

## Rapid report

# An arsenate tolerance gene on chromosome 6 of rice

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Received: 19 March 2004

Accepted: 15 April 2004

doi: 10.1111/j.1469-8137.2004.01109.x

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**Key words:** Arsenic tolerance, phosphate, *Oryza*, QTL, *Oryza glaberrima*, Straightthead.

### Summary

- The genetics of arsenic tolerance in plants has not been extensively studied and no arsenic tolerance gene has been genetically mapped.
- Screening 20 diverse genotypes of rice for reduced root growth in 13.3  $\mu\text{M}$  arsenate identified marked differences in tolerance. The most sensitive variety, Dawn, is known to be highly susceptible to straightthead, a condition linked to arsenic contamination of soil.
- Screening 108 recombinant inbred lines of the Bala  $\times$  Azucena mapping population revealed the presence of a major gene, *AsTol*, which mapped between markers RZ516 and RG213 on chromosome 6.
- This gene is a good target for further characterisation. It should prove valuable for investigations into the physiological and molecular mechanism behind arsenic tolerance in plants and may lead to strategies aimed at breeding for arsenic contaminated regions.

© *New Phytologist* (2004) **163**: 45–49

### Introduction

The genetics of arsenic uptake and accumulation has not been extensively studied in plants. Naturally occurring resistance to high soil arsenic, which has been observed in some species, has been shown to be caused by the reduction of phosphate as well as arsenate uptake (Meharg & Hartley-Whitaker, 2002) (arsenate is an analogue of phosphate and enters the plant through phosphate transporters). While studies on the grass *Holcus lanatus* (Yorkshire Fog) have indicated the presence of a single major gene conferring tolerance to arsenate (Macnair *et al.*, 1992), which reduces arsenate and phosphate uptake (Wright *et al.*, 2000), no natural arsenic resistance gene has been mapped. Recently an arsenic resistance gene has been

identified in *Arabidopsis* by induced mutation, but this does not appear to match the known mechanism of natural arsenic resistance (Lee *et al.*, 2003).

If arsenic tolerance genes can be identified in crop plants and be shown to reduce arsenic uptake in the plant, they should be useful in alleviating the toxic effects of arsenic caused by water and soil contamination. The digging of tube-wells for drinking water supply into aquifers with high arsenic concentration in southeast Asia has been described as the greatest mass poisoning in human history (Smith *et al.*, 2000), with an estimated 51 million people or more exposed to elevated arsenic in their drinking water in Bangladesh and West Bengal alone (Pearce, 2003).

Irrigation with arsenic contaminated water has resulted in soils also contaminated with high levels of arsenic (Meharg &

Rahman, 2003). Rice grain collected in districts of Bangladesh with high arsenic levels in paddy soils had 10-fold higher than the 'normal' level of  $c. 0.2 \text{ mg kg}^{-1}$  arsenic (Meharg & Rahman, 2003). Glasshouse and field studies have shown that arsenic levels in grain increase with increasing arsenic in irrigation water/soil (Xie & Huang, 1998; Abedin *et al.*, 2002). Modelling suggests that rice could contribute a substantial amount to the total arsenic consumption in contaminated areas (Meharg & Rahman, 2003). Because arsenic cannot be removed from contaminated soil, there is a need to produce locally adapted rice varieties for Bangladesh which have low arsenic accumulation.

Here we screen diverse rice germplasm for tolerance to arsenate using the standard methodology and subsequently use a mapping population to locate a natural arsenate tolerance gene.

## Materials and Methods

A total of 20 diverse rice varieties were obtained from either the International Rice Research Institute (IRRI), The Philippines or from Didier Tharreau, CIRAD, France. These include parents of several rice mapping populations and some US varieties known to be tolerant or susceptible to straighthead (a plant disorder linked to arsenic contamination). One variety (CG14) is of the species *Oryza glaberrima* while the rest are *O. sativa*.

A mapping population of 205 bulked F6 recombinant inbred lines (RILs) was produced from a cross of varieties Bala and Azucena (original parental seed obtained from IRRI) by single-seed descent as described in Price *et al.* (2000). A total of 108 randomly selected lines was used in this study.

