

OsHAC1;1 and OsHAC1;2 Function as Arsenate Reductases and Regulate Arsenic Accumulation

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Running title: OsHAC1;1 and OsHAC1;2 Regulate Arsenic Accumulation in Rice

One sentence summary: OsHAC1;1 and OsHAC1;2 function as arsenate reductases that play an important role in restricting As accumulation in rice shoots and grain when plants are exposed to arsenate.

Footnotes:

The study was funded by the Natural Science Foundation of China (31520103914 to F.J.Z. and D.E.S.; 31471462 to D.Y.C), the Ministry of Science and Technology Key R&D program (2016YFD0100704 to D.Y.C. and T.Z.), Strategic Priority Research Program of Chinese Academy of Sciences (XDA08020108-4 to D.Y.C), the special fund for agro-scientific research in the public interest (201403015 to F.J.Z.), the Ministry of Education (IRT1256 to F.J.Z.), and the US National Institutes of Health (<http://www.nih.gov/>) (grant 2R01GM078536 and 2P4ES007373-19A1 to D.E.S.).

Author contributions: F.J.Z. and D.Y.C. designed research; S.S., T.W., Z.C., Z.T. and Z.W. performed research; S.S., T.W., D.Y.C. and F.J.Z. analyzed data; F.J.Z., D.Y.C., S.S. and D.E.S. wrote the paper with contributions of all the authors.

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ABSTRACT

Rice is a major dietary source of the toxic metalloid arsenic (As). Reducing its accumulation in rice grain is of critical importance to food safety. Rice roots take up arsenate and arsenite depending on the prevailing soil conditions. The first step of arsenate detoxification is its reduction to arsenite, but the enzyme(s) catalyzing this reaction in rice remains unknown. Here, we identify OsHAC1;1 and OsHAC1;2 as arsenate reductases in rice. *OsHAC1;1* and *OsHAC1;2* are able to complement an *Escherichia coli* mutant lacking the endogenous arsenate reductase and to reduce arsenate to arsenite. *OsHAC1;1* and *OsHAC1;2* are predominantly expressed in roots, with *OsHAC1;1* being abundant in the epidermis, root hairs and pericycle cells while *OsHAC1;2* is abundant in the epidermis, outer layers of cortex and endodermis cells. Expression of the two genes was induced by arsenate exposure. Knocking out *OsHAC1;1* or *OsHAC1;2* decreased the reduction of arsenate to arsenite in roots, reducing arsenite efflux to the external medium. Loss of arsenite efflux was also associated with increased As accumulation in shoots. Greater effects were observed in a double mutant of the two genes. In contrast, overexpression of either *OsHAC1;1* or *OsHAC1;2* increased arsenite efflux, reduced As accumulation and enhanced arsenate tolerance. When grown under aerobic soil conditions overexpression of either *OsHAC1;1* or *OsHAC1;2* also decreased As accumulation in rice grain, whereas grain As increased in the knockout mutants. We conclude that OsHAC1;1 and OsHAC1;2 are arsenate reductases that play an important role in restricting As accumulation in rice shoots and grain.

INTRODUCTION

Arsenic (As) is a toxic metalloid and is listed as a class-one carcinogen (National Research Council, 2001). Humans are exposed to As mainly through drinking water and food. Rice, the staple food for more than half of the world population, is the most important dietary source of As for populations in south and southeast Asia (Mondal and Polya, 2008; Meharg et al., 2009; Zhao et al., 2010; Li et al., 2011), and there is evidence linking high As exposure in rice with genotoxic effects in humans (Banerjee et al., 2013). A study on pregnant women in the US also found a significant association between rice consumption and urinary As excretion, a biomarker of As exposure (Gilbert-Diamond et al., 2011). Pre-cooked milled rice is a common ingredient of baby food and high As levels in some baby rice products present a particular concern (Meharg et al., 2008). It is therefore of critical importance to reduce As accumulation in rice grain.

Rice roots take up arsenate [As(V)] or arsenite [As(III)] depending on the prevailing soil conditions. As(III) is the main As species in anaerobic flooded paddy soil. However, rice grown under upland or water-saving cultivation conditions experience long periods when field soils becomes aerobic. Under such aerobic conditions As(V) is the main form of As rice roots are exposed to (Xu et al., 2008). In addition, As(III) can also be oxidized in the rhizosphere even during flooded paddy cultivation due to the release of oxygen from rice roots (Liu et al., 2006; Seyfferth et al., 2010; Zhao et al., 2010). As(III) and As(V) are taken up into roots by the silicic acid and phosphate transporters, respectively (Abedin et al., 2002; Ma et al., 2008; Wu et al., 2011). As(III) is detoxified by complexation with phytochelatins (Ha et al., 1999; Raab et al., 2005; Liu et al., 2010) and transported into the vacuoles via ABCC transporters (Song et al., 2010; Song et al., 2014). Vacuolar sequestration of As(III)-thiol complexes helps restrict the translocation of As to rice grain (Song et al., 2014; Chen et al., 2015). The first step of As(V) detoxification is the reduction to As(III). Most plant species have an inherently high As(V) reduction capacity because As(III) is found to be the predominant As species in plants exposed to As(V) (Dhankher et al., 2006; Xu et al., 2007). Reduction of As(V) to As(III) allows the latter to be detoxified via the

mechanisms of phytochelatin complexation and vacuolar sequestration. Importantly, As(III) can also be extruded into the external environment following As(V) uptake, with As(III) efflux typically accounting for 60 – 80% of the As(V) uptake by roots of rice and other plant (Xu et al., 2007; Liu et al., 2010; Zhao et al., 2010). Therefore, As(III) efflux is an efficient way for reducing the cellular As burden without the risk of losing phosphate, a chemical analogue of As(V).

Despite the importance of As(V) reduction in plant As metabolism and detoxification, rather little was known about the enzymes catalyzing the reduction reaction until recently. Earlier studies suggested that plant ACR2 proteins, which are homologs of the yeast (*Saccharomyces cerevisiae*) As(V) reductase and belong to CDC25 phosphatases, may be responsible for As(V) reduction in plant cells (Bleeker et al., 2006; Dhankher et al., 2006; Ellis et al., 2006; Duan et al., 2007). However, these studies are mainly based on heterologous functional assays in *Escherichia coli* or yeast, which may not reflect the in planta functions of the genes. In the study of Dhankher et al. (2006), RNAi silencing of the *Arabidopsis thaliana* ACR2 was found to lead to As(V) sensitivity and As hyperaccumulation in shoots. However, these observations could not be reproduced in studies using T-DNA insertion ACR2 null mutants (Liu et al., 2012; Chao et al., 2014). Recently, Chao et al. (2014) and Sanchez-Bermejo et al. (2014) independently identified a new As(V) reductase in *A. thaliana*, named HAC1 (for High As Content 1) or ATQ1 (for Arsenate Tolerance QTL 1). The protein is a member of the rhodanase-like family, but lacks the HCX₅R active site found in the yeast ACR2 (Mukhopadhyay and Rosen, 2001). HAC1/ATQ1 is able to reduce As(V) to As(III) both *in vitro* and in planta. Weak or null alleles of *HAC1/ATQ1* in *A. thaliana* accessions are associated with decreased tolerance to As(V) (Chao et al., 2014; Sanchez-Bermejo et al., 2014) and elevated As accumulation in shoots (Chao et al., 2014). Knockout mutants of *HAC1* have greatly decreased As(III) efflux to the external medium following As(V) uptake, which causes As hyperaccumulation in shoots (Chao et al., 2014). This study therefore demonstrates a crucial role of HAC1 in mediating As(V) reduction and limiting As accumulation in the above-ground tissues.

There are more than 10 *AtHAC1*-like genes in the rice genome (Supplemental Fig.

S1), but their functions have not been characterized. Here, we show that OsHAC1;1 and OsHAC1;2, close homologs of AtHAC1, function as As(V) reductases and play an important role in regulating As accumulation in rice shoots and grain.

RESULTS

OsHAC1;1 and OsHAC1;2 Function as Arsenate Reductase

We identify two genes encoding rhodanase-like proteins (Loc_Os02g01220 and Loc_Os04g17660) from rice (*Oryza sativa* cv. Nipponbare) which have a high homology (84% and 81% similarity in the amino acid sequence, respectively) with *A. thaliana* AtHAC1. These two genes share 90% amino acid sequence similarity and are thereafter named *OsHAC1;1* and *OsHAC1;2*, respectively (Supplemental Fig. S1). To test if OsHAC1;1 and OsHAC1;2 are able to reduce As(V) to As(III), we expressed the two rice genes in a strain of *E. coli* lacking the endogenous arsenate reductase ArsC. This mutant strain is sensitive to As(V) because it is not able to reduce the absorbed As(V) to As(III) to allow the latter to be extruded from the cell (Oden et al., 1994). Heterologous expression of either *OsHAC1;1* or *OsHAC1;2* restored the growth of the *E. coli* strain in the LB medium in the presence 1 mM As(V) (Fig. 1A). Furthermore, As speciation analysis using HPLC-ICP-MS showed the production of As(III) in the medium in the presence 10 μ M As(V) by the *E. coli* Δ arsC strain expressing *OsHAC1;1* or *OsHAC1;2*, in contrast to the empty vector control that produced no detectable As(III) (Fig. 1B).

The Expression Patterns and Subcellular Localization of *OsHAC1;1* and *OsHAC1;2* in Rice

To investigate the expression patterns of the two *HAC1* genes in rice, we created stable transgenic rice lines expressing *OsHAC1;1-GFP* and *OsHAC1;2-GFP* chimeric protein constructs driven by their native promoters. Based on the GFP signals, we found that both OsHAC1;1 and OsHAC1;2 predominantly accumulate in root, with the epidermis and the pericycle cells, as well as root hairs in the mature zone of roots, showing particularly strong accumulation of OsHAC1;1 (Fig. 2). In contrast,

OsHAC1;2 is more abundantly found in the epidermis, the exodermis, the outer layer of cortex and the endodermis cells (Fig. 2). The expression patterns are similar between the different root zones from the root tip to the mature zone. For both gene constructs, very faint signals of GFP were observed in the shoot tissues (data not shown).

