



HAL
open science

Zinc availability modulates plant growth and 1 immune responses via AZI1

Nadia Bouain, Santosh B. Satbhai, Chorpet Saenchai, Guilhem Desbrosses, Pierre Berthomieu, Wolfgang Busch, Hatem Rouached

► To cite this version:

Nadia Bouain, Santosh B. Satbhai, Chorpet Saenchai, Guilhem Desbrosses, Pierre Berthomieu, et al.. Zinc availability modulates plant growth and 1 immune responses via AZI1. 2017. hal-01777544

HAL Id: hal-01777544

<https://hal.science/hal-01777544v1>

Preprint submitted on 24 Apr 2018

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

1 **Zinc availability modulates plant growth and immune responses via *AZII***

2 Nadia Bouain¹, Santosh B. Satbhai^{2,3}, Chorpet Saenchai¹, Guilhem Desbrosses¹, Pierre

3 Berthomieu¹, Wolfgang Busch^{2,3*}, Hatem Rouached^{1*¶}

4

5 1-Biochimie et Physiologie Moléculaire des Plantes, Institut National de la Recherche Agronomique, Centre

6 National de la Recherche Scientifique, Université Montpellier 2, Montpellier SupAgro. Bat 7, 2 place Viala,

7 34060 Montpellier cedex 2, France.

8 2- Gregor Mendel Institute (GMI), Austrian Academy of Sciences, Vienna Biocenter (VBC), Dr. Bohr-

9 Gasse 3, 1030 Vienna, Austria

10 3 – Plant Molecular And Cellular Biology Laboratory, Salk Institute for Biological Studies, La Jolla, CA

11 92037, USA

12

13 [¶] Present address: Department of Plant Biology, Carnegie Institution for Science, 260 Panama Street,

14 Stanford, CA 94305, USA.

15

16 *To whom correspondence should be addressed:

17

18 **Dr. Hatem ROUACHED**

19 UMR Biochimie & Physiologie Moléculaire

20 des Plantes - CNRS-INRA-SUPAGRO-UM,

21 Cedex 2 Montpellier, 34060

22 France

23 Phone: +33 (0)4 99 61 31 54

24 Fax : +33 (0) 4 67 52 57 37

25 E.mail: hatem.rouached@inra.fr

26

35

27 **Dr. Wolfgang BUSCH**

28 Salk Institute for Biological Studies

29 10010 N Torrey Pines Rd • La Jolla, CA

30 92037

31 Phone: +1 (858) 453-4100 x1765

32

33 E.mail: wbusch@salk.edu

34

36

37 **Abstract.**

38 **Zinc is an essential micronutrient for all living organisms and is involved in a plethora**
39 **of processes including growth and development, and immunity. However, it is unknown**
40 **if there is a common genetic and molecular basis underlying multiple facets of zinc**
41 **function. Here we used natural variation in *Arabidopsis thaliana* to study the role of zinc**
42 **in regulating growth. We identify allelic variation of the systemic immunity gene *AZII***
43 **as a key for determining root growth responses to low zinc conditions. We further**
44 **demonstrate that this gene is important for modulating root growth depending on the**
45 **zinc and defence status. Finally, we show that the interaction of the immunity signal**
46 **azelaic acid and zinc level to regulate root growth is conserved in rice. This work**
47 **demonstrates that there is a common genetic and molecular basis for multiple zinc**
48 **dependent processes and that nutrient cues can determine the balance of plant growth**
49 **and immune responses in plants.**

50

51 **Key words.**

52 **GWAS, root growth, low zinc condition, signalling, azelaic acid, immunity**

53

54

55

56 **Introduction.**

57 Zinc (Zn) is an essential micronutrient for humans, animals, and plants^{1,2,3,4}. It is of particular
58 importance for the function of numerous metalloenzymes that are involved in a plethora of
59 processes such as energy metabolism, nucleic acid and protein synthesis, and protein
60 catabolism⁵. These key biological processes can be adversely altered in situations in which Zn
61 availability is limited. Low Zn manifests itself at physiological and molecular levels, and can
62 cause deleterious effects such as growth retardation and malfunction of immune responses.
63 Recent studies in mammalian systems have shifted the focus on the role of Zn from simply a
64 nutrient to a signalling molecule that fine-tunes intracellular signalling events (for an
65 overview see⁶) and an important player in nutritional immunity⁷. In particular, in the role of
66 Zn for host defence, a complex role is emerging. On one hand, Zn is actively depleted from
67 infection sites restricting the ability of the pathogen to proliferate^{8,9} and on the other hand
68 high Zn levels are generated by the host that contribute to kill the pathogen¹⁰. However,
69 despite its fundamental importance, it remains unclear whether there is a common molecular
70 basis for these multiple functions involving Zn and whether the signalling and immune related
71 functions of Zn are also relevant for plants.

