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RESEARCH PAPER

Genetic engineering low-arsenic and low-cadmium rice grain

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Abstract

Rice is prone to take up the toxic elements arsenic (As) and cadmium (Cd) from paddy soil through the transporters for other essential elements. Disruption of these essential transporters usually adversely affects the normal growth of rice and the homeostasis of essential elements. Here we report on developing low-As and low-Cd rice grain through the co-overexpression of *OsPCS1*, *OsABCC1*, and *OsHMA3* genes under the control of the rice *OsActin1* promoter. Co-overexpression of *OsPCS1* and *OsABCC1* synergistically decreased As concentration in the grain. Overexpression of *OsPCS1* also decreased Cd concentration in the grain by restricting the xylem-to-phloem Cd transport in node I, but paradoxically caused Cd hypersensitivity as the overproduced phytochelatins in *OsPCS1*-overexpressing plants suppressed OsHMA3-dependent Cd sequestration in vacuoles and promoted Cd transport from root to shoot. Co-overexpression of *OsHAM3* and *OsPCS1* overcame this suppression and complemented the Cd hypersensitivity. Compared with non-transgenic rice control, co-overexpression of *OsABCC1*, *OsPCS1*, and *OsHMA3* in rice decreased As and Cd concentrations in grain by 92.1% and 98%, respectively, without causing any defect in plant growth and reproduction or of mineral nutrients in grain. Our research provides an effective approach and useful genetic materials for developing low-As and low-Cd rice grain.

Keywords: Arsenic, cadmium, OsABCC1, OsHMA3, OsPCS1, phytochelatin, rice, vacuolar sequestration.

Introduction

Cadmium (Cd) and arsenic (As) are a toxic metal and metalloid, respectively, that may cause acute and chronic adverse health effects in humans (Hughes, 2002; Fowler, 2009). Rice, feeding more than half the world's population, is a major dietary source of Cd and As (Clemens and Ma, 2016; Zhao and Wang, 2019). Compared with other cereal crops, rice is likely to accumulate more As and Cd due to its growth environment and efficient uptake and transportation systems for the two toxic elements (Ma *et al.*, 2008; Xu *et al.*, 2008; Sui *et al.*, 2018). It is a great challenge to simultaneously control Cd and As accumulation in the rice grain by using traditional approaches, including breeding low-grain arsenic rice varieties, and water and nutrient management. However, these strategies all have their own limitations. Complicated interactions between genetics and environmental factors make the genotype selection of low-As rice varieties difficult (Norton *et al.*, 2009a, b; Ahmed *et al.*, 2010; Duan *et al.*, 2017). More importantly, the accumulation of Cd and As exhibited opposite correlations with the heading date among a broad range of rice cultivars, making it challenging to select rice varieties with both low Cd and low As (Duan *et al.*, 2017). Anaerobic conditions markedly reduce the availability of Cd, due to the formation of insoluble

cadmium sulfide (CdS). In contrast, anaerobic conditions promote the reduction of As(V) to As(III), which is more bioavailable than As(V). Therefore, As and Cd exhibit opposing reactions to water management in rice (Clemens and Ma, 2016). For example, sprinkler irrigation, a water management technique, reduced As but enhanced Cd accumulation in rice grain (Moreno-Jimenez *et al.*, 2014). In addition, silicon fertilization decreases total As in straw by 78%, but only by 16% in grain (Li *et al.*, 2009).

Over the past few decades, significant progress has been made in understanding the molecular mechanisms involved in As and Cd uptake, translocation, and detoxification in rice (Clemens and Ma, 2016; Zhao and Wang, 2019). The uptake and xylem loading of As(III) is mainly mediated by the silicon transporters, OsLsi1 and OsLsi2, respectively (Ma *et al.*, 2008). In the cytosol, non-coded peptide phytochelatins (PCs), synthesized from glutathione (GSH) by phytochelatin synthase (PCS) (Grill *et al.*, 1985; Ha *et al.*, 1999), chelate As(III) to form PC–As(III) complexes which are then sequestered into the vacuoles via a tonoplast transporter OsABCC1. This process plays an important role in As detoxification in rice (Song *et al.*, 2014; Hayashi *et al.*, 2017). In nodes, OsABCC1 is localized in the tonoplast of phloem companion cells in vascular bundles, and limits As transport in the grain by sequestering As into the vacuoles (Song *et al.*, 2014; Hayashi *et al.*, 2017). In rice, OsNramp5 is a major transporter for Cd uptake in the root, and it is mainly expressed in the root and localized to the plasma membrane of the distal side of the exodermis and endodermis (Ishikawa *et al.*, 2012; Sasaki *et al.*, 2012). In addition, the plasma membrane-localized transporters OsNramp1 and OsCd1 are also involved in root Cd uptake (Yan *et al.*, 2019; Chang *et al.*, 2020). After the uptake, Cd is transported into the vacuoles of root cells by the tonoplast transporter OsHMA3, which effectively inhibits Cd loading into the xylem and long-distance Cd translocation in the shoot; thereby overexpression of *OsHMA3* greatly reduces Cd accumulation in rice grain (Ueno *et al.*, 2010; Miyadate *et al.*, 2011). In addition, OsHMA2 and OsLCT1 are responsible for Cd distribution in nodes (Uraguchi *et al.*, 2011; Takahashi *et al.*, 2012; Yamaji *et al.*, 2013). Besides the cell-to-cell pathway, Cd can also enter plants through the apoplastic pathway, where water and solutes can move towards the stele through the extracellular space in the root. Under Cd stress, rice can enhance the apoplastic barrier development close to the root tip to reduce Cd transfer into the xylem via passive diffusion (Qi *et al.*, 2020).

Several efforts have been made to manipulate transporter genes to mitigate the accumulation of As or Cd in rice grains. However, As and Cd share transporters with other essential elements in rice, and the loss of function of these transporter genes leads to an adverse effect on plant growth, development, reproduction, or homeostasis of essential elements (Ma *et al.*, 2007, 2008; Sasaki *et al.*, 2012). For example, Nramp5 is an essential transporter for Cd and Mn uptake. Knockout of *OsNramp5* resulted in an impaired growth and yield, especially in low-Mn conditions (Sasaki *et al.*, 2012), although this phenomenon was not observed in a different study (Ishikawa *et al.*, 2012). Loss of function of silicon transporter genes *OsLsi1* and *OsLsi2* significantly reduced As accumulation in the grain, but also affected the normal growth of plants owing to Si deficiency (Ma *et al.*, 2007, 2008). Overexpression of the endogenous *OsPCS1* in rice significantly reduced As concentration in the grain (Hayashi *et al.*, 2017). However, the heteroexpression of the wheat PCS gene (*TaPCS1*) in rice led to Cd hypersensitivity, although the mechanism causing this phenomenon is not yet clear (Wang *et al.*, 2012). Thus, it is necessary to tackle the contradiction to utilizing PCS genes to control the accumulation of As and Cd in the rice grain. So far, no study has focused on reducing As and Cd concentrations in the rice grain simultaneously by genetic or genetic engineering approaches. Our objective was to generate low-As and low-Cd rice grain by enhancing As and Cd sequestration in vacuoles of cells in the root or other vegetative tissues without causing any pleiotropic phenotype, yield penalty, or change in the concentrations of mineral nutrients in rice.

Materials and methods

Genes, constructs, and rice transformation

Constructs for gene overexpression in rice were made based on the binary vector pCAMBIA1305.1 (accession no. AF304545). Briefly, the cauliflower mosaic virus (CaMV) 35S promoter upstream of the GUS*Plus*™ gene in the T-DNA region of pCAMBIA1305.1 was replaced by a 1414 bp promoter derived from the rice *OsActin1* gene (Os03g0718100) (Reece *et al.*, 1990). The coding region of the GUS*Plus*™ gene was substituted by the coding regions derived from the cDNA clones of *OsHMA3* (Os07g0232900) or *OsPCS1* (Os05g0415200), or the genomic clone of *OsABCC1* (Os04g0620000) to yield binary constructs pCHMA3-C, pCPCS1-C, and pCABCC1-G that harbour promoter fusion genes P_{Actin1}:*cHMA3*:T_{Nos}, P_{Actin1}:*cPCS1*:T_{Nos}, and P_{Actin1}:*gABCC1*:T_{Nos}, respectively (Supplementary Fig. S1). All constructs were introduced into the *Agrobacterium tumefaciens* strain AGL1 and used for rice transformation. The *Agrobacterium*-mediated transformation of T5105 was performed according to the procedures described previously, with slight modification (Hiei *et al.*, 1994); the 1 mg l^{-1} kinetin (KT) and 0.2 mg l^{-1} 1-naphthaleneacetic acid (NAA) in the rice regeneration medium N6S3-CH were replaced with 1 mg l^{-1} 6-benzylaminopurine (BA) and 1 mg l^{-1} NAA.

Plant materials, growth conditions, and As and/or Cd treatment

The rice cultivar used in this study was T5105, an improved aromatic *indica* rice in the genetic background of Thai fragrance KTML 105 (Luo and Yin, 2013). T5105 and transgenic plants were grown in pots filled with 5 kg of soil in a greenhouse at 24–33 °C under natural light. The control soil used in this study contained background levels of As at 2.09 mg kg^{-1} and Cd at 0.44 mg kg^{-1}. For soil treatment, the control soil was supplemented with 10 mg kg^{-1} As supplied as $NaAsO_2$ and/or 3 mg kg^{-1} Cd supplied as $CdSO_4$. Rice seedlings were grown in control soil in the nursery for 28 d. They were then transplanted onto soils with or without As and/or Cd treatment and grown to maturity in the greenhouse. Rice seeds and straws were harvested and dried for inductively coupled plasma (ICP)-MS analysis.

Southern blotting analysis

Genome DNA from transgenic rice was extracted using the HP plant DNA mini kit (Omega BIO-TEK). About 2 μg of DNA was digested with restriction enzymes *Hin*dIII and *Bam*HI (NEB). DNA fragments were separated on a 0.8% (w/v) agarose gel by gel electrophoresis. The fragments were then blotted from the agarose gel onto a Hybond-N^+ membrane (GE Healthcare). Digoxigenin (DIG)-labelled specific nucleic acid probes for the *hpt* gene were amplified by PCR using DIG DNA labelling Mix (Roche) and the primer pairs listed in Supplementary Table S1. Southern blot hybridization and detection of the DIG-labelled probes were performed according to the manufacturer's instruction using the DIG-High Prime DNA Labeling and Detection Starter Kit II (Roche). The ChemiDoc Touch imaging system (Bio-Rad) was used to detect the chemiluminescent signal.

Quantitative real-time PCR

Total RNA was extracted from leaf tissues of 8-week-old plants using a Favorprep plant total RNA purification mini kit (FAVORGEN), followed by DNA digestion using DNaseI (Roche). The first-strand cDNAs were synthesized from 1 μg of total RNA using a cDNA synthesis kit (Bio-Rad). Quantitative real-time PCR (qRT-PCR) with three technical replicates and three biological replicates was performed on a CFX96 real-time system (Bio-Rad) using SYB FAST qPCR Master Mix (KAPA Biosystems). The qRT-PCR results were normalized against rice elongation factor gene *OsEF-1α* (Os03g0178000) and rice ubiquitin gene *OsUBQ5* (Os01g0328400), respectively. The relative gene expression levels were determined using the $2^{-\Delta\Delta Ct}$ relative quantification method with the expression level in T5105 arbitrarily set to 1 (Livak and Schmittgen, 2001). The oligo DNA primers for qRT-PCR of different genes are listed in Supplementary Table S1.

Rice genome sequencing and identification of the T-DNA insertion sites

Genomic DNA was isolated from homozygous transgenic rice seedlings. High-throughput genome sequencing containing clean reads with 50-fold sequencing depth was conducted at BGI Genomics Co., Ltd. The T-DNA insertion sites were identified by aligning to the relevant regions of the T-DNA constructs, spanning from the left border to the right border, with Burrows–Wheeler Aligner (BWA) software and samtools. The T-DNA insertion sites were further confirmed by PCR.

Test of rice seedlings for tolerance to As and/or Cd

Rice seeds were surface-sterilized and germinated on half-strength Murashige and Skoog (MS) medium in Phytatray II vessels (Sigma-Aldrich) at 25 °C in a tissue culture room with a photoperiod of 16 h light and 8 h darkness. Two-week-old rice seedlings were transferred to half-strength MS medium containing different concentrations of $NaAsO_2$ (0–100 μM) and/or $CdSO_4$ (0–40 μM), and cultured for another 14 d. The root tissues of treated seedlings were washed three times with 5 mM $CaCl_2$ and deionized water, respectively. They were photographed before the shoot length was measured. The seedling samples were dried at 70 °C in an oven for 7 d and the dry weight of the seedlings was measured.

Measurement of the total concentration of phytochelatins in rice tissues

The non-protein thiols (NPTs) were extracted and determined according to previous reports, with minor modification (Schat and Kalff, 1992; Devi and Prasad, 1998). About 200 mg of fresh root or shoot tissues were ground in liquid nitrogen, and NPTs were extracted with 1 ml of buffer of a 5% (w/v) sulfosalicylic acid (SSA) solution with 6.3 mM diethylenetriaminepentaacetic acid (DTPA) (pH <1) at 0 °C. The homogenate was centrifuged at 10 000 *g* for 10 min at 4 °C. The concentration of NPTs in the supernatant was measured immediately. An aliquot of 300 μl of supernatant was mixed with 650 μl of 0.5 M K_2HPO_4, and the absorbance at 412 nM was measured after a 2 min incubation. The mixture was then added with 26.6 μl of DTNB solution [6 mM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), 0.143 M K_2HPO_4, 6.3 mM diethylenetriaminepentaacetic acid (DTPA), pH 7.5] and incubated for 2 min. The absorbance at 412 nM was measured again. The increase in absorbance was corrected for the absorbance of DTNB, and GSH was used as standard. The concentration of free GSH in the supernatants was measured using the Glutathione Fluorescent Detection Kit (Invitrogen). The total concentration of PCs was calculated as the total concentration of NPTs minus free GSH.

Element analysis by inductively coupled plasma mass spectrometry

The concentrations of As, Cd, Co, Cu, Fe, Mn, Se, and Zn in rice grain (de-husked but unpolished brown rice) and straw tissues were determined by ICP-MS. The roots and shoots of seedling plants, grains, and different straw tissues of adult plants at the harvest stage were collected and dried in an oven at 70 °C for 7 d. About 0.1 g of dried rice seeds or straw tissues were pre-digested with 3 ml of concentrated HNO_3/H_2O_2 mixture (5:1, v/v) overnight at room temperature, and then were digested in a microwave oven (Ethos One, Milestone Technologies) for 4 h. After dilution, the concentrations of elements in the digested solution were determined by ICP-MS (7700S, Agilent Technologies, USA). The rice flour NIST SRM 1568b was used for certified reference material (CRM) to assess the precision and accuracy of analysis procedures.

