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Expressing *ScACR3* in Rice Enhanced Arsenite Efflux and Reduced Arsenic Accumulation in Rice Grains

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Regular Paper

Arsenic (As) accumulation in rice grain poses a serious health risk to populations with high rice consumption. Extrusion of arsenite [As(III)] by ScAcr3p is the major arsenic detoxification mechanism in *Saccharomyces cerevisiae*. However, ScAcr3p homolog is absent in higher plants, including rice. In this study, *ScACR3* was introduced into rice and expressed under the control of the *Cauliflower mosaic virus* (CaMV) 35S promoter. In the transgenic lines, As concentrations in shoots and roots were about 30% lower than in the wild type, while the As translocation factors were similar between transgenic lines and the wild type. The roots of transgenic plants exhibited significantly higher As efflux activities than those of the wild type. Within 24 h exposure to 10 μM arsenate [As(V)], roots of *ScACR3*-expressing plants extruded 80% of absorbed As(V) to the external solution as As(III), while roots of the wild type extruded 50% of absorbed As(V). Additionally, by exposing the As-containing rice plants to an As-lacking solution for 24 h, about 30% of the total As derived from pre-treatment was extruded to the external solution by *ScACR3*-expressing plants, while about 15% of As was extruded by wild-type plants. Importantly, *ScACR3* expression significantly reduced As accumulation in rice straws and grains. When grown in flooded soil irrigated with As(III)-containing water, the As concentration in husk and brown rice of the transgenic lines was reduced by 30 and 20%, respectively, compared with the wild type. This study reports a potential strategy to reduce As accumulation in the food chain by expressing heterologous genes in crops.

Keywords: Arsenic • Efflux • Rice • *ScACR3*.

Abbreviations: As, arsenic; CaMV, *Cauliflower mosaic virus*; DMA, dimethylarsinic acid; EF1α, elongation factor 1α; ICP-MS, inductively coupled plasma mass spectroscopy; PC, phytochelatin.

Introduction

Arsenic (As) is a class one carcinogen present ubiquitously in the environment (International Agency for Research on Cancer 2004). Mining activities, use of arsenical herbicides and insecticides, and irrigation with As-contaminated groundwater result in As accumulation in paddy soil, especially in South and Southeast Asia (Islam et al. 2004, Norra et al. 2005). Rice is particularly efficient in As accumulation compared with other cereal crops (Williams et al. 2007, Su et al. 2010), because of anaerobic conditions in paddy soil (Takahashi et al. 2004, Xu et al. 2008) and due to sharing the highly efficient silicon (Si) uptake pathway (Ma et al. 2008, Zhao et al. 2009). Uptake of As by rice plants and accumulation in grains would present a food safety problem. A number of studies have shown that consumption of rice constitutes a large proportion of the dietary intake of inorganic As for populations whose staple food is rice (Mondal and Polya 2008, Brammer and Ravenscroft 2009, Meharg et al. 2009). In addition, As causes significant yield losses due to As phytotoxicity (Panaullah et al. 2009). Moreover, rice straw, containing much higher levels of As than grain, is widely used as cattle feed, thus presenting another route of As entry into the food chain (Rahman et al. 2008, Panaullah et al. 2009). Therefore, there is an urgent need to develop mitigation strategies in reducing As accumulation in rice plants.

To prevent As accumulation and survive in As-contaminated environments, several strategies have been developed by organisms. In plants, strategies usually include inhibition of As uptake by roots (Meharg et al. 1992, Bleeker et al. 2003), chelation by phytochelatins (PCs) and sequestration into intracellular compartments (Pickering et al. 2000, Li et al. 2004, Tong et al. 2004, Kang et al. 2010), efflux from root cells (Xu et al. 2007) and transformation into less toxic organic As compounds (Zhao et al. 2009). In microbes, the most effective strategy of As detoxification is extrusion of As(III) outside of the cells

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(Bhattacharjee and Rosen 2007, Wysocki et al. 2010). ArsB, an antiporter of As(III), constitutes a major detoxification pathway for arsenicals in *Escherichia coli* (Rosen 1997, Rosen 2002, Meng et al. 2004). In *Saccharomyces cerevisiae*, ScAcr3p, an As(III) plasma membrane transporter, mediates the efflux of As(III) and confers tolerance to arsenicals (Bobrowicz et al. 1997, Ghosh et al. 1997, Wysocki et al. 1997, Maciaszczyk-Dziubinska et al. 2010). Expression of the *ScACR3* gene in yeast cells increases resistance to As(III) by 10-fold and decreases As accumulation, while cells lacking *ScACR3* are highly sensitive to both As(III) and As(V) (Bobrowicz et al. 1997, Ghosh et al. 1997, Wysocki et al. 1997).

Recently, two genes similar to *ScACR3* were isolated from *Petteris vittata* (Indriolo et al. 2010), *PvACR3* and *PvACR3;1*. PvACR3 was able to complement the As-sensitive phenotypes of yeast cells deficient in *ScACR3* and exhibited As(III) efflux activity. Gametophytes with knocked-down expression of *PvACR3* were sensitive to As(III). These results indicate that PvACR3 potentially functions as an As(III) efflux transporter like ScAcr3p (Indriolo et al. 2010). However, only ferns and some other gymnosperms have ACR3 homologs, which are absent in angiosperms, including rice (Indriolo et al. 2010).

