Arsenic Shoot-Grain Relationships in Field Grown Rice Cultivars

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Arsenic (As) accumulation in rice grains is a risk to human health. The mechanism of transfer of As from the shoot into the grain during grain filling is unknown at present. In this study As speciation in the shoot and grains at maturity were examined, and the relationships between phosphorus (P) and As, and silicon (Si) and As were established in a wide range of cultivars grown in As contaminated field trials in Bangladesh and China. No correlations were observed between shoot and grain speciation, with the inorganic form comprising 93.0–97.0% of As in the shoot and 63.0–83.7% in the grains. The percentage of dimethylarsinic acid (DMA) was between 1.4 and 6.6% in the shoot and 14.6 and 37.0% in the grains; however, the concentrations were comparable, ranging from 0.07 to 0.26 mg kg⁻¹ in the shoots and 0.03 to 0.25 mg kg⁻¹ in the grains. A positive correlation was observed between shoot As and shoot Si, however, no correlation was observed between shoot Si and grain As. A significant negative correlation was observed between shoot P and grain As concentrations. These results suggest that the translocation of As into the grain from the shoots is potentially using P rather than Si transport mechanisms. The findings also indicate that inorganic As and DMA translocation to the grain differ considerably.

Introduction

Rice (*Oryza sativa* L.) grain is problematic with respect to arsenic (As) accumulation; its anaerobic growth conditions

mean that As is more available from the soil (1), leading to rice being much more efficient at assimilating As into the shoot and grain compared with other cereal crops (2). In rice grain As is dominated by inorganic As (As_i) and dimethylarsinic acid (DMA) (3). The concentration of As, in particular As_i, in rice grains has been identified as a risk to human health for countries where rice is a dietary staple (4–9). Also, rice straw containing high concentrations of As is widely used as cattle feed, and is therefore another means of As entry into the food chain (10–12).

Little is currently known about mechanisms of As translocation to the grain. As_i is readily assimilated by rice roots (13, 14), yet is strongly retained in the roots and less readily translocated to above ground tissues (15). As_i enters the roots either as arsenite taken up by rice roots through the highly efficient Si transport pathway (16), or as arsenate via arsenate/phosphate cotransporters (13). Assimilated arsenate is then reduced to arsenite, possibly through arsenate reductases (17). Arsenite is strongly coordinated with sulphhydryl (-SH) containing moieties such as phytochelatins (PCs) (18), which are induced upon As exposure (19, 20); As-PC complexes are thought to be stored in vacuoles (21, 22). Conversely, DMA is poorly taken up by roots (10, 15, 23) yet can reach high concentration in rice grain (3). The source of DMA in the grain is still an uncertainty, either from soil via root uptake or from in planta biomethylation. However, efficient above ground translocation of DMA (15) may explain its preferential accumulation in the grain; DMA may be efficiently internally transported due to its poor -SH coordination, unlike for inorganic arsenite and monomethylarsonous acid (MMA(III)) (18).

Grain As must be derived from either direct xylem transport from roots or remobilization of shoot As pools through phloem during grain filling. Direct evidence for the involvement of these pathways is technically difficult to ascertain for different As species, but has been better studied using analogues of As. Arsenite and arsenate behave as analogues of silicic acid and phosphate (Pi), respectively, in terms of plant transport. Si is not readily accumulated in the grain but rather deposited in the husk, which is primarily xylem fed (24). Phosphate is taken up by plant roots via the xylem and then translocated to other parts of the plant, depending on metabolic needs (25).

Previous studies looking at relationships between soil As and grain As, and shoot As and grain As, have observed that grain As saturates when there are high concentrations of either soil or shoot As (2, 26, 27). In order to understand plant transport of As to grain and the human risk posed by that grain, characterizing As speciation as well as total grain As is crucial. This study ascertains the role of genetic and environmental variation in grain As and how this interacts with As speciation, as well as explores the relationships of plant As with elements that are analogues of As species, Pi, and silicic acid.