Isolation of intact protoplasts and vacuoles from rice mesophyll cells

The protoplasts were isolated from rice mesophyll cells as described previously (Trinidad *et al.*, 2021). In brief, the shoot tissues from 10-day-old rice seedlings germinated and grown in half-strength MS medium were cut into 0.5 cm strips. The protoplasts were released from the strips by adding protoplast isolation buffer [0.6 mannitol, 10 mM MES, 10 mM $CaCl_2$, 0.1% BSA (w/v), 1.5 % (w/v) cellulase RS (C0615, Sigma, USA), and 0.75% (w/v) pectinase RS (P2401, Sigma, USA)] followed by incubation in the dark with gentle shaking at 28 °C for 4 h. The protoplasts were collected by centrifugation at 150 *g* and 20 °C for 5 min using a swinging bucket rotor with slow acceleration and slow deceleration setting. The pellet was washed twice with W5 buffer (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, and 2 mM MES) and re-collected by spinning at 100 *g* for 3 min. Vacuoles were isolated from rice mesophyll cells using a modified method (Frangne *et al.*, 2002). Lysis buffer [0.2 M mannitol, 10% Ficoll-400, 15 mM EDTA (pH 8.0), 5 mM sodium phosphate (pH 8.0)] pre-warmed to 37 °C was added to the protoplasts. The protoplasts were resuspended gently by being pipetted up and down 5–8 times and lysed by incubation in a warm water bath at 37 °C for 5–10 min. The vacuoles released from protoplasts were purified by centrifugation on a three-step Ficoll-400 gradient. One volume of lysed protoplast suspension was overlaid with two volumes of Ficoll-400 solution (5% w/v), prepared by mixing one volume of lysis buffer and one volume of vacuole buffer (30 mM KCl, 20 mM HEPES-KOH, pH 7.5, 0.4 M betaine, 15 mg ml^{-1} BSA, and 1 mM DDT). One volume of vacuole buffer was then layered on the top of the gradient carefully but quickly. The vacuoles were collected on the interface between the 5% Ficoll-400 solution and vacuole buffer after centrifugation at 1500 *g* for 20 min.

Collection of xylem sap and phloem exudate, and mineral analysis

To analyse the Cd concentration in xylem sap and phloem exudate, rice plants at 10 d after flowering were transferred to quarter-strength MS medium containing 10 μM Cd for 4 d. Xylem sap collection was performed as previously described (Kan *et al.*, 2019). Rice stalks were cut at 1 cm above node I, and the cut surface was cleaned with deionized H_2O. A tube containing absorbent cotton was placed on the cut end of node I and the xylem sap was absorbed by the cotton for 6 h. The cotton was centrifuged at 12 000 *g* for 10 min to collect the xylem sap. The xylem sap was subjected to Cd determination using ICP-MS. Phloem exudate collection was performed according to previous reports (King and Zeevaart, 1974; Uraguchi *et al.*, 2011). Rice internode I with panicle removed was cut 1 cm above node I, and then the lower cut end was recut under 20 mM EDTA (pH 7.5) solution. The lower cut end of internode I was immediately immersed in 200 μl of EDTA solution, and the detached internode I was kept in darkness at 25 °C and high relative humidity (>85%) for 3 h. The EDTA solution containing phloem exudates was subjected to Cd determination using ICP-MS.

Statistical analysis

Data were analysed using two-tailed Student's *t*-test (*$P<0.05$ or **$P<0.01$) or one-way ANOVA followed by LSD test (significance level of $P<0.05$). All analyses were performed using IBM SPSS statistics 19.

Results

Generation and characterization of transgenic lines

To investigate the effect of overexpression of individual *OsPCS1*, *OsABCC1*, or *OsHMA3* genes and their combinations on As and/or Cd accumulation in rice grain, we generated independent transgenic lines in the T5105 genetic background that harboured cDNA or a genomic clone of the *OsPCS1*, *OsABCC1*, or *OsHMA3* genes under the control of the rice *OsActin1* promoter (P_{Actin1}:*cPCS1*:T_{Nos}, P_{Actin1}:*gABCC1*:T_{Nos}, or P_{Actin1}:*cHMA3*:T_{Nos}) (Supplementary Fig. S1). For each gene, three independent transgenic lines were selected for detailed characterization. They were PCS1-L1, PCS1-L2, and PCS1-L4 for the P_{Actin1}:*cPCS1*:T_{Nos} gene, ABCC1-L3, ABCC1-L27, and ABCC1-L31 for the P_{Actin1}:*gABCC1*:T_{Nos} gene, and HMA3-L1, HMA3-L3, and HMA3-L12 for the P_{Actin1}:*cHMA3*:T_{Nos} gene. These transgenic lines harboured single-copy and intact T-DNA fragments free of vector backbone sequence and showed overexpression of the transgenes (Supplementary Figs S2A–C, S3A–C, S4A–C). Compared with the non-transgenic control T5105, they showed no significant difference in growth, development, or reproduction (Supplementary Figs S2D–G, S3D–G, S4D–G). The representative transgenic lines PCS1-L1, ABCC1-L27, and HMA3-L3 were selected for gene pyramiding and further studies (Supplementary Figs S2G, S3G, S4G). Whole-genome sequencing analysis revealed that the T-DNA fragments were inserted in the intergenic regions between Os01g0688300 and Os01g0688400 in PCS1-L1, between Os03g0811400 and Os03g0811500 in ABCC1-L27, and between Os08g0300100 and Os08g0300200 in HMA3-L3 (Supplementary Fig. S5).

Co-overexpression of OsPCS1 and OsABCC1 in rice synergistically decreases As concentration in grain and provides enhanced As tolerance

T5105, *OsPCS1*-overexpressing lines (PCS1-L1, PCS1-L2, and PCS1-L4), and *OsABCC1*-overexpressing lines (ABCC1-L3, ABCC1-L27, and ABCC1-L31) were grown in As-contaminated soil, and the As concentration in grain and straw was measured by ICP-MS. The As concentration in the grain of the *OsPCS1*- and *OsABCC1*-overexpressing lines ranged from 20.5% to 29.5% and from 46.9% to 64.1% of that of T5105, respectively (Fig. 1A, B). The As concentration in straw tissues was measured in the representative transgenic lines PCS1-L1, ABCC1-L31, and T5105. Compared with T5105, both PCS1-L1 and ABCC1-L31 accumulated more As in root, nodes (node I and node II), internode II, and leaf II, but similar or lower As concentration in the tissues above node I, including internode I, flag leaf, rachis, and husk (Fig. 1C). These results demonstrate that the overexpression of *OsPCS1* or *OsABCC1* in rice increases As accumulation in vegetative tissues, especially in root and nodes, and decreases As allocation to panicle and seed. An *OsPCS1*- and *OsABCC1*-co-expressing line (AP) was developed through the cross between PCS1-L1 and ABCC1-L31. The As concentration in the grain of AP was 10.1% that of T5105, which was lower than that of PCS1-L1 or ABCC1-L31 (Fig. 1D). The result indicates that co-overexpression of *OsPCS1* and *OsABCC1* synergistically decreases As concentration in grain. The seedling of *OsPCS1*- and *OsABCC1*-overexpressing lines as well as AP were tested in As(III)-containing MS medium. Compared with T5105, all transgenic lines showed enhanced tolerance to As(III) in the concentration range 75–100 μM (Supplementary Fig. S6). Among the transgenic lines tested, AP showed the highest enhanced tolerance with the longest shoot length and the greatest dry weight per plant (Supplementary Fig. S6G–I). AP also had the highest As concentration in root and the lowest As concentration in shoot among these plants (Fig. 1E, F). The results demonstrate that the overexpression of *OsPCS1* or *OsABCC1* in rice promotes As accumulation in root and decreases As transport to the shoot, which results in enhanced tolerance to As stress, and co-overexpression of *OsPCS1* and *OsABCC1* in rice enhances this process.

Overexpression of OsPCS1 in rice decreases grain Cd concentration but paradoxically leads to Cd hypersensitivity at the seedling stage

The *OsPCS1*-overexpressing lines PCS1-L1, PCS1-L2, and PCS1-L4 were also tested in Cd-contaminated soil. The Cd concentration in the grain of the three *OsPCS1*-overexpressing lines was 58.9–62.6% of that in the grain of

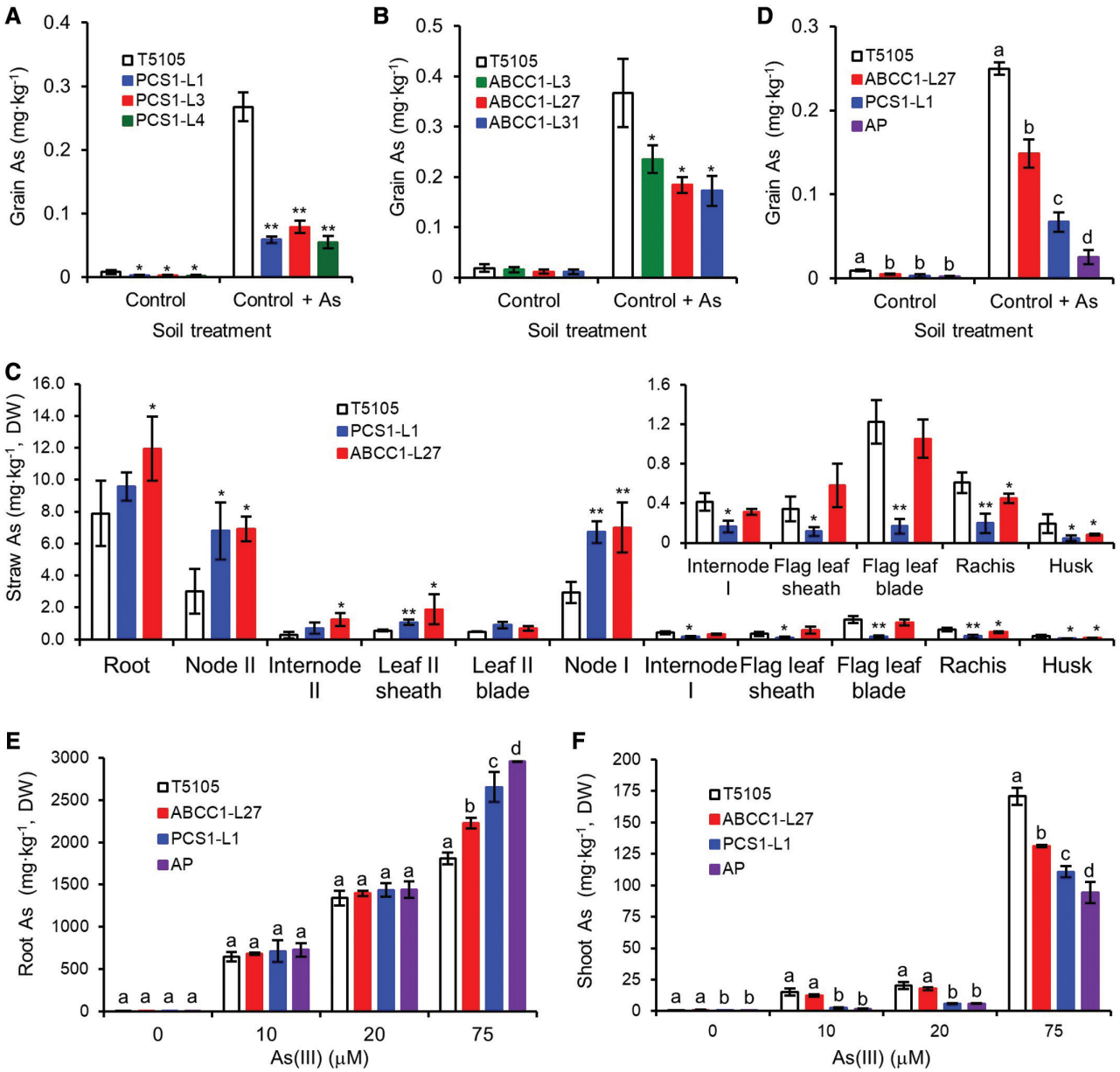

Fig. 1. Co-overexpression of *OsPCS1* and *OsABCC1* under the control of the *OsActin1* promoter in rice synergistically decreases As concentration in grain. (A and B) As concentration in the grain of T5105, *OsPCS1*-overexpressing lines (A), and *OsABCC1*-overexpressing lines (B) grown in As-contaminated soil. (C) As concentration in different straw tissues of T5105, PCS1-L1, and ABCC1-L27 grown in As-contaminated soil. (D) As concentration in the grain of T5105, PCS1-L1, ABCC1-L27, and AP grown in As-contaminated soil. (E and F) As concentration in root (E) and shoot (F) of seedlings of T5105, ABCC1-L27, PCS1-L1, and AP at 14 d after As treatment. The data (mean ±SD) were obtained from three independent experiments using separately grown plants. The asterisks in (A–C) indicate a significant difference between T5105 and transgenic lines (*P<0.05; **P<0.01 by Student's t-test). The different letters in (D–F) indicate a significant difference calculated by one-way ANOVA followed by LSD test at P<0.05. T5105, non-transgenic control; AP, an *OsPCS1*- and *OsABCC1*-co-overexpressing line with transgenes derived from PCS1-L1 and ABCC1-L27.