Although *ScACR3* homolog is absent in the angiosperm genome, rapid As(III) efflux by plant roots has been observed in a number of plant species (Xu et al. 2007, Logoteta et al. 2009, Zhao et al. 2010a). The rapid As(III) efflux by roots may contribute to an important mechanism of As detoxification in plants, and enhancing As efflux by roots would be a potential strategy to increase As tolerance and decrease As accumulation in food crops (Tripathi et al. 2007, Verbruggen et al. 2009, Zhao et al. 2009, Zhu and Rosen 2009). Aquaporins have been thought to be the candidate transporters responsible for As(III) efflux in plants (Bienert et al. 2008, Isayenkov and Maathuis 2008, Zhao et al. 2009). However, in rice, Lsi1 is an aquaporin and involved in bi-directional As(III) transport, which accounts for only 15–20% of the total efflux (Zhao et al. 2010a), suggesting the existence of other efflux transporters in rice roots. In addition to the passive efflux through aquaporins, Xu et al (2007) have suggested the possibility of an ScAcr3p-like mechanism for active As(III) efflux in plants. However, so far, the key membrane transporter proteins responsible for As(III) efflux in plant roots are not known.

Since ScAcr3p is an efficient As(III) efflux transporter of yeast, and since a similar As detoxification mechanism exists in rice plants, we hypothesized that expression of *ScACR3* in rice could enhance As(III) efflux in rice roots and thus reduce As accumulation in rice plants. In this study, *ScACR3* under the control of the *Cauliflower mosaic virus* (CaMV) 35S promoter was introduced into rice. Under both hydroponic solution and flooded soil conditions, As accumulation in seedlings and grains of *ScACR3*-expressing lines was significantly lower than that in the wild type.

Results

Expression of *ScACR3* in rice plants

ScACR3 was cloned from *S. cerevisiae* and driven by the CaMV 35S promoter in rice (**Fig. 1A**). Eight independent transgenic lines were established, and T_1 generation plants were used. Integration of *ScACR3* into the rice genome was verified by germinating seeds on hygromycin-selective plates and further by PCR with *ScACR3*-specific primers. The accumulation of *ScACR3* transcript levels in rice leaves was determined by quantitative PCR (**Fig. 1B**). For quantitative PCR, mRNA was prepared from three individual plants of each line and the transcript level was determined independently. *ScACR3* transcript was not detected in wild-type plants, while it was accumulated in all transgenic plants (**Fig. 1B**). The expression level of *ScACR3* varied among different transgenic lines. Among the eight lines, three lines with high accumulation of *ScACR3* transcripts (lines 2, 6 and 7) were chosen for the following experiments.

ScACR3 expression proportionally reduced As accumulation in rice seedling

To investigate the effects of *ScACR3* expression on As accumulation in rice seedlings and As translocation from roots to shoots, rice seedlings of transgenic lines and the wild type were treated with As(III). When the rice plants were treated with 0, 5, 10, 20, 40 and 60 μM As(III) for 24 h, toxicity

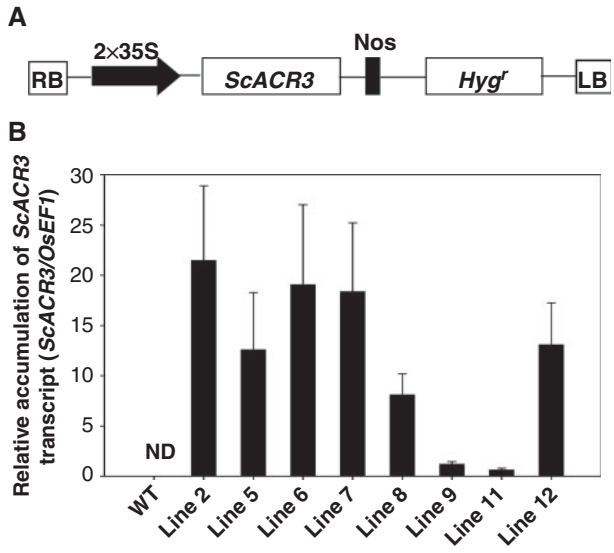

Fig. 1 Diagram of the *ScACR3* expression construct and determination of *ScACR3* transcript accumulation in transgenic plants. (A) Schematic diagram of the *ScACR3* expression construct. *ScACR3* was driven by the CaMV 35S promoter in the pMDC32 vector. (B) Relative accumulation of *ScACR3* transcripts levels in transgenic plant leaves. Total RNAs were independently prepared from three individual plants of each line; the values were shown as a ratio relative to Line 9. Data are means ± SD ($n = 3$), line 2–line 12 are independent transgenic lines, WT indicates the wild type, ND means not detectable.

