A feedback loop between CaWRKY41 and H2O2 coordinates the response to Ralstonia solanacearum and excess cadmium in pepper

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Abstract

WRKY transcription factors have been implicated in both plant immunity and plant responses to cadmium (Cd); however, the mechanism underlying the crosstalk between these processes is unclear. Here, we characterized the roles of CaWRKY41, a group III WRKY transcription factor, in immunity against the pathogenic bacterium Ralstonia solanacearum and Cd stress responses in pepper (Capsicum annuum). CaWRKY41 was transcriptionally up-regulated in response to Cd exposure, R. solanacearum inoculation, and H2O2 treatment. Virus-induced silencing of CaWRKY41 increased Cd tolerance and R. solanacearum susceptibility, while heterologous overexpression of CaWRKY41 in Arabidopsis impaired Cd tolerance, and enhanced Cd and zinc (Zn) uptake and H2O2 accumulation. Genes encoding reactive oxygen species-scavenging enzymes were down-regulated in CaWRKY41-overexpressing Arabidopsis plants, whereas genes encoding Zn transporters and enzymes involved in H2O2 production were up-regulated. Consistent with these findings, the ocp3 (overexpressor of cationic peroxidase 3) mutant, which has elevated H2O2 levels, displayed enhanced sensitivity to Cd stress. These results suggest that a positive feedback loop between H2O2 accumulation and CaWRKY41 up-regulation coordinates the responses of pepper to R. solanacearum inoculation and Cd exposure. This mechanism might reduce Cd tolerance by increasing Cd uptake via Zn transporters, while enhancing resistance to R. solanacearum.

Keywords: Capsicum annuum, CaWRKY41, cadmium, H2O2, Ralstonia solanacearum, reactive oxygen species.

Introduction

Plants are frequently exposed to various biotic and abiotic stresses in their natural habitats. A variety of defense response mechanisms have evolved that protect the plant against particular stresses. These mechanisms are mediated by complex signaling pathways, which must be coordinately and tightly regulated. Common signaling pathways such as MAPK cascades...
(Rodriguez et al., 2010; Meng and Zhang, 2013) and pathways involving calcium (Knight, 2000; Bose et al., 2011) and reactive oxygen species (ROS; Qi et al., 2017) are ubiquitously involved in plant responses to various biotic or abiotic stresses, suggesting that they coordinate these responses. However, the exact roles of most of these signaling components and how they are functionally linked are poorly understood.

ROS, including the superoxide radical \( \text{O}_2^\cdot \), hydrogen peroxide \( \text{H}_2\text{O}_2 \), hydroxyl radical \( \cdot \text{OH} \), and singlet oxygen \( \text{O}_2(\cdot) \), are partially reduced forms of molecular oxygen \( \text{O}_2 \) that typically result from the transfer of one, two, or three electrons to \( \text{O}_2 \). \( \text{H}_2\text{O}_2 \) is the most stable ROS, with a relatively long half-life (~1 ms in the cell), and often acts as an intracellular and intercellular signal that triggers downstream responses (Baxter et al., 2014; Camejo et al., 2016). ROS homeostasis is modulated by various enzymes; ROS production in multiple subcellular locations is associated with the activities of NADPH oxidases [or respiratory burst oxidase homologs (RBOHs)], glycylated oxidases, and peroxidases (Mittler, 2002; Suzuki et al., 2011; Marino et al., 2012; Gupta et al., 2017). ROS are scavenged by the antioxidant system, including non-enzymatic antioxidants such as ascorbic acid and glutathione, and several antioxidant enzymes, such as catalase (CAT), ascorbate peroxidase (APX), monodehydroascorbate reductase, dehydroascorbate reductase, glutathione reductase, glutathione peroxidase, and glutathione-S-transferase (Romero-Puertas et al., 2007; Dinakar et al., 2010). The production and decomposition of ROS are balanced under non-stress conditions. However, under various environmental stress conditions, this balance frequently breaks down, resulting in a burst of ROS (Lv et al., 2017). Although excess ROS cause oxidative injury, these molecules also act as second messengers that regulate physiological and developmental processes in plants under both stress and non-stress conditions (Apel and Hirt, 2004; Baxter et al., 2014; Qi et al., 2017).

Accumulating evidence indicates that ROS bursts are crucial regulators of plant immunity (Torres et al., 2006; Mersmann et al., 2010; Vellosoillo et al., 2010). The perception of pathogen-associated molecular patterns by pattern recognition receptors, and of specific pathogen effectors (either directly or indirectly) by specific nucleotide-binding leucine-rich repeat receptors, triggers ROS bursts in the plant through the activation of RBOHs and peroxidases (Schwizer et al., 2017). ROS bursts are thought to reinforce the cell wall around points of infection and activate downstream responses including defense gene expression, the production of antimicrobial compounds, and the hypersensitive response (Alvarez et al., 1998; Torres et al., 2006). Virulent pathogens possess effectors that are capable of suppressing ROS bursts in various ways and thereby suppressing downstream immune responses during infection (Shidore et al., 2017). Thus, ROS may act as overlapping components in pathogen-associated molecular pattern-triggered immunity and effector-triggered immunity, and serve as crucial nodes connecting these processes (Tsuda and Katagiri, 2010; Adachi et al., 2015).

ROS bursts are also a primary effect of exposure to excess cadmium (Cd). This element, which is released into the agricultural ecological system as a result of urbanization and industrialization, is considered to be one of the most toxic heavy metals in the environment (Gupta et al., 2017). Cd is thought to induce the formation of ROS indirectly by inhibiting the activity of antioxidant enzymes, impairing the respiratory chain, or displacing copper and iron ions from metalloenzymes and interfering with the redox status of the cell (Valko et al., 2005). ROS production in response to Cd exposure may cause oxidative injury to plants, but the exact roles of ROS in the plant response to Cd exposure are poorly understood. As ROS are associated with the plant response to pathogen infection and Cd toxicity, these processes are thought to be linked via ROS. Indeed, treatment with salicylic acid (SA), a defense-signaling molecule, alleviates Cd toxicity in barley (Hordeum vulgare) seedlings (Metrally et al., 2003). Moreover, Cd concentrations close to the toxicity threshold induce defense-signaling pathways mediated by SA and jasmonic acid (Cabot et al., 2013). However, the exact roles of ROS in plant responses to Cd tolerance and whether and how plant immunity and responses to Cd stress are coordinated by ROS, remain to be elucidated.

