

Maize OXIDATIVE STRESS2 Homologs Enhance Cadmium Tolerance in Arabidopsis through Activation of a Putative SAM-Dependent Methyltransferase Gene¹

Lilong He, Xiaoling Ma, Zhenzhen Li, Zhengli Jiao, Yongqing Li*, and David W. Ow*

Plant Gene Engineering Center, South China Botanical Garden, Chinese Academy of Sciences, Guangzhou 510650, China (L.H., X.M., Z.L., Z.J., Y.L., D.W.O.); and University of Chinese Academy of Sciences, Beijing 100049, China (L.H., X.M., Z.L., Z.J.).

Previously the Arabidopsis (*Arabidopsis thaliana*) zinc finger protein OXIDATIVE STRESS2 (AtOXS2) and four OXS2-like (AtO2L) family members were described to play a role in stress tolerance and stress escape. For stress escape, *SOC1* was a target of AtOXS2. However, for stress tolerance, the downstream targets were not identified. We cloned two OXS2 homolog genes from sweet corn, *ZmOXS2b* and *ZmO2L1*. Both genes are transiently inducible by Cd treatment. When expressed in Arabidopsis, each enhances tolerance against cadmium. Further analysis showed that *ZmOXS2b* and *ZmO2L1* proteins enhance Cd tolerance in Arabidopsis by activating at least one target gene, that encoding a putative S-adenosyl-L-Met-dependent methyltransferase superfamily protein (AT5G37990), which we named *CIMT1*. This activation involves the *in vivo* interaction with a segment of the *CIMT1* promoter that contains a BOXS2 motif previously identified as the binding element for AtOXS2. More importantly, *CIMT1* is induced by Cd treatment, and overexpression of this gene alone was sufficient to enhance Cd tolerance in Arabidopsis. The connection of *ZmOXS2b* and *ZmO2L1* to Arabidopsis *CIMT1* suggests a similar network may exist in maize (*Zea mays*) and may provide a clue to possibly using a *CIMT1* maize homolog to engineer stress tolerance in a major crop.

Cadmium (Cd) is among the most serious heavy metal pollutants (Nriagu and Pacyna, 1988; Patra et al., 2004) and is readily absorbed by leafy vegetables and the grain crop rice. Chronic dietary intake of Cd is associated with various health problems including cancer and cardiovascular diseases (McLaughlin et al., 1999; Waalkes, 2003; Wagner, 1993). An excess amount of Cd in soil reduces the efficiency of plant photosynthesis, absorption of water, and growth (Küpper et al., 2007; Prasad, 1995; Sandalio et al., 2001). Cd inhibition of root elongation leads to lower uptake and transport of nutrients and water from root to shoot (Chen et al., 2003), and in shoot, Cd leads to reduced leaf conductance, CO₂ uptake, and stomatal opening (Perfus-Barbeoch et al., 2002).

At the cellular level, Cd affects DNA repair, DNA methylation, gene transcription, and translation (Hartwig and Schwerdtle, 2002; Takiguchi et al., 2003; Waisberg et al., 2003). Cd can also induce concentration-dependent oxidative stress, and in response, plants use enzymatic and nonenzymatic scavenging mechanisms to maintain cellular function (Mittler et al., 2004; Sytar et al., 2012). Chelation, extrusion, and sequestration are among several mechanisms involved in Cd detoxification (Clemens et al., 1999; Kim et al., 2007; Li et al., 1997; Yadav, 2010).

The Arabidopsis (*Arabidopsis thaliana*) zinc finger protein OXS2 (OXIDATIVE STRESS2) was previously found to induce stress escape through the direct activation of at least one floral integrator gene, *SOC1*, by binding the BOXS2 cis-element in its promoter (Blanvillain et al., 2011). Because a loss of function in *AtOXS2* is more sensitive to stress, *AtOXS2* was also proposed to play a role in alleviating stress tolerance. However, overexpression of *AtOXS2* alone could not enhance stress tolerance. The lack of an enhanced tolerance phenotype makes it difficult to use *AtOXS2* overexpression lines to identify possible downstream stress-responsive genes. In contrast, we found that overexpression in Arabidopsis of the maize (*Zea mays*) OXS2 homologs *ZmOXS2b* and *ZmO2L1* (*OXS2-Like1*) was able to enhance Cd tolerance. Therefore, we describe in this study an RNA-seq transcriptome profiling of Cd-treated Arabidopsis wild type and *ZmOXS2b* or *ZmO2L1* transgenic lines and the identification of a group of differentially expressed genes (DEGs). Of eight highly differentially expressed genes tested,

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* Address correspondence to liyongqing@scbg.ac.cn or dow@scbg.ac.cn.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: David W. Ow (dow@scbg.ac.cn).

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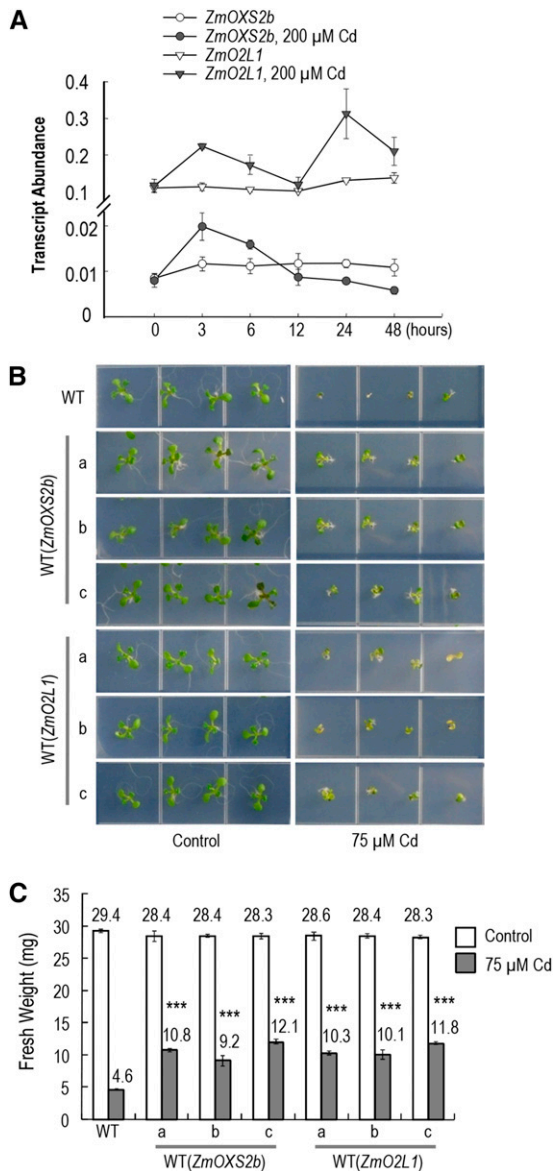


Figure 1. ZmOXS2 family members in stress response. A, *ZmOXS2b* and *ZmO2L1* transcript abundance in maize (relative to EF1- α control) determined by qRT-PCR. Fifteen-day-old maize seedlings exposed to 0 or 200 μM CdCl₂. Error bars indicate \pm SD from three independent experiments. B, Arabidopsis plants grown on 0.5 \times MS plates horizontally without or with 75 μM CdCl₂ for 11 d. Three independent homozygous lines transgenic for each gene shown. Representative data from three reproducible experiments with T3 seedlings. C, Average fresh weight of 20 T4 seedlings (11 d old) grown on 0.5 \times MS without or with 75 μM CdCl₂ (measured in batches of five seedlings). Error bars indicate \pm SD from three independent experiments. *P* value of Student's *t* test: transgenic plants compared with wild-type plants. ****P* < 0.001

DEG23 (AT5G37990) was the only one that enhanced Cd tolerance in Arabidopsis when overexpressed. DEG23 encodes a root specific putative member of the *S*-adenosyl-L-Met (SAM)-dependent methyltransferases superfamily, which we named CIMT1 (for Cd-inducible methyltransferase 1). Although CIMT1

can be activated by ZmOXS2b or ZmO2L1, it is not activated by AtOXS2. This may help explain why AtOXS2 overexpression does not enhance Cd tolerance. From a crop improvement perspective, the link of ZmOXS2b and ZmO2L1 to CIMT1 suggests a possible connection of ZmOXS2b and ZmO2L1 to CIMT1 homolog(s) in maize that can potentially be used for engineering stress tolerance in a major crop plant.

