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Original Research Article

Promoter *pLsi1*-driven *PvACR3* expression in rice enhances arsenic phytoextraction in paddy soils

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ABSTRACT

Arsenic (As) contamination in paddy soils is a global problem, threatening rice production and food safety. Hyperaccumulator plants have garnered significant attention for their potential to remove pollutants from contaminated soil. However, no natural hyperaccumulators have been found for the phytoremediation of As-contaminated paddy soils under flooding conditions. One promising strategy is to genetically engineer *Oryza sativa* (rice) to hyperaccumulate As for effective phytoremediation of paddy soil. A key challenge remains in increasing metal accumulation without compromising tolerance. Here, *PvACR3* from the As hyperaccumulator *Pteris vittata* was introduced under the control of a rice root-specific promoter *pLsi1* to create high-As-accumulating and tolerant transgenic remediation rice. The remediation rice strains exhibited robust growth, with shoot As concentration reaching up to 451–557 mg/kg in a hydroponic experiment with 20 μM $NaAsO_2$ treatment, and 45.9–80.3 mg/kg in pot experiments with moderately As-contaminated paddy soils. Compared to wild-type rice, the *pLsi1::PvACR3* transgenic rice removed 23.5 times more As from the same paddy soils. By harvesting rice shoots before grain filling, the soil pore water As was almost completely depleted, and the acid-soluble and reducible fractions of As were significantly reduced. This study presents the first transgenic remediation rice characterized by high As accumulation, tolerance, and adaptability to paddy soils under flooding conditions for effective phytoremediation.

1. Introduction

The contamination of arsenic (As) in paddy soils has become a worldwide problem because it can lead to high As concentrations in rice grains and endangers human health [1–5]. Developing in situ remediation strategies for paddy soils directly addresses this urgent food safety crisis [6]. Hyperaccumulators can accumulate high concentrations of specific metals or metalloids and feature prominently in phytoremediation [7,8]. *Pteris vittata* is recognized as the most promising natural hyperaccumulator of As [9,10]. However, the limited adaptability of *P. vittata* to long-term flooding restricts its application for soil phytoremediation in paddy fields.

Genetically modified heavy metal hyperaccumulating plants designed for phytoextraction offer solutions to the limitations of natural hyperaccumulators [11–13]. Rice (*Oryza sativa*) has emerged as a promising candidate for phytoextraction due to its versatility in being cultivated in both flooded and dry fields. It offers several advantages as a phytoremediation plant, including large biomass, short growth period, and well-established agronomic management and machinery for harvesting [14–17]. However, natural rice varieties have limited capacity to

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remove As because it primarily accumulates in the roots, which significantly compromises the efficacy due to the challenges associated with root removal [15]. To date, no transgenic remediation rice strain with high As accumulation in shoots for phytoextraction has been developed.

Current knowledge gaps that need to be addressed are whether functional genes from *P. vittata* have the same effect in transgenic rice. Some genes like *PvACR3*, *PvACR3;1*, *PvACR3;2*, *PvACR3;3*, *PvTIP4;1* and *PvPht1;3* expressed in *Arabidopsis*, tobacco or rape were proven to improve As accumulation or resistance [18–22]. Among them, *PvACR3* has high potential for engineering As-tolerant and hyperaccumulating plants for phytoextraction, as it enhances As translocation from roots to shoots without causing severe toxicity in *Arabidopsis thaliana* and *Nicotiana benthamiana* [18,23,24]. However, the effect in transgenic rice needs further investigation.

Challenges also exist in balancing resistance and accumulation in rice's easy-to-harvest parts [25]. A potential solution to this problem is to select an appropriate promoter that can regulate the extent of downstream gene expression and localization to an intensity that does not significantly affect the growth but increases heavy metal accumulation. This approach has been tried widely to improve crop quality, such as using a tissue-specific promoter P_{D540} to combat sheath blight and a stress-induced promoter *OsNAC6* to avoid the effect of constitutive expression of *OsTZF5* on plant growth and development [26,27]. The effectiveness of creating high-As-accumulating remediation plants is still unknown.

In this study, we expressed *PvACR3* in rice driven by a pan-tissue expression promoter *pUbi* (maize *Ubiquitin* promoter) and root-tissue specific promoter *pLsi1* (rice *OsLsi1* promoter) to create high-As-accumulating remediation rice strains [28,29]. Following hydroponic culturing and soil pot experiments, we showed that the *pLsi1::PvACR3* transgenic rice can tolerate, accumulate, and remove large amounts of As in the shoots, thus offering great potential for phytoremediation of As-contaminated paddy soils.

2. Materials and methods

2.1. Synthesis of the PvACR3 gene and pLsi1 promoter

The two-month-old seedlings of *P. vittata* preserved in our laboratory (collected from an As-contaminated mine site in Hubei Province, China) were used for *PvACR3* synthesis [30,31]. Total RNA was extracted from the leaves of *P. vittata* using a plant RNA extraction kit (Vazyme, Nanjing, China), and then immediately converted into cDNA using a reverse transcription kit (Vazyme, Nanjing, China). The full-length coding DNA sequence (CDS) of *PvACR3* was cloned from cDNA using primers (Table S1) designed according to the nucleotide sequence GI number *310768479*. Total genomic DNA of *O. sativa* (Zhonghua 11 cultivar) was extracted from seedlings using a plant DNA extraction kit (Vazyme, Nanjing, China). *pLsi1* was an upstream 2500 bp sequence of *OsLsi1* (LOC_Os02g51110) cloned from genomic DNA using the primers shown in Table S1. The cloned sequences were verified after sequencing by the BGI gene sequencing platform and alignment by DNAMAN 8.0. The verified PCR products were stored in −20 °C.

