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Article

The *TaWRKY22–TaCOPT3D* Pathway Governs Cadmium Uptake in Wheat

Xiaojuan Liu [1], Hongcheng Wang [2], Fang He [1], Xuye Du [2], Mingjian Ren [1,*] and Yinguang Bao [3,*]

[1] College of Agriculture, Guizhou University, Guiyang, Guizhou Province, China
[2] School of Life Sciences, Guizhou Normal University, Guiyang, Guizhou Province, China
[3] State Key Laboratory of Crop Biology, Agronomy College, Shandong Agricultural University, Tai'an, Shandong Province, China
*Corresponding author: rmj72@163.com (R.M.J.); baoyinguang@163.com (B.Y.G.)

Abstract: Cadmium (Cd) is a toxic non–essential element to plants, and its accumulation in crops has significant adverse effects on human health. The cross–talk between copper (Cu) and Cd has been reported, but the molecular mechanisms remain unknown. Here, the function of wheat *Cu transporter 3D* (*TaCOPT3D*) in Cd tolerance was investigated. The *TaCOPT3D* transcripts significantly accumulated in wheat roots under Cd exposure. To test whether *TaCOPT3D* was involved in response to Cd stress, overexpressing lines with altered expression of *TaCOPT3D* transporters were compared to wild–type (WT) plants. The results showed that, under 20 μM Cd treatment, *TaCOPT3D*–overexpression lines exhibited more biomass and lower Cd accumulation in roots, shoots, and grains compared to WT plants. In addition, the *TaCOPT3D*–overexpression lines demonstrated less reactive oxygen species (ROS) and a greater amount of active antioxidant enzymes under Cd conditions than WT plants. Moreover, *TaCOPT3D*–overexpression lines highly reduced Cd accumulation under exposure to 20 μM Cu. The regulation pathway of *TaCOPT3D* in response to Cd stress was investigated, and transcription factor (TF) *TaWRKY22*, which targeted the *TaCOPT3D* promoter, was identified. Therefore, *TaCOPT3D* can serve as a candidate gene for decreasing Cd accumulation in wheat through genetic engineering.

Keywords: wheat; *TaCOPT3D*; Cd uptake; *TaWRKY22*; transcriptional regulation

1. Introduction

Cadmium (Cd) is a non–essential element for plants and has become one of the most toxic pollutants in water and soil worldwide [1]. Cd interferes with many physiological and metabolic processes in plants, such as disrupting electron transport, decreasing chlorophyll concentration, causing cell death, and inhibiting nutrient absorption and distribution [2, 3]. In agricultural production, excess Cd inhibits plant growth and development and reduces crop yield and quality [4]. Additionally, Cd accumulates in the edible parts of plants poses a potential risk to humans through the food chain [5]. The cultivation of crop cultivars with low Cd accumulation capacities is an essential strategy to ensure food security. To reduce the risk of Cd entering the food chain, it is critical to identify candidate genes related to Cd uptake and transport in crops.

Several studies have made progress on the molecular aspects related to Cd transporters in plants [6, 7]. Cd enters into root cells via a complex pathway and is easily absorbed by roots and can be transferred to shoots. However, there is no specific transporter for Cd uptake and transport [8]. As such, transporters responsible for the uptake of essential elements are involved in Cd transport, including iron–regulated transporter (IRTs), zinc–regulated transporter/IRT–like proteins (ZIPs), natural resistance–associated macrophage proteins (NRAMPs), and heavy metal ATPases (HMAs) [9, 10]. Cd has a similar structure to several necessary elements (such as Fe, Mn, and Zn) and can replace these elements in the enzyme [11]. Importantly, enzyme activity is destroyed when Cd enters the cells, leading to the inhibition of growth, metabolic abnormality, and even death [12]. In addition,

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Cd entering plants causes the production of reactive oxygen species (ROS) that damage protein and DNA, leading to oxidative stress [13]. Under Cd stress conditions, the activity of antioxidant enzymes, including peroxidase (POD), superoxide dismutase (SOD), hydrogen oxidation enzymes (CAT), and glutathione reductase (GR), is increased, thereby enhancing the scavenging of intracellular ROS and reducing oxidative damage [14].

