

Would the real arsenate reductase please stand up?

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Over the last two decades a debate has been simmering within a group of plant biologists about the true identity of the enzyme that reduces arsenate to arsenite in plants. *Would the real arsenate reductase please stand up?* This debate was initiated with the release of the *Arabidopsis thaliana* genome allowing comparisons to be made between yeast and plants, progressed through the release of rice genome, the use of gene knockout, knockdown and overexpression, and ended with the application of forward genetics. This has not been an inconsequential debate. Inorganic arsenic is classified as a non-threshold class-1 human carcinogen. Further, its elevated level in rice (*Oryza sativa*) produced in Bangladesh, China, and India poses a real risk of cancer given the high consumption of rice typical of many Southeast Asian countries. Rice products (such as baby food) and juices (such as apple and grape) can also contain inorganic arsenic. In this issue of *New Phytologist* Xu *et al.* (pp. ??) provide new and exciting evidence for the identity and function of an enzyme that plays an important role in controlling arsenic accumulation in rice. This enzyme was identified by Xu and co-workers based on the simple assumption that genes involved in arsenic processing in plants would, when disabled, cause the plant to become more sensitive to the toxic effects of arsenic. Using this simple assumption, Xu and co-workers generated a large population of rice plants which contained randomly mutated genes. By screening this population for individual plants with increased sensitivity to arsenic, and by applying a mapping-by-sequencing approach, they were able to efficiently identify genes that play an important role in this process. What this revealed is that a gene encoding an enzyme that chemically reduces arsenate (As^{V}) to arsenite (As^{III}) is required for resistance of rice to the toxic effects of arsenate. In itself, such a discovery was not a surprise as it builds on almost 20-years of research into how plants cope with arsenate, which is toxic because it mimics phosphate and disrupts high-energy phosphate metabolism. However, the work of Xu *et al* provides strong evidence that it is the HAC-like class of arsenate reductases rather than the ACR2-like class that govern this critical step in how plants process arsenate into arsenic. Extending what we knew in *A. thaliana* into rice, allowing us to broaden our conclusions from dicots to monocots.

It is hard to say when a scientific debate starts given that each hypothesis arises out of previous conclusions, experimentation and discussion. But, in this case the publication of Pickering *et al.*, (2000) is as good a place as any to start. X-ray absorption spectroscopy (XAS) provides a unique tool for studying the chemical form of an element with minimal sample preparation, and it has been used to good effect in plants. Using this technique Pickering and co-workers were able to directly observe that plant roots when exposed to arsenate were able to convert essentially all the arsenate they accumulated into arsenite. The mechanism of this reduction was unknown at the time. In the same year an arsenate reductase (ACR2) was discovered in *Saccharomyces cerevisiae* (yeast) (Mukhopadhyay *et al.*, 2000), and shown to play a critical role in tolerance of yeast to arsenate. This mechanism involves the conversion of arsenate to arsenite which is then extruded from cells by a specific arsenite efflux protein. With the release of the completed *A. thaliana* genome in 2000 and the rice genome in 2005 it became possible to ask the question, does *A. thaliana* and rice contain a gene similar to the yeast gene encoding ACR2. Not surprisingly several groups did just that and identified the sequence called ACR2 in *A. thaliana* and ACR2;1 and ACR2;2 in rice. All studies provided convincing evidence that ACR2 in *A. thaliana*, and ACR2;1 and ACR2;2 in rice are arsenate reductase, from both heterologous expression in *E. coli* and using purified recombinant enzyme (Duan *et al.*, 2005 & 2007, Bleeker *et al.*, 2006, Dhanker *et al.*, 2006 and Ellis *et al.*, 2006). The discrepancies started to arise when evidence was sought for the *in planta* function of these genes. Using the *A. thaliana* loss-of-function *acr2-1* T-DNA insertional allele (SALK_143282) Duan *et al.*, (2005) published that loss of function of ACR2

leads to the complete loss of measurable arsenate reductase activity in root extracts, whereas Bleeker et al., (2006) using the same allele observed only a 36% reduction. Further, Bleeker et al., (2006) observed a significant reduction in accumulation of arsenic in shoots of *A. thaliana* in *acr2-1*. This was contrasted with a 10- to 16-fold increase in shoot arsenic in an *ACR2* knockdown line generated by RNAi (*ACR2Ri*) in *A. thaliana* reported by Dhanker et al., (2006). Such results promoted lively discussion at the *New Phytologist* sponsored meeting *Arsenic: unraveling its metabolism and speciation in plants*, held in Aberdeen, UK, June 2008 (Salt & Norton, 2008). Four years past before Liu et al., (2012) published convincing genetic evidence using two independent *A. thaliana* loss-of-function T-DNA alleles *acr2-1* and *acr2-2* (GABI-Kat 772G06) showing that loss of function of *ACR2* has no effect on conversion of arsenate to arsenite, accumulation of arsenic in roots or shoots, or efflux of arsenite from roots. It was concluded that the function of *ACR2* as an arsenate reductase *in planta* is completely redundant. After 12-years of work it appeared the field was back where it started, arsenate is reduced to arsenite in plant roots but we do not know how.