RESULTS

OXS2 Homologs Induced by Cd in Maize

An initial BLAST analysis with Arabidopsis OXS2 protein sequence found two homologous proteins in the maize genome. Based on the degree of homology to AtOXS2 (Supplemental Fig. S1A), and consistent with rice OXS2 proteins, we named the two maize homologs ZmOXS2b (ACN25172, 45.4% identity with AtOXS2) and ZmO2L1 (OXS2-Like1, NP_001145979, 42.1% identity with AtOXS2) (<http://www.ebi.ac.uk/Tools/psa>). In July 2014, a new maize homolog sequence (XP_008665236) was deposited into the database, and we refer to it as ZmOXS2a since it is most closely related to rice OXS2a (Supplemental Fig. S1A). However, since this work started before the discovery of ZmOXS2a, it is not included in this study.

We cloned the protein coding sequences of *ZmOXS2b* and *ZmO2L1* from the genome of the South China sweet corn cultivar FengTian 1 and found a few coding sequence differences from those of the NCBI (Supplemental Table S1). Similar to the AtOXS2 family proteins, both ZmOXS2 homologs have two ANKYRIN repeats and two zinc finger domains (Supplemental Fig. S1B).

To test whether expression of *ZmOXS2b* and *ZmO2L1* responds to stress, maize seedlings were grown in nutrient solution without or with 200 μM CdCl₂. Leaves were collected at different time points after Cd treatment. Quantitative reverse transcription PCR (qRT-PCR) data show that the mRNA abundance for both genes was steady in the absence of Cd and with *ZmO2L1* expressing at a higher level (Fig. 1A). In the presence of Cd, expression increased by 2-fold 3 h after treatment, but returned to basal level at the 12-h time point. At the 24- and 48-h time points, *ZmO2L1* expression again climbed and dipped, while that of *ZmOXS2b* gradually dropped to its lowest level. Although the mRNA abundance after extended Cd treatment is difficult to interpret, it is clear that both genes respond to Cd within 3 h with accumulation of mRNA.

ZmOXS2b and ZmO2L1 Enhance Cd Tolerance in Arabidopsis

ZmOXS2b or *ZmO2L1* was fused behind the *Cauliflower mosaic virus* 35S RNA promoter in a binary vector to yield p35S::*ZmOXS2b* or p35S::*ZmO2L1*, respectively, for transformation into Arabidopsis (cv Columbia). In addition, p35S::*ZmOXS2b* was also transformed

into mutant *oxs2-1* and p35S::*ZmO2L1* into mutant *o2l1-1*. Approximately 10 independent transgenic lines were obtained for each transformation. Unlike overexpression of *AtOXS2* that caused early flowering (Blanvillain et al., 2011), we did not observe early flowering with the four classes of transgenic lines: WT(*ZmOXS2b*), WT(*ZmO2L1*), *oxs2-1*(*ZmOXS2b*), and *o2l1-1* (*ZmO2L1*). Three independent lines from each class were randomly selected and homozygous plants obtained for further analysis. RT-PCR verified the expression of *ZmOXS2b* and *ZmO2L1* and the transgenic plants were compared to the wild type control against several types of stresses: Cd (75 μ M), diamide (1 and 2 mM), NaCl (100 and 150 mM), mannitol (100 and 200 mM), abscisic acid (1.5 and 3 μ M), heat (37°C, 3 h), and cold (4°C, 3 h). In the absence of stress, or for the tested stress conditions other than Cd, the phenotype of the transgenic plants was indistinguishable from that of the untransformed control. In Cd (75 μ M), the root length, shoot growth, and biomass of WT(*ZmOXS2b*) and WT(*ZmO2L1*) plants were greater than the untransformed controls (Fig. 1, B and C; Supplemental Fig. S2A). Likewise, the root length and shoot growth of *oxs2-1*(*ZmOXS2b*) and *o2l1-1*(*ZmO2L1*) plants were also greater than *oxs2-1* and the wild type (Supplemental Fig. S2, B and C).

Differentially Regulated Genes Identified from RNA-Seq Analysis

The Cd tolerance phenotype associated with constitutive expression of *ZmOXS2b* or *ZmO2L1* is likely due to a gene expression change caused by the putative transcription factors. As *AtOXS2* is known to activate promoters of some flowering genes (Blanvillain et al., 2011), it would seem plausible that *ZmOXS2b* and *ZmO2L1* may recognize heterologous promoters from a similar protein-structure/DNA sequence interaction. To examine if the gene expression pattern has changed, an RNA-seq analysis was conducted on Cd-treated plants comparing WT(*ZmOXS2b*) and WT(*ZmO2L1*) against the wild-type control.

The expression profile of WT(*ZmOXS2b*) compared to the wild-type control is hereafter referred to as comparison 1 or C1; likewise, WT(*ZmO2L1*) compared to the wild type is C2. DEGs with statistically significant change (up-regulated by at least 1.67-fold or down-regulated by at least 0.59-fold, NOISeq Q value over 0.8; Tarazona et al., 2011) in either C1 or C2 were selected (Supplemental Table S2), consisting of 86 DEGs in C1 and 69 DEGs in C2. However, since 30 of them are common to both C1 and C2 (Fig. 2A), the total number of DEGs is 125. Gene Ontology analysis shows that many of these DEGs are involved in oxidoreductase activity, arsenate reductase activity, and iron ion binding (Supplemental Table S3). In C1, 61 DEGs are up-regulated and 25 down-regulated in WT(*ZmOXS2b*). Thirty-eight of these 86 DEGs are listed as involved in stress response, defense response, or metal ion

transport (Supplemental Table S2). In C2, 52 DEGs are up-regulated and 17 down-regulated in WT(*ZmO2L1*). Twenty-eight of these 69 DEGs in C2 are listed as involved in stress responses, defense response, or metal ion transport (Supplemental Table S2). Given that 30 DEGs are common to C1 and C2, we narrowed the focus to this smaller group. The 30 members were named DEG1 to DEG30 from top to bottom in the clustering analysis map (Fig. 2B). They behave similarly in both transgenic lines except that DEG30 (AT5G39110) was down-regulated in WT(*ZmOXS2b*) but up-regulated in WT(*ZmO2L1*) (Fig. 2B). Among these 30 DEGs, 14 are annotated to be stress related or metal ion transport genes (Supplemental Table S2).