A key step in plant responses to diverse stresses is the transcriptional reprogramming of a multitude of defense-associated genes by various transcription factors (TFs). WRKY proteins, which are characterized by the presence of one or two highly conserved WRKY domains, constitute one of the largest TF families. WRKY TFs are important positive and negative regulators of plant growth and development, and of defense responses to environmental stimuli (Eulgem et al., 2000; Rushton et al., 2010). While this large family of TFs is mainly involved in regulating plant immune responses (Sarris et al., 2015), a few WRKY members, including Tamaria hispida WRKY7 (Yang et al., 2016) and Zea mays WRKY4 (Hong et al., 2017), have been implicated in plant responses to Cd toxicity. In addition, some WRKY TFs are involved in more than one biological process, suggesting that WRKYs are crucial nodes in the crosstalk between plant immunity and other biological processes (Rushton et al., 2010). Moreover, the expression of most group III WRKY genes is modified in response to pathogen attack and treatment with SA (Kalde et al., 2003). As recent studies have shown that group III WRKY genes play important roles in plant responses to abiotic stress (Li et al., 2013; Ding et al., 2014; Chen et al., 2017), we reasoned that these genes might be involved in the crosstalk between plant responses to pathogen attack and abiotic stress, possibly coordinating plant responses to these stresses.

Pepper (Capsicum annuum) is a solanaceous vegetable crop widely grown around the world. Blight and bacterial wilt caused by the soil-borne pathogens Phytophthora capsici and Ralstonia solanacearum, respectively, frequently reduce pepper production. Heavy metal contamination is another factor that inhibits pepper growth. Heavy metal residues are present in soils as a result of sewage irrigation and the use of heavy-metal-containing products such as pesticides and fertilizers. A better understanding of how pepper responds to heavy metal contamination would lay the foundations for developing effective countermeasures.

In the present study, we investigated the transcriptional responses of group III WRKYs to Cd toxicity and R. solanacearum inoculation. We also investigated the responses of these...
genes to iron (Fe) deficiency, because Cd toxicity-induced chlorosis resembles Fe deficiency-induced chlorosis (Sun et al., 2015; Chen et al., 2016; Li et al., 2016), and plant responses to Fe deficiency are related to responses to excess Cd (Nakanishi et al., 2006; Han et al., 2014; Mendoza-Cozatl et al., 2014). Among the eight group III WRKY genes we examined, only CaWRKY41 was synergistically up-regulated in pepper plants challenged by Cd toxicity, Fe deficiency, or R. solanacearum inoculation. We identified a positive feedback loop between CaWRKY41 and H₂O₂ accumulation during the response to R. solanacearum inoculation and excess Cd exposure in pepper.

**Materials and methods**

**Plant materials and growth conditions**

Seeds of pepper (Capsicum annuum) 8# (an inbred line provided by the pepper breeding group at Fujian Agriculture and Forestry University) and CM334 (Mexican landrace of C. annuum cv. CM334), and tobacco (Nicotiana benthamiana) were imbibed in sterile water at 25±2 °C overnight and sown in a steam-sterilized soil mix (peat moss, vermiculite, and perlite; 2:1:1 by volume) in plastic pots. Pepper plants were grown in a growth room maintained at 25±2 °C with a light intensity of ~100 µmol photons m⁻² s⁻¹ and a relative humidity of 70%, under a ½ Murashige and Skoog (MS) medium (PhytoTechnology, product ID for 3 days and then sown on vertically placed Petri dishes containing genic Arabidopsis seeds were treated by exposure to 4 °C in darkness for 3 days and then sown on vertically placed Petri dishes containing modified one-fifth Hoagland solution. The initial nutrient solution contained the macronutrients KNO₃ (1 mM), Ca(NO₃)₂·4H₂O (1 mM), MgSO₄·7H₂O (1.4 mM), and KH₂PO₄ (0.2 mM), and the micronutrients Fe-EDTA (20 µM), H₃BO₃ (3 µM), (NH₄)₆Mo₇O₂₄ (1 µM), MnCl₂ (0.5 µM), ZnSO₄ (0.4 µM), and CuSO₄ (0.2 µM). The pH of the solution was adjusted to 5.8, and the nutrient solution was renewed every 3 days.

For Arabidopsis thaliana cultivation, wild-type (WT; Col-0), ep3 (Coego et al., 2005), CaWRKY41-OE1, and CaWRKY41-OE4 transgenic Arabidopsis seeds were treated by exposure to 4 °C in darkness for 3 days and then sown on vertically placed Petri dishes containing ½ Murashige and Skoog (MS) medium (PhytoTechnology, product ID M524) supplemented with 1% (w/v) sucrose and 0.8% agar (Sigma, cat. no. A1296) in the absence or presence of heavy metals or other supplements in a growth chamber maintained at 22±2 °C with a light intensity of ~100 µmol photons m⁻² s⁻¹ and a relative humidity of 70%, under a 16 h light/8 h dark cycle.

**Phylogenetic analysis of group III WRKY TFs across three plant species**

The WRKY TFs were described previously (Eulgem et al., 2000). The amino acid sequences of proteins and domains of group III CaWRKYs, SiWRKYs, and AtWRKYs from the C. annuum, Solanum lycopersicum, and A. thaliana genomes were downloaded from Plant TFDB V4.0 (http://planttfdb.cbi.pku.edu.cn/index.php).

**Pathogens and inoculation procedures**

Ralstonia solanacearum strain FJ150501 was isolated from pepper plants showing symptoms of bacterial wilt infection in Guangdong Province, China. For soil-drenching inoculation, PYL-279 and PYL-279-wrky41 pepper plants grown in pots, with the roots partially and mechanically damaged, were inoculated with a 10⁸ cfu/ml (OD₆₀₀=0.8) suspension of R. solanacearum. A disease index (from 0 to 5) was scored daily in the R. solanacearum-inoculated pepper plants, as follows: 0 (no wilting), 1 (1 to 20% wilting), 2 (21 to 40% wilting), 3 (41 to 60% wilting), 4 (61 to 80% wilting), and 5 (81 to 100% wilted or dead). The average values reported are based on three independent replicates, each comprising six plants. Electrolyte leakage was measured in pepper leaves at 0, 24, and 48 h post inoculation. For suspension inoculation, pepper plants were grown in 1.2 l black plastic beakers containing one-fifth Hoagland solution. Eight of the lateral roots were removed from each plant with a pair of scissors, and the plants were then transferred to one-fifth Hoagland solution with 10³ cfu/ml R. solanacearum suspension.