Copper transporter (COPT) is known to perform Cu acquisition and transport in eukaryotes [15]. The functions of the members of the COPT are well known to include the regulation of Cu transport and homeostasis in *Arabidopsis* [16]. Several COPTs are involved in the transport of ions other than Cu. For example, *Arabidopsis* COPT2 is involved in the uptake of Au, while COPT5 participates in the response to Fe deficiency [17, 18]. In rice, the expression patterns of COPT are positively regulated by Fe, Mn, or Zn [19]. These works suggest that COPT may be involved in the uptake and transport of multiple ions in plants. However, whether *COPT* is involved in the response to Cd stress remains unclear. In the present study, the wheat *COPT* gene *Cu transporter 3D* (*TaCOPT3D*), which acts in response to Cd stress, was identified. Transcription factor TaWRKY22 binds to the promoter of *TaCOPT3D* and regulates its expression. The overexpression of *TaCOPT3D* increased Cd concentration in root tissues but decreased Cd concentration in grain. In addition, the expression of *TaHMA3* was highly induced in transgenic wheat under Cd stress. The results of this study will help achieve a full understanding of the effect of *TaCOPT3D* in response to Cd stress and its underlying mechanisms, which may provide a basis for the breeding of Cd–resistant wheat with low Cd concentrations in edible parts.

2. Materials and methods

2.1. Plant materials and treatments

The wheat cultivar Bobwhite, as the wild–type (WT) line, was used for the genetic transformation. For the hydroponic experiment, Bobwhite seeds were germinated in ddH_2O and transferred to Hoagland's nutrient solution in a growth chamber with a day/night temperature regime of 25/20°C and a photoperiod of 16 h at a photosynthetically active. When the seedlings reached the trefoil stage, the two following treatments were applied to the wheat plants: (I) a control treatment of 0 mM Cd; and (II) a Cd treatment of 20 μM Cd (in the form of $CdCl_2$·5 H_2O). The seedlings were collected at 0 h, 1 h, 3 h, 6 h, 12 h, 24 h, and 14 d, then stored at −80°C after liquid nitrogen refrigeration for the determination of enzyme activity assay, gene expression analysis, and ion concentration detection. For the pot experiment, germinated seeds were maintained at 4°C for 14 d and then transplanted into pots and grown in a phytotron with a 12 h photoperiod of cool white fluorescent light and an indoor temperature of 26°C. These pots were further divided into two groups: (I) a control group of 1 kg soil; and (II) an experimental group of 1 kg soil with 1 mg Cd (in forms of $CdCl_2 \cdot 5H_2O$). Before planting wheat, 200 g of compound fertilizer was added to each pot. The plants were irrigated with 2 L water every 2 weeks until completely ripened. At the end of the experiment, 10 seedlings were selected from each treatment of each line to measure the seed Cd concentration.

2.2. Analysis of gene expression

The total RNA from the root and shoot tissue was extracted using an RNApure Plant Kit (CwBio, Beijing, China) following the manufacturer's instructions. A total of 2 μg of RNA was employed to synthesize the first–strand cDNA using a SuperRT cDNA Synthesis Kit (CwBio, Beijing, China). The expression of the genes was analyzed using an UltraSYBR One Step RT–qPCR Kit (CwBio, Beijing, China) in a real–time polymerase chain reaction (PCR) system (LightCycler 96, Roche, Basel, Switzerland). The wheat *β–actin* was used as an internal control and the relative expression levels of genes were calculated by the $2^{-\triangle\triangle CT}$ method [20].