Validation of Differential Expression

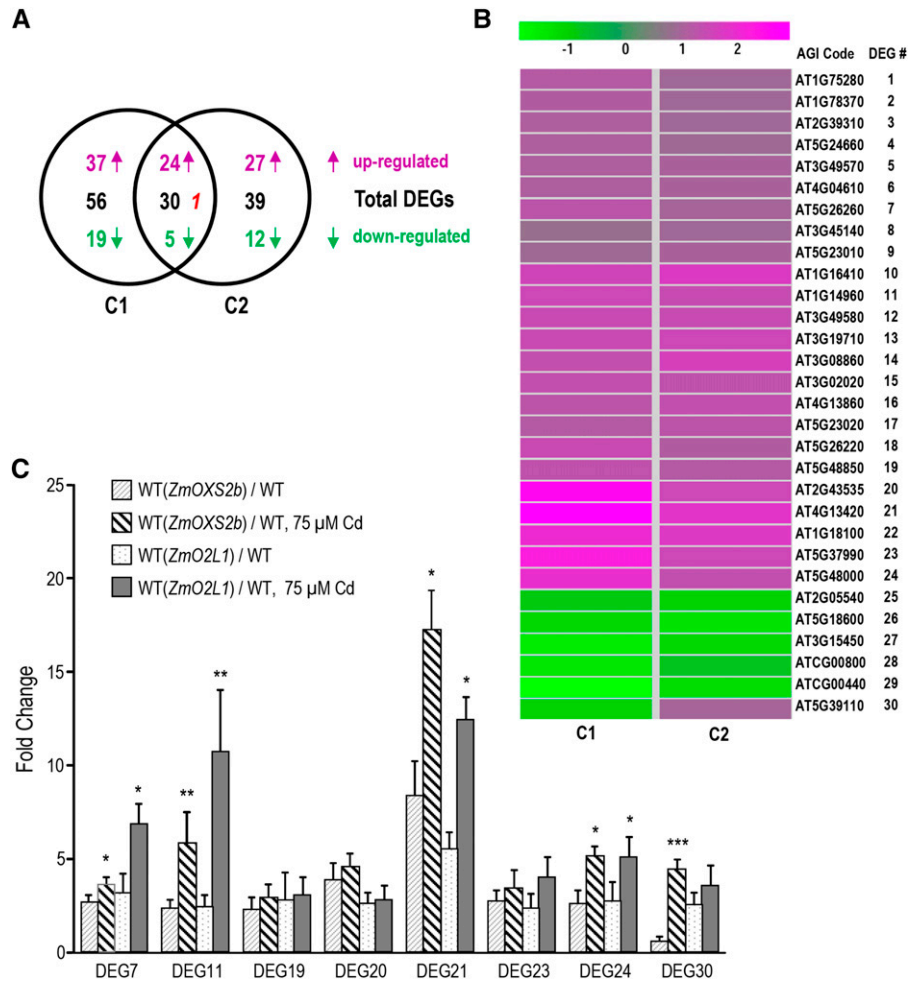
To verify the expression pattern of the RNA-seq analysis, qRT-PCR was conducted on the 30 common DEGs with the same tissues used for RNA-seq. The expression pattern obtained was similar to the RNA-seq data for most of the genes except the fold change differed in a few instances (Supplemental Table S4). In the qRT-PCR ranking, eight DEGs among the top 10 DEGs that show the most dramatic change in gene expression (3.6- to 13.9-fold; Supplemental Table S4) are common in C1 and C2. They are DEG7 (AT5G26260), DEG11 (AT1G14960), DEG19 (AT5G48850), DEG20 (AT2G43535), DEG21 (AT4G13420), DEG23 (AT5G37990), DEG24 (AT5G48000), and DEG30 (AT5G39110).

WT(*ZmOXS2b*), WT(*ZmO2L1*), and wild-type control plants were again cultured on 0.5 \times Murashige and Skoog media without or with 75 μ M CdCl₂ for 11 d for qRT-PCR analysis of these eight specific DEGs. Even in the absence of Cd treatment, all but DEG30 in WT (*ZmOXS2b*) were up-regulated in the transgenic lines (Fig. 2C). All were induced by Cd treatment in the wild-type control (Supplemental Fig. S3), but the induction is more significant in the WT(*ZmOXS2b*) and WT (*ZmO2L1*) plants (Fig. 2C). This shows that *ZmOXS2b* and *ZmO2L1* have further elevated the expression of at least seven genes that are normally up-regulated by Cd in Arabidopsis.

ZmOXS2b and ZmO2L1 Activate BOXS2-Containing Promoters

AtOXS2 has been shown previously to bind a 9-bp CT-rich motif named BOXS2 and that it can activate several BOXS2-containing promoters (Blanvillain et al., 2011). Among these eight genes, the promoters of DEG11, DEG21, and DEG23, but not the other five DEGs, contain sequences similar to a BOXS2 motif (Fig. 3A). We fused the firefly luciferase reporter gene (*luc*) to a 2-kb promoter including its 5' untranslated region (UTR) fragment from each of the eight DEG candidates to generate all eight promoter-*luc* fusions. Each of these constructs was transiently introduced into tobacco leaf

Figure 2. RNA-seq analysis and qRT-PCR verification. A, Venn diagram of DEGs. C1 = WT(*ZmOXS2b*) versus the wild type; C2 = WT(*ZmO2L1*) versus the wild type. Total numbers of DEGs; up- and down-regulated DEGs shown in magenta and green lettering, respectively. Red italic lettering indicates one gene (DEG30) down-regulated in C1 but up-regulated in C2. B, Heat map of clustering analysis of the 30 DEGs in the intersection of C1 and C2. Expression ratios shown as \log_2 values. Magenta represents increased expression; green represents decreased expression compared to control. C, Expression patterns of eight DEG candidates. Vertical axis shows fold enrichment of relative transcript levels between transgenic and wild-type plants. Data obtained with T3 generation seedlings. Error bars represent \pm sd from three independent experiments. *P* value of Student's *t* test: stressed plants compared with unstressed plants. **P* < 0.05; ***P* < 0.01; ****P* < 0.001



tissue by agroinfiltration, along with p35S::*ZmOXS2b*, p35S::*ZmO2L1*, or an empty vector control. In this transactivation assay, enhanced expression of pDEG-*luc* by p35S::*ZmOXS2b* and p35S::*ZmO2L1* was seen only with the three BOXS2-containing constructs (Fig. 3A). None of the other five DEG promoters were affected, except that the DEG30 promoter showed repression by p35S::*ZmOXS2b* and p35S::*ZmO2L1*.

A chromatin immunoprecipitation-quantitative PCR (ChIP-qPCR) analysis was performed to test the *in vivo* interaction of these promoters in transgenic wild-type Arabidopsis producing FLAG-tagged *ZmOXS2b* or *ZmO2L1*, which showed similar Cd tolerance as the non-FLAG-tagged lines (data not shown). Following immunoprecipitation with anti-FLAG antibody, two pairs of primers were used for each promoter corresponding to fragments F1-F16 (Fig. 3, A and B). Positive interaction for *ZmOXS2b* and *ZmO2L1* was found for F3, F9, and F12 (Fig. 3, A and B), but not for the other fragments, including the *ACT2* (At3g18780) promoter used as negative control. Interestingly, only these three fragments span across DNA segments that encompass the BOXS2 element. It is tempting to

suggest that *ZmOXS2b* and *ZmO2L1* bind the BOXS2 element, as was found with *AtOXS2*.

Overexpression of DEG23 Enhances Cd Tolerance in Arabidopsis

Each of the eight DEG candidates was fused to the *Cauliflower mosaic virus* 35S RNA promoter for expression in Arabidopsis. Approximately 10 independent transgenic lines were generated for each candidate. None showed an aberrant phenotype that differs from wild-type plants under normal growth conditions. Three independent lines of each genotype were randomly selected for testing. Under normal conditions, qRT-PCR analysis found 4- to 33-fold overexpression in these T2 generation lines compared to the wild-type control (Supplemental Fig. S4). When tested on Cd (75 μ M), only plants overexpressing DEG23 (AT5G37990) grew stronger with more biomass than the wild-type control (Fig. 4, A and B; Supplemental Fig. S2D). However, the 75 μ M concentration of Cd in synthetic media is too high for culturing the plants beyond the small plantlet stage.

