitation (ChIP) assays. **B** The direct binding of *WRKY12* to the W-box of the *GSH1, GSH2, PCS1*, and *PCS2* promoters using ChIP-real-time PCR assay. Input DNAs were used as internal control. Data present the means ± SE ($n = 3$). Bars with different lowercase letters are significantly different at $P < 0.05$ (Tukey's test)

BSO co-existed, the growth of the mutants was the same as that of the WT plants. When treated with Cd stress, compared with WT plants, the concentrations of GSH and total PCs were decreased in *WRKY12*-OE plants and increased in the mutants. It is well known that the genes, including *GSH1, GSH2, PCS1*, and *PCS2*, involved in the synthesis of GSH and PCs are closely related with this phenomenon. Overexpression of *GSH1, GSH2, PCS1*, and *PCS2* enhanced Cd tolerance by increasing the contents of GSH and PCs (Zhu et al. 1999a, b; Cazalé and Clemens 2001; Gasic and Korban 2007; Brunetti et al. 2011; Kühnlenz et al. 2014). Co-overexpressing *GSH1* and *PCS1* increased the tolerance and accumulation of Cd in *Arabidopsis thaliana* (Guo et al. 2008). In our study, we found that the transcription levels of these genes were induced in the *wrky12* mutants, while they were the opposite in the *WRKY12*-OE plants. Therefore, *WRKY12*-mediated Cd tolerance is dependent on the synthesis of GSH and PCs, and *WRKY12* is a negative regulator.

The WRKY TFs respond to abiotic and biotic stresses as either positive or negative regulators. They can activate or repress the transcriptions of stress-related and co-regulated genes by directly recognizing and binding to the W-box, which contains a core sequence TGAC present in the promoters of those genes (Eulgem and Somssich 2007). Promoter sequence analysis revealed that W-box is enriched in the promoters of PCs synthesis genes. ChIP-qPCR assays demonstrated that WRKY12 directly bound to the W-box in the promoter of *GSH1*, but not in the promoter of *GSH2,*

PCS1, and *PCS2*. Interestingly, the GUS staining assays showed that WRKY12 repressed the activities of the promoters of *GSH1, GSH2, PCS1*, and *PCS2*. These results suggest that *WRKY12* directly regulates the expression of *GSH1* but indirectly suppresses the expression of *GSH2, PCS1*, and *PCS2*.

Moreover, we further studied the relationship between *WRKY12* and *PDR8* using qRT-PCR. The results showed that *PDR8* was not involved in *WRKY12*-mediated Cd tolerance. Previously, we found that *WRKY13* positively regulates Cd stress by activating the expression of *PDR8* (Sheng et al. 2018). Moreover, we also demonstrated that a GSH-dependent PC synthesis pathway is not involved in the mechanism of WRKY13-mediated Cd tolerance (Sheng et al. 2018). Thus, the opposite role of *WRKY12* and *WRKY13* in the regulation of plant Cd tolerance is though different pathways, although they both belong to the same subgroup within the *WRKY* gene family.

We propose a possible working mode for *WRKY12* in regulating Cd accumulation and tolerance (Fig. 8): *WRKY12* directly targets *GSH1* and indirectly represses the expressions of PC synthesis-related genes to negatively regulate Cd tolerance.

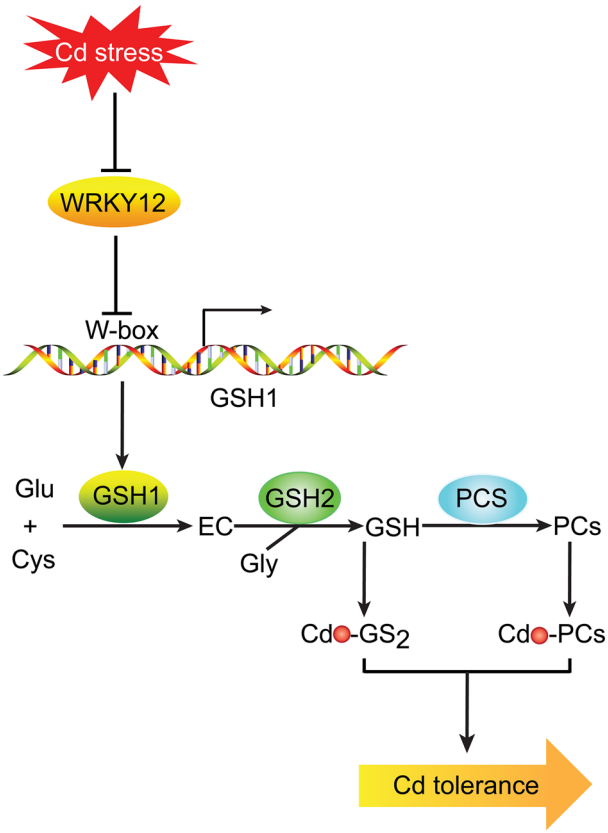

Fig. 8 A working model of the role of *WRKY12* in plant responses to Cd stress. Cd stress inhibits the expression of *WRKY12*, releasing its binding to the promoter of *GSH1* and co-ordinately facilitating phytochelatin (PC) synthesis-related gene expression. This results in increased contents of glutathione (GSH) and PCs, which leads to increased Cd tolerance

