Journal of Experimental Botany, Vol. 59, No. 8, pp. 2277–2284, 2008
doi:10.1093/jxb/ern098 Advance Access publication 2 May, 2008
This paper is available online free of all access charges (see http://jxb.oxfordjournals.org/open_access.html for further details)

Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

Rice–arsenate interactions in hydroponics: a three-gene model for tolerance

Gareth J. Norton[1,*], Meher Nigar[1], Paul N. Williams[1], Tapash Dasgupta[2], Andrew A. Meharg[1] and Adam H. Price[1]

[1] *Department of Plant and Soil Science, Institute of Biological and Environmental Sciences, University of Aberdeen, Aberdeen AB24 3UU, UK*

[2] *Department of Genetics and Plant Breeding, University of Calcutta, 35 BC Road, Kolkata 700 019 West Bengal, India*

Received 7 December 2007; Revised 5 February 2008; Accepted 7 March 2008

Abstract

In this study, the genetic mapping of the tolerance of root growth to 13.3 μM arsenate [As(V)] using the Bala×Azucena population is improved, and candidate genes for further study are identified. A remarkable three-gene model of tolerance is advanced, which appears to involve epistatic interaction between three major genes, two on chromosome 6 and one on chromosome 10. Any combination of two of these genes inherited from the tolerant parent leads to the plant having tolerance. Lists of potential positional candidate genes are presented. These are then refined using whole genome transcriptomics data and bioinformatics. Physiological evidence is also provided that genes related to phosphate transport are unlikely to be behind the genetic loci conferring tolerance. These results offer testable hypotheses for genes related to As(V) tolerance that might offer strategies for mitigating arsenic (As) accumulation in consumed rice.

Key words: Arsenate tolerance, candidate genes, epistatic interactions, *Oryza sativa*.

Introduction

In the majority of plants investigated, arsenate [As(V)] tolerance is achieved by the suppression of high affinity arsenate/phosphate co-transport (Meharg and Hartley-Whitaker, 2002). The most detailed studies are on the native grass *Holcus lanatus* where it has been found that a single allele, present in all populations but at increased frequency in populations from arsenic (As)-contaminated soils, suppresses high affinity arsenate/phosphate co-transport (Meharg and Hartley-Whitaker, 2002). This adaptive tolerance is also reliant on constitutive detoxification of As within the plant [i.e. As(V) reduction and phytochelatin complexation] (Meharg and Hartley-Whitaker, 2002). However, other models of plant tolerance to As(V) have been found. As(V) tolerance in the heather *Calluna vulgaris* is achieved by its mycorrhizal symbiont *Hymenoscyphus ericae* which reduces As(V) to arsenite [As(III)] and then effluxes this As(III), a tolerance mechanism found in prokaryotes (Sharples *et al*., 2000), and very recently reported in tomato and rice (Xu *et al*., 2007). As-hyperaccumulating ferns of the *Pteris* genus behave quite differently from all other plants. The mechanism of how they tolerate high As burdens in their tissues is not well understood, although tolerance is not due to enhanced phytochelatin production or metabolism of inorganic As to organic species (Raab *et al*., 2004). The role of methylation as a detoxification mechanism in plants has not been fully investigated, although it is clear that >10% of a plant's As burden can be dimethyl arsenate [DMA(V)] depending on As exposure concentrations and on nutrition (Raab *et al*., 2005).

Dasgupta *et al*. (2004) reported the first genetic mapping of interactions with this toxin in plants. This revealed a segregation which appeared to indicate a single gene which was termed *AsTol* and mapped to a marker-poor region of rice chromosome 6. Subsequent addition of markers in that region indicated a conflict between new marker data and the supposed single *AsTol* gene. Here a new three-gene model of tolerance is presented. From

* To whom correspondence should be addressed. E-mail: g.norton@abdn.ac.uk

the position of these three major genes it was possible to produce a list of candidate genes, and, by integrating microarray analysis that has been reported in a companion paper (Norton *et al*., 2008), it was possible to narrow these lists down further. Also, physiological experiments are presented to examine the role of As(V) transport and phosphate as a mechanism of tolerance.

Materials and methods

Arsenate tolerance in recombinant inbred population

The data used to map As(V) tolerance were previously described in Dasgupta *et al*. (2004). Briefly, seeds of 108 recombinant inbred lines of the Bala×Azucena population (fully described in Price *et al*., 2000) were germinated for 3 d at 37 °C and then floated on alkathene beads within 100 ml beakers filled with either phosphate-free nutrient solution containing 0.1 mM Mg^{2+} and SO_4^{2-}, 0.2 mM Ca^{2+} and K^+, and 0.6 mM NO_3^- (control), or the same nutrient solution supplemented with di-sodium hydrogen As(V) (treatment) at a concentration of 13.3 μM. The seedlings were grown in controlled conditions at 25 °C with 12 h day length (300 μmol m^{-2} s^{-1} PAR). After 1 week the maximum length of the main root of plants was measured. The tolerance index was calculated as the percentage of root length in As(V) compared with control. The population was divided into tolerant or sensitive genotypes (based on the observed segregation as described in Dasgupta *et al*., 2004) using a separation of 40% tolerance (i.e. sensitive <40%, tolerant >40%) to provide a qualitative score (0 = sensitive, 1 = tolerant).

