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Mapping quantitative trait loci associated with arsenic accumulation in rice (*Oryza sativa*)

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Summary

- The quantitative trait loci (QTLs) associated with arsenic (As) accumulation in rice were mapped using a doubled haploid population established by anther culture of F1 plants from a cross between a Japonica cultivar CJ06 and an Indica cultivar TN1 (*Oryza sativa*).
- Four QTLs for arsenic (As) concentrations were detected in the map. At the seedling stage, one QTL was mapped on chromosome 2 for As concentrations in shoots with 24.4% phenotypic variance and one QTL for As concentrations in roots was detected on chromosome 3. At maturity, two QTLs for As concentrations in grains were found on chromosomes 6 and 8, with 26.3 and 35.2% phenotypic variance, respectively.
- No common loci were detected among these three traits. Interestingly, the QTL on chromosome 8 was found to be colocated for As concentrations in grain at maturity and shoot phosphorus (P) concentrations at seedling stage.
- These results provide an insight into the genetic basis of As uptake and accumulation in rice, and will be useful in identifying genes associated with As accumulation.

Key words: arsenic (As), doubled haploid (DH) lines, quantitative trait loci (QTLs), rice (*Oryza sativa*)

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Introduction

Arsenic (As) is a toxic metalloid, which can enter the environment through natural processes and anthropogenic activities (Yan, 1994; Abedin *et al.*, 2002). As-contaminated groundwater and food pose a serious health risk to people, especially in Southeast Asia, in countries such as Bangladesh and China (Huang *et al.*, 1992; Smith *et al.*, 1992; Chen *et al.*, 1995; Mandal *et al.*, 1997; Liao *et al.*, 2005). Paddy soils and irrigation water are contaminated with high concentrations of As in some areas in these regions. The background concentrations of As in soils vary widely, but typically range from 4 to 8 mg As kg^{-1}. However, in Bangladesh, as a result of irrigation with As-contaminated groundwater, As concentrations in paddy soils can reach values as high as 57 mg As kg^{-1} (Alam & Sattar, 2000), and even 83 mg As kg^{-1} (Ullah, 1998). Large areas of Bangladesh, West Bengal and Vietnam have to rely on As-contaminated groundwater for irrigation of crops, such as rice (Meharg & Hartley-Whitaker, 2002).

Rice is the staple food for populations in South-East Asia. However, the buildup of As in paddy soils and irrigation water have led to the elevation of As concentrations in rice grains. In districts of Bangladesh with high As concentrations in paddy soils, the As concentration in rice grains was up to above 2 mg kg^{-1} (Meharg & Rahman, 2003). In Chenzhou, Hunan province of China, concentrations between 0.5 and 7.5 mg As kg^{-1} were found in rice grains as a result of metal mining activities in the area (Liao *et al.*, 2005). Arsenic accumulated in grains can be transferred to humans through the food chain, causing serious health problems. For Asian populations, As ingestion through rice consumption has become one of the major sources of As exposure (Meharg, 2004), and there is thus an urgent need to reduce As concentration in rice grains. Nevertheless, As concentration varies greatly among

rice cultivars (Liu *et al.*, 2004, 2006; Williams *et al.*, 2005); for example, the inorganic As concentration in grains was 0.35 mg kg^{-1} in the cultivar of YY-1, and only 0.15 mg kg^{-1} in 94D-22 when grown under the same conditions. These findings suggested the possibility of breeding cultivars with lower As concentrations in rice plants by conventional and molecular methods. Therefore, the exploitation of rice cultivars that accumulate lower concentrations of As in the grain is a promising technology for reducing As contamination in rice.