Materials and Methods

Total grain As concentrations and grain As speciation of these experimental materials have previously been published in refs 28 and 29, whereas the shoot composition data and grain P analysis have not. Relevant As characterization in the soils of these sites can also be found in refs 28 and 29. The Bangladeshi sites are contaminated through irrigation of As-contaminated tube well water during the dry season. The Chenzhou site in China is contaminated through base metal mining, whereas the Qiyang site is naturally elevated in As (29).

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Field Sites. The field trials at the four sites are described in refs 28 and 29. Briefly, there were two field sites each in Bangladesh (Faridpur and Sonargaon) and China (Chenzhou and Qiyang), with experiments involving 76 rice cultivars at the Bangladeshi field sites and 84 rice cultivars at the Chinese field sites. Cultivars consisted of parental lines used to generate rice genetic mapping populations (referred to as parents), local landraces, and locally improved cultivars; between the four field sites there were 16 common cultivars (all parents of mapping populations). The cultivars were grown in a random setup in triplicate, in a split-block design as described in refs 28 and 29. Shoots were sampled during the vegetative growth stage (48 days after sowing) and at grain harvest.

Analysis of Total As and P. Trace element grade reagents were used for all digests, and for quality control replicates of certified reference material (CRM) (beech leaf [BCR-100]) and rice flour [NIST 1568a]) were used; spikes and blanks were also included. Shoot samples were cut into 0.5 cm long pieces and 0.2 g weighed into 50 mL polyethylene centrifuge tubes. Grain samples were dehulled and 0.2 g weighed into 50 mL polyethylene centrifuge tubes. Samples were digested with concentrated HNO_3 and H_2O_2 as described in ref 30. Total As and P analysis was performed by ICP-MS (Agilent Technologies 7500). As and P standards with the appropriate ranges were made from 1000 mg L^{-1} and 10 000 mg L^{-1} ICP-MS grade stock solutions respectively. All samples and standards contained 10 $\mu\text{g L}^{-1}$ indium for the Bangladeshi samples, or 10 $\mu\text{g L}^{-1}$ rhodium for the Chinese samples, as the internal standard. Analysis was performed as described in ref 30.

Determination of As Speciation. As speciation in the shoots at grain harvest was performed on a subset of rice cultivars from the Faridpur field site, and for grain speciation an extra set of grain samples were analyzed from the Sonargaon field site. This subset consisted of 8 local landrace cultivars, 6 BRRI cultivars, and 11 cultivars used as parents of mapping populations. The local landraces were divided into red and brown bran rice. Speciation analysis followed protocols previously described in ref 28. Shoot samples were oven-dried, powdered, and 0.1 g weighed into a 50 mL polyethylene centrifuge tube. Grain samples were powdered and 0.2 g weighed into 50 mL polyethylene centrifuge tube. Samples and CRM (rice flour [NIST 1568a]) were extracted with 1% HNO_3 as described in ref 30. DMA, MMA, and As_i were determined by anion exchange HPLC-ICP-MS. Details of the protocol for As speciation analysis is given in ref 30.

Shoot Si Analysis. The same subset of cultivars for which shoot As speciation at Faridpur was determined were used for Si analysis. Samples were prepared for Si analysis as described in ref 31. Dried powdered shoot samples or CRM (Poplar [NCS DC 73350]) (0.02 g) were weighed into polyethylene centrifuge tubes, and 0.6 mL of H_2O_2 and 1.5 mL of 50% NaOH were added. The samples were heated for 1 h at 90 °C in a water bath, vortexed, and then heated for 1 h in an autoclave (123 °C, 0.15 MPa). Samples were diluted by mass to 20 mL with deionized water and diluted a further 1:5 with deionized water. The standard ammonium molybdate method for Si analysis by flow injection spectrophotometer was adapted to neutralize the 0.29 M NaOH sample matrix with 0.29 M HCl, and the ammonium molybdate was omitted from the reaction. The reaction products were then measured at a wavelength of 410 nm.

Statistical Analysis. Analysis of variance (ANOVA), general linear models, and correlations were performed using Minitab 15 Statistical Software.