2.2. Vector construction and transgenic rice generation

To dissect the tissue-specific regulatory role of *PvACR3* in As accumulation and detoxification, two transgenic constructs were generated: *pUbi::PvACR3* (constitutive overexpression) and *pLsi1::PvACR3* (root-specific expression). The *pLsi1* promoter was selected due to its dual-layer expression in root exodermis and endodermis cells under flooded conditions, which aligns with the natural spatial regulation of *OsLsi1* [29]. In contrast, the maize ubiquitin promoter *pUbi* served as a constitutive control based on its well-established pan-tissue expression profile and high transcriptional stability in monocots [32]. Both constructs were assembled using the binary vector *pUN1301*. The *pUbi::PvACR3* was generated by inserting *PvACR3* into *pUN1301* by recombination between BamHI and KpnI. The *pLsi1::PvACR3* was generated from *pUbi::PvACR3* by replacing *pUbi* with *pLsi1*.

To determine the tissue expression pattern of *pUbi* and *pLsi1* in rice, the fusion of *pUbi* and *pLsi1* with the *GUS* (β-glucosidase) reporter gene was created by replacing *p35S* upstream of GUS in *pUN1301* using the ClonExpress II One Step Cloning Kit (Vazyme, Nanjing, China).

All the vectors were transformed into *Escherichia coli DH5α*, and positive single clones were selected and verified by the BGI gene sequencing platform and alignment by DNAMAN 8.0. The recombinant vectors were then transformed into the calli of Zhonghua 11 rice by Agrobacterium-mediated transformation [33]. The stable homozygous positive transgenic plants were identified in the T3 generation via segregation analysis.

2.3. GUS staining analysis for tissue localization

The roots and leaves of *pUbi::GUS* and *pLsi1::GUS* transgenic rice were collected 4 weeks after seeding. *GUS* histochemical staining was performed for 8 h according to the *GUS* Stain Kit (Solarbio, Beijing, China) and then subdivided into sections for observation by confocal microscopy (Carl Zeiss; LSM510Meta).

2.4. RT-qPCR

The expression levels of the *PvACR3* gene were determined by RT-qPCR. Total RNA was extracted from the roots and leaves of 3-week-old rice seedlings planted in Kimura B nutrition solution using the Plant Total RNA Kit (Sigma–Aldrich). First-strand cDNA was synthesized from 2 μL of total RNA using the HiScript II 1-Step RT-PCR Kit (Vazyme Biotech, Nanjing, China) with RT-PCR templates. The cDNA of *PvACR3* was amplified by PCR for 30 cycles using *PvACR3*-specific primers, as shown in Table S1. The length of the target fragment detected by RT-qPCR was the full length of the *PvACR3* coding sequence (1140 bp). *OsActin* was amplified for 30 cycles as an expression control using the *OsActin*-specific primers in Table S1.

2.5. Luciferase assay

To determine the activity of *pUbi* and *pLsi1*, the promoter sequence was inserted upstream of the luciferase gene in *pGreenII 0800-LUC* to form a recombinant vector. The verified correct recombinant vectors *pUbi::Luc* and *pLsi1::Luc* were transformed into *Agrobacterium tumefaciens GV3101* and injected into 4-week-old tobacco leaves [34]. Luminescence images were obtained by a plant CCD imaging system with IndiGO software and the relative luciferase activity (luminescence intensity per cm^2 leaf area) was measured following Wang et al. [35].

2.6. Subcellular localization of PvACR3

To determine the subcellular localization of *PvACR3*, *pLsi1::PvACR3-GFP* and *pUbi::PvACR3-GFP* fusion vectors were constructed using the *pSAT6-eGFP* backbone. Rice protoplasts were isolated from 7-day-old etiolated seedlings (Zhonghua 11) via enzymatic digestion according to the manufacturer's instructions (Coolaber, Beijing, China). For electroporation, 200 μL of protoplast (2×10^5 cells/mL) were mixed with 20 μg of plasmid DNA and transfected using a Gene Pulser Xcell™ system (300 V, 250 μF, 10 ms pulse). After 16-h incubation in darkness at 25 °C, GFP fluorescence was visualized with a confocal microscope (Zeiss LSM710NLO). Co-localization analysis was performed using FV10-ASW software.

2.7. Hydroponic and soil experiments

Seeds of wild type (WT) and transgenic rice were surface-sterilized with 70% alcohol (1 min) and 30% H_2O_2 (20 min), then soaked in

deionized water for 24 h before germination in darkness at 35 °C for 3 days [15]. For hydroponic experiments, uniform seedlings were transferred to Kimura B solution containing 0, 5, or 20 μM $NaAsO_2$ for 2 weeks (solution replaced every 3 days) under controlled conditions (16/8 h light/dark, 32 °C/28 °C).

For soil experiments, two As-contaminated soils were used: natural paddy soil (38.5 mg/kg total As) and artificially amended soil (44.2 mg/kg total As, prepared by adding 5 mg/kg $NaAsO_2$ and aging for 30 days). Soil properties, including total As, pH, CEC, SOC, total N, P, K, and available P and K of the soil, are measured and shown in Table S2 [36,37]. Then, each pot (20 cm × 15 cm) was filled with 3.0 kg of soil, pre-flooded (2–3 cm water) for 7 days, and then transplanted with four-week-old rice seedlings (2 plants/hill, 3 hills/pot). A constant flood depth of 2–3 cm was maintained throughout the growing period. Basal fertilizers, including $CO(NH_2)_2$ and K_2HPO_4, were applied at 0.4 g/kg pre-transplant and 0.6 g/kg during the jointing and filling stages. Plants were grown for 16 weeks under controlled conditions (16/8 h light/dark, 32 °C/28 °C, 60% RH, 3000 lx).

2.8. Sample collection and analysis

Pore water was collected biweekly using soil solution samplers (19.21.21F, 10 cm length, Rhizosphere Research Products, Wageningen, Netherlands) inserted at 45° near seedlings and subsequently analyzed by ICP-MS [38]. At harvest, plants were photographed, measured for root length, height, and biomass, and then separated into roots and shoots. The rinsed plants were heated at 105 °C for 30 min and dried at 65 °C for 24 h, then weighed and ground to determine the As content digested by HNO_3 and H_2O_2 (EPA Method 3051a) at 180 °C. All digested materials were diluted to 50 mL for total As analysis by ICP-MS.