To date, the genetics of As uptake and accumulation in rice has not been extensively studied. So far only one published paper has been associated with QTL-mediated As tolerance on chromosome 6 (Dasgupta *et al.*, 2004). To understand the genetic mechanism behind phenotypic complexity, QTL mapping based on high-density molecular linkage maps is used, which is the first step and a powerful genetic approach to identify the number, position and effects of genetic factors that contribute to phenotypic variation (An *et al.*, 2006). To facilitate genetic mapping and map-based cloning of QTLs, novel mapping populations such as backcross inbred lines (BILs) (Xue *et al.*, 2006), chromosome segment substitution lines (CSSLs) (Ebitani *et al.*, 2005) and doubled haploid (DH) lines (Sogawa *et al.*, 2004) have been developed in plants. Using these lines or populations, researchers have identified QTLs related to agronomic traits such as aluminum, selenate and arsenic tolerance (Dasgupta *et al.*, 2004; Xue *et al.*, 2006; Zhang *et al.*, 2006b); phosphorus efficiency (Wissuwa & Ae, 2001); nitrogen uptake (An *et al.*, 2006); and cadmium and zinc accumulation (Deniau *et al.*, 2006). Although the genetic analysis of rice is much more developed than that of other crops (Song *et al.*, 2007; Uauy *et al.*, 2006; Perata & Voesenek, 2007), so far there is no useful genetic information on controlling the As concentrations in rice plants. The objective of this study was therefore to locate QTLs controlling As concentrations in rice plants, which is the first step in understanding the genetic mechanisms that control As concentration in rice plants.

Materials and Methods

Plant cultures and treatments

A DH rice population established by anther culture of F1 plants from a cross between a Japonica cultivar CJ06 (*Oryza sativa* L.) and an Indica cultivar TN1 (*Oryza sativa* L.) was employed in this study (Sogawa *et al.*, 2004; Zhang *et al.*, 2006a), which segregates for submergence tolerance (Zhang *et al.*, 2006a) and whitebacked planthopper resistances (WBPH) (Sogawa *et al.*, 2005). In this study, 94 DH lines and the two parents were used for QTL analysis at the seedling stage and the mature stage. However, 82 lines were mature with grains and 12 lines were dead or ripe without grains as a result of the weather and management in the maturity experiment.

A pot experiment was carried out in a glasshouse at Jiaxing Academy of Agricultural Sciences, Zhejiang province in southeast China. The paddy clay soil (0–20 cm) was collected from Jiaxing city (N 30°50′08.2″E 120°43′03.7″), with 4.61 μg kg^{-1} As, 1.27 mg kg^{-1} Cd, 30.49 mg kg^{-1} Pb and pH 5.6. Air-dried soil was thoroughly crushed and blended with *c.* 10 mg As kg^{-1} ($Na_2AsO_4 \cdot 12H_2O$) as solution. The As-contaminated soil was then thoroughly air-died and passed through a 2 mm sieve.

After 2 wk of soil equilibration, uniform 3-wk-old rice seedlings of 94 lines and the parents were transplanted into 3.5 l polyethylene pots containing 1.5 kg of the soil (one plant per pot) for the seedling stage experiment and 5 l polyethylene pots containing 3 kg of the soil (one plant per pot) for the mature stage experiment. There were no drainage holes at the bottom of all these pots. Each genotype was replicated three times at one growth stage and the pots were randomized in the glasshouse. Fertilizer was applied to each pot with 0.33 g potassium chloride and 0.56 g superphosphate as top dressing at the seedling stage. Urea of 2.58 g per pot was applied at rates of 30, 30, 20 and 20%, respectively, as top dressing at early tillering (10 d after transplanting), mid-tillering (20 d after transplanting), late-tillering (35 d after transplanting), and early panicle differentiation stage.

After 35 d of growth in the pots for the seedling stage experiment, plants were harvested. Shoots and roots were separated, and iron plaque on fresh root surfaces was extracted with DCB solution (Liu *et al.*, 2004). Then shoots and roots were oven-dried at 70°C for 72 h and then weighed. After grain ripening for the mature stage experiment, the plants were harvested. Brown rice was separated from the rice straw and grain chaff, and also oven-dried and weighed.

Analysis of As and P concentrations

Oven-dried plant (shoot, root and brown rice) materials were digested in nitric acid on a heating block (Digestion Systems of AIM500, AI Scientific, Brisbane, Australia). The concentrations of As in roots, shoots and grains were measured by an atomic fluorescence spectrometry (AF-610 A, Beijing Ruili Analytical Instrument Co., Beijing, China). The concentrations of phosphorus (P) in roots and shoots were measured by an inductively coupled plasma optical emission spectrometer (ICP-OES Optima 2000 DV, Perkin-Elmer, Wellesley, MA, USA). A reagent blank and standard reference plant material (GBW07605, from the National Research Center for Standard Materials in China) were included to verify the precision of analytical procedures.

