

1 **Dissecting the components controlling root-to-shoot arsenic translocation in**
2 *Arabidopsis thaliana*

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5 Chengcheng Wang^{1 a}, GunNam Na^{2 a}, Eduardo Sanchez Bermejo³, Yi Chen⁴, Jo Ann
6 Banks^{2 b}, David E Salt^{3 b} and Fang-Jie Zhao^{1 b}

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8 ¹ *State Key Laboratory of Crop Genetics and Germplasm Enhancement, College of*
9 *Resources and Environmental Sciences, Nanjing Agricultural University, Nanjing,*
10 *210095, China.*

11 ² *Department of Botany and Plant Pathology, Purdue University, West Lafayette, IN*
12 *47907, USA*

13 ³ *School of Biosciences, University of Nottingham, Sutton Bonington Campus,*
14 *Loughborough, Leicestershire LE12 5RD, UK.*

15 ⁴ *Department of Metabolic Biology, John Innes Centre, Norwich Research Park,*
16 *Norwich NR4 7UH, UK*

17

18 Authors for correspondence:

19 Fang-Jie Zhao

20 Tel: +86 25 8439 6509

21 Email: Fangjie.Zhao@njau.edu.cn

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23 ^a These authors contributed equally to this work.

24 ^b These authors contributed equally as senior authors to the study.

25

26 **Summary**

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28 • Arsenic (As) is an important environmental and food-chain toxin. We investigated
29 the key components controlling As accumulation and tolerance in *Arabidopsis*
30 *thaliana*.

31

32 • We tested the effects of different combinations of gene knockout, including
33 arsenate reductase (*HAC1*), γ -glutamyl-cysteine synthetase (γ -*ECS*), PC synthase
34 (*PCSI*) and phosphate effluxer (*PHO1*), and heterologous expression of the
35 As-hyperaccumulator *Pteris vittata* arsenite efflux (*PvACR3*) on As tolerance,
36 accumulation, translocation and speciation in *A. thaliana*.

37

38 • Heterologous expression of *PvACR3* markedly increased As tolerance and root to
39 shoot As translocation in *A. thaliana*, with *PvACR3* being localised to the plasma
40 membrane. Combining *PvACR3* expression with *HAC1* mutation led to As
41 hyperaccumulation in the shoots, whereas combining *HAC1* and *PHO1* mutation
42 decreased As accumulation. Mutants of γ -*ECS* and *PCSI* were hypersensitive to
43 As and had higher root to shoot As translocation. Combining γ -*ECS* or *PCSI* with
44 *HAC1* mutation did not alter As tolerance or accumulation beyond the levels
45 observed in the single mutants.

46

47 • *PvACR3* and *HAC1* have large effects on root to shoot As translocation. Arsenic
48 hyperaccumulation can be engineered in *A. thaliana* by knocking out *HAC1* gene
49 and expressing *PvACR3*. *PvACR3* and *HAC1* also affect As tolerance, but not to
50 the extent of γ -*ECS* and *PCSI*.

51

52 **Key words:** arsenic, arsenate reductase, arsenite efflux, arsenic accumulation,
53 tolerance, *Arabidopsis thaliana*, *Pteris vittata*

54 **Introduction**

55

56 Arsenic (As) is a toxic metalloid widely distributed in the environment. The transfer
57 of As from soil to the edible parts of crop plants is of great concern as dietary
58 exposure to As can present a significant risk to human health (Meharg *et al.*, 2009;
59 Zhao *et al.*, 2010). Arsenic is present in soil primarily as arsenate [As(V)] or
60 arsenite [As(III)] depending on the prevailing redox conditions. These As species
61 are taken up inadvertently by plant roots via phosphate transporters in the case of
62 As(V) (Shin *et al.*, 2004; González *et al.*, 2005; Wang *et al.*, 2016) or silicic acid
63 transporters (Ma *et al.*, 2008) and some aquaporin channels in the case of As(III)
64 (Isayenkov & Maathuis, 2008; Kamiya *et al.*, 2009; Xu *et al.*, 2015). Although
65 As(V) and As(III) are taken up readily by roots, their translocation from roots to
66 shoots is limited in most plant species studied to date (Raab *et al.*, 2007; Zhao *et al.*,
67 2009). A small number of fern species are able to hyperaccumulate As in the
68 above-ground parts (Ma *et al.*, 2001; Zhao *et al.*, 2002). These plants are
69 characterised by an exceptionally high ability to transport As from roots to the
70 above-ground tissues (Su *et al.*, 2008; Zhao *et al.*, 2009). Thus, the translocation of
71 As from roots to shoots appears to be the bottleneck controlling As accumulation in
72 the above-ground tissues, although the underlying mechanisms remain unclear.

73 One possible determinant of the As translocation efficiency is the capacity of
74 As(V) reduction in the roots. Recent studies have identified a new class of As(V)
75 reductases in plants, named HAC1 or ATQ1, that play an important role in
76 controlling As accumulation in the shoots (Chao *et al.*, 2014; Sanchez-Bermejo *et*
77 *al.*, 2014; Shi *et al.*, 2016; Xu *et al.*, 2017). In *Arabidopsis thaliana*, loss of function
78 of *HAC1* leads to an approximately 50-fold increase in As accumulation in the
79 shoots during short-term As(V) feeding hydroponic experiments and a 10-fold
80 increase in shoot As concentration after 5-weeks growth in a potting mix spiked
81 with 7.5 mg kg⁻¹ As(V) (Chao *et al.*, 2014). In rice, *oshac1;1 oshac1;2* double
82 mutant and *oshac4* single mutant had 2.3 and 3 fold, respectively, higher As
83 concentrations in the shoots compared with wild-type (WT) (Shi *et al.*, 2016; Xu *et*

84 *al.*, 2017). There are several possible reasons for the observed effect of the HACs
85 on the root to shoot As translocation. First, HACs reduces As(V) to As(III) to allow
86 the latter to be extruded to the external medium, thus decreasing the cellular As
87 content in the roots available for xylem loading (Chao *et al.*, 2014; Shi *et al.*, 2016;
88 Xu *et al.*, 2017). Second, decreased As(V) reduction in the roots of the HACs
89 mutants may allow As(V) to be loaded into the xylem efficiently via phosphate
90 transporters, although this hypothesis has not been tested. Third, As(V) reduction
91 catalysed by HACs allows the As(III) to be complexed by thiol compounds and
92 subsequently sequestered in the vacuoles in the roots (Song *et al.*, 2010; Song *et al.*,
93 2014), therefore decreasing the mobility of As. There is some evidence that the
94 complexation of As(III) with phytochelatins (PCs) decreases As(III) mobility from
95 roots to shoots in *A. thaliana*, with the shoot to root As concentration ratio in the PC
96 mutant *cad1;3* and the glutathione (GSH) mutant *cad1;2* being 5 – 10 fold higher
97 than wild-type plants (Liu *et al.*, 2010). In the As hyperaccumulator *P. vittata*, As(V)
98 remains the predominant As species in the roots after As(V) exposure and there is
99 also very little As(III)-thiol complexation (Zhao *et al.*, 2003; Zhang *et al.*, 2004;
100 Pickering *et al.*, 2006); both of which may contribute to the high efficiency of As
101 translocation in *P. vittata*.

