Maize OXIDATIVE STRESS 2 homologs enhance cadmium tolerance in Arabidopsis through activation of a putative SAM-dependent methyltransferase gene

Lilong He1,2, Xiaoling Ma1,2, Zhenzhen Li1,2, Zhengli Jiao1,2, Yongqing Li1* and David W. Ow1*

1Plant Gene Engineering Center, South China Botanical Garden, Chinese Academy of Sciences, Guangzhou 510650, China
2University of Chinese Academy of Sciences, 19 Yuquan Road, Beijing 100049, China.

*Correspondence: David W. Ow email: dow@scbg.ac.cn; Yongqing Li email: liyongqing@scbg.ac.cn

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ABSTRACT

Previously the *Arabidopsis thaliana* zinc finger protein OXIDATIVE STRESS 2 (AtOX2S2) and four OXS2-like (AtO2L) family members were described to play a role in stress
tolerance and stress escape. For stress escape, SOCI was a target of AtOX2. However, for stress tolerance, the downstream targets were not identified. We cloned two OXS2 homolog genes from sweet corn, ZmOX2b and ZmO2L1. Both genes are transiently inducible by Cd treatment. When expressed in Arabidopsis, each enhances tolerance against cadmium. Further analysis showed that ZmOX2b and ZmO2L1 proteins enhance Cd tolerance in Arabidopsis by activating at least one target gene, that encoding a putative S-adenosyl-L-methionine-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 AtOX2. 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 ZmOX2b and ZmO2L1 to Arabidopsis CIMT1 suggests a similar network may exist in maize, and may provide a clue to possibly using a CIMT1 maize homolog to engineer stress tolerance in a major crop.
INTRODUCTION

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 (Kupper 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 non-enzymatic 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 zinc finger protein OXS2 (OXIDATIVE STRESS 2) 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 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, 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-methionine-dependent methyltransferases superfamily, which we named CIMT1 for Cd inducible methyltransferase 1. Although *CIMT1* can be activated by ZmOX2b or ZmO2L1, it is not activated by AtOX2. This may help explain why AtOX2 overexpression does not enhance Cd tolerance. From a crop improvement perspective, the link of ZmOX2b and ZmO2L1 to *CIMT1* suggests a possible connection of ZmOX2b 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 (Basic Local Alignment Search Tool) 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 Like 1, 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 the current 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 hours after treatment, but returned to basal level at the 12 hour time point. At the 24 and 48 hour 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 hours with accumulation of mRNA.

ZmOXS2b and ZmO2L1 Enhance Cd Tolerance in Arabidopsis

ZmOXS2b or ZmO2L1 was fused behind the CaMV 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 4 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 µM), diamide (1, 2 mM), NaCl (100, 150 mM), mannitol (100, 200 mM), ABA (1.5, 3 µM), 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 µM), the root length, shoot growth and biomass of WT(ZmOXS2b) and WT(ZmO2L1) plants were greater than the untransformed controls (Fig. 1B, C and 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 WT (Supplemental Fig. S2B, 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 WT control.

The expression profile of WT(ZmOXS2b) compared to the WT control is hereafter referred to as comparison 1 or C1; likewise, WT(ZmO2L1) compared to WT is C2. Differentially expressed genes (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.
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, 8 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 WT control plants were again cultured on 1/2MS media without or with 75 μM CdCl2 for 11 days for qRT-PCR analysis of these 8 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 WT 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 7 genes that are normally up-regulated by Cd in Arabidopsis.
ZmOX2b and ZmO2L1 Activate BOXS2-Containing Promoters

AtOX2 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 8 genes, the promoters of DEG11, DEG21 and DEG23, but not the other 5 DEGs, contain sequences similar to a BOXS2 motif (Fig. 3A). We fused the firefly luciferase reporter gene (luc) to a 2kb promoter including its 5’ UTR fragment from each of the 8 DEG candidates to generate all 8 promoter-luc fusions. Each of these constructs was transiently introduced into tobacco leaf tissue by agro-infiltration, along with p35S::ZmOX2b, p35S::ZmO2L1 or an empty vector control. In this transactivation assay, enhanced expression of pDEG-luc by p35S::ZmOX2b and p35S::ZmO2L1 was seen only with the three BOXS2 containing constructs (Fig. 3A). None of the other 5 DEG promoters were affected, except that the DEG30 promoter showed repression by p35S::ZmOX2b and p35S::ZmO2L1.