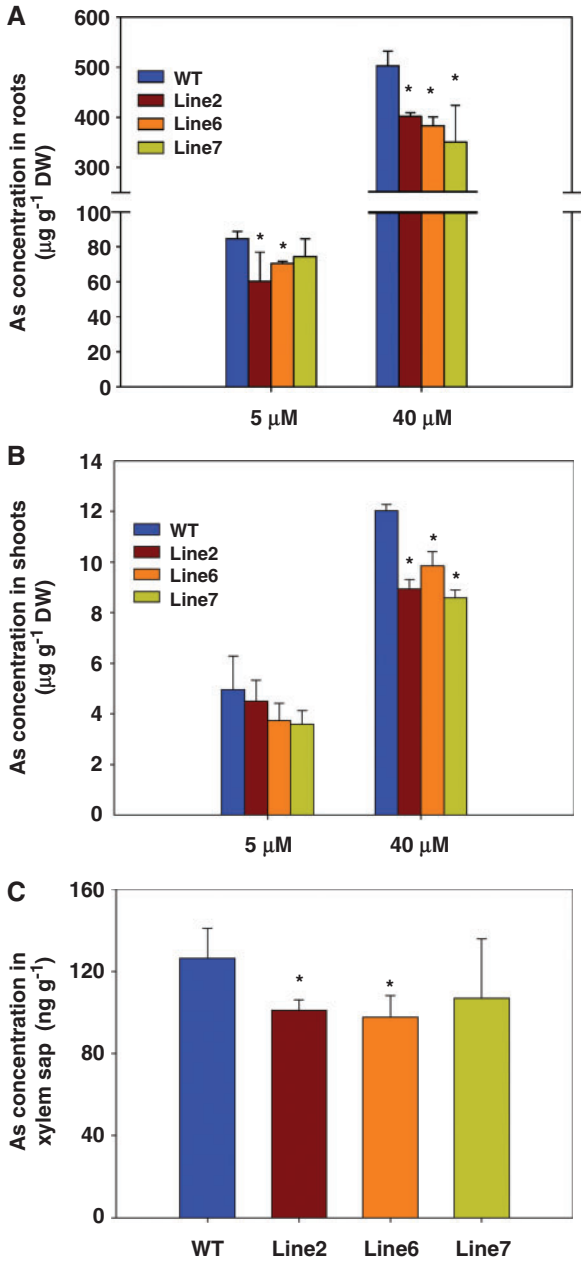

Fig. 2 As accumulation in roots (A) and shoots (B) of rice plants after exposure to 5 or 40 μM As(III) for 24 h, and the As concentration in the xylem sap (C) of rice plants after exposure to 40 μM As(III) for 24 h. Data are means ± SD ($n = 4$); asterisks indicate a significant difference from the wild type ($P < 0.05$).

symptoms were apparent with 60 μM As(III) treatment, but not at the lower concentrations (data not shown). Therefore, concentrations of 5 μM (low) or 40 μM (high) were chosen for As(III) treatments. After 24 h low or high As(III) treatments, plants were harvested and xylem sap was collected, and the As concentration in roots (**Fig. 2A**), shoots (**Fig. 2B**) and xylem sap (**Fig. 2C**) was determined. At the time of the collection there were no visible symptoms in the plants treated with 40 μM As(III).

Accumulation of As in plants treated with both low and high As(III) was examined. Under low As(III) treatment, As concentrations in roots of lines 2 and 6 were significantly lower than those of the wild type, being decreased by 28 and 15%, respectively, compared with the wild type, while the decrease in line 7 was not statistically significant (**Fig. 2A**). The As concentrations in shoots of *ScACR3*-expressing lines were also lower than in those of the wild type, whereas the decrease in all these lines was not statistically significant compared with the wild type (**Fig. 2B**). Under the high As(III) treatment, the As concentration in roots of lines 2, 6 and 7 was decreased by 20, 23 and 30%, respectively, and in shoots by 35, 29 and 42%, respectively, compared with the wild type (**Fig. 2A, B**). The As concentrations in both roots and shoots of *ScACR3* expressing lines were significantly lower than those of the wild-type. These results indicate that the expression of *ScACR3* reduced As accumulation in rice seedlings exposed to both low and high As(III) conditions.

The As concentrations in xylem sap and As translocation factors were analyzed. Xylem sap was collected from the plants treated with 40 μM As(III) for 24 h. In the xylem sap of lines 2 and 6, As concentrations were significantly lower than those of the wild type, being decreased by 20% and 23%, respectively, while the decrease in line 7 was not statistically significant (**Fig. 2C**). As translocation factors were calculated as the shoot/root As concentration ratio. There was no significant difference in As translocation factors between *ScACR3*-expressing lines and the wild type, under either low or high As(III) treatments (**Supplementary Fig. S1**). These results suggest that the expression of *ScACR3* reduces the As concentration in xylem but is unlikely to be directly involved in xylem loading of As.

ScACR3 expression reduced As accumulation by extruding As to the external solution

To investigate the mechanism of reduced As accumulation in the *ScACR3*-expressing plants, an As efflux experiment was performed. In this experiment, rice plants were treated with 5 or 40 μM As(III) for 24 h, and then transferred to As-free nutrient solution for another 24 h (as shown in **Fig. 3A**). As efflux activities were determined 2, 4, 6, 8, 10 and 24 h after transfer to As-free solution (**Fig. 3B, C**). After 24 h in the As-free solution, As in plants (**Fig. 3D, E**) and the distribution of As between plants and solution were analyzed (**Fig. 3F, G**).

The As efflux activity was calculated as the amount of As in the solution divided by the root biomass. Roots of the *ScACR3*-expressing lines exhibited significantly higher As efflux activities than those of the wild type, after both low and high As pre-treatments (**Fig. 3B, C**). After 24 h in the As-free solution, As efflux activity of lines 2, 6 and 7 pre-treated with low As(III) were about 30, 23 and 28% higher than in the wild type, respectively. Similarly, with high As(III) pre-treatment, the efflux activity of transgenic lines was also 30% higher than that of the