2.3. Plasmid construction and plant transformation

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The open reading frame (ORF) of *TaCOPT3D* was amplified and inserted into the pCambia3300 vector using the *BamH* I and *Kpn* I sites to achieve the *Ubi*:*TaCOPT3D* construct. All binary vectors harboring the desired constructs were transferred into strain EHA105 and transformed into the wheat cultivar Bobwhite using *Agrobacterium*–mediated transformation [21].

2.4. Detection of antioxidant enzyme activity

The determination of antioxidant enzymes, including superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT), was conducted using test kits (Solarbio, Beijing, China) according to the manufacturer's instructions.

2.5. Measurements of Cd^{2+} flux

WT and transgenic wheat plants were used for ion flux measurements. Trefoil–stage wheat seedlings were treated with Hoagland's nutrient solution or 20 μM Cd within Hoagland's nutrient solution for 24 h. The net Cd^{2+} flux was measured in wheat root meristematic tissues using non–invasive micro–test technology as described by Zhang et al. [22].

2.6. Measurement of Cd concentrations

The Cd concentrations of seedlings and grains were measured using inductively–coupled plasma–mass spectroscopy according to previous research [22]. The Cd fluorescence in root tissues was visualized using the Leadmium Green fluorescent probe (Invitrogen, CA, USA) according to the manufacturer's protocol.

2.7. Yeast one–hybrid (Y1H) assay

The cDNA library was constructed in our previous work [21]. Y1H library screening was performed according to Lin et al. [23]. The Y1H assay was performed with the Y187 strain as described by the kit manufacturer (MATCHMAKER One Hybrid System; Clontech, Palo Alto, CA, USA). The pHIS2–*TaCPOT3Dpro* was generated with the *TaCPOT3D* promoter and the pHIS2 reporter vector, and the pHIS2–*TaCPOT3Dpro* and pGADT7–*TaWRKY22* plasmids were co–transformed into the Y187 yeast strain and co–cultured on a medium without Trp and Leu. Then, the co–transformed strain was screened on a medium lacking Leu, Trp, and His supplemented with 90 mM 3–AT for 3 d.

2.8. Electrophoretic mobility shift assay (EMSA)

An oligonucleotide probe of the *TaCOPT3D* promoter was synthesized and labeled with biotin at the 3' end by Sangon (Shanghai, China). The *TaWRKY22* coding sequence was cloned into the pGEX4T–1 vector and subsequently subjected to the EMSA assay using the LightShift Chemiluminescent EMSA Kit (Pierce, Rockford, IL, USA).

2.9. Transient expression assays in tobacco leaves

Transient expression assays were performed in tobacco leaves as described by Shang et al. [24]. The *TaCPOT3Dprom* was cloned into pGreen II 0800–LUC to generate the reporter. The *TaWRKY22* ORF was inserted into pGreen II 62–SK to generate the effector. Images were captured using the NightOWL II LB983 apparatus.

2.10. Statistical analysis

Differences were detected with Student's *t* test or one-way ANOVA assays. Each experiment was performed with at least three independent biological replicates. $P \leq 0.05$ was considered significant.

2.11. Primers

All primers used in this study are listed in Table S1.

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3. Results

3.1. Transcription profile of TaCOPT3D in wheat under Cd treatment

Real–time quantitative PCR (RT–qPCR) analysis showed that *TaCOPT3D* was particularly expressed in the root tissue of wheat (Fig. 1a). In addition, Cd exposure significantly increased the *TaCOPT3D* transcript levels in wheat (Fig. 1b).

3.2. Subcellular localization of TaCOPT3D

The TaCOPT3D–GFP fusion was constructed under the control of the CaMV 35S promoter, and *Agrobacterium*–mediated transient expression in tobacco leaves was used for protein expression. TaCOPT3D–GFP was localized mainly in the membrane (Fig. 2), suggesting that TaCOPT3D probably functioned in the cell membrane.