Post-harvest, rhizosphere soils were air-dried and ground to <2 mm and <0.25 mm. Total As was determined after triple-acid digestion (HNO_3-HF-$HClO_4$), while bioavailable As was measured in the solution sampled using Zr-oxide DGT samplers (LSLZ-NP, DGT® Research, Nanjing, China) [39,40]. As speciation was analyzed by a modified BCR sequential extraction method [41,42]. All extracts were diluted with HNO_3 and stored at 4 °C before analysis by ICP-MS.

2.9. Statistical analysis

Three replicates of all samples were used to perform the statistical analysis. Data are presented as the mean of three replicates with standard deviation (mean ± SD). All treatment means were compared by Tukey's test of one-way analysis of variance (ANOVA) in SPSS 16.0 software. Differences were considered significant at $p < 0.05$. The figures were drawn by Origin Lab 2018.

3. Results

3.1. Promoter pLsi1-driven PvACR3 expressed in the rice root plasma membrane

To develop a transgenic rice capable of decontaminating As from paddy soil, As-accumulating rice was engineered using a heterologous transgenic approach (Fig. S1). *PvACR3*, an As(III) transporter present in *P. vittata* but absent in rice, was selected as the downstream module source (Figs. S2–S4). The *PvACR3* driven by *pLsi1* and *pUbi* was thus designed to facilitate the transport of As(III) to the vasculature. Finally, for each *pLsi1::PvACR3* and *pUbi::PvACR3* transgenic rice, two homozygous lines of the T3 generation (namely L1 and L2) were obtained for further research on As accumulation and tolerance in As-contaminated soils.

In order to further investigate the downstream gene *PvACR3* tissue location and gene expression patterns driven by *pLsi1* and *pUbi*, the GUS histochemical analysis, RT–qPCR and the assessment of luciferase activity were performed. As shown in Fig. 1A, *pLsi1::GUS* gene expression was observed only in rice roots, while *pUbi::GUS* gene expression was observed in almost all tissues, including roots and shoots, confirming that *pLsi1* is a rice root-specific expression promoter [29]. The RT–qPCR results were consistent with *GUS* staining results, confirming that the *PvACR3* gene was expressed only in the roots driven by *pLsi1* but was expressed in both roots and shoots regulated by *pUbi* (Fig. 1B). Furthermore, the RT–qPCR results showed that the expression level of *PvACR3* in roots driven by *pLsi1* was almost 50% lower than the expression level driven by *pUbi*. This result was supported by the results of luciferase activity driven by *pUbi* and *pLsi1* (Fig. 1C), in which *pLsi1::luciferase* showed a lower fluorescence intensity than *pUbi::luciferase*, implying that *pLsi1* is a promoter with weaker priming capability.

To clarify the subcellular localization of *PvACR3* in rice, two *PvACR3-GFP* fusion plasmids driven by *pUbi* and *pLsi1* were transiently transformed into rice protoplasts. Both the fluorescence signals of *pLsi1::PvACR3::GFP* and *pUbi::PvACR3::GFP* overlapped with the plasma membrane of protoplasts from rice roots, while fluorescence was observed everywhere in the vector *pUbi::GFP*, suggesting that *PvACR3* was localized to the plasma membrane in rice (Fig. 1D).

3.2. pLsi1::PvACR3 rice enhances arsenite tolerance, accumulation, and translocation in hydroponics

To investigate the capacity of *pLsi1::PvACR3* rice to tolerate and accumulate As, we determined the growing status and As concentrations in WT and transgenic rice exposed to arsenite ($NaAsO_2$), the main form of As in flooded paddy soil, under hydroponic conditions.

pLsi1::PvACR3 expression in rice significantly increased As tolerance. In the presence of 0, 5, or 20 μM $NaAsO_2$, plant growth varied based on the As concentration (Fig. 2A, Fig. S6). Without As exposure, *pLsi1::PvACR3* rice appeared similar to the WT, with no significant changes in plant height, root length, and biomass, indicating that the expression of *PvACR3* conferred inconspicuous changes in plant growth. In the 5 and 20 μM $NaAsO_2$ treatment, *pLsi1::PvACR3* rice showed higher As tolerance compared to WT, with a significantly larger fresh shoot biomass and longer observable root length ($p < 0.05$). These findings indicate that *pLsi1::PvACR3* has the potential for higher As tolerance compared with WT and *pUbi::PvACR3* rice.

The expression of *pLsi1::PvACR3* in rice significantly increased As concentration in rice shoots. Under 5 and 20 μM $NaAsO_2$ treatments, WT plants showed the highest root As concentrations ($p < 0.05$), whereas transgenic lines displayed significantly reduced root As levels (Fig. 2B-C, Fig. S6A–B). In contrast, shoot As accumulation demonstrated a reverse trend. WT shoots exhibited the lowest As concentrations (7.24 and 23.5 mg/kg under 5 and 20 μM treatments, respectively; $p < 0.05$), consistent with their minimal total As translocation to aerial tissues (Fig. 2D and E). Strikingly, *pLsi1::PvACR3* lines exhibited 37.1–50.3-fold and 19.2–23.7-fold higher shoot As concentrations than WT under 5 μM (268–364 mg/kg) and 20 μM (452–557 mg/kg) treatments, respectively ($p < 0.05$).

pLsi1::PvACR3 expression in rice also dramatically increased the transfer factor (TF, the ratio of As concentration in shoot to root) of As. In the presence of 5 μM $NaAsO_2$, the TF of the *pLsi1::PvACR3* transgenic rice sharply increased from 0.00252 to 0.779–1.104 compared with the WT, and in the presence of 20 μM $NaAsO_2$, the TF of the *pLsi1::PvACR3* transgenic rice increased from 0.00449 to 0.248–0.390 compared with the WT (Fig. 2F and G). These results indicate that the *pLsi1::PvACR3* rice significantly promoted As translocation from roots to shoots.