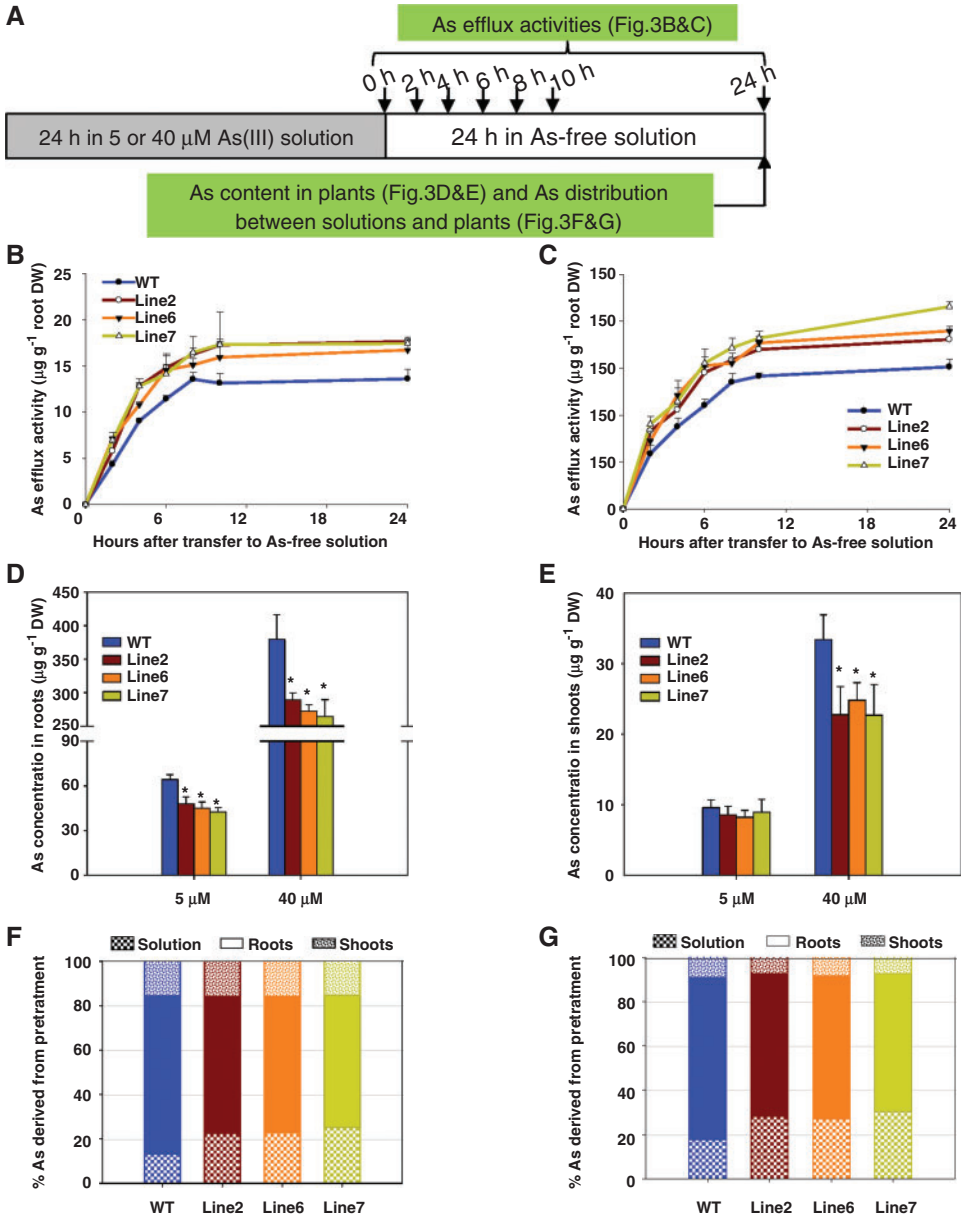

Fig. 3 As-containing roots efflux As to the external solution. (A) Schematic diagram of As treatments. Rice plants were pre-treated with 5 μM (A) or 40 μM As(III) for 24 h, then exposed to As-free solution for another 24 h. (B and C) As efflux activities of rice roots pre-treated with 5 μM (B) or 40 μM As(III) (C). (D and E) As concentration in roots (D) and shoots (E) after 24 h exposure to As-free solution. (F and G) As distribution between the solution and plants pre-treated with 5 μM (F) or 40 μM As(III) (G) after 24 h exposure to As-free solution. Data are means ± SD (n = 4); asterisks indicate a significant difference from the wild type ($P < 0.05$).

wild type. These results indicate that expression of *ScACR3* in rice enhances As efflux activity of rice roots.

After exposure to the As-free solution for 24 h, rice plants were harvested and As concentrations in roots and shoots were determined. In roots, *ScACR3* expression significantly reduced As concentrations under both low and high As(III) pre-treatment, being decreased by 24–35% compared with the wild type (**Fig. 3D**). In shoots, the As concentration of *ScACR3*-expressing lines was about 30% lower than in the wild type under high As(III) pre-treatment, while the decrease was not significant under low As(III) pre-treatment (**Fig. 3E**). These results further showed that *ScACR3* expression reduced As accumulation in rice seedlings.

After 24 h in the As-free solution, the As concentration in the external solution (**Fig. 3B, C**) and shoots (compare **Figs. 2B** and **3E**) was increased, while As in roots was decreased (compare **Figs. 2A** and **3D**). These data indicate that during exposure to the As-free solution, As in roots was re-distributed in two directions: uploaded to shoots and extruded to the external solution. To understand the difference in As re-distribution

between wild-type and *ScACR3*-expressing plants, the proportions of As in the external solution and plants were calculated (the sum of As in the solution and in plants was calculated as total As derived from pre-treatment). With 5 μM As(III) pre-treatment, wild-type plants extruded about 13% of As derived from pre-treatment to the external solution within 24 h, and retained 72% in roots and 15% in shoots, while *ScACR3*-expressing plants extruded a larger proportion of As to the external solution (about 25%), and retained less As in plants (60% in roots and 15% in shoots) (**Fig. 3F**). With 40 μM As(III) pre-treatment, about 15% of As derived from pre-treatment was extruded to the solution by wild-type plants, and about 75 and 10% was retained in roots and shoots, respectively, while about 30% of As was extruded to the solution by *ScACR3*-expressing plants, and 61 and 9% was retained in roots and shoots, respectively (**Fig. 3G**). These results showed that *ScACR3*-expressing plants reduced As accumulation in plants by extruding more As to the external solution than the wild type.