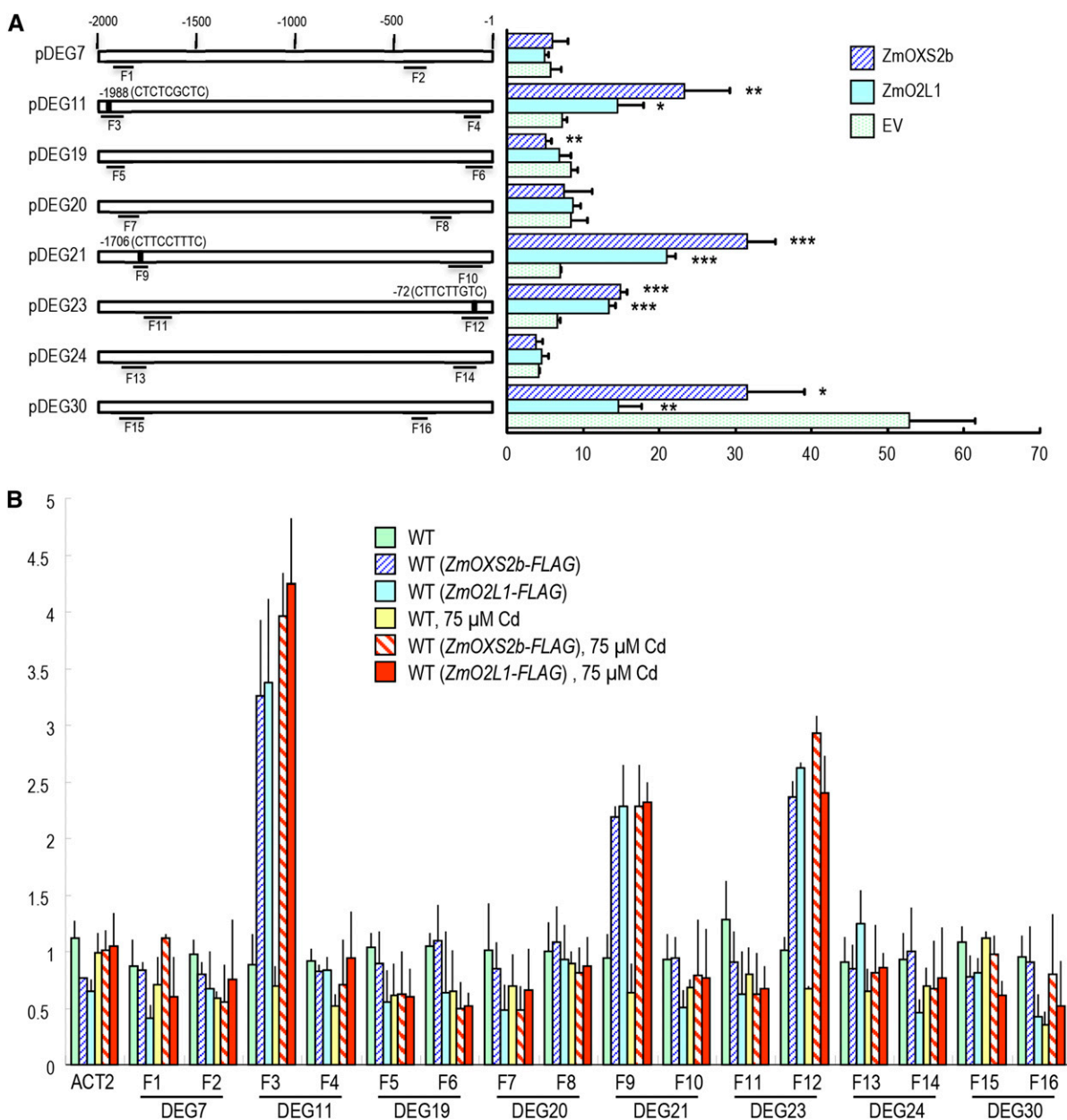


Figure 3. ZmOXS2b and ZmO2L1 activate and interact with BOXS2-containing promoters. A, ZmOXS2b and ZmO2L1 activation of DEG promoters (promoter fragment include 5' UTR) determined by infiltration mediated transient expression assay. x axis is the ratio of LUC to rLUC activity 2 d after infiltration. Black boxes in promoters indicate putative BOXS2 motifs. Numbers indicate position of starting nucleotide of each BOXS2 relative to translation start. Error bars show \pm SD from three independent experiments. *P* value of Student's *t* test: ZmOXS2b or ZmO2L1 compared with empty vector. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. B, ZmOXS2b and ZmO2L1 interact with BOXS2-containing promoters DEG11, DEG21, and DEG23. ChIP-qPCR to test in vivo interaction of promoters (including 5' UTR) with ZmOXS2b or ZmO2L1 in tissues from the wild type, WT(*ZmOXS2b*-FLAG), and WT(*ZmO2L1*-FLAG) treated with or without 75 μ M Cd. Promoter or 5' UTR segments tested are labeled F1-F16 (shown in A). CP (crossing point) value of immunoprecipitated DNA fractions with α -FLAG or no antibody control (NoAb) normalized to CP value of input DNA fractions for the same qPCR assay. y axis is the ChIP signals calculated as the enrichment relative to the no antibody control (No Ab). Error bars indicate \pm SD from three independent experiments on T2 seedlings.

Therefore, we grew plants in soil for 3 weeks and treated them with 75 μ M Cd for another week before harvest for Cd measurement. However, WT(DEG23)

did not show a statistically significant difference in Cd content compared to wild-type, *oxs2-1*, *o2l1-1*, WT (*ZmOXS2b*), or WT(*ZmO2L1*) (Supplemental Fig. S5A).

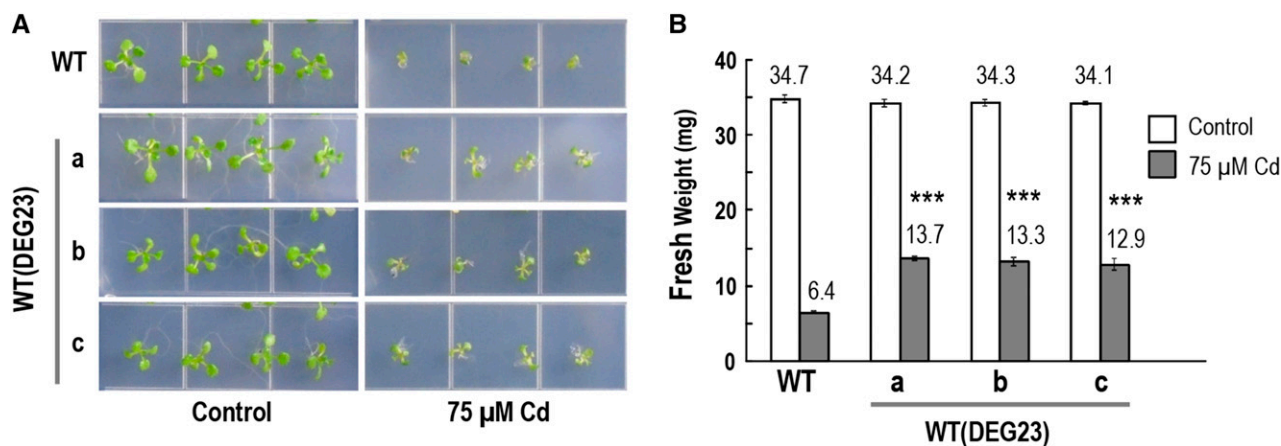


Figure 4. Overexpression of DEG23 (*CIMT1*) confers Cd Tolerance in Arabidopsis. A, Plants grown on 0.5× MS plates horizontally without or with 75 μM CdCl₂ for 12 d. Three independent transgenic lines shown. Representative data from three independent experiments on T2 seedlings. B, Average fresh weight of 20 seedlings (12 d old) grown on 0.5× MS without or with 75 μM CdCl₂ (measured in batches of five seedlings). Error bars indicate ±SD from three independent experiments. *P* value of Student's *t* test: transgenic plants compared with wild-type plants. ****P* < 0.001.

Previously, Huang et al. (2012) reported differences in Cd accumulation between wild type and transgenic Arabidopsis when grown in soil with 5 μM Cd. We also tried this same treatment, but as before, a difference in Cd accumulation in root, shoot, or seed from the wild-type level was not found among WT(DEG23), WT(*ZmOXS2b*), and WT(*ZmO2L1*) (Supplemental Fig. S5B). It does not appear that Cd tolerance mediated by DEG23, *ZmOXS2b*, and *ZmO2L1* is due to a difference in Cd accumulation.

The fact that overexpression of DEG23 on its own is sufficient to enhance Cd tolerance indicates that this gene plays a role in ameliorating the toxic effects of Cd. Since DEG23 encodes a putative member of the superfamily of *S*-adenosyl-*L*-Met-dependent methyltransferases, we named the gene *CIMT1* (*Cd-Inducible Methyltransferase 1*).

ZmOXS2 Family and AtOXS2 Interacts with Distinct and Overlapping BOXS2-Containing Promoters

To explore in greater detail of the interaction between the *CIMT1* promoter and *ZmOXS2b* or *ZmO2L1*, four more pairs of primers were used against the *CIMT1* (DEG23) promoter corresponding to fragments F17-F20 (Fig. 5A). Again, ChIP-qPCR showed positive signal for *ZmOXS2b* or *ZmO2L1* interaction only with the BOXS2-containing fragments F12 and F20, and this interaction is independent on Cd treatment (Fig. 5A).