Improving the map and genetic analysis

Additional markers in specific regions were added to the map by PCR, either using microsatellite markers or designing primers to amplify across InDel polymorphisms revealed by Shen *et al*. (2004). Genetic mapping was conducted on both the quantitative percentage tolerance data and the qualitative tolerance score. The molecular map includes a total of 163 markers covering a total length of 1720 cM. Genetic loci were identified by composite interval mapping using the programme QTLCartographer version 1.15 (CJ Basten, BS Weir, and Z-B Zeng, Department of Statistics, North Carolina State University). Background markers for composite interval mapping were selected by 'forward stepwise regression with backward elimination' using the default threshold. The default window size of 10 cM was used.

Arsenate uptake kinetics

From seedlings of varieties of Azucena and Bala grown in the absence of As(V) as described above, roots were excised at the basal node. These were then incubated in aerated test solutions composed of 5.0 mM MES (pH 5.0) with different concentrations of As(V) for 20 min. The roots were then rinsed and incubated for 10 min in ice-cold phosphate solution containing 1 mM K_2HPO_4. Fresh weights of the roots were recorded. Root samples were placed into quartz glass digestion tubes, steeped in 2.5 ml of 70% nitric acid, and allowed to stand overnight at room temperature. The samples were digested at 120 °C until clear and then evaporated to dryness at 140 °C. The residue was resuspended in 1.2% nitric acid to a weight of 10 g. Indium (100 μl of 100 μg l^{-1}) was added as the internal standard. Total As concentration was determined using an ICP-MS 7500 (Agilent Technologies) (for full details, see Abedin *et al*., 2002).

Interactions between arsenate and phosphate

Parental rice varieties Azucena and Bala were tested for As(V) tolerance in nutrient solution containing As(V) (0, 13.3, or 133 μM) as described above, but in a range of phosphate concentrations (0, 0.05, 0.5, and 5 mM), each adjusted to pH 5.5. Tolerance was calculated independently at each phosphate concentration since phosphate alone had a moderate but significant effect on root length. In addition, a phosphate loading experiment was performed in which Azucena and Bala plants were grown for a week in pots containing 1.0 l of nutrient solution with 0 mM and 0.3 mM phosphate as described above. Root lengths were measured before transferring the plants into fresh phosphate-free nutrient solution. Half of the new pots contained As(V) at 6.65 μM, so that for both genotypes tolerance could be tested on plants that had been loaded with phosphate and plants that had not. Tolerance was calculated on the basis of root growth in the second week.

Compilation of gene list, assignment of probe sets, and array data

Gene lists were compiled using the Institute of Genomic Research (www.tigr.org) Pseudomolecules release 5. Transposons and retrotransposons were removed from the lists. Expression data were assigned to the genes and probe sets from the array data (series GSE4471) described in the companion paper (Norton *et al*., 2008).

Results

Adding markers and re-mapping arsenate tolerance

The original data for this population indicated that As(V) tolerance within the 108 recombinant inbred lines (RILs) had a bimodal distribution, with ranges of 18–35% indicative of sensitive genotypes and ranges of 45–70% indicative of tolerant genotypes, with no genotypes in between (see Fig. 3 in Dasgupta *et al*., 2004). Thus, the discrete segregation looked like that of classic single gene inheritance. The original mapping using a tolerance score as a marker had placed a single As(V) tolerance locus on the top of chromosome 6 in a 29.4 cM region between markers *RZ516* (genome sequence position 10.7 cM) and *RG213* (at ~33.5 cM). The mapping software MapMaker 3.0 (Lander *et al*., 1987; Lincoln *et al*., 1992) indicated that there was a likelihood (log of odds, LOD) of 16 for the placement of the *AsTol* marker on chromosome 6, and it was 10^5 times more likely (LOD of 5) to be between *RZ516* and *RG213* than anywhere else on the chromosome. However, adding molecular markers to this region revealed a conflict between molecular marker data and the sensitive/tolerance score. Thus some plants that were tolerant nonetheless inherited marker *PO499E08* (genome sequence position 13.8 cM) and *RM253* (at ~19.1 cM) from the sensitive parent Azucena. The results of quantitative trait loci (QTLs) analysis with the quantitative tolerance percentage with the new map (not presented) revealed three distinct LOD peaks at markers *RZ516*, *RG213*, and *RG257*; while using the tolerance score (0 or 1) the peaks were similarly located except that on chromosome 10 it was 2 cM below *RG257*. From this

analysis and examination of the genotypes of RILs, it is apparent that there are three genes involved, not one. Single marker analysis of variance of tolerance percentage or tolerance score, using the genotype at each of the three closest markers together (i.e. three-way ANOVA), revealed remarkably highly significant effects for each marker alone, and for the three together (Table 1), but not for any two-marker combinations. The tolerance of all but one RIL can be predicted by its marker genotype at each of these three markers (Table 2). The table indicates that of the eight marker classes, four are tolerant and four are susceptible, which explains the apparent single gene nature of the inheritance as reported previously. A plant must inherit only two of the three loci from Bala to be tolerant, but it can be any two. There is one individual in the 96 (for which all marker and tolerance data are available) in which the genotype does not match the phenotype. Thus RIL256 has the marker class ABB, but is sensitive to As(V). This has been verified in three separate repeats of the tolerance test. This RIL does not appear to be recombinant between marker *RG213* and its flanking markers but there is a recombination between *RG257* and the next marker on chromosome 10, *RM5689* (at 26.1 cM). This suggests that the gene *AsTol10* is just below *RG257*. To summarize, there are three tolerance genes, two on chromosome 6 and one on chromosome 10. These three loci for As(V) tolerance have been assigned the nomenclature *AsTol6.1*, *AsTol6.2*, and *AsTol10*. Inheritance of any two of these loci from the tolerant parent Bala confers tolerance.

