The concentration of arsenic (As) in rice grains has been identified as a risk to human health. The high proportion of inorganic species of As (As\textsubscript{i}) is of particular concern as it is a nonthreshold, class 1 human carcinogen. To be able to breed rice with low grain As, an understanding of genetic variation and the effect of different environments on genetic variation is needed. In this study, 13 cultivars grown at two field sites each in Bangladesh, India, and China are evaluated for grain As. There was a significant site, genotype, and site by genotype interaction for percentage As\textsubscript{i} in rice grains. Correlations were observed only between sites in Bangladesh and India, not between countries or within the Chinese sites. For seven cultivars the As\textsubscript{i} was speciated which revealed significant effects of site, genotype, and site by genotype interaction for percentage As\textsubscript{i}. Breeding low grain As cultivars that will have consistently low grain As and low As\textsubscript{i} over multiple environments using traditional breeding approaches may be difficult, although CT9993-5-10-1-M, Lemont, Azucena, and Te-qing in general had low grain As across the field sites.

**Introduction**

The major arsenic (As) species in rice grain are inorganic (As\textsubscript{i}), arsenite and arsenate, and dimethylarsinic acid (DMA). The level of As\textsubscript{i} in rice grains has been identified as a risk to human health wherever rice is a dietary staple (2–6), which is ca. 50% of the world’s population (5, 7, 8). As\textsubscript{i} is a risk to human health because it is a nonthreshold, class 1 human carcinogen (9). It has also been demonstrated that the bioavailability of rice grain As\textsubscript{i} in the mammalian gut is high (10), and concerns regarding human intake of As, from rice have recently been highlighted (11).

Having characterized the extent of As consumption associated with As in rice (7), focus must now be turned to ways of mitigating the amount of As entering the food-chain via rice (12). There are two major ways to do this. One would be to improve paddy management practices to limit As entering fields, removing As already present, or altering the bioavailability/accumulation of As present in soil (13–16). Such changes to farming practice may meet cultural resistance, have financial consequences and could potentially have major implications for yield. The second approach, breeding low As rice, is perhaps the simplest and most cost-effective as seed can be distributed centrally, ensuring widespread uptake, and breeding would ensure that low As cultivars are adapted to local climate, edaphic factors, and flood management, etc. (17).

Previously a study looking at genetic variation in rice grain As concentration in Bangladeshi landraces, improved cultivars, and a set of diverse cultivars, identified a 4.6-fold range in grain As concentration for the cultivars grown at a field site with high soil As, and a 4.0-fold range in grain As concentration for the cultivars grown at a field site with lower soil As (12). For these two sites, a negative relationship between total As and percentage As\textsubscript{i} was also observed (12), suggesting that the total As concentration drives the percentage between As\textsubscript{i} and organic As.

Here, the way in which the genetic diversity in grain As concentrations and speciation of diverse rice cultivars interacted with the environment was evaluated, by growing a set of cultivars at six sites across three countries (Bangladesh, India, and China).

**Materials and Methods**

**Bangladeshi Field Sites.** The field trial for the two Bangladeshi field sites at Faridpur and Sonargaon is described in ref 12. Briefly, a field trial was conducted, with rice germinated in December 2007 and harvested in May 2008, at two field locations in Bangladesh, in Faridpur (latitude 23° 35.105’ and longitude 90° 36.37’). Both sites had a history of dry season (boro) irrigation with As contaminated water extracted from tubewells. The Faridpur field site had an average soil As content of 29.6 ± 7.2 mg kg\textsuperscript{-1}, and a tubewell water As concentration of 198 ± 31 μg L\textsuperscript{-1}. The Sonargaon field site had an average soil As content of 10.3 ± 2.2 mg kg\textsuperscript{-1}, and a tubewell water As content of 331 ± 13 μg L\textsuperscript{-1}. The field site in Faridpur was under continually flooded conditions, with irrigation every three days. The field site in Sonargaon was irrigated every two days, which resulted in alternative wet–dry cycles. The field sites were fertilized with 70 kg N/ha (split over three equal applications), 20 kg P/ha, 50 kg K/ha, 15 kg...
S/ha, and 2 kg Zn/ha. The field sites were also sprayed with a single application of Furadan.