Previously, we reported that AtOXS2 activates BOXS2-containing promoters but overexpression of AtOXS2 did not confer Cd tolerance (Blanvillain et al., 2011). To examine whether AtOXS2 might interact with the three BOXS2-containing promoters (F3 in DEG11 promoter, F9 in DEG21 promoter, and F12 in *CIMT1* promoter; Fig. 5B) that showed

interaction with *ZmOXS2b* and *ZmO2L1*, a ChIP-qPCR was carried out with the *oxs2-1* mutant transgenic for a 35S promoter-driven *AtOXS2:FLAG* construct. As shown in Figure 5B, interaction was not detected with fragments F3 or F12, but with F9, and the interaction was not affected by Cd stress (Fig. 5B). This suggests that DEG21 could be a target of AtOXS2. Indeed, expression of DEG21, but not the other DEGs, was elevated by overexpression of *AtOXS2* (Fig. 6A). However, since overexpression of DEG21 alone could not enhance Cd tolerance, it is consistent with the previous finding that *AtOXS2* overexpression could not enhance Cd tolerance (Blanvillain et al., 2011). The lack of AtOXS2 recognition of the *CIMT1* F12 fragment is also consistent with the data that AtOXS2 could not elevate expression of *CIMT1* (Fig. 6A).

CIMT1 Expression Is Cd Inducible Only in Shoot

To check the tissue-specific expression of *CIMT1*, the plant lines WT(*ZmOXS2*), WT(*ZmO2L1*), WT(*CIMT1*), and wild-type control were grown without or with CdCl₂. With 75 μM Cd, the root lengths were rather short, especially with the wild-type control, and this made it difficult to separate the roots from the shoots. Hence, we grew the plants at 25 μM Cd. The wild-type control shows that Cd up-regulates *CIMT1* mRNA abundance (~20×) in shoot but down-regulates its abundance (~30%) in root (Fig. 6B). WT(*ZmOXS2*), WT(*ZmO2L1*), and WT(*CIMT1*) all showed a similar pattern of regulation, although compared to the wild type, higher mRNA abundance is found in the absence or presence of Cd treatment. This suggests that overexpression did not alter the overall pattern, but strengthened transcriptional

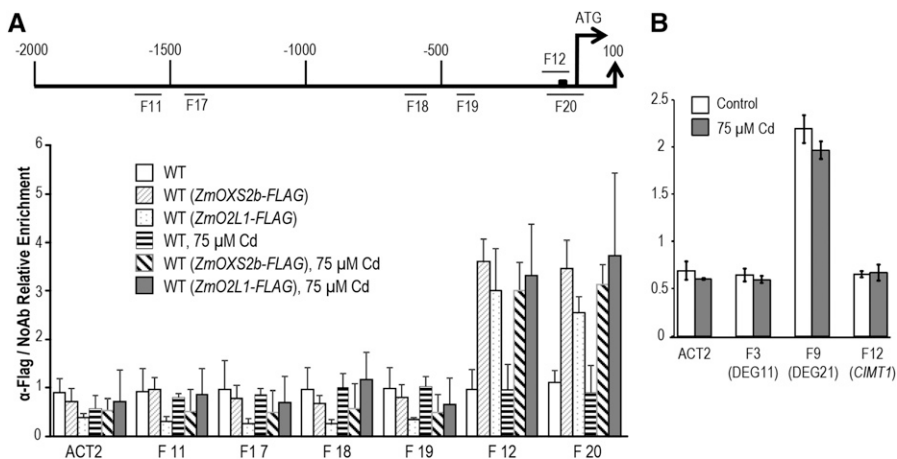


Figure 5. Only one BOXS2-containing promoter interacts with all three proteins: ZmOXS2b, ZmO2L1, and AtOXS2. ChIP with FLAG-tagged ZmOXS2b, ZmO2L1, or AtOXS2 performed on tissues from plants treated without or with 75 μ M Cd. Primers against *ACT2* promoter used as negative control. A, ChIP with FLAG-tagged ZmOXS2b or ZmO2L1. F11, F17, F18, F19, F12, and F20 indicate *CIMT1* (DEG23) promoter segments tested with qPCR. Black box indicates putative BOXS2 motif CTCTTGTC. Numbers indicate position of starting nucleotide of BOXS2 relative to translation start. B, ChIP with FLAG-tagged AtOXS2 shows binding to BOXS2-containing fragment F9 in DEG21 promoter, but not those within F3 of DEG11 or F12 of *CIMT1* (see Fig. 3A). Data show average \pm SD of three independent experiments on T2 seedlings.

output. Given that *CIMT1* mRNA in wild-type plants is down-regulated in root in response to Cd, it is possible that the higher root mRNA abundance in the transgenic plants may not be physiologically relevant, as

compared to the elevated shoot transcript level, which were up-regulated by as much as 2.5-fold in WT (*CIMT1*). Alternatively, or additionally, it is possible that the higher shoot *CIMT1* transcript abundance in

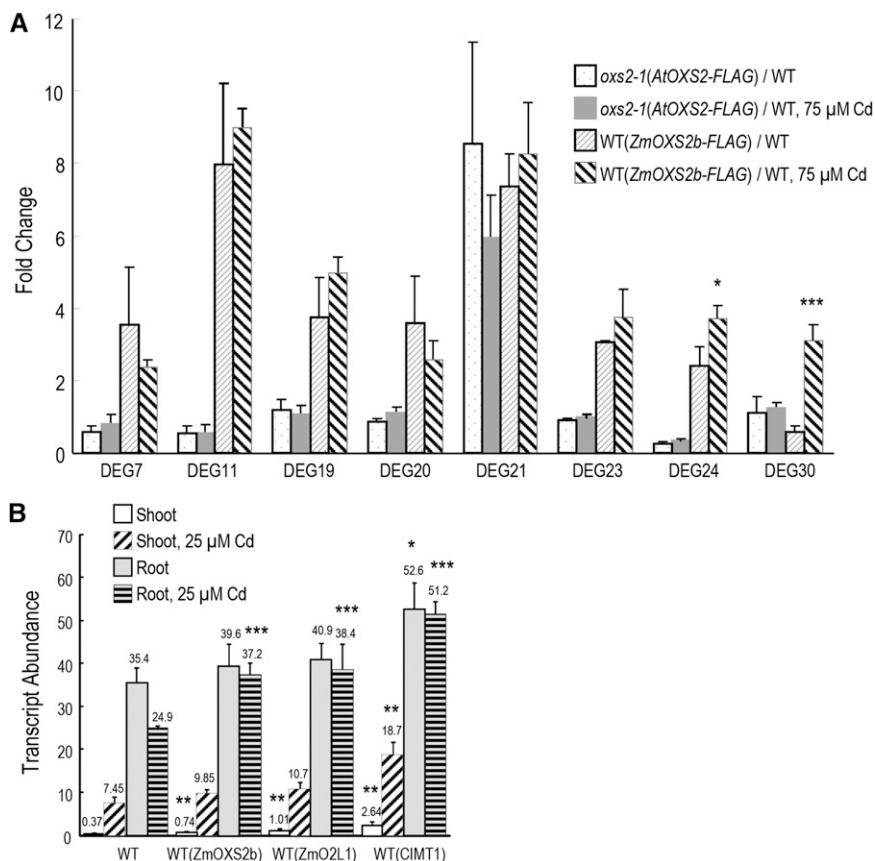


Figure 6. Expression pattern of DEGs and organ-specific analysis of *CIMT1*. Transcript abundance in Arabidopsis is determined by qRT-PCR. A, Whole seedlings grown for 11 d without or with 75 μ M CdCl₂ were assayed for fold change of DEG transcription between transgenic plants over wild-type control. *P* value of Student's *t* test: stressed plants compared with unstressed plants. B, Shoot and root expression of *CIMT1* (relative to *ACT1* control) in 11-d-old Arabidopsis seedlings exposed to 0 or 25 μ M CdCl₂; T5 for WT(*ZmOXS2b*) and WT(*ZmO2L1*) and T3 for WT(*CIMT1*). Error bars indicate \pm SD from three independent experiments. *P* value of Student's *t* test: WT(*ZmOXS2b*), WT(*ZmO2L1*), and WT(*CIMT1*) compared with the wild type. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

the absence of Cd might prime the plant for a more robust stress response.