In contrast, the *pUbi::PvACR3* rice exhibited reduced As tolerance under $NaAsO_2$ stress. At 5 μM $NaAsO_2$, shoot fresh biomass decreased by 72.2% with a corresponding 61.7% reduction in plant height. This effect intensified at 20 μM $NaAsO_2$, showing 79.9% biomass loss and 61.2% height reduction (Fig. S6). However, the *pUbi::PvACR3* lines showed even more pronounced enhancement, reaching 946–1059 mg/kg (5 μM) and 2112–2428 mg/kg (20 μM), corresponding to 131–146-fold and 89.9–103-fold increases over WT ($p < 0.05$) (Fig. S7C–D). What's more, the TF of *pUbi::PvACR3* lines reached up to 9.13–17.9 in the 5 μM

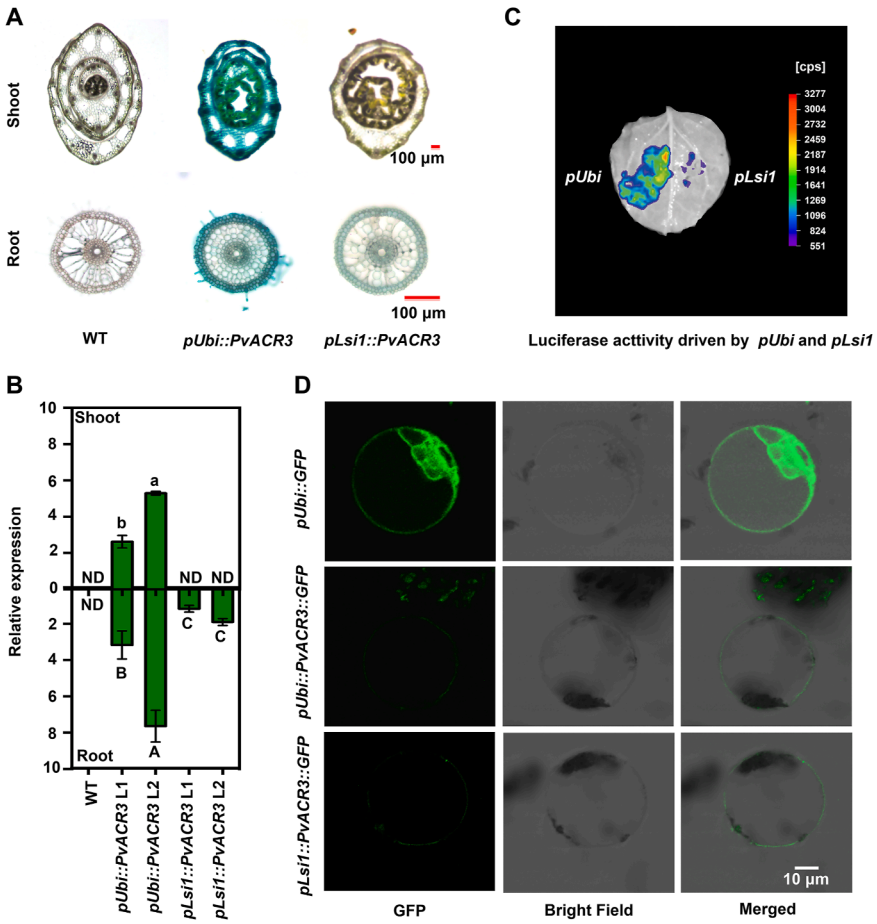

Fig. 1. Expression profile of *PvACR3* in rice strains. (A) *GUS* staining of *pUbi::GUS* and *pLsi1::GUS* in rice root and shoot; (B) RT-qPCR analysis of *PvACR3* expression of *pUbi::PvACR3* and *pLsi1::PvACR3* rice in root and shoot. *OsActin* were used as the internal reference genes; L1 and L2 represent different transgenic lines. Different letters indicate significant differences ($p < 0.05$); (C) Imaging of luciferase expression activity regulated by *pLsi1* and *pUbi*; (D) The subcellular localization of *PvACR3* in rice. ND, not detected; GFP, green fluorescent protein; WT, wild type.

$NaAsO_2$ treatment and 1.36–1.81 in the 20 μM $NaAsO_2$ treatment (Fig. S7E–F). These results demonstrate that shoot As accumulation depends on promoter activity, with the constitutive *pUbi* driving more pronounced *PvACR3*-mediated effects than the root-specific *pLsi1*. The elevated *PvACR3* abundance under *pUbi* likely enhances As(III) efflux from root cells to the apoplast, promoting xylem loading and subsequent shoot translocation.

3.3. *pLsi1::PvACR3* rice enhances As resistance and accumulation in contaminated paddy soils

To examine the phytoextraction potential of the engineered transgenic rice in As-contaminated soils, two polluted paddy soils containing 38.5 and 44.2 mg/kg total As were selected for pot experiments under greenhouse conditions (Table S2). The experiments were carried out for 112 days. Plants were harvested at the booting stage before the grain filling stage for As concentration determination.

In pot experiments, *pUbi::PvACR3* transgenic rice died quickly in the seedling stage, with shoot As concentrations reaching 398–477 mg/kg (Fig. S8). In contrast, *pLsi1::PvACR3* transgenic rice developed better, with no significant differences in plant height and biomass in aboveground tissues compared to WT (Fig. 3A,C, Fig. S9). Furthermore, As concentrations in the aerial parts of the *pLsi1::PvACR3* transgenic rice were significantly higher than those in WT, with 11.4–19.4-fold more As in L1 and 13.7–20.7-fold more As in L2 (Fig. 3B,D). These results indicate that *pLsi1::PvACR3* transgenic rice is capable of balancing As accumulation and resistance in contaminated soils, showing the potential for phytoremediation. The *pLsi1::PvACR3* transgenic rice accumulated 92.5%–96.2% of the total As in the aerial parts, whereas 91.7% of the As remained in the roots of wild-type rice (Fig. 3E). As a result, the bioconcentration factor (BCF, the ratio of As concentration in plant to soil) and TF of As in *pLsi1::PvACR3* rice were increased markedly and reached 1.15–1.23 and 2.93–5.70 (Fig. 3F and G), respectively, meeting the requirement that the BCF and TF of a hyperaccumulator be greater than 1 [43].