To test arsenate tolerance, seeds were allowed to germinate for 3 d at  $37^\circ\text{C}$  and then floated on alkathene beads within 250 ml beakers filled with either phosphate-free nutrient solution containing  $0.1 \text{ mM Mg}^{2+}$  and  $\text{SO}_4^{2-}$ ,  $0.2 \text{ mM Ca}^{2+}$  and  $\text{K}^+$  and  $0.6 \text{ mM NO}_3^-$  (control), or the same nutrient solution supplemented with di-sodium hydrogen arsenate (treatment). The seedlings were grown in controlled conditions at  $25^\circ\text{C}$  with a 12 h day length ( $300 \mu\text{mol m}^{-2} \text{ s}^{-1}$  PAR). After 1 wk the maximum length of the root of plants was measured. The tolerance index was calculated as the percentage of root length in arsenate compared to the control. In experiment 1, one replicate beaker containing 15–20 seedlings at six concentrations of arsenate was used to characterise the dose–response for varieties Azucena and Bala. The data for each plant in a beaker was averaged and the standard error calculated for Fig. 1. In experiment 2, the response of 20 rice varieties to  $13.3 \mu\text{M}$  arsenate was tested. In order to estimate sources of variation, two replicate beakers each containing 10 plants were used for both control and treatment. The 40 pair-wise comparisons of beaker means revealed a regression of 94% indicating that the vast majority of variance was between individual plants in beakers, rather than between beakers (Fig. 2). This conclusion was confirmed by three-way analysis of variance (factors:

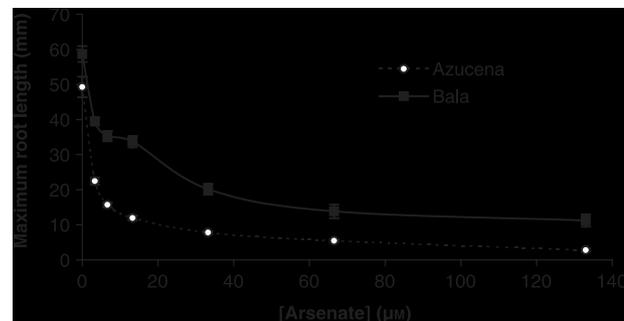


Fig. 1 Dose–response of maximum root length to increasing concentrations of arsenate for rice varieties Bala and Azucena. Bar, SE.

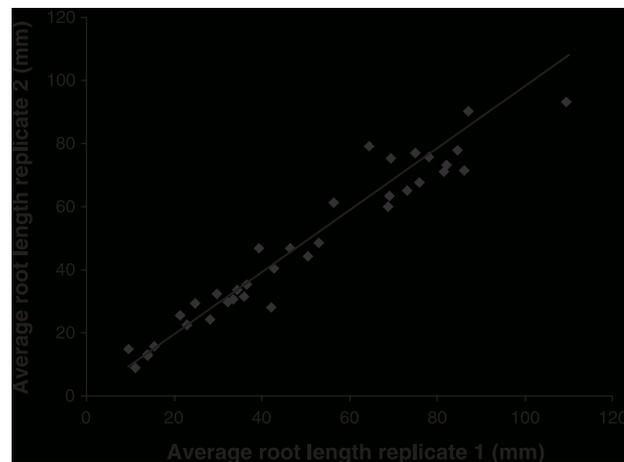


Fig. 2 Plot between the average root length observed in each of two replicate beakers used in experiment 2 (20 variety screen). The line is at  $y = x$ .

genotype, treatment and replicate beaker) which indicated that variance due to replicate beaker and its interaction with the other factors was an order of magnitude lower than the error variance. The tolerance values presented in Table 1 represent the average and standard deviation of arsenate tolerance index for each plant in the arsenate treatment calculated by dividing its root length by the average root length of all 20 control plants (from two beakers). In experiment 3, 108 lines of the mapping population were tested, with one control and one treatment beaker used for each line. In addition, four replicate beakers for the two parental varieties Azucena and Bala were used. In the mapping population, the distribution of tolerance indicated two discrete classes, and a value of  $> 40\%$  was taken to indicate tolerance, and  $< 40\%$  to indicate sensitivity to arsenate.