3.3. Overexpression of TaCOPT3D increased the cadmium tolerance of wheat

To further determine the function of *TaCOPT3D* in the regulation of wheat Cd tolerance, we generated transgenic wheat plants that overexpressed *TaCOPT3D*. A plant binary vector driven by maize *Ubiquitin* (*Ubi*) promoter was constructed (Fig. S1a). Two independent transgenic lines (OE1 and OE2) were obtained (Fig. S1b). The expression of TaCOPT3D in transgenic lines was further investigated and the *TaCOPT3D*–overexpression lines clearly increased *TaCOPT3D* expression to about 2–fold that of the WT (Fig. S1c).

To explore Cd stress tolerance conferred by the expression change of *TaCOPT3D*, Cd tolerance in wheat plants was studied through hydroponic and pot experiments. Under normal conditions, no obvious phenotypic differences were observed between transgenic lines and WT plants (Figs. 3a and b). Under the hydroponic test of the 20 μM Cd treatment, both the WT and transgenic lines showed a degree of growth inhibition. However, the transgenic lines exhibited stronger growth than the WT (Fig. 3a). Cd stress treatments were performed using transgenic lines and WT plants in the vegetative and reproductive stages of wheat growth through a pot experiment (Fig. 3b). Under normal conditions, there was no difference between WT and transgenic lines. Nevertheless, under Cd stress, the *TaCOPT3D*–overexpression lines showed much higher Cd tolerance than the WT lines (Fig. 3b). The Cd concentrations in the roots, shoots, and grains of WT and transgenic lines after Cd treatment were further compared. As shown in Fig. 3c, the transgenic lines had lower Cd concentration than the WT line in these tissues.

Taken together, these results suggest that the *TaCPOT3D* gene functions as a positive regulator of Cd tolerance in wheat.

3.4. Overexpression of TaCOPT3D enhanced the antioxidant capacity in transgenic wheat under Cd stress

Abiotic stress induces the accumulation of ROS in plants. H_2O_2 and $O_2^{\cdot-}$ accumulation levels in the root tissues of WT and transgenic lines under hydroponic conditions were investigated. There were no significant differences in the root H_2O_2 and $O_2^{\cdot-}$ levels of WT and transgenic lines without Cd stress (Figs. 4a and b). Under Cd stress, both H_2O_2 and $O_2^{\cdot-}$ levels were induced in the WT and transgenic lines; however, the H_2O_2 and $O_2^{\cdot-}$ levels were significantly higher in the transgenic lines than in WT (Figs. 4a and b).

We further examined the activities of antioxidant enzymes (SOD, CAT, and POD) between the transgenic and WT lines. As indicated in Figs. 4c–e, there were no differences in the antioxidant enzyme activities between the WT and transgenic lines under control conditions. While the Cd stress increased the activities of all tested antioxidant enzymes in the WT and transgenic lines, the activities were induced significantly higher in transgenic lines than in WT.

Physiological indicators, including malondialdehyde (MDA) concentration, electrolyte leakage, and chlorophyll concentration, were examined. The overexpression of *TaCOPT3D* increased chlorophyll concentration (Fig. 4f). The chlorophyll concentration was correlated with higher net photosynthesis [22], and the results suggested that the

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transgenic plants could maintain better photosynthesis under Cd stress. MDA concentration and electrolyte leakage were closely correlated with the degree of cell membrane damage under abiotic stress [25]. In the present work, the MDA concentration increased in all samples under Cd stress treatments compared with the plants under normal conditions; however, the MDA concentration of transgenic lines was significantly lower than that of WT plants under Cd stress conditions (Fig. 4g). In addition, the electrolyte leakage was lower in transgenic wheat seedlings compared with the WT seedlings under Cd stress (Fig. 4h). The results above suggested that the overexpression of *TaCOPT3D* increased Cd tolerance and inhibited Cd accumulation in wheat. In addition, the lower MDA concentration and electrolyte leakage of transgenic wheat lines under Cd stress reflected a lower degree of damage to the plant cell membranes.