To investigate the subcellular localization of the OsHAC1;1 and OsHAC1;2, we isolated protoplasts from the transgenic rice roots expressing *OsHAC1;1-GFP* or *OsHAC1;2-GFP*. For both gene constructs, the GFP signals were localized in the cytoplasm (Supplemental Fig. S2). To further investigate the subcellular localization of the two proteins, we constructed N-terminal OsHAC1;1 or OsHAC1;2 fusions with GFP with expression driven by the cauliflower mosaic virus 35S promoter, and transfected the derived expression vector into rice protoplasts. We observed that both the OsHAC1;1::GFP and OsHAC1;2::GFP fusion proteins are localized in the cytoplasm and nucleus (Fig. 3). Because of the relatively small molecular sizes of the two proteins, the possibility of their diffusion from the cytoplasm to the nucleus cannot be ruled out.

We used quantitative real-time PCR (qRT-PCR) to investigate the expression pattern of *OsHAC1;1* and *OsHAC1;2* in response to As(V) exposure. Three-week-old rice plants (cv. Nipponbare) were exposed to 0 or 10 μ M As(V) in a nutrient solution without phosphate for up to 24 h. Phosphate was withdrawn during this short-term experiment to facilitate As(V) uptake. In the control treatment (no As(V)), *OsHAC1;1* and *OsHAC1;2* were predominantly expressed in roots and moderately in shoots, and there were no temporal changes in their relative expression levels over the 24 h time course (Fig. 4). Exposure to As(V) significantly enhanced the expression of both *OsHAC1;1* and *OsHAC1;2* during the first 12 h, with *OsHAC1;1* showing a greater response than *OsHAC1;2* (Fig. 4). In contrast, exposure to 10 μ M As(III) decreased the mRNA levels of *OsHAC1;1* and *OsHAC1;2* in both roots and shoot (Supplemental Fig. S3).

Knocking Out *OsHAC1;1* or *OsHAC1;2* Affects Arsenate Reduction and Arsenic

Accumulation in Rice

To investigate the in planta function of OsHAC1;1 and OsHAC1;2, we created two independent knockout lines of *OsHAC1;1*, *hac1;1-1* and *hac1;1-2*, in the cv. Zhonghua 11 background using CRISPR-Cas9 technology (Supplemental Fig. S4). We also obtained two independent homozygous T-DNA insertion mutants of *OsHAC1;2* (*hac1;2-1* and *hac1;2-2* in the cv. Zhonghua 11 and Dongjin background, respectively; Supplemental Fig. S4). In addition, we obtained a homozygous double mutant *hac1;1 hac1;2* in the cv. Zhonghua 11 background by crossing *hac1;1-1* with *hac1;2-1*. Analysis using qRT-PCR showed that the expressions of *OsHAC1;1* and *OsHAC1;2* were abolished in these mutants (Supplemental Fig. S4). If OsHAC1;1 and OsHAC1;2 play a role in As(V) reduction, knocking out of *OsHAC1;1* or *OsHAC1;2* may impact As speciation in the plants. To test this hypothesis, mutant and wild-type (WT) plants were exposed to 10 μ M As(V) for 48 h and As speciation in roots and shoots were determined using HPLC-ICP-MS. *hac1;1-1*, *hac1;1-2*, *hac1;2-1* and *hac1;1 hac1;2* and their common WT (Zhonghua 11) were compared in the same experiment, and *hac1;2-2* and its WT (Dongjin) in a separate experiment. As(III) and As(V) were the only two As species detected in the plant tissues, with As(III) being the predominant As species. The extraction method does not preserve As(III)-thiol complexes (Raab et al., 2005; Liu et al., 2010), which would be dissociated and determined as As(III) by the method used. Knocking out of *OsHAC1;1* resulted in 3.1 times higher As(V) concentration in roots compared with WT, while the mutation in *OsHAC1;2* increased root As(V) concentration by 40 – 80% (Fig. 5A and C). In the double mutant, root As(V) concentration was 4.7 times higher than WT. All mutants had significantly higher concentrations of both As(V) and As(III) in shoots than WT plants (Fig. 5B and D). Compared with WT, *hac1;1*, *hac1;2* and the double mutant had 170%, 150 – 190% and 230%, respectively, higher total As (sum of As(V) and As(III)) concentration in shoots. The effect of *OsHAC1;1* or *OsHAC1;2* mutations is also evident from the changes in As(III) as a percentage of the total As in roots, decreasing from 82% in WT (Zhonghua 11) to 62%, 72% and 57% in *hac1;1*, *hac1;2-1* and the double mutant, respectively, and from 77% in WT (Dongjin) to 71% in *hac1;2-2*. In the shoot tissues,

As(III) as a percentage of the total As also decreased from 93% in WT (Zhonghua 11) to 90% in the three single mutants and 81% in the double mutant. These results support a role for *OsHAC1;1* and *OsHAC1;2* in As(V) reduction in rice roots, with the double mutant having a greater effect than the single mutants and the single mutants of *OsHAC1;1* having a greater effect than those of *OsHAC1;2*.

Chao et al. (2014) showed that As(III) efflux from roots to the external medium diminished greatly in mutants with a loss of *AtHAC1* function, resulting in a markedly increased As accumulation in *A. thaliana* shoots. To test if mutations in *OsHAC1;1* or *OsHAC1;2* also affect As(III) efflux in rice, plants were exposed to 10 μ M As(V) for 48 h. As(V) uptake and As(III) efflux were estimated by measuring the changes in As speciation in the culture solution. There were no differences in As(V) uptake between mutants and WT (Fig. 5E and G). In contrast, As(III) efflux from roots was significantly decreased in all single mutants compared with WT plants, and a larger decrease was found in the double mutant than the single mutants (Fig. 5F and H). The As(III) efflux efficiency, calculated as a ratio of As(III) efflux to As(V) uptake, was 0.80 – 0.83 in WT, 0.56 – 0.66 in the single mutants and 0.32 in the double mutant, respectively. A decreased As(III) efflux to the external medium could explain the enhanced As accumulation in mutant shoots.

In a further experiment, we tested the effect of *OsHAC1;1* or *OsHAC1;2* mutations on As accumulation over a range of As(V) concentration from 2 to 20 μ M. Knocking out of *OsHAC1;1* or *OsHAC1;2* resulted in a significant increase in As accumulation in roots at all As(V) concentrations and in shoots at all but the 2 μ M As(V) treatment (Fig. 6A and B).

Furthermore, we observed no significant change in the concentrations of As(V) and As(III) in roots or shoots of single or double mutants compared with WTs when plants were exposed to 10 μ M As(III) for 48 h (Supplemental Fig. S5), suggesting that the effect of *OsHAC1;1* and *OsHAC1;2* is specific to As(V).

Overexpression of *OsHAC1;1* or *OsHAC1;2* Increases Arsenate Reduction and Decreases Arsenic Accumulation

To further investigate the role of *OsHAC1;1* and *OsHAC1;2* in As metabolism, we overexpressed *OsHAC1;1* and *OsHAC1;2* in rice (cv. Nipponbare) using the Ubiquitin promoter. Three independent lines for each gene were selected for further investigation. qRT-PCR analysis showed that all overexpressing lines had greatly enhanced expression of *OsHAC1;1* or *OsHAC1;2* in roots compared with WT plants (Fig. 7A and E). After exposure to 10 μ M As(V) for 48 h, there was no significant difference in As(V) uptake between different transgenic plants and WT plants (Supplemental Fig. S6). In contrast, transgenic plants overexpressing *OsHAC1;1* or *OsHAC1;2* had 34 – 50% and 20 – 28%, respectively, larger As(III) efflux into the external medium than WT plants (Fig. 7B and F). As(III) efflux as a proportion of As(V) uptake increased from 0.65 in WT to 0.83 in the *OsHAC1;1* overexpression lines, and from 0.76 in WT to 0.90 in the *OsHAC1;2* overexpression lines. As a result, overexpression of *OsHAC1;1* or *OsHAC1;2* significantly decreased the concentrations of As(III), As(V) and total As in both shoots and roots compared with WT plants, with the effect being greater on shoot As concentration than on root As concentration (Fig. 7C, D, G and H).

In a further experiment, plants were exposed to a range of As(V) concentrations varying from 2 to 20 μ M for 48 h. Arsenic accumulation in roots and shoots were determined. Overexpression of *OsHAC1;1* or *OsHAC1;2* resulted in a significant decrease in root As concentration at all four As(V) exposure concentrations, as well as a significant decrease in shoot As concentration in all but the 2 μ M As(V) treatment (Fig. 6C and D).

In contrast, overexpression of *OsHAC1;1* or *OsHAC1;2* had no significant effect on As(V) and As(III) concentrations in roots and shoots compared with WT when plants were exposed to 10 μ M As(III) (Supplemental Fig. S7).

Knockout or Overexpression of *OsHAC1;1* or *OsHAC1;2* Affects As Speciation in Xylem Sap

If *OsHAC1;1* or *OsHAC1;2* plays a role in As(V) reduction in roots, knocking out or overexpression of these genes may affect As speciation in xylem sap. To test this

hypothesis, we analyzed As speciation in xylem sap collected from plants exposed to 10 μ M As(V) for 24 h. As(III) was found to be the predominant species of As in xylem sap. The concentrations of both As(V) and As(III) in the xylem sap from *OsHAC1;1* or *OsHAC1;2* single mutants were significantly higher than those from WT, whereas the double mutant also had a significantly higher As(V) concentration than the single mutants (Fig. 8A). The percentage of As(III) in the xylem sap total As was higher in WT (80%) than in single mutants (73 – 76%) or double mutant (66%). In contrast, transgenic plants overexpressing either *OsHAC1;1* or *OsHAC1;2* showed lower As(V) and As(III) concentrations in the xylem sap compared with WT (Fig. 8B). The differences were significant in all except one of the *OsHAC1;2* overexpression lines for As(V) concentration. These results are consistent with a role of *OsHAC1;1* and *OsHAC1;2* in As(V) reduction in rice roots.