102 Another key determinant of the root to shoot As translocation is xylem loading.
103 Both As(III) and As(V) are found in the xylem sap, although there is usually more
104 As(III) than As(V) even when plants are exposed to As(V) (reviewed in Zhao *et al.*,
105 2009). As(V) may be loaded into the xylem via phosphate transporters such as
106 PHO1, but *pho1* mutants did not show decreased As accumulation in the shoots
107 compared with WT plants (Quaghebeur & Rengel, 2004), suggesting that As(III) is
108 the main As species loaded into the xylem in WT plants. As(III) can be loaded into
109 the xylem via NIP3;1 and NIP7;1 in *A. thaliana* (Xu *et al.*, 2015; Lindsay &
110 Maathuis, 2016). In rice, the silicic acid efflux transporter Lsi2 is also able to
111 transport As(III) out of the cells toward the stele for xylem loading (Ma *et al.*, 2008).
112 A high expression of Lsi2 and its polar localisation on the proximal side of the
113 endodermal cells probably explain the relatively high translocation of As(III) in rice

114 (Ma *et al.*, 2008). In *P. vittata*, As(III) was found to be the main form of As in the
115 xylem sap (Su *et al.*, 2008). However, how As(III) is loaded into the xylem in this
116 As hyperaccumulator is still unknown. Indriolo *et al.* (2010) identified an As(III)
117 efflux transporter, PvACR3, that plays an important role in As(III) tolerance by
118 transporting As(III) into the vacuoles in the gametophyte of *P. vittata*. Intriguingly,
119 heterologous expression of *PvACR3* in *A. thaliana* increased As translocation from
120 roots to shoots (Chen *et al.*, 2013). Moreover, PvACR3 was found to be localised in
121 the plasma membrane in transgenic *A. thaliana*, suggesting a role of PvACR3 in
122 mediating As(III) efflux for xylem loading in this heterologous system (Chen *et al.*,
123 2013). Heterologous expression of the yeast *ScACR3* in rice or *A. thaliana* was
124 found to increase As(III) efflux to the external medium, but the effects on As
125 distribution between roots and shoots were inconsistent (Ali *et al.*, 2012; Duan *et al.*,
126 2012).

127 In the present study, we tested the effects of different combinations of gene
128 knockout and heterologous expression on As accumulation, especially the mobility
129 of As during root-to-shoot translocation, and As tolerance in *A. thaliana*. The genes
130 tested included the As(V) reductase (*HAC1*), γ -glutamyl-cysteine synthetase
131 (γ -*ECS*), PC synthase (*PCSI*), phosphate effluxer (*PHO1*) and *PvACR3*. We first
132 tested the effect of *PvACR3* expression in the WT or *hac1* mutant background. This
133 was followed by experiments investigating whether PHO1 affects As accumulation
134 in the shoots differently in the WT or *hac1* background. Finally, we tested the effect
135 of reduced thiol synthesis caused by γ -*ECS* or *PCSI* mutation in combination with
136 *HAC1* mutation. Our results show that *HAC1* mutation combined with *PvACR3*
137 expression dramatically increases As accumulation in *A. thaliana* shoots.

138

139 **Materials and Methods**

140

141 **Plant materials**

142

143 Plant materials used included *A. thaliana* wild-type Columbia-0 (Col-0) and *cad1-3*

144 (PC-deficient mutant), and *cad2-1* (GSH-deficient mutant) (Howden *et al.*, 1995a;
145 Howden *et al.*, 1995b), two T-DNA insertion knockout mutants of *HAC1*
146 (GABI_868F11, SM_3_38332 for *hac1-1* and *hac1-2*, respectively) (Chao *et al.*,
147 2014), and *pho1-2* mutant (Delhaize & Randall, 1995). All single mutants are in the
148 Col-0 background. Double mutants *cad2-1 hac1*, *cad1-3 hac1* and *pho1-2 hac1*
149 were generated by crossing respective single mutants. Homozygous double mutants
150 were identified by PCR genotyping of the F₂ progeny (Supplementary Fig. S1,
151 Table S1).

152 To generate the *35S::PvACR3* construct, *PvACR3* (UniProt #FJ751631) was
153 cloned into pCC0869, a pBI121-derived plant transformation vector containing 35S
154 CaMV promoter. The *PvACR3* gene was PCR amplified with the 5' primer 5'-
155 GCTCTAGAATGGAGAACTCAAGCG-3' (XbaI) and the 3' primer 5'- TCCCCC
156 GGGCTAAACAGAAGGCCCTTC-3' (SmaI) using cDNA derived from
157 arsenate-grown gametophytes of *P. vittata*, and the resulting PCR fragment
158 confirmed by sequencing. The resulting fragment was inserted into XbaI and
159 SmaI-linearized pCC0869 vector. *A. thaliana* (Col-0) was transformed with the
160 *35S::PvACR3* construct using *Agrobacterium* and the floral dip method (Clough &
161 Bent, 1998). Homozygous lines were identified in the T3 generation via segregation
162 analysis.

163 Two approaches were used to generate *HAC1* mutation and *PvACR3* expression
164 lines. First, a *PvACR3* expression line (E8) in the Col-0 background was crossed to
165 *hac1-1* and *hac1-2*. Homozygous *hac1-1 PvACR3* and *hac1-2 PvACR3* were
166 identified from F2 progeny by PCR genotyping. Second, *PvACR3* was expressed in
167 *hac1* directly. *PvACR3* was cloned into the 2X35S promoter cassette of pMDC32
168 between AscI and PacI restriction sites by recombination of the following primers:
169 5'-CGGGCCCCCTCGAGGCGCGCCATGGAGAACTCAAGCGCGGAGC-3'
170 (AscI) and 5'-
171 CCGCTCTAGAACTAGTTAATTAATAACTAAACAGAAGGCCCTTCC-3' (PacI),
172 using the ClonExpressTM II one step cloning kit (Vazyme). The binary vector
173 pMDC32-*PvACR3* was transformed into *Agrobacterium* strain GV3101 by

174 freeze-thaw method. The *Agrobacterium* culture was used to transform *A. thaliana*
175 *hac1-2* by *Agrobacterium*-mediated dip floral transformation (Clough & Bent,
176 1998). Homozygous lines were identified in the T3 generation, and three lines with
177 relatively high levels of *PvACR3* expression in the roots were selected.

178

179 Arsenic tolerance assays

180

181 *A. thaliana* seeds were surface-sterilized and sown on agar plates containing MS
182 medium (1/2 MS salts, 2% sucrose, pH 5.6, solidified with 1% agar) amended with
183 various concentrations of As(V) or As(III). Each line was replicated in 5 plates. All
184 plates were placed at 4°C in the dark for 2 days to synchronize germination. The
185 plates were then placed vertically in a growth chamber at 22°C with a 16-h light/8-h
186 dark regime. Root length and shoot fresh weight were determined after 2-week
187 growth.

188

189 Arsenic uptake and speciation

190

191 Different lines of *A. thaliana* were grown hydroponically with 1/5 strength
192 Hoagland nutrient solution (Liu *et al.*, 2010). Four-week old plants were exposed to
193 5 µM As(V) or As(III) for 24 h, with 4 replicates for each line. Phosphate was
194 withheld in the As(V) experiments to facilitate As(V) uptake. As(V) uptake and
195 As(III) efflux were estimated by measuring the changes in As speciation in the
196 nutrient solution as described previously (Liu *et al.*, 2010). Roots and shoots were
197 harvested for analysis of As speciation and concentration.