Results

CRM recoveries and speciation recoveries are presented in the Supporting Information (SI). All shoot concentrations

for As, P, and Si refer to the shoots at grain harvest unless stated otherwise.

Shoot and Grain As at the Faridpur Field Site. Total grain As in the cultivars grown at the Faridpur field sites has previously been reported in ref 28. The total As concentrations were determined in the shoots of all the cultivars at harvest grown at the Faridpur field site. Among the 76 cultivars, the As concentrations in the shoots ranged from 1.9 to 11.3 mg kg^{-1} with an average of 4.8 mg kg^{-1} . There was a significant genotype effect on shoot As ($P < 0.001$, $F = 3.91$).

When taken as a whole, there was no correlation between As in the shoots and As in the grains at the Faridpur field site. However, when the data were grouped into rice cultivars with red bran and cultivars with brown bran, a positive quadratic correlation ($P < 0.001$, $r = 0.579$) was observed between As in the shoots and grains with cultivars with brown bran; no correlation was observed for the cultivars with red bran (Figure 1A). There was, however, a significant difference across all cultivars ($P < 0.001$, $F = 4.19$) in the ratio of grain to shoot As concentration.

During the early growth stage at the Faridpur field site there was a genotypic difference in shoot As concentration ($P < 0.001$, $F = 2.35$). Concentrations in the early shoot ranged from 5.5 to 11.8 mg kg^{-1} with an average concentration of 8.3 mg kg^{-1} . There was no correlation between shoot As in the early shoots and the shoots at harvest (SI Figure S1).

Shoot and Grain P at the Faridpur Field Site. The shoot P concentration of the cultivars at harvest ranged from 212 to 1306 mg kg^{-1} for the 76 cultivars, with an average concentration of 477 mg kg^{-1} . There was a significant genotype effect for shoot P ($P < 0.001$, $F = 3.95$). Grain P ranged from 1836 to 3338 mg kg^{-1} , with an average concentration of 2538 mg kg^{-1} for the cultivars. There was a significant genotype effect in grain P ($P < 0.001$, $F = 3.3$). Within the grain there was less than a 2-fold range in P concentration, which is a smaller range compared to the shoots. For both shoot and grain P there was a significant subgroup effect ($P < 0.001$, $F = 10.48$ for the shoots and $P = 0.004$, $F = 4.78$ for the grains); in both cases the landraces with red bran have the lowest average P concentration. It is worth noting that the landraces with red bran also have the highest average grain As (28). A positive correlation was observed between shoot and grain P for all cultivars ($P < 0.001$, $r = 0.553$) (Figure 1C). Negative correlations were observed between shoot P and shoot As ($P < 0.014$, $r = -0.279$) (Figure 1D), grain P and grain As ($P < 0.001$, $r = -0.494$) (Figure 1E), and shoot P and grain As ($P < 0.001$, $r = -0.708$) (Figure 1B).

During the early growth stage there was a small genotypic difference in shoot P concentration ($P = 0.01$, $F = 1.56$). Concentration in the early shoots ranged from 1010 to 1721 mg kg^{-1} with an average concentration of 1372 mg kg^{-1} . There was a significant positive correlation ($P = 0.002$, $r = 0.351$) between shoot P in the early shoots and the shoots at harvest for the cultivars (Figure 1F).

A hyperbolic relationship was observed for the molar ratio of P/As in the shoots and grain As ($P < 0.001$, $r = 0.706$); cultivars which had a smaller P/As molar ratio in the shoots had a higher concentration of As in the grain (SI Figure S2).

Shoot Si. Si analysis was performed on the same subset of cultivars at the Faridpur site in which the As was speciated ($n = 25$). The concentration of Si in the shoots at harvest ranged from 2.8 to 6.1% with an average concentration of 4.4%. There was a significant genotype effect for Si concentration in the shoots ($P < 0.001$, $F = 4.86$). A positive correlation was observed between shoot Si concentration and shoot As ($P = 0.003$, $r = 0.609$) for the cultivars analyzed (Figure 2); there was no correlation between shoot Si and grain As.

