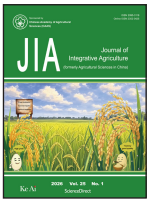
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LETTER

Overexpression of *OsCAX2* in *indica* rice reduces cadmium accumulation in grains without yield loss

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Highlights

- OsCAX2 is localized to tonoplast, and cadmium induces its expression.
- *OsCAX2* overexpression reduces cadmium concentration in *indica* rice grains by 49.1%.

Cadmium (Cd) exposure poses significant health risks to humans, and the International Agency for Research on Cancer has classified it as a Group I carcinogen. Cadmium undergoes minimal metabolism in the human body; consequently, prolonged Cd^{2+} exposure can cause severe damage to multiple organs including the liver, kidneys, lungs, bones, and immune system (Shao *et al.* 2024). Rice, one of the three global staple crops, and Cd exposure in humans primarily occurs the consumption of contaminated rice grains. The contribution of rice to the total dietary Cd intake is over 50% for non-smoking Asian populations (Chen *et al.* 2018; Shi *et al.* 2020). Current mitigation strategies focus on soil remediation and breeding low Cd accumulating cultivars (Zhao *et al.* 2022). Key regulatory genes such as *OsHMA3* and *OsNRAMP5* have been identified: enhanced-function haplotypes of *OsHMA3* reduce grain Cd accumulation by 50–90% without yield loss, while *OsNRAMP5* knockout achieves >90% Cd reduction but severely impairs manganese uptake (Ueno *et al.* 2010; Sasaki *et al.* 2012, 2014; Tang *et al.* 2017; Lu *et al.* 2019; Zhou *et al.* 2019). Additionally, *OsLCT1*, which regulates Cd^{2+} transport to rice grains, serves as a potential target for gene editing strategies (Uraguchi *et al.* 2011). Southern China's *indica* rice-growing regions face elevated Cd contamination risks due to soil acidification, industrial pollution, and the inherently higher Cd accumulation capacity of *indica* than *japonica* subspecies (Guo *et al.* 2010; Zhu *et al.* 2016; Yang *et al.* 2018). This study demonstrates that overexpression of a Ca^{2+}/H^{+} exchanger-encoding gene *OsCAX2* in the *indica* rice cultivar 'Huanghuazhan (HHZ)', the most widely cultivated cultivar in southern China, significantly reduced Cd^{2+} concentration in brown rice.

The cation/H^{+} exchanger (CAX) family is a class of cation transporters that transport divalent metal ions, including calcium (Ca), manganese (Mn), and Cd, to perform essential biological functions. These transport proteins primarily localize to the membrane systems in plant cells (Kamiya *et al.* 2005; Korenkov *et al.* 2007; Wu *et al.* 2011). In rice, tonoplast-localized OsCAX1a and OsCAX1c, as well as plasma membrane-localized OsCAX4, have been demonstrated to possess Cd^{2+} transport activity through yeast heterologous expression assays (Zou *et al.* 2021). Overexpression of the tonoplast protein genes *AtCAX1*, *AtCAX2*, *TuCAX1a*, and *TuCAX1* in different plants effectively reduces Cd^{2+} translocation to shoots, thereby enhancing plant tolerance to Cd^{2+} toxicity (Korenkov *et al.* 2007; Qiao *et al.* 2019). Phylogenetic analysis reveals the highest amino acid sequence homology between OsCAX2 and AtCAX2 (Appendix A). Notably, transient expression assays in rice protoplasts revealed that the OsCAX2:GFP fusion protein was specifically localized to the tonoplast (Fig. 1-A), suggesting that overexpression of *OsCAX2* may confer similar tolerance to Cd^{2+} toxicity through vacuolar compartmentalization.