Overexpression of *OsHAC1;1* or *OsHAC1;2* Enhances Tolerance To Arsenate

Because overexpression of *OsHAC1;1* or *OsHAC1;2* increased As(III) efflux to the external medium, we hypothesized that the overexpression lines might be more tolerant to As(V). In a short-term root elongation assay, root elongation of rice seedlings during 24 or 48 h under different As(V) concentrations was measured. The assay was conducted in the absence of phosphate to heighten the toxicity of As(V). Because the response patterns were similar between the 24 and 48 h exposure, only the data of 24 h exposure are shown (Fig. 9; Supplemental Fig. S8). Root growth of WT seedlings was inhibited by more than 90% by 2.5 μ M As(V) and completely arrested by 4 μ M As(V). In contrast, *OsHAC1;1* or *OsHAC1;2* overexpression lines had significantly larger root elongation than WT in the presence of 2.5 – 4 μ M As(V) (Fig. 9), indicating an increased tolerance to As(V).

***OsHAC1;1* and *OsHAC1;2* Affect Grain As Accumulation in Soil-grown Rice**

The experiments described above were conducted in hydroponic cultures with young rice plants. To determine if *OsHAC1;1* and *OsHAC1;2* play a role in regulating As accumulation in rice grain, plants were grown up to maturity in a paddy soil amended

with an environmentally relevant dose of As(V) (20 mg kg⁻¹) (Zhao et al., 2010). The soil was irrigated regularly with free drainage, aerobic conditions under which As(V) was likely to be the main species of As in the soil solution (Xu et al., 2008). Under aerobic conditions, *hac1;1* and *hac1;2* mutants had 36% and 20%, respectively, higher As concentration in the brown rice than WT (Fig. 10A), whereas the *OsHAC1;1* and *OsHAC1;2* overexpression lines had approximately 20% lower grain As concentration than WT (Fig. 10B). Grain yield and straw biomass were not significantly different between the mutants and WT or between the overexpressing lines and WT (data not shown).

DISCUSSION

In the present study, we show that OsHAC1;1 and OsHAC1;2 function as As(V) reductases and are involved in the reduction of As(V) to As(III) in rice plants. OsHAC1;1 and OsHAC1;2 are the closest homologs of the *A. thaliana* AtHAC1/ATQ1, which has been discovered recently as representing a new type of As(V) reductase in plants (Chao et al., 2014; Sanchez-Bermejo et al., 2014). Similar to *AtHAC1*, both *OsHAC1;1* and *OsHAC1;2* were able to complement the As(V) sensitive *E. coli* mutant lacking the endogenous As(V) reductase ArsC (Fig. 1). The *E. coli* mutant expressing either *OsHAC1;1* or *OsHAC1;2* was able to reduce As(V) and extrude As(III) into the external medium, which is a key mechanism of As(V) detoxification widely employed by microorganisms (Rosen, 2002). Further evidence for a role of OsHAC1;1 and OsHAC1;2 in As(V) reduction in rice plants can be seen in the altered As speciation in the mutants or overexpression lines of the two genes; mutations in either *OsHAC1;1* or *OsHAC1;2* resulted in an increased proportion of As(V) and a decreased proportion of As(III) in rice roots and xylem saps, whereas overexpression of either gene produced the opposite effect (Figs. 5, 7, 8). The effect was greater when both genes were knocked out in the double mutant.

We show that OsHAC1;1 protein accumulates predominantly in the root epidermis, root hairs and the stele, and OsHAC1;2 accumulates mainly in the exodermis, the outer layer of cortex and the stele (Fig. 2). By mediating As(V) reduction in the root

epidermis, root hairs and possibly also the exodermis, OsHAC1;1 and OsHAC1;2 enable As(III) efflux from the outer layers of root cells to the external medium. In agreement with previous studies (Xu et al., 2007; Zhao et al., 2010), As(III) efflux was found to represent a large proportion of As(V) influx in WT plants (c. 80%). This proportion decreased to c. 60% in the single mutants and to c. 30% in the double mutant (Fig. 5). Efflux of As(III) following As(V) uptake is critical for controlling As accumulation in plant tissues. Decreased As(III) efflux in the mutants leads to more As accumulation in both roots and shoots (Figs. 5 and 6). In contrast, overexpression of either *OsHAC1;1* or *OsHAC1;2* increased As(V) reduction and As(III) efflux to the external medium, resulting in decreased As accumulation in both roots and shoots (Figs. 6 and 7). Furthermore, the localization of OsHAC1;1 and OsHAC1;2 in the stele implies a role of the two proteins in regulating As translocation from roots to shoots possibly by blocking xylem loading of As(V) via phosphate transporters such as PHO1 (Poirier et al., 1991; Secco et al., 2010). Increased proportions of As(V) in the xylem sap of the knockout mutants (Fig. 8) are consistent with this interpretation. A previous study showed that As(III) is preferentially stored in the vacuoles of the pericycle and endodermal cells of rice roots (Moore et al., 2011), supporting the notion that these cells are important in regulating the root to shoot translocation of As. It is possible that OsHAC1;1 and OsHAC1;2 also contribute to As(V) reduction in the shoots. However, the altered phenotypes of As accumulation in the knockout mutants and the overexpression lines can be attributed primarily to the function of the two enzymes in the roots.

The presence of substantial amounts of As(III) in the roots and shoots of the *oshac1;1 oshac1;2* double mutant indicates the presence of other As(V) reduction mechanisms in plants. There are more than 10 *HAC1*-like genes in the rice genome (Supplemental Fig. S1), some of which may also play a role in As(V) reduction. Another possibility is that As(V) could be reduced non-enzymatically by glutathione (Delnomdedieu et al., 1994), although the reaction may be slow. However, As(V) can participate in phosphorylation reactions (Byers et al., 1979), forming arsenate esters which are more easily reduced by thiols such as glutathione (Gregus et al., 2009).

Unlike AtHAC1, OsHAC1;1 and OsHAC1;2, some of these additional As(V) reduction processes may not be linked to As(III) efflux, either because they do not interact with efflux transporters or are localized in cells not suited for As(III) efflux to the external medium (Chao et al., 2014). The presence of multiple As(V) reduction mechanisms explains why not only As(V), but also As(III), is elevated in the xylem sap and shoots of the mutants (Figs. 5 and 8).

OsHAC1;1 and OsHAC1;2 mediated As(V) reduction is also required for tolerance to As(V) as it likely lessens the cellular burden of As through efficient As(III) efflux. Furthermore, As(V) reduction allows the product As(III) to be complexed with phytochelatins and subsequently sequestered in the vacuoles (Zhao et al., 2009; Liu et al., 2010; Song et al., 2010). The expressions of *OsHAC1;1* and *OsHAC1;2* were strongly induced by As(V) exposure (Fig. 4), which is consistent with a role of the two genes in As(V) detoxification.

OsHAC1;1 appears to play a greater role in controlling As accumulation than OsHAC1;2. This difference could be attributed to a higher expression of *OsHAC1;1* (Fig. 4). The strong localization of OsHAC1;1 in the epidermis and root hairs would also make it more efficient in enabling As(III) efflux, as has been observed for AtHAC1 in *A. thaliana* (Chao et al., 2014). Although *OsHAC1;1* and *OsHAC1;2* are similar to AtHAC1 in a number of aspects discussed above, the impact of *OsHAC1;1* or *OsHAC1;2* single mutation is not as large as that of AtHAC1 knockout reported by Chao et al. (Chao et al., 2014). This difference can be explained by a degree of functional redundancy between *OsHAC1;1* and *OsHAC1;2*, which is clearly demonstrated by larger effects in the double mutant (Figs. 5 and 8). In addition, there are other *HAC1*-like genes in rice (Supplemental Fig. S1) whose functions remain to be investigated. Although OsHAC1;1 and OsHAC1;2, as well as AtHAC1, function as As(V) reductases, their primary metabolic functions, if any, remain unknown. It is also intriguing that the rice genome contains a considerable number of *HAC1*-like genes. We observed no growth or developmental phenotypes in *oshac1;1*, *oshac1;2* or *athac1* mutants under non-As stressed conditions.

Previously, OsACR2, a CDC-25 protein and a homolog of the yeast As(V) reductase

ScACR2, has been suggested to be involved in As(V) reduction in rice (Duan et al., 2007). However, this study was based on heterologous expression of *OsACR2* in yeast and *in vitro* characterization of the *OsACR2* enzyme. No knockout or knockdown lines of *OsACR2* were included in the study of Duan et al. (2007). Whether *OsACR2* plays a role in As(V) reduction in rice plants remains unclear. In the case of *A. thaliana*, the report by Dhankher et al. (2006) that silencing *AtACR2* by RNA interference leads to As hyperaccumulation in the shoots could not be confirmed by recent studies using two independent T-DNA insertional knockout mutants of the gene (Liu et al., 2012; Chao et al., 2014). Because *AtACR2* and *AtHAC1* share sequence identity within the region used by Dhankher et al. (2006) to knock down expression of *AtACR2* by RNA interference, this sequence may also have suppressed *AtHAC1* expression in their RNAi lines, thus resulting in decreased As(V) tolerance and As hyperaccumulation in the shoots (Chao et al., 2014). Nahar et al. (2012) reported increased As(V) sensitivity and As accumulation in the shoots of a single T-DNA line (SALK_005882C) with a T-DNA insertion to the neighboring gene (At5g03452) of *AtACR2* (At5g03455), which appeared to knockdown the expression of *AtACR2*.

Although lowland rice is typically grown under flooded conditions, paddy water is usually drained periodically during the rice growing season. Upland rice often experiences dry periods due to water shortage. There is also an increasing trend of using aerobic to save water usage and to reduce greenhouse gas emissions from paddy fields (Bouman et al., 2005; Linqvist et al., 2015). All these agronomic factors lead to aerobic soil conditions under which As(V) is expected to be the dominant As species present in the soil solution and taken up by rice roots. Consequently, As(V) reductases may play an important role in As accumulation in rice grain when plants are exposed to the aerobic soil conditions that can occur when rice is grown under normal field conditions. We tested this hypothesis by growing mutants and overexpression lines of *OsHAC1;1* and *OsHAC1;2* under aerobic soil conditions to maturity. Under the experimental conditions, loss-of-function mutants of *OsHAC1;1* or *OsHAC1;2* had significantly higher concentration of As in rice grain than WT, whereas overexpression lines contained significantly lower levels of As than WT (Fig. 10). We therefore

conclude that in the field, when rice roots are exposed to irregular oxidizing and reducing cycles, the ability to specifically reduce As(V) to As(III) through the action of the OsHAC1 arsenate reductases is important to restrict As accumulation in rice grain.