A ChIP-qPCR analysis was performed to test the in vivo interaction of these promoters in transgenic WT Arabidopsis producing FLAG-tagged ZmOX2b 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. 3A, B). Positive interaction for ZmOX2b and ZmO2L1 was found for F3, F9 and F12 (Fig. 3A, 3B), but not for the other fragments, including the ACT2 (At3g18780) promoter used as negative control. Interestingly, only these 3 fragments span across DNA segments that encompass the BOXS2 element. It is tempting to suggest that ZmOX2b and ZmO2L1 bind the BOXS2 element, as was found with AtOX2.

Overexpression of DEG23 enhances Cd tolerance in Arabidopsis

Each of the 8 DEG candidates was fused to the CaMV 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 WT control (Supplemental Fig.
When tested on Cd (75 µM), only plants overexpressing DEG23 (AT5G37990) grew stronger with more biomass than the WT control (Fig. 4A, B, Supplemental Fig. S2D). However, the 75 µM concentration of Cd in synthetic media is too high for culturing the plants beyond the small plantlet stage. Therefore, we grew plants in soil for 3 weeks and
treated them with 75 µM Cd for another week before harvest for Cd measurement.

However, WT(DEG23) did not show a statistically significant difference in Cd content compared to WT, oxs2-1, o2l1-1, WT(ZmOXs2b) or WT(ZmO2L1) (Supplemental Fig. S5A).

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 WT
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-methionine-dependent
methyltransferases, we name the gene *CIMT1* (*Cadmium 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, 4 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 3 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-l*
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(*ZmOX2*)
WT(ZmO2L1), WT(CIMT1) and WT control were grown without or with CdCl2. With 75 µM Cd, the root lengths were rather short, especially with the WT control and this made it difficult to separate the roots from the shoots. Hence, we grew the plants at 25 µM Cd. The WT control shows that Cd up-regulates CIMT1 mRNA abundance (~20X) in shoot, but
down-regulates its abundance (~30%) in root (Fig. 6B). WT(ZmOX2), WT(ZmO2L1) and WT(CIMT1) all showed a similar pattern of regulation, although compared to the WT, 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 output. Given that CIMT1 mRNA in WT 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 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 relieve 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 current 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. To identify 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 all can enhance stress tolerance in the heterologous organism *S. pombe* (data not shown), we had 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 14-17 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). Neither 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 methyltransferase, 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 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, 16 hour light/8 hour 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 1/2MS solid media without or with diamide (1, 2 mM), NaCl (100, 150 mM), Cd (25 µM, 75 µM),
mannitol (100, 200 mM), ABA (1.5, 3.0 µM). Heat shock treatment was conducted in a 37°C chamber for 3 hours and cold treatment at 4°C for 3 hours with 10-day-old plants.

Sweet corn (cv. FengTian1) was germinated (5 days in water, 4 days without water) and grown in MS hydroponic cultures with 30 min aeration twice per day in a controlled environment at 22°C, 16 hour light/8 hour dark photoperiod. After 5 days of growth in hydroponic culture, plantlets were placed into fresh culture without or with 200 µM Cd. Leaf samples were collected at time points 0 h, 3 h, 6 h, 12 h, 24 h 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 L per pot (pot size 0.4 L) of CdCl₂ with indicated concentrations of CdCl₂ solution followed by normal watering without Cd. Whole seedlings were collected one 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 by 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**

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 8 DEGs were PCR cloned with one pair of primers against *Arabidopsis* genomic DNA, with 5′- and 3′ UTR included. For DEG21 (AT4G13420) which is over 5,500 bp (genomic DNA, UTR’s included), we divided the PCR amplification into three parts and fused them together using In-Fusion® HD Cloning Kit (Cat# 011614, Clontech). All primers were designed according to information from the TAIR website (http://www.arabidopsis.org). Each was inserted into the *XbaI* 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* ORF fragments.
without stop codon were PCR amplified with primers ZmOX2b-2F and ZmOX2b-2R or
ZmO2L1-2F and ZmO2L1-2R. The fragments were cloned into binary vector
pCambia1305 that contains a FLAG tag after the XbaI site. To make the
35S::AtOX2-FLAG construct, AtOX2 coding region without its stop codon was PCR
amplified from genomic DNA with primers AtOX2-2F and AtOX2-2R and inserted between
KpnI and PstI sites on pCambia1305. Promoters including 5′UTR of the 8 DEG candidates
(2000 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 XbaI site of pCambia3300 using In-Fusion® HD
Cloning Kit (Cat# 011614, Clontech).