These tolerance loci are not an artefact of root growth in general, since QTL analysis performed using the control root length measurements revealed no root growth QTLs on chromosome 6 and a single putative root growth QTL on chromosome 10. However, this was 20 cM below the major tolerance gene on chromosome 10.

Table 1. *The* F *statistic and probability obtained from three-way analysis of variance for the variables tolerance value or sensitive/tolerance (S/T) score, with factors the genotype (Azucena or Bala) at three identified markers*

Source	% Tolerance		S/T score	
	F value	*P*	*F* value	*P*
RZ516	134.8	<0.001	444.7	<0.001
RG213	102.7	<0.001	376.4	<0.001
RG257	113.2	<0.001	376.4	<0.001
RZ516×RG213	2.5	0.115	0.7	0.401
RZ516×RG257	0.1	0.773	0.7	0.401
RG213×RG257	2.4	0.126	0.7	0.401
RZ516×RG213×RG257	109.4	<0.001	376.4	<0.001

Table 2. *Arsenate tolerance of mapping population of Dasgupta* et al. *(2004) grouped into eight classes based on genotype at the marker nearest the three tolerance genes (A = Azucena, B = Bala)*

Genotype at marker[a]			*n*	Arsenate tolerance (%) (±SD)	Sensitive/ tolerance score (0 or 1)	Comment
RZ516 (6;10.7)	RG213 (6;~33.5)	RG257 (10;21.8)				
A	A	A	11	24.2±3.3	0±0	All sensitive
A	A	B	10	26.1±2.4	0±0	All sensitive
A	B	A	4	23.7±4.1	0±0	All sensitive
A	B	B	12	49.3±8.2	0.92±0.29	11/12 tolerant
B	A	A	7	23.2±2.3	0±0	All sensitive
B	A	B	16	53.7±6.0	1±0	All tolerant
B	B	A	11	54.9±6.1	1±0	All tolerant
B	B	B	19	53.4±6.1	1±0	All tolerant

[a] The numbers in parentheses are the marker chromosome and position in cM on the genome sequence. There is one plant whose marker genotype (ABB) indicates it should be tolerant but it is in fact sensitive, hence the variability in this marker class.

Genes within 2.5 cM of markers

The locations of these three tolerance genes can be given with a known degree of confidence based on the number of genotypes used to locate them. The position of *AsTol6.1* can be assessed only in individuals which have both an Azucena and a Bala allele for the other two genes (i.e. marker classes AAB, ABA, BAB, and BBA), which is 41 genotypes (Table 2), which gives a theoretical accuracy of 100/41 or 2.4 cM. For *AsTol6.2* (marker classes AAB, BAB, ABA, and BBA), the accuracy is 100/40 or 2.5 cM. For *AsTol10* (marker classes ABA, BAA, BAB, and ABB), the accuracy is 100/39 or 2.6 cM. The location on the sequence map of rice is known for each of the three markers. *RZ516* is on bacterial artificial chromosome (BAC) clone P0710H01 at 10.7 cM on chromosome 6, *RG213* is on BAC P0516A04 at ~33.5 cM on chromosome 6, and *RG257* is on BAC OJA1208D02 at ~21.8 cM on chromosome 10. Using the sequence information at TIGR, the annotated genes on each BAC can be listed within 2.5 cM of each marker. This size of 2.5 cM is justified and errs on the side of caution because the genetic distance in the Bala×Azucena map is larger than that of the RGP map that is used for physical to genetic sequence alignment [by 2.4-fold for *AsTol6.1*, by 1.25-fold for *AsTol6.2*, and by 2.5-fold for *AsTol10* (based on genetic positions of flanking markers in both maps)]. For *AsTol6.1*, 17 BAC clones span the 5 cM region of the chromosome centred on this marker containing 284 potential candidate genes (see Supplementary Table S1 at *JXB* online), of which 172 have a known function or known functional domain, 63 are classified as 'expressed protein', and 48 are 'hypothetical proteins'. For *AsTol6.2*, 5 cM covers 10 BACs with 150 genes (see Supplementary Table S2 at *JXB* online), of which 107 have known function or functional domain, 12 are 'expressed proteins', and 31 are 'hypothetical proteins'. For *AsTol10*, 5 cM covers 27 BACs with 296 genes (see Supplementary

Table S3 at *JXB* online), of which 120 have known function or functional domain, 34 are 'expressed proteins', and 142 are 'hypothetical proteins'. It must be noted, however, that there remains a gap in the sequence of chromosome 10 at 23.1 cM from BACs B1082A06 to OSJNBa0032N04 that is within the target region. Initially, given the nature of the three-gene interaction (any two from the tolerant parent gives tolerance), the same gene was looked for in each location based on annotation. Genes annotated as 'Phi-1 phosphate-induced protein, putative' and 'ATP-binding protein' are located in each list. A number of genes are present in two of the three lists. Genes annotated as 'F-box domain-containing protein' are present in the gene list *AsTol6.1* with a single gene in the *AsTol10* gene list, although the array data suggest that the gene in the *AsTol10* gene list is not expressed. A gene annotated as a 'NBS-LRR disease resistance protein' is present in both the *AsTol6.1* and *AsTol10* candidate gene lists, although the expression data suggest that the gene on chromosome 10 is not expressed. A single gene annotated as a 'nucleic acid-binding protein' is present in both the gene lists on chromosome 6, and the array data suggest that both these genes are expressed.