Shoot to Grain Transfer at Four Field Sites. Across the four field sites there were a total of 16 cultivars in common.

FIGURE 1. Shoot and grain As and P concentrations in the cultivars at the Faridpur field site. (A) Correlation between shoot As at harvest and grain As (trend line is only for cultivars with brown bran); (B) Correlation between shoot P at harvest and grain P; (C) Correlation between grain As and grain P; (D) Correlation between shoot As at harvest and shoot P at harvest; (E) Correlation between grain As and shoot P at harvest; (F) Correlation between shoot P during vegetative growth stage and shoot P at harvest. Triangle symbols are parent cultivars, circles are BRRI cultivars and squares are landraces. Open symbols are cultivars with brown bran and filled symbols are cultivars with red bran.

The mean and standard deviation of the grain/shoot ratio of As concentrations in the cultivars at the field sites were 0.150 ± 0.042 , 0.069 ± 0.022 , 0.049 ± 0.011 , and 0.044 ± 0.018 for the Sonargaon, Faridpur, Qiyang, and Chenzhou sites, respectively, where shoots were obtained at harvest. At the four sites there were significant site and genotype effects on shoot to grain ratio ($P < 0.001$, $F = 96.9$ and $P < 0.001$, $F = 3.3$, respectively) for these cultivars, however there was no significant site by genotype interaction ($P = 0.373$). The shoot and grain As concentrations of the cultivars grown at all four sites are presented in Figure 3; there is a positive quadratic correlation ($P < 0.001$, $r = 0.823$) between grain As and shoot As across the multiple field sites.

Speciation of Shoot As. At harvest for the Faridpur field site the percentage As_i in the shoots ranged from 92.9 to 97.3% and there was no genotype effect in percentage As_i ($P = 0.679$, $F = 0.83$). Percentage DMA ranged from 1.4 to 6.6%, and percentage MMA ranged from 0.3 to 4.1%; there was no genotypic variation in either ($P = 0.666$, $F = 0.84$, and $P = 0.198$, $F = 1.36$ respectively). There was a positive correlation between total shoot As concentration and percentage As_i ($P = 0.006$, $r = 0.538$) (Figure 4A), and a negative correlation between percentage DMA and shoot grain As ($P = 0.013$, $r = -0.490$) (Figure 4C). There was no correlation between DMA concentration (and percentage DMA) in the shoots and grain (SI Figure S3A and B). There was no correlation

FIGURE 2. Correlation between total shoot As and shoot percentage Si at harvest at the Faridpur field site. Triangle symbols are parent cultivars, circles are BRRI cultivars and squares are landraces. Open symbols are cultivars with brown bran and filled symbols are cultivars with red bran.

FIGURE 3. Concentration of As in grain and shoots at harvest for the 16 cultivars in common at all four field sites. Cultivars grown at Bangladeshi field sites are represented by circular symbols (open for the Sonargaon field site and filled for the Faridpur field site), cultivars grown at the Chinese field sites are represented by square symbols (open for the Qiyang field site and filled for the Chenzhou field site).

between As_i concentration (and percentage As_i) in the shoots and those in the grain (SI Figure S3C and D). There was no linear correlation between total As in the shoots and the grain using all data (SI Figure S3E), however, as with the whole data set, if the red bran rice cultivars are removed a positive quadratic correlation is observed ($P = 0.045$, $r = 0.553$).

Discussion

Over a wide range of environments total As in the grain has been correlated with total As in the shoots at harvest, for a single rice cultivar, and modeled by a hyperbolic relationship (27). Here, however, for a large number of cultivars grown within a single environment (the Faridpur site), there was a significant genotype effect in the grain to shoot As ratio, but poor correlation between the concentration of As within the grain and shoots at harvest (Figure 1A), although this was improved slightly when the red bran landraces were removed. This suggests that there is genetic regulation for both grain and shoot As but that the two are only closely related when there is a low concentration of shoot As. This is further emphasized by the analysis of the same cultivars across the