198 *PvACR3 hac1* and WT seeds were germinated on 1/2 MS medium without As
199 for 1 week. Seedlings were transplanted into an As contaminated soil for 3 weeks.
200 The soil contained 101 mg kg⁻¹ total As due to nearby mining activities. Each pot
201 was filled with 0.3 kg soil and planted with either 4 *A. thaliana* plants or 1 plant of
202 *P. vitatta* (2-3 frond stage), with 4 replicates per line. *PvACR3 hac1* and WT plants
203 were also grown in trays containing a vermiculite based potting compost spiked

204 with 10 mg kg⁻¹ As(V), with 5 replicates per line. Plants were grown in a growth
205 chamber at 22°C with a 16-h light/8-h dark regime. *A. thaliana* plants were
206 harvested 3 weeks after transplanting, whilst *P. vittata* plants were harvested 3 and 6
207 weeks after transplanting.

208

209 RNA extraction and Semi-quantitative RT-PCR

210

211 Total RNAs were extracted from 10-day-old *A. thaliana* roots and shoots using the
212 RNeasy plant Mini Kit (Biotech). Reverse transcription was carried out using the
213 R233-01 kit (Vazyme). Semi-quantitative RT-PCR was done in a reaction mixture
214 of 20 µl of 2 X Taq Master Mix (Vazyme) for 30 cycles. *Actin2* was used as the
215 reference gene (primer, forward 5'-TCACAGCACTTGCACCAAGCA-3', reverse
216 5'-AACGATTCCTGGACCTGCCTCA-3'). *HAC1* and *PvACR3* were PCR
217 amplified using the primer sets 5'-GAAGATGTTGAGACCGTTGATGTTT-3'
218 (forward) and 5'-TCACTTTCAAGTTTCAAGTGCCGAT-3' (reverse) for *HAC1*
219 and 5'-ATGGAGAACTCAAGCGCG-3' (forward) and
220 5'-GACCCACCCAGCATTTCAT -3' (reverse) for *PvACR3*.

221

222 Subcellular localisation of PvACR3

223

224 To investigate the subcellular localization of PvACR3 expressed in *A. thaliana*, a
225 *35S::PvACR3-GFP* fusion construct was generated. The *Aequorea coerulea* GFP
226 (*AcGFP*) coding region was amplified from plasmid pUC-AcGFP and inserted into
227 the *Sma*I and *Eco*RI sites of the plasmid pGreen 0299 plant transformation vector to
228 make pGreen-*AcGFP* constructs. The full-length *PvACR3* gene was amplified from *P.*
229 *vittata* cDNAs generated from RNA isolated from arsenate treated gametophytes
230 using the primers 5'-GCTCTAGAATGGAGAACTCAAGCG-3' (*Xba*I, forward) and
231 5'-TCCCCGGGAACAGAAGGCCCTTCCTC-3' (*Sma*I, reverse) then cloned in
232 frame to *AcGFP*. The final construct was confirmed by sequencing. The construct was
233 introduced to *A. thaliana* (Col-0) by *Agrobacterium*-mediated transformation. Stably

234 transformed *A. thaliana* plants expressing *PvACR3-GFP* were selected for GFP
235 fluorescence analysis. Images were acquired using a Zeiss LSM 710 laser confocal
236 microscope (Carl Zeiss Co., Germany). To label *A. thaliana* seedlings with the plasma
237 membrane dye FM4-64 (T13320, Invitrogen), 5-day-old whole seedlings grown on
238 petri dishes were incubated with 1 μ M FM4-64 in water for 15 min. Seedlings were
239 rinsed in distilled water and imaged immediately. To further confirm the subcellular
240 localisation of PvACR3, total microsomal membrane fractions were isolated from
241 4-week-old whole transgenic *A. thaliana* expressing *PvACR3*. Aqueous two-phase
242 extractions performed as previously described (Indriolo *et al.*, 2010).

243

244 Determination of As speciation and total As concentration

245

246 At the end of As exposure, roots were desorbed of the apoplastic As in an ice-cold
247 solution containing 1 mM K_2HPO_4 , 0.5 mM $Ca(NO_3)_2$ and 5 mM MES (pH 5.5) for
248 10 min (Xu *et al.*, 2007). Roots and shoots were rinsed with deionized water,
249 blotted dry and weighed. Plant samples were ground in liquid nitrogen to a fine
250 powder. Subsamples (~ 0.1 g) of the ground materials were extracted with 10 ml of
251 a phosphate buffer solution (2 mM NaH_2PO_4 , 0.2 mM Na_2-EDTA , pH 5.5). Arsenic
252 species in the nutrient solution and in the root and shoot extracts were determined
253 using high-performance liquid chromatography linked to inductively coupled
254 plasma mass spectrometry (HPLC-ICP-MS; NexIon 300x, Perkin-Elmer), as
255 described previously (Xu *et al.*, 2007). For the determination of total As
256 concentration in plant samples, plant tissues were washed with deionized water and
257 dried at 65°C for 2 d. Dried plant samples were digested with 5 ml mix acids of
258 $HNO_3/HClO_4$ (vol:vol = 85:15) in a digestion block. The digests were diluted with
259 2% HNO_3 and As concentrations were determined using ICP-MS (Perkin Elmer
260 NexION 300x).

261

262 Determination of non-protein thiols

263

264 After 24 h exposure to As(V), *A. thaliana* roots and shoots were separated and
265 immediately frozen in liquid nitrogen. Non-protein thiol compounds (Cys, GSH,
266 PC₂, PC₃ and PC₄) were extracted and quantified using a HPLC method with
267 monobromobimane (mBBr) derivatization as described previously (Minocha *et al.*,
268 2008).

269

270 Statistical analysis

271

272 One-way or two-way analysis of variance (ANOVA) was performed to test the
273 significance of treatment effects, followed by comparisons of treatment means
274 using Tukey's HSD test. Data were transformed logarithmically before ANOVA to
275 stabilize the variance where necessary. For the As tolerance assays, the
276 dose-response data were fitted to a log-logistic equation to estimate the effect
277 concentration causing 50% inhibition (EC₅₀).

278

279 **Results**

280

281 Heterologous expression of *PvACR3* enhances As tolerance and accumulation in *A.*
282 *thaliana*

283

284 Two independent lines of transgenic *A. thaliana* (Col-0) expressing *PvACR3* (E4 and
285 E8) were selected for As(V) and As(III) tolerance assays (Supplementary Fig. S2). In
286 the absence of As(III), no significant differences in root growth or shoot fresh weight
287 between WT and transgenic lines were observed (Fig. S2c, d). In the presence of toxic
288 As(III) concentrations (25 - 100 μ M As(III)), both lines grew significantly better than
289 WT plants (Fig. S2b, c, d). The EC₅₀ values were estimated from the fitted
290 dose-response curves (Table 1). Compared with WT, the expression of *PvACR3*
291 increased the EC₅₀ of As(III) by approximately 2 – 3 fold. The two *PvACR3*
292 transgenic lines also displayed enhanced tolerance to As(V) compared with WT plants
293 (Fig. S2b, e, f). The As(V) EC₅₀ values for the two *PvACR3* transgenic lines were 4.0

294 – 5.8 times of those for WT based on root growth, and 2.1 – 2.6 times those for WT
295 based on shoot biomass (Table 1). These results demonstrate that the expression of
296 *PvACR3* confers both As(III) and As(V) tolerance in *A. thaliana*.