Phytoremediation efficiency was further estimated by calculating the total amount of As removed by the rice (Fig. 3H). In the soils mildly contaminated with As, *pLsi1::PvACR3* rice shoots accumulated 1.11–1.55 mg As/hill (Fig. 3H), which was more than 33-fold higher compared with WT in the actual contaminated soil pots. Based on this finding, it is estimated that 121 mg As/m^2 in the top 20 cm surface soil can be removed from paddy soil in one season by harvesting the aboveground portions of the plant only, with a plant density of 756,000 plants/hm^2 and based on an amount of 1.55 mg As/hill (Fig. 3I) [15]. Although the wild type high As-accumulating rice reported in other research also showed high As removal capacity (Fig. 3I), the As was enriched in roots, which will limit phytoremediation because of the risk of further release of As into the soil environment and lack of ideal method for removing root systems in practical application [15]. Besides, when *P. vittata* seedlings were planted in the same contaminated soils without flooding under 60% field capacity for 112 days, the hyperaccumulator shoots accumulated only 0.822 mg As/plant, which was 26.5% and 48.2% lower than the amount of As removed by the *pLsi1::PvACR3* rice shoots (Table S3). A previous study found that in a contaminated agricultural field with soil As content of 29.3 mg/kg, the hyperaccumulator *P. vittata* could only remove an estimated 35.3 mg As/m^2 from the top 20 cm of soil [44]. The *pLsi1::PvACR3*

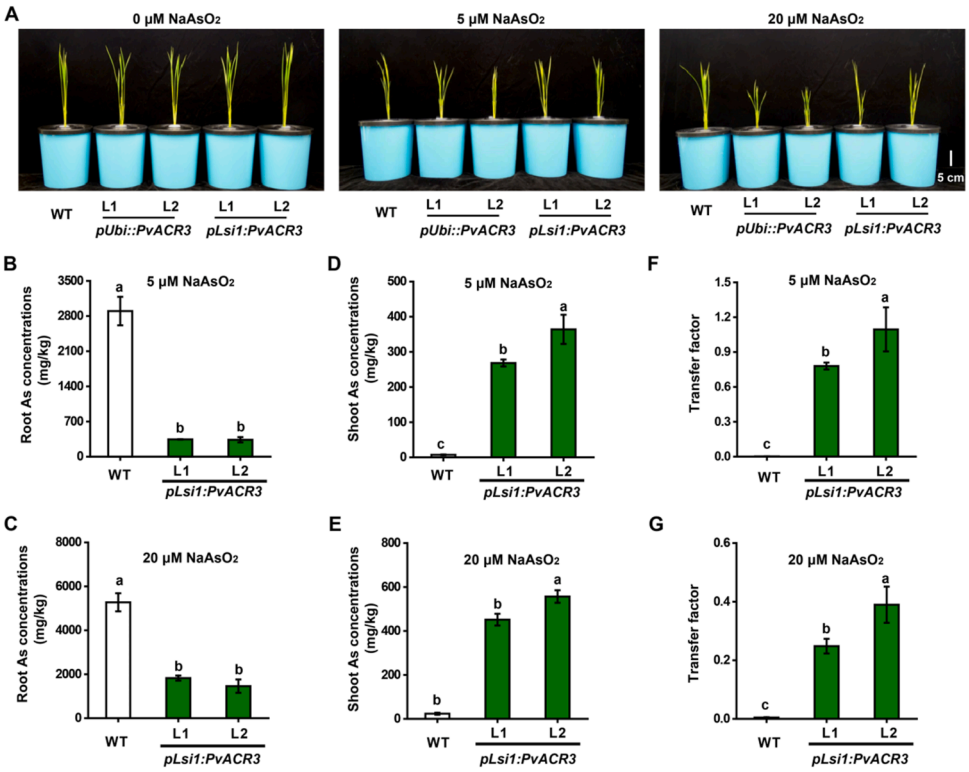

Fig. 2. As tolerance and accumulation characteristics of WT and different types of transgenic rice strains under varying $NaAsO_2$ exposure. (A) Rice phenotypes; (B–E) Root and shoot As concentrations; (F–G) Transfer factors. Different letters indicate significant differences ($p < 0.05$).