3.5. TaCPOT3D influences the Cd^{2+} flux in wheat roots

The transient Cd^{2+} flux was recorded using a non–invasive micro–test technique. The influx of Cd^{2+} in *TaCPOT3D*–overexpression lines was less than that of the WT line under Cd treatment (Figs. 5a and b). The results indicated that the overexpression of *TaCPOT3D* decreased Cd enrichment by inhibiting net Cd^{2+} flux.

3.6. A Low concentration of Cu decreased Cd accumulation in transgenic wheat

Cu reduced the Cd uptake in plant roots, however, high copper concentrations can damage plant cells [16]. Although the overexpression of *TaCOPT3D* reduces Cd uptake in wheat, the interactions between exogenous Cu and Cd that impact the uptake of Cd in transgenic lines are still unknown. As shown in Fig. 6, Cd accumulation in the roots of transgenic wheat lines was decreased in the presence of 20 μM or 100 μM exogenous Cu. However, Cd accumulation in transgenic wheat was higher when 100 μM of exogenous Cu was added than with the addition of 20 μM of exogenous Cu. This was probably because 100 μM Cu damaged the cells and the roots lost the ability to inhibit the absorption and transportation of Cd in wheat.

3.7. Transcription factor TaWRKY22 binds to the TaCPOT3D promoter

To identify the TF regulating *TaCOPT3D*, a cDNA library from Cd–treated wheat seedlings was constructed and a yeast one–hybrid (Y1H) assay was performed with the *TaCOPT3D* promoter as bait. A total of 27 positive colonies were sequenced and a WRKY TF was characterized. A BLAST search for this sequence (www.ncbi.nlm.nih.gov/BLAST) revealed that the TF was *TaWRKY22*. The Y1H experiment was performed to further confirm the screening results (Fig. 7a).

Next, EMSA was conducted to confirm the TaWRKY22–binding ability of *TaCOPT3Dprom*. As indicated in Fig. 7b, the TaWRKY22–*TaCOPT3Dprom* complex was observed. Comparison of the *in vivo* and *in vitro* results suggested that TaWRKY22 is bound to the *TaCOPT3Dprom*.

A transcriptional activity assay in tobacco leaves was performed to analyze whether TaWRKY22 activated *TaCOPT3D* transcription. As indicated in Fig. 7c, TaWRKY22 promoted the expression of the reporter gene Luc driven by the *TaCOPT3D* promoter. The results showed that TaWRKY22 could directly activate the *TaCOPT3D* promoter through *in vivo* transcription.

4. Discussion

The *COPT* genes involved in Cu, Fe, and Zn absorption have been extensively studied. However, it is unclear how *COPT* genes act in response to Cd stress in plants. The present study explored the effects of *TaCOPT3D* on Cd stress in wheat. We further found that *TaCOPT3D* was strongly regulated by the *TaWRKY22* TF. Thus, the results of this study preliminarily clarified the wheat Cd stress transcriptional pathway *TaWRKY22*–*TaCOPT3D*.