297 We next investigated the effect of *PvACR3* expression on As accumulation in *A.*
298 *thaliana*. Plants were grown hydroponically and then exposed to 5 μ M As(III) or
299 As(V) for 2 days. In the As(III) experiment, the two *PvACR3* transgenic lines
300 accumulated significantly lower concentrations of As (by 35 – 45%) in the roots
301 than WT plants, but accumulated 25 – 34 fold higher As concentrations of As in the
302 shoots (Fig. 1a, b). The ratio of shoot to root As concentration was 0.12 – 0.20 in
303 the transgenic lines, compared with 0.003 in WT plants. Similar results were
304 obtained in the As(V) experiment, with the transgenic lines accumulating 28 – 50%
305 lower total As in the roots, but 29 – 37 fold higher total As in the shoots than WT
306 plants (Fig. 1c, d). The shoot to root As concentration ratio was 0.3 – 0.6 in the
307 transgenic lines, compared with 0.008 in WT plants. Arsenic speciation in root and
308 shoot tissues was determined in the As(V) experiment. As(III) was the predominant
309 As species in WT plants, accounting for 92% and 94% of the total As in the roots
310 and shoots, respectively, indicating an efficient As(V) reduction in *A. thaliana*.
311 Expression of *PvACR3* decreased the As(III)% in the roots to 88 – 89%, but
312 increased the As(III)% in the shoots to 95 – 96%.

313 Because *PvACR3* encodes an As(III) efflux transporter (Indriolo *et al.*, 2010),
314 we also estimated As(III) efflux from roots to the external medium following As(V)
315 uptake in the As(V) exposure experiment. We found no significant differences
316 between WT and *PvACR3* transgenic plants in either As(V) uptake or As(III) efflux,
317 with As(III) efflux accounting for 82 – 85% of the As(V) uptake after 1 day of As(V)
318 exposure (Fig. 1e).

319 To determine whether the expression of *PvACR3* in the root, the shoot or both
320 is important in conferring As tolerance in *A. thaliana*, four types of reciprocal grafts
321 between WT (Col-0) and *PvACR3*-E8 plants were generated. No significant
322 differences in root growth or shoot fresh weight between the four different graft
323 types grown in the absence of As(III) were observed (Fig. 2). Self-grafted WT

324 plants showed decreased root growth and shoot fresh weight in the presence of 25
325 μM As(III) while self-grafted *PvACR3*-E8 plants did not. When WT shoot scions
326 were grafted to *PvACR3*-E8 rootstocks, the resulting plants showed As tolerance
327 comparable to that of self-grafted *PvACR3*-E8 plants grown in the presence of 25
328 μM As(III) (Fig. 2). However, when *PvACR3*-E8 shoot scions were grafted to WT
329 rootstocks, the root growth and shoot fresh weight of the resulting plants were
330 similar to those of self-grafted Col-0 plants grown in the presence of As(III). This
331 experiment demonstrates that As tolerance in *PvACR3* expressing plants is driven
332 by the expression of *PvACR3* in the root and not the shoot.

333

334 *PvACR3* is localised to the plasma membrane in transgenic *A. thaliana* plants

335

336 The subcellular localisation of the *PvACR3* protein in *A. thaliana* was determined by
337 expressing *PvACR3:AcGFP* under the control of CaMV 35S promoter. Leaves and
338 roots from four independent transformed lines were incubated in the plasma
339 membrane dye FM4-64. Green fluorescence from AcGFP was observed to co-localise
340 with the red fluorescence of FM4-64 in both leaves and roots (Supplementary Fig.
341 S3a), indicating that the *PvACR3:AcGFP* fusion protein localises to the plasma
342 membrane. Furthermore, *PvACR3:AcGFP* appears to be preferentially expressed in
343 the endodermis and the stele of the transgenic *A. thaliana* roots (Supplementary Fig.
344 S4).

345 The plasma membrane localisation of *PvACR3:AcGFP* was also confirmed by
346 protein immunoblotting using antibodies to GFP after membrane purification using
347 two-phase extraction (Supplementary Fig. S3b). Membranes collected in the upper
348 phase of the extraction system are enriched in plasma membrane relative to the lower
349 phase as shown by the enrichment of the P-type ATPase plasma membrane marker
350 and the depletion of the V-type ATPase vacuolar membrane marker in the upper phase.
351 The plasma membrane enriched upper phase is also enriched in AcGFP confirming
352 the plasma membrane localisation of the *ACR3:AcGFP* fusion protein.

353

354 Combining *HAC1* mutation with *PvACR3* expression leads to As
355 hyperaccumulation in *A. thaliana*
356
357 Because mutation of *HAC1* also results in a large increase in As accumulation in the
358 shoots (Chao *et al.*, 2014), we tested the combined effect of *HAC1* mutation with
359 *PvACR3* expression. We first crossed *PvACR3*-E8 with two T-DNA insertion *HAC1*
360 knockout lines and obtained homozygous lines combining *PvACR3* expression with
361 *HAC1* mutation. The As(V) tolerance results of *PvACR3*-E8 *hac1-1* and
362 *PvACR3*-E8 *hac1-2* are similar, so only the dataset of the former is shown here.
363 Consistent with the results shown in Fig. S2 and those reported by Chao *et al.*
364 (2014), *PvACR3* expression increased As(V) tolerance in *A. thaliana*, whereas *hac1*
365 mutants were more sensitive to As(V) than WT plants (Supplementary Fig. S5,
366 Table 1). In this experiment, the highest As(V) concentration (250 μ M) did not
367 significantly inhibit root or shoot growth of *PvACR3*-E8; therefore the exact EC₅₀
368 could not be estimated (Table 1). Expression of *PvACR3* in *hac1* mutants enhanced
369 the As(V) tolerance to a level that was similar to or higher than that of WT plants,
370 but lower than that of *PvACR3*-E8 plants, especially at the high (250 μ M) As(V)
371 concentration (Fig. S5, Table 1).

372 We then determined As accumulation and speciation in hydroponically grown
373 plants exposed to 5 μ M As(V) for 1 day. *HAC1* mutation resulted in a 35% decrease
374 in the root As(III) concentration, but a 16 – 24 fold increase in the root As(V)
375 concentration, compared with WT (Fig. 3a). The percentage of As(III) in the root
376 (relative to total As) decreased from 90% in WT plants to 19 – 27% in *hac1* mutants,
377 which is indicative of a loss of function of a key As(V) reductase. *PvACR3*
378 expression in the Col-0 background decreased As(III) concentration in the roots by
379 74%, but had little effect on the As(V) concentration. Combining *PvACR3*
380 expression with *HAC1* mutation decreased both As(III) and As(V) concentrations in
381 the roots compared with the *hac1* mutants. *HAC1* mutation, *PvACR3* expression and
382 the combination of the two genetic events produced striking phenotypes in As
383 accumulation *A. thaliana* shoots. Compared with WT (Col-0), *HAC1* mutation and

384 *PvACR3* expression increased shoot As concentration by 18 - 19 and 58 fold,
385 respectively, whereas combining the two events increased shoot As concentration
386 by 114 – 117 fold (Fig. 3b). The increase in the shoot As concentration in
387 *PvACR3*-E8 *hac1* plants was more than the additive effect of *PvACR3*-E8 and *hac1*
388 alone. In all lines, As(III) was the predominant As species in the shoots (>90%). In
389 this experiment, the shoot to root As concentration ratio increased from 0.002 in
390 WT to approximately 0.015 in *hac1* mutants and 0.24 in *PvACR3*-E8 *hac1* plants.
391 We also determined As(III) efflux from the roots to the external medium following
392 As(V) uptake. In agreement with a previous study (Chao *et al.*, 2014), *HAC1*
393 mutation greatly decreased As(III) efflux to the external medium (Fig. 3c). By
394 contrast, *PvACR3* expression had little effect on this process in either the Col-0 or
395 *hac1* background.