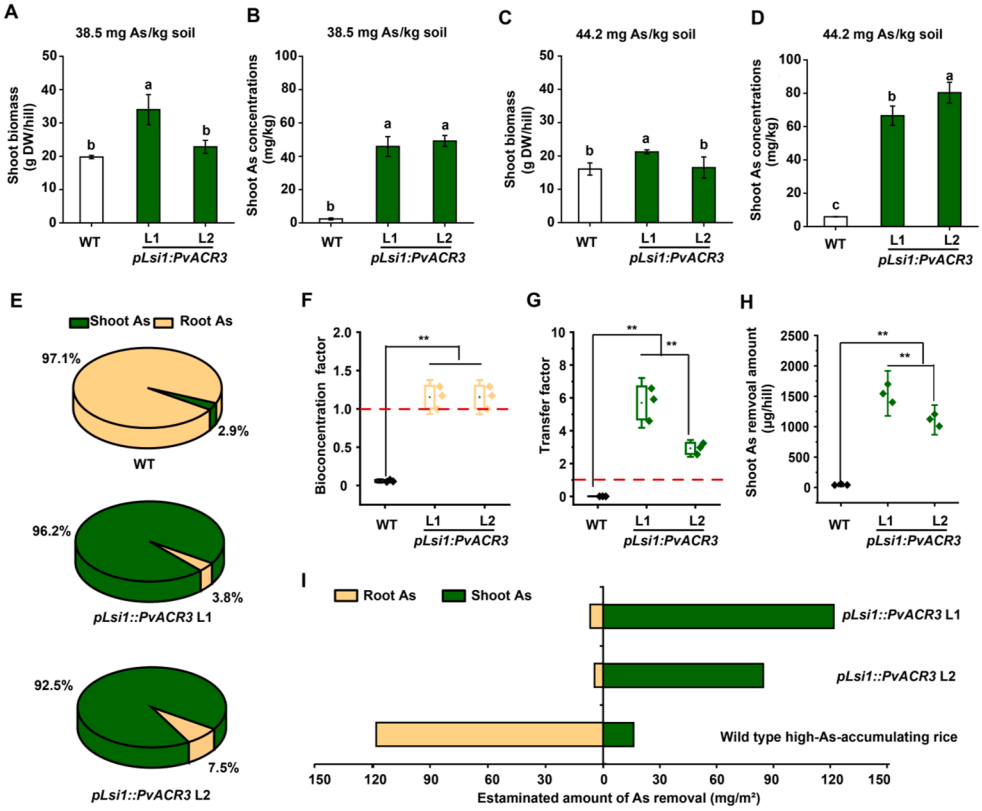

Fig. 3. Growth characteristics and As removal capacity of WT and *pLsi1::PvACR3* rice in As-contaminated paddy soils. (A–D) Shoot biomass and As concentrations of rice grown in 38.5 and 44.2 mg/kg total As-contaminated soils. Different letters indicate significant differences ($p < 0.05$). (E–H) As distribution in rice, bioconcentration factor (BCF), transfer factor (TF), and shoot As removal amount in 38.5 mg/kg total As-contaminated soils, $^{**}p < 0.01$; (I) As removal amount estimated based on field planting density. DW, dry weight.

rice thus showed a more effective removal of As from soil, even only by harvesting the shoots. Thus, we successfully engineered a remediation rice with the potential for simplifying its harvesting process by accumulating As in shoots.

3.4. *pLsi1::PvACR3 rice efficiently reduces bioavailable As before booting*

The residual concentration of As in the rhizosphere soil after phytoremediation was measured to evaluate the As removal efficiency. Phytoremediation using *pLsi1::PvACR3* transgenic rice resulted in a marked depletion of bioavailable As pools in rhizosphere soils. Comparative analysis revealed two-tiered reductions: relative to untreated controls, acid-soluble As decreased by 58.8%–69.1% and reducible As by 28.5%–43.7% (Fig. 4A). More critically, the transgenic line outperformed WT rice by achieving additional reductions of 30.2%–49.7% and 20.3%–49.7% in these mobile fractions, respectively. The oxidizable As fraction showed no statistically significant variation across treatments ($p > 0.05$). This selective depletion of adsorbed and Fe/Mn oxide-bound As fractions aligns with the enhanced root-to-shoot translocation of As mediated by *PvACR3* expression. By facilitating translocation of As(III) in aerial tissues through its *PvACR3* transporter activity, the transgenic lines effectively decouple As from iron redox cycling in the rhizosphere, thereby minimizing As re-mobilization risks.

To further evaluate the bioavailable As that was most easily taken up by plants after phytoremediation, a diffusive gradient in thin-films (DGT) method that simulates the root absorption process was applied [37]. The initial DGT-As concentrations were 1095–1211 μg/kg. After phytoremediation with *pLsi1::PvACR3* rice, the DGT-As concentrations decreased by 51.5%–66.3% compared to the unplanted control soils (CK), and 22.0%–46.8% compared to WT (Fig. 4B). This comparison highlights that the growth of *pLsi1::PvACR3* rice efficiently removes the bioavailable As from the soils.

The As concentration in the pore water of paddy fields is another key parameter for assessing the efficiency of phytoremediation [45]. To determine the optimal timing for achieving the best phytoremediation effect in the shortest possible period of time, levels of As in pore water of CK, rhizosphere soils of WT, and *pLsi1::PvACR3* rice were monitored throughout the experiment, as shown in Fig. 4C. In unplanted control soils, the As concentrations in pore water surged from 226 to 346 μg/L to 920–1635 μg/L after 2 weeks of flooding, indicating an increase in the bioavailable As under flooded conditions. During the seedling, tillering, and jointing stages, the As levels in the rhizosphere soils decreased continuously in the presence of growing transgenic rice plants. Specifically, from days 28–84, the As concentrations in the soil solution fell dramatically to 15.3–53.9 μg/L, which was significantly lower than those in the control soils without rice planting. This finding indicates rapid As uptake by the transgenic rice plants during the seedling to jointing stages. In the booting stage, pore water As concentrations remained below the detection limit, indicating that As had been absorbed and depleted. Therefore, harvesting rice plants prior to the booting stage can be suggested to shorten the pratice time for efficient phytoextraction.

4. Discussion

4.1. *pLsi1::PvACR3 cross-species engineering balances As accumulation and tolerance in rice*

Transgenic engineering of plants for As phytoremediation has emerged as a promising strategy for soil decontamination. However, current advancements remain confined to model plants or dry-farming crops under controlled laboratory settings, leaving a critical gap in aquatic phytoremediation systems such as paddy fields. To address the critical need for paddy field adaptation, this study pioneers rice as the transgenic recipient for wetland phytoremediation applications.

In this study, both *pUbi*- and *pLsi1*-driven *PvACR3* localized in the plasma membrane in rice (Fig. 1D). This finding aligns with prior observations in *Arabidopsis*, where plasma membrane-localized *PvACR3* dramatically reshaped As spatial dynamics [18]. Strikingly, *PvACR3* displays species-dependent subcellular targeting. While it functions as a vacuolar membrane-localized As(III) sequestrator in *P. vittata* gametophytes [46], it localizes to the plasma membrane in angiosperms such as *Arabidopsis* [18] and rice (Fig. 1D). This evolutionary divergence fundamentally reprograms As trafficking routes. In angiosperms, plasma membrane-anchored *PvACR3* drives root-to-shoot As(III) translocation through apoplastic efflux and enhanced xylem loading.