QTL analysis

A molecular-marker linkage map containing a total of 178 molecular markers chosen from the available 227 markers was constructed by China National Rice Research Institute in

Hangzhou, and covered 1674.8 cM with 9.4 cM per marker using Mapmarker/EXP 3.0b (Sogawa *et al.*, 2005; Zhang *et al.*, 2006a). Analyses of QTL and epistatic interactions between the identified QTL and all other markers were evaluated using QTLNetwork 2.0 (Yang *et al.*, 2005). The threshold for QTL detection was set at a LOD value of 3.0.

Broad-sense heritability (h_b^2) was estimated for each trait using the following equation:

$$h_b^2 = \mathrm{VG}/(\mathrm{VG} + \mathrm{VE}) \times 100\%$$

(VG, variance between DH lines; VE, variance within DH lines (Reymond *et al.*, 2006)).

Paired-samples *t*-test was used to detect differences between the means of CJ06 and TN1. Paired-samples *t*-test, variance analysis and correlation coefficients between traits were analyzed using SPSS11.5 for Windows.

Results

At the seedling stage, As concentration in roots of CJ06 was 134.60 mg kg^{-1} and in roots of TN1 was 141.15 mg kg^{-1}, and ranged from 73.31 to 242.56 mg kg^{-1} among the 94 DH lines and the two parents. Shoot As concentrations were 18.05 mg kg^{-1} in CJ06 and 12.98 mg kg^{-1} in TN1, and ranged from 10.46 to 22.52 mg kg^{-1}. There were no significant differences in As concentrations in roots and shoots between the two parental varieties. At maturity, concentrations in grain ranged from 0.16 to 0.95 mg As kg^{-1} among the 82 DH lines and the two parents. The grain As concentration of CJ06 (0.27 mg kg^{-1}) was significantly lower than that of TN1 (0.66 mg kg^{-1}) ($P < 0.001$). The frequency distribution of As concentrations in tissue showed a continuous phenotypic variation and transgressive segregation in both parental directions at the seedling stage, and the set of 82 DH lines also segregated clearly for As concentrations in brown rice (Fig. 1). At the seedling stage, As concentrations in shoots were significantly positively correlated with P concentrations in shoots (Table 1). The correlation between As concentrations in brown rice and P concentrations in shoots at the seedling stage were also significantly positive.

As shown in Fig. 2, one QTL for As concentrations in roots was mapped on chromosome 3 (defined as AsR) at the interval of RM1350-RM3919, explaining 18.2% of the variation in root As concentrations (Table 2). One QTL associated with As concentrations in shoots was identified and mapped on chromosomes 2 (AsS), flanked by markers RM318 and RM450. Two QTLs were detected on chromosomes 6 (AsSe1, flanked by RM3-RM162) and 8 (AsSe2, flanked by RM1111-RM310) in controlling As concentrations in brown rice, explaining 26.3 and 35.2% of the phenotypic variance, respectively. The positive values of additive effect for AsS, AsSe1 and AsSe2 (Table 2) indicate that the trait-enhancing alleles are all originated from the parent TN1. For AsR, the trait-enhancing