396 We also generated *PvACR3* expression lines in the *hac1* mutant (*hac1-2*)
397 directly by transgenesis. Three independent lines of *PvACR3 hac1-2* (E6, E7, E11)
398 were selected for further experiments (Supplementary Fig. S6a). The three
399 transgenic lines, *hac1-2* and Col-0 were grown for 3 weeks in a vermiculite-based
400 potting medium amended with or without 10 mg kg⁻¹ As(V) (Fig. S6b). The
401 addition of 10 mg kg⁻¹ As(V) inhibited shoot fresh weight of Col-0 and *hac1-2* by
402 28% and 41%, respectively. The inhibition on the three lines of *PvACR3 hac1-2*
403 ranged from 31% to 52%, which was not significantly different from that in *hac1-2*
404 (Fig. 4a). Compared with Col-0, *hac1-2* contained 8.5 fold higher total As
405 concentration in the shoots (Fig. 4b). Expression of *PvACR3* in *hac1-2* enhanced As
406 accumulation in the shoots much further, to 17 – 28 times of that in Col-0. The
407 bioaccumulation factors (shoot to soil As concentration ratio) were 0.2, 2.0 and 4.0
408 – 6.1 in Col-0, *hac1-2* and *PvACR3 hac1-2*, respectively.

409 To assess the As accumulation ability of *PvACR3 hac1-2* plants in soil, the
410 three transgenic lines, *hac1-2*, Col-0 and *P. vittata* were grown in an
411 As-contaminated soil. Shoot biomass after 3-week growth was not significantly
412 different between different *A. thaliana* lines (Fig. 5a). Compared with Col-0, shoot
413 As concentration was 9.7 and 35 – 39 times higher in *hac1-2* and the three lines of

414 *PvACR3 hac1-2*, respectively (Fig. 5b). After *P. vittata* was transplanted into the
415 same soil for 3 or 6 weeks, there was no significant increase in the frond biomass
416 (Fig. 5c). There was also no significant increase in As concentration in the fronds 3
417 weeks after transplanting compared with the initial As concentration at the time of
418 transplanting (Fig. 5d). Six weeks after transplanting, As concentration in *P. vittata*
419 fronds increased from the initial level of 14 mg kg⁻¹ to 54 mg kg⁻¹ (Fig. 5d). The net
420 increase in frond As concentration was comparable to the shoot As concentrations
421 of the *PvACR3 hac1-2* lines grown in the same soil over 3 weeks.

422

423 Combining *HAC1* and *PHO1* mutation decreases As accumulation in *A. thaliana*
424 shoots

425

426 Because *hac1* mutants accumulated large amounts of As(V) in the roots (Fig. 3a),
427 we hypothesized that As(V) may be loaded into the xylem in the roots via the PHO1
428 phosphate exporter for long-distance transport to the shoots. To test this hypothesis,
429 we crossed *pho1-2* mutant (Delhaize & Randall, 1995) with *hac1-1* mutant (Chao *et*
430 *al.*, 2014) to generate a double mutant. In hydroponic culture with a normal level of
431 phosphate concentration (0.1 mM), the *pho1-2* mutant plants were smaller than WT,
432 with approximately 70% and 40% inhibition of the shoot and root biomass,
433 respectively (Fig. 6a). This phenotype is similar to that reported before (Delhaize &
434 Randall, 1995; Rouached *et al.*, 2011). The *hac1-1* mutant was also smaller than
435 WT (by approximately 20% in both the shoot and root biomass). The *hac1-1 pho1-2*
436 double mutant showed the same growth phenotype as *pho1-2* (Fig. 6a). Arsenic
437 speciation in roots and shoots was determined after plants were exposed to 5 μM
438 As(V) for 1 day in the absence of phosphate. The *pho1-2* mutant showed no
439 significant differences from Col-0 in the total concentrations of As in the roots and
440 shoots, and there were also no significant differences in As speciation with As(III)
441 accounting for 95 – 98% of the total As (Fig. 6b, c). By contrast, the *hac1-1* mutant
442 had 2.2 and 57 times higher As concentration in the roots and shoots, respectively,
443 compared with Col-0. The increased root As concentration in *hac1-1* was mainly in

444 the form of As(V), accounting for 58% of the total As, whereas most of the
445 increased shoot As concentration was in the form of As(III). The As accumulation
446 phenotype in *hac1-1* was reduced by 85% in the *hac1-1 pho1-2* double mutant,
447 although total As concentrations in the roots and shoots of the double mutant were
448 still significantly higher than those of Col-0 (by 20% and 6 fold, respectively) (Fig.
449 6b, c). The As(III)% in the roots of the double mutant was 59%, which was lower
450 than Col-0 and *pho1-2* (95 – 96%) but higher than *hac1-2* (42%). The results
451 suggest that *PHO1* mutation had no impact on As accumulation in the Col-0
452 background, but greatly suppressed As translocation from the roots to the shoots in
453 the *hac1-2* background.

454

455 Effects of GSH and PC mutants in combination with HAC1 mutation on As
456 tolerance and accumulation

457

458 It is well known that GSH and PCs are crucial for As detoxification through the
459 formation of As(III)-thiol complexes (Ha *et al.*, 1999; Pickering *et al.*, 2000; Liu *et*
460 *al.*, 2010). Moreover, the sequestration of the As(III)-thiol complexes in the root
461 vacuoles reduces the root to shoot translocation of As in *A. thaliana* Col-0 (Liu *et*
462 *al.*, 2010). Here, we investigated the effects of combining mutations that reduce
463 GSH (*cad2-1*) or PC (*cad1-3*) concentrations (Howden *et al.*, 1995a; Howden *et al.*,
464 1995b; Cobbett *et al.*, 1998) with *hac1-1* and *hac1-2* mutants (Chao *et al.*, 2014) on
465 As tolerance and accumulation. As the double mutants of *cad2-1 hac1-1* and *cad1-3*
466 *hac1-1* behaved similarly to *cad2-1 hac1-2* and *cad1-3 hac1-2*, respectively, only
467 one set of data are presented. In addition to genotyping based on the mutated genes,
468 we also determined the concentrations of Cys, GSH and PCs in the roots and shoots
469 of WT, single and double mutants after exposure to 5 μ M As(V) for 1 day. The
470 results are consistent with expectations, with *cad2-1* and *cad2-1 hac1-2* containing
471 lower levels of GSH and PCs than WT and *cad1-3* and *cad1-3 hac1-2* containing
472 almost no PCs (Supplementary Fig. S7).