Our study has shed light on the mechanism of As(V) reduction in rice, a staple food crop with an unusually high contribution to dietary As intake by humans. Our results and those of Chao et al. (2014) and Sanchez-Bermejo et al. (2014) on *A. thaliana* show that As(V) reduction is a key step in As metabolism that controls the accumulation of As in the above-ground tissues of plants. Our results point to a possible strategy for limiting grain As accumulation in rice cultivated under conditions in which the soil is aerobic for extended periods of time. Such a strategy would involve enhancing As(V) reductase activities in rice roots to both enhance As(III) efflux and limit its xylem loading and transport.

MATERIALS AND METHODS

Plant Materials

Rice (*Oryza sativa ssp. japonica*) cv. Nipponbare, Zhonghua 11 or Dongjin were used as wild-types in the present study and for rice transformation. A T-DNA insertion mutant line *oshac1;2-1* (RMD_03Z11FF65) in the Zhonghua11 background was obtained from Huazhong Agricultural University, China. We obtained another T-DNA insertion mutant line *oshac1;2-2* (PFG_3A-02094) in the Dongjin background from Zhejiang University. The location of the T-DNA insertion in the mutant was determined by DNA sequence analysis using PCR. A homozygous T-DNA insertion line was identified by PCR using gene-specific primers in conjunction with T-DNA border primers (Supplemental Table S1). Two independent mutants of *OsHAC1;1*, *oshac1;1-1* and *oshac1;1-2*, were generated using the CRISPR/Cas9 technology (see below). Overexpression lines of *OsHAC1;1* and *OsHAC1;2* were generated in the cv. Nipponbare background (see below). *hac1;1-1* and *hac1;2-1* (both in the Zhonghua 11 background) were crossed to generate a double mutant. A homozygous double mutant line was identified by PCR using gene-specific primers and sequencing.

Plant Growth Conditions

Rice seeds were surface sterilized in a 30% (v/v) hydrogen peroxide solution for 30 min, washed, and germinated for 3 d at 37 °C in the dark. Ten-days-old seedlings were transferred to a 1/2 strength Kimura nutrient solution. The composition of the nutrient solution was as follows (in mM): 0.091 KH₂PO₄, 0.273 MgSO₄, 0.182 (NH₄)₂SO₄, 0.091 KNO₃, 0.183 Ca(NO₃)₂, 0.003 H₃BO₃, 0.0005 MnCl₂, 0.001 (NH₄)₆Mo₇O₂₄, 0.0004 ZnSO₄, 0.0002 CuSO₄, 0.02 Fe(III)-EDTA. The pH of the solution was adjusted to 5.5. The nutrient solution was renewed every 2 d. Hydroponic experiments were conducted inside a growth room with a 14 h/10 h light/dark period, 250 μmol m⁻² s⁻¹ light intensity, 25/20 °C day/night temperatures, and a relative humidity at approximately 70%. Arsenic treatments were started by adding As(V) (Na₃AsO₄) or As(III) (NaAsO₂) to the nutrient solution at target concentrations.

A soil pot experiment was conducted with mutants, overexpression lines and their WT. A paddy soil was collected from an experimental farm of Nanjing Agricultural University. The soil contains 12 mg As kg⁻¹ and has a pH of 6.6. Basal fertilizers (120 mg N kg⁻¹ as NH₄NO₃, 25 mg S kg⁻¹ soil as MgSO₄, 30 mg P kg⁻¹ soil and 75.5 mg K kg⁻¹ soil as K₂HPO₄) were added to the soil and mixed thoroughly. The soil was amended with 20 mg As(V) kg⁻¹. Twelve kg soil were placed in a 15-liter plastic pot. The water management regimes with the soil was maintained under aerobic conditions. Each pot contained one seedling each of *oshac1;1-1*, *oshac1;1-2*, *oshac1;2-1* mutants and their WT (Zhonghua 11), or two overexpression lines each of *OsHAC1;1* and *OsHAC1;2* and their WT (Nipponbare). There were four replicated pots for each treatment. Plants were harvested at grain maturity.

RNA Extraction and Transcriptional Analysis by Quantitative Real-time PCR

Total RNA were extracted from shoots and roots using the RNeasy plant mini kit (Biotech). Reverse transcription was carried out using the R233-01 kit (Vazyme). Quantitative Real-time (qRT) PCR analysis was performed with a Real-Time PCR Detection system (Bio-Rad CFX96) in a reaction mixture of 20 μL of SYBR Green

master mix (SYBR Green Master Mix; Vazyme; <http://www.vazyme.com>). *OsActin* (accession No. AB047313) was used as the reference genes. Expression of each gene was calculated as $2^{-\Delta CT}$ relative to *OsActin*. The qRT-PCR program was set as follows: 95 °C, 3min; (95 °C, 15s; 58 °C, 30s; 72 °C, 15s) \times 39; 60 – 90 °C for melting curve detection. Accession numbers of the rice genes investigated in the present study and primer sequences are given in Supplemental Table S1.

Construction of pOsHAC1;1:OsHAC1;1-GFP and pOsHAC1;2:OsHAC1;2-GFP Fusion Proteins and Microscopy Observation

We modified the binary vector pHB (Mao et al., 2005) to construct the expression vectors for expressing the fusion proteins of OsHAC1;1-GFP and OsHAC1;2-GFP driven by the *OsHAC1;1* and *OsHAC1;2* promoter, respectively. Firstly, we replaced the 35S promoter of pHB with a small fragment containing three restriction sites *EcoR* I, *Sal* I and *Hind* III by the two enzymes *EcoR* I and *Hind* III, to form the vector pHMS. A fragment fused with GFP coding sequence and a linker (ggaggaggaggaggagga) coding a 6x Glycine peptide was inserted into the *Pst* I and *Xba* I site of pHMS to form the vector pHMS-GFP. The *OsHAC1;1* genomic fragment including 2.3 kb promoter region and gene body with the stop codon replaced with TTA and the *OsHAC1;2* genomic fragment including 1.5 kb promoter region and gene body with the stop codon replaced with TTG were amplified from the genomic DNA of rice variety Zhonghua 11 by using primers listed in the Supplemental Table S1. The *OsHAC1;1* genomic fragment was inserted in frame into the pHMS-GFP vector by the *Hind* III and *Pst* I restriction enzymes, while the *OsHAC1;2* genomic fragment was homologously recombined in frame into the pHMS-GFP vector with One Step Pcr Cloning Kit (Shawnxin Biotech. Co. Ltd, Shanghai). The expression vectors were transformed into rice variety Zhonghua 11 mediated by *Agrobacterium tumeraciens* strain EHA105. The positive transgenic lines were observed for GFP signal under stereo fluorescence microscope (LEICA M165 FC, Leica Co. Ltd) and confocal microscope (LEICA TCS SP8, Leica Co. Ltd) in the Core Facility Center of Shanghai Institute of Plant Physiology and Ecology. To check the GFP signal inside of the root,

the roots of the transgenic lines were cross sectioned by free hand, and the hand sections were screened under confocal microscope (LEICA TCS SP8, Leica Co. Ltd). To observe the subcellular localization, protoplasts were isolated from the roots of transgenic rice expressing *OsHAC1;1-GFP* or *OsHAC1;2-GFP*. Roots were cut into segments and placed in an enzyme digestion solution (MES pH 5.7, 10 mM mannitol, 0.5 M cellulose, 1.5% RS cellulase, 0.75% macerozyme R-10, 10 mM CaCl₂, 0.1% bovine serum albumin) for 4 h in the dark at 28°C with gentle shaking (80 rpm). Thereafter, an equal volume of W5 solution (2 mM MES, pH5.7; 5 mM KCl; 154 mM NaCl; 125 mM CaCl₂) was added, followed by vigorous shaking by hand for 10 s. Protoplasts were released by filtering through 40 µm nylon meshes into a round bottom tube with 3 – 5 washes of W5 solution. The pellet was collected by centrifugation at 140 g for 7 min and re-suspended with 1 ml W5 solution. The GFP signals in the isolated protoplasts were examined using a confocal microscope.

Construction of OsHAC1;1-GFP and OsHAC1;2-GFP Fusion Proteins, Transient Expression in Rice Protoplasts and Subcellular Localization of OsHAC1;1-GFP and OsHAC1;2-GFP

The full-length cDNAs of *OsHAC1;1* and *OsHAC1;2* without the stop codon were amplified and sequenced. The fragments were cloned into the pS1GFP-8 vector driven by the cauliflower mosaic virus 35S promoter. Then, 0.2 mL of protoplast suspension (approximately 2105 cells) was transfected with DNA for various constructs (10 mg each). After transfection, cells were cultured in a protoplast medium (0.4 M mannitol, 4 mM MES [pH 5.7], 4 mM KCl, sterilized) overnight (approximately 12 h). The fluorescence of Acridine orange (a nucleus-selective dye) and GFP in the cells were analyzed with a 543-nm helium-neon laser and a 488-nm argon laser, respectively, using a confocal laser scanning microscope (LSM410; Carl Zeiss).