**Plasmid construction and plant transformation**

To construct the vector p35S::CaWRKY41, the full-length open reading frame was cloned into pDONR207 and transferred into the pGWB2 expression vector (Invitrogen, USA). To construct the reporter vector (pCaWRKY41::GUS) for histochemical β-glucuronidase (GUS) analysis, the promoter of CaWRKY41 was amplified via PCR from pepper genomic DNA and cloned into the pMDC163 vector (Invitrogen). The constructs 35S::CaWRKY41 and pCaWRKY41::GUS were transformed into Agrobacterium tumefaciens strain GV3101 using the freeze-thaw method. A. tumefaciens-mediated transformation of Arabidopsis was performed using the floral dip method (Clough and Bent, 1998), and transgenic plants were identified by sowing seeds on ½ MS agar plates containing 50 mg l⁻¹ hygromycin and selecting hygromycin-resistant seedlings.

**Subcellular localization and transcriptional activity analysis**

The coding region of CaWRKY41 without the stop codon was cloned into the pCambia1300-GFP/C vector by In-Fusion Cloning (Clontech, USA). The pCambia1300-CaWRKY41-GFP construct was transformed into A. tumefaciens GV3101 and infiltrated into the fully expanded leaves of 5-week-old N. benthamiana plants. At 2 days post inoculation, green fluorescent protein (GFP) fluorescence was observed by confocal laser-scanning microscopy (Zeiss LSM710, Germany). For the transactivation assay, the open reading frames of CaWRKY41 (1–329) and the mutant genes CaWRKY41 (61–329), CaWRKY41 (131–329), and CaWRKY41 (192–329) were generated by PCR with specific primer pairs and cloned into pGBK77 (Clontech) to generate various CaWRKY41 constructs (BD–CaWRKY41–1,–2 and –3). Transcriptional activation activity was determined in yeast cells transformed with these constructs grown on SD medium lacking Trp for 3 days, and a colony—luciferase filter assay (X-gal assay) was performed.

**Virus-induced gene silencing**

CaWRKY41-silenced pepper plants were generated using tobacco rattle virus-based gene silencing (VIGS) as described previously (Dang et al., 2013). Briefly, a specific 328 bp fragment of CaWRKY41 was identified by homologous searching via BLAST analysis against the genome sequences of pepper cultivars CM334 (http://peppergenome.snu.ac.kr/) and Zunla-1 (http://peppersequence.genomics.cn/page/species/blat.jsp). The fragment was cloned into the entry vector pDONR207 and then into the PLY279 vector. The vectors (PLY-279 and PYL-279-wrky41) were separately transformed into A. tumefaciens GV3101 cells, which were subsequently mixed with A. tumefaciens cells harboring PYL-192 and injected into fully expanded pepper seedling cotyledons. PYL-279-wrky41 pepper plants were subjected to experimental analysis, with PYL-279 plants (transformed with empty vector) serving as a control. Levels of H₂O₂ and of the expression of various genes were measured in CaWRKY41-silenced PLY-279 and PYL-279-wrky41 pepper plants grown in liquid culture.

**Treatment of plants with Cd and exogenous application of H₂O₂**

To test the effect of Cd on seed germination and growth in Arabidopsis, seeds were treated by exposure to 4 °C in darkness for 3 days and then grown on ½ MS medium containing 25 µM, 50 µM, or 100 µM CdSO₄ for 8 days. To measure the expression of various genes in plants in the presence of excess Cd supply, 7-day-old Arabidopsis seedlings were transferred to ½ MS medium without or with 25 µM CdSO₄, cultured for 6 or 72 h, and harvested for use. To investigate the expression of the eight CaWRKY group III genes, pepper plants at the six-leaf stage grown in liquid culture
were treated with Cd stress (2.5, 5, 25, and 60 μM CdSO₄) and Fe deficiency (0 μM Fe-EDTA). Pepper plants at the six-leaf stage were sprayed with H₂O₂ (1 mM) and incubated for 0, 1, 3, 6, 12, 24, 36, and 48 h, and leaf tissue was harvested for CaWRKY expression analysis.

Histochemical staining

Leaves were stained with Trypan blue and 3, 3′-diaminobenzidine (DAB) as described previously (Dang et al., 2013, 2014; Cai et al., 2015). For GUS staining, seedlings or tissues were incubated overnight in GUS staining solution (1 mg ml⁻¹ X-Gluc, 1 mM K₃Fe(CN)₆, 1 mM K₄Fe(CN)₆, 50 mM sodium phosphate buffer pH 7.0, 10 mM Na₂EDTA, and 0.1% Triton X–100) at 37 °C, destained several times in 75% (v/v) ethanol, and observed under a stereomicroscope (Leica, Germany).

Measurement of H₂O₂ and Cd contents and enzyme activity

Seedlings were grown on ½ MS medium for 7 days, treated with 25 μM CdSO₄ for 3 and 5 days, and sampled for H₂O₂ and Cd analysis and enzymatic assays. For H₂O₂ measurements, seedlings were harvested, ground in liquid nitrogen, and examined using an Amplex Red H₂O₂-peroxidase Assay Kit (Molecular Probes). This one-step assay uses Amplex Red reagent (10-acyetyl-3,7-dihydroxyphenoxazine) to detect H₂O₂. Briefly, approximately 80 mg of sample was processed and measured using an H₂O₂ standard curve. The fluorescence emission spectrum (590 nm) was detected at an excitation wavelength of 530 nm using a Tecan Infinite 200 Pro (Tecan, Switzerland).

To measure the Cd contents in roots and shoots, the roots were rinsed three times (for 4 min each time) with Milli-Q water to remove Cd attached to the root surface. The root and shoot samples were weighed and digested with 0.5 ml (for roots) and 1 ml (for shoots) concentrated HNO₃. Each sample was adjusted to 10 ml with Milli-Q water and then filtered through filter paper. Cd in the samples was detected by inductively coupled plasma-atomic emission spectrometry (IRIS/AP Optical Emission Spectrometer, Thermo Scientific, USA). The experiment was performed in three biological replicates.