**Indian Field Sites.** A field trial was conducted, with rice germinated in December 2007 and harvested in May 2008, at two field locations in West Bengal, India. The first field site was in Nonaghata (latitude 23°42′ and longitude 88°44′) and the second was in De Ganga (latitude 22°87′ and longitude 88°76′). Both sites had a history of dry season (boro) irrigation with As contaminated water extracted from tubewells, which has been identified as a major source of As contamination of food substances in India (18–20). The tubewell in the Nonaghata field was installed in 1997, and had an average soil As content (measured by taking 24 samples systematically across the trial area) of 6.3 ± 1.3 mg kg⁻¹, and a tubewell water As concentration of 14.9 ± 4.1 µg L⁻¹. At the De Ganga field site the tubewell was installed in 1994, and had an average soil As content (measured by taking 24 samples systematically across the trial area) of 17.9 ± 4.0 mg kg⁻¹, and a tubewell water As concentration of 131 ± 8.8 µg L⁻¹. Both field sites were maintained under continually flooded conditions, with irrigation every day. The field sites were fertilized with 70 kg N/ha, 35 kg P/ha, and 35 kg K/ha (split over two applications).

**Chinese Field Sites.** A field trial was conducted, with rice germinated in April 2008 and harvested in August 2008, at two field locations in China. The first field site was located in Deng Jia-tang, in the southwest of Chenzhou city, Hunan province (latitude 25°35′ and longitude 113°00′). This region of China has a history of As contamination, from mining and mining associated industries (21). The region where this field site was has a number of metal exploitation factories, especially smelting factories. This field site had an average soil As concentration (measured by taking seven samples systematically across the trial area) of 65.6 ± 2.5 mg kg⁻¹, the As concentration in the water inlet was 602 ± 314 µg L⁻¹. The second field site was in Qiyang city, Hunan province (latitude 26°45′, and longitude 111°52′). At the Qiyang site the contamination was derived from geologically elevated levels of As, and the average soil As content (measured by taking six samples systematically across the trial area) was 64.6 ± 4.7 mg kg⁻¹, the As concentration in the water inlet was 218 ± 86 µg L⁻¹. At both sites fertilizer was applied three times during the field trial. On the day of transplanting the seedlings 200 kg/ha of compound fertilizer (N, P, K) was applied. Eight days after transplanting, 60 kg/ha of urea was applied and 30 days after transplanting, 100 kg/ha compound fertilizer was applied.

**Plant Material.** A set of 76 cultivars was grown at the Bangladeshi field sites (12) consisting of Bangladesh Rice Research Institute (BRRRI) improved cultivars, diverse cultivars previously used to generate permanent rice mapping populations, and local landraces (12).

At the two Indian field sites a total of 89 cultivars were grown (for a full list of genotypes see Supporting Information (SI) Table S1), consisting of Indian improved cultivars, Indian landraces, and diverse cultivars previously used to generate permanent rice mapping populations.

At the two Chinese field sites a total of 84 cultivars were grown (for a full list of genotypes see SI Table S2), consisting of Chinese improved cultivars and diverse cultivars previously used to generate permanent rice mapping populations.

Plants were sown in a randomized complete block design with three replicates. In each replicate each genotype was planted in a single row of 2 m with 10 hills, each hill (one seedling) 20 cm apart, and each row 20 cm apart. To separate the test genotypes, two hills of a check cultivar were planted at each end of the 10-hill test rows. Between every row of test genotypes one row of the check cultivar was planted. From the four field sites fully described in this study and two field sites previously described in ref 12, a total of 13 cultivars were common at all six field sites.

**Analysis of Total As.** Trace element grade reagents were used for all digests, and for quality control replicates of certified reference material (CRM) (rice flour (NIST 1568a)) were used; spikes and blanks were also included. Rice grain samples were dehusked and 0.2 g weighed into 50 mL polyethylene centrifuge tubes. Samples were digested with concentrated HNO₃ and H₂O₂, as described in ref 22. Total As analysis was performed by ICP-MS (Agilent Technologies 7500). As standards with the appropriate ranges were made from 1000 µg L⁻¹ ICP-MS grade As stock solution. All samples and standards contained 10 µg L⁻¹ indium for the Indian and Bangladeshi samples or 10 µg L⁻¹ rhodium for the Chinese samples as the internal standard. Analysis was performed as described in ref 22.