However, *PvACR3,* driven by different promoters, has different functions in rice. The constitutive expression of *pUbi::PvACR3* in rice

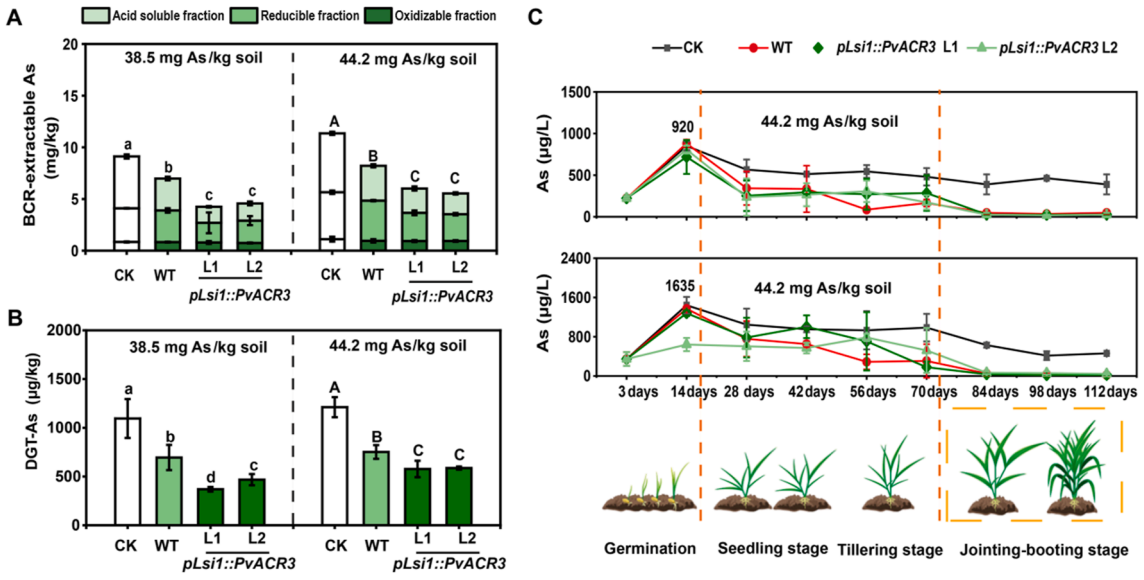

Fig. 4. Residual As in soils after phytoremediation by WT and *pLsi1::PvACR3* transgenic rice and dynamic changes in pore water As during cultivation. (A) Concentrations of acid soluble, reducible and oxidizable As fractions. Significant differences refer to the total BCR-extractable As (sum of all fractions); (B) Bioavailable As concentrations; (C) Dynamic changes of As concentrations in pore water. Different letters indicate statistically significant differences ($p < 0.05$). BCR, modified BCR (Community Bureau of Reference) sequential extraction method; CK, unplanted control soil; DGT, Diffusive Gradients in Thin-films.

triggered lethal As hyperaccumulation (Fig. S8), likely due to ectopic overexpression in shoot tissues. Notably, although *pUbi::PvACR3* lines achieved elevated shoot As accumulation (398–477 mg/kg), their constitutive expression disrupted spatial control of As metabolism (Fig. 1). In fact, in hydroponics, significantly decreased As tolerance was observed in *pUbi::PvACR3* rice under $NaAsO_2$ treatments (Fig. 2). However, due to the short time of hydroponics, *pUbi::PvACR3* rice has not died although it was seriously poisoned. However, in the long-term pot experiment, *pUbi::PvACR3* rice died at the tillering stage because of the long-term toxicity, resulting in poorer As accumulation ability in the pot experiment (Fig. S8). Two key mechanisms might account for this toxicity [1]: ubiquitous *PvACR3* expression in shoots may redirect As (III) efflux into metabolically active tissues (e.g., mesophyll cells), elevating cytosolic free As(III) levels and exacerbating oxidative stress [2]; chronic As(III) accumulation could deplete glutathione (GSH) and phytochelatins (PCs), essential thiol compounds for As chelation [47]. Furthermore, ectopic *PvACR3* activity in aerial tissues may bypass nodal As filtration systems, specifically *OsABCC1*-mediated vacuolar sequestration of As(III)-PC complexes [48], thereby overwhelming cellular detoxification capacity.

In contrast to this detrimental constitutive expression, wild-type rice employs a sophisticated uptake mechanism via *Lsi1*-encoded silicon/arsenite influx transporters, which are polarized to the plasma membrane of root exodermal and endodermal cells [29]. Inspired by this natural spatial regulation, we engineered root-specific *PvACR3* expression using the *Lsi1* promoter (*pLsi1*). This strategy successfully restricted *PvACR3* to root cell plasma membranes (Fig. 1D) while maintaining strict tissue specificity (Fig. 1A and B). Root-confined *PvACR3* activity conferred dual advantages [1]: apoplastic/rhizospheric As(III) efflux (Fig. 2B and C) reduced cytosolic As burden, preserving nutrient uptake and stress resilience [2]; redirected As flux potentiated xylem loading, amplifying shoot accumulation without exceeding endogenous detoxification thresholds for lower promoter activity. This engineered redistribution synergizes with native detoxification pathways, including (i) *OsABCC1*-mediated vacuolar sequestration of As(III)-PC complexes in nodal tissues—a critical checkpoint allowing selective As entry into xylem prior to shoot filtration, and (ii) GSH-dependent thiol chelation [47,48]. By hierarchically integrating transgenic transport enhancement with endogenous buffering systems, our tissue-specific expression paradigm maximizes As translocation efficiency while minimizing physiological interference, underscoring the necessity of spatial precision in phytoremediation strategies.

4.2. *pLsi1::PvACR3 rice serves as a powerful tool for As-contaminated paddy soils phytoextraction*

Paddy fields represent a major site of As contamination. The restoration of large-scale As-contaminated paddy fields has several unavoidable difficulties. The prolonged flooding conditions in paddy soils make it difficult for most dryland hyperaccumulators to survive. The efficiency of phytoremediation is closely related to the soil environment, and flooded conditions significantly increase As bioavailability. It is best to repair As-contaminated paddy soils under flooded conditions in order to remove more bioavailable As. Besides, high repair efficiency, along with shortened time and simplified follow-up treatment, is required when large-scale popularization and application in paddy soils. In this study, the designed transgenic rice was proven to effectively solve those problems.