For enzymatic activity analysis, approximately 80 mg of sample was ground in liquid nitrogen using a TissueLyser II, and milled samples were homogenized in phosphate buffer (600 μl, 50 mM, pH 7.0) and centrifuged at 3000 × g at 4 °C for 10 min. Then, peroxidase (POD), CAT, and APX activity were analyzed using an ELISA kit (Shanghai Bangyi Biotechnology Co. Ltd, China) according to the manufacturer’s instructions. Microtiter plate wells were coated with purified POD, CAT, and APX antibody, to make a solid-phase antibody, and then samples were added to the wells together with an antibody labeled with horseradish peroxidase, and an antibody–antigen–enzyme complex formed. Substrate solution was added after thorough washing, and then, using a blank well as the zero control, the absorbance was measured at 450 nm in a Tecan Infinite 200 Pro Plate Reader (Tecan).

RNA extraction and reverse transcription–quantitative PCR (RT–qPCR)

Total RNA was isolated from Arabidopsis and pepper tissues using a TaKaRa MiniBEST Universal RNA Extraction Kit (TaKaRa, Dalian, China). RNA (1 μg) was used as a template to synthesize cDNA with a TaKaRa PrimeScript RT-PCR Kit (TaKaRa) according to the manufacturer’s instructions. Gene expression levels were measured on a CFX96 Real-Time PCR System (Bio-Rad, USA) using SYBR® Premix Ex Taq™ II (TaKaRa); specific primers are listed in Supplementary Table S1 at JXB online. Arabidopsis UBQUITIN10 (AtUBQ10) and pepper Actin1 (CaActin1) were used for normalization.

Results

Phylogenetic analysis of CaWRKY group III genes

To identify the phylogenetic relationships among the eight CaWRKY group III genes, we compared their nucleotide sequences to those of WRKY genes from tomato and Arabidopsis. We constructed an unrooted phylogenetic tree based on an alignment of the amino acid sequences of the group III WRKY proteins and domains from the three plant species using the neighbor-joining method. Based on this analysis, WRKYs from pepper share higher sequence similarity with WRKYs from tomato than with those from Arabidopsis (see Supplementary Fig. S1). Detailed information about the CaWRKY group III genes is provided in Supplementary Tables S2 and S3.

Expression analysis of eight CaWRKY group III genes during exposure to excess Cd or Fe deficiency

Cd is a highly toxic heavy metal that is readily absorbed by plant roots, loaded into the xylem, and transported to leaves, leading to the generation of ROS (Valko et al., 2005; Perez-Chaca et al., 2014; Keunen et al., 2015). ROS production has been detected in sunflower (Helianthus annuus L.) and maize (Z. mays) under conditions of Fe deficiency (Ranieri et al., 2001; Sun et al., 2007).

In the present study, H₂O₂ accumulation was detected in DAB-stained pepper leaves after 24, 36, and 48 h of Cd stress and Fe deficiency treatments (see Supplementary Fig. S2A, B). Similar to the response to Cd stress, the newly emerged leaves of pepper plants at the eight-leaf stage displayed yellowing after Fe deficiency treatment (Supplementary Fig. S2C, D). To identify the group III WRKY TFs involved in Cd stress, we measured the expression of the eight group III WRKY genes by RT–qPCR analysis in pepper plants exposed to Cd stress or Fe deficiency. CaWRKY41 and CaWRKY53a expression significantly increased under Cd stress in both the roots and leaves of pepper plants (Fig. 1A). Furthermore, CaWRKY41, CaWRKY53, and CaWRKY54 expression markedly increased under Fe deficiency treatment in both roots and leaves (Fig. 1). Therefore, among the eight group III WRKY genes in pepper, only CaWRKY41 expression was up-regulated by both Cd toxicity and Fe deficiency in roots and leaves, pointing to the involvement of CaWRKY41 in the response of pepper to excess Cd and Fe deficiency, which might be associated with the production of H₂O₂.

CaWRKY41 is up-regulated in response to Cd and H₂O₂

To further investigate the involvement of CaWRKY41 in the response of pepper to Cd toxicity, we measured the time course and dose-responsive patterns of CaWRKY41 expression in response to Cd stress by RT–qPCR analysis. After exposure to excess Cd, CaWRKY41 expression was strongly enhanced, peaking at 12 h post treatment (HPT) in the leaves and 1 HPT in the roots (Fig. 2A, B). CaWRKY41 expression was also increased in response to treatment with 2.5, 5, and 60 μM Cd compared with the control (Fig. 2C). Additionally, CaWRKY41 expression was significantly up-regulated in response to exogenous application of H₂O₂ (Fig. 2D). However, the CaWRKY41 expression in pepper leaves triggered by excess Cd was reduced when samples were treated with the H₂O₂ scavenger ascorbic acid (Fig. 2E, Supplementary Fig. S2E).
To confirm the expression pattern of CaWRKY41, we generated pCaWRKY41::GUS transgenic Arabidopsis plants. Seven-day-old pCaWRKY41::GUS seedlings were transferred to ½ MS medium without or with excess Cd for 12 h and then stained to analyze GUS activity. When pCaWRKY41::GUS seedlings were transferred to conditions of excess Cd, increased GUS activity was observed in the shoot and root (Fig. 2F, G). When pCaWRKY41::GUS seedlings were grown under normal conditions, GUS staining was consistently detected in the roots, shoots, mature leaves, and petioles (Supplementary Fig. S3A–G). Intensive GUS staining was also observed in the flowers (Supplementary Fig. S3H, I) but not in the siliques (Supplementary Fig. S3J). These results imply that CaWRKY41 might be involved in the response of pepper to excess Cd and H2O2 accumulation.

Analysis of the subcellular localization and transcriptional activity of CaWRKY41

As the function of a given protein is closely related to its subcellular localization, we investigated the subcellular localization of CaWRKY41 in transiently transformed N. benthamiana leaves harboring the open reading frame of this gene, without the translation terminator, driven by the 35S promoter and fused to the GFP gene. The CaWRKY41-GFP fusion protein was exclusively localized to the nuclei of epidermal cells when heterologously expressed in N. benthamiana (Supplementary Figs S3K and S4A).

In addition, we assayed the transcriptional activity of CaWRKY41 in yeast via a transcriptional activation assay. The expression of the LacZ reporter gene driven by the GAL4 upstream activating sequence was significantly increased by the presence of the BD-CaWRKY41 fusion protein in yeast, but LacZ expression was not induced in the negative control (Supplementary Fig. S4B). These results indicate that CaWRKY41 is a nuclear protein with transcriptional activity.