The tolerant RILs were ascribed a Bala genotype while the sensitive ones were classified with an Azucena genotype to allow the tolerance reaction to be treated as a genetic marker. The arsenate tolerance gene was placed on the existing map using MapMaker 3.0 (Lander *et al.*, 1987; Lincoln *et al.*, 1992) with the Haldane algorithm. The location of the adjacent RFLP markers on the genetic map of the Rice Genome

**Table 1** Tolerance to 13.3  $\mu\text{M}$  arsenate of 20 diverse rice varieties calculated as  $100 \times \text{root length with arsenate} / \text{root length without arsenate}$ 

Variety	Subspecies ( <i>O. sativa</i> unless stated)	Tolerance (%) $\pm$ SD
CO39	Indica	60.2 $\pm$ 12.4
ZYQ 8	Indica	53.0 $\pm$ 12.6
IR64	Indica	52.8 $\pm$ 12.4
Nortai	Unknown	44.3 $\pm$ 9.6
Bala	Indica	43.7 $\pm$ 13.4
Zhong 156	Indica	43.3 $\pm$ 6.5
Nipponbare	Japonica	41.2 $\pm$ 7.2
Gumei 2	Indica	41.1 $\pm$ 10.8
Kasalath	Indica	38.3 $\pm$ 12.5
Teqing	Indica	37.8 $\pm$ 7.4
Labelle	Unknown	37.8 $\pm$ 10.2
Lebonnet	Unknown	35.6 $\pm$ 11.6
Starbonnet	Unknown	35.5 $\pm$ 9.5
CG14	<i>O. glaberrima</i>	33.5 $\pm$ 5.2
Moroberekan	Japonica	30.1 $\pm$ 6.7
Belle Patna	Unknown	28.9 $\pm$ 7.7
JX 17	Japonica	24.0 $\pm$ 13.8
Lemont	Japonica	21.6 $\pm$ 6.9
Azucena	Japonica	20.7 $\pm$ 5.3
Dawn	Unknown	18.3 $\pm$ 2.3

Project (RGP) was obtained from the Gramene website (<http://www.gramene.org>) for marker C76 or from locating the position of BAC clones containing sequence matching markers RZ516 and RG213 on the emerging sequence information being compiled by The Institute of Genetic Research (<http://www.tigr.org>).

## Results

### Dose–response of Azucena and Bala (experiment 1)

Varieties Bala and Azucena were grown in five different arsenate concentrations plus a zero control (Fig. 1). In this experiment, Azucena had slightly shorter roots than Bala (49 mm vs 59 mm) in the absence of arsenate. Root length in Azucena was more severely affected by arsenate than Bala, falling to 45% in 3.3  $\mu\text{M}$  arsenate (compared to 67% in Bala). In the presence of 13.3  $\mu\text{M}$  arsenate, root length had been reduced to 24% in Azucena and 57% in Bala. This was considered a suitable dose to screen diverse genotypes.

### Tolerance of 20 diverse rice varieties (experiment 2)

After screening 20 rice varieties for tolerance to 13.3  $\mu\text{M}$  arsenate, a large range of responses was observed (Table 1) with highly significant varietal difference in arsenate tolerance ( $P \leq 0.001$ ;  $F = 26.9$ ;  $df = 19$ ). The variety most tolerant to

13.3  $\mu\text{M}$  arsenate was CO39, with a tolerance of 60.2% (i.e. a 40% reduction in root length). The most sensitive variety was Dawn with a tolerance of 18.2% (or an 82% reduction in root length). Between these extremes, there was a continuous range of responses without any clear distinction into tolerant and sensitive varieties. In general, the *O. sativa* varieties from the indica subspecies were more tolerant than those from the japonica subspecies. Because of this, the parents of a number of mapping populations appear to be contrasting in tolerance (e.g. IR64 and Azucena, Bala and Azucena, and ZYQ 8 and JX 17). Notably, however, the parents of the Nipponbare  $\times$  Kasalath population were not different indicating that not all japonica varieties are highly sensitive. Six varieties from the US were included because they have been shown to have contrasting resistance to the straighthead disorder, a physiological condition thought to be related to the build-up of arsenic containing compounds in soil following prolonged use of the herbicide monosodium methanearsonate (MSMA) in the USA (Wells & Gilmour, 1977). These are the straighthead resistant varieties Nortai, Labelle and Belle Patna (resistance score 2 on a 1–9 scale), moderately resistant varieties Lebonnet and Starbonnet (resistance score 3), and the susceptible variety Dawn (resistance score 9). The arsenate tolerance matched the straighthead resistance to some extent. Thus the straighthead susceptible variety Dawn was the most sensitive to arsenate, while the straighthead resistant and moderately resistant varieties were among the most arsenate tolerant. The exception was Belle Patna which was rather sensitive to arsenate despite being straighthead resistant.