Generation of *OsHAC1;1* Knockout Mutants

We used the CRISPR/cas9 technology to generate *oshacl;1* knockout lines in the cv. Zhonghua11 background using the protocol described previously (Feng et al.,

2013). Firstly, we chose the sequence 5'-TGGCGCCTCCCTATGAAACC-3' in the first exon of *OsHAC1;1* as the target region and designed two oligos CAS9-*OsHAC1;1*F and CAS9-*OsHAC1;1*R (Supplemental Table S1). The two oligos were annealed and ligated with vector SK-OsU6-2-85-sgRNA restricted by enzyme *Bbs* I to form the transition vector SK-OsU6-2-85-*OsHAC1;1*-sgRNA. The transition vector was then restricted with *Kpn* I and *Hind* III to harvest a 476-bp fragment containing OsU6 promoter and guide RNA. Meanwhile, the vector SK-35S-CAS9-NOS was restricted with *Hind* III and *EcoR* I to harvest a 5.5-kb fragment containing 35S promoter, CAS9 coding gene and a NOS terminator. The two fragments were subsequently ligated with linearized pCambia1300 with restriction enzymes *Kpn* I and *EcoR* I to form the final expression binary vector. The final vector was transformed into rice variety Zhonghua 11 mediated by *A. tumefaciens* strain EHA105. At the T₀ generation, all positive transgenic lines were genotyped with the primers *HAC1;1*-CAS9SF and *HAC1;1*-CAS9SF (Supplemental Table S1). Heterozygous knock-out mutants were picked and their T₁ progenies were further genotyped for homozygous knock-out mutants.

Generation of *OsHAC1;1* and *OsHAC1;2* Overexpression Lines

To generate *OsHAC1* and *OsHAC1;2* overexpression lines, the full-length coding sequence of *OsHAC1;1* and *OsHAC1;2* were amplified and sequenced using the specific primers listed in Supplemental Table S1. The fragments were digested with *BamH* I and *Spe* I and ligated to the pTCK303 vector (Wang et al., 2004). The verified vectors were used for generating transgenic plants of *OsHAC1;1* and *OsHAC1;2* in the cv. Nipponbare background. We obtained 25 transgenic lines for each gene. Three lines each were selected randomly for hydroponic and soil pot experiments.

Functional Complementation of *OsHAC1;1* and *OsHAC1;2* in *Escherichia coli*

For prokaryotic expression of *OsHAC1;1* and *OsHAC1;2*, the full-length coding sequences of *OsHAC1;1* and *OsHAC1;2* were amplified using gene-specific primers (Supplemental Table S1). The fragments were cloned into the prokaryotic expression

vector pET-29a and verified by sequencing. The vector was transformed into *E. coli* $\Delta arsC$ mutant WC3110 (a strain lacking the endogenous arsenate reductase) and its wild-type W3110 for complementation. The $\Delta arsC$ mutant (WC3110) and its WT (W3110) with *pET-29a* empty vector, *pET-29a-OsHAC1;1* or *pET-29a-OsHAC1;2* were cultured at 37 °C overnight. All cultured strains were diluted to OD_{600 nm}=0.5 and 1 mL was inoculated into 100 ml of LB liquid media containing 1 mM IPTG and different concentrations of As(V). Cells were cultured at 16 °C. The cell density was measured at OD_{600 nm} using a spectrophotometer at different time points. The LB medium containing 10 μ M As(V) was collected at 72 h and filtered through a 0.22 μ m membrane filter before As speciation analysis using HPLC-ICP-MS.

Analysis of Total As Content and As Speciation

For the determination of total As concentration in plant samples, plant tissues were washed with deionized water for three times and dried at 70 °C for three days. Dried plant samples were digested with 5 mL mix acids of HNO₃/HClO₄ (85:15) in a digestion block. The digests were diluted with 2% HNO₃ and As concentrations were determined using ICP-MS (Perkin Elmer NexION 300x, Waltham, MA, US). As speciation in nutrient solutions, xylem saps and plant extracts was determined using HPLC-ICP-MS (Liu et al., 2010). Plant roots were rinsed briefly in an ice-cold desorption solution containing 1 mM K₂HPO₄, 0.5 mM Ca(NO₃)₂ and 5 mM MES (pH 6.0) and immersed in 1 liter of the same solution for 10 min to remove apoplastic As. Roots were blotted dry, weighed, and frozen in liquid nitrogen. Plant shoots were rinsed with deionized water, blotted dry, weighed, and frozen in liquid nitrogen. Shoots and roots were ground in liquid nitrogen to fine powder with a mortar and pestle. The finely ground materials were extracted with 10 mL phosphate-buffer solution containing 2 mM NaH₂PO₄ and 0.2 mM Na₂-EDTA (pH 6.0) for 1 h under sonication in a 4°C room (Xu et al., 2007). The extract was filtered through 0.22 μ m before analysis. Arsenic species were separated using an anion-exchange column (Hamilton PRP X-100, fitted with a guard column; Reno, NV, US) with a mobile phase of 6.0 mM NH₄H₂PO₄, 6.0 mM NH₄NO₃, and 0.2 mM Na₂EDTA (pH 6.0), run isocratically at 1

ml min⁻¹. The solution from the separation column was mixed continuously with an internal standard solution (Indium) before being introduced into the ICP-MS. The instrument was set up in the kinetic energy discrimination mode with helium as the collision gas to reduce polyatomic interferences. Signals at $m/z^{75}\text{As}$ and ^{115}In were collected with a dwell time of 300 ms; the In counts were used to normalize the As counts. Arsenic species in the sample were quantified by external calibration curves using peak areas.

Sequence data from this study can be found in the GenBank under accession numbers NP_001045596 and NP_001052130 for *OsHAC1;1* and *OsHAC1;2*, respectively.

Supplemental Data

The following supplemental materials are available:

Supplemental Figure S1. Sequence analysis of *HAC* genes in rice and *Arabidopsis thaliana*.

Supplemental Figure S2. Subcellular localization of *OsHAC1;1* and *OsHAC1;2* in protoplasts isolated from transgenic rice plants expressing *pHAC1;1:OsHAC1;1-GFP* or *pHAC1;2:OsHAC1;2-GFP*.

Supplemental Figure S3. Exposure to As(III) decreases the expression of *OsHAC1;1* and *OsHAC1;2*.

Supplemental Figure S4. Knockout mutants of *OsHAC1;1* (*hac1;1-1*, *hac1;1-2*) and *OsHAC1;2* (*hac1;2-1*, *hac1;2-2*).

Supplemental Figure S5. Knocking out *OsHAC1;1* or *OsHAC1;2* has no significant effect on As(III) uptake and As accumulation in rice.

Supplemental Figure S6. Overexpression of *OsHAC1;1* or *OsHAC1;2* has no significant effect on As(V) uptake.

Supplemental Figure S7. Overexpression of *OsHAC1;1* or *OsHAC1;2* has no significant effect on As(III) uptake and As accumulation in rice.

Supplemental Figure S8. Root elongation of *OsHAC1;1* and *OsHAC1;2* overexpression lines and wild-type plants exposed to different concentrations of As(V).

Supplemental Table S1. The primers used in this study.

Figure Captions:

Figure 1. *OsHAC1;1* and *OsHAC1;2* encode arsenate reductases. (A) Expression of *OsHAC1;1* or *OsHAC1;2* suppresses the As(V) sensitivity of the *E. coli* mutant lacking the *arsC* arsenate reductase. Strains were grown at 16°C and cell density measured at OD600 nm after exposure to 1 mM As(V) for 0 – 72 h. WT = *E. coli* wild type (W3110); Δ *arsC* = *arsC* mutant in WC3110; Vector = empty pET29a; *arsC-HAC1;1*=pET-29a vector containing *OsHAC1;1*; Δ *arsC-HAC1;2*=pET-29a vector containing *OsHAC1;2*. (B) Production of As(III) in LB medium after *E. coli* expressing the empty vector (EV), *OsHAC1;1* or *OsHAC1;2* was exposed to 10 μ M As(V). n.d.= not detected.

Figure 2. Expression patterns of *OsHAC1;1* and *OsHAC1;2* revealed by the accumulation of the OsHAC1;1-GFP or OsHAC1;2-GFP fusion proteins driven by their native promoters. Roots were cut by hand at different zones: RT, root tip; EZ, elongation zone; MZ, mature zone. Scale bar=100 μ m.

Figure 3. Subcellular localization of OsHAC1;1 and OsHAC1;2. Representative microscopic images of rice protoplasts expressing the OsHAC1;1-GFP (top panel) or OsHAC1;2-GFP (middle panel) fusion protein, or eGFP (bottom panel) driven by the cauliflower mosaic virus 35S promoter. Scale Bars = 5 μ m.

Figure 4. Induction of *OsHAC1;1* and *OsHAC1;2* expression in roots (A) and shoots revealed by quantitative real-time PCR. Plants were exposed to 0 or 10 μ M As(V) for 24 h. Expression of each gene was calculated as $2^{-\Delta CT}$ relative to *OsActin*. Data are means \pm S.D. (n=3 biological replicates).

Figure 5. Knocking out *OsHAC1;1* or *OsHAC1;2* affects As(V) reduction and As accumulation in rice. (A-D) As speciation in roots (A, C) and shoots (B, D) after wild-type and knockout single or double mutants were exposed to 10 μ M As(V) for 48 h. (E-H) Uptake of As(V) (E, G) and efflux of As(III) (F, H) after wild-type and mutant plants were exposed to 10 μ M As(V) for 48 h. WT1, cv Zhonghua 11; WT2, cv

Dongjin. Data are means \pm S.E. ($n=4$ biological replicates). Different letters above bars represent significant difference at $P<0.05$.

Figure 6. Knockout or overexpression *OsHAC1;1* or *OsHAC1;2* affects As accumulation in rice. Arsenic concentration in roots and shoots of knockout mutant and wild-type (WT1, Zhonghua 11) plants (A, B) and the overexpression lines and wild-type (WT3, Nipponbare) plants (C, D). Plants were exposed to different As(V) concentrations for 48 h. *Ox1;1-1*, *Ox1;1-2*, *Ox1;1-3* represent independent overexpression lines of *OsHAC1;1*. *Ox1;2-1*, *Ox1;2-2*, *Ox1;2-3* represent independent overexpression lines of *OsHAC1;2*. Data represents means \pm S.E. ($n=4$ biological replicates). * indicates significant difference from WT at $P<0.05$. DW= dry weight.