473 Tolerance to As(V) was assessed by measuring root and shoot biomass after

474 plants were grown on agar plates amended with 0 – 250 μ M As(V). Consistent with
475 previous reports (Ha *et al.*, 1999; Liu *et al.*, 2010), *cad2-1* and *cad1-3* mutants were
476 hypersensitive to As(V), with EC₅₀ being at least 10 times lower than WT based on
477 root growth and approximately 20 times lower than WT based on shoot growth
478 (Supplementary Fig. 8, Table 1). *hac1* mutants were also more sensitive than Col-0,
479 but not to the extent of *cad2-1* and *cad1-3* mutants. The *cad2-1 hac1-2* and *cad1-3*
480 *hac1-2* double mutants showed the same As(V) sensitivity as the *cad2-1* and *cad1-3*
481 single mutant, respectively, with similar EC₅₀ values between the single and double
482 mutants (Table 1).

483 After exposure to 5 μ M As(V) for 1 day, *hac1-2* accumulated 2 and 12 fold
484 more As in the roots and shoots, respectively, than Col-0 (Fig. 7a, b). The *cad2-1*
485 and *cad1-3* mutants accumulated less As in the roots (~50%) but more As in the
486 shoots (~3 fold) than Col-0. The *cad2-1 hac1-2* and *cad1-3 hac1-2* double mutants
487 behaved similarly to the *hac1-2* single mutant, with the exception that *cad1-3*
488 *hac1-2* had 25% lower shoot As concentration than *hac1-2*. With regard to As
489 speciation, *HAC1* mutation markedly decreased the ability of roots to reduce As(V)
490 to As(III), resulting in a decrease in the proportion of As(III) in the total As from
491 85% in Col-0 to 18% in *hac1-2* (Fig. 7a). *cad2-1* and *cad1-3* mutants also had lower
492 As(III)% (76% and 80%, respectively) in the roots than WT. Combining either
493 *cad2-1* or *cad1-3* with *hac1-2* further decreased the As(V) reduction ability,
494 decreasing the As(III)% in the *cad2-1 hac1-2* and *cad1-3 hac1-2* roots to 1.7% and
495 14%, respectively. Most of the As in the shoots was in the form of As(III) (93 –
496 100%), with little difference between Col-0, single and double mutants (Fig. 7b).

497

498 **Discussion**

499

500 Combining *HAC1* mutation with *PvACR3* expression leads to As
501 hyperaccumulation in *A. thaliana*

502

503 Previous studies have shown that *HAC1* mutation (Chao *et al.*, 2014) or expression

504 of *PvACR3* in the Col-0 background of *A. thaliana* (Chen *et al.*, 2013) markedly
505 increases As accumulation in the above-ground tissues of *A. thaliana*. Here, we
506 show that a combination of these two genetic events leads to As hyperaccumulation
507 in the shoots of *A. thaliana* (Figs. 3-5). The combined effect is more than additive
508 of the two events alone. Moreover, when *hac1 PvACR3* plants were grown in an As
509 contaminated soil for 3 weeks, they accumulated As in the shoots to levels
510 comparable to the As hyperaccumulating fern *P. vittata* grown on the same soil for 6
511 weeks after transplanting (Fig. 5).

512 Consistent with the previous study (Chao *et al.*, 2014), *HAC1* mutation leads to
513 a greatly decreased As(III) efflux from the roots to the external medium following
514 As(V) uptake and a markedly increased As translocation from the roots to the
515 shoots (Fig. 3). This effect has also been observed in the rice mutants of *OsHAC1;1*,
516 *OsHAC1;2* and *OsHAC4*, which are homologous genes of *HAC1* (Shi *et al.*, 2016;
517 Xu *et al.*, 2017). By contrast, expression of the *P. vittata* As(III) efflux transporter
518 gene *PvACR3* in *A. thaliana* did not increase the efflux of As(III) to the external
519 medium (Figs. 1 and 3). This result is different from the study of Chen *et al.* (2013),
520 which reported increased As(III) efflux to the external medium in the *PvACR3*
521 expressing lines. A closer examination of the data by Chen *et al.* (2013) reveals that
522 the amount of As(III) extruded into the medium was barely detectable in both Col-0
523 and transgenic lines, suggesting that their experimental method was not optimized
524 to allow a reliable determination of As(V) uptake and As(III) efflux. Also different
525 from the study of Chen *et al.* (2013) is our observation of a much larger
526 enhancement of root to shoot As translocation and shoot As accumulation in the
527 *PvACR3* transgenic lines (Fig. 1). This enhancement occurred regardless of whether
528 As was supplied to plants in the form of As(V) or As(III) (Fig. 1). Although
529 *PvACR3* was shown to be a vacuolar As(III) transporter in the gametophyte of *P.*
530 *vittata* (Indriolo *et al.*, 2010), *PvACR3* was found to be localised to the plasma
531 membrane in both the root and leaf cells of *A. thaliana* plants heterologously
532 expressing *PvACR3:GFP* (Supplementary Fig. S3) (also Chen *et al.*, 2013). In
533 transgenic *A. thaliana* lines, *PvACR3* likely acts as a plasma membrane localised

534 As(III) efflux transporter for the loading of As(III) into the xylem. The fact that
535 PvACR3 did not enhance As(III) efflux to the external medium could be explained
536 by a preferential accumulation of the protein in the endodermis and the stele in the
537 roots of the transgenic plants (Supplementary Fig. S4). Although the CaMV35S
538 promoter used to drive the expression of *PvACR3:GFP* is a constitutive promoter, it
539 is not unusual that such a promoter can lead to a preferential expression in the
540 vascular tissues (Benfey *et al.*, 1989). Thus, decreasing As(III) efflux to the external
541 medium by disrupting *HAC1* and enhancing As(III) efflux to the xylem by
542 expressing *PvACR3* are sufficient to induce As hyperaccumulation in *A. thaliana*
543 shoots. These are the two key traits postulated to explain As hyperaccumulation in *P.*
544 *vittata* (Su *et al.*, 2008; Zhao *et al.*, 2009). In fact, As speciation analysis of *P.*
545 *vittata* roots exposed to As(V) showed relatively low percentages (13 – 19%) of
546 As(III) (Zhao *et al.*, 2003), suggesting that As(V) reduction is limited in *P. vittata*
547 roots, which is similar to *A. thaliana hac1* mutants. It would be interesting to
548 investigate whether *P. vittata* possesses functional HAC1-like enzymes.

549 Despite HAC1 playing an important role in As(V) reduction in *A. thaliana*
550 roots (Figs. 3, 6, 7), there likely exist other As(V) reductases or non-enzymatic
551 As(V) reduction mechanisms that contribute to As(V) reduction in the *hac1* mutants
552 (Chao *et al.*, 2014). As(III) produced by these additional reduction mechanisms is
553 then loaded into the xylem via PvACR3, as well as indigenous As(III) transporters
554 such as AtNIP3;1 (Xu *et al.*, 2015). The action of PvACR3 may also enhance As(V)
555 reduction in the roots by alleviating the feedback inhibition of As(III), thus
556 explaining decreased concentrations of not only As(III) but also As(V) in the roots
557 of *hac1 PvACR3* plants (Fig. 3). Different from *hac1 PvACR3* plants, *hac1 pho1*
558 double mutant had a much lower As concentration in the shoots than *hac1* single
559 mutant (Fig. 6). The phenotype of *hac1 pho1* double mutant with a markedly
560 decreased As accumulation in the shoots suggests that As(V) is also loaded into the
561 xylem via PHO1. This mechanism is important in the *hac1* background because of
562 the build-up of As(V) in the roots, but not in the Col-0 background because most of
563 the As(V) taken up was reduced to As(III) (Figs. 3 and 6). Therefore, xylem loading

564 of As(V) mediated by PHO1 is important for shoot As accumulation in *hac1* plants,
565 whereas As(III) is likely to be the predominant form of As transported to the shoots
566 in Col-0 and *hac1 PvACR3* plants. In the shoots, however, most of the As was in the
567 form of As(III) even in the *hac1* background, suggesting a strong capacity of As(V)
568 reduction that is little unaffected by *HAC1* mutation. As(V) reduction in the shoots
569 may be mediated by other As(V) reductases or non-enzymatic reactions (Chao *et al.*,
570 2014).