CaWRKY41 silencing increases Cd tolerance and reduces H2O2 accumulation in pepper

The induction of CaWRKY41 expression by excess Cd points to its involvement in the Cd stress response. To test this possibility, we examined the effect of VIGS of CaWRKY41 on the response of pepper to Cd stress. To avoid possible off-target silencing, we inserted a specific 328 bp fragment of CaWRKY41 into the PYL-279-wrky41 vector [tobacco rattle virus (PYL-279); wrky41] to silence CaWRKY41 in pepper. CaWRKY41 was expressed at a level approximately 3.8- and 3.2-fold lower in CaWRKY41-silenced plants than in control plants (PYL-279), in the presence and absence of Cd stress, respectively (Fig. 3A), respectively, indicating that we had successfully silenced CaWRKY41 via VIGS.

Upon exposure to Cd stress, PYL-279-wrky41 plants and detached leaves consistently exhibited attenuated Cd stress-induced chlorosis compared with controls (Fig. 3B–D). CaWRKY41-silenced leaves also accumulated less H2O2 than control leaves under Cd stress (Fig. 3E). Consistently, genes encoding antioxidant enzymes, including CAT (CaCAT1), superoxide dismutase (CaSOD1), copper zinc superoxide dismutase (CaCSD2), and APX (CaAPX1 and CaAPX2), were up-regulated at 24 HPT with Cd stress in the youngest leaves of PYL-279-wrky41 plants compared with the control. However, no difference in the expression of these genes was detected between the youngest leaves of PYL-279 and PYL-279-wrky41 under normal growth conditions (Fig. 4). These results suggest that CaWRKY41 negatively regulates Cd tolerance, likely by mediating the accumulation of H2O2 through the transcriptional regulation of antioxidant genes.

Overexpression of CaWRKY41 increases sensitivity to Cd in Arabidopsis in an H2O2-dependent manner

To confirm the results of the CaWRKY41-silencing experiments, we performed a gain-of-function analysis by ectopically overexpressing CaWRKY41 in Arabidopsis. None of the seven CaWRKY41-overexpressing T1 homozygous transgenic Arabidopsis lines exhibited significant differences in seed germination, seedling growth, or development compared with WT plants under normal conditions (Supplementary Fig. S4C, D), although, as expected, CaWRKY41-overexpressing plants exhibited high expression of CaWRKY41, as revealed by semi-quantitative PCR (Supplementary Fig. S4E). We randomly selected two independent overexpressing lines
(CaWRKY41-OE1 and CaWRKY41-OE4) for further analysis. These CaWRKY41-OE lines were more sensitive than the WT to Cd stress (Fig. 5A–D), and had lower fresh weights and shorter roots (Fig. 5E, F).

Next, we compared the growth status of CaWRKY41-OE1 and OE4 plants with that of WT plants exposed to excess Cd, or to no Cd, via rapid noninvasive chlorophyll fluorescence imaging. Under normal conditions, there was no marked difference in the fluorescence characteristics of WT and CaWRKY41-OE plants (Supplementary Fig.S5A, C, E); however, under Cd stress, CaWRKY41-OE1 and OE4 plants exhibited lower chlorophyll fluorescence parameters than WT plants (Supplementary Fig. S5 B, D, F). Furthermore, higher Cd (Fig. 5G) and zinc (Zn) (Supplementary Fig. S6A, B) contents were detected in both the roots and shoots of CaWRKY41-overexpressing plants (OE1 and OE4) than in those of the WT after 3 or 5 days of Cd treatment. By contrast, the Fe contents in roots and shoots were similar in CaWRKY41 and WT plants (Supplementary Fig. S6C, D). Additionally, the CaWRKY41-OE lines were more sensitive than the WT plants to excess Zn (Supplementary Fig. S6 E, F).

The reduced accumulation of H$_2$O$_2$ in CaWRKY41-silenced leaves compared with control plants under Cd stress suggests that H$_2$O$_2$ might be involved in CaWRKY41-mediated responses.
CaWRKY41 and H₂O₂ alter R. solanacearum and Cd tolerance

Fig. 3. CaWRKY41 silencing enhances tolerance to Cd stress in pepper. (A) CaWRKY41 expression in PYL-279 (control) and PYL-279-wrky41 pepper leaves. (B) Less yellowing was observed in PYL-279-wrky41 compared with PYL-279 pepper leaves. Pepper plants were grown in one-fifth Hoagland solution. When photobleaching was observed in PYL-279-pds leaves, PYL-279-wrky41 and PYL-279 plants were transferred to fresh nutrient solution containing 50 µM CdSO₄ for 4 days. (C, D) Leaves from PYL-279 and PYL-279-wrky41 cultured on 1/5 MS medium without (C) or with (D) 25 µM CdSO₄ for 4 days. (E) H₂O₂ production observed after 3, 3′-diaminobenzidine staining in leaves of PYL-279 and PYL-279-wrky41 plants at 3 days post treatment with 25 µM CdSO₄. (This figure is available in colour at JXB online.)

to Cd in pepper. To investigate this possibility, we analyzed the effect of CaWRKY41 overexpression on H₂O₂ accumulation in Arabidopsis plants subjected to Cd stress. H₂O₂ levels were higher in the leaves of CaWRKY41-overexpressing lines (OE1 and OE4) than in those of the WT, as revealed by DAB staining and direct H₂O₂ measurements (Fig. 6A, B). Accordingly, the activities of the ROS-scavenging enzymes POD, CAT, and APX were reduced in OE1 and OE4 plants compared with WT plants (Fig. 6C–E). By contrast, higher expression of genes associated with ROS production, such as AtRBOHC (Macho et al., 2012), AtRBOHD (Li et al., 2014; Kadota et al., 2015), AtRBOHE, and AtRBOHF (Chaouch et al., 2012) (Fig. 7A–D), and lower expression of the ROS-scavenging enzyme genes AtCAT1, AtAPX1, AtSOD1, AtSOD2, and AtGST2 (Fig. 7E–I), were detected in OE1 and OE4 plants compared with WT plants at 6 and 72 HPT with Cd. These results suggest that the enhanced accumulation of ROS including H₂O₂ in response to CaWRKY41 overexpression might be due to enhanced ROS production and reduced ROS scavenging, and that elevated H₂O₂ levels might contribute to Cd sensitivity in pepper plants.

To test this possibility, we examined whether there was a relationship between H₂O₂ accumulation and Cd sensitivity in the A. thaliana ocp3 (overexpressor of cationic peroxidase 3) mutant, which harbors a T-DNA insertion in a homeodomain TF gene involved in increased H₂O₂ production in healthy plants (Coego et al., 2005). Mutant ocp3 plants exhibited shorter primary roots than WT plants under Cd stress (Supplementary Fig. S7), supporting the notion that Cd sensitivity is associated with H₂O₂ accumulation. Collectively, these results suggest that the CaWRKY41-mediated Cd sensitivity observed in transgenic Arabidopsis is caused by H₂O₂ accumulation due to increased H₂O₂ production and reduced H₂O₂ scavenging.