In order to determine if any of the genes lacking annotation have any similarity across lists, a local protein database was created containing all the protein sequences of genes described as 'expressed protein' and 'hypothetical protein'. Each sequence was then BLASTed against the database to look for sequences with homology within the different lists. A total of 19 different matches with expected scores below 0.0001 was observed (Table 3). A number of genes that have homology to genes in one list also have homology to genes in the other list. Examples of these are Os10g23940 which has 35.6% homology to Os06g12000 and 37.7% homology to Os06g06330, and Os06g11150 which has 32.3% homology to Os06g06330 which shares 41% homology to Os10g23890. As well as these genes that show a degree of homology over the three gene lists, two clusters of genes annotated as 'expressed protein' and 'hypothetical protein' share homology on two different gene lists. One cluster is at the top of the gene list for *AsTol6.1*, where several genes annotated as 'expressed' are present on BACs P0679C08 and P0001H02. These show sequence homology to a second cluster of 'expressed' genes on chromosome 10, on clones OJA1208D02 and OSJNBa0091J06.

Table 3. *Percentage homology between genes annotated as 'expressed protein' and 'hypothetical protein' within the three candidate gene lists*

Gene 1	Gene 2	Percentage homology	Length[a]	Expected value
Os06g04310	Os10g22730	33.81	139	1.00E-17
Os06g04330	Os10g22740	27.51	309	4.00E-16
Os06g04470	Os10g22740	30.97	113	1.00E-10
Os06g05140	Os10g23830	39.71	68	5.00E-10
Os06g04360	Os10g22740	32.17	115	2.00E-09
Os06g04260	Os10g22740	33.05	118	2.00E-09
Os06g04340	Os10g22740	33.33	84	1.00E-06
Os06g12000	Os10g23940	35.62	73	1.00E-06
Os10g23840	Os06g10940	35.48	93	2.00E-06
Os06g04350	Os10g22740	27.83	115	4.00E-06
Os06g04330	Os10g22720	23.44	192	5.00E-06
Os06g06330	Os06g11150	32.29	96	1.00E-04
Os06g04340	Os10g22720	27.19	114	2.00E-04
Os06g05970	Os06g11980	29.17	72	2.00E-04
Os06g04210	Os10g24090	35.71	70	2.00E-04
Os06g06330	Os10g23940	37.74	53	2.00E-04
Os10g24000	Os06g11820	38.67	75	5.00E-04
Os10g23890	Os06g06330	41.03	39	5.00E-04
Os06g06330	Os10g24180	35.59	59	6.00E-04

[a] Number of amino acids that the homology spans.

Expression of genes within the positional candidate list

Of the 284 positional candidate genes for *AsTol6.1*, 252 genes have one or more assigned probe sets. The number of genes expressed for each treatment–genotype combination in this candidate region ranged between 61% and 68%, with 15 genes being significantly up-regulated and 12 genes being down-regulated in Azucena in response to As(V), whereas in Bala eight genes were up-regulated and five down-regulated in response to As(V). A total of 31 genes were differentially expressed between Azucena and Bala under control conditions, while under As(V) treatment 35 genes were differentially expressed between the parental genotypes (see Supplementary Table S1 at *JXB* online).

For *AsTol6.2*, 131 of the 150 genes have a matching probe set(s) and between 47% and 56% of the genes were expressed. In Azucena, nine genes were up-regulated by As(V) and five were down-regulated, whereas in Bala five genes were up-regulated and two down-regulated. The number of genes differentially expressed between Azucena and Bala was one under control conditions and two after As(V) treatment, and in all cases the expression was higher in Bala (see Supplementary Table S2 at *JXB* online).

Of the 334 candidate genes for *AsTol10*, 78 do not have corresponding Affymetrix probe sets. Between 38% and 44% of those genes that do have probe sets were expressed in the four different treatment–genotype combinations. A total of seven genes were up-regulated and 11 down-regulated in Azucena. In Bala, two genes were up-regulated and seven down-regulated. Eleven genes were differentially expressed between Azucena and Bala under control conditions, and 20 genes after As(V) treatment (see Supplementary Table S3 at *JXB* online).