T5105 (Fig. 2A). Compared with T5105, the representative *OsPCS1*-overexpressing line PCS1-L1 had significantly higher Cd concentration in nodes (node I and node II) and internode II than T5105 (Fig. 2B). Notably, although PCS1-L1 had a lower Cd concentration in the grain than T5105, it accumulated more Cd in the husk and rachis than the non-transgenic control (Fig. 2A, B). In rice, the Cd distribution between the xylem and phloem in node I determines the Cd concentration in husk and grain (Tanaka *et al.*, 2007; Kato *et al.*, 2010). To examine whether the overexpression of *OsPCS1* affected Cd distribution between xylem and phloem in node I, we measured Cd and potassium (K) (as a control) concentrations in xylem sap and phloem exudate from internode I in PCS1-L1 and T5105. Compared with T5105, PCS1-L1 had significantly

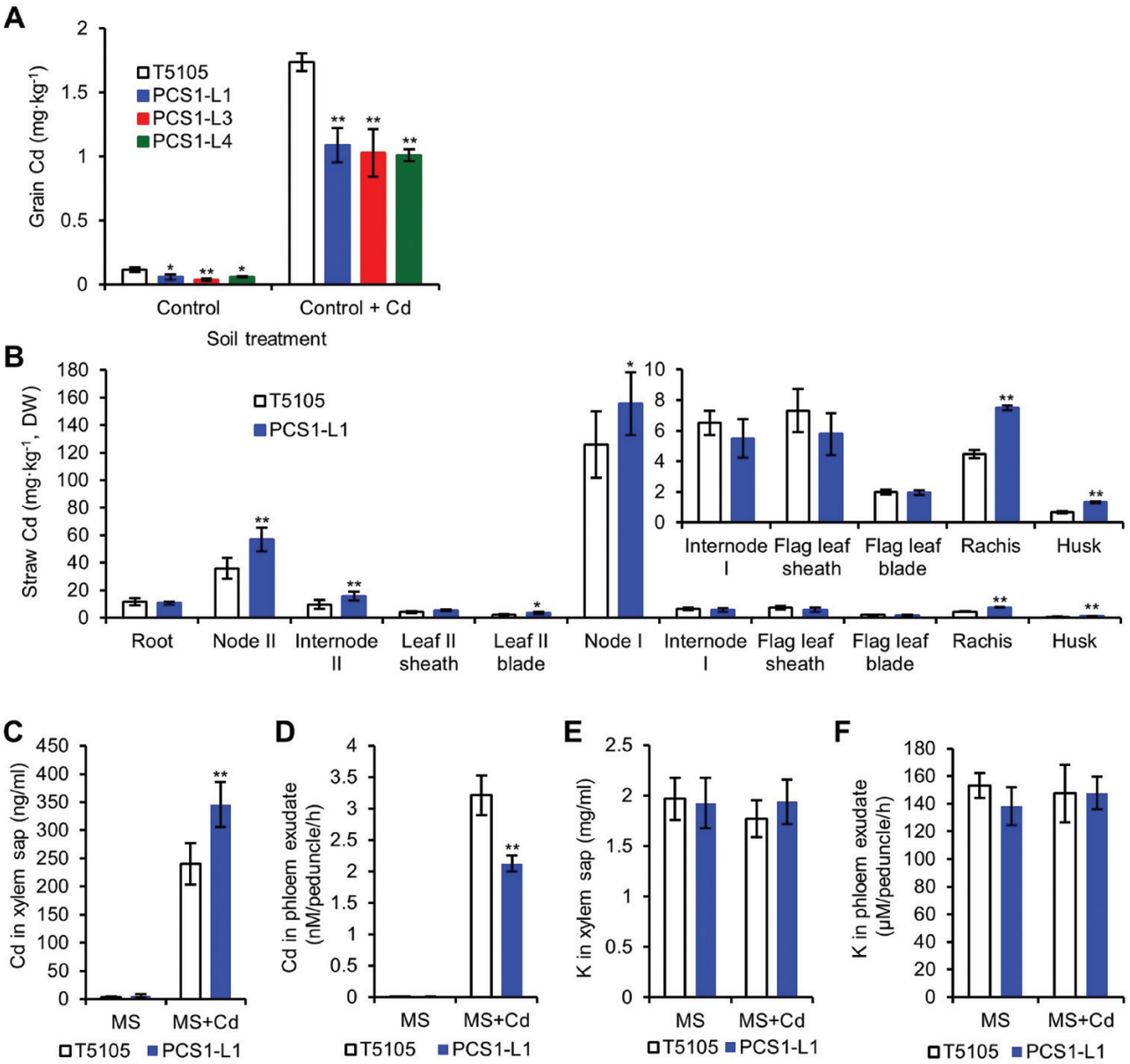

Fig. 2. Grain Cd concentration was significantly decreased in *OsPCS1*-overexpressing lines. (A) Grain Cd concentration of T5105 and *OsPCS1*-overexpressing lines grown in control soil or Cd-contaminated soil. (B) Cd concentration in different straw tissues of T5105 and PCS1-L1 grown in Cd-contaminated soil. (C and D) Cd concentration in xylem sap (C) and phloem exudate (D) from internode I treated in MS medium or Cd-containing MS medium. (E and F) Potassium (K) concentration in xylem sap (E) and phloem exudate (F) from internode I treated in MS medium or Cd-containing MS medium. The data (mean ±SD) were obtained from three independent experiments using separately grown plants. The asterisks in (A–D) indicate a significant difference between T5105 and the *OsPCS1*-overexpressing lines (*P<0.05; **P<0.01 by Student's t-test).

higher Cd concentration in xylem sap but much lower Cd concentration in phloem exudate (Fig. 2C, D). However, there was no significant difference in the K concentration in xylem sap and phloem exudates between PCS1-L1 and T5105 in internode I (Fig. 2E, F). The results indicated that overexpression of *OsPCS1* enhanced Cd accumulation in the rachis and husk but decreased Cd allocation to grain by suppressing Cd xylem-to-phloem transport in node I.

The *OsPCS1*-overexpressing lines were then tested for Cd tolerance on Cd-containing MS medium. It was unexpected to observe that the *OsPCS1*-overexpressing lines showed hypersensitivity to Cd stress at the seedling stage (Fig. 3A). They had a significantly shorter shoot length and lower dry weight per plant than T5105 (Fig. 3B, C). To investigate if the overproduced PCs caused this paradoxical phenotype in the *OsPCS1*-overexpressing lines, we measured the total PC concentration

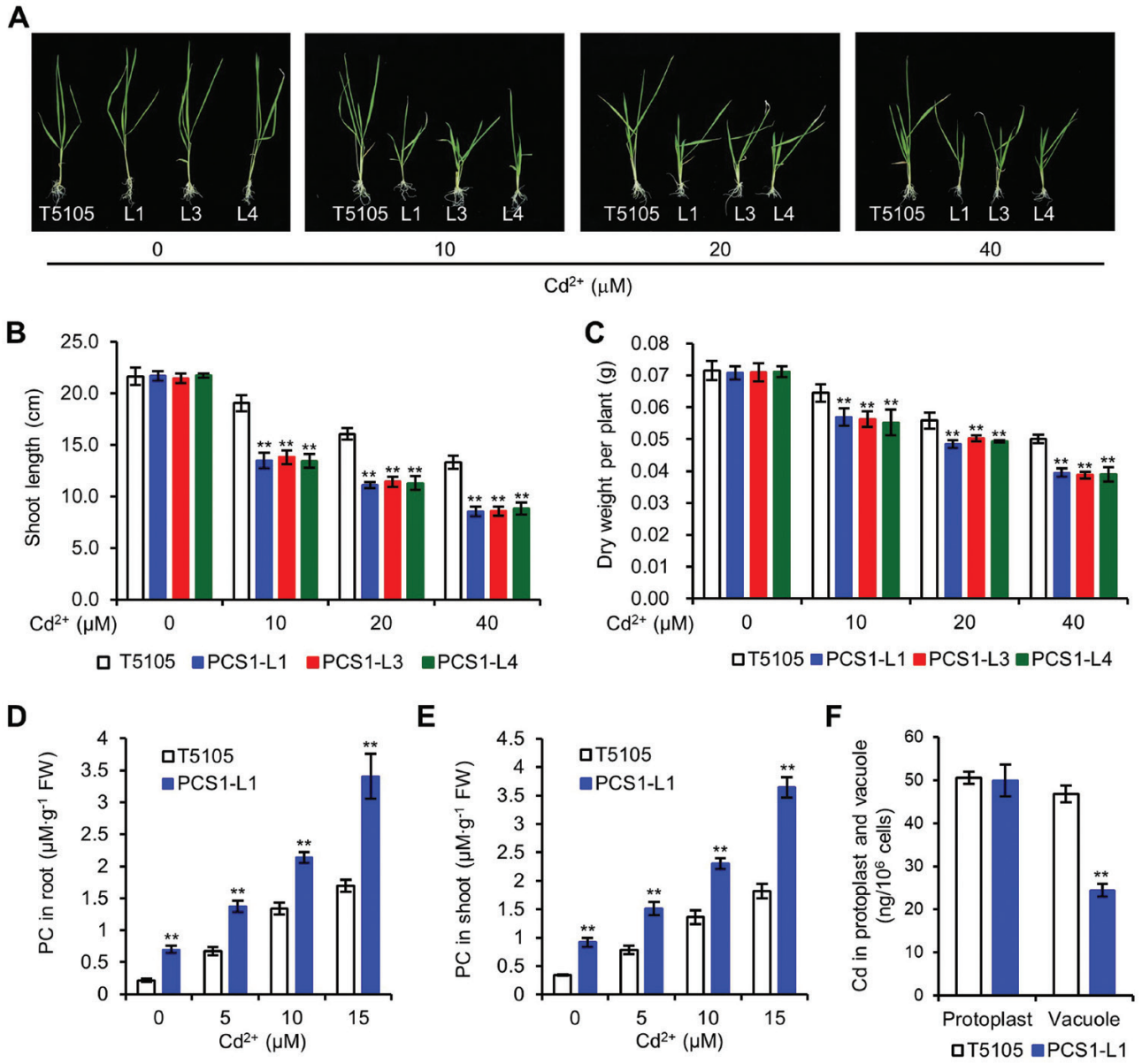

Fig. 3. Overexpression of *OsPCS1* in rice causes Cd hypersensitivity and suppresses vacuolar Cd sequestration. (A) Seedlings of T5105 and *OsPCS1*-overexpressing lines at 14 d after Cd treatment. (B and C) Shoot length (B) and dry weight (C) of T5105 and *OsPCS1*-overexpressing lines at 14 d after Cd treatment. (D and E) PC concentration in root (D) and shoot (E) of T5105 and PCS1-L1 seedlings at 14 d after Cd treatment. (F) Cd concentration in vacuoles and protoplasts isolated from T5105 and PCS1-L1 seedlings grown in Cd-containing medium for 10 d. The data (mean ±SD) in (B–F) were obtained from three independent experiments using separately grown plants. The asterisks in (B–F) indicate a significant difference between T5105 and the *OsPCS1*-overexpressing lines (*P<0.05; **P<0.01 by Student's t-test). T5105, non-transgenic control; L1, PCS1-L1; L3, PCS1-L3; L4, PCS1-L4.

in PCS1-L1 and T5105. The total PC concentrations in the shoot and root of PCS1-L1 were 2.7- and 3.2-fold that of T5105, respectively (Fig. 3D, E). In addition, the total PC concentration in both PCS1-L1 and T5105 was proportionally increased after Cd treatment (Fig. 3D, E). To further investigate if the overproduced PCs in PCS1-L1 suppressed Cd sequestration in vacuoles, we isolated protoplasts and vacuoles from the shoot tissues of PCS1-L1 and T5105 treated with Cd and measured their Cd concentration (Supplementary Fig. S7). PCS1-L1 and T5105 had comparable Cd concentrations in their isolated protoplasts; however, the Cd concentration in the purified vacuoles of PCS1-L1 was only 52.2% that of T5105 (Fig. 3F). The results indicate that the overproduced PCs in the *OsPCS1*-overexpressing lines suppressed vacuolar Cd sequestration and caused Cd hypersensitivity at the seedling stage. It should be mentioned that this Cd hypersensitivity was not

observed with the *OsPCS1*-overexpressing lines grown in soil, in which the Cd treatment was applied on 4-week-old rice plants after they were transplanted onto Cd-contaminated soil.

Co-overexpression of OsHMA3 *in PCS1-L1 complements its Cd hypersensitivity by enhancing vacuolar Cd sequestration in roots*

To investigate if *OsHMA3* expression could complement the Cd hypersensitivity in PCS1-L1, we generated three independent *OsHMA3*-overexpressing lines (HMA3-L1, HMA3-L3, and HMA3-L12). The Cd concentration in the grain of the three *OsHMA3*-overexpressing lines grown in Cd-contaminated soil was only 1.1–2.8% of that in the grain of T5105 (Fig. 4A). To test the effect of co-overexpression of *OsHMA3* and *OsPCS1* on Cd accumulation in grain and straw, the representative *OsHMA3*-overexpressing line HMA3-L3 was crossed with PCS1-L1 to develop an *OsHMA3*- and *OsPCS1*-co-overexpressing line (HP). The Cd concentration in the grain of HP grown in Cd-contaminated soil was comparable with that of HMA3-L3 and both were significantly lower than that of T5105 or PCS1-L1 (Fig. 4B). Like HMA3-L3, HP had a significantly increased Cd concentration in the root and a notably lower Cd concentration in other aerial vegetative tissues compared with T5105 or PCS1-L1 (Fig. 4C). In addition, compared with T5105, the *OsHMA3*-overexpressing lines showed an enhanced tolerance to Cd stress at the seedling stage (Supplementary Fig. S8). Like HMA3-L3, HP had a longer shoot and higher dry weight per plant than PCS1-L1 or T5105 (Fig. 4D–F), suggesting that co-overexpression of *OsHMA3* and *OsPCS1* complemented the Cd hypersensitivity of PCS1-L1 at the seedling stage. The Cd concentration in the root of PCS1-L1 was comparable with that of T5105, and the Cd concentration in the shoot of PCS1-L1 was slightly higher than that of T5105 (Fig. 4G, H). However, like HMA3-L3, HP had a significantly higher Cd concentration in the root and notably lower Cd concentration in the shoot than PCS1-L1 or T5105 (Fig. 4G, H). The results collectively demonstrate that the co-overexpression of *OsHMA3* and *OsPCS1* in rice complements the Cd hypersensitivity caused by the overproduced PCs in *OsPCS1*-overexpressing lines by promoting Cd accumulation in the root and decreasing Cd allocation to aerial parts of the straw and grain.

Co-overexpression of OsPCS1, OsABCC1, *and* OsHMA3 *in rice significantly decreases both As and Cd concentrations in grain*

To reduce As and Cd concentrations in grain, we developed an *OsPCS1*-, *OsABCC1*-, and *OsHMA3*-co-overexpressing line by pyramiding the P_{Actin1}:*cPCS1*:T_{Nos} gene from PCS1-L1, the P_{Actin1}:*gABCC1*:T_{Nos} gene from ABCC1-L27, and the P_{Actin1}:*cHMA3*:T_{Nos} gene from HMA3-L3, and designated the rice line as PAH. PAH had comparable agronomic traits, including plant architecture, plant height, seed size, panicle morphology, seed-setting rate, and 100-grain weight, to T5105 (Fig. 5A–E). When grown in the As- and Cd-contaminated soil, the As concentration in the brown rice of PAH was 0.03 ± 0.002 mg kg^{-1}, which was 7.9% of that of T5105 at 0.34 ± 0.02 mg kg^{-1}, while the Cd concentration in the brown rice of PAH was 0.04 ± 0.01 mg kg^{-1}, which was 2.0% of that of T5105 at 1.81 ± 0.14 mg kg^{-1} (Fig. 5F, G). Both As and Cd concentrations in the brown rice of PAH were far below the current FAO/WHO limit for As in brown rice at 0.35 mg kg^{-1} and Cd in polished rice at 0.40 mg kg^{-1}, respectively (Codex-Alimentarius-Commission, 1995). The concentrations of mineral elements, including cobalt (Co), copper (Cu), iron (Fe), manganese (Mn), selenium (Se), and zinc (Zn), were also measured in the grain of PAH, PCS1-L1, ABCC1-L27, HMA3-L3, and T5105. All transgenic lines had concentrations of the mineral elements comparable with T5105 (Supplementary Fig. S9). PAH also showed a higher enhanced tolerance to both As and Cd stress compared with PCS1-L1, ABCC1-L27, or HMA3-L3 when they were cultured in As- and Cd-containing MS medium (Supplementary Fig. S10A–C). In addition, PAH accumulated more As and Cd in the root but less As and Cd in the shoot than T5105 (Supplementary Fig. S10D–G). The results demonstrate that the co-overexpression of *OsPCS1*, *OsABCC1*, and *OsHMA3* in rice efficiently promotes As and Cd accumulation in the root and significantly decreases As and Cd allocation in the grain without causing any defect in plant growth and reproduction or change in the concentrations of mineral nutrients in the grain.