Overexpression of CaWRKY41 increases Cd levels in Arabidopsis by activating Zn transporters

Since we detected higher levels of Cd but not Fe in both the roots and shoots of CaWRKY41-OE plants compared with WT upon excess Cd supply, we reasoned that the enhanced Cd sensitivity in response to CaWRKY41 overexpression might be due to enhanced uptake of Cd. A Cd-specific transporter has not yet been identified, and Cd is thought to be transported by Fe and Zn transporters in plants (Saraswat and Rai, 2011; Barabasz et al., 2016). Therefore, we reasoned that, since Fe levels were not elevated in CaWRKY41-OE Arabidopsis plants...
Fig. 4. Expression of genes encoding ROS-scavenging enzymes determined by RT-qPCR analysis in CaWRKY41-silenced plants 0, 6, and 24 h after treatment with 25 µM CdSO₄. Data represent the mean ±SE of three biological replicates. Asterisks indicate significant differences compared with control plants (Student's t-test; *P<0.05, **P<0.01).

Fig. 5. Overexpression of CaWRKY41 reduces tolerance to Cd stress in transgenic Arabidopsis plants. (A–D) Seedling growth in WT, CaWRKY41-OE1, and CaWRKY41-OE4 lines on ½ MS medium containing (A) 0, (B) 25, (C) 50, and (D) 100 µM CdSO₄. Representative photographs were taken 8 days after germination. (E) Fresh weight and (F) root length in WT, CaWRKY41-OE1, and CaWRKY41-OE4 plants exposed to Cd stress. (G) Cd concentration in the shoots and roots of WT, CaWRKY41-OE1, and CaWRKY41-OE4 plants after 3 and 5 days of treatment. Data represent the mean ±SE of three biological replicates. Different letters indicate significant differences compared with the control (Tukey's test; lowercase letters indicate P<0.05 and uppercase letters indicate P<0.01). (This figure is available in colour at JXB online.)
Fig. 6. \(\text{H}_2\text{O}_2\) accumulation and ROS-scavenging enzymatic activity in response to Cd stress. (A) \(\text{H}_2\text{O}_2\) production observed via 3, 3'-diaminobenzidine staining in leaves of WT, CaWRKY41-OE1, and CaWRKY41-OE4 plants at 24 h post treatment with 50 \(\mu\text{M}\) CdSO\(_4\). CK, control untreated. (B) Seedling \(\text{H}_2\text{O}_2\) content. DPT, days post treatment. (C) Peroxidase (POD) activity. (D) Catalase (CAT) activity. (E) Ascorbate peroxidase (APX) activity. For B–E, 7-day-old WT, CaWRKY41-OE1, and CaWRKY41-OE4 seedlings were transferred to \(\frac{1}{2}\) MS medium without or with 25 \(\mu\text{M}\) CdSO\(_4\) for 3 or 5 days before analysis. Data represent the mean ±SE of three biological replicates. Different letters indicate significant differences compared with the control (Tukey’s test; lowercase letters indicate \(P<0.05\) and uppercase letters indicate \(P<0.01\)). (This figure is available in colour at JXB online.)

Fig. 7. Expression of genes encoding ROS-producing and ROS-scavenging enzymes detected by RT-qPCR analysis in WT, CaWRKY41-OE1, and CaWRKY41-OE4 plants at 0, 6, and 72 h post treatment with Cd. (A–D) Expression of ROS-producing enzyme genes (A) \(\text{AtRBOHC}\), (B) \(\text{AtRBOHD}\), (C) \(\text{AtRBOHE}\), and (D) \(\text{AtRBOHF}\). (E–I) Expression of ROS-scavenging enzyme genes (E) \(\text{AtCAT1}\), (F) \(\text{AtAPX1}\), (G) \(\text{AtSOD1}\), \(\text{AtSOD2}\), and (H) \(\text{AtGST2}\). Data represent the mean ±SE of three biological replicates. Different letters indicate significant differences compared with the control (Tukey’s test; lowercase letters indicate \(P<0.05\) and uppercase letters indicate \(P<0.01\)).
compared with control plants, Cd might enter CaWRKY41-OE Arabidopsis plants via Zn transporters.

To test this hypothesis, we measured the expression of genes encoding Zn transporters, including AtZIP1 (Kawachi et al., 2009), AtZIP3 (Gustin et al., 2009), AtZIP4 (Gustin et al., 2009), AtZIP5 (Gustin et al., 2009), and AtZIP9 (Gustin et al., 2009) in CaWRKY41-OE Arabidopsis plants. Only AtZIP3, AtZIP4, and AtZIP9 (Supplementary Fig. S8 B, C, E), were up-regulated in these plants compared with controls; the expression of the other genes did not differ from those of controls upon exposure to excess Cd. These results suggest that increased Cd uptake might be due at least in part to the enhanced expression of genes encoding Zn transporters.

Silencing of CaWRKY41 confers reduced resistance to R. solanacearum inoculation

Our results indicate that H$_2$O$_2$, which has been implicated in plant immunity (Levine et al., 1994; Alvarez et al., 1998; Torres et al., 2006), is involved in CaWRKY41-mediated Cd sensitivity. NADPH oxidases, which contribute to ROS production, have frequently been shown to be involved in plant immunity (Kadota et al., 2015). Thus, we reasoned that CaWRKY41 might also participate in plant immunity.