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The uptake, transportation, and accumulation of metal ions in plants depend on ion transporters [26]. The NRAMP ATP–binding cassette (ABC) transporter, HMAs, yellow–stripe–like (YSL) proteins, and ZIP metal transporters have been identified as putative metal ion transporters [27]. Transporters capable of binding metal ions and the transport of ions through ion channels are located on the cellular membrane of each plant cell [28]. There are specific Cd transporters in plants. Previous reports have demonstrated that Cd entry into plant cells occurs via transporters for other divalent metal ions [8]. A previous study has reported that NRAMP5 in rice (*OsNRAMP5*) functions as a metal transporter for Mn and Cd uptake [29, 30]. The overexpression of *OsNRAMP5* blocked the radial transport of Cd from the epidermis to the xylem and reduced cadmium accumulation in rice grains [29]. *TpNRAMP5* from *Triticum polonicum* enhanced the accumulation of Cd, Co, and Mn, but not Zn and Fe [8]. COPT acts as a high–affinity Cu transporter in plants, and some COPTs are also involved in Cd response [19]. *Arabidopsis thaliana COPT5* mutants (*copt5*) are more sensitive to Cd stress than WT plants, and ethylene biosynthesis diminishes in the presence of Cd [31]. Yuan et al. reported 7 members of *COPTs* in rice, all of which were characterized for their functions in Cu transport [19]. In addition, these COPTs cannot mediate Fe and Zn uptake, suggesting that COPTs facilitate the selective absorption of Cu, Fe, and Zn [19]. In the present work, the overexpression of *TaCOPT3D* increased the Cd tolerance of transgenic lines. In addition, Cd absorption diminishes further under exogenous Cu application (Figs. 3 and 6). In yeast cells, Cd has been shown to modify Cu deficiency responses [32]. In rice, Cu could effectively alleviate the stress induced by Cd and increased the rice biomass and ripening rate; however, 10 μM $CuSO_4$ significantly increased the Cd concentration in rice grains [33]. Similar results in *A. thaliana* showed that the Cd concentration in shoot tissue increased significantly when grown in a medium supplemented with 0.1 mM Cu [31]. In the present work, 20 μM Cu significantly decreased the Cd absorption in the WT and *TaCOPT3D* transgenic wheat lines in the presence of 20 μM Cd, while Cd accumulation in the WT line was increased under 100 μM of exogenous Cu (Fig. 6). This may have occurred because the appropriate amount of Cu increased the root activity and reduced Cd uptake. In addition, 100 μM Cu damaged root tissue cells and reduced Cd stress responsiveness.

Transcriptional regulation may be an adaptation to respond to different levels of abiotic and biotic stresses [34]. Plants often respond to environmental stimuli by activating specific TFs, and the TFs then bind to the promoter sequences of target genes to initiate the transcription of downstream genes to respond to environmental changes [35]. In the present work, Y1H screening using the *TaCOPT3D* promoter as bait was used to identify a TF that targeted the *TaCOPT3D* promoter (Fig. 7a). The results demonstrated that TaWRKY22 could activate the gene expression of the *TaCOPT3D* promoter (Figs. 7c and d). WRKY proteins constitute a large family of TFs in plants involved in plant growth, development, and responses to biotic and abiotic stresses [36]. The WRKY TF specifically binds to the W–box or W–box–like elements containing the TGAC core sequence [37]. Numerous transcriptional regulation pathways have been shown to serve as important mechanisms in response to heavy metal stress in plants [37–39]. WRKY13 activates PDR8 expression to positively regulate Cd tolerance in *Arabidopsis* [36]. *Arabidopsis* A4 heat shock TF *HsfA4a* regulates Cd tolerance by activating the expression of the metallothionein gene [40]. In addition, *OsWRKY22* promotes aluminum (Al) tolerance via the activation of *OsFRDL4* expression in rice [35]. In the present work, it was demonstrated that TaWRKY22 bound the *TaCOPT3D* promoter using EMSA and luciferase reporter assay (Fig. 7b and c). The pathway *TaWRKY22–TaCOPT3D* constitutes a regulatory pathway involved in Cd response in wheat.

5. Conclusion

This work revealed the function of *TaCOPT3D* in the Cd response of wheat. The overexpression of *TaCOPT3D* activated the ROS scavenging system, which resulted in enhanced Cd tolerance. Moreover, the overexpression of *TaCOPT3D* decreased the Cd but

increased the Cu absorption in wheat, resulting in reduced Cd accumulation. A WRKY TF, *TaWRKY22*, was found to regulate the expression of *TaCOPT3D* by binding the W–box in the TaCOPT3D promoter. Thus, *TaCOPT3D* may be a candidate gene for producing safe wheat grains in Cd–contaminated soil.

Author Contributions: LXJ, HF and DXY conceived and designed the experiments; DXY and WHC collected the plant samples, LXJ and HF performed the experiments; and LXJ conducted bioinformatics analysis. LXJ, RMJ and BYG wrote the paper. All authors read and approved the final manuscript.

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