Figure 7. Overexpression of *OsHAC1;1* or *OsHAC1;2* increases As(III) efflux and decreases As accumulation in rice shoots. (A, E), The expression levels of *OsHAC1;1* (A) or *OsHAC1;2* (E) in wild-type (WT3, Nipponbare) and transgenic lines by qRT-PCR. (B, F), Efflux of As(III) after wild-type and overexpression lines of *OsHAC1;1* (B) or *OsHAC1;2* (F) were exposed to 10 μ M As(V) for 48 h. (C, D, G, H), As speciation in roots (C, G) and shoots (D, H) after wild-type and overexpression lines of *OsHAC1;1* (C, D) or *OsHAC1;2* (G, H) were exposed to 10 μ M As(V) for 48 h. Data are means \pm S.E. ($n=4$ biological replicates). Different letters above bars represent significant difference at $P<0.05$.

Figure 8. Knockout or overexpression of *OsHAC1;1* or *OsHAC1;2* affects As speciation in xylem sap. Concentrations of As(V) and As(III) in xylem sap of knockout single or double mutants and wild-type (WT1, Zhonghua 11) plants (A) and the overexpression lines and wild-type (WT3, Nipponbare) plants (B). Plants were exposed to 10 μ M As(V) for 24 h. Data represents means \pm S.E. ($n=4$ biological replicates). Different letters above bars represent significant difference at $P<0.05$.

Figure 9. Overexpression of *OsHAC1;1* or *OsHAC1;2* enhances As(V) tolerance. Root growth of rice seedlings during 24 h under different As(V) concentrations was measured. *Ox1;1-1*, *Ox1;1-2*, *Ox1;1-3* represent independent overexpression lines of *OsHAC1;1*. *Ox1;2-1*, *Ox1;2-2*, *Ox1;2-3* represent independent overexpression lines of

OsHAC1;2. Data represents means \pm S.E. ($n=10$ biological replicates). * indicates significant difference from WT (WT3, Nipponbare) at $P<0.05$.

Figure 10. Knockout or overexpression of *OsHAC1;1* or *OsHAC1;2* affect arsenic accumulation in rice grain under aerobic soil conditions. (A) As concentration in brown rice of *OsHAC1;1* or *OsHAC1;2* knockout mutants and wild-type (WT1, Zhonghua 11) plants. (B) As concentration in brown rice of *OsHAC1;1* or *OsHAC1;2* overexpression lines and wild-type (WT3, Nipponbare) plants. Plants were grown in a soil amended with 20 mg As(V) kg^{-1} under aerobic conditions and rice grain were harvested at maturity. Data are means \pm S.E. ($n=4$ biological replicates). Different letters above bars represent significant difference at $P<0.05$.

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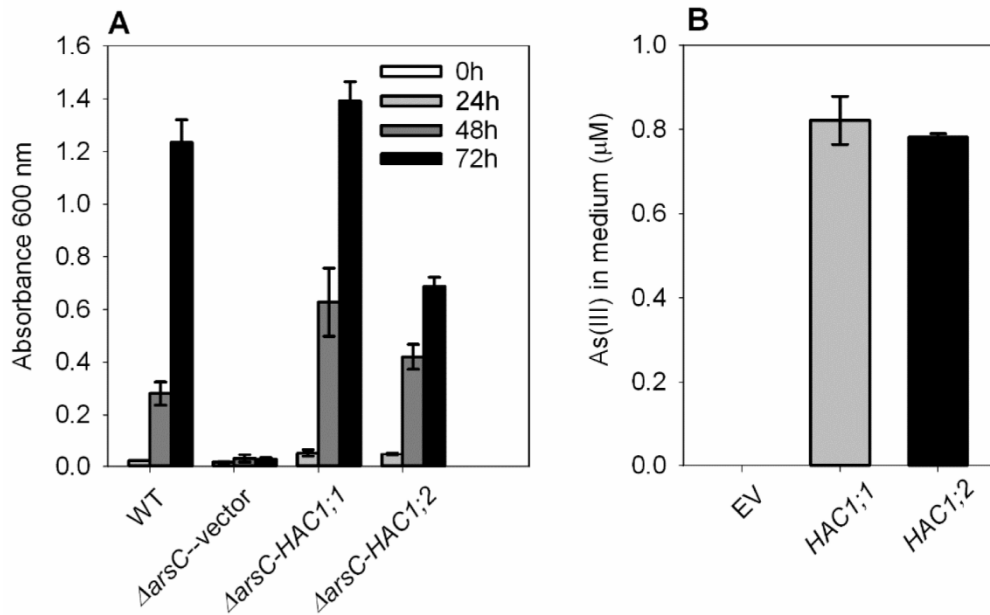


Figure 1. *OsHAC1;1* and *OsHAC1;2* encode arsenate reductases. (A) Expression of *OsHAC1;1* or *OsHAC1;2* suppresses the As(V) sensitivity of the *E. coli* mutant lacking the *arsC* arsenate reductase. Strains were grown at 16°C and cell density measured at OD600 nm after exposure to 1 mM As(V) for 0 – 72 h. WT = *E. coli* wild type (W3110); Δ arsC = *arsC* mutant in WC3110; Vector = empty pET29a; *arsC-HAC1;1*=pET-29a vector containing *OsHAC1;1*; *arsC-HAC1;2*=pET-29a vector containing *OsHAC1;2*. (B) Production of As(III) in LB medium after *E. coli* expressing the empty vector (EV), *OsHAC1;1* or *OsHAC1;2* was exposed to 10 μM As(V). n.d.= not detected.

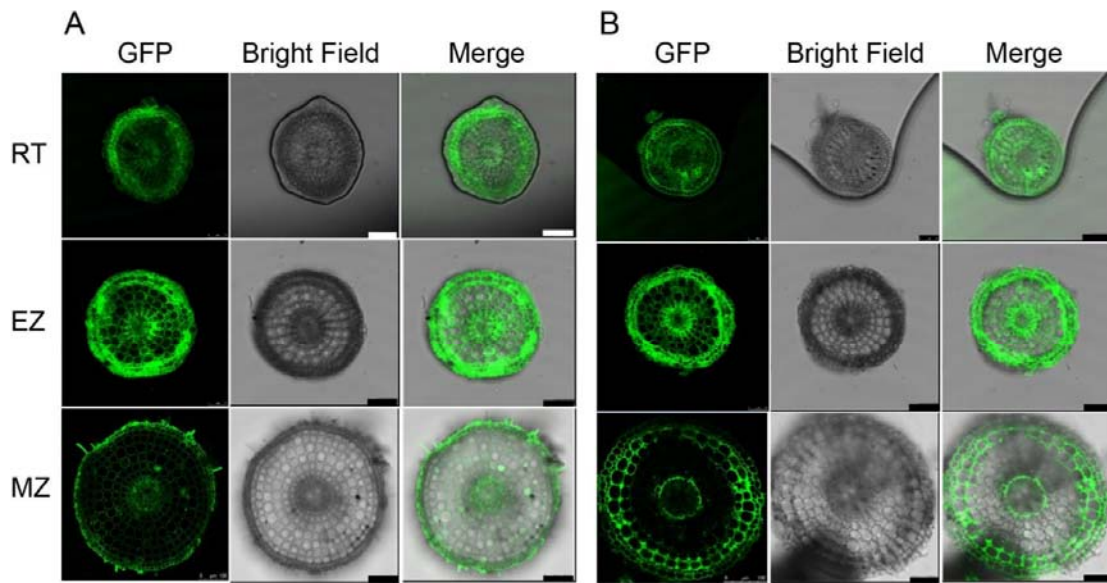


Figure 2. Expression patterns of *OshAC1;1* and *OshAC1;2* revealed by the accumulation of the *OshAC1;1*-GFP or *OshAC1;2*-GFP fusion proteins driven by their native promoters. Roots were cut by hand at different zones: RT, root tip; EZ, elongation zone; MZ, mature zone. Scale bar=100 μ m.

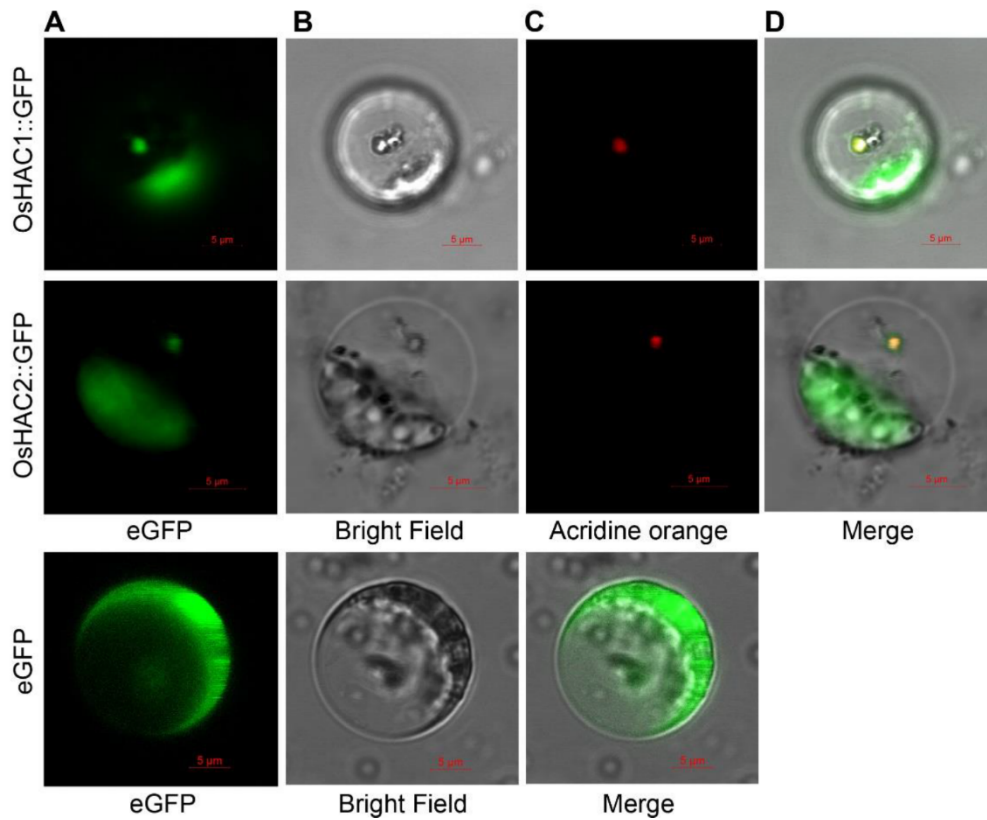


Figure 3. Subcellular localization of OshAC1;1 and OshAC1;2. Representative microscopic images of rice protoplasts expressing the OshAC1;1-GFP (top panel) or OshAC1;2-GFP (middle panel) fusion protein, or eGFP (bottom panel) driven by the cauliflower mosaic virus 35S promoter. Scale Bars = 5 µm.