571 Another possible contributing factor in As hyperaccumulation in *P. vittata* is
572 that most of the As in the roots is not complexed with thiol compounds and hence is
573 highly mobile for root to shoot translocation (Zhao *et al.*, 2009). The shoot to root
574 As concentration ratio was significantly higher in the *cad1-3* and *cad2-1* mutants
575 than in Col-0 (Fig. 7), consistent with a higher As mobility in these mutants.

576 However, this effect is far smaller than that caused by either *HAC1* mutation or
577 *PvACR3* expression (Figs. 3 and 7). Combining thiol mutants with *HAC1* mutation
578 also did not increase the root to shoot As translocation beyond the level observed in
579 the *hac1* mutant (Fig. 7). These results suggest that the effect on root to shoot As
580 translocation ranks in the following order: As(III) xylem loading mediated by
581 *PvACR3* > loss of function of *HAC1* > limited As(III)-thiol complexation in roots.

582

583 Comparisons of the contribution of GSH, PCs, *HAC1* and *PvACR3* to As tolerance

584

585 The hypersensitivity of *cad1-3* and *cad2-1* to As(V) or As(III) demonstrates the
586 critical roles of PCs and GSH in As tolerance in *A. thaliana* (Ha *et al.*, 1999; Li *et al.*
587 *et al.*, 2006; Liu *et al.*, 2010) (also Fig. S8, Table 1). *HAC1* is also important for the
588 tolerance to As(V), but not to As(III) (Chao *et al.*, 2014; Sanchez-Bermejo *et al.*,
589 2014). However, the *hac1* mutant showed As(V) sensitivity at a much higher As(V)
590 concentration than either *cad1-3* or *cad2-1*, even though *cad1-3* and *cad2-1*
591 accumulated much lower levels of As in both roots and shoots than *hac1* (Fig. S8,
592 Table 1). Moreover, *hac1 cad1-3* and *hac1 cad2-1* were no more sensitive to As(V)
593 than *cad1-3* and *cad2-1* single mutant, respectively. These results support the notion

594 that internal detoxification of As via complexation with thiol compounds represents
595 a fundamental mechanism of As tolerance in As nonhyperaccumulating plants that
596 is required even at relatively low levels of As exposure, whereas HAC1 mediated
597 As(V) reduction and subsequent As(III) efflux confers As(V) tolerance only at high
598 levels of As(V) exposure. Interestingly, *hac1 cad2-1* roots contained As almost
599 exclusively in the form of As(V) after exposure to 5 μ M As(V) for 1 day (Fig. 7),
600 demonstrating that the double mutant has lost most of the As(V) reduction capacity.
601 This result suggests that GSH is required as a reductant for either enzymatic or
602 non-enzymatic As(V) reduction.

603 Unlike As non-hyperaccumulators, only a very small proportions of As in *P.*
604 *vittata* is complexed with thiol compounds (Webb *et al.*, 2003; Zhao *et al.*, 2003;
605 Zhang *et al.*, 2004; Pickering *et al.*, 2006), suggesting a limited role of PCs in As
606 tolerance in the hyperaccumulator. Vacuolar sequestration of As(III) appears to be
607 the key mechanism of As tolerance in *P. vittata*, with PvACR3 playing an important
608 role in this process (Indriolo *et al.*, 2010). Expression of *PvACR3* in *A. thaliana*
609 significantly increased its tolerance to both As(V) and As(III) (Fig. S2). This result
610 is in agreement with the study by Chen *et al.* (2013). However, their suggestion that
611 *PvACR3* enhances As tolerance by extruding As(III) from root cells to the external
612 medium is not supported by our data (Fig. 1). Instead, *PvACR3* likely mediates
613 xylem loading of As(III), thus reducing the build-up of As(III) in the root cells. It
614 has been shown that in cowpea (*Vigna unguiculata*) root growth is particularly
615 sensitive to As(V) exposure with As preferentially accumulating in the root apex,
616 causing damage to the meristem (Kopittke *et al.*, 2012). By exporting As(III) from
617 the roots to the less sensitive shoot tissues, *PvACR3* may enhance the overall As
618 tolerance of the plant. This hypothesis is supported by the grafting experiment,
619 which shows that *PvACR3* expressed in the root alone is sufficient to explain the
620 enhanced As tolerance in *PvACR3* expressing *A. thaliana* plants (Fig. 2). *PvACR3*
621 expression in the *hac1* background also partially rescued the As(V) sensitive
622 phenotype caused by *HAC1* mutation (Fig. S5), which may be attributed to
623 decreased As(III) concentration in the roots (Fig. 3). Despite increased As tolerance

624 in *PvACR3* expressing *A. thaliana*, the level of tolerance is still below that in *P.*
625 *vittata*, suggesting the existence of other tolerance mechanisms. Of the genes tested
626 in the present study, their relative importance to As tolerance ranks in the following
627 order: thiol production by PCS1 and γ -ECS > xylem loading of As(III) by
628 *PvACR3* > As(V) reduction by HAC1.

629

630 Taken together, our study has demonstrated that As hyperaccumulation can be
631 engineered in *A. thaliana* by knocking out *HAC1* gene and expressing *PvACR3*. The
632 same approach may be applied to high biomass plant species for the purpose of
633 phytoremediation of As-contaminated soil. Expression of *PvACR3* also enhances As
634 tolerance, though not to the level found in *P. vittata*.

635

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642

643 **Author contributions**

644 F-J.Z., J.A.B. and D.E.S. designed the research. C.W., G.N.N., E.S.B. and Y.C.
645 performed the experiments and analysed the data. F-J.Z., C.W., J.A.B. and D.E.S.
646 wrote the paper with contributions from all the authors.

647

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789 role of phytochelatins in arsenic tolerance in the hyperaccumulator *Pteris*
790 *vittata*. *New Phytologist* **159**: 403-410.