Around this time genome-wide association (GWA) mapping was emerging as a powerful tool for the use of natural genetic variation for the discovery of gene function. By assessing associations genome-wide between high-density SNP markers and leaf arsenic concentrations in over three hundred wild-collected accessions of *A. thaliana* grown in a controlled environment Chao et al., (2014) made a breakthrough. Using this new approach, they were able to identify a new type of arsenate reductase and called it High Arsenic Concentration1 (*HAC1*). Independently, Sanchez-Bermejo et al. (2014) identified the same gene as casual for an arsenate tolerance QTL they had identified in a synthetic recombinant *A. thaliana* population, and which they termed arsenate tolerance QTL1 (*ATQ1*). This novel arsenate reductase has only limited homology with yeast *ACR2*. Loss of function of *HAC1* in *A. thaliana* causes a clear decrease in the ability of roots to convert arsenate to arsenite, leading to increased arsenate accumulation in both roots and shoots. Strikingly, *HAC1* is also necessary for the efflux of arsenite from roots required for arsenate detoxification. It was suggested that *HAC1* is necessary for the reduction of arsenate to arsenite to create a specific pool of arsenite for efflux. This could occur through arsenate reduction and arsenite efflux being in close physical proximity. The expression of *HAC1* primarily in the epidermis and root hairs fits well with this role in arsenite efflux. With the identification of *HAC1* in *A. thaliana* an explanation emerged for the contradictory results regarding the role of *ACR2* in arsenate reduction (Dhanker et al. 2006; Liu et al. 2012). Since *ACR2* and *HAC1* share sequence identity within the region used to knock down expression of *ACR2* by RNA interference (RNAi) in *A. thaliana* (Dhanker et al. 2006), this RNAi sequence, targeted at *ACR2*, may have also suppressed *HAC1* expression. This would have led to the phenotypes observed by Dhanker et al. (2006) being due to the knockdown of *HAC1* and not *ACR2*. This would explain why the increased arsenic accumulation and sensitivity observed by Dhanker et al. (2006) using RNAi to knockdown *ACR2* actually matched those for *hac1* (Chao et al. 2014) and not those observed when *ACR2* was knocked out by T-DNA insertion (Liu et al., 2012).

Chao et al., (2014) also noted that *HAC1* homologues exist in rice. With this door open, genetic evidence rapidly emerged that *HAC1;1* and *HAC1;2* play similar roles in rice as *HAC1* in *A. thaliana*, being involved in both reduction of arsenate to arsenite and efflux of arsenite from roots (Shi et al. 2016). It was also reported that the rice genome contains 12 *HAC*-like genes (Shi et al. 2016). It is within this context that the work of Xi et al. (2017) was published on the identification of *HAC4* in rice. Based on the strength of its expression *HAC4* is the dominant arsenate reductase in rice roots, and its expression in the epidermis and exodermis is ideally suited to its role in reducing arsenate to arsenite for efflux of arsenite out of the root.

What is now emerging is a picture in which various *HAC* arsenate reductases have overlapping function in the different cell-types of the rice root to both drive arsenite efflux from the root and limit the translocation of arsenic to the shoot (Figure 1). *HAC1;1* and *HAC4* primarily work in the outer cell

layers of the root (root hair, epidermis and exodermis) to maintain a pool of arsenite for efflux. HAC1;2 in the epidermis, exodermis, outer layer of the cortex and endodermis perhaps in part to limit arsenate radial transport by conversion to arsenite and loading into the vacuole as a complex with phytochelatins (Zhao et al. 2010). HAC1;1 maintains a low concentration of arsenate in the pericycle to prevent its efficient loading into the xylem via phosphate transporters, as arsenate is a chemical analogue of phosphate. Moreover, given that there are at least a further eight uncharacterised HACs in rice, several of which are highly expressed in nodes and leaves (Xi et al. 2017), this is clearly not the end of the HAC story, but rather just the beginning.

Key words

Arsenic, arsenate, arsenite, arsenate reductase, *Arabidopsis thaliana*, rice, efflux, uptake, transport, ACR2, HAC1.

Figure Legends

Figure 1. Schematic of the proposed cell-type specific roles of HAC proteins in arsenic chemical transformation and transport in rice roots. Proposed functions of HAC proteins in the chemical transformations and transport of arsenic during its radial transport from the soil, across the root and into the central vascular system for transport to the shoot. Blue circles, arsenate uptake transporter; red circle, arsenite efflux transporter; As(V), arsenate; As(III), arsenite; PC-As(III), phytochelatin-arsenic(III) complex; CS, Casparian strip.

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