Quantitative reverse transcription polymerase chain

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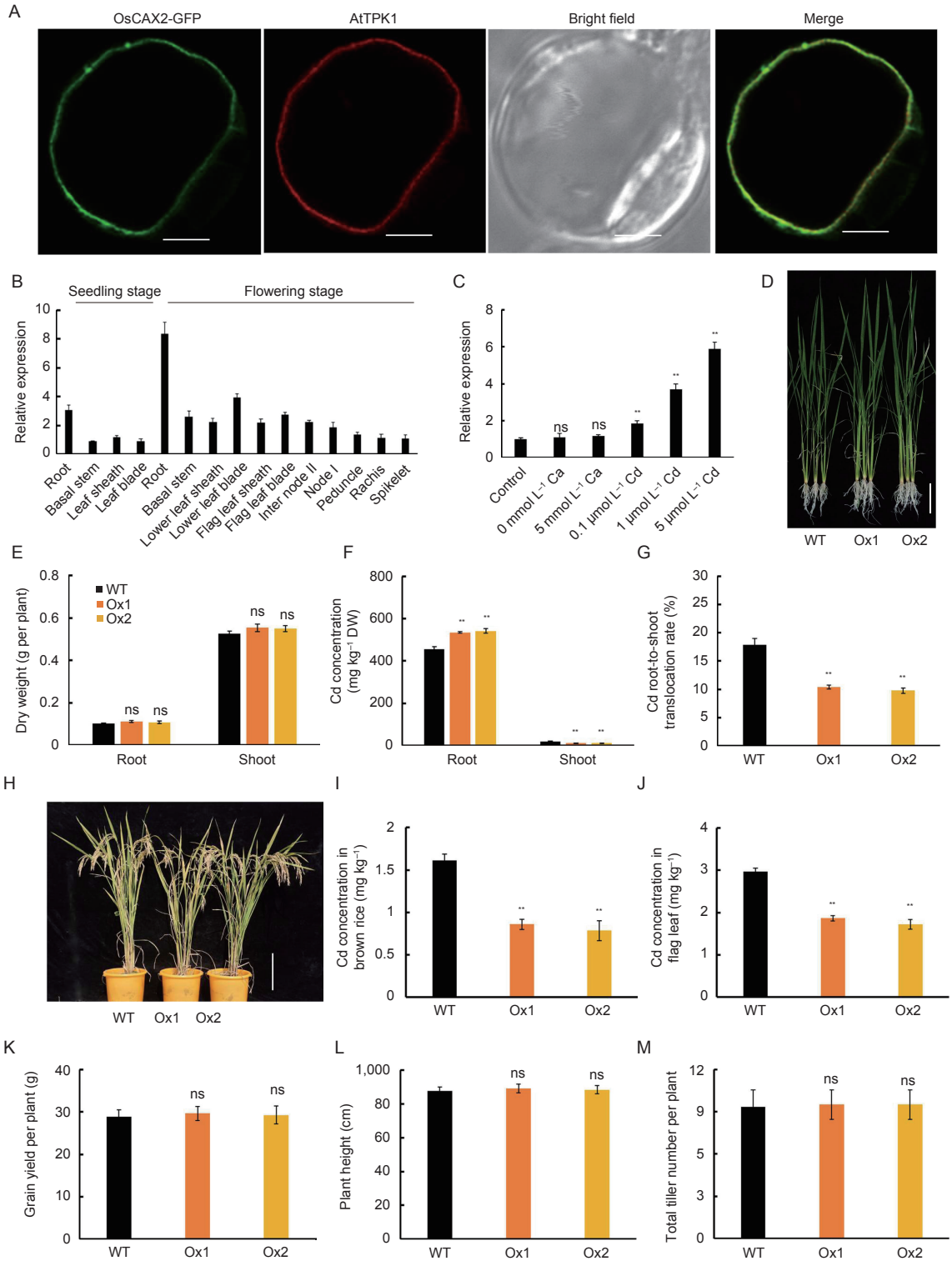


Fig. 1 Overexpression of *OsCAX2* reduces cadmium (Cd) accumulation in *indica* rice. A, subcellular localization of OsCAX2; red fluorescence indicates the subcellular localization of the vacuolar membrane-localized AtTPK1:RFP fusion protein, bar=5 μm. B, relative expression of *OsCAX2* in different tissues during the seedling and flowering stages of rice. The relative expression was calculated using the $2^{-\Delta C_T}$ method. Bars mean SD (n=3). C, relative expression of *OsCAX2* in roots under exposure to varying concentrations of Ca^{2+} and Cd^{2+}. The relative expression was calculated using the $2^{-\Delta\Delta C_T}$ method. Bars mean SD (n=3). D, phenotypes of overexpression lines and wild-type (WT) rice under 0.1 μmol L^{-1} Cd^{2+} treatment, bar=15 cm. Biomass (E), Cd concentration (F), and Cd translocation (G) in overexpression lines and WT rice under 0.1 μmol L^{-1} Cd^{2+} treatment. H, phenotypes of overexpression lines and WT rice at maturity stage under 1 mg kg^{-1} Cd^{2+} treatment, bar=30 cm. Cd concentration in brown rice (I) and flag leaves (J) of overexpression lines and WT rice under 1 mg kg^{-1} Cd^{2+} treatment. Grain yield per plant (K), plant height (L), and tiller number (M) of overexpression lines and WT rice under 1 mg kg^{-1} Cd^{2+} treatment. Bars mean SD (n=5). Asterisks denote statistically significant differences within the group compared with the WT rice, **, P<0.01, and ns indicates statistically non-significant differences (one-way ANOVA, Dunnett's test).