Discussion

In Arabidopsis, two vacuolar Cd sequestration pathways have been identified. One is the PC-dependent pathway mediated by ABCC-type transporters (AtABCC1 and AtABCC3) and the other is the PC-independent pathway mediated by ATPase (AtHMA3) (Morel *et al.*, 2009; Park *et al.*, 2012; Brunetti *et al.*, 2015). However, the knockout of *OsABCC1* in rice did not affect Cd toxicity, indicating that OsABCC1 is not involved in Cd detoxification (Song *et al.*, 2014). In addition, knockdown or knockout of *OsPCS1* or *OsPCS2* in rice showed little effect on Cd detoxification (Hayashi *et al.*, 2017; Yamazaki *et al.*, 2018). These findings suggested that the PC-dependent vacuolar Cd sequestration might not be the major Cd detoxification pathway in rice. On the other hand, a loss-of-function mutation of OsHMA3 resulted in an increased rate of root-to-shoot Cd transport and a high Cd concentration in rice grain (Ueno *et al.*, 2010; Miyadate *et al.*, 2011). Overexpression of *OsHMA3* in rice enhanced Cd sequestration in root cells, reduced root-to-shoot Cd transport, effectively decreased Cd allocation in grain, and provided enhanced Cd tolerance (Fig. 4) (Ueno *et al.*, 2010; Sasaki *et al.*, 2014; Shao *et al.*, 2018). The results collectively demonstrate that the OsHMA3-mediated

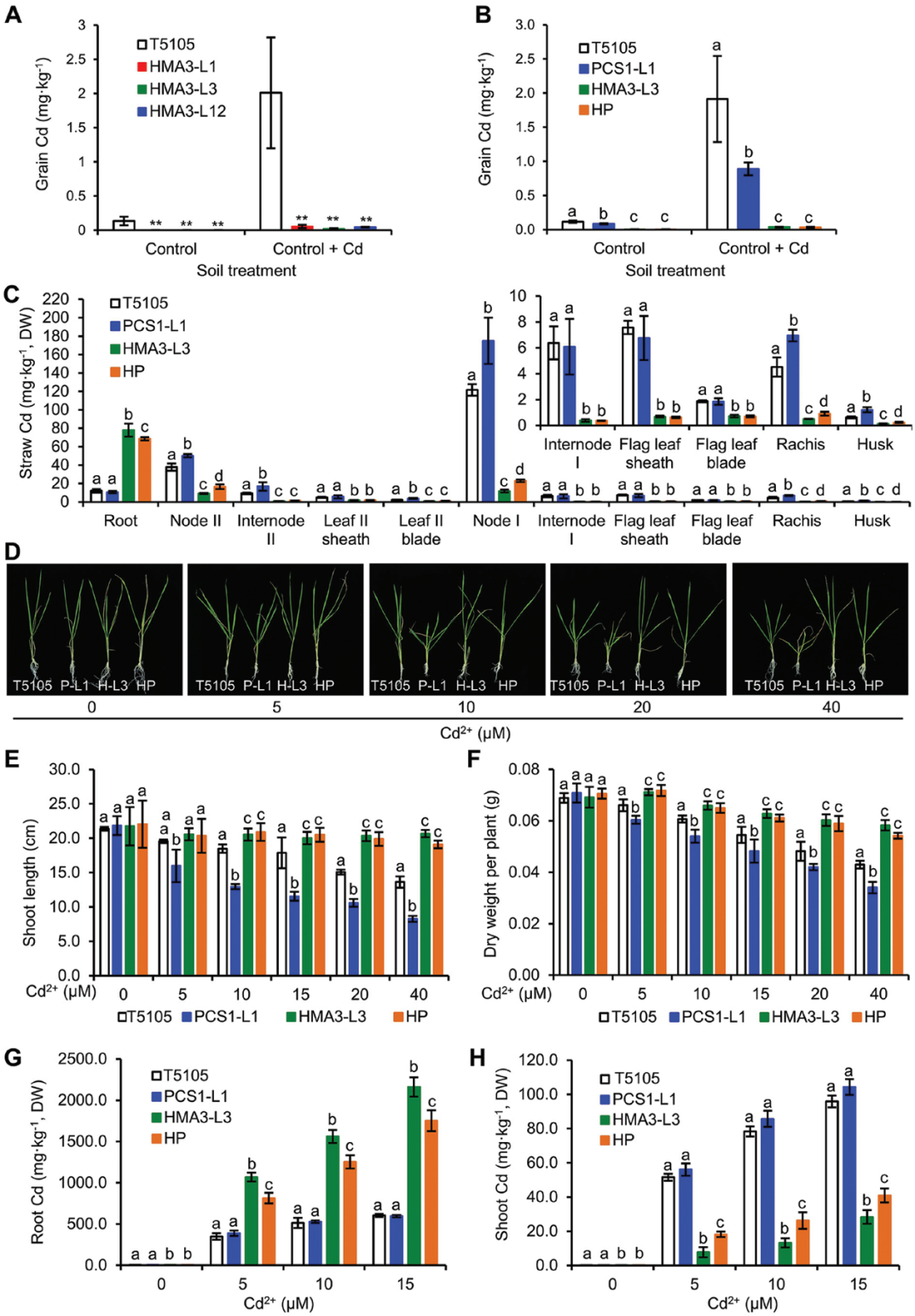

Fig. 4. Co-overexpression of *OsHMA3* and *OsPCS1* in rice complemented Cd hypersensitivity of PCS1-L1 and significantly reduced Cd concentration in grain. (A) Grain Cd concentration of T5105 and the *OsHMA3*-overexpressing lines grown in control soil or Cd-contaminated soil. (B) Grain Cd concentration of T5105, PCS1-L1, HMA3-L3, and HP grown in control soil or Cd-contaminated soil. (C) Cd concentration in different straw tissues of T5105, PCS1-1, HMA3-L3, and HP grown in Cd-contaminated soil. (D) Seedlings of T5105, PCS1-L1, HMA3-L3, and HP at 14 d after Cd treatment. (E and F) Shoot length (E) and DW (F) of T5105, PCS1-L1, HMA3-L3, and HP at 14 d after Cd treatment. (G and H) Cd concentration in root (G)

and shoot (H) of T5105, PCS1-L1, HMA3-L3, and HP at 14 d after Cd treatment. The data (mean ±SD) in (A–C) and (E–H) were obtained from three independent experiments using separately grown plants. The asterisks in (A) and (B) indicate a significant difference between T5105 and OsHMA3-L3 (**P<0.01 by Student's t-test). The different letters in (C) and (E–H) indicate a significant difference calculated by one-way ANOVA followed by LSD test at P<0.05. T5105, non-transgenic control; P-L1, PCS1-L1; H-L3, HMA3-L3; HP, an *OsHMA3*- and *OsPCS1*-co-overexpressing line with transgenes derived from HMA3-L3 and PCS1-L1.

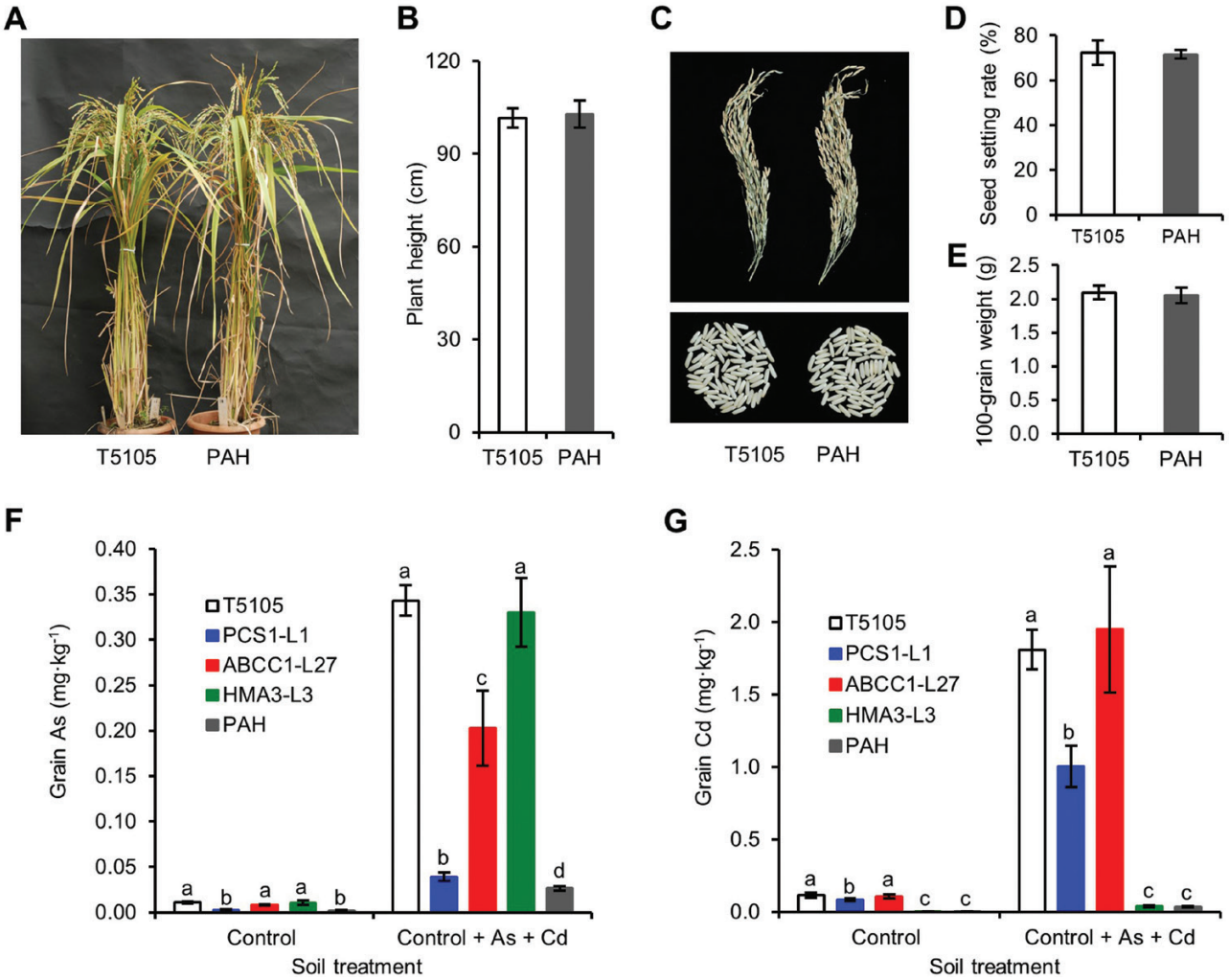

Fig. 5. Co-overexpression of *OsPCS1*, *OsABCC1*, and *OsHMA3* in rice significantly reduces As and Cd concentration in grain. (A) Morphological phenotype of T5105 and PAH at 120 d after sowing. (B) Plant height of T5105 and PAH at 120 d after sowing. (C) Panicles and unpolished rice grain of T5105 and PAH. (D) Seed setting rate of T5105 and PAH. (E) One hundred grain weight of T5105 and PAH. (F and G) As concentration (F) and Cd concentration (G) in the grain of T5105, PCS1-L1, ABCC1-L27, HMA3-L3, and PAH grown in control soil or As- and Cd-contaminated soil. The data (mean ±SD) in (B) and (D–G) were obtained from three independent experiments using separately grown plants. The different letters in (F) and (G) indicate a significant difference calculated by one-way ANOVA followed by LSD test at P<0.05. T5105, non-transgenic control; PAH, an *OsPCS1*-, *OsABCC1*-, and *OsHMA3*-co-overexpressing line with transgenes derived from PCS1-L1, ABCC1-L27, and HMA3-L3.

Cd sequestration into vacuoles of root cells is a major Cd detoxification pathway in rice.

In a previous study, heterologous overexpression of the wheat PCS gene *TaPCS1* in wild tobacco *Nicotiana glauca* significantly enhanced Cd tolerance (Gisbert *et al.*, 2003). However, overexpression of *TaPCS1* in rice led to Cd hypersensitivity despite an increased level of PCs (Wang *et al.*, 2012). Cd hypersensitivity was also reported in Arabidopsis overexpressing *AtPCS1* (Lee *et al.*, 2003; Li *et al.*, 2004). However, the mechanism of Cd hypersensitivity resulting from PCS overexpression was unclear. A previous study speculated that the Cd hypersensitivity of Arabidopsis plants overexpressing AtPCS1 was due to the toxicity of PCs as they existed at a high level compared with GSH (Lee *et al.*, 2003), whereas others suggested that the downstream

processing of PC–Cd complexes differs between tobacco and Arabidopsis (Li *et al.*, 2004). In this study, we found that overexpression of *OsPCS1* in T5105 also caused Cd hypersensitivity (Fig. 3A–C). Further studies revealed that overexpression of *OsPCS1* in PCS1-L1 caused a slightly increased Cd concentration in the shoot, but a significantly decreased Cd concentration in vacuoles of shoot cells at the seedling stage (Figs 3F, 4H). Based on these results, we speculate that the overproduced PCs in *OsPCS1*-overexpressing lines compete with OsHMA3 for Cd in the cytosol by forming PC–Cd complexes and suppressing OsHMA3-dependent vacuolar Cd sequestration in the root. In addition, the overproduced PCs or PC–Cd complexes in root cells also promoted Cd transport from root to shoot (Fig. 4G, H) (Gong *et al.*, 2003). Both processes may contribute to Cd hypersensitivity in *OsPCS1*-overexpressing lines at the seedling stage. To address this issue, we developed the *OsHMA3*- and *OsPCS1*-co-overexpressing line HP. The overexpressed OsHMA3 proteins in HP, which may have either a higher amount or a higher affinity for binding Cd than PCs, overcome this suppression and complement the Cd hypersensitivity by efficiently sequestrating Cd into vacuoles of root cells and decreasing Cd transport from the root to the shoot and grain (Fig. 4).