For subcellular localization vectors, ZmOX2b and ZmO2L1 ORF fragments without stop
codon were PCR amplified with primers ZmOX2b-3F and ZmOX2b-3R or ZmO2L1-3F
and ZmO2L1-3R. ZmOX2b and ZmO2L1 were inserted between KpnI and SpeI or XbaI
and BglII in pGFP vector, respectively. CIMT1 ORF without stop codon was PCR amplified
with primers CIMT1 F and CIMT1 R and cloned into SacI and BamHI in frame with eGFP in
vector pCambia3300.

qRT–PCR
RNA extraction was conducted using a plant RNA kit (Cat# R5105, GBCBIO
Technologies). Reverse transcription was conducted using PrimeScript™ RT reagent Kit
with gDNA Eraser (Cat# RR047A, TaKaRa). qPCR was conducted with SYBR® Premix Ex
Taq™ (Cat# DRR820A, TaKaRa) on LightCycler® 480 II (Roche). Zea mays elongation
factor 1-alpha (EFL-α, NM_001112117) and Arabidopsis ACTIN1 (ACT1, AT2G37620) was
used as internal control.

Tranactivation Assay
Nicotiana benthamiana plants were grown in soil in a controlled environment at 28°C, with
a 14 hour light/10 hour 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 hours at
28°C. 100 μL of near-saturation Agrobacterium were inoculated to 5 mL fresh LB medium containing 10 μg/ml rifampicin, 50 μg/ml kanamycin, 10 mM MES (pH 5.6) buffer, 20 μM acetylsyringone and grown for more than 8 hours at 28°C. Cells were collected by centrifugation (4000 rpm, 10 min), re-suspended to an OD600 of 0.8 infiltration medium (10 mM MgSO4, 200 μM acetylsyringone, 10 mM MES) and incubated at room temperature for 3 hours. Infiltration medium contains 3 Agrobacterium strains: 1 ml of transcription activator strain (p35S::ZmOXS2b, p35S::ZmO2L1 or empty vector), 100μL 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 days. Luciferase values measured with Dual-Luciferase® Reporter Assay System (Cat# E1910, Promega).

Chromatin Immuno-Precipitation

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, 0.1M sucrose) for 1 h at 20 psi on ice and placed on ice for another 1h. The reaction was stopped by adding glycine 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 pre-cold mortar to make a relatively thick slurry with M1 buffer (10mM potassium phosphate pH 7.0, 0.1 M NaCl, 10 mM beta-mercaptoethanol, 1 M hexylene glycol, 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 beta-mercaptoethanol, 1 M hexylene glycol, 10 mM MgCl2, 0.5% Triton X-100) supplemented with protease inhibitor cocktail (Cat# 78410, Thermo), then washed with 1ml M3 buffer (10 mM potassium phosphate pH 7.0, 0.1 M NaCl, 10 mM beta-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, 0.5% sarkosyl) and vortexed for 30 s. Chromatin complexes were sonicated to DNA fragment sizes 250-500 bp (cycles of 5 s sonication/5 s pause, 9 min) and centrifuged at top speed.
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, 0.05% SDS) and incubated without or with anti-FLAG (1:500) and Dynabeads® Protein A (Cat# 10002D, Life Technologies AS)/protein G (Cat# 10004D, Life Tecnolgies AS) 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, 10 mM EDTA) followed by reverse cross-link and purification. DNA fragments were quantified by qPCR with SYBR® Premix ExTaq™ Mix (Cat# DRR820A, TaKaRa). CP (crossing point) value of immuno-precipitated 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**

WT, WT(ZmOXS2b) and WT(ZmO2L1) were grown on 1/2MS plates supplemented with 75 μM CdCl2 for 11 days. Whole seedlings were collected and sent to BGI-Tech (Shenzhen, China) for RNA-seq analysis. Total RNA isolation, library construction, sequencing and basic data analysis were carried out by BGI-Tech. NCBI database accession numbers of RNA-seq samples are SAMN04259680, SAMN04259681, SAMN04259682, SAMN04259683, SAMN04259684, SAMN04259685.