DISCUSSION

Previous research in *Arabidopsis* has led to our current model on OXS2 regulation of oxidative stress (Blanvillain et al., 2011). In this working model, stress induces OXS2 translocation from the cytoplasm to the nucleus. Nuclear OXS2 along with other family members activate the stress tolerance pathway to alleviate the stress. At a higher stress level, nuclear OXS2 autoactivates its own promoter to produce higher levels of the transcription factor commensurate to the stress challenge. Upon relief of stress, OXS2 accumulates in the cytoplasm where it is needed to resume vegetable growth. In the case of a very high level of stress, where tolerance pathways are unable to cope with the damage, nuclear OXS2 activates the stress escape response, inducing reproduction to insure survival of the species over that of the individual. Much has been learned of the molecular details of this stress escape pathway. In transactivation assays, OXS2 has been shown to activate several flowering genes: the floral integrators *SOC1* and *LFY*, and the floral identity gene *AP1*. In ChIP-qPCR assays, OXS2 binds to the *SOC1* promoter and to a fragment that encompasses a BOXS2 motif. Mutation analysis shows that stress-induced expression of *SOC1* depends on OXS2 and other family members.

In contrast, not much is known on how OXS2 activates the stress tolerance pathway. Presumably, there are target genes that OXS2 acts on, and a goal of this study was to find these stress tolerance pathway targets. The most logical route is to continue with the research on the *Arabidopsis* OXS2 family of proteins. However, since we were eager to embrace translational research to a major crop plant, we isolated the two maize homologs. Identifying target genes in maize would be the ultimate goal, but since generating transgenic maize takes much greater effort, we regressed back to using transgenic *Arabidopsis* for a first analysis. Given that OXS2 family members of *Arabidopsis*, maize, and rice (*Oryza sativa*) all can enhance stress tolerance in the heterologous organism *Schizosaccharomyces pombe* (data not shown), we considered it possible that all of them recognize similar target genes in evolutionarily conserved stress tolerance pathways.

Indeed, the RNA-seq analysis of overexpressed maize OXS2 genes revealed a number of DEGs in the *Arabidopsis* transgenic lines, among which at least 88 are up-regulated. Finding many genes is consistent with an expectation that stress tolerance requires the activity of numerous proteins to alleviate the stress challenge. It would also justify the need for upstream regulators to orchestrate their expression. Therefore, it was quite surprising to find that expression of a single target gene, *CIMT1*, was sufficient to enhance Cd tolerance to a level similar to that from expression of

ZmOXS2b or *ZmO2L1*. This effect may be due to the much higher dosage of expression. In *ZmOXS2b* and *ZmO2L1* expressing lines, *CIMT1* was activated by as much as 5-fold (Fig. 2C; Supplemental Table S4), whereas in the *CIMT1* overexpression lines, this gene transcript was elevated by 14- to 17-fold (Supplemental Fig. S4).

As shown by ChIP-qPCR, *CIMT1* is indeed a target of *ZmOXS2b* and *ZmO2L1*. Moreover, these proteins interact with a segment of the *CIMT1* promoter that contains a BOXS2 motif. However, a follow-up ChIP-qPCR experiment failed to show *AtOXS2* interaction with this promoter (Fig. 5B), nor was *CIMT1* mRNA up-regulated in the *AtOXS2*-overexpressing line (Fig. 6A). This at least partially explains why overexpressing *AtOXS2* fails to confer Cd tolerance in *Arabidopsis*, since it cannot activate this gene. However, this does not rule out *CIMT1* as a target of other members of the *AtOXS2* family.

CIMT1 is a root-specific protein under normal growth condition (Baerenfaller et al., 2008), but its shoot mRNA can be induced by Cd treatment (Fig. 6B). *CIMT1* is one of 25 SAM-dependent methyltransferases in *Arabidopsis*. Highly conserved in all branches of life, SAM-dependent methyltransferases catalyze the transfer of methyl groups from SAM to a broad range of substrates including DNA, proteins, and small metabolites. In plants, stress hormones such as salicylic acid and jasmonic acid are both substrates of these enzymes (Lee et al., 2007; Seo et al., 2001). With a Rossmann-like superfold, *CIMT1* belongs to the Class I methyltransferases, the largest group of methyltransferases catalyzing the majority of methylation reactions in all kinds of organisms. As far as we are aware, this particular member has not been functionally characterized in *Arabidopsis*. Hence, this research provides direct evidence of its involvement in plant heavy metal tolerance. We presume that a similar homolog would exist in maize. However, with over 20 homologs in the database, some effort is needed to sort out which maize homolog(s) might play a similar role in response to Cd stress. Perhaps we could follow the clue that its promoter would likely be regulated by *ZmOXS2b* and *ZmO2L1*, and thus through ChIP-qPCR with these proteins, we may be able to fish out the true ortholog(s).

As reported previously (Blanvillain et al., 2011), subcellular localization patterns differ among members of *AtOXS2* family proteins. Only *AtOXS2* and *AtO2L1* translocate from the cytoplasmic to the nucleus during stress. *AtO2L2* is largely cytoplasmic, while *AtO2L3* and *AtO2L4* are mainly in the nucleus. As for the subcellular localization of *ZmOXS2b*, *ZmO2L1*, and *AtCIMT1*, a protoplast transient expression assay of GFP-fused proteins in *Arabidopsis* showed that all three proteins were found in both the cytoplasm and the nucleus, and this pattern is not altered by Cd treatment (Supplemental Fig. S6). Nuclear *ZmOXS2b* and *ZmO2L1* fit their role in transcription, but whether there is also a cytoplasmic role is not clear. For *AtCIMT1*, as a putative methyl transferase, its subcellular localization

pattern could raise the possibility of substrate(s) in both the nucleus and cytoplasm.

To conclude, we have shown that maize *OXS2* family proteins can enhance Cd tolerance in Arabidopsis, resulting in the differential expression of a large number of genes. From this large collection of leads, we have at least narrowed down one gene, *CIMT1*, as a target of the maize transcription factors. More importantly, from a biotechnology point of view, its expression alone can enhance Cd tolerance. This redirects our focus to finding a similar *CIMT1* in maize that may someday be engineered for higher stress tolerance in a major crop.

MATERIALS AND METHODS

Plant Treatments

Arabidopsis (*Arabidopsis thaliana*) wild-type Col-0 (SALK_6000) and mutants *oxs2-1* (SALK_037470) and *o2l1-1* (SALK_020612) have been described (Blanvillain et al., 2011). Arabidopsis plants were grown in a controlled environment at 22°C in a 16-h-light/8-h-dark photoperiod.

Plant transgene expression constructs were transformed into *Agrobacterium tumefaciens* strain GV3101 by the floral dip procedure. Transgenic plants were selected with phosphinothricin (10 µg/mL) or hygromycin (50 µg/mL). Seeds used for phenotypic assays were harvested at the same time. The growth assay was performed on plates with 0.5× MS solid media without or with diamide (1 and 2 mM), NaCl (100 and 150 mM), Cd (25 and 75 µM), mannitol (100 and 200 mM), and abscisic acid (1.5 and 3.0 µM). Heat shock treatment was conducted in a 37°C chamber for 3 h and cold treatment at 4°C for 3 h with 10-d-old plants.

Sweet corn (cv FengTian1) was germinated (5 d in water, 4 d without water) and grown in MS hydroponic cultures with 30 min aeration twice per day in a controlled environment at 22°C with a 16-h-light/8-h-dark photoperiod. After 5 d of growth in hydroponic culture, plantlets were placed into fresh culture without or with 200 µM Cd. Leaf samples were collected at 0, 3, 6, 12, 24, and 48 h. Three leaves from independent plants were mixed as one sample, flash frozen with liquid nitrogen, and stored at -80°C.

For Cd accumulation measurement, Arabidopsis plants were grown in soil for 3 weeks (for 75 µM CdCl₂ treatment) and 4 weeks (for 5 µM CdCl₂ treatment) and then bottom flooded once with 0.4 liters per pot (pot size 0.4 liters) of CdCl₂ with indicated concentrations of CdCl₂ solution followed by normal watering without Cd. Whole seedlings were collected 1 week after 75 µM CdCl₂ treatment. As for plants treated with 5 µM CdCl₂ shoots, roots and seeds were collected for sampling. Cd²⁺ content was measured with the 7700X ICP-MS (Agilent).