ScACR3 expression enhanced As(III) efflux activity of roots

To confirm further that expression of *ScACR3* in rice could enhance As(III) efflux activity of rice roots, rice plants were treated with 10 μM As(V), and then As(V) and As(III) concentrations in the solution were monitored over a 24 h period.

As(V) uptake activity and As(III) efflux activity were calculated from the changes in the amount of As in the solution according to root biomass. There was no significant difference in As(V) uptake activity between the *ScACR3*-expressing lines and the wild type, except for line 6 in which the activity was significantly higher than in other lines at the time points of 8 and 24 h (**Fig. 4A**). In addition, during the 24 h period, As(V) concentrations in solutions of transgenic lines and the wild type were similar (**Supplementary Fig. S2B**). These results indicate that *ScACR3* expression did not affect the As(V) uptake activity of rice roots.

Regarding the As(III) efflux activity, *ScACR3*-expressing lines were significantly higher than those of the wild type during the whole time course examined, except for line 7 at 2 and 4 h. At 24 h, As(III) efflux activity of lines 2, 6 and 7 was higher than that of the wild type by about 56, 106 and 74%, respectively (**Fig. 4B**), resulting in a faster increase in As(III) concentrations in the nutrient solutions (**Supplementary Fig. S2A**). After 24 h, As(III) concentrations in the solutions of *ScACR3*-expressing lines were about 2-fold higher than those of the wild-type (**Supplementary Fig. S2A**), and the percentage of As (III) in the solutions was about 30% higher than that of the wild type (**Supplementary Fig. S2D**). In addition, total As in solutions of *ScACR3*-expressing lines was significantly higher than in the wild type (**Supplementary Fig. S2C**).

To take into account the variation in As(V) uptake, As(III) efflux as a percentage of As(V) uptake was calculated (**Fig. 4C**) as described by Zhao et al. (2010a). During the whole time course examined, *ScACR3*-expressing lines extruded 60–80% of absorbed As(V) to the external solution as As(III), while only 25–50% was extruded by the wild type. This result showed that *ScACR3* expression increased As(III) efflux capacity in rice roots by about 30%.

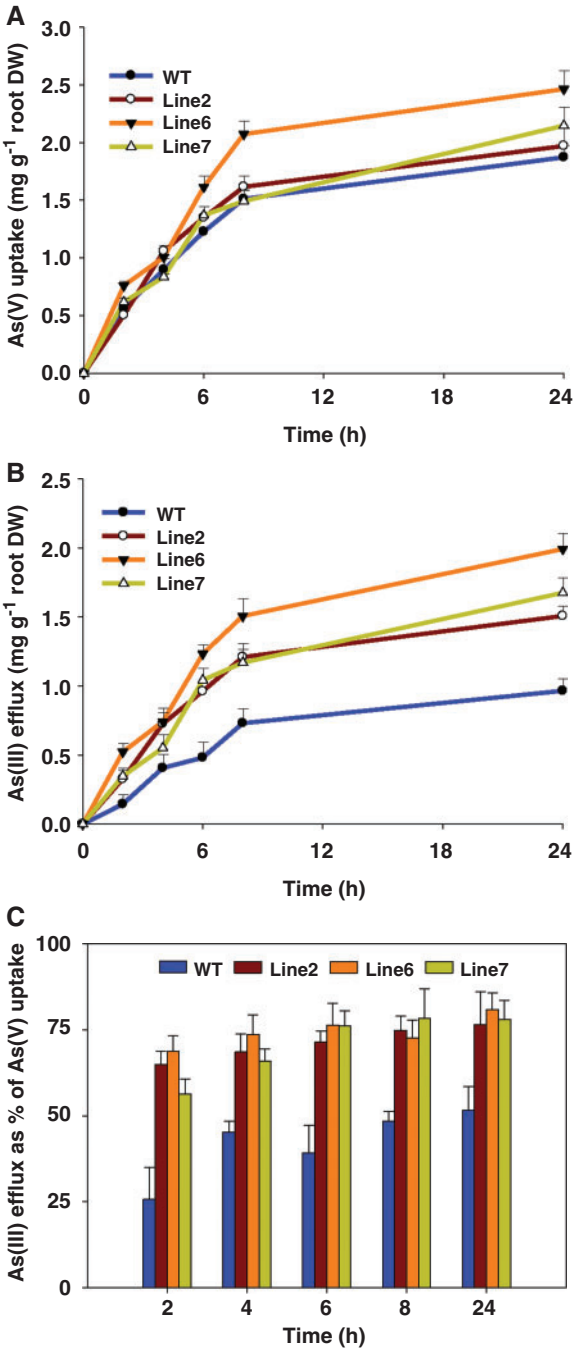

Fig. 4 As(V) uptake (A) and As(III) efflux (B) of the rice treated with 10 μM As(V) for the indicated time, and As(III) efflux as a percentage of As(V) uptake at the indicated time (C). Data are means ± SD ($n = 4$); asterisks indicate a significant difference from the wild type ($P < 0.05$).