Materials and methods

Plant materials and growth condition

All seeds of *Arabidopsis thaliana* (L.) Heynh. (including wild-type [WT] ecotype *Col-0, wrky12* mutants, and transgenic plants) were surface-sterilized and planted on half-strength Murashige–Skoog (1/2 MS) media (Murashige and Skoog 1962) containing 1% sucrose and 1% agar [Sangon Biotech] (pH 5.8). The seeds were vernalized in the dark at 4 °C for 3 days and then grown in a controlled culture room under long day (16/8 h light/dark) at 22 °C (light intensity of 100 μmol m^{-2} s^{-1}).

The T-DNA insertion lines for *WRKY12* (*wrky12-1*:CS435919; *wrky12-2*:CS 374453) were obtained from the *Arabidopsis* Biological Resource Center (ABRC) at Ohio State University, USA. The T-DNA insertion sites were confirmed by PCR amplification using the primers showed in Supplementary Table S1.

Generation of transgenic plants

To generate *WRKY12*-overexpressing (OE) and *WRKY12*-green fluorescent protein (GFP) plants, full-length *WRKY12* cDNA was amplified with specific primers (primers are listed in Supplementary Table S1), cloned into the pCAMBIA1301 or pXB94 (pART27 with expanded restriction sites, 35S promoter, and GFP reporter) at the *Xba*I and *Hind*III restriction sites (named *35S::WRKY12* and *35S::WRKY12::GFP*), and transformed into *Arabidopsis* using the floral dip method (Clough and Bent 1998). The obtained homozygous lines were selected for further study.

Transcript analysis by qRT-PCR

Plant total RNA was extracted from the seedlings, grown as described above using TRIzol reagent (Invitrogen), and the first-stand cDNA was synthesized by a RevertAid™ First Strand cDNA Synthesis Kit (Fermentas). Quantitative real-time PCR was conducted using SYBR Green qPCR SuperMix-UDG (Invitrogen) according to the instructions provided for the Bio-Rad iCycler iQ system. The PCR amplifications for each sample were quantified at least in triplicate and normalized using glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene as an internal control. The primers used are listed in Supplementary Table S1.

Chemical treatments

For Cd tolerance assay, following a 3-day growth, *Arabidopsis* seedlings were transplanted to 1/2 MS medium in the absence or presence of indicated concentrations of $CdCl_2$. The root lengths and FW were measured after 10 days of cultivation. All the tests were conducted in triplicate, and approximately 30 plants were used for each measurement.

To test the function of GSH under *WRKY12*-mediated Cd tolerance, buthionine sulfoximine (BSO, Sigma Aldrich), a GSH synthesis-inhibitor, was used to treat the *wrky12* mutants cultivated on media with or without $CdCl_2$.

Analysis of Cd content

Cd content assay was performed as described by Kim et al. (2006). Briefly, the roots and aboveground parts of the plants (WT, *wrky12* mutants, and *WRKY12*-OE) grown on 1/2 MS medium containing $CdCl_2$ for 2 weeks were harvested apart, digested, and the digested samples were analysed using an atomic absorption spectrometer (Solaar M6; Thermo Fisher).

Measurement of GSH and PC contents

Two-week-old WT, *wrky12* mutant, and *WRKY12*-OE plants were treated or not with $CdCl_2$ for 24 h and then sampled for determination of GSH and PC contents. The specific method was carried out as described previously (Chen et al. 2015).

Cloning and transient expression assay

The full-length promoters of *GSH1, GSH2, PCS1*, and *PCS2* were PCR-amplified from *Arabidopsis* genomic DNA using specific primers (Supplementary Table S1). These promoters were cloned into the vector pXB93 (pART27 with expanded restriction sites and *β*-glucuronidase [GUS] reporter), named *ProGSH1::GUS, ProGSH2::GUS, ProPCS1::GUS*, and *ProPCS2::GUS*. These four constructs were co-transformed into epidermal cells of *Nicotiana benthamiana* with the *35S:WRKY12* construct. Samples were then collected and labelled, and GUS staining was performed as described previously (Xu et al. 2006).

ChIP-qPCR assay

Chromatin immunoprecipitation coupled with real-time PCR (ChIP-qPCR) assays were performed as previously described (Kaufmann et al. 2010) with an anti-GFP antibody (Abmart). In brief, 3 g of 10-day-old *35S::WRKY12:GFP* plants as the experimental group and 35S::GFP plants as the control group were harvested and fixed. The fixed plant tissues were sonicated with ultrasonic cell disruption and divided into three parts: one part was used for input DNA, and the two other parts were incubated with anti-GFP antibody. The relative concentrations of DNA fragments were analysed by qRT-PCR using primers listed in Supplementary Table S1.

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Author Contributions SC conceived the original research plans; YH, XZ, XW and JO performed the experiments; SC, YH, and TF and LJ designed the experiments and analysed the data; TF and SC wrote the article with contributions of all the authors.

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