**Determination of As Speciation.** For grain speciation a subset of the test cultivars was selected, consisting of cultivars used as parents of mapping populations. The grain samples were dehusked, powderized, and 0.2 g weighed into a 50 mL polyethylene centrifuge tube. Samples were extracted with 1% HNO₃ for the Indian and Chinese samples, and with 3% HNO₃ for the Bangladeshi samples. Certified Reference Material (CRM) (rice flour (NIST 1568a)) was used for all digests, and for quality control replicates of CRM. DMA and As were all landrace Indian cultivars: AS203/2, Jubali Ahu, GP 35, Koimurali, and Bon Ahu. The cultivars with the lowest grain As at the Nonaghata field site had a mix of backgrounds. Three were parents of mapping populations: Dawn, Bala, and Azucena; one was an...
Indian improved cultivar, CN 1646-1; and the other was Satya, an Indian landrace. The cultivars with the highest grain As were Nipponbare and CO 39, which are both parents of mapping populations, Nayanmoni and Balonbichi, which are Indian landraces, and Badami which is an improved Indian cultivar.

**Chinese Field Sites.** At the Chenzhou field site, 80 of the 84 cultivars established and produced grain. The total grain As varied from 0.27 to 0.74 mg kg\(^{-1}\), with a mean of 0.41 mg kg\(^{-1}\). At this site there was a significant genotype effect \((P < 0.001, F = 2.5)\) for total grain As. A total of 77 of the 84 cultivars established, flowered, and produced grain at the Qiyang site. The grain As ranged from 0.37 to 0.85 mg kg\(^{-1}\), with a mean of 0.58 mg kg\(^{-1}\). There was a significant genotype effect for grain As at this site \((P < 0.001, F = 10.4)\). There was no significant correlation \((P = 0.993, r = 0.001)\) for total grain As in the 76 cultivars in common between the two Chinese field sites (Figure 1).

At the Chenzhou site, the five cultivars with the lowest grain As were four parents of mapping populations: Koshihikari, Millyang 23, Akihikari, and Bala, and the other was a Chinese cultivar, Tyou618. The cultivars with the highest grain As were three parents of mapping populations: IR 62266-42-6-2, IRAT 109, and Azucena, as well as two Chinese cultivars: Xiangzaoxian 143 and Jinyou 706. The five lowest grain As cultivars at the Qiyang site were three parents of mapping populations: Lemont, IRAT 109, and Azucena, and two Chinese cultivars: Zhunliangyou 527 and Fengyuanyou 326. The five cultivars with the highest total grain As at the Qiyang site were three Chinese cultivars: Weiyou 46, Fengyuanyou 299, and Longping 001, and two parents of mapping populations, Dawn and IR 62266-42-6-2.

**Genetic Variation of Genotypes for Total Grain As for Cultivars Common Across All 6 Sites.** A total of 13 genotypes (all diverse cultivars used to generate permanent mapping populations (full list of As concentrations for the genotypes is presented in SI Table S3)) were present at the two Indian and the two Chinese sites described above, as well as the two Bangladeshi field sites (12). The field sites which yielded the lowest to highest average grain As for the 13 cultivars were (1) Sonargaon (Bangladesh), (2) Nonagatha (India), (3) De Ganga (India), (4) Faridpur (Bangladesh), (5) Chenzhou (China), and (6) Qiyang (China) (SI Figure S1). As well as the 13 genotypes present at all the sites, an additional nine genotypes were present at four or more field sites (SI Table S3).

Analysis of variance of the 13 genotypes at the six field sites indicated that the site \((P < 0.001, F = 153)\) as well as genotype \((P < 0.001, F = 9.7)\) had a significant effect on grain As. There was also a significant site by genotype interaction \((P < 0.001, F = 5.1)\) for total grain As.

The only significant correlations between the six sites for the 13 genotypes were between the two Bangladeshi sites (Faridpur and Sonargaon) \((P = 0.005, r = 0.724)\), and between the two Indian sites (Nonagatha and De Ganga) \((P = 0.012, r = 0.674)\). No other significant correlations were detected.

**Grain As Speciation.** There was a significant negative correlation between total grain As and percentage As for cultivars grown at the Bangladesh \((P < 0.001, r = 0.483)\) and Indian \((P = 0.017, r = -0.539)\) field sites, but no correlation for the cultivars grown in China \((P = 0.192, r = 0.304)\). For the cultivars grown in Bangladesh and India there was a corresponding positive correlation between DMA and total grain As \((P < 0.001, r = 0.572\) and \(P = 0.015, r = 0.547\) respectively). There was no correlation between percentage DMA and total grain As for the cultivars grown in China. However, when the Chinese data were split into individual field sites there were significant correlations between percentage As and total grain As, and percentage DMA and total grain As. At the Chenzhou site there was a significant negative correlation between percentage As and total grain As \((P = 0.02, r = -0.714)\), and a corresponding positive correlation between percentage DMA and total grain As \((P = 0.018, r = 0.723)\). At the Qiyang site there was a positive correlation between percentage As in the grain and total grain As \((P = 0.007, r = 0.789)\), and a corresponding negative correlation between percentage DMA and total grain As \((P = 0.006, r = -0.799)\) (Figure 2). Percentage DMA and As in the mapping parent cultivars at the six field sites is presented in Figure 3. Out of the 13 cultivars present at all field sites, seven had spectiation data for all sites. The average percentage As and percentage DMA for these cultivars across the six sites is presented in SI Figure S2. The two Bangladeshi and two Indian sites varied little in the average percentage of DMA and As, whereas the two Chinese sites have higher percentages of As than the site in Qiyang.