four field sites. For the 16 cultivars there was a significant genetic as well as environmental effect, with the environmental effect dominating. It is worth noting that the cultivars within the site with the lowest average grain As among the 16 cultivars (Sonargaon, Bangladesh) had the highest grain to shoot ratio, that is, the cultivars had a higher proportion of As in the grain compared to the As concentration in the shoots. The site with the second lowest grain As also had the second highest grain to shoot ratio. This trend is in agreement with previous studies where the grain to shoot ratio across multiple environments is observed as a hyperbolic relationship, with a decrease in translocation efficiency alongside increasing shoot As accumulation (2, 26, 27). In other words, the higher the shoot As concentration, the lower the proportion (but not the amount) of As that reaches the grain.

The As speciation in shoots at harvest was in agreement with Abedin et al (10), who found that in rice >95% of shoot As was in the As_i form, either arsenate or arsenite, at harvest. No genotypic variation in percentage shoot As_i or DMA was observed in the 25 genotypes at the Faridpur site. However, the significant correlation between percentage As_i and total shoot As (Figure 4A) suggests that the percentage of As_i in the shoot is mechanistically linked to the total concentration of As within the shoot. Interestingly, the correlations between total grain As and percentage grain As_i , and total shoot As and percentage shoot As_i , are in opposite directions. The opposite trend for total shoot and grain As versus percentage DMA in shoot and grain was also observed (Figure 4C and D). While As_i concentrations were ~12-fold higher in shoots compared to grain at the Faridpur field site, the concentrations of DMA in shoots and in grain were similar, ranging from 0.07 to 0.26 $mg\ kg^{-1}$ in shoots and from 0.03 to 0.25 $mg\ kg^{-1}$ in grain, and there was no correlation between grain and shoot DMA concentration (nor percentage DMA) in the subset of genotypes for which As speciation was determined (SI Figure S3). As the concentrations of DMA in both the shoots and the grain are in the same range, it may suggest that DMA is unloaded at a similar rate into these two compartments. This contrasts with As_i concentrations in the grain, which were lower than in the shoot, suggesting much less efficient grain unloading of As_i . The relatively efficient in planta transport of DMA compared to As_i is in agreement with the previously identified poor -SH coordination of DMA (18).

It is not known if the DMA is synthesized in planta or taken up directly from soil. So far, the pathway and enzymology for As methylation in plants has not been established (22), but in bacteria and fungi the Challenger Pathway is recognized as the process by which As is methylated (32). In the Challenger pathway arsenite is methylated by S-adenosylmethyltransferase to form MMA(V), which is reduced to MMA(III). The methylation process is then repeated in bacteria and fungi with MMA(III) being methylated to form DMA(V), which is reduced to DMA(III), which is further methylated to trimethylarsine oxide and finally reduced to the volatile gas trimethylarsine. Recently, Li et al. (34) showed that rice roots were able to reduce MMA(V) to MMA(III). However, evidence for As methylation in plants is rather limited; methylation activity has been demonstrated in crude cell extracts from *Agrostis capillaris* leaves (but not roots) (33). The authors found MMA; however, over longer assays DMA did accumulate. There are no reports that plants produce volatile species of As, the terminal products of the Challenger Pathway (22).

The positive correlation between shoot As and shoot Si (Figure 2) suggests a shared mechanism for the transport of As and Si, as silicic acid, into the shoots. This is in agreement with the recent identification of two silicic acid transporters which also transport arsenite (16). In a subsequent study using a mutant of one these transporters (Lsi1, an aquaporin)

FIGURE 4. Relationships between total As concentrations and percentage As_i and DMA at the Faridpur field site. (A) Correlation between total shoot As and percentage shoot As_i; (B) Correlation between total grain As and percentage grain As_i; (C) Correlation between total shoot As and percentage shoot DMA; (D) Correlation between total grain As and percentage grain DMA. Triangle symbols are parent cultivars, circles are BRRI cultivars and squares are landraces. Open symbols are cultivars with brown bran and filled symbols are cultivars with red bran.