The nodes in rice play an important role in As storage and redistribution, where OsABCC1 sequesters PC–As(III) complexes into vacuoles of phloem companion cells and restricts As transport to the panicle and grain (Song *et al.*, 2014; Chen *et al.*, 2015). In this study, when grown in As-contaminated soil, T5105 accumulated a higher As concentration in node I and node II than other straw tissues except for the root, while PCS1-L1 or ABCC1-L27 individually had a higher As concentration in the two nodes than T5105 (Fig. 1C). Due to the collaborative function of OsABCC1 and OsPCS1 in sequestration of As into vacuoles, co-overexpression of *OsPCS1* and *OsABCC1* in AP and PAH can reduce the As concentration in grain to the maximum extent (Figs 1D, 5F). In rice, the dominant source of Cd for grain is phloem-derived Cd, whereas that for husk it is xylem-derived Cd (Tanaka *et al.*, 2007; Yoneyama *et al.*, 2010). Thus, an efficient intervascular xylem-to-phloem transport is necessary for Cd remobilization from shoot to grain, and this process mainly occurs in node I (Yoneyama *et al.*, 2010;Yamaguchi *et al.*, 2012). In other words, the Cd storage and redistribution at node I determine the Cd allocation in grain (Fujimaki *et al.*, 2010; Uraguchi *et al.*, 2011; Yamaguchi *et al.*, 2012; Yamaji *et al.*, 2013). In another study, it was found that the grain low-Cd rice variety YaHui2816 has a higher PC concentration in nodes than the grain high-Cd rice variety, indicating that PCs could participate in the Cd remobilization process (Guo *et al.*, 2020). In this study, PCS1-L1 had a higher PC concentration than T5105, which was further increased by Cd stress (Fig. 3D, E). Like the grain low-Cd rice variety YaHui2816 (Guo *et al.*, 2020), PCS1-L1 accumulated a significantly higher Cd concentration in node I and node II than T5105 when grown in Cd-contaminated soil (Fig. 2A, B). Notably, overexpression of *OsPCS1* only reduced Cd in the grain, but accumulated more Cd in the other parts of panicles, including rachises and husks (Fig. 2B). Further investigation revealed that PCS1-L1 had a significantly higher Cd concentration in xylem sap but a much lower Cd concentration in phloem exudate from internode I than T5105 (Fig. 2C, D). Taken together, we speculate that the overproduced PCs restricted Cd xylem-to-phloem transport in node I, which increased Cd transport to husks through the xylem but decreased Cd allocation to grain through the phloem (Fig. 2A, B). However, the detailed mechanism remains to be revealed of how the overproduced PCs restricted xylem-to-phloem Cd transport in node I of rice.

It was noted that the grain As concentration in the *OsABCC1*-overexpressing lines in this study was not consistent with that in a previous report, in which overexpression of *OsABCC1* under control of the maize ubiquitin gene 1 (*ZmUbi1*) promoter did not affect As concentration in the rice grain (Deng *et al.*, 2018). One of the reasons for this difference could result from the different promoters used in the two studies. A previous study demonstrated that the *OsActin1* promoter has relatively lower overall activity in seed endosperm than the *ZmUBi1* promoter in rice seeds (Park *et al.*, 2010). The low activity of the *OsActin1* promoter in endosperm might decrease As sequestration in vacuoles of endosperm cells, while its high activity in vegetative tissues and organs promoted As sequestration and accumulation in straw. In this scenario, the utilization of the *OsActin1* promoter to drive *OsPCS1*, *OsABCC1*, and *OsHMA3* expression in transgenic rice in this study should also contribute to lower grain As and/or Cd concentration. While the transgenic rice needs to go through safety evaluation as well as other formalities before commercialization, the use of biotechnology is a practical and effective strategy to reduce As and Cd concentration in the rice grain simultaneously.

Supplementary data

The following supplementary data are available at *JXB* online.

Fig. S1. Diagram of the binary constructs and genes used in this study.

Fig. S2. Generation of *OsPCS1*-overexpressing lines.

Fig. S3. Generation of *OsABCC1*-overexpressing lines.

Fig. S4. Generation of *OsHMA3*-overexpressing lines.

Fig. S5. Nucleotide sequences of the junction regions at the T-DNA insertion sites in the transgenic lines.

Fig. S6. Co-overexpression of *OsPCS1* and *OsABCC1* in rice synergistically confers enhanced tolerance to As.

Fig. S7. Light microscopy images on isolated protoplasts and purified vacuoles for Cd concentration measurement.

Fig. S8. Overexpression of *OsHMA3* in rice confers enhanced tolerance to Cd.

Fig. S9. Concentrations of nutrient elements in the grain of T5105, PCS1-L1, ABCC1-L27, HMA3-L3, and PAH.

Fig. S10. Co-overexpression of *OsPCS1*, *OsABCC1*, and *OsHMA3* in rice provides an enhanced tolerance to both As and Cd stress.

Table S1. Oligo primers used in this study.

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Author contributions

ZY: conceptualization; ZY and YG: design and writing; YG, JT, and DT: conducting the experiments. All authors read and approved the article for publication.

Conflict of interest

Temasek Life Sciences Laboratory Ltd has filed a patent on the method of generating low-As and Low-Cd rice grains as described in this study (Singapore provisional patent application No: 10202260511R; Filing date: 20 December 2022) with ZY and YG as the inventors.

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Data availability

All data supporting the findings of this study are available within the paper and within its supplementary data published online. The transgenic rice lines developed herein are available for non-commercial research purposes based on signing a Material Transfer Agreement defined by the Intellectual Property Office of Temasek Life Sciences Laboratory Ptd, Singapore.

References

Ahmed ZU, Panaullah GM, Gauch H, McCouch SR, Tyagi W, Kabir MS, Duxbury JM. 2010. Genotype and environment effects on rice (*Oryza sativa* L) grain arsenic concentration in Bangladesh. Plant and Soil **338**, 367–382.

Brunetti P, Zanella L, De Paolis A, Di Litta D, Cecchetti V, Falasca G, Barbieri M, Altamura MM, Costantino P, Cardarelli M. 2015. Cadmium-inducible expression of the ABC-type transporter AtABCC3 increases phytochelatin-mediated cadmium tolerance in Arabidopsis. Journal of Experimental Botany **66**, 3815–3829.

Chang JD, Huang S, Yamaji N, Zhang W, Ma JF, Zhao FJ. 2020. OsNRAMP1 transporter contributes to cadmium and manganese uptake in rice. Plant, Cell & Environment **43**, 2476–2491.

Chen Y, Moore KL, Miller AJ, McGrath SP, Ma JF, Zhao FJ. 2015. The role of nodes in arsenic storage and distribution in rice. Journal of Experimental Botany **66**, 3717–3724.

Clemens S, Ma JF. 2016. Toxic heavy metal and metalloid accumulation in crop plants and foods. Annual Review of Plant Biology **67**, 489–512.

Codex-Alimentarius-Commission. 1995. Codex Alimentarius—international food standards. FAO/WHO. CXS 193-1995 (Amended in 2019), 46–48.

Deng F, Yamaji N, Ma JF, Lee SK, Jeon JS, Martinoia E, Lee Y, Song WY. 2018. Engineering rice with lower grain arsenic. Plant Biotechnology Journal **16**, 1691–1699.

Devi SR, Prasad M. 1998. Copper toxicity in *Ceratophyllum demersum* L. (Coontail), a freefloating macrophyte: response of antioxidant enzymes and antioxidants. Plant Sciences **138**, 157–165.

Duan G, Shao G, Tang Z, Chen H, Wang B, Tang Z, Yang Y, Liu Y, Zhao F-J. 2017. Genotypic and environmental variations in grain cadmium and arsenic concentrations among a panel of high yielding rice cultivars. Rice **10**, 1–13.

Fowler BA. 2009. Monitoring of human populations for early markers of cadmium toxicity: a review. Toxicology and Applied Pharmacology **238**, 294–300.

Frangne N, Eggmann T, Koblischke C, Weissenböck G, Martinoia E, Klein M. 2002. Flavone glucoside uptake into barley mesophyll and Arabidopsis cell culture vacuoles energization occurs by H+-antiport and ATP-binding cassette-type mechanisms. Plant Physiology **128**, 726–733.

Fujimaki S, Suzui N, Ishioka NS, Kawachi N, Ito S, Chino M, Nakamura S. 2010. Tracing cadmium from culture to spikelet: noninvasive imaging and quantitative characterization of absorption, transport, and accumulation of cadmium in an intact rice plant. Plant Physiology **152**, 1796–1806.

Gisbert C, Ros R, De Haro A, Walker DJ, Pilar Bernal M, Serrano R, Navarro-Aviñó J. 2003. A plant genetically modified that accumulates Pb is especially promising for phytoremediation. Biochemical and Biophysical Research Communications **303**, 440–445.

Gong J-M, Lee DA, Schroeder JI. 2003. Long-distance root-to-shoot transport of phytochelatins and cadmium in Arabidopsis. Proceedings of the National Academy of Sciences, USA **100**, 10118–10123.

Grill E, Winnacker E-L, Zenk MH. 1985. Phytochelatins: the principal heavy-metal complexing peptides of higher plants. Science **230**, 674–676.

Guo J, Zhang X, Ye D, Huang H, Wang Y, Zheng Z, Li T, Yu H. 2020. Crucial roles of cadmium retention in node for restraining cadmium transport from straw to ear at reproductive period in a grain low-cadmium rice line (*Oryza sativa* L). Ecotoxicology and Environmental Safety **205**, 111323.

Ha SB, Smith AP, Howden R, Dietrich WM, Bugg S, O'Connell MJ, Goldsbrough PB, Cobbett CS. 1999. Phytochelatin synthase genes from Arabidopsis and the yeast *Schizosaccharomyces pombe*. The Plant Cell **11**, 1153–1164.

Hayashi S, Kuramata M, Abe T, Takagi H, Ozawa K, Ishikawa S. 2017. Phytochelatin synthase OsPCS1 plays a crucial role in reducing arsenic levels in rice grains. The Plant Journal **91**, 840–848.

Hiei Y, Ohta S, Komari T, Kumashiro T. 1994. Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. The Plant Journal **6**, 271–282.

Hughes MF. 2002. Arsenic toxicity and potential mechanisms of action. Toxicology Letters **133**, 1–16.

Ishikawa S, Ishimaru Y, Igura M, Kuramata M, Abe T, Senoura T, Hase Y, Arao T, Nishizawa NK, Nakanishi H. 2012. Ion-beam irradiation, gene identification, and marker-assisted breeding in the development of low-cadmium rice. Proceedings of the National Academy of Sciences, USA **109**, 19166–19171.

Kan M, Yamazaki K, Fujiwara T, Kamiya T. 2019. A simple and high-throughput method for xylem sap collection. Biotechniques **67**, 242–245.

Kato M, Ishikawa S, Inagaki K, Chiba K, Hayashi H, Yanagisawa S, Yoneyama T. 2010. Possible chemical forms of cadmium and varietal differences in cadmium concentrations in the phloem sap of rice plants (*Oryza sativa* L). Soil Science and Plant Nutrition **56**, 839–847.

King R, Zeevaart J. 1974. Enhancement of phloem exudation from cut petioles by chelating agents. Plant Physiology **53**, 96–103.

Lee S, Moon JS, Ko T-S, Petros D, Goldsbrough PB, Korban SS. 2003. Overexpression of Arabidopsis phytochelatin synthase paradoxically leads to hypersensitivity to cadmium stress. Plant Physiology **131**, 656–663.

Li RY, Stroud JL, Ma JF, McGrath SP, Zhao FJ. 2009. Mitigation of arsenic accumulation in rice with water management and silicon fertilization. Environmental Science & Technology **43**, 3778–3783.

Li Y, Dhankher OP, Carreira L, Lee D, Chen A, Schroeder JI, Balish RS, Meagher RB. 2004. Overexpression of phytochelatin synthase in Arabidopsis leads to enhanced arsenic tolerance and cadmium hypersensitivity. Plant and Cell Physiology **45**, 1787–1797.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta}C_T$ method. Methods **25**, 402–408.

Luo Y, Yin Z. 2013. Marker-assisted breeding of Thai fragrance rice for semi-dwarf phenotype, submergence tolerance and disease resistance to rice blast and bacterial blight. Molecular Breeding **32**, 709–721.

Ma JF, Yamaji N, Mitani N, Tamai K, Konishi S, Fujiwara T, Katsuhara M, Yano M. 2007. An efflux transporter of silicon in rice. Nature **448**, 209–212.

Ma JF, Yamaji N, Mitani N, Xu XY, Su YH, McGrath SP, Zhao FJ. 2008. Transporters of arsenite in rice and their role in arsenic accumulation in rice grain. Proceedings of the National Academy of Sciences, USA **105**, 9931–9935.

Miyadate H, Adachi S, Hiraizumi A, *et al*. 2011. OsHMA3, a P1B-type of ATPase affects root-to-shoot cadmium translocation in rice by mediating efflux into vacuoles. New Phytologist **189**, 190–199.

Morel M, Crouzet J, Gravot A, Auroy P, Leonhardt N, Vavasseur A, Richaud P. 2009. AtHMA3, a P1B-ATPase allowing Cd/Zn/co/Pb vacuolar storage in Arabidopsis. Plant Physiology **149**, 894–904.

Moreno-Jimenez E, Meharg AA, Smolders E, Manzano R, Becerra D, Sanchez-Llerena J, Albarran A, Lopez-Pinero A. 2014. Sprinkler irrigation of rice fields reduces grain arsenic but enhances cadmium. Science of the Total Environment **485-486**, 468–473.

Norton GJ, Duan G, Dasgupta T, *et al*. 2009a. Environmental and genetic control of arsenic accumulation and speciation in rice grain: comparing a range of common cultivars grown in contaminated sites across Bangladesh, China, and India. Environmental Science & Technology **43**, 8381–8386.

Norton GJ, Islam MR, Deacon CM, *et al*. 2009b. Identification of low inorganic and total grain arsenic rice cultivars from Bangladesh. Environmental Science & Technology **43**, 6070–6075.

Park J, Song WY, Ko D, Eom Y, Hansen TH, Schiller M, Lee TG, Martinoia E, Lee Y. 2012. The phytochelatin transporters AtABCC1 and AtABCC2 mediate tolerance to cadmium and mercury. The Plant Journal **69**, 278–288.

Park S-H, Yi N, Kim YS, Jeong M-H, Bang S-W, Choi YD, Kim J-K. 2010. Analysis of five novel putative constitutive gene promoters in transgenic rice plants. Journal of Experimental Botany **61**, 2459–2467.

Qi X, Tam NF-y, Li WC, Ye Z. 2020. The role of root apoplastic barriers in cadmium translocation and accumulation in cultivars of rice (*Oryza sativa* L) with different Cd-accumulating characteristics. Environmental Pollutio **264**, 114736.

Reece KS, McElroy D, Wu R. 1990. Genomic nucleotide sequence of four rice (*Oryza sativa*) actin genes. Plant Molecular Biology **14**, 621–624.

Sasaki A, Yamaji N, Ma JF. 2014. Overexpression of *OsHMA3* enhances Cd tolerance and expression of Zn transporter genes in rice. Journal of Experimental Botany **65**, 6013–6021.

Sasaki A, Yamaji N, Yokosho K, Ma JF. 2012. Nramp5 is a major transporter responsible for manganese and cadmium uptake in rice. The Plant Cell **24**, 2155–2167.