The *pLsi1::PvACR3* rice strains demonstrate an exceptional capacity for As phytoextraction in paddy soils, surpassing previously reported strains. Compared to the well-known hyperaccumulator *P. vittata*, these transgenic lines showed significantly greater As removal efficiency. Under identical non-flooded conditions (60% field capacity, 112 days), *P. vittata* accumulated only 0.822 mg As/plant in shoots, which was 26.5%–48.2% lower than *pLsi1::PvACR3* lines (Table S3). More importantly, unlike *P. vittata* which is restricted to well-drained soils, the transgenic rice maintains this superior performance under flooded paddy conditions while producing higher biomass, a critical advantage for field-scale remediation. Besides, when compared to wild-type rice cultivars with naturally high As uptake, these transgenic lines solve a fundamental limitation. While these conventional cultivars accumulate As primarily in roots (Fig. 3I), posing risks of As re-release and complicating root disposal in practical applications [15], the *pLsi1::PvACR3* rice strains exhibit 92.5%–96.2% of the total As accumulated in the aerial parts (Fig. 3E). This engineered trait enables safer and more efficient As removal through above-ground harvest.

The primary benefits of utilizing these *pLsi1::PvACR3* rice strains for phytoremediation are the enhanced rapid depletion of the bioavailable As pool in soil. In this study, we observed the dynamic consumption of pore water As without rebound by the engineered high-As-accumulating remediation rice. This is because the *pLsi1::PvACR3* rice strains can rapidly take up and transfer As from the roots to the aboveground parts, while simultaneously accelerating the decrease of adsorbed and iron/manganese-bonded fractions of As in the soils. Overall, phytoremediation by these transgenic remediation rice strains can greatly reduce the soil extractable As fractions and then risks to terrestrial ecosystems, and provides a feasible solution for the improvement of As-contaminated paddy soils [49,50].

The striking 11.4–20.7-fold enhancement of root-to-shoot As translocation in *pLsi1::PvACR3* transgenic lines compared to wild-type plants underscores its unparalleled capacity for As phytoextraction. This capacity parallels the role of *OsLsi2*, a silicon/arsenite efflux transporter critical for root-to-shoot As flux, as mutations in *OsLsi2* reduce shoot As accumulation by 80% [29]. Similarly, systemic As distribution relies on phloem-localized transporters like *INTs* (inositol transporters), which mediate long-distance As transport to grains [51]. However, these transporters operate within distinct regulatory frameworks. For instance, while *OsLsi2* knockout reduces shoot and grain As by 70%–80% [29], overexpression of *OsNIP1;1* and *OsNIP3;3* (aquaporin-like channels) limits xylem loading, decreasing shoot As by 50%–60% [52]. Conversely, disruption of phloem-specific transporters such as *AtINT2*, *AtINT4* in *Arabidopsis* reduces seed As accumulation by 70% [51], highlighting their pivotal role in grain As partitioning. Notably, vacuolar sequestration strategies exhibit opposing effects: *PvACR3;1* suppresses shoot As by 55%–61% through root vacuolar trapping [27], while *OsABCC1* reduces grain As by 40%–50% via compartmentalization of As(III)-phytochelatin complexes in phloem vacuoles [48]. These contrasts emphasize a fundamental dichotomy in As transporter functionality, enhancing mobilization for phytoremediation (e.g., *PvACR3*) while others restrict food-chain transfer via sequestration (e.g., *PvACR3;1*, *OsABCC1*). To reconcile these opposing outcomes, future studies should prioritize tissue-specific engineering. For example, coupling root-specific *PvACR3* expression (to maximize As extraction) with grain-specific *OsABCC1* activation (to block As entry into edible tissues) could optimize both phytoremediation efficiency and food safety. Such dual-targeted approaches may resolve the current trade-off between environmental decontamination and agricultural risk mitigation.

While demonstrating high remediation potential, future studies should establish safety protocols for field application, including containment measures, food risk assessment, and standardized disposal methods for As-laden biomass. Rice possesses unique agronomic value as the sole cereal crop capable of thriving in a wide range of ecosystems, such as drylands, paddy fields, and aquatic environments [15], thus rendering it an ideal candidate for integrating soil remediation with food production. However, realizing this dual functionality requires stringent prevention of As transfer to edible grains. Previous studies proved that As accumulation in rice predominantly occurs during vegetative growth phases [53]. Our current protocol mitigates this risk by developing a non-food remediation protocol (harvesting at pre-grain-filling stage), effectively decoupling phytoremediation from grain development, while maintaining remediation efficiency through high-shoot biomass yield. Nevertheless, for large-scale application to become feasible, subsequent

problems that should be considered include avoiding As accumulation in grains and appropriate treatment of the As-enriched shoots, such as incineration for As capture or converting them to construction composites. The harvested rice straw could achieve As encapsulation in cement matrices, enabling closed-loop biomass processing and preventing As entry into the agricultural commodity streams. This warrants future research to assist the implementation of this strategy.

CRediT authorship contribution statement

Xia Zhu: Writing – original draft, Visualization, Validation, Methodology, Formal analysis, Data curation, Conceptualization. **Huili Yan:** Writing – original draft, Visualization, Validation, Methodology, Conceptualization. **Chen Tu:** Writing – review & editing, Visualization, Conceptualization. **Ruijie Li:** Visualization, Formal analysis, Data curation. **Han Zhang:** Formal analysis, Data curation. **Yuan Li:** Writing – review & editing, Visualization. **Shuai Yang:** Writing – review & editing, Data curation. **Fangjie Zhao:** Writing – review & editing, Methodology. **Willie J.G.M. Peijnenburg:** Writing – review & editing. **Mi Ma:** Conceptualization. **Zhenyan He:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Yongming Luo:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

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