### Mapping the arsenate tolerance gene (experiment 3)

A total of 108 recombinant inbred lines of the Bala  $\times$  Azucena mapping population were screened together with four replicate measurements of the parental varieties. The mapping population had an average tolerance of 42.7% ( $\pm 14.8$  SD;  $n = 108$ ) while for Bala and Azucena the value was  $53.4 \pm 2.6$  and  $21.6 \pm 2.8$ , respectively ( $n = 4$ ). Observing the frequency distribution of the population (Fig. 3) it is clear that there are two distinct populations that can be classified into tolerant ( $\geq 40\%$  root length) and sensitive ( $\leq 40\%$  root length). The population divides 67 : 41 tolerant: sensitive. Converting the tolerance score to a marker genotype allowed the gene to be mapped to chromosome 6, 9.9 cM below RZ516 and 19.4 cM above RG213 (Fig. 4). The deviation of the segregation ratio of tolerants to sensitives from the expected 1 : 1 matches a slight segregation distortion observed in the closest marker. Thus for RZ516, the ratio of Bala to Azucena genotypes within the mapping population is 122 : 71. Since the location of the flanking markers in the Rice Genome Project genetic map is known, it can be concluded that the arsenic tolerance gene is at 12.3 cM in the Bala  $\times$  Azucena map and about 18 cM in the map-based sequence emerging from the International Rice Genome Sequencing Project.

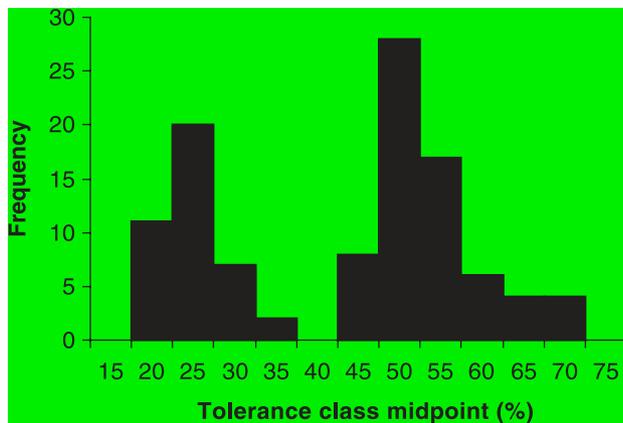


Fig. 3 Frequency distribution of arsenate tolerance in the Bala × Azucena mapping population divided into classes of 5%.

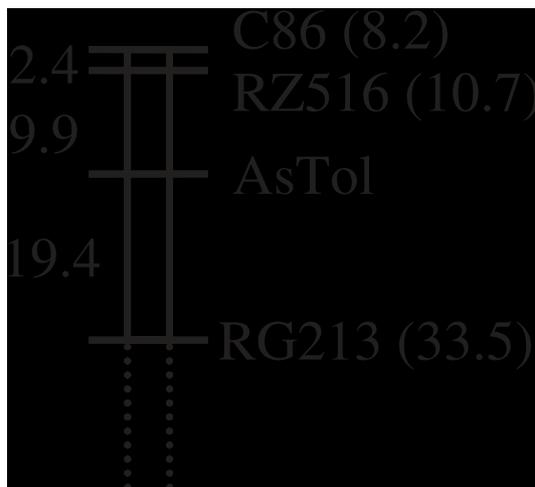


Fig. 4 Linkage map showing the location of the *AsTol* gene on the top of chromosome 6. Numbers to the left of the chromosome are the distance between loci in cm. The names of RFLP markers are on the right and their position in the International Rice Genome Sequencing Project's map-based sequence is indicated in brackets.