Schat H, Kalff MM. 1992. Are phytochelatins involved in differential metal tolerance or do they merely reflect metal-imposed strain? Plant Physiology **99**, 1475–1480.

Shao JF, Xia J, Yamaji N, Shen RF, Ma JF. 2018. Effective reduction of cadmium accumulation in rice grain by expressing *OsHMA3* under the control of the *OsHMA2* promoter. Journal of Experimental Botany **69**, 2743–2752.

Song WY, Yamaki T, Yamaji N, Ko D, Jung KH, Fujii-Kashino M, An G, Martinoia E, Lee Y, Ma JF. 2014. A rice ABC transporter, OsABCC1, reduces arsenic accumulation in the grain. Proceedings of the National Academy of Sciences, USA **111**, 15699–15704.

Sui F-Q, Chang J-D, Tang Z, Liu W-J, Huang X-Y, Zhao F-J. 2018. Nramp5 expression and functionality likely explain higher cadmium uptake in rice than in wheat and maize. Plant and Soil **433**, 377–389.

Takahashi R, Ishimaru Y, Shimo H, Ogo Y, Senoura T, Nishizawa NK, Nakanishi H. 2012. The OsHMA2 transporter is involved in root-to-shoot translocation of Zn and Cd in rice. Plant, Cell & Environment **35**, 1948–1957.

Tanaka K, Fujimaki S, Fujiwara T, Yoneyama T, Hayashi H. 2007. Quantitative estimation of the contribution of the phloem in cadmium transport to grains in rice plants (*Oryza sativa* L). Soil Science and Plant Nutrition **53**, 72–77.

Trinidad JL, Longkumer T, Kohli A. 2021. Rice protoplast isolation and transfection for transient gene expression analysis. Methods in Molecular Biology **2238**, 313–324.

Ueno D, Yamaji N, Kono I, Huang CF, Ando T, Yano M, Ma JF. 2010. Gene limiting cadmium accumulation in rice. Proceedings of the National Academy of Sciences, USA **107**, 16500–16505.

Uraguchi S, Kamiya T, Sakamoto T, Kasai K, Sato Y, Nagamura Y, Yoshida A, Kyozuka J, Ishikawa S, Fujiwara T. 2011. Low-affinity cation transporter (OsLCT1) regulates cadmium transport into rice grains. Proceedings of the National Academy of Sciences, USA **108**, 20959–20964.

Wang F, Wang Z, Zhu C. 2012. Heteroexpression of the wheat phytochelatin synthase gene (*TaPCS1*) in rice enhances cadmium sensitivity. Acta Biochimica et Biophysica Sinica **44**, 886–893.

Xu XY, McGrath SP, Meharg AA, Zhao FJ. 2008. Growing rice aerobically markedly decreases arsenic accumulation. Environmental Science & Technology **42**, 5574–5579.

Yamaguchi N, Ishikawa S, Abe T, Baba K, Arao T, Terada Y. 2012. Role of the node in controlling traffic of cadmium, zinc, and manganese in rice. Journal of Experimental Botany **63**, 2729–2737.

Yamaji N, Xia J, Mitani-Ueno N, Yokosho K, Feng Ma J. 2013. Preferential delivery of zinc to developing tissues in rice is mediated by P-type heavy metal ATPase OsHMA2. Plant Physiology **162**, 927–939.

Yamazaki S, Ueda Y, Mukai A, Ochiai K, Matoh T. 2018. Rice phytochelatin synthases OsPCS1 and OsPCS2 make different contributions to cadmium and arsenic tolerance. Plant Direct **2**, e00034.

Yan H, Xu W, Xie J, *et al*. 2019. Variation of a major facilitator superfamily gene contributes to differential cadmium accumulation between rice subspecies. Nature Communications **10**, 2562.

Yoneyama T, Gosho T, Kato M, Goto S, Hayashi H. 2010. Xylem and phloem transport of Cd, Zn and Fe into the grains of rice plants (*Oryza sativa* L) grown in continuously flooded Cd-contaminated soil. Soil Science and Plant Nutrition **56**, 445–453.

Zhao F-J, Wang P. 2019. Arsenic and cadmium accumulation in rice and mitigation strategies. Plant and Soil **446**, 1–21.

Genetic engineering low-arsenic and low-cadmium rice grain

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I. Supplementary figures and figure legends

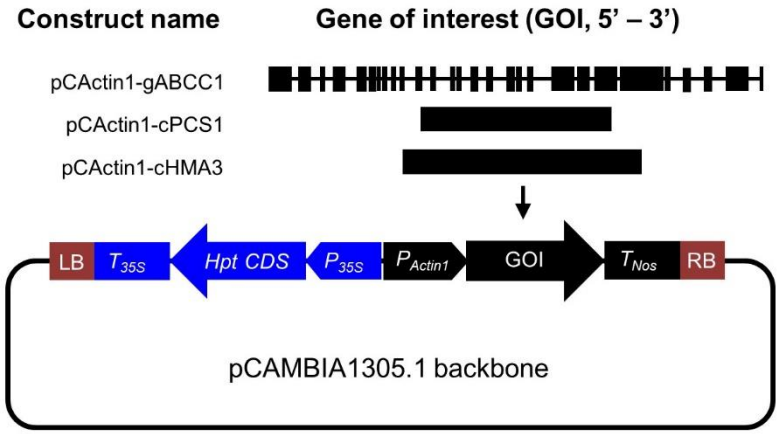

Fig. S1. Diagram of the binary constructs and genes used in this study. The lower panel of the diagram shows the genes in the T-DNA regions of binary constructs with a backbone derived from pCAMBIA1305.1. The upper panel of the diagram shows the genes of interest that were used to replace GUSPlus in pCAMBIA1305.1. The cDNA clones of the *OsHMA3* and *OsPCS1* genes were used to make constructs pCActin1-cHMA3 and pCActin1-cPCS1, respectively, whereas the genomic clone of the *OsABCC1* gene was used to make construct pCActin1-gABCC1. In all constructs, the CaMV35S promoter in pCAMBIA1305.1 was replaced with the rice *Actin1* gene promoter. The diagram was not drawn to scale. Abbreviation: LB, left border; T_{35S}, CaMV 35S terminator; *Hpt CDS*, the coding region of the hygromycin phosphotransferase gene; P_{35S}, 2x CaMV35S promoter; P_{Actin1}, rice *Actin1* gene promoter; GOI, gene-of-interest; T_{Nos}, nopaline synthase gene terminator; RB, Right border.

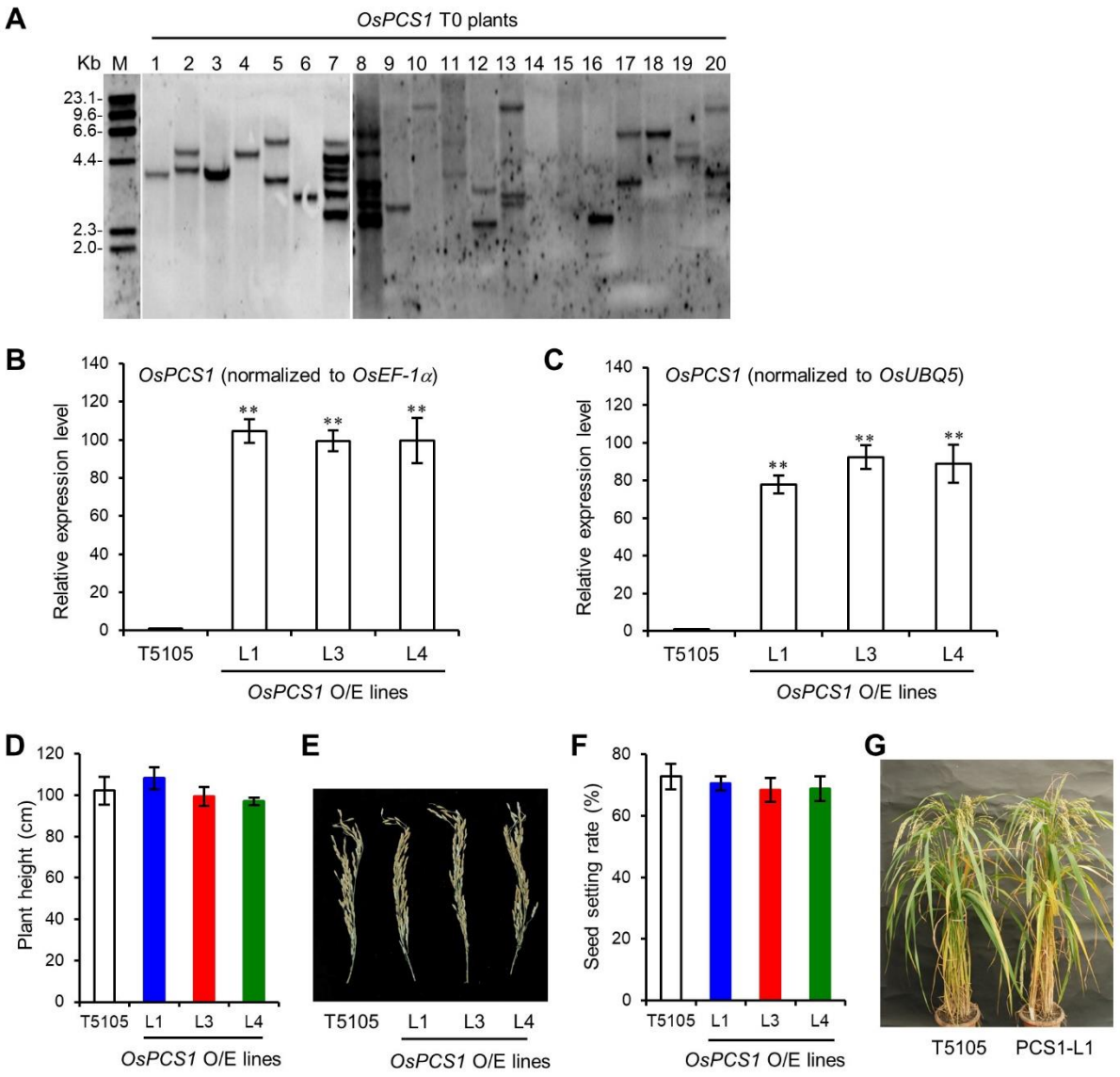

Fig. S2. Generation of *OsPCS1*-overexpressing lines. (**A**) Detection of the copy number of T-DNA in *OsPCS1* transgenic plants (T0) by Southern blot analysis. M, molecular marker. (**B**) and (**C**) Expression levels of the *OsPCS1* gene in T5105 and three homozygous *OsPCS1* overexpressing lines detected by qRT-PCR. The results were normalized against levels of *OsEF-1α* (**B**) and *OsUBQ5* (**C**), respectively, and the expression level in T5105 was arbitrarily set to 1. The asterisks indicate a significant difference between T5105 and transgenic lines (**$P < 0.01$ by Student's *t* test). (**D**) Plant height of T5105 and the *OsPCS1*-overexpressing lines. L1 (PCS1-L1), L3 (PCS1-L3) and L4 (PCS1-L4) are three independent *OsPCS1*-overexpressing lines. (**E**) Morphology of rice panicles. (**F**) Seed setting rates of T5105 and the *OsPCS1*-overexpressing lines. (**G**) Morphological phenotype of T5105 and PCS1-L1. Plants were photographed at 120 d after sowing. The data (mean ± SD) in (**B**), (**C**), (**D**) and (**F**) were obtained from 3 independent experiments using separately grown plants. No significant difference in (**D**) and (**F**) was observed between T5105 and the *OsPCS1*-overexpressing lines ($P > 0.05$ by Student's *t* test).

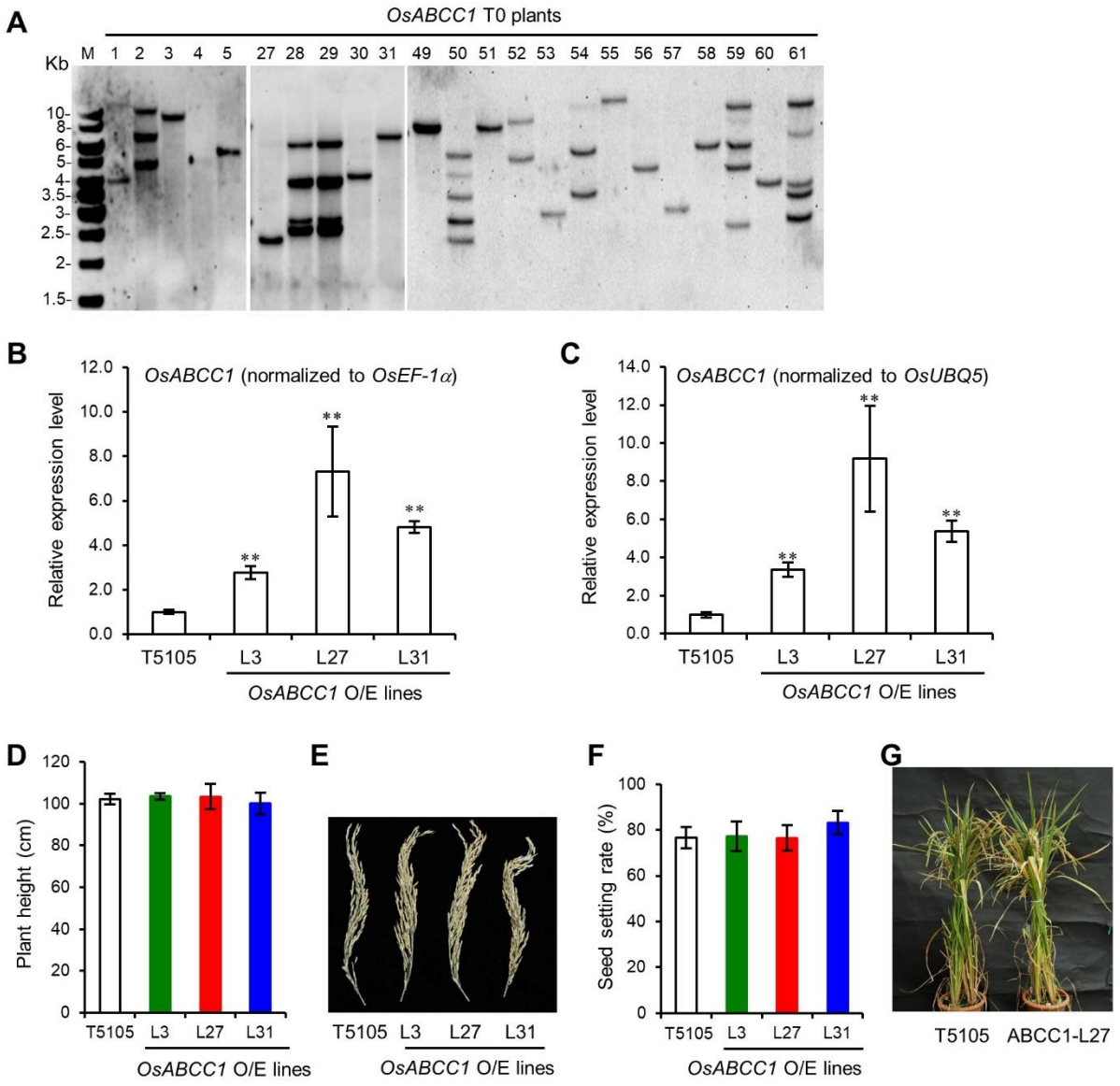

Fig. S3. Generation of *OsABCC1*-overexpressing lines. (**A**) Detection of the copy number of T-DNA in *OsABCC1* transgenic plants (T0) by Southern blot analysis. M, molecular marker. (**B**) and (**C**) Relative expression levels of the *OsABCC1* gene in T5105 and three homozygous *OsABCC1* overexpressing lines detected by qRT-PCR. The results were normalized against levels of *OsEF-1α* (**B**) and *OsUBQ5* (**C**), respectively, and the expression level in T5105 was arbitrarily set to 1. The asterisks indicate a significant difference between T5105 and transgenic lines (**$P < 0.01$ by Student's *t* test). (**D**) Plant height of T5105 and the *OsABCC1*-overexpressing lines. L3 (ABCC1-L3), L27 (ABCC1-L27) and L31 (ABCC1-L31) are three independent *OsABCC1*-overexpressing lines. (**E**) Morphology of rice panicles. (**F**) Seed setting rates of T5105 and the *OsABCC1*-overexpressing lines. (**G**) Morphological phenotype of T5105 and ABCC1-L27. Plants were imaged at 120 d after sowing. The data (mean ± SD) in (**B**), (**C**), (**D**) and (**F**) were obtained from 3 independent experiments using separately grown plants. No significant difference in (**D**) and (**F**) was observed between T5105 and the *OsABCC1*-overexpressing lines ($P > 0.05$ by Student's *t* test).