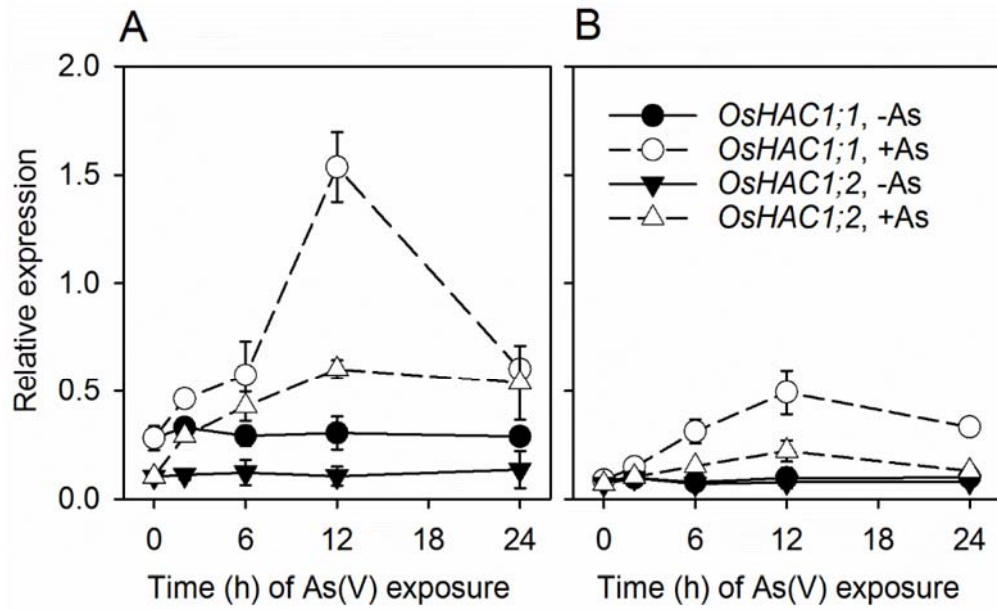


Figure 4. Induction of *OsHAC1;1* and *OsHAC1;2* expression in roots (A) and shoots (B) revealed by quantitative real-time PCR. Plants were exposed to 0 or 10 μM As(V) for 24 h. Expression of each gene was calculated as $2^{-\Delta\text{CT}}$ relative to *OsActin*. Data are means \pm S.D. (n=3 biological replicates).

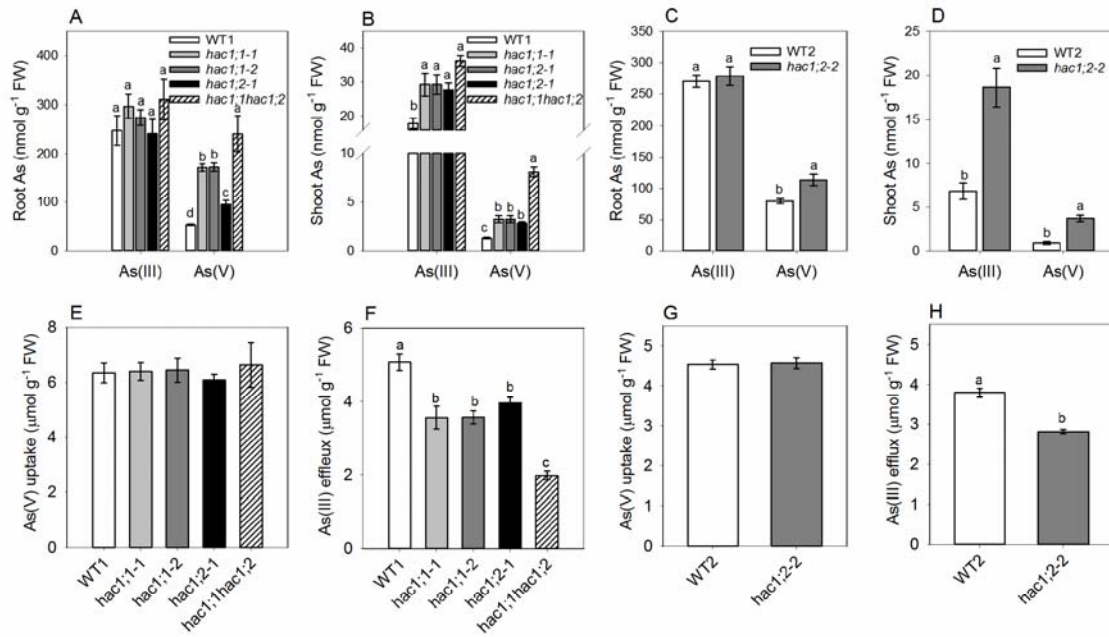


Figure 5. Knocking out *OsHAC1;1* or *OsHAC1;2* affects As(V) reduction and As accumulation in rice. (A-D) As speciation in roots (A, C) and shoots (B, D) after wild-type and knockout single or double mutants were exposed to 10 μM As(V) for 48 h. (E-H) Uptake of As(V) (E, G) and efflux of As(III) (F, H) after wild-type and mutant plants were exposed to 10 μM As(V) for 48 h. WT1, cv Zhonghua 11; WT2, cv Dongjin. Data are means ± S.E. (*n*=4 biological replicates). Different letters above bars represent significant difference at *P*<0.05.

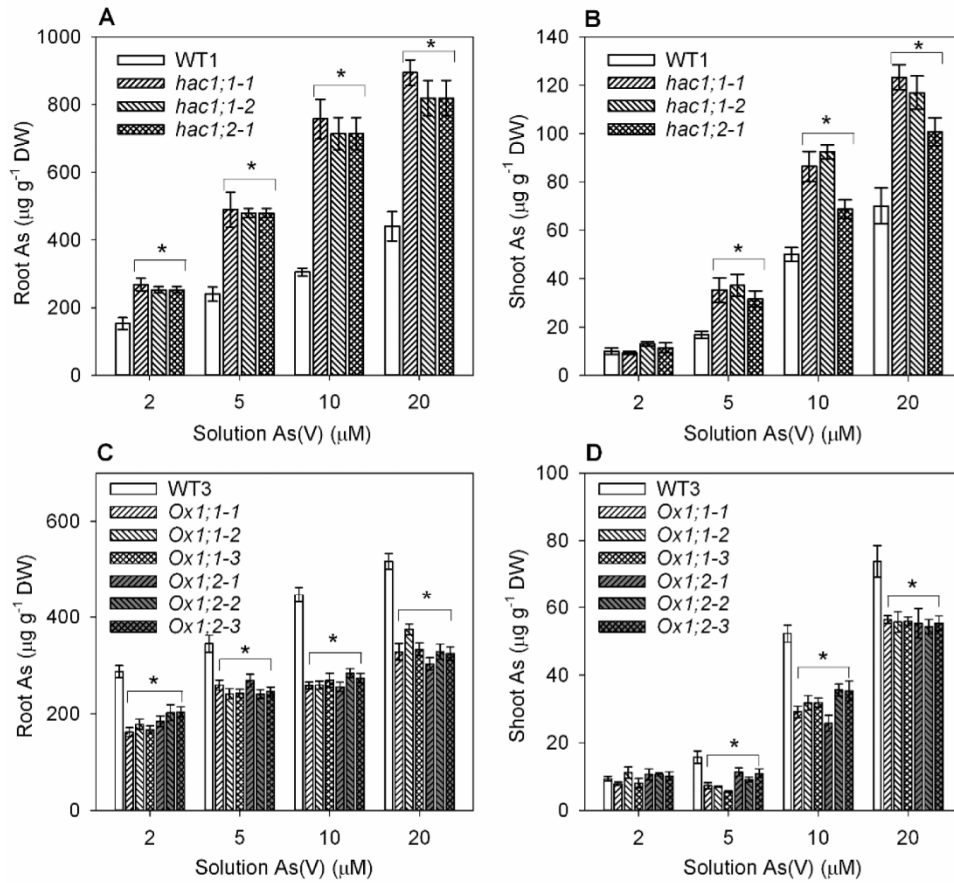


Figure 6. Knockout or overexpression *OshAC1;1* or *OshAC1;2* affects As accumulation in rice. Arsenic concentration in roots and shoots of knockout mutant and wild-type (WT1, Zhonghua 11) plants (A, B) and the overexpression lines and wild-type (WT3, Nipponbare) plants (C, D). Plants were exposed to different As(V) concentrations for 48 h. *Ox1;1-1*, *Ox1;1-2*, *Ox1;1-3* represent independent overexpression lines of *OshAC1;1*. *Ox1;2-1*, *Ox1;2-2*, *Ox1;2-3* represent independent overexpression lines of *OshAC1;2*. Data represents means \pm S.E. ($n=4$ biological replicates). * indicates significant difference from WT at $P<0.05$. DW= dry weight.