Protoplast of Arabidopsis were prepared and transfected according to standard protocol by Dr. Jen Sheen's lab (http://molbio.mgh.harvard.edu/sheenweb/protocols_reg.html).

Molecular Constructs

For expression constructs *ZmOXS2b* and *ZmO2L1*, DNA was PCR amplified from sweet corn (cv FengTian1) genomic DNA with primer sets *ZmOXS2b*-1F/*ZmOXS2b*-1R and *ZmO2L1*-1F/*ZmO2L1*-1R, respectively (all primers listed in Supplemental Table S5). Seven of the eight DEGs were PCR cloned with one pair of primers against Arabidopsis genomic DNA, with 5' and 3' UTRs included. For DEG21 (AT4G13420), which is over 5,500 bp (genomic DNA, UTRs included), we divided the PCR amplification into three parts and fused them together using In-Fusion HD cloning kit (catalog no. 011614; Clontech). All primers were designed according to information from the TAIR website (<http://www.arabidopsis.org>). Each was inserted into the *Xba*I site of binary vector pCambia3300 (<http://www.cambia.org>) to yield p35S::*ZmOXS2b*, p35S::*ZmO2L1*, and p35S::DEGs. For binary vector with FLAG, *ZmOXS2b* and *ZmO2L1* open reading frame (ORF) fragments without stop codon were PCR amplified with primers *ZmOXS2b*-2F and *ZmOXS2b*-2R or *ZmO2L1*-2F and *ZmO2L1*-2R. The fragments were cloned into binary vector pCambia1305 that contains a FLAG tag after the *Xba*I site. To make the 35S::*AtOXS2*-FLAG construct, *AtOXS2* coding region without its stop codon was PCR amplified from

genomic DNA with primers *AtOXS2*-F and *AtOXS2*-R and inserted between *Kpn*I and *Pst*I sites on pCambia1305. Promoters including 5' UTR of the eight DEG candidates (2,000 bp upstream of ATG) were PCR cloned and fused to a firefly luciferase ORF. Likewise, a double enhancer 35S promoter was fused to the Renilla luciferase ORF. Each of the luciferase fusions was inserted into the *Xba*I site of pCambia3300 using In-Fusion HD cloning kit (catalog no. 011614; Clontech).

For subcellular localization vectors, *ZmOXS2b* and *ZmO2L1* ORF fragments without stop codon were PCR amplified with primers *ZmOXS2b*-3F and *ZmOXS2b*-3R or *ZmO2L1*-3F and *ZmO2L1*-3R. *ZmOXS2b* and *ZmO2L1* were inserted between *Kpn*I and *Spe*I or *Xba*I and *Bgl*III in pGFP vectors, respectively. *CIMT1* ORF without stop codon was PCR amplified with primers *CIMT1* F and *CIMT1* R and cloned into *Sac*I and *Bam*HI in frame with eGFP in vector pCambia3300.

qRT-PCR

RNA extraction was conducted using a plant RNA kit (catalog no. R5105; GBCBio Technologies). Reverse transcription was conducted using PrimeScript RT reagent kit with gDNA Eraser (catalog no. RR047A; TaKaRa). qPCR was conducted with SYBR Premix Ex Taq (catalog no. DRR820A; TaKaRa) on LightCycler 480 II (Roche). Maize (*Zea mays*) elongation factor 1-α (*EF1-α*; NM_001112117) and Arabidopsis ACTIN1 (*ACT1*, AT2G37620) were used as internal controls.

Transactivation Assay

Nicotiana benthamiana plants were grown in soil in a controlled environment at 28°C, with a 14-h-light/10-h-dark photoperiod. Infiltration was done on 5- to 6-week-old plants. Single clones of GV3101 carrying different vectors were inoculated to LB medium containing 10 µg/mL rifampicin and 50 µg/mL kanamycin and grown for more than 24 h at 28°C. One hundred microliters of near-saturation *Agrobacterium tumefaciens* was inoculated to 5 mL fresh LB medium containing 10 µg/mL rifampicin, 50 µg/mL kanamycin, 10 mM MES (pH 5.6) buffer, and 20 µM acetosyringone and grown for more than 8 h at 28°C. Cells were collected by centrifugation (4,000 rpm, 10 min), resuspended to an OD₆₀₀ of 0.8 infiltration medium (10 mM MgSO₄, 200 µM acetosyringone, and 10 mM MES), and incubated at room temperature for 3 h. Infiltration medium contains three *A. tumefaciens* strains: 1 mL of transcription activator strain (p35S::*ZmOXS2b*, p35S::*ZmO2L1*, or empty vector), 100 µL promoter strain (DEG promoter-*luc*), and 5 µL reference strain (35S-*rLUC*). Infiltration carried out with healthy *N. benthamiana* leaves using a 1-mL syringe without needle. After infiltration, plants were kept in a dark chamber with high humidity for one night and then put back to a normal growth room for 2 d. Luciferase values measured with Dual-Luciferase Reporter Assay System (catalog no. E1910; Promega).

Chromatin Immunoprecipitation

Fresh tissue (1 g, whole seedlings) was infiltrated in 25 mL of 1% formaldehyde MC buffer (10 mM potassium phosphate, pH 7.0, 50 mM NaCl, and 0.1 M Suc) for 1 h at 20 p.s.i. on ice and placed on ice for another 1 h. The reaction was stopped by adding Gly powder to 0.15 M final concentration and incubated at 4°C for 40 min. Samples were washed twice with MC buffer at 4°C for 40 min. Tissues were ground in a cold mortar to make a relatively thick slurry with M1 buffer (10 mM potassium phosphate, pH 7.0, 0.1 M NaCl, 10 mM β-mercaptoethanol, 1 M hexylene glycol, and 1 mM PMSF), centrifuged at top speed at 4°C for 3 min, and the pellet washed thoroughly four times with 1 mL M2 buffer (10 mM potassium phosphate, pH 7.0, 0.1 M NaCl, 10 mM β-mercaptoethanol, 1 M hexylene glycol, 10 mM MgCl₂, and 0.5% Triton X-100) supplemented with protease inhibitor cocktail (catalog no. 78410; Thermo), then washed with 1 mL M3 buffer (10 mM potassium phosphate, pH 7.0, 0.1 M NaCl, and 10 mM β-mercaptoethanol). The pellet was resuspended in 0.75 mL of sonication buffer (10 mM potassium phosphate, pH 7.0, 0.1 M NaCl, 10 mM EDTA, pH 8.0, and 0.5% Sarkosyl) and vortexed for 30 s. Chromatin complexes were sonicated to DNA fragment sizes 250 to 500 bp (cycles of 5-s sonication/5-s pause, 9 min) and centrifuged at top speed for 5 min at 4°C. Chromatin supernatants were kept in IP buffer (50 mM HEPES, pH 7.5, 150 mM KCl, 5 mM MgCl₂, 1% Triton X-100, and 0.05% SDS) and incubated without or with anti-FLAG (1:500) and Dynabeads Protein A (catalog no. 10002D; Life Technologies)/protein G (catalog no. 10004D; Life

Technologies) mixtures (1:1) overnight at 4°C with gentle agitation. After several rounds of washing with IP buffer, the pellets were eluted using elution buffer (50 mM Tris, pH 8.0, 1% SDS, and 10 mM EDTA) followed by reverse cross-link and purification. DNA fragments were quantified by qPCR with SYBR Premix ExTaq Mix (catalog no. DRR820A; TaKaRa). The CP (crossing point) value of immunoprecipitated DNA fractions with α -FLAG or no antibody control (NoAb) normalized to CP value of input DNA fractions for the same qPCR assay. The ChIP signals were calculated as the relative enrichment in signal relative to the NoAb control. All experiments were repeated three times.

RNA-Seq Library Construction and Sequencing

The wild type, WT(*ZmOXS2b*), and WT(*ZmO2L1*) were grown on 0.5× MS plates supplemented with 75 μ M CdCl₂ for 11 d. Whole seedlings were collected and sent to BGI-Tech for RNA-seq analysis. Total RNA isolation, library construction, sequencing, and basic data analysis were carried out by BGI-Tech.