To test this possibility, we monitored changes in CaWRKY41 expression in response to R. solanacearum inoculation. CaWRKY41 was strongly induced by R. solanacearum inoculation, with peak expression detected at 6 h post inoculation (Supplementary Fig. S9A). In addition, analysis of CaWRKY41-silenced pepper plants, in which CaWRKY41 expression was approximately 3.2- and 3.3-fold lower than the control (PYL-279), under pathogen inoculation and non-inoculation conditions, respectively (Supplementary Fig. S9B), showed that CaWRKY41 silencing increased susceptibility to R. solanacearum compared with PYL-279 plants at 5, 7, and 9 days post inoculation (Fig. 8A). Consistently, PYL-279-wrky41 plants had a higher disease index, higher rate of R. solanacearum growth, and higher level of electrolyte leakage compared with PYL-279 plants (Fig. 8B–D). In addition, more serious symptoms of bacterial wilt were observed in the detached youngest leaves of

![Fig. 8. CaWRKY41 silencing enhances susceptibility to Ralstonia solanacearum FJ150501. (A) Appearance of PYL-279 and PYL-279-wrky41 pepper plants at 0, 5, 7, and 9 days post inoculation (DPI) with R. solanacearum. (B) Disease index scored daily for R. solanacearum-inoculated PYL-279 and PYL-279-wrky41 pepper plants. (C) Bacterial growth and (D) conductivity (as a measure of electrolyte leakage) in PYL-279 and PYL-279-wrky41 pepper leaves following R. solanacearum inoculation. HPI, hours post inoculation. Data represent the mean ±SE of three biological replicates. Asterisks indicate significant differences compared with control plants (Student’s t-test: *P<0.05, **P<0.01). (E) Effect of R. solanacearum on leaves isolated from PYL-279 and PYL-279-wrky41 plants. R. solanacearum was collected from stem exudates or the vascular portions of infected pepper leaves, and the appearance of symptoms was observed 72 HPI. CK, control untreated. (F) Decreased H$_2$O$_2$ levels and cell death in the leaves of PYL-279-wrky41 pepper plants compared with PYL-279 24 h after inoculation with R. solanacearum. (This figure is available in colour at JXB online.)](https://academic.oup.com/jxb/article/70/5/1581/5289166)
**CaWRKY41** and H$_2$O$_2$ alter *R. solanacearum* and Cd tolerance | 1591

**Discussion**

Although plant immunity and Cd tolerance have been intensively studied in the past few decades, and several proteins have been implicated in both of these processes (Mirouze et al., 2006; Kim et al., 2007; Kuhnelz et al., 2015; Campe et al., 2016; Peris-Peris et al., 2017), little is known about the connections between the two processes. In the present study, we provide evidence that both immunity and Cd uptake in pepper are coordinately regulated by CaWRKY41 and are dependent on the ROS signaling pathway.

**Responses of pepper to R. solanacearum inoculation and Cd are coordinately regulated by CaWRKY41**

We analyzed the expression of eight group III WRKY genes in the roots and leaves of pepper plants grown in the presence of excess Cd or under Fe deficiency, since the response of plants to Fe deficiency was previously shown to be related to the response to excess Cd (Nakanishi et al., 2006; Han et al., 2014; Mendoza-Cozatl et al., 2014). Among these eight genes, only CaWRKY41 was up-regulated in roots and leaves by both excess Cd exposure and Fe deficiency (Fig. 1). In addition, CaWRKY41 was induced by *R. solanacearum* inoculation (Supplementary Fig. S9A), pointing to a role for CaWRKY41 in the crosstalk between the response to excess Cd exposure and *R. solanacearum* inoculation in pepper. Gain- and loss-of-function analyses confirmed this speculation: CaWRKY41-silenced pepper plants showed substantially enhanced sensitivity to *R. solanacearum* inoculation (Fig. 8A), as also revealed by lighter Trypan blue staining compared with PYL-279 plants when challenged with *R. solanacearum* (Fig. 8F). In addition, the growth rate of *R. solanacearum* and the disease index (indicative of the severity of symptoms of infection) was higher in CaWRKY41-silenced pepper plants than in PYL-279 plants (Fig. 8B, C). Moreover, the leaves of CaWRKY41-silenced pepper plants showed enhanced tolerance to Cd (Fig. 3B–D), while CaWRKY41-overexpressing Arabidopsis plants exhibited enhanced sensitivity to Cd (Fig. 5A–D), with these plants having a lower fresh weight and shorter primary root than WT plants (Fig. 5E, F).

Together, our findings indicate that CaWRKY41 is a positive regulator of immunity and a negative regulator of Cd tolerance in pepper. Crosstalk between biotic and abiotic stress responses is thought to be involved in coordinately regulating plant responses to multiple environmental stresses (Fujita et al., 2006; Wu et al., 2009). Although the synergistic effect of Cd and Botrytis infection on PDF1.2 expression (Cabot et al., 2013) and the differential regulation of Cd uptake in response to SA application in plants (Kovacik et al., 2009) have been previously reported, little is known about the crosstalk between Cd toxicity and pathogen responses. Furthermore, members of the WRKY TF family have been implicated in plant immunity, but only a few WRKY TFs, such as *T. hispida* WRKY7 (Yang et al., 2016) and *Z. mays* WRKY4 (Hong et al., 2017), have been shown to positively regulate plant tolerance to Cd toxicity. The results of the current study strongly suggest that CaWRKY41 plays a role in the crosstalk between the response of pepper to *R. solanacearum* infection and excess Cd exposure.

R. solanacearum inoculation and excess Cd activate a positive feedback loop between CaWRKY41 expression and H$_2$O$_2$ accumulation

Although bursts of ROS including H$_2$O$_2$ have been shown to be involved in plant responses to pathogen attack (Torres et al., 2006; Vellosillo et al., 2010) and exposure to Cd toxicity (Garnier et al., 2006; Heyno et al., 2008), and the role of H$_2$O$_2$ as a signaling molecule in plant immunity is well established (Alvarez et al., 1998; Qi et al., 2017), the role of H$_2$O$_2$ in plant responses to Cd toxicity has remained elusive.

The results of the current study indicate that both exposure to excess Cd and *R. solanacearum* inoculation trigger H$_2$O$_2$ accumulation in pepper plants. The enhanced H$_2$O$_2$ accumulation might induce the expression of CaWRKY41, as exogenous application of H$_2$O$_2$ significantly increases CaWRKY41 expression (Fig. 2D), which in turn triggers H$_2$O$_2$ accumulation...
in Arabidopsis under Cd stress, as revealed by DAB staining and direct H$_2$O$_2$ measurements (Fig. 6A, B). These results suggest that there is a positive feedback loop between CaWRKY41 expression and H$_2$O$_2$ accumulation during the response to R. solanacearum inoculation and excess Cd exposure in pepper. Similar positive feedback loops are common in plant responses to pathogens or other abiotic stresses and are believed to be crucial for amplifying defense signaling (Wang et al., 2014; Cai et al., 2015; Shen et al., 2016; Guo et al., 2017; Ren et al., 2018).

In plants, H$_2$O$_2$ is a general signaling molecule in the response to pathogen or abiotic stresses and is coupled with large-scale transcriptional reprogramming (Yang et al., 2013). However, it is unclear how H$_2$O$_2$ signaling is linked to specific TFs. It was recently reported that oxidation of the BRASSINAZOLE-RESISTANT1 (BZR1) transcription factor can be induced by H$_2$O$_2$, and that this plays a major role in regulating gene expression (Tian et al., 2018).