it was demonstrated that it also mediates the uptake of undissociated methylated As species (35). It has also been demonstrated that the application of Si fertilization decreased the total As concentration in straw and grain by 78 and 16%, respectively, and specifically decreasing arsenite uptake (34). However, in the present study no correlation was observed between shoot Si and grain As. This may suggest that although the uptake of arsenite and methylated As into shoots is mediated by the Si uptake mechanisms, translocation of As from shoots to grain, especially the unloading of As into the grain, may involve other transporters. This would explain why Si fertilizer suppressed As accumulation in the shoots much more (78%) than in grain (16%) (34). Furthermore, it has been reported that Si accumulates in the husk (palea and lemma) but not inside the grain (24). Bogdan and Schenk (36) recently identified a negative correlation between shoot Si and shoot As, as well as a negative correlation between shoot Si and grain As, for a single rice cultivar from 68 different fields. These results suggest that environmental variation in available soil Si leads to variation in straw Si, and that this competes with the uptake of As, which is in agreement with the Si fertilization experiments (34). This negative correlation does not conflict with the positive one detected in the present study. All the cultivars were grown in the same field site at Faridpur, and were therefore exposed to similar soil Si environments. Thus the positive relationships observed between shoot As and Si are predominantly due to genetic differences between the cultivars, and could be explained by the existence of cultivars that have higher or lower efficiency

of taking up Si, and therefore As, through a common transport mechanism.

Previously, phosphate transporters have been identified as mechanisms of arsenate assimilation in rice, as the two ions are analogues (13, 14). Genes involved in rice phosphate transport have also been shown to be regulated by arsenate exposure (37). Evidence from mapping of quantitative trait loci (QTL) has identified a region on chromosome 8 of rice, that is responsible for increasing shoot P concentration and decreasing grain As concentration (38). Additionally, in a different rice mapping population, a region on chromosome 5 has been identified as increasing shoot P concentration and decreasing shoot As concentration (39). These relationships at the genetic level indicate that there is a relationship between P and As.

The strong negative correlation between shoot P and grain As (Figure 1B) may indicate that As utilizes the P transport mechanisms in rice for transport into the grain, that is, P is outcompeting As. Note that the Bangladeshi red rice cultivars are the highest in grain As accumulation and also the most P efficient—having the lowest shoot biomass P status—yet are the highest yielding cultivars at Bangladeshi sites investigated (28). Also worth noting is the difference between the concentration of P in the shoots during the vegetative stage and at harvest; the concentration of P in the cultivars during the vegetative stage had less than a 2-fold range, whereas at harvest the range was greater than 6-fold (Figure 1F). This would suggest that there is a large variation between the cultivars in P remobilisation from the shoots to the grain.

In situ synchrotron X-ray absorption spectrometry and extraction/HPLC-ICP-MS analysis demonstrate that As speciation in rice grain is dominated by free arsenite (9, 24), which is not a phosphate analogue, and by DMA. As speciation has not been determined robustly in rice shoots because extraction followed by HPLC-ICP-MS allows uncoordinated arsenate and arsenite to interconvert (10). It is well-known that arsenite is readily -SH coordinated by PCs, and is likely to be stored in vacuoles, as determined by both extraction/HPLC-ICP-MS of intact As-PC complexes (40) and by energy dispersive X-ray microanalyses (EDXA) in a range of plants (41). Free arsenate and arsenite are in equilibrium that is driven toward arsenite by the reducing conditions of plant cells, maintained by arsenate reductases or other nonenzymatic pathways (17, 22, 42). However, even if arsenite predominates, arsenate that is formed may be readily translocated from shoot to grain during P mobilization. If indeed arsenate is the main form of As transported to the grain, then it must be reduced to arsenite in the grain tissue to account for the fact that arsenite dominates grain inorganic species. These hypotheses relating shoot P to grain As need to be tested further.

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Supporting Information Available

CRM recoveries. Information on the relationship between shoot arsenic concentration during vegetative growth and at grain harvest, relationship between the molar ratio P/As in shoots and grain As, and correlation between total shoot arsenic and arsenic species. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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