Screening of DEGs and Expression Pattern Analysis of DEGs (Heat Map)

Expression level calculated as RPKM (reads per kilobase per million reads) according to Mortazavi et al. (2008) was compared between transgenic plants (with either *ZmOXS2b* or *ZmO2L1*) and wild-type control. NOISeq approach was used to evaluate the significance of the gene expression differences (Tarazona et al., 2011). Clustering software was used to perform cluster analysis of gene expression patterns (de Hoon et al., 2004). Assessment of RNA-seq quality, screening of DEGs, and expression pattern analysis of DEGs were carried out by BGI-Tech.

Other Customized Data Analysis for RNA-Seq

Gene Ontology analysis was carried out by BGI-Tech. BOXS2 analysis in Arabidopsis genome was conducted by GENE DENOVO with transcription factor binding site software (<http://tfbs.genereg.net>).

Accession Numbers

Sequence data for the RNA-seq samples can be found in the NCBI database under the following accession numbers: SAMN04259680, SAMN04259681, SAMN04259682, SAMN04259683, SAMN04259684, and SAMN04259685.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. OXS2 homolog proteins in maize.

Supplemental Figure S2. Overexpression of *ZmOXS2s* or *DEG23(CIMT1)* enhances cadmium tolerance.

Supplemental Figure S3. Expression of eight DEG candidates in response to Cd treatment.

Supplemental Figure S4. Relative transcription of DEGs in WT(DEGs)/wild type.

Supplemental Figure S5. Cd accumulation in different genetic backgrounds

Supplemental Figure S6. Subcellular localization of AtCIMT1, *ZmOXS2b*, and *ZmO2L1* fused to GFP and transiently expressed in Arabidopsis protoplasts.

Supplemental Table S1. Sequence differences found between *ZmOXS2* homologs from sweet corn and information from NCBI.

Supplemental Table S2. Summary of differentially expressed genes discovered by RNA-seq.

Supplemental Table S3. Significantly enriched Gene Ontology terms of DEGs.

Supplemental Table S4. RNA-seq and qRT-PCR values of 30 DEGs in intersection of C1 and C2.

Supplemental Table S5 Primer sequences.

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LITERATURE CITED

- Baerenfaller K, Grossmann J, Grobei MA, Hull R, Hirsch-Hoffmann M, Yalovsky S, Zimmermann P, Grossniklaus U, Gruissem W, Baginsky S (2008) Genome-scale proteomics reveals Arabidopsis thaliana gene models and proteome dynamics. *Science* **320**: 938–941
- Blanvillain R, Wei S, Wei P, Kim JH, Ow DW (2011) Stress tolerance to stress escape in plants: role of the OXS2 zinc-finger transcription factor family. *EMBO J* **30**: 3812–3822
- Chen YX, He YF, Luo YM, Yu YL, Lin Q, Wong MH (2003) Physiological mechanism of plant roots exposed to cadmium. *Chemosphere* **50**: 789–793
- Clemens S, Kim EJ, Neumann D, Schroeder JI (1999) Tolerance to toxic metals by a gene family of phytochelatin synthases from plants and yeast. *EMBO J* **18**: 3325–3333
- de Hoon MJL, Imoto S, Nolan J, Miyano S (2004) Open source clustering software. *Bioinformatics* **20**: 1453–1454
- Hartwig A, Schwerdtle T (2002) Interactions by carcinogenic metal compounds with DNA repair processes: toxicological implications. *Toxicol Lett* **127**: 47–54
- Huang J, Zhang Y, Peng JS, Zhong C, Yi HY, Ow DW, Gong JM (2012) Fission yeast HMT1 lowers seed cadmium through phytochelatin-dependent vacuolar sequestration in Arabidopsis. *Plant Physiol* **158**: 1779–1788
- Kim DY, Bovet L, Maeshima M, Martinoia E, Lee Y (2007) The ABC transporter AtPDR8 is a cadmium extrusion pump conferring heavy metal resistance. *Plant J* **50**: 207–218
- Küpper H, Parameswaran A, Leitenmaier B, Trtílek M, Setlík I (2007) Cadmium-induced inhibition of photosynthesis and long-term acclimation to cadmium stress in the hyperaccumulator *Thlaspi caerulescens*. *New Phytol* **175**: 655–674
- Lee J, Nam J, Park HC, Na G, Miura K, Jin JB, Yoo CY, Baek D, Kim DH, Jeong JC, et al (2007) Salicylic acid-mediated innate immunity in Arabidopsis is regulated by SIZ1 SUMO E3 ligase. *Plant J* **49**: 79–90
- Li ZS, Lu YP, Zhen RG, Szczypka M, Thiele DJ, Rea PA (1997) A new pathway for vacuolar cadmium sequestration in *Saccharomyces cerevisiae*: YCF1-catalyzed transport of bis(glutathionato)cadmium. *Proc Natl Acad Sci USA* **94**: 42–47
- McLaughlin MJ, Parker DR, Clarke JM (1999) Metals and micronutrients - food safety issues. *Field Crops Res* **60**: 143–163
- Mittler R, Vanderauwera S, Gollery M, Van Breusegem F (2004) Reactive oxygen gene network of plants. *Trends Plant Sci* **9**: 490–498
- Mortazavi A, Williams BA, McCue K, Schaeffer L, Wold B (2008) Mapping and quantifying mammalian transcriptomes by RNA-Seq. *Nat Methods* **5**: 621–628
- Nriagu JO, Pacyna JM (1988) Quantitative assessment of worldwide contamination of air, water and soils by trace metals. *Nature* **333**: 134–139
- Patra M, Bhowmik N, Bandopadhyay B, Sharma A (2004) Comparison of mercury, lead and arsenic with respect to genotoxic effects on plant systems and the development of genetic tolerance. *Environ Exp Bot* **52**: 199–223
- Perfus-Barbeoch L, Leonhardt N, Vavasseur A, Forestier C (2002) Heavy metal toxicity: cadmium permeates through calcium channels and disturbs the plant water status. *Plant J* **32**: 539–548
- Prasad MNV (1995) Cadmium toxicity and tolerance in vascular plants. *Environ Exp Bot* **35**: 525–545
- Sandalio LM, Dalurzo HC, Gómez M, Romero-Puertas MC, del Río LA (2001) Cadmium-induced changes in the growth and oxidative metabolism of pea plants. *J Exp Bot* **52**: 2115–2126

- Seo HS, Song JT, Cheong JJ, Lee YH, Lee YW, Hwang I, Lee JS, Choi YD** (2001) Jasmonic acid carboxyl methyltransferase: a key enzyme for jasmonate-regulated plant responses. *Proc Natl Acad Sci USA* **98**: 4788–4793
- Sytar O, Kumar A, Latowski D, Kuczynska P, Strzalka K, Prasad MNV** (2012) Heavy metal-induced oxidative damage, defense reactions, and detoxification mechanisms in plants. *Acta Physiol Plant* **35**: 985–999
- Takiguchi M, Achanzar WE, Qu W, Li G, Waalkes MP** (2003) Effects of cadmium on DNA-(Cytosine-5) methyltransferase activity and DNA methylation status during cadmium-induced cellular transformation. *Exp Cell Res* **286**: 355–365
- Tarazona S, García-Alcalde F, Dopazo J, Ferrer A, Conesa A** (2011) Differential expression in RNA-seq: a matter of depth. *Genome Res* **21**: 2213–2223
- Waalkes MP** (2003) Cadmium carcinogenesis. *Mutat Res* **533**: 107–120
- Wagner GJ** (1993) Accumulation of cadmium in crop plants and its consequences to human health. *Adv Agron* **51**: 173–212
- Waisberg M, Joseph P, Hale B, Beyersmann D** (2003) Molecular and cellular mechanisms of cadmium carcinogenesis. *Toxicology* **192**: 95–117
- Yadav SK** (2010) Heavy metals toxicity in plants: An overview on the role of glutathione and phytochelatins in heavy metal stress tolerance of plants. *S Afr J Bot* **76**: 167–179