Arsenate uptake kinetics and interactions with phosphate

In order to gain a better appreciation of the type of gene that might confer resistance, the possibility was investigated that tolerance was conferred by altered arsenate/

phosphate transport as has been demonstrated for other grasses such as *H. lanatus* (Meharg and Macnair, 1992). As(V) uptake was saturated at ~20 μM As(V) in agreement with Abedin *et al.* (2002), but importantly there was no apparent difference between Bala and Azucena (Fig. 1). The results of conducting the tolerance test in a range of phosphate concentrations are shown in Fig. 2. It is clear that phosphate of 0.5 mM and 5 mM offered protection from As(V) inhibition of root growth, but there was a highly significant ($P > 0.001$) phosphate by genotype interaction indicating that 0.5 mM phosphate was much more effective in protecting Bala than Azucena. Crucially, the amount of phosphate that protects in the tolerance test is much higher than the As(V) concentration (500 μM versus 13.3 μM), and much higher than the concentrations that protect other grasses such as wheat from arsenate-induced root growth inhibition which are in the 10–50 μM range (unpublished data).

Testing tolerance after 1 week's growth with or without phosphate also revealed a highly significant effect of phosphate on As(V) tolerance, but not an interaction with genotype (Fig. 3). In other words, phosphate loading affords a degree of tolerance in rice, but does not remove the genetic difference in tolerance between Azucena and Bala.

Discussion

The rice varieties Azucena and Bala have previously been shown to differ in their level of As(V) tolerance, with Azucena being more affected by As(V) across the range tested (3–130 μM) (Dasgupta *et al.*, 2004). The data used here to determine the three-gene model were from Dasgupta *et al.* (2004), where an As(V) concentration of 13.3 μM was used as it gave the clearest difference in As(V) tolerance between the two parents of the mapping population.

The relevance of this tolerance test (despite its widespread use in studying the interaction between plants and As) has yet to be confirmed in the field situation, where there is a need to reduce As uptake and translocation to the grain. However, Dasgupta *et al.* (2004) indicated

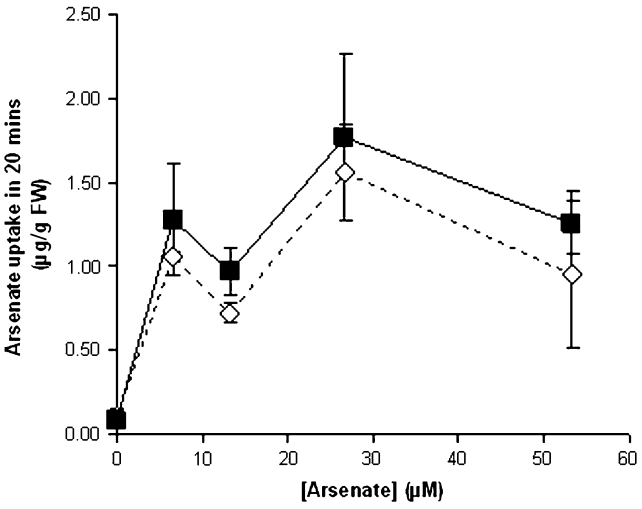

Fig. 1. Arsenate uptake of root tips after 20 min in a range of arsenate solutions. Filled symbols and the solid line are Azucena, and open symbols and the dashed line are Bala. The bar is the standard error (n=3).

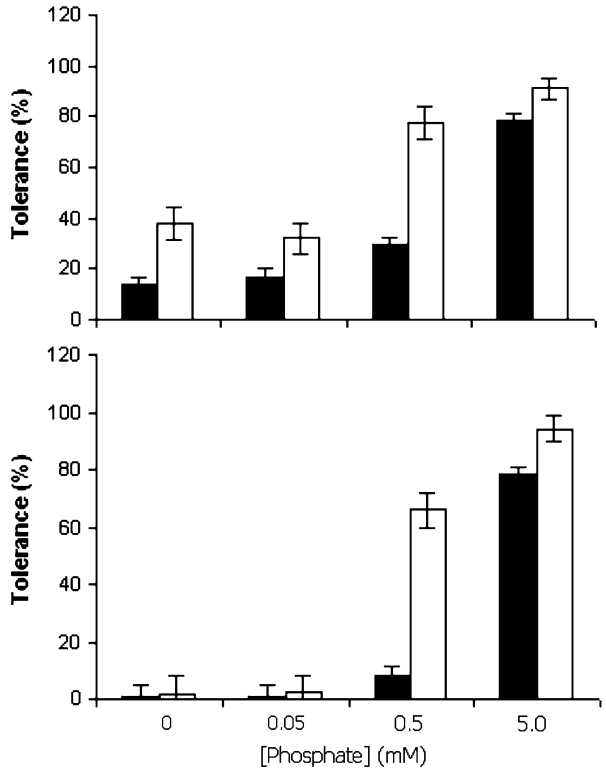

Fig. 2. Arsenate tolerance of Azucena (filled bars) and Bala (open bars) at a range of phosphate concentrations in 13.3 μM (upper) and 133 μM (lower) arsenate. The error bar is the standard error.

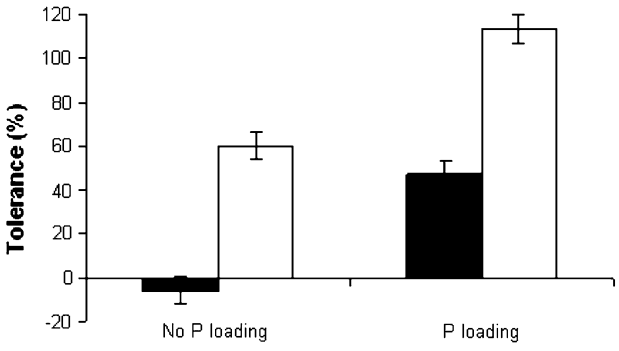

Fig. 3. Tolerance to 6.65 μM arsenate of Azucena (filled bars) and Bala (open bars) which had previously been grown for a week in nutrient solution with or without 0.3 mM phosphate. The error bar is the standard error.

a reasonable agreement between As(V) tolerance and resistance to the physiological disease 'straighthead' which is thought to be related to arsenic-containing compounds. It must also be acknowledged that insights into the molecular interactions between plants and As compounds may offer opportunities to alter natural processes such as As transport or metabolism as a strategy for reducing the impact of As contamination of paddy fields.