Further research is required to elucidate the mechanism underlying H$_2$O$_2$-mediated transcriptional modulation of CaWRKY41 expression during the response to Cd stress and R. solanacearum infection in pepper. H$_2$O$_2$ accumulation was attributed to its enhanced production and reduced degradation due to the enhanced expression of CaWRKY41, since the genes encoding NADPH oxidases (associated with ROS production), including AtRBOHC (Macho et al., 2012), AtRBOHD (Li et al., 2014; Kadota et al., 2015), AtRBOHE, and AtRBOHF (Chaouch et al., 2012) were up-regulated in Arabidopsis plants overexpressing CaWRKY41 (Fig. 7A–D). These results are consistent with the finding that NADPH oxidases differentially regulate ROS production and are significantly up-regulated by Cd exposure (Gupta et al., 2017). Furthermore, H$_2$O$_2$ accumulation has been found to be dependent on or closely correlated to NADPH oxidase (Foreman et al., 2003). By contrast, genes encoding antioxidant enzymes, including POD, CAT, and APX (Smeets et al., 2013), were significantly down-regulated in response to CaWRKY41 overexpression in Arabidopsis (Fig. 6C–E, Fig. 7E–I). Similarly, it was reported that repression of CATALASE2 (CAT2) resulted in H$_2$O$_2$ accumulation, and that inhibition of H$_2$O$_2$ degradation conferred enhanced disease resistance (Yuan et al., 2017).

We speculate that exposure to excess Cd triggers H$_2$O$_2$ accumulation, and that H$_2$O$_2$, and therefore the expression of CaWRKY41, might confer Cd sensitivity and resistance to R. solanacearum. In support of this notion, the Arabidopsis ocp3 mutant, which produces high levels of H$_2$O$_2$ and exhibits its increased resistance to the necrotrophic pathogens Botrytis cinerea and Plectosphaerella cucumerina (Coego et al., 2005), exhibited enhanced sensitivity to excess Cd compared with
control plants in the present study (Supplementary Fig. S7). In addition, Cd exposure repressed the growth of *R. solanacearum* in inoculated pepper plants (Supplementary Fig. S9C). By contrast, *R. solanacearum* inoculation increased Cd uptake by the roots and leaves of pepper plants exposed to excess Cd (Supplementary Fig. S9G, H). Together, these results strongly suggest that H$_2$O$_2$ accumulation increases plant immunity and plant sensitivity to excess Cd.

**CaWRKY41 likely mediates Cd sensitivity by enhancing Cd uptake via enhanced Zn transporter activity**

Increased Cd uptake or reduced levels of Cd detoxification result in cellular damage in plants (Schutzenbühel et al., 2001). We found that Cd levels in both the roots and shoots of *CaWRKY41*-overexpressing Arabidopsis plants were significantly higher than those of WT plants (Fig. 5G), indicating that the susceptibility of *CaWRKY41*-overexpressing Arabidopsis plants to Cd stress is due to their high Cd contents.

Our findings suggest that the enhanced Cd contents might be due to the up-regulation of various Zn transporter genes, such as *AtZIP3*, *AtZIP4*, and *AtZIP9*, by *CaWRKY41* (Supplementary Fig. S8B, C, E). Indeed, uptake of Cd by Zn and Fe transporters has previously been suggested (Saraswat and Rai, 2011; Barabasz et al., 2016), and Fe content was found to increase in Arabidopsis roots and to vary in accordance with the period and concentration of Cd treatment (Gupta et al., 2017). However, although *CaWRKY41* was activated by Fe deficiency, the Fe content of *CaWRKY41*-overexpressing Arabidopsis plants did not significantly differ from that of control plants (Supplementary Fig. S6C, D). It is puzzling from an evolutionary point of view why *CaWRKY41* would positively regulate disease resistance in pepper plants but promote the absorption of Cd and enhance sensitivity to Cd, which might reduce the adaptability of the plant to a heavy-metal-contaminated environment. We speculate that *CaWRKY41* might have evolved to coordinate plant immunity and the absorption of essential ions, including Zn, by modulating the activity of specific ion transporters. Indeed, Zn is required for the functioning of Zn binding motif-containing proteins associated with disease resistance, including WRKY TFs (Duan et al., 2007), Rar1 (Shirasu et al., 1999; Musckett et al., 2002; Wang et al., 2017), and R proteins (Yang et al., 2010; Bi et al., 2011), which play important roles in plant immunity. However, some of these ion transporters can be hijacked by Cd, which has only recently been released into the environment as a result of modern industrial practices, suggesting that plants have not yet evolved a counterstrategy to distinguish between Zn and Cd.

Based on these findings, we propose a working model (Fig. 9) in which H$_2$O$_2$ accumulation and the expression of *CaWRKY41*, as well as a positive feedback loop between these processes, are induced by *R. solanacearum* infection or excess Cd exposure. The increase in H$_2$O$_2$ accumulation and *CaWRKY41* expression enhance plant immunity and sensitivity to excess Cd exposure by increasing Cd uptake via Zn transporters.

**Supplementary data**

Supplementary data are available at JXB online.

Fig. S1. Phylogenetic analysis of eight pepper group III WRKY proteins and Arabidopsis and tomato group III WRKY proteins.

Fig. S2. Cd stress and Fe deficiency promotes H$_2$O$_2$ accumulation.

Fig. S3. GUS expression in transgenic pCaWRKY41::GUS Arabidopsis plants under normal growth conditions.

Fig. S4. *CaWRKY41* is a transcriptional activator localized to the nucleus.

Fig. S5. Analysis of the effects of Cd stress on plant growth using chlorophyll fluorescence imaging before the appearance of visible effects on plant growth.

Fig. S6. Effect of Cd treatment on Zn concentrations in Arabidopsis.

Fig. S7. The Arabidopsis *apq3* mutant shows reduced tolerance to Cd stress.

Fig. S8. RT–qPCR analysis of the ZIP members involved in Zn uptake.

Fig. S9. Cd inhibits *R. solanacearum* growth and *R. solanacearum* infection increases Cd uptake.

Table S1. Sequences of primers used in this study.

Table S2. *CaWRKY41* group III genes.

Table S3. Analysis of the C/S-elements in the 2 kb promoter fragment of *CaWRKY41* group III genes.

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