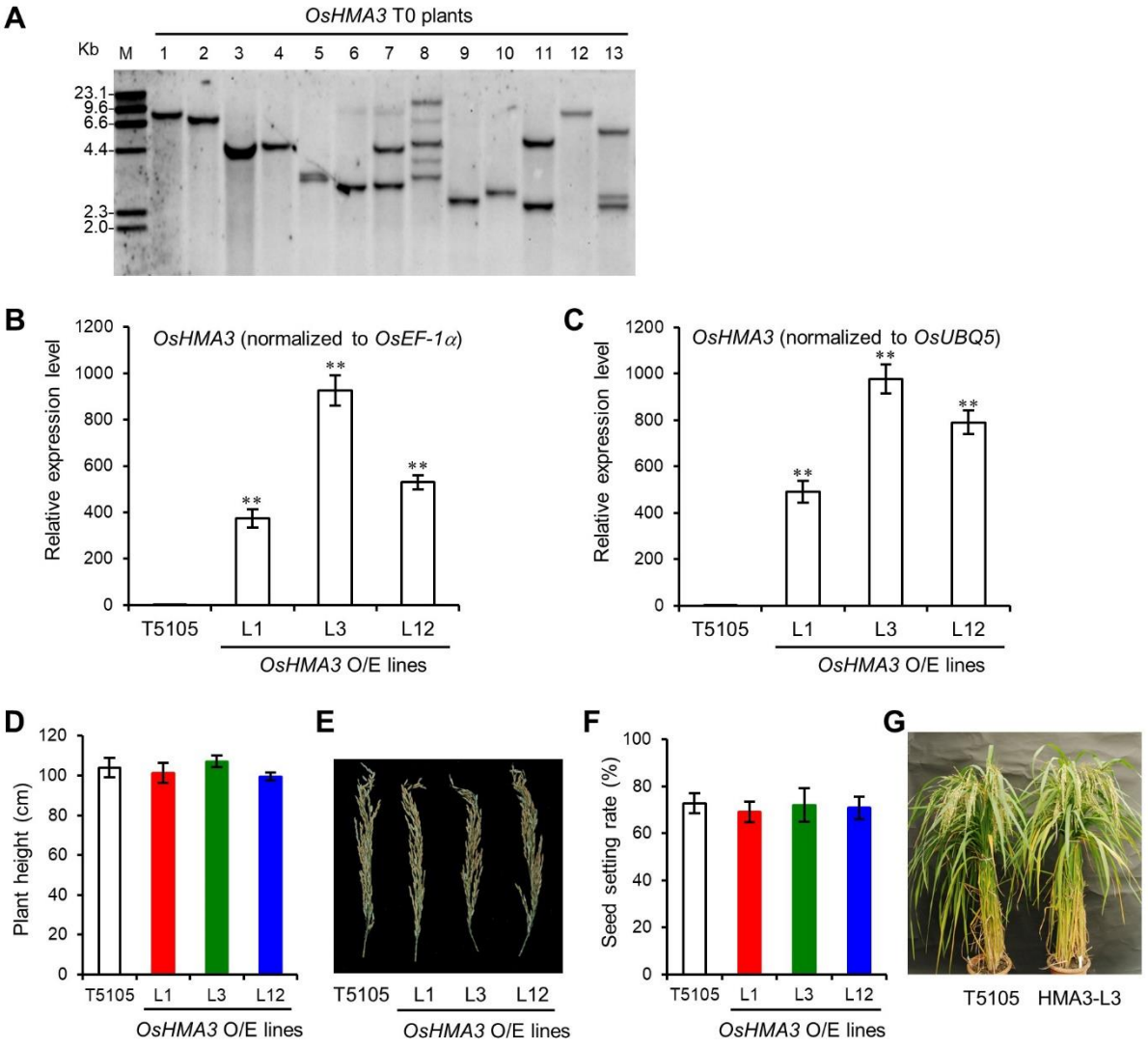

Fig. S4. Generation of *OsHMA3*-overexpressing lines. (**A**) Detection of the copy number of T-DNA in *OsHMA3* transgenic plants (T0) by Southern blot analysis. M, molecular marker. (**B**) and (**C**) Relative expression levels of the *OsHMA3* gene in T5105 and three homozygous *OsHMA3* overexpressing lines detected by qRT-PCR. The results were normalized against levels of *OsEF-1α* (**B**) and *OsUBQ5* (**C**), respectively, and the expression level in T5105 was arbitrarily set to 1. The asterisks indicate a significant difference between T5105 and transgenic lines (***P* < 0.01 by Student's *t* test). (**D**) Plant height of T5105 and the *OsHMA3*-overexpressing lines. L1 (HMA3-L1), L3 (HMA3-L3) and L12 (HMA3-L12) are three independent *OsHMA3*-overexpressing lines. (**E**) Morphology of rice panicles. (**F**) Seed setting rates of T5105 and the *OsHMA3*-overexpressing lines. (**G**) Morphological phenotype of T5105 and HMA3-L3. Plants were imaged at 120 d after sowing. The data (mean ± SD) in (**B**), (**C**), (**D**) and (**F**) were obtained from 3 independent experiments using separately grown plants. No significant difference in (**D**) and (**F**) was observed between T5105 and the *OsHMA3*-overexpressing lines (*P* > 0.05 by Student's *t* test).

Transgenic line	Nucleotide sequences of the junction region at the T-DNA insertion site
PCS1-L1	Os01g0688300.....ccctgcgtgctgcaaatataTTGTGGTGTAAACAAATTGA.....TAAACTATCAGTG TTTGAACgctgctactactaacactgc.....Os01g0688400
ABCC1-L27	Os03g0811400.....tcccttctctcccagccaacTTATATTGTGGTGTAAACAA.....ATCGGGAATTAAA CTATCAGtgtccctttcttcttcctct.....Os03g0811500
HMA3-L3	Os08g0300100.....aggcatggtctcaatgcttaGACAACTTAATAACACATTG.....GCAATTATACATTT AATACGtggattcaaaagatatgcag.....Os08g0300200

Fig. S5. Nucleotide sequences of the junction regions at the T-DNA insertion sites in the transgenic lines**.** The DNA sequences of the inserted T-DNA fragments are shown in uppercase letters. The genomic flanking DNA sequences are displayed in lowercase letters. The accession numbers of rice genes that flank the T-DNA fragments are also indicated. PCS1-L1, ABCC1-L27 and HMA3-L3 are the selected overexpression lines for *OsPCS1*, *OsABCC1* and *OsHMA3*, respectively.

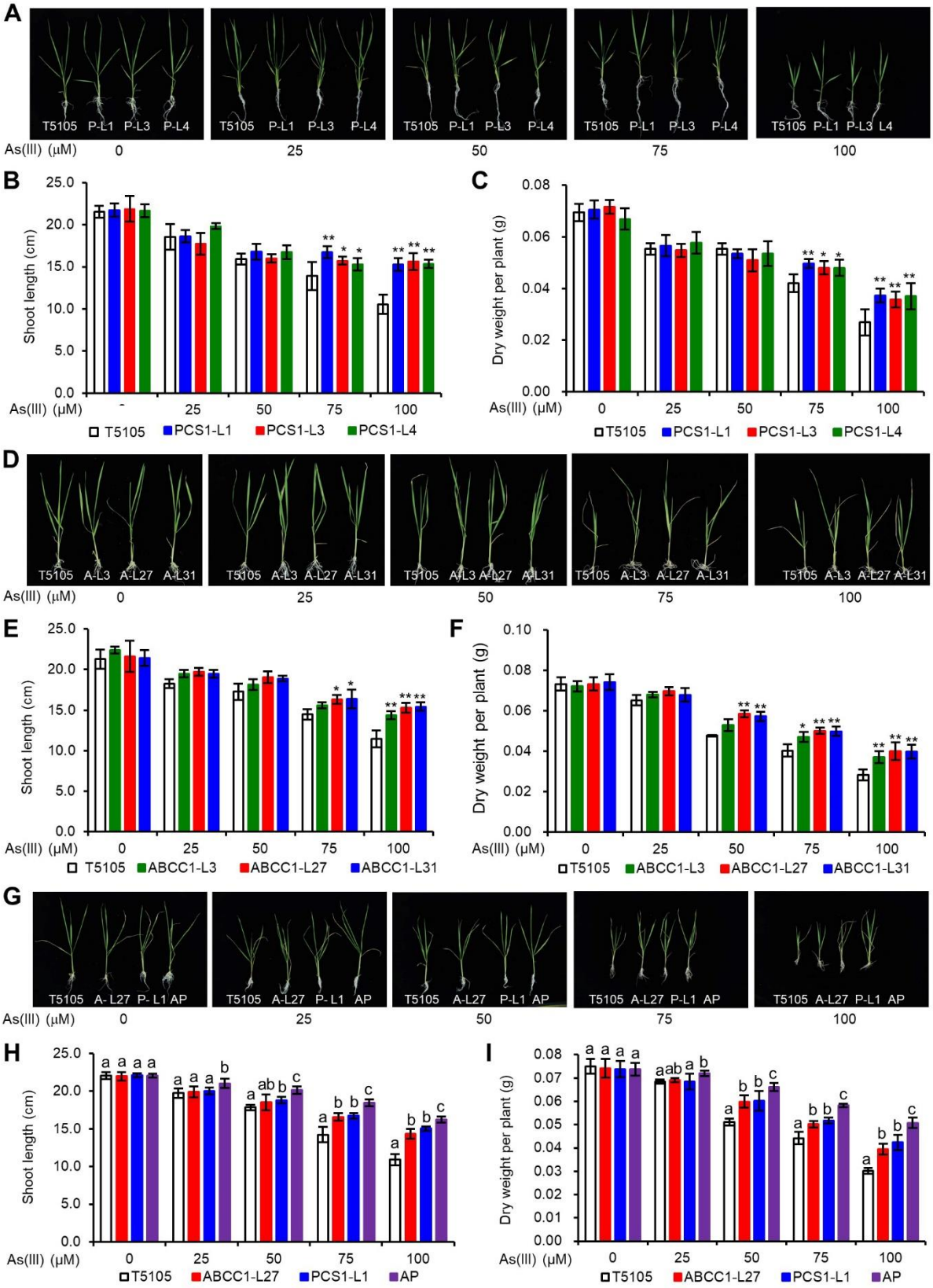

Fig. S6. Co-overexpression of *OsPCS1* and *OsABCC1* in rice synergistically confers enhanced tolerance to As. (**A**) Seedlings of T5105 and *OsPCS1* over-expressing lines at 14 d after As treatment. (**B**) and (**C**) Shoot length (**B**) and dry weight (**C**) of T5105 and *OsPCS1* over-expressing lines at 14 d

after As treatment. (**D**) Seedlings of T5105 and *OsABCC1*-overexpressing lines at 14 d after As treatment. (**E**-**F**) Shoot length (**E**) and dry weight (**F**) of T5105 and *OsABCC1*-overexpressing lines at 14 d after As treatment. (**G**) Seedlings of T5105, ABCC1-L27, PCS1-L1, AP at 14 d after As treatment. (**H**) and (**I**) Shoot length (**H**) and dry weight (**I**) of T5105, ABCC1-L27, PCS1-L1, AP at 14 d after As treatment. The data (mean ± SD) in (**B**), (**C**), (**E**), (**F**), (**H**) and (**I**) were obtained from 3 independent experiments using separately grown plants. The asterisks in (**B**), (**C**), (**E**) and (**F**) indicate a significant difference between T5105 and *OsPCS1* or *OsABCC1* overexpressing lines (**P* < 0.05; ***P* < 0.01 by Student's *t* test). The different letters in (**H**) and (**I**) indicate significant differences calculated by one-way ANOVA followed by LSD's test at *P* < 0.05. T5105, non-transgenic control; P-L1, PCS1-L1; P-L3, PCS1-L3; P-L4, PCS1-L4; A-L3, ABCC1-L3, A-L27, ABCC1-L27; A-L31, ABCC1-L31; AP, an *OsABCC1* and *OsPCS1* co-overexpressing line with transgenes derived from ABCC1-L27 and PCS1-L1.