With the improvement of the Bala×Azucena genetic map, a three-gene model for As tolerance has been elucidated that reflects a highly unusual three-gene epistasis. All three alleles that confer As(V) tolerance are from the Bala parent. There is another possible explanation for this apparent epistatic interaction, which is the combined effect of three additive genes with a threshold response to gene dose. In this model, the three genes would have the same or equivalent products, and, if at least two of the tolerant parent alleles were present, the plant would have sufficient product to be tolerant. In this model, one might expect a different dose requirement with different As(V) challenges, so it would be interesting to test the dose response of different allelic combinations in the RILs to As(V).

Linking the genetic map to the sequenced rice genome, it has been possible to construct a candidate gene list for the three tolerance loci. Within the three candidate gene lists, one gene annotation, Phi-1, appears in all three lists. For *AsTol6.1*, the Phi-1 gene (Os06g04250) is expressed in both varieties and both treatments. For *AsTol6.2*, three genes are annotated as Phi-1 (Os06g11660, Os06g11680, and Os06g11700), but only Os06g11700 is expressed. One Phi-1 gene is annotated in the *AsTol10* candidate gene list but has no corresponding Affymetrix probe set. The Phi-1 gene was identified as a phosphate-induced gene in tobacco BY-2 cells, localized in the cytoplasm, and possibly involved in phosphorylation (Sano *et al.*, 1999). The Phi-1 genes are good candidate genes, but further validation will be needed. To validate these genes as candidate genes, sequencing and detailed expression analysis will be needed, to determine if there is any allelic variation between Bala and Azucena. The observation that an ATP-binding protein is present in all three lists is interesting since disruption of ATP metabolism is a recognized mechanism of As(V) toxicity (Meharg and Hartley-Whitaker 2002). However, the sequence similarity at the protein level between loci from the different lists is very low (<10%), and the 'ATP-binding protein' on chromosome 10 has very low levels of expression according to the transcriptomic data.

For a gene within the target region to be a good candidate for As tolerance it must be expressed (in at least one genotype, in at least one environment) and there must be a significant difference in either gene expression between the genotypes (most importantly under treatment) or functional allelic variation (i.e. in the protein sequence). As no evidence of sequence diversity among the candidate genes is presented, the focus is on genes that are differentially expressed (Table 4). For the *AsTol6.1* candidate gene list, 21 genes have different levels of expression between Azucena and Bala. A total of 13 of these genes are annotated as 'expressed proteins'. The other eight genes have a diverse range in functions (Table 4), but two are noteworthy. The Phi-1 gene, already identified as a good candidate due to the presence of this gene in all three lists, has a greater level of expression in Azucena than Bala under As(V) treatment, further strengthening its candidacy. Another good candidate in this list is the *S*-adenosylmethionine-dependent methyltransferase/methyltransferase/thiopurine *S*-methyltransferase which has greater levels of expression in Bala compared with Azucena under As(V) treatment. In the mammalian pathway of As detoxification (the Challanger pathway), a key enzyme is an *S*-adenosylmethionine-dependent methyltransferase that converts As(III) to monomethyl arsenate [MMA(V)] and converts monomethyl arsenite [MMA(III)] to DMA(V) (Aposhian *et al.*, 2004; Thomas *et al.*, 2004). Although the rice *S*-adenosylmethionine-dependent methyltransferase/methyltransferase/thiopurine *S*-methyltransferase does not show strong homology to the mammalian arsenate/monomethyl methyltransferase gene, it has conserved regions common to the mammalian genes. Candidate genes involved in As methylation are very interesting as they may offer strategies for reducing the toxicity of As, since organic forms of As are less toxic than inorganic ones (World Health Organization, 2001).

For the *AsTol6.2* locus there are two genes that are differentially regulated between genotypes under As(V) treatment. These are an aminoacylase-1 and an aquaporin NIP4.1. Both have higher levels of expression in Bala. The aquaporin gene is of interest as this class of gene has been implicated in the transport of As(III) into roots (Meharg and Jardine, 2003).