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ScACR3 expression reduced As accumulation in rice grains

To investigate further how *ScACR3* expression affects As accumulation in mature plants, rice plants were grown in soil irrigated with As(III)-containing water until maturation. *ScACR3* expression significantly decreased As accumulation in both husk and brown rice (**Fig. 5A, B**). The As concentration in brown rice of lines 2, 6 and 7 decreased by 26, 20 and 14%, respectively, compared with the wild type, and in husk it decreased by 29, 19 and 30%, respectively. *ScACR3* expression also decreased As accumulation significantly in flag leaves, but the difference was not statistically significant in stems. These results established that expression of *ScACR3* significantly reduced As accumulation in mature rice, including grains.

The As species in brown rice were determined (**Fig. 5C**). In the brown rice, As(V), As(III) and dimethylarsinic acid (DMA) were detectable, and about 50% of total As was DMA, 40% was As(III) and 10% was As(V). There was no significant difference in As species patterns between the *ScACR3*-expressing lines and the wild type.

Discussion

Consumption of rice constitutes a major route of entry of inorganic As into the food chain, especially for people whose staple food is rice (Ohno et al. 2007, Mondal and Polya 2008). Mitigation measures to reduce As accumulation in rice grain are urgently needed. In the present study, we showed that expression of *ScACR3* in rice enhanced As(III) efflux activity of rice roots and thus decreased As accumulation in rice plants.

Although wild-type rice plants exhibited high As efflux activities as previously reported (Xu et al. 2007, Zhao et al. 2010a), expression of *ScACR3* significantly enhanced As efflux activities of rice roots. By treating rice plants with As(III), we found that plants expressing *ScACR3* extruded more As to the external solution than the wild type. We also observed that the transgenic plants accumulated low levels of As in roots compared with those in the wild type (**Fig. 3B–G**). Furthermore, by treating rice plants with As(V), we found that the As(III) concentration in solutions of *ScACR3*-expressing lines increased faster than in the wild type (**Supplementary Fig. S2**), and the As(III) efflux activities were enhanced by about 30% compared with the wild type (**Fig. 4**). These results indicate that ScACR3 functions as an active As(III) efflux transporter in the transgenic rice plants. It is known that As is conjugated to PCs in plant cells after As exposure, but free As is also present in roots cells. In roots of *Arabidopsis thaliana*, about 30% of As in the roots is not bound to thiol compounds after 3 d exposure to 10 μM As(V) (Liu et al. 2010). In addition, we think that it is unlikely that a PC–As complex is the form in which As is excreted from roots cells, because in *A. thaliana*, a PC–As complex is not available for root efflux and root to shoot translocation (Liu et al. 2010). In this study, the As(III) efflux rate of the wild type [50% of As(V) uptake] was lower than that reported by Zhao et al (2010a) (about 80%); this may be due to the difference in As(V) treatments. In this study, plants were treated with 10 μM As(V), while Zhao et al (2010a) used 5 μM As(V), and treatment with higher As(V) concentrations may result in higher As(V) uptake.

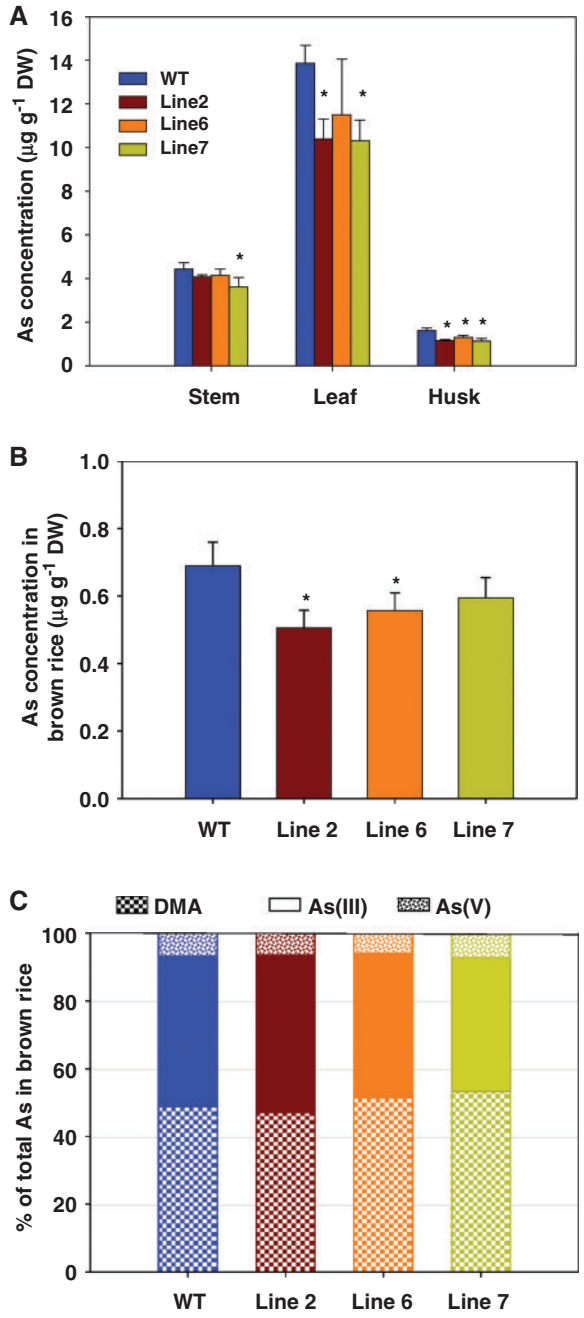

Fig. 5 As accumulation in mature rice tissues (A) and brown rice (B), and As species as a percentage of total As in brown rice (C). Plants were grown in As-free soil and irrigated with 40 μM As(III) solution every 4 d from flowering to grain maturation. Data are means ± SD ($n = 4$); asterisks indicate a significant difference from the wild type ($P < 0.05$).