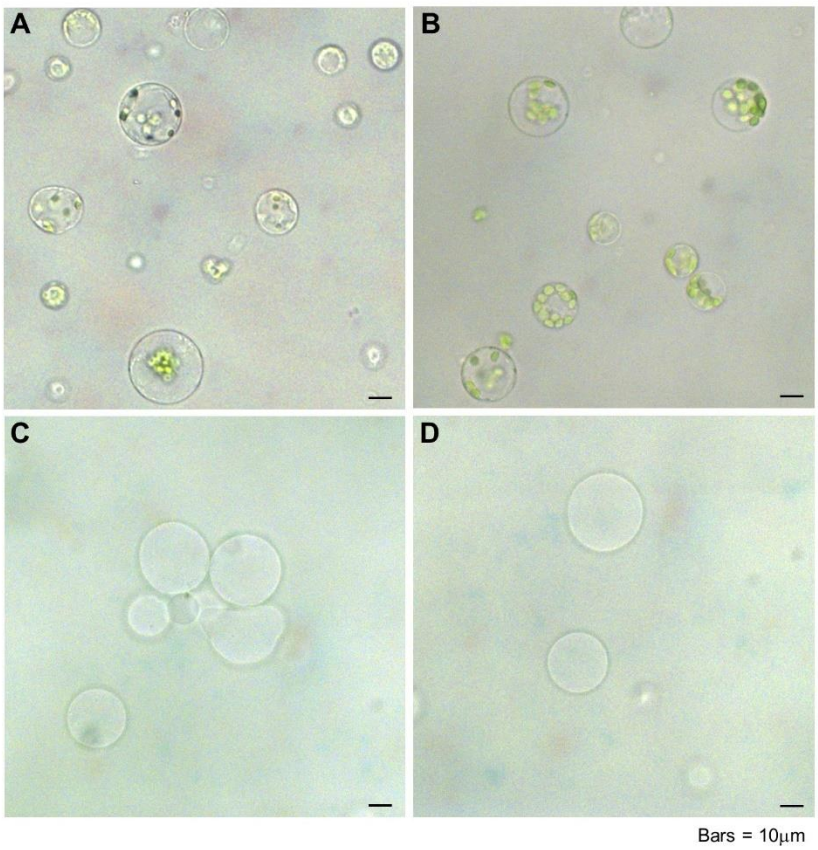

Fig. S7. Light microscopy images on isolated protoplasts and purified vacuoles for Cd concentration measurement. (**A**) and (**B**) Protoplasts isolated from T5105 (**A**) and PCS1-L1 (**B**) shoots. (**C**) and (**D**) Purified vacuoles isolated from T5105 (**C**) and PCS1-L1 (**D**) protoplasts**.**

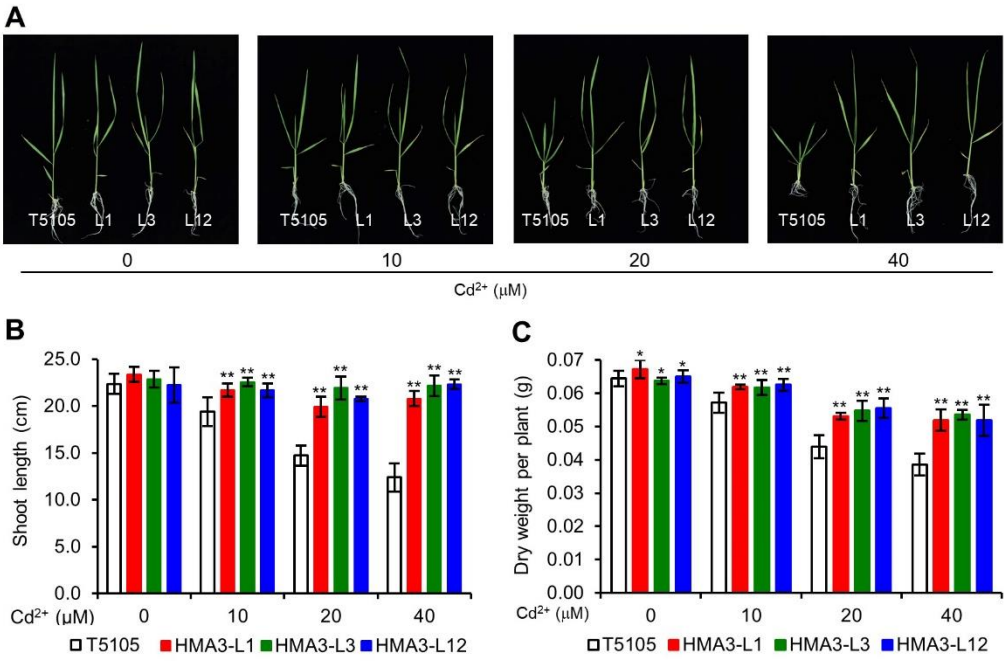

Fig. S8. Overexpression of *OsHMA3* in rice confers enhanced tolerance to Cd. (**A**) Seedlings of T5105 and *OsHMA3*-overexpressing lines at 14 d after Cd treatment. (**B**) and (**C**) Shoot length (**B**) and dry weight (**C**) of T5105 and *OsHMA3*-overexpressing lines at 14 d after Cd treatment. The data (mean ± SD) in (**B**) and (**C**) were obtained from 3 independent experiments using separately grown plants. The asterisks in (**B**) and (**C**) indicate a significant difference between T5105 and the *OsHMA3*-overexpressing lines (*$P < 0.05$; **$P < 0.01$ by Student's *t* test). T5105, non-transgenic control; L1, HMA3-L1; L3, HMA3-L3; L12, HMA3-L12.

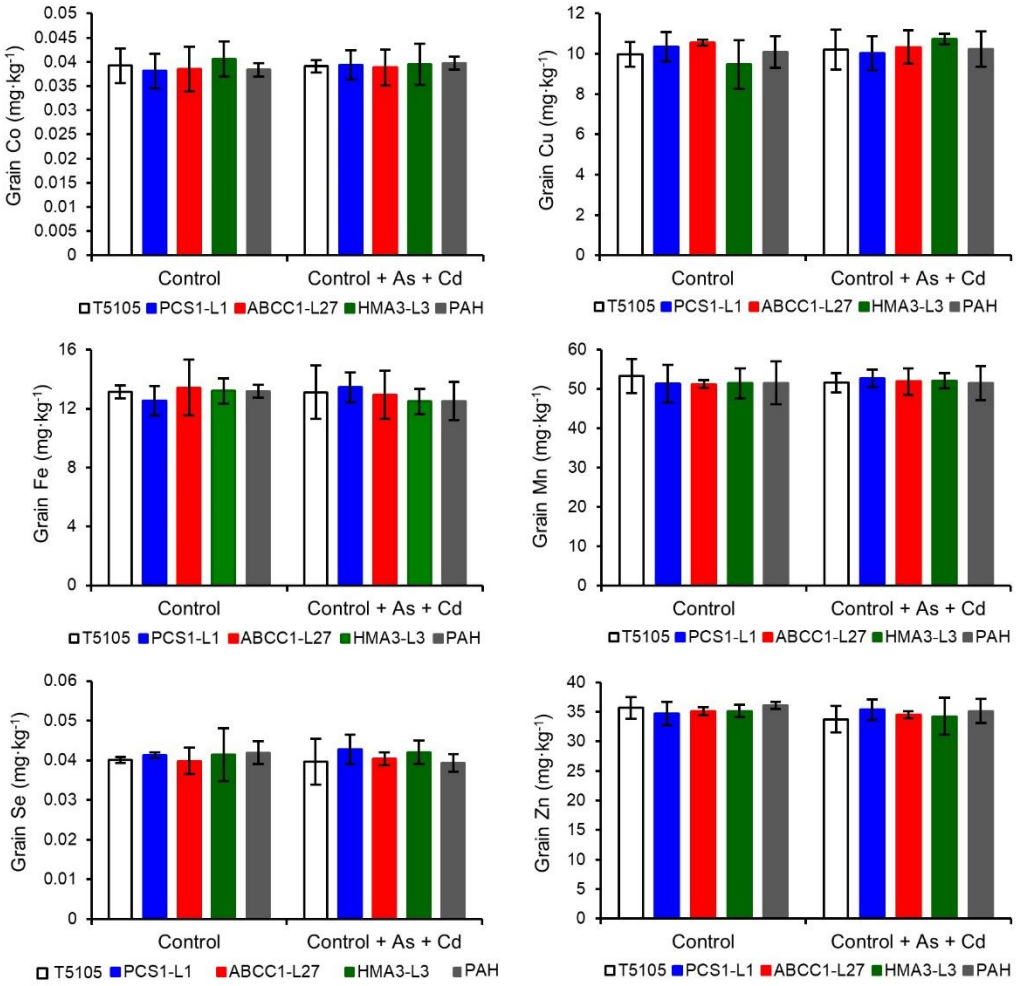

Fig. S9. The Concentration of nutrient elements in the grain of T5105, PCS1-L1, ABCC1-L27, HMA3-L3 and PAH. Rice plants were grown in control soil or As- and Cd-contaminated soil. The concentrations of Co, Cu, Fe, Mn, Se and Zn in rice grain were determined by ICP-MS. The data (mean ± SD) were obtained from 3 independent experiments using separately grown plants. No significant difference was detected between T5105 and transgenic lines by LSD's test at $P > 0.05$.

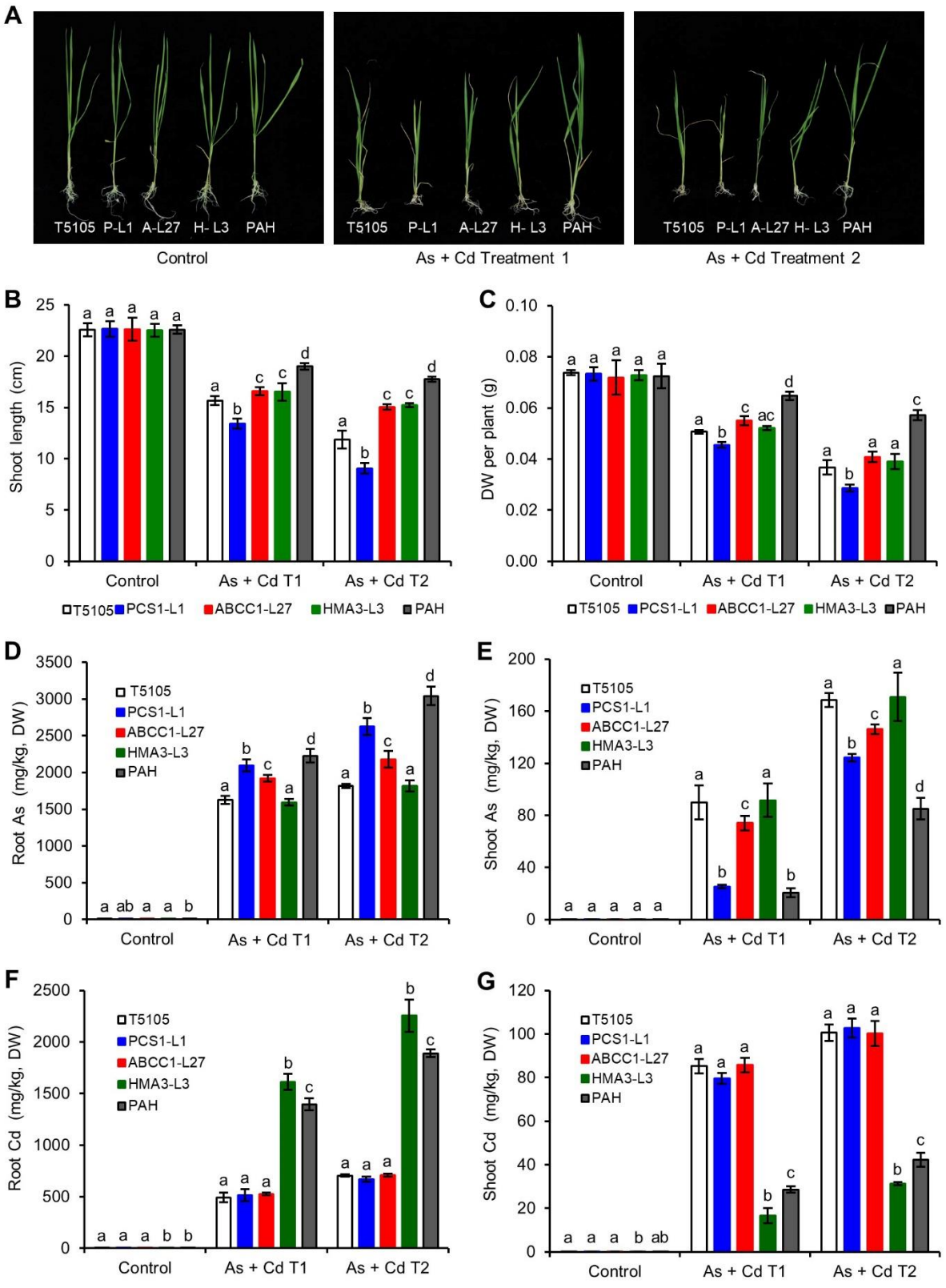

Fig. S10. Co-overexpression of *OsPCS1*, *OsABCC1* and *OsHMA3* in rice provides an enhanced tolerance to both As and Cd stress. (**A**) Seedlings of rice lines at 14 d after treatment with both As and Cd. (**B**) and (**C**) Shoot length (**B**) and dry weight per plant (**C**) of rice lines. (**D**) and (**E**) As concentration in roots (**D**) and shoots (**E**) of rice lines. (**F**) and (**G**) Cd concentration in roots (**F**) and shoots (**G**) of

rice lines. The data (mean ± SD) in (**B**) to (**G**) were obtained from 3 independent experiments using separately grown plants. Different letters indicate significant differences calculated by one-way ANOVA followed by LSD's test at $P < 0.05$. As + Cd Treatment 1 (As + Cd T1), half-strength MS medium containing 50 μM $NaAsO_2$ + 10 μM $CdSO_4$; As + Cd Treatment 2 (As + Cd T2), half-strength MS medium containing 75 μM $NaAsO_2$ and 20 μM $CdSO_4$. T5105, not-transgenic control; P-L1, PCS1-L1; A-L27, ABCC1-L27; H-L3, HMA3-L3; PAH, an *OsPCS1*, *OsABCC1* and *OsHMA3* co-overexpression line with transgenes derived from PCS1-L1, ABCC1-L27 and HMA3-L3.

II. Supplementary table

Table S1. Oligo primers used in this study

Name of primer	DNA sequences (5' to 3')	Purpose
ABCC1-qPCR-F	AACAGTGGCTTATGTTCCTCAAG	qRT-qPCR
ABCC1-qPCR-R	AACTCCTCTTTCTCCAATCTCTG	qRT-qPCR
PCS1-qPCR-F	AGCCCAAGTAAAGAGGCTAAC	qRT-qPCR
PCS1-qPCR-R	TACAACAGGGCTGCTTAGAAC	qRT-qPCR
HMA3-qPCR-F	CAGAACAGCAGGTCGAAGAC	qRT-qPCR
HMA3-qPCR-R	CCATTGCTCAAGGCCATCT	qRT-qPCR
EF-qPCR-F	GCACGCTCTTCTTGCTTTC	qRT-PCR
EF-qPCR-R	AGGGAATCTTGTCAGGGTTG	qRT-PCR
UBQ5-qPCR-F	AACCACTTCGACCGCCACT	qRT-PCR
UBQ5-qPCR-R	GTTCGATTTCCTCCTCCTTCC	qRT-PCR
HPT-F	AGCCTGAACTCACCGCGACGT	DNA probe
HPT-R	TACTTCTACACAGCCATCGGTCCA	DNA probe