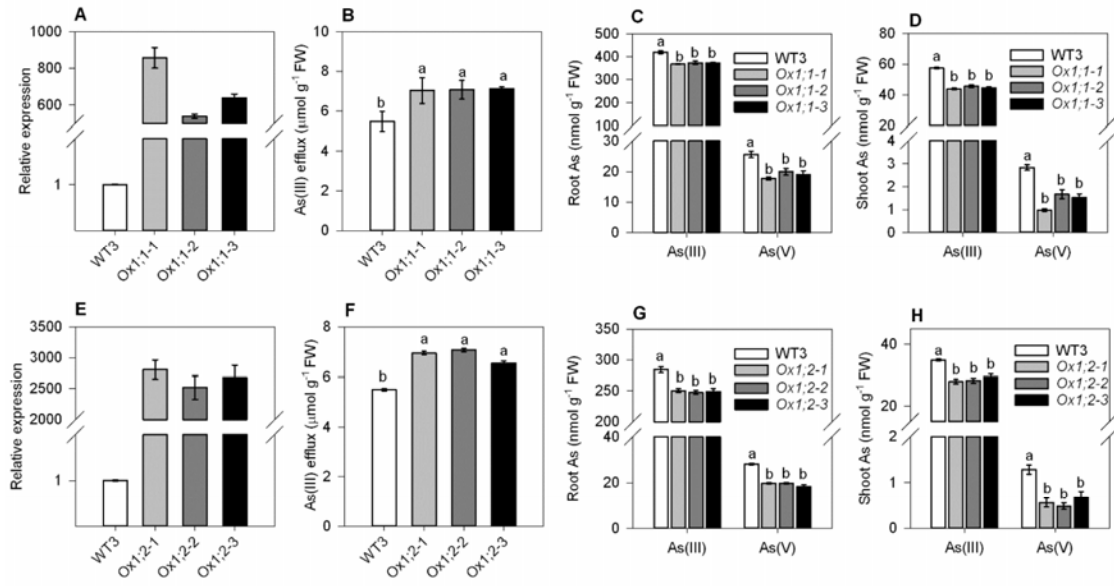


Figure 7. Overexpression of *OshAC1;1* or *OshAC1;2* increases As(III) efflux and decreases As accumulation in rice shoots. (A, E) The expression levels of *OshAC1;1* (A) or *OshAC1;2* (E) in wild-type (WT3, Nipponbare) and transgenic lines by qRT-PCR. (B, F) Efflux of As(III) after wild-type and overexpression lines of *OshAC1;1* (B) or *OshAC1;2* (F) were exposed to 10 μM As(V) for 48 h. (C, D, G, H) As speciation in roots (C, G) and shoots (D, H) after wild-type and overexpression lines of *OshAC1;1* (C, D) or *OshAC1;2* (G, H) were exposed to 10 μM As(V) for 48 h. Data are means \pm S.E. ($n=4$ biological replicates). Different letters above bars represent significant difference at $P<0.05$.

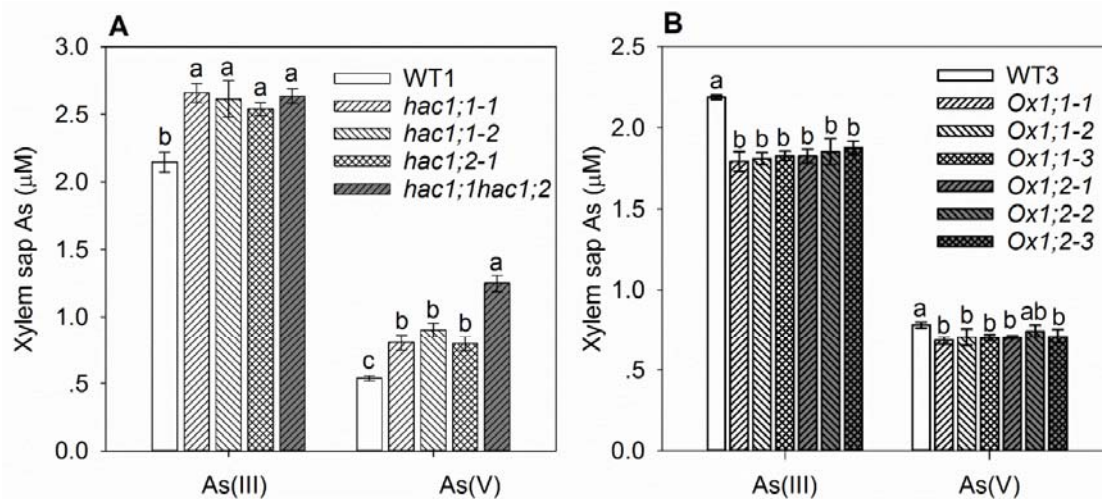


Figure 8. Knockout or overexpression of *OshAC1;1* or *OshAC1;2* affects As speciation in xylem sap. Concentrations of As(V) and As(III) in xylem sap of knockout single or double mutants and wild-type (WT1, Zhonghua 11) plants (A) and the overexpression lines and wild-type (WT3, Nipponbare) plants (B). Plants were exposed to 10 μ M As(V) for 24 h. Data represents means \pm S.E. ($n=4$ biological replicates). Different letters above bars represent significant difference at $P<0.05$.

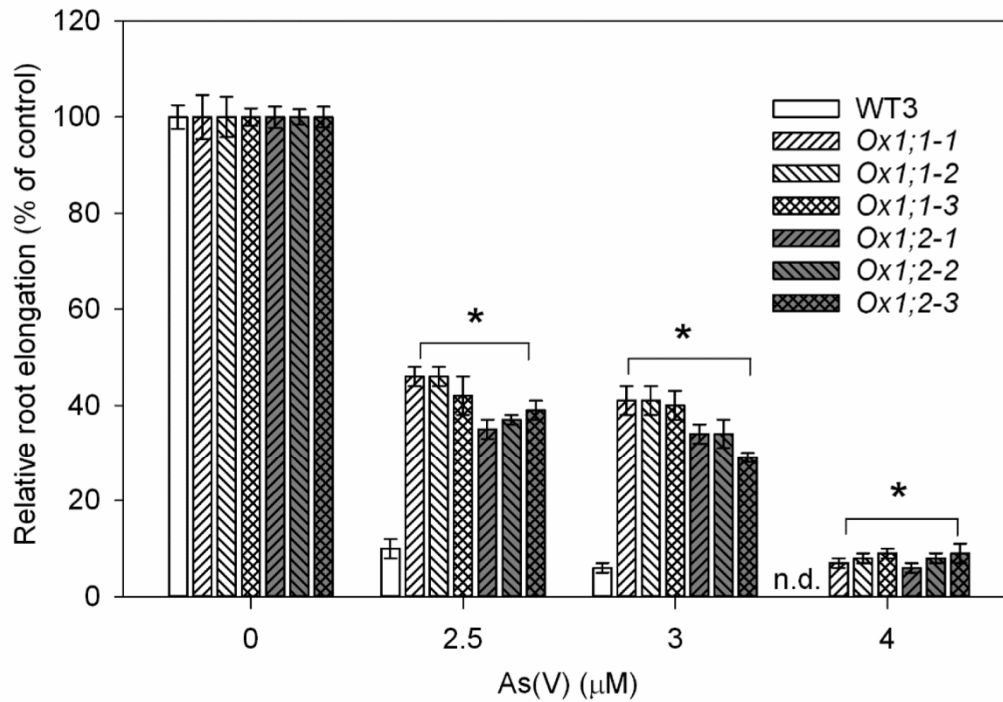


Figure 9. Overexpression of *OshAC1;1* or *OshAC1;2* enhances As(V) tolerance. Root growth of rice seedlings during 24 h under different As(V) concentrations was measured. *Ox1;1-1*, *Ox1;1-2*, *Ox1;1-3* represent independent overexpression lines of *OshAC1;1*. *Ox1;2-1*, *Ox1;2-2*, *Ox1;2-3* represent independent overexpression lines of *OshAC1;2*. Data represents means \pm S.E. ($n=10$ biological replicates). * indicates significant difference from WT (WT3, Nipponbare) at $P<0.05$.

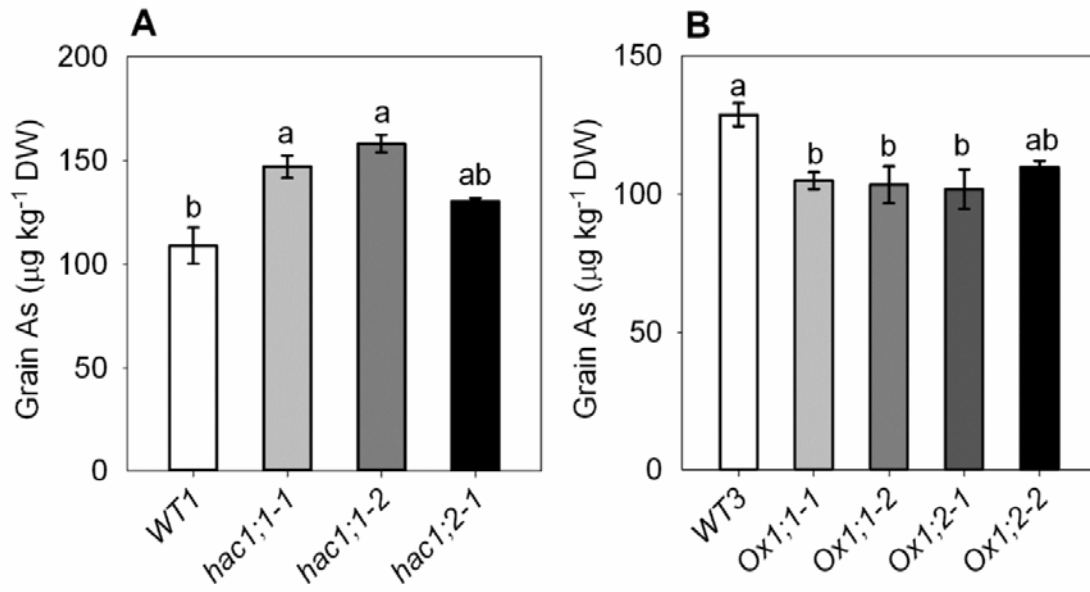


Figure 10. Knockout or overexpression of *OsHAC1;1* or *OsHAC1;2* affect arsenic accumulation in rice grain under aerobic soil conditions. (A) As concentration in brown rice of *OsHAC1;1* or *OsHAC1;2* knockout mutants and wild-type (WT1, Zhonghua 11) plants. (B) As concentration in brown rice of *OsHAC1;1* or *OsHAC1;2* overexpression lines and wild-type (WT3, Nipponbare) plants. Plants were grown in a soil amended with 20 mg As(V) kg^{-1} under aerobic conditions and rice grain were harvested at maturity. Data are means \pm S.E. ($n=4$ biological replicates). Different letters above bars represent significant difference at $P<0.05$.