Five genes have significant differences in gene expression under As(V) treatment in the candidate gene region on chromosome 10. Two have higher levels of gene expression in Bala (hypothetical protein and peptide transporter PTR2), and three have higher levels of expression in Azucena [glutathione *S*-transferase (GST), cellulase-containing protein, and protein CutA]. The protein CutA has been identified as a copper-binding protein in *Arabidopsis* (Burkhead *et al.*, 2003). The role of GSTs during As(V) treatments seems to be significant as a large number of these genes are up-regulated during As(V) exposure [see companion paper (Norton *et al.*, 2008)], and identifying one within a target region may be of significance. It is also noteworthy to mention that a GST is also present in the chromosome 6.2 candidate gene list, and this gene is induced in both genotypes by As(V) treatment but does not show varying

Table 4. *Genes which show significant differential gene expression between genotypes during As(V) treatment*

Gene	TIGR gene annotation	Affymetrix probe set	Azucena signal[a]	Bala signal[a]	Adjusted *P*-value[b]
AsTol6.1					
Os06g04190	Rad1-like protein	Os.51161.1.S1_at	27.3	12.5	2.68E-02
Os06g04250	Phi-1-like phosphate-induced protein	Os.49653.1.S1_at	98.9	52.8	1.89E-02
Os06g04330	Expressed protein	Os.34404.1.S1_at	182.9	23.9	1.14E-04
Os06g04360	Expressed protein	Os.51695.1.S1_at	51.6	6.3	1.29E-03
Os06g04380	Aminomethyltransferase	Os.6080.1.S1_at	259.0	68.6	3.07E-02
Os06g04470	Expressed protein	Os.22672.1.S1_at	377.2	49.9	5.41E-05
Os06g04480	Expressed protein	Os.56019.1.S1_at	23.2	13.0	2.27E-02
Os06g04680	Expressed protein	Os.49667.1.S1_at	96.5	47.7	3.65E-02
Os06g04699	Expressed protein	Os.7474.1.S1_at	71.9	4.8	4.66E-05
Os06g04840	Leucine-rich repeat receptor protein kinase EXS precursor	Os.53844.1.S1_at	17.2	8.4	1.96E-02
Os06g04970	Expressed protein	Os.17050.1.A1_at	67.9	276.1	2.39E-03
Os06g05410	Expressed protein	Os.10862.1.S1_at	311.3	6.2	1.57E-03
Os06g05420	Expressed protein	Os.50399.1.S1_at	286.7	1.6	6.72E-06
Os06g05430	Expressed protein	Os.50383.1.S1_at	83.2	5.2	1.11E-07
Os06g05440	Expressed protein	Os.10736.1.S1_at	138.2	4.8	4.47E-05
Os06g05510	Expressed protein	Os.55293.1.S1_at	17.1	221.7	6.88E-04
Os06g05900	Hexaprenyldihydroxybenzoate methyltransferase	Os.51932.1.S1_at	191.0	70.2	7.17E-03
Os06g05940	Lipopolysaccharide-modifying protein	Os.56370.1.S1_at	23.9	4.3	4.91E-02
Os06g06040	*S*-Adenosylmethionine-dependent methyltransferase/ methyltransferase/thiopurine *S*-methyltransferase	Os.21058.1.S1_s_at	240.6	435.2	3.47E-02
Os06g06210	Expressed protein	Os.17072.1.S1_at	62.7	18.5	7.89E-03
Os06g06380	Disease resistance protein RPM1	Os.33898.1.S1_at	1.6	30.5	5.52E-05
AsTol6.2					
Os06g10770	Aminoacylase-1	Os.4905.4.S1_at	25.4	471.0	5.16E-04
Os06g12310	Aquaporin NIP4.1	Os.17147.1.S1_x_at	256.0	652.9	3.49E-02
AsTol10					
Os10g21970	Hypothetical protein	Os.25164.1.A1_at	4.3	42.6	1.01E-03
Os10g22310	Glutathione *S*-transferase	Os.46885.1.S1_at	33.5	6.3	4.86E-02
Os10g22560	Peptide transporter PTR2	Os.46546.1.S1_at	17	89.2	3.15E-02
Os10g22570	Cellulase-containing protein	Os.26719.1.S1_at	116.8	17.5	9.77E-04
Os10g23204	Protein CutA	Os.9220.1.S1_at	1277.7	561.5	1.81E-03

[a] Values presented after normalization using the MAS5.0 software.
[b] Adjusted *P*-value calculated from GCRMA normalized data, and corrected using a Benjamini and Hochberg correction.

levels of expression between the genotypes. It would be interesting to test gene expression of RILs with the eight different allelic combinations of the three As tolerance loci on arrays. These experiments would allow confirmation of the candidate genes and facillitate the identification of gene networks common to the different alleles.

Recently, four QTLs have been identified for As concentration in rice (Zhang *et al*., 2008). However, there were no QTLs for As concentration detected on chromosome 10, and the QTL detected on chromosome 6 for seed As concentration is not in the same location as either of the tolerance loci detected in this study.

The phosphate–arsenic interaction data clearly indicate that the phosphorus status affects the toxicity of As(V), but suggest that this does not derive from competition with As(V) at the phosphate transporter, but rather reflects another mechanism of interaction between As and phosphorus. This is also supported by the array data which indicate that the phosphate transporter (OsPT2) is significantly down-regulated by As(V), but to a similar degree in both varieties [see companion paper (Norton *et al*., 2008)]. It is interesting to note that As-resistant *Chlamydomonas* mutants have higher phosphate uptake than wild types (Kobayashi *et al*., 2005), an observation that clearly indicates there is much that is unknown about phosphate–arsenic interactions.

Conclusions

In summary, the mapping of an As(V) tolerance gene was refined to reveal a remarkable three-gene epistatic interaction. Linking the genetic map to the sequenced rice genome, it was possible to construct a candidate gene list for the three tolerance loci.