The mean tolerance score for the tolerant ( $56.4\% \pm 6.1$ ) and sensitive ( $25.2\% \pm 3.9$ ) classes are close to those for the tolerant and sensitive parents, but the standard deviation is larger providing evidence for a small degree of quantitative variation. Composite interval mapping of the residual variation revealed a quantitative trait locus (QTL) at marker C449 on chromosome 12 of LOD 5.2 and an additive effect of 2.2%, the greater tolerance coming from the Azucena parent.

## Discussion

### Tolerance to arsenate linked to straighthead resistance

It is shown here that the highly straighthead susceptible variety Dawn is very sensitive to arsenate. This variety suffered 61%

and 93% reductions in panicle weight when grown in flooded fields treated with 1.1 or 11.2 kg/ha arsenic applied as monosodium methanearsenate (MSMA), while the other US varieties used here showed reductions of no more than 20% and 49% in the respective treatments (Wells & Gilmour, 1977). Since the straighthead disorder is thought to be linked to soil arsenic content, there is some evidence that the sensitivity to arsenate revealed here reflects a demonstrable susceptibility to arsenic in the field.

### An arsenate tolerance gene on chromosome 6

The data presented here indicate the first mapping of an arsenate tolerance gene in plants. This gene is located near the top of chromosome 6, at 12.4 cm on the Bala × Azucena map, which is approximately 18 cm on the Rice Genome Project map. Wissuwa *et al.* (1998) and Wissuwa & Ae (2001) working with a Kasalath × Nipponbare mapping population and near isogenic lines produced from it have characterised a QTL for plant biomass under phosphorous-deficient conditions and for phosphorus uptake in the same region. That QTL was located 2 cm below marker C498 which is at 15.5 on the RGP map, indicating the presence of a gene affecting phosphorus uptake at approximately 17.5 cm. We believe the coincidence of the arsenate tolerance gene and the phosphorus uptake QTL is noteworthy since arsenate is an analogue of phosphate and is transported by the same mechanism (Meharg & Hartley-Whitaker, 2002). Thus it is quite possible that the phosphate uptake QTL and the arsenate tolerance gene reflect pleiotropic actions of the same gene. The link between arsenic tolerance and phosphate uptake and use has been observed in the grass species *H. lanatus*, where an arsenic resistance gene has been identified which is present at high frequency in populations growing on non-arsenic-contaminated soils (Macnair *et al.*, 1992). This gene has been shown to have a pleiotropic effect on phosphate uptake and use, where resistant plants had reduced phosphate uptake by the roots, poorer P use efficiency, lower vegetative growth and earlier and more abundant flowering compared to non-tolerants (Wright *et al.*, 2000). This *H. lanatus* gene is postulated to be a transcription factor regulating the expression of phosphate transport proteins (Meharg & Macnair, 1992).

In addition to the naturally occurring genes observed in rice and *H. lanatus*, an arsenate tolerance gene has also been identified in *Arabidopsis* by the generation of an induced mutant (Lee *et al.*, 2003). This mutant showed normal arsenate uptake but increased phosphate uptake and the authors observed that high phosphate reduced arsenate toxicity in wild-type plants, implicating increased phosphate transport in the mechanism of tolerance. Data on the impact of this rice gene on arsenic and phosphate uptake will be required in order to determine whether its action matches that of the *Arabidopsis* or *H. lanatus* gene or represents a novel mode of action.

In conclusion, an arsenic tolerance gene has been identified and mapped to chromosome 6 in rice. The colocation with a phosphate uptake QTL in another population of rice provides circumstantial evidence for a mechanism involving the behaviour of arsenate as a phosphate analogue, in agreement with the behaviour of an arsenic resistance gene found in populations of *H. lanatus*. However, whether the gene does alter arsenic or phosphorus uptake needs to be confirmed. Most importantly, if this gene is to prove useful in breeding applications, the gene must be shown to reduce straw and grain arsenic in plants grown in arsenic contaminated soils under field conditions.

## Acknowledgements

The authors would like to thank Brigitte Courtois (formerly IRRI, presently CIRAD, Montpellier) for the supply of bulked seeds of the mapping population. The mapping population was originally developed using funding from the Plant Sciences Programme of the Department for International Development, UK.

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