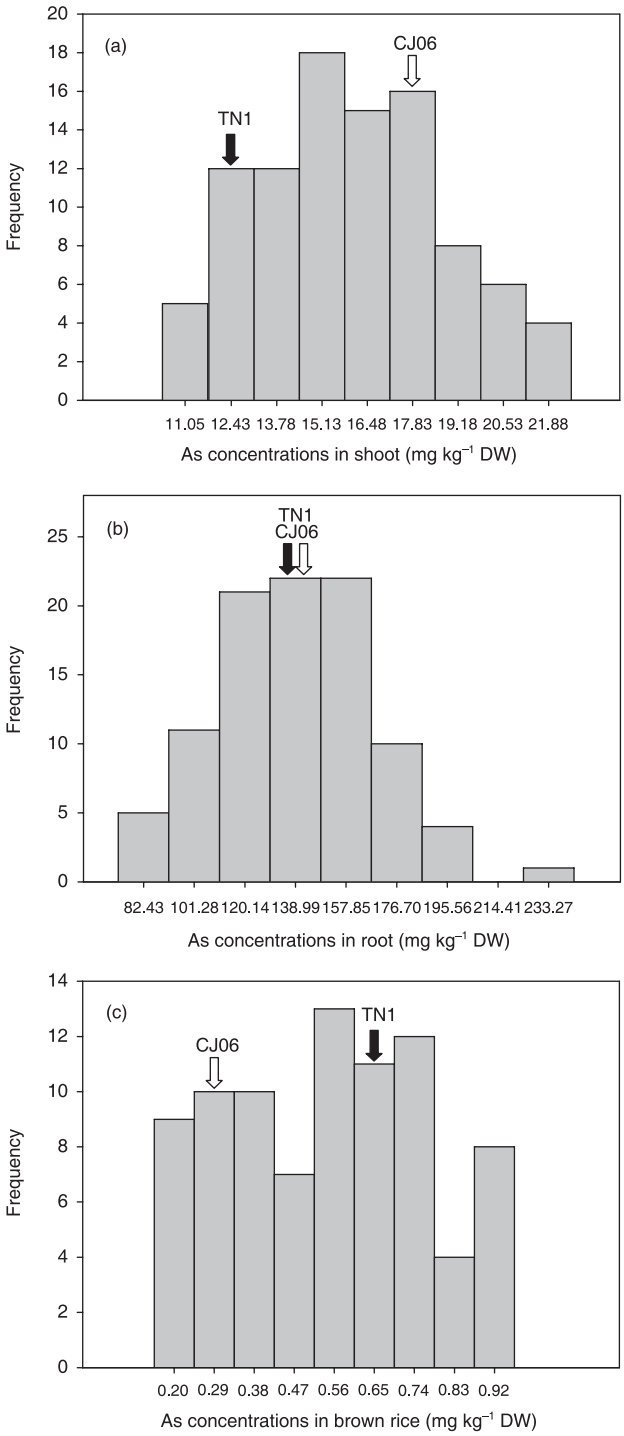

Fig. 1 Frequency distributions of arsenic (As) concentrations in shoots (a) and roots (b) at the seedling stage ($n = 96$), and As concentration in grains (c) at maturity ($n = 84$) of the rice (*Oryza sativa*) doubled haploid (DH) lines and the parents. Each value of the concentrations represents the mean of three replicates. TN1, Indica cultivar; CJ06, Japonica cultivar.