Across the six sites there were both a significant site and genotype effect in percentage As \((P < 0.001, F = 32.5, \text{ and } P < 0.001, F = 6.6\) respectively) and percentage DMA \((P < 0.001, F = 45.5, \text{ and } P < 0.001, F = 6.8\) respectively), as well as a significant site by genotype interaction for both.
percentage As, \( P < 0.001, F = 6.0 \) and percentage DMA, \( P < 0.001, F = 6.0 \).

**Discussion**

It is now becoming apparent that rice grain is a major source of As for those on rice diets, particularly subsistence rice diets (7). An understanding of the genetic variation in grain As concentration is essential in breeding new low As cultivars. In addition, knowledge of genetic variation for grain As across multiple environments needs to be developed.

In India the irrigation of rice fields with As contaminated water (from tubewells) has led to increased As concentrations in food substances, including rice (18–20). At the two Indian field sites there was a wide range in grain As for the cultivars. At the De Ganga field site there was a 7.5-fold range in grain As and at the Nonaghata site a 14.5-fold range in grain As. These ranges are much larger than previously identified in the two Bangladeshi field sites, where only a 4 and 4.5-fold change were observed (12). As with the two Bangladeshi field sites, there was a positive correlation between the Indian field sites, suggesting that the observed genetic variation is stable across these two environments.

The Hunan region of China (the region in which the Chenzhou field site was located) has been identified as a...
region producing rice with high concentrations of grain As (21). At the two Chinese field sites the range in grain As was narrower at the Chenzhou (2.7-fold) and Qiyang sites (2.3-fold) than the two Indian and two Bangladeshi field sites (12). Unlike the Bangladeshi and Indian field sites, there was no correlation between the two Chinese field sites (Figure 1); there were also a number of cultivars at direct opposites in terms of grain As concentrations between the two sites. For example, Azucena and IRAT 109 were two of the five highest grain As cultivars at the Chenzhou site, but at the Qiyang site they were among the lowest grain As cultivars. However, some cultivars were consistent at both sites, for example the cultivar IR 626-42-6-2 was among the five highest cultivars for grain As at both Chinese field sites. These variations in total grain As at the two Chinese sites for the same cultivars are not fully understood at present, a contribution to this variation may be that the two sources of As contamination are quite different; at the Chenzhou site the contamination is from mining and smelting area runoff, whereas at the Qiyang site the As is naturally geologically elevated. A wide range of factors are known to affect As uptake and translocation in plants including arsenic speciation, soil redox, water and fertilizer management, presence of iron, phosphate and silicon, and iron plaque formation in the aerated root zone (23, 24). At present it is unknown if there is any variation in the As species in the soil and water at these sites (if the As is in a methylated and/or reduced form), and how these species affect over the rice growing period.