Enhanced As(III) efflux activity of roots resulted in reduced As accumulation in *ScACR3*-expressing plants grown in hydroponic solution (**Figs. 2A, B, 3D, E**). After 24 h 40 μM As(III) treatment, As accumulation in roots and shoots was reduced by 20–30 and 29–42%, respectively, compared with the wild type. Furthermore, during As(V) treatment, total As in the solution of the wild type decreased more rapidly than in the solution of the transgenic lines. After 24 h, the As concentration in the solution of the wild type was about 25% lower than that in the solution of *ScACR3*-expressing lines (**Supplementary Fig. S2C**). Less As was left in the solution of the wild type, suggesting that more As was accumulated in the wild type than in transgenic plants. These results showed that *ScACR3* expression reduced As accumulation in plants treated with both As(III) and As(V). Regarding the effects of *ScACR3* expression on As translocation, *ScACR3* expression decreased the As concentration in the xylem sap, while it did not significantly affect the translocation factor (**Fig. 2C**. **Supplementary Fig. S1**). This result indicates that *ScACR3* expression proportionally reduced As accumulation in rice plants, and *ScACR3* is unlikely to be directly involved in As loading to the xylem.

In roots, *ScACR3* expression significantly reduced As accumulation under both 5 and 40 μM As(III) treatment, whereas in shoots the decrease was not significant under low As(III) treatment (**Figs. 2B**, **3E**). This ambiguous difference under low As treatment may be explained by PC complexes. Under low As(III) treatment, most cellular As(III) was coordinated with PCs and then sequestrated into the root vacuole (Raab et al. 2005). This might result in a similar free As(III) level in the wild type and transgenic lines. In contrast, under high As(III) treatment, the amount of As(III) may exceed the capacity of PCs and the free As(III) level in roots of the wild type could be higher than in transgenic plants, and thus more As was transported to shoots of wild-type plants.

The accumulation of As in rice grains contributes to the risk for the food chain. Thus reduction of As accumulation in rice grains is very important. In this study, *ScACR3* expression decreased As accumulation in brown rice by about 20% compared with the wild type (**Fig. 5B**). In addition, *ScACR3* expression significantly reduced As accumulation in rice straw and husk (**Fig. 5A**), which are widely used as cattle feed. These results indicate that *ScACR3* expression could be a potential strategy to reduce As entry into the food chain. Recently, genetic engineering has been thought to be a potential strategy for both phytoremediation and food safety (Tripathi et al. 2007, Zhao et al. 2009, Zhu et al. 2009, Zhao et al. 2010). For the purpose of food safety, it is necessary to develop crops with low uptake and/or less loading to edible parts. Ma et al. (2008) found that disruption of the Si transporter, Lsi2, decreased As accumulation in rice grains to 50–60% of that of the wild type. However, As(V) and As(III) share transport systems with essential or beneficial elements, such as phosphorus (P) and Si (Ma et al. 2009, Zhao et al. 2009, Wu et al. 2011). Therefore, reducing As accumulation in rice grains by blocking the As entry system would severely affect the plant growth and the yield of crops. In the present study, As(III) extrusion through expressing *ScACR3* decreased As accumulation in brown rice significantly, while it did not affect rice plant growth (data not shown).

Although expression of *ScACR3* reduced As accumulation in brown rice significantly, the As concentration in brown rice could still be high under high As conditions. Therefore, to minimize As loading and toxicity to/in rice grain, it would be helpful to manipulate more genes for the simultaneous optimization of As detoxification, such as genes involved in As sequestration in roots and/or As volatilization by leaves (Zhu et al. 2009, Zhao et al. 2010b, Meng et al. 2011). In addition, to reduce As accumulation in rice grains to acceptable levels, genetic engineering could also be coupled with selection of appropriate rice varieties and management of agronomic practices, such as water management or Si fertilization (Xu et al 2008, Arao et al. 2009, Li et al. 2009, Norton et al. 2009).

In conclusion, expression of *ScACR3* in rice enhanced As(III) efflux activity by 30%, and thus significantly reduced As accumulation in rice seedlings. Importantly, *ScACR3* expression reduced As accumulation in rice husk and brown rice by 30 and 20%, respectively. This study suggests that expressing *ScACR3* in rice is a potential strategy to reduce As accumulation in rice grain and rice straw.

Materials and Methods

Plasmid construction and rice transformation

ScACR3 was amplified from genomic DNA of *S. cerevisiae* by using the primers: 5′-CCGCTCGAGATGTCAGAAGATCAAAA AAGTG-3′ and 5′-CCAAAGCTTAGTGGTATTATTCATTGGTG CCC-3′ (underlining indicates *Xho*I and *Hin*dIII sites). The resulting fragment was digested with *Xho*I and *Hin*dIII, cloned into the pENTR2B vector (Invitrogen), and subsequently transferred to the binary vector pMDC32 by using LR clonase (Invitrogen) (Curtis and Grossniklaus 2003).

The binary vector was introduced into *Agrobacterium tumefaciens* strain EHA101 and used for transformation of *Oryza sativa* L. cv. Nipponbare. *Agrobacterium*-mediated transformation of rice plants was performed as described by Toki (1997). T_1 plants were used in this study.