reaction (RT-qPCR) analysis revealed that *OsCAX2* was predominantly expressed in rice roots during the seedling and flowering stages (Fig. 1-B). The expression pattern of *OsCAX2* was examined under various Cd^{2+} concentrations to assess its response to Cd^{2+} stress. The analysis demonstrated enhanced *OsCAX2* expression with increasing Cd^{2+} concentrations. Notably, the expression of *OsCAX2* remained unaffected by Ca^{2+} concentrations (Fig. 1-C). This result aligns with previous studies indicating that among the five OsCAX family proteins in rice, OsCAX2 uniquely failed to confer Ca^{2+} tolerance in yeast heterologous expression systems (Kamiya *et al.* 2005).

To examine whether *OsCAX2* can reduce Cd accumulation in rice grains, we overexpressed *OsCAX2* from Nipponbare in the *indica* rice cultivar HHZ background. Sequence alignment revealed that the nucleotide sequence of *OsCAX2* and its corresponding protein sequence differ by only six bases and three amino acids between *indica* and *japonica* rice (Appendix B). Through RT-qPCR analysis, two independent transgenic lines with significantly elevated expression levels of *OsCAX2* (designated Ox1 and Ox2) were identified (Appendix C). Under hydroponic culture conditions, 2-wk-old seedlings of wild-type and overexpression lines were exposed to 0.1 μmol L^{-1} Cd^{2+} stress for 10 d, followed by phenotypic observation and statistical analysis. The results indicated that overexpression of *OsCAX2* did not induce significant phenotypic changes in rice plants, such as root or shoot dry weights (Fig. 1-D and E). However, analysis of Cd concentrations revealed that overexpression of *OsCAX2* significantly increased Cd accumulation in roots while substantially reducing Cd levels in shoots (Fig. 1-F). This result corresponds with Liu *et al.* (2025), who reported that overexpression of *OsCAX2* in *japonica* rice cultivar 'Zhonghua 11' also resulted in reduced Cd concentration in shoots and decreased root-to-shoot Cd translocation. Their observation of smaller rice seedlings and reduced Cd concentration in roots of overexpression lines differs from our findings in *indica* rice. Whether this inconsistency arises from differences in transformation varietal backgrounds or variations in treatment method (Liu *et al.* used 0.5 μmol L^{-1} Cd^{2+} for 5 days), requires further investigation. Given that *OsCAX2* localizes to the tonoplast, we hypothesize that its overexpression enhances sequestration of absorbed Cd within root cell vacuoles. Analysis of root-to-shoot Cd translocation showed a 43.7% reduction upon *OsCAX2* overexpression (Fig. 1-G). Consistent with the expression patterns and yeast tolerance experiments, overexpression of *OsCAX2* did not significantly affect Ca concentrations (Fig. 1-C; Appendix D; Kamiya *et al.* 2005). These results suggest that *OsCAX2* overexpression may significantly decrease Cd accumulation in grains of the cultivar HHZ.

To validate the effect of *OsCAX2* overexpression on Cd accumulation in rice grains, we applied 1 mg kg^{-1} Cd^{2+} to the soil during the experiment, and both wild-type and overexpression lines were cultivated until maturity (Fig. 1-H). Measurement of Cd concentration in brown grains and flag leaves revealed that the Cd concentrations in grains of overexpression lines decreased by 49.1% compared to the wild type, with a concurrent 39.7% reduction in flag leaves Cd concentration (Fig. 1-I and J). This finding indicates that *OsCAX2* significantly inhibits Cd translocation to grains by regulating Cd transport pathways. Analysis of yield-related traits demonstrated no statistical difference in grain yield per plant between overexpression lines and the wild type (Fig. 1-K). Important agronomic characteristics including plant height, tiller number, panicle weight, seed-setting rate, biomass per plant, and grain-to-straw ratio remained unaltered (Fig. 1-L and M; Appendix E). These results confirm that *OsCAX2* overexpression reduces Cd accumulation in grains by approximately 50% while maintaining normal rice growth and development, providing both a theoretical foundation and crucial targets for screening of *indica* rice germplasm to develop low Cd-accumulating rice varieties.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

Declaration of generative AI and AI-assisted technologies in the writing process

The authors declare that they did not use AI in the preparation and writing of this manuscript.

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