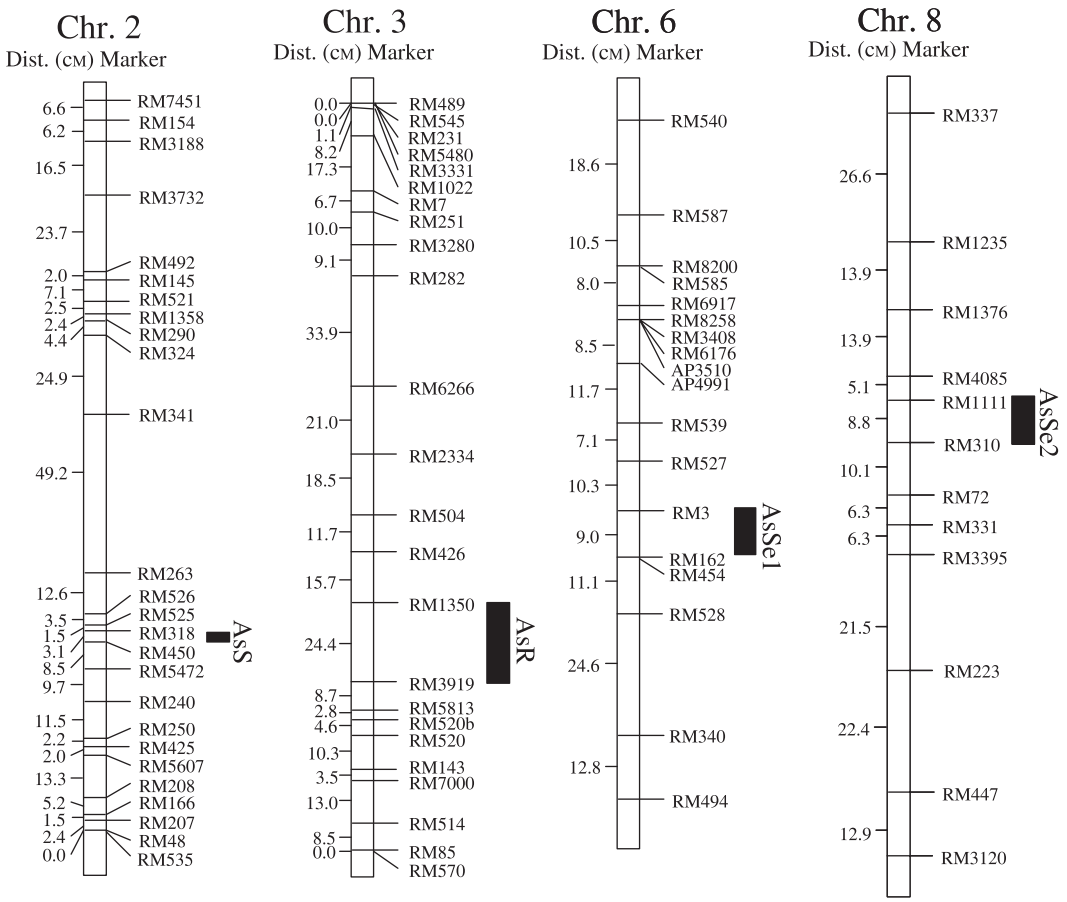

Fig. 2 Location of all the significant quantitative trait loci (QTLs) for arsenic (As) accumulation in the rice (*Oryza sativa*) doubled haploid (DH) lines. Bars, genomic regions with LOD > 3.0.

Table 1 Correlations between arsenic (As) and phosphorus (P) concentrations in shoots and roots at seedling stage, and brown rice at maturity (n = 84) of the rice (*Oryza sativa*) doubled haploid (DH) lines and the parents

Trait	AsS	AsR	AsSe	PS	PR
PSe (mg kg^{-1} DW)	0.08	0.21	–0.09	0.05	0.29**
PR (mg g^{-1} DW)	0.03	0.34**	–0.06	0.45**	
PS (mg g^{-1} DW)	0.25*	0.24*	0.32**		
AsSe (mg kg^{-1} DW)	0.11	0.11			
AsR (mg kg^{-1} DW)	0.50**				

*,**Correlation is significant at the 0.05 and 0.01 levels, respectively. Each value of the concentrations represents the mean of three replicates.
AsS, As concentrations in shoots; AsR, As concentrations in roots; AsSe, As concentrations in brown rice; PS, P concentrations in shoots; PR, P concentrations in roots; PSe, P concentrations in brown rice.

allele was derived from CJ06. AsSe2, the QTL on chromosome 8, was detected to colocate with the QTL for P accumulation in shoots (PS) at the seedling stage with a LOD value of 4.97, which explained 26.1% of variation in shoot P concentration. No common loci (LOD > 3.0) were detected among the traits of AsS, AsR, PS and PR. Moreover, epistatic interactions were not significant either for the four QTLs detected for As concentrations or for the QTLs with other markers in the map.

Discussion

In this study, we measured As accumulation in roots and shoots at the seedling stage and in brown rice at maturity of the parental cultivars (CJ06/TN1) and their DH lines' exposure to *c.* 10 mg As kg^{-1} (freshly spiked) in a pot experiment. Judging from the transgressive segregation of As concentrations in rice seedlings and grains, it appears that As accumulation in these lines and in the parents was a quantitatively inherited trait and could be controlled by multiple genes under our experimental conditions. The distribution patterns for shoot and grain As concentrations were similar (Fig. 1), while that for root As concentration was different, with little difference between the two parents. In general, root As is not a good measure of As uptake and metabolism, partly because residual As in the root surface after iron plaque removal may interfere

Table 2 Quantitative trait loci (QTLs) detected for arsenic (As) and phosphorus (P) concentrations in the rice (*Oryza sativa*) doubled haploid (DH) lines and the parents

Trait	QTL	Chr	Marker interval	LOD	PVE (%)	h_b^2	Add. effect
AsS (mg kg^{-1} DW)	AsS	2	RM318-RM450	5.7**	24.4	33.0	–1.38
AsR (mg kg^{-1} DW)	AsR	3	RM1350-RM3919	3.6**	18.2	47.8	11.18
AsSe (mg kg^{-1} DW)	AsSe1	6	RM3-RM162	3.1**	26.3	97.5	–0.081
	AsSe2	8	RM1111-RM310	5.5**	35.2		–0.092
PS (mg g^{-1} DW)	PS1	8	RM1111-RM310	5.0**	26.1	59.0	0.27
PR (mg g^{-1} DW)	PR	12	RM270-RM17	3.0*	15.3	91.8	0.10