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

Expression level calculated as RPKM (Reads Per Kb 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

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

Supplemental Material

The following supplemental materials are available

1. Supplemental Figure S1. OXS2 homolog proteins in maize.
2. Supplemental Figure S2. Overexpression of ZmOXS2s or DEG23(CIMT1) enhances cadmium tolerance.
3. Supplemental Figure S3. Expression of 8 DEG candidates in response to Cd treatment.
4. Supplemental Figure S4. Relative transcription of DEGs in WT(DEGs) / WT.
5. Supplemental Figure S5. Cd accumulation in different genetic backgrounds
6. Supplemental Figure S6. Subcellular localization of AtCIMT1, ZmOXS2b and ZmO2L1 fused to GFP and transiently expressed in *Arabidopsis* protoplasts.
7. Supplemental Table S1. Sequence differences found between ZmOXS2 homologs from sweet corn and information from NCBI.
8. Supplemental Table S2. Summary of differentially expressed genes discovered by RNA-seq.
9. Supplemental Table S3. Significantly enriched Gene Ontology (GO) terms of DEGs.
10. Supplemental Table S4. RNA-seq and qRT-PCR values of 30 DEGs in intersection of C1 and C2.
11. Supplemental Table S5. Primer sequences.

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FIGURE LEGENDS

Figure 1. ZmOXS2 family members in stress response. A, ZmOXS2b and ZmO2L1 transcript abundance in maize (relative to EF1-a control) determined by qRT–PCR. Fifteen-days-old maize seedlings exposed to 0 or 200 µM CdCl2. Error bars indicate ± SD from three independent experiments. B, Arabidopsis plants grown on 1/2MS plates horizontally without or with 75 µM CdCl2 for 11 days. Three independent homozygous lines transgenic for each gene shown. Representative data from 3 reproducible experiments with T3 seedlings. C, Average fresh weight of 20 T4 seedlings (11-day-old) grown on 1/2MS without or with 75 µM CdCl2 (measured in batches of 5 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

Figure 2. RNA-seq analysis and qRT-PCR verification. A, Venn diagram of differentially expressed genes (DEGs). C1= WT(ZmOXS2b) VS WT; C2= WT(ZmO2L1) VS WT. Total numbers of DEGs, up- and down-regulated DEGs shown in magenta and green lettering, respectively. Red italic lettering indicate 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 log2 values. Magenta color represents increased expression, green color decreased expression compared to control. C, Expression patterns of 8 DEG candidates. Vertical axis shows fold enrichment of relative transcript levels between transgenic and WT plants. Data obtained with T3 generation seedlings. Error bars represent ± 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

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 two days 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 ± 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 WT, WT(ZmOXS2b-FLAG) and WT(ZmO2L1-FLAG) treated with or without 75 μM Cd. Promoter or 5’UTR segments tested are labeled F1-F16 (shown in A). CP (crossing point) value of immuno-precipitated 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 ± SD from three independent experiments on T2 seedlings.

Figure 4. Overexpression of DEG23 (CIMT1) confers Cd Tolerance in Arabidopsis. A, Plants grown on 1/2MS plates horizontally without or with 75 μM CdCl₂ for 12 days. Three independent transgenic lines shown. Representative data from three independent experiments on T2 seedlings. B, Average fresh weight of 20 seedlings (12-day-old) grown on 1/2MS without or with 75 μM CdCl₂ (measured in batches of 5 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.

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 μM Cd. Primers against ACT2 promoter used as negative control. A, ChIP with FLAG-tagged ZmOXS2b or ZmO2L1. F11, F17, F18, F19, F12, F20 indicate CIMT1 (DEG23) promoter segments tested with qPCR. Black box indicates putative BOXS2 motif CTTCTTGTC. 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 ±SD of three independent experiments on T2 seedlings.

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 days without or with 75 μM CdCl₂ were assayed for fold change of DEG transcription between transgenic plants over WT 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-day-old Arabidopsis
seedlings exposed to 0 or 25 µM CdCl₂; T5 for WT(ZmOXS2b) and WT(ZmO2Li) and T3 for WT(CIMT1). Error bars indicate ± SD from three independent experiments. P value of Student's t-test: WT(ZmOXS2b), WT(ZmO2Li) and WT(CIMT1) compared with WT. *: P<0.05; **: P<0.01; ***: P<0.001.