791

792 Table 1. The effect concentration of As(III) or As(V) causing 50% inhibition of root or shoot growth
 793 ($EC_{50} \pm SE$) of Col-0, mutants and *PvACR3* expression lines of *Arabidopsis thaliana*

Lines	As(V) EC_{50} (μM)		As(III) EC_{50} (μM)	
	Root length	Shoot biomass	Root length	Shoot biomass
Col-0	76.5 \pm 15.4	88.9 \pm 155.9	12.9 \pm 3.4	17.2 \pm 8.9
<i>PvACR3</i> -E4	307.4 \pm 56.3	230.0 \pm 58.3	29.6 \pm 5.0	36.9 \pm 7.5
<i>PvACR3</i> -E8	444.0 \pm 36.1	185.6 \pm 81.5	38.9 \pm 4.1	42.0 \pm 17.6
Col-0	243.6 \pm 14.3	106.9 \pm 10.3		
<i>hac1-2</i>	90.9 \pm 17.7	93.8 \pm 21.7		
<i>PvACR3</i> -E8 <i>hac1-2</i>	222.4 \pm 55.4	253.4 \pm 80.4		
Col-0	> 250 ^b	163.3 \pm 34.3		
<i>cad2-1</i>	21.8 \pm 1.0	8.2 \pm 1.4		
<i>hac1-2</i>	92.8 \pm 11.5	94.8 \pm 11.1		
<i>cad2-1 hac1-2</i>	19.2 \pm 1.3	7.8 \pm 1.6		
Col-0	> 250 ^b	208.7 \pm 33.5		
<i>cad1-3</i>	23.2 \pm 1.4	8.4 \pm 1.4		
<i>hac1-2</i>	147.9 \pm 31.9	136.8 \pm 18.4		
<i>cad1-3 hac1-2</i>	24.3 \pm 10.3	11.4 \pm 1.4		

794 ^a Experiment 1 and Experiments 2-4 used different agars, which contained different phosphate
 795 concentrations and therefore resulted in different EC_{50} values for Col-0.

796 ^b Where the highest As dose did not result in a significant inhibition, EC_{50} could not be estimated and
 797 was considered to be higher than the largest As concentration in the experiment.

798

799 **List of Figures:**

800

801 **Fig. 1** Arsenic accumulation in transgenic *A. thaliana* expressing *PvACR3*. (a, b) Total
802 As concentration in roots (a) and shoots (b) of Col-0 and *PvACR3* expressing lines
803 after plants were exposed to 5 μM As(III) for 2 days. (c, d) The concentrations of
804 As(III) and As(V) in roots (c) and shoots (d) of Col-0 and *PvACR3* expressing lines
805 after plants were exposed to 5 μM As(V) for 2 days in the absence of phosphate. (e)
806 As(V) uptake and As(III) efflux to the external solution after plants were exposed to 5
807 μM As(V) for 1 day in the absence of phosphate. Data are means \pm SE (n = 4). The %
808 values in (e) represents As(III) efflux as a % of As(V) uptake. Different letters above
809 bars indicate significant differences at $P < 0.05$.

810

811 **Fig. 2** Arsenic tolerance of reciprocally grafted *A. thaliana* plants expressing *PvACR3*.
812 Reciprocally grafted seedlings were grown on plates in the absence of arsenic (a) or in
813 the presence of 25 μM arsenite (b); self grafted Col-0 (Col-0/Col-0); self grafted
814 *PvACR3* expressing line (ACR3/ACR3); *PvACR3* expressing line shoot scion with
815 Col-0 rootstock (ACR3/Col-0); Col-0 shoot scion with *PvACR3* expressing line
816 rootstock (Col-0/ACR3). Root growth (c) and shoot fresh weight (d) in the presence
817 of 25 μM arsenite relative to control treatment. Data point are mean \pm SE (n = 4).
818 Same letter within graphs represents lines that are not significantly different ($P > 0.05$).
819 *PvACR3*-E8 lines was used as a *PvACR3* expressing transgenic line.

820

821

822 **Fig. 3** Effect of combining *PvACR3* expression with *HAC1* mutation on As
823 accumulation and speciation in *A. thaliana*. (a, b) Arsenic speciation in the roots (a)
824 and shoots (b) of different *A. thaliana* lines grown in hydroponic culture and exposed
825 to 5 μM As(V) for 1 day without phosphate. (c) As(V) uptake and As(III) efflux into
826 the nutrient solution after 1-day exposure to 5 μM As(V) without phosphate. Data are
827 means \pm SE (n = 4). The % values in (c) represents As(III) efflux as a % of As(V)

828 uptake. Different letters above bars indicate significant differences at $P < 0.05$.

829

830 **Fig. 4** Arsenic accumulation in transgenic *A. thaliana hac1* mutant expressing
831 PvACR3 grown in a potting medium. (a) Shoot dry weights of different lines as
832 affected by the addition of 10 mg kg⁻¹ As(V). (b) Total As concentration in the shoots
833 of different lines grown in a potting medium amended with 10 mg kg⁻¹ As(V). Data
834 are means \pm SE (n=4). The values inside open bars represent the percentage values
835 relative to the control. Different letters above bars represent significant difference at P
836 < 0.05 .

837

838 **Fig. 5** Arsenic accumulation in transgenic *A. thaliana hac1-2* mutant expressing
839 PvACR3 and *P. vittata* grown in an As-contaminated soil. (a) Shoot dry weights of
840 different *A. thaliana* lines. (b) Total As concentrations in the shoots of different lines
841 of *A. thaliana* 3 weeks after transplanting. (c) Frond dry weights of *P. vittata*. (d) Total
842 As concentrations in the fronds of *P. vittata* at the time of transplanting and 3 or 6
843 weeks after transplanting. Data are means \pm SE (n = 4). Different letters above bars
844 represent significant difference at $P < 0.05$.

845

846 **Fig. 6** Effect of combining *PHO1* and *HAC1* mutation on As accumulation in *A.*
847 *thaliana*. (a) Shoot and root fresh weights of Col-0, *hac1-1*, *pho1-2* and *hac1-1*
848 *pho1-2* plants of *A. thaliana*. (b, c) Arsenic speciation in the roots (b) and shoots (c) of
849 different *A. thaliana* lines grown in hydroponic culture and exposed to 5 μ M As(V)
850 for 1 day without phosphate. Data are means \pm SE (n = 4). Different letters above bars
851 indicate significant differences at $P < 0.05$.

852

853 **Fig. 7** Arsenic speciation in the roots (a) and shoots (b) of Col-0, *hac1*, *cad2-1*,
854 *cad1-3* and the double mutants of *hac1 cad2-1* and *hac1 cad1-3* after exposure to 5
855 μ M As(V) (without phosphate) for 1 day. Data are means \pm SE (n=4). Different letters
856 above bars represent significant difference at $P < 0.05$.

857

858 **Supporting Information**

859 **Fig. S1** Genotyping of various mutants and expressing lines used in the present study.

860 **Fig. S2** Arsenate and arsenite tolerance of transgenic *A. thaliana* expressing *PvACR3*.

861 **Fig. S3** Subcellular localisation of *PvACR3* in *A. thaliana*.

862 **Fig. S4** Preferential expression of *PvACR3* in the endodermis and stele of *A. thaliana*
863 roots.

864 **Fig. S5** Effect of combining *PvACR3* expression with *HAC1* mutation on arsenate
865 tolerance in *A. thaliana*.

866 **Fig. S6** Expression levels of *PvACR3* and *HAC1* genes in *A. thaliana hac1* mutant
867 expressing *PvACR3* and the growth phenotypes of different lines grown in a potting
868 medium with or without 10 mg kg⁻¹ As(V).

869 **Fig. S7** The concentrations of non-protein thiols in the roots and shoots of Col-0,
870 *cad2-1*, *cad1-3*, *hac1-2*, *cad2-1hac1-2* and *cad1-3 hac1-2* of *A. thaliana*.

871 **Fig. S8** Arsenate tolerance in *hac1*, *cad2-1*, *cad1;3* and double mutants of *cad2-1*
872 *hac1* and *cad1;3 hac1*.

873 **Table S1** Primers used for genotyping of different lines of *A. thaliana*.

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