Evidence is provided that genes related to phosphate transport are not likely to be behind the genetic loci conferring tolerance in Bala, since Bala is more tolerant in a range of environments with a wide range of phosphate availabilities. These results will be used to characterize candidate genes for As(V) tolerance further in rice. Some candidates offer possibilities of allowing modification of As metabolism in rice, such that its toxicity is reduced.

Supplementary data

Supplementary data (Tables S1–S3) can be found at *JXB* online.

Table S1. List of genes within 5 cM of *RZ516* (*AsTol6.1*).
Table S2. List of genes within 5 cM of *RG213* (*AsTol6.2*).
Table S3. List of genes within 5 cM of *RG257* (*AsTol10*).

Acknowledgements

GJN was employed on a BBSRC grant (BB/C509931/1). MN is supported by a Commonwealth Scholarship (BDCA-2004-5), and PNW was supported by a BBSRC (UK) studentship.

References

Abedin J, Cresser MS, Meharg AA, Feldmann J, Cotter-Howells J. 2002. Arsenic accumulation and metabolism in rice (*Oryza sativa* L.). *Environmental Science and Technology* **36,** 962–968.

Aposhian VH, Zakharyan RA, Avram MD, Sampayo-Reyes A, Wollenberg ML. 2004. A review of the enzymology of arsenic metabolism and a new potential role of hydrogen peroxide in the detoxication of the trivalent arsenic species. *Toxicology and Applied Pharmacology* **198,** 327–335.

Burkhead JL, Abdel-Ghany SE, Morrill JM, Pilon-Smits EAH, Pilon M. 2003. *The Arabidopsis thaliana CUTA* gene encodes an evolutionarily conserved copper binding chloroplast protein. *The Plant Journal* **34,** 856–867.

Dasgupta T, Hossain SA, Meharg AA, Price AH. 2004. An arsenate tolerance gene on chromosome 6 of rice. *New Phytologist* **163,** 45–49.

Kobayashi I, Fujiwara S, Shimogawara K, Sakuma C, Shida Y, Kaise T, Usuda H, Tsuzuki M. 2005. High intracellular phosphorus contents exhibit a correlation with arsenate resistance in *Chlamydomonas* mutants. *Plant and Cell Physiology* **46,** 489–496.

Lander ES, Green P, Abrahamson J, Barlow A, Daly MJ, Lincoln SE, Newburg L. 1987. Mapmaker: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. *Genomics* **1,** 174–181.

Lincoln S, Daly M, Lander E. 1992. *Mapping genes controlling quantitative traits with MAPMAKER/QTL 1.1,* 2nd edn. Whitehead Institute Technical Report.

Meharg AA, Hartley-Whitaker J. 2002. Arsenic uptake and metabolism in arsenic resistant and nonresistant plant species. *New Phytologist* **154,** 29–43.

Meharg AA, Jardine L. 2003. Arsenite transport into paddy rice (*Oryza sativa*) roots. *New Phytologist* **157,** 39–44.

Meharg AA, Macnair MR. 1992. Polymorphism and physiology of arsenate tolerance in *Holcus lanatus* L. from an uncontaminated site. *Plant and Soil* **146,** 219–225.

Norton GJ, Lou-Hing DE, Meharg A, Price AH. 2008. Rice–arsenate interactions in hydroponics: whole genome transcriptional analysis. *Journal of Experimental Botany* **59,** (in press).

Price AH, Steele KA, Moore BJ, Barraclough PB, Clark LJ. 2000. A combined RFLP and AFLP linkage map of upland rice (*Oryza sativa* L.) used to identify QTLs for root-penetration ability. *Theoretical and Applied Genetics* **100,** 49–56.

Raab A, Feldmann J, Meharg AA. 2004. The nature of arsenic–phytochelatin complexes in *Holcus lanatus* and *Pteris cretica*. *Plant Physiology* **134,** 1113–1122.

Raab A, Schat H, Meharg AA, Feldmann J. 2005. Uptake, translocation and transformation of arsenate and arsenite in sunflower (*Helianthus annuus*): formation of arsenic–phytochelatin complexes during exposure to high arsenic concentrations. *New Phytologist* **168,** 551–558.

Sano T, Kuraya Y, Amino S, Nagata T. 1999. Phosphate as a limiting factor for the cell division of tobacco BY-2 cells. *Plant and Cell Physiology* **40,** 1–8.

Sharples JM, Meharg AA, Chambers SM, Cairney JWG. 2000. Symbiotic solution to arsenic contamination. *Nature* **404,** 951–952.

Shen Y, Jiang H, Jin J, *et al*. 2004. Development of genome-wide DNA polymorphism database for map-based cloning of rice genes. *Plant Physiology* **135,** 1198–1205.

Thomas DJ, Waters SB, Styblo M. 2004. Elucidating the pathway for arsenic methylation. *Toxicology and Applied Pharmacology* **198,** 319–326.

World Health Organization. 2001. *WHO Fact sheet number 210*. Geneva: WHO.

Xu XY, McGrath SP, Zhao FJ. 2007. Rapid reduction of arsenate in the medium mediated by plant roots. *New Phytologist* **176,** 590–599.

Zhang J, Zhu YG, Zeng DL, Cheng WD, Qian Q, Duan GL. 2008. Mapping quantitative trait loci associated with arsenic accumulation in rice (*Oryza sativa*). *New Phytologist* **177,** 350–355.