*,**, genome-wise significance at the 0.05 and 0.01 levels, respectively.
AsS, As concentrations in shoots; AsR, As concentrations in roots; AsSe, As concentrations in brown rice; PS, P concentrations in shoots; PR, P concentrations in roots; Chr, chromosome name; PVE (%), percentage phenotypic variation explained; h_b^2, heritability of the trait; Add. effect, additive effect.

with the measurement. Overall, these results are significant, as this is the first report on a genetic analysis of As accumulation in plants.

By combining molecular marker data with the value of As concentrations in seedlings, we found that the QTL AsR with 18.2% of the phenotypic variance, or AsS with 24.4% of the phenotypic variance, was the only QTL with LOD > 3.0 for the trait. The heritabilities of AsR and AsS (47.8 and 33.0%, respectively) were low in this experiment. Thus, the actual genetic variance of the trait attributable to major QTL should be larger, and the influence of environmental variance and measurement errors on the heritability of As concentrations in the seedlings was high, since the total phenotypic variance included both environmental variance and measurement errors (Zhang *et al.*, 2006b). Furthermore, we found that another minor QTL for As concentrations in roots colocated with the QTL regulating P concentrations in roots on chromosome 12 (flanked by RM270-RM17), but the LOD value was lower than 3.0, although As concentrations in roots at seedling stage were positively related to P concentrations in roots. These results could be explained by the fact that arsenate, an analogue of phosphate, is transported by the same mechanism (Meharg & Hartley-Whitaker, 2002; Quaghebeur & Rengel, 2003), and since the high environmental variance, some common loci for As concentrations in tissue at the seedling stage could not be detected in this experiment. The QTLs for As concentrations in grain (AsSe1 and AsSe2) explained > 50% of this trait variation without epistatic interactions from other QTLs, which might have major effects on As accumulation in grains. The heritability for grain As is relatively high (97.5%, Table 2); this could be for two reasons: homogeneous environmental conditions in the glasshouse; and the high precision of As detection using an atomic fluorescence spectrometer, because a high heritability (91%) of the primary root length response to P environment had been reported (Reymond *et al.*, 2006).

In previous studies, an As tolerance QTL on chromosome 6 coincided with a P uptake gene that had been found in two different rice mapping populations (Wissuwa *et al.*, 1998; Dasgupta *et al.*, 2004). For our results, we found a colocation of QTL for grain As concentrations (AsSe2) at maturity and P concentrations in shoots (PS) at seedling stage. This may indicate that arsenate accumulation in plants is closely related to phosphate uptake, as arsenate can compete with phosphate transport from roots to shoots (Meharg & Hartley-Whitaker, 2002; Quaghebeur & Rengel, 2003), an interaction that influences As translocation to the grain. Since the location of markers flanking the loci can be found in the Rice Genome Project genetic map, we searched the markers flanked AsSe1 and the As tolerance gene (AsTol) (Dasgupta *et al.*, 2004) on chromosome 6 at the websites of Gramene (http://www.gramene.org) and TIGR (http://www.tigr.org) to detect their positions in the rice genome. The results showed that the nearest marker (RM162) to the As accumulation gene (AsSe1) is at *c.* 24.0 cM, and the nearest marker to AsTol is at *c.* 6.2 cM on chromosome 6; therefore AsTol is unlikely to be a gene associated with the QTL AsSe1. In addition, OsACR2.2, an arsenate reductase gene identified in rice roots (Duan *et al.*, 2007), was *c.* 0.4 cM on chromosome 3 where AsR was located as identified in the present study. The nearest marker (RM3919) to AsR was at *c.* 19.6 cM on chromosome 3, indicating that OsACR2.2 could not be in the interval of the QTL associated with AsR.

Results obtained in this study show that As accumulation in rice is a complex quantitatively inherited trait. The molecular markers tightly linked to the four QTLs detected for seedling and maturity stages in this study might be the first important findings in the development of rice cultivars with low straw and grain As through marker-assisted selection (MAS). This study is likely to be the first step towards possible isolation of the genes responsible for low grain As.

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