Plant culture and treatment

Rice seeds were surface-sterilized with bleach and deionized-exchanged water, and then sown on agar medium containing 0.5 mM $CaCl_2$ without (wild type) or with 50 μg ml^{-1} hygromycin (transgenic plants). One week after germination, hygromycin-resistant plants were further confirmed by PCR using *ScACR3*-specific primers 5′-CAGATTGCTGGAGGAGA C-3′ and 5′-GGTAAGACTTCCCAAACG-3′. Verified seedlings were transferred to hydroponic solution in a 1.5 liter container for 2 weeks with modified Kimura solution as described by Xu et al (2007). Nutrient solutions were renewed every 3 d. Plants were grown in a greenhouse under natural light conditions (30°C/25°C, day/night).

As accumulation in rice seedlings

Uniform 1-week-old rice seedlings were transferred from agar plates to 1.5 liter vessels, four plants (one plant of each line) per vessel. Two weeks after transplanting, plants were treated with both low As [5 μM As(III)] and high As [40 μM As(III)] for 24 h. Each treatment was replicated in four vessels. For xylem sap collection, rice stems were cut at 1 cm above the roots, then the cut surfaces were rinsed with deionized water and blotted dry. Xylem exudates were collected for 1 h. After harvest, rice shoots were rinsed with deionized water, and roots were rinsed with ice-cold desorption solution as described by Xu et al (2007). For As analysis, seedling samples were oven dried at 65°C until a constant weight was reached. Xylem sap was directly digested with HNO_3.

As efflux from As-containing rice roots to As-free solution

Uniform 1-week-old rice seedlings were transferred from agar plates to 0.5 liter vessels, one plant per vessel. Each line was replicated in four vessels. Two weeks after transplanting, plants were treated with 5 or 40 μM As(III) for 24 h. After the treatments, rice roots were rinsed briefly with deionized water, followed by desorption solution for 10 min. Plants were then placed into fresh As-free nutrient solution. Aliquots of 0.5 ml of nutrient solution were removed from each vessel at 2, 4, 6, 8, 10 and 24 h, and solutions were diluted with 0.08 N HNO_3 containing 10 ppb Ge, and As concentrations were determined. After 24 h efflux, plant roots and shoots were harvested for As determination.

As(III) efflux from rice roots during As(V) treatment

Uniform 1-week-old rice seedlings were transferred from agar plates to 0.5 liter vessels, one plant per vessel. Each line was replicated in four vessels. Two weeks after transplanting, plants were treated with 10 μM As(V) and without P as described by Xu et al (2007). At 2, 4, 6, 8, 10 and 24 h after As(V) treatments, aliquots of 450 μl of nutrient solution were taken from each vessel and mixed with 50 μl of 2 mM Na_2-EDTA (pH 6.0). Solutions were kept on ice, and filtered through a 0.45 μm filter. As species in the solutions were analyzed within 24 h after collection.

As accumulation in grains

Four uniform seedlings of each line were selected for this experiment. Plants were grown in 1.5 liter vessels (one plant per vessel) containing 1.5 kg of an As-free mixture of soil and vermiculite (2 : 1, v/v). Four vessels (one for each line) were put in one tray randomly and re-arranged every 2 weeks. Each line was replicated in four trays. A water layer about 2–3 cm above the soil surface was maintained throughout the experiment.

From the flowering stage, plants were watered with As(III) solution (40 μM, 5 liters per tray). The irrigation of As(III) solution was carried out every 4 d until plants were harvested. At the mature stage, shoots from 6–8 cm above the soil were taken as stem samples, and flag leaves were taken as leaf samples. Stem and leaf samples were washed with distilled water, and then oven dried at 65°C until a constant weight was reached. Grains were air-dried, and then separated into husk and brown rice.

Quantification of transcripts by real-time PCR

After growing in hydroponic solution for 7 d, total RNA was isolated from 0.1 g (FW) of leaves using an RNeasy plant mini kit (Qiagen), and DNase treatment was performed with an RNase-free DNase set (Qiagen). RNA was converted into cDNA using the PrimeScript RT reagent kit (TaKaRa). cDNA was diluted by 10-fold and used for real-time PCR analysis by using SYBR Premix Ex TaqII (TaKaRa). Transcript accumulation was quantified as a value relative to elongation factor 1α (EF1α, internal standard) transcript accumulation. RNA samples were independently prepared from three individual plants of each line. The primer sequences used for *ScACR3* were: 5′-CAGATTG CTGGAGGAGACAATG-3′ and 5′-GAAGTATTCAGGTGGTCA TGAG-3′. The primer sequences used for EF1α (internal standard) were: 5′-AGGTCAAGTCGGTTGAGATG-3′ and 5′-AGGGT CATCCTTGGAGTTG-3′.

Analysis of total As and As species

For total As analysis, oven-dried samples or air-dried husk and brown rice were digested with concentrated HNO_3 at 120°C. After complete digestion, the sample was dissolved and diluted. Determination of the As concentration was performed by using inductively coupled plasma mass spectroscopy (ICP-MS) with ^{72}Ge as an internal standard, and the mass 75 was monitored as the As signal (Kamiya et al. 2009). Filtered nutrient solutions were diluted and then directly subjected to As concentration determination by ICP-MS.

The As species from brown rice and nutrient solution were determined by HPLC/ICP-MS as described by Arao et al (2011) and Kuramata et al. (2011). The HPLC with a Super IC-Anion HS column (5 μm, 4.6 mm i.d. × 150 100 mm) was used to separate As species. The isocratic mobile phase system consists of 10 mM ammonium acetate.

Supplementary data

Supplementary data are available at PCP online.

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