For the 13 cultivars grown at all six field sites, correlations were detected only between the two Bangladeshi field sites and the two Indian field sites. This lack of correlation is also supported by 2-way ANOVA analysis which indicated that the largest factor in grain As was due to the field site, then genotype, and a significant site by genotype interaction was detected, suggesting that different genotypes behave different at different field sites. As the source of As contamination (and geographical location) for the two Bangladeshi and two Indian fields are similar, the absence of correlation between the Indian field sites and the Bangladeshi field sites is quite surprising. This suggests that variation in grain As may be altered not only by the source of As but also by field management practices and potentially other soil properties (e.g., phosphate and silicate concentrations). As well as the 13 genotypes present at all the sites an additional nine genotypes were present at four or more field sites (SI Table S3). Ranking these genotypes from lowest to highest grain As concentration, a number of potentially promising genotypes can be identified for use as breeding material. Four cultivars, CT9993-5-10-1-M, Lemont, Azucena, and Te-qing, have the lowest average ranking of the 22 genotypes. These genotypes in general performed well (i.e., had low grain As) at most sites, however in one or two sites they did not perform as well (SI Table S3). For example, Lemont was present at all field sites and was ranked the lowest grain As cultivar at two sites, second lowest cultivar at two sites, the 8th and 15th lowest at the other sites; these variations are reflected in the genotype by environment interaction. At present these four cultivars represent the best material to initiate breeding programs due to their average performance across all the field sites. Genotype and site by genotype interactions in speciation were also observed for the genotypes present at all six field sites (Figure 3). Genetic variation in speciation has previously been observed in a soil-bead system (25) and in field rice grown in Bangladesh (12), however, by using the same genotypes across multiple field sites a detailed study on how speciation varies within genotypes across multiple environments is possible. For example, the total grain As was very similar at the two Indian field sites (0.521 mg kg\textsuperscript{-1} at the De Ganga site and 0.522 mg kg\textsuperscript{-1} at the Nonaghata site) for the cultivar Nipponbare, but the percentage As\textsubscript{i} varied; at the Nonaghata field site the percentage As\textsubscript{i} was 50.6% compared to 76.8% at the De Ganga field site. For the Indian field sites, and for the Qiyang site, there was a negative correlation between total As and percentage As\textsubscript{i}, which is in alignment with the Bangladeshi field sites (12), however at the Chenzhou field site there was a positive correlation between total As and percentage As\textsubscript{i}. This suggests that there are strong environmental factors that affect the percentage of As\textsubscript{i}. It must be noted that the Chenzhou site had the lowest percentage As\textsubscript{i} compared to all other sites (average of 61.0% (SI Figure S2)); this is in contradiction to a previous study with data from the Hunan province (the region in which the Chenzhou field site is), where much higher percentages of As\textsubscript{i} were observed (an average of 84.4%) (26). These discrepancies between experiments only highlight the environmental impact on As\textsubscript{i}. Breeding grain with low total As which contains a lower proportion of As, is essential, as in Bangladesh, West Bengal, and Vietnam, rice has been identified as a major source of As\textsubscript{i}, (2–6). However, these results indicate that there is a large variation imposed on the percentage of each As species due to the environment in which the plants are grown. Therefore, breeding for low As traits must consider local conditions, and bred cultivars must be widely tested for given agronomic conditions, to identify the conditions under which low As traits are exhibited.

When assessing the risk of As entering the food chain via rice As consumption is the key parameter. For China the standard for As\textsubscript{i} is 150 µg kg\textsuperscript{-1} in rice (11), at the Chenzhou and Qiyang field sites, for the seven cultivars which were speciated at all sites, all had a concentration 150 µg kg\textsuperscript{-1} As\textsubscript{i} or greater, with the highest concentration of As\textsubscript{i} three times above the standard (SI Table S4). In India and Bangladesh there is no standard at present but the WHO standard is 2 µg kg\textsuperscript{-1} day\textsuperscript{-1} from all sources (27). For a 50 kg person consuming 0.45 kg of rice per day, typical body mass and rice consumption rate for this region (8), a rice concentration of >222 µg kg\textsuperscript{-1} As\textsubscript{i} would exceed the WHO standard. At the Indian and Bangladesh field sites the seven speciated cultivars in common across all field sites exceeded 111 µg kg\textsuperscript{-1}, and 5, 2, and 1 cultivars exceeded 222 µg kg\textsuperscript{-1} As\textsubscript{i}; at the Faridpur, De Ganga and Nonaghata sites respectively (SI Table S4). It should be noted that the values presented here are for whole grain and not polished rice, therefore a decrease in As\textsubscript{i}, maybe expected for polished rice (28), however, the cultivars which were speciated at all six field sites were parents of mapping populations and these were the cultivars with some of the overall lowest concentrations of As\textsubscript{i} identified in this study, therefore the cultivars which have even higher concentrations of As are potentially even more problematic.

Variations across different environments will pose difficulty to breeding low grain As cultivars that will have consistently low grain As (and low As\textsubscript{i}), over multiple environments using traditional breeding approaches, although we identified a number of genotypes that in general had low grain As across the multiple field sites. These cultivars will be a good starting point for both traditional breeding and the detection of targets using genetic approaches.

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Supporting Information Available
Information on the average grain arsenic concentration for the cultivars grown at the Chinese and Indian field sites is available as well as information on the ranking of the cultivars in common at all field sites. Data is also available on the average total As content and average As speciation at all six
field sites. This material is available free of charge via the Internet at http://pubs.acs.org.

**Literature Cited**


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