72

73 Plants are the first link in the chain of human Zn nutrition and therefore studying Zn related
74 processes in plants is of particular relevance. Land plants acquire Zn at the root–soil interface
75 and multiple processes are crucial for efficient Zn acquisition. While Zn transport is clearly
76 very important for Zn acquisition and homeostasis⁴, other processes also play pronounced
77 roles for efficient growth under Zn limited conditions. For instance, when responses of two
78 lines of rice that displayed a contrasting tolerance to -Zn were quantified, these lines didn't
79 differ in Zn-transporter activity but mostly in their maintenance of root growth and the
80 exudation rates of organic acids¹¹. A similar correlation of increased root growth and

81 increased tolerance to $-Zn$ conditions had also been described in wheat¹². Overall, this
82 indicated that Zn levels in the environment are perceived by the plant and lead to distinct
83 changes in root growth that might be important for adaptive responses to low Zn conditions.
84 Nevertheless, so far, neither a role for Zn as signal, nor the genetic and molecular bases of
85 root growth changes upon $-Zn$ conditions in plants has been clearly established yet.

86
87 One important biotic stress response process that Zn and its availability has been shown to be
88 involved in animals¹³ as well as in plants is disease resistance¹⁴. One gene that is involved in
89 the response to biotic and abiotic stresses in *Arabidopsis thaliana* is *AZII* (*AZELAIC ACID*
90 *INDUCED 1*, At4g12470)^{15, 16,17}. It encodes for a lipid transfer protein (LTP)-like protein and
91 belongs to the *EARLY ARABIDOPSIS ALUMINIUM-INDUCED GENE1* (*EARLII*) gene
92 subfamily¹⁸. *AZII* was named after its unique response to the systemically active compound
93 azelaic acid (AzA, a nine-carbon dicarboxylic acid)¹⁹. In the *Arabidopsis* genome, *AZII*
94 clusters in a tandem array on chromosome 4 with three other *EARLII*-type genes, namely
95 At4g12480 (*EARLII*), AT4G12490 (*ELHYPRP2* (*EARLII-LIKE HYBRID PROLINE-RICH*
96 *PROTEIN 2*) and AT4G12500. Among these genes, the role of *AZII* for long-distance signals
97 related to systemic acquired resistance (SAR) is the best documented so far. In addition to
98 *AZII*, SAR involves an important hormone, namely salicylic acid (SA). SA accumulates upon
99 pathogen attack²⁰, and leads to the induction of *PATHOGENESIS-RELATED GENE 1* (*PRI*,
100 SA marker gene)²¹. In *Arabidopsis thaliana*, mutation of *AZII* causes a specific loss of
101 systemic immunity triggered by pathogens¹⁹. Beyond its role in biotic stress responses, the
102 involvement of *AZII* in response to abiotic stress, such as the regulation of seedling growth
103 under salt stress, was demonstrated¹⁷. But, whether *AZII* is involved in the response to
104 nutrient levels that potentially affect the plants capability to defend itself or the capability for
105 pathogens for infection (e.g. low Zn), remains unexplored.

106

107 Here, we approach study the genetic basis of low exogenous Zn levels on root growth by
108 exploring natural genetic variation. We find that there is heritable natural variation of root
109 growth responses to low Zn and that natural allelic variation of the immune gene *AZELAIC*
110 *ACID INDUCED* (*AZII*) determines a significant proportion of this response. We further
111 reveal an intriguing evolutionary conserved interaction between exogenous Zn levels and
112 AzA dependent defence pathways to regulate root growth.

113 114 **Results**

115 **The *AZII* gene is involved in the root growth response to low Zn conditions.**

116 To identify genetic components that regulate plant growth upon low Zn (-Zn) conditions, we
117 determined the length of the primary root of 230 genetically diverse natural accessions of
118 *Arabidopsis thaliana* (Figure S1A) from the RegMap population²² grown on +Zn and -Zn
119 medium over 7 days (Table S1-2). Importantly, while still being correlated, the root growth of
120 the vast majority of accessions clearly differed in +Zn and -Zn conditions (Figure S1B, C).
121 To assess whether these root growth responses were specifically due to the -Zn treatments, we
122 determined mRNA levels of four Zn-deficiency responsive marker genes *ZIP3*, *ZIP5*, *ZIP12*
123 ²³ and *PHO1;H3*²⁴ in the Col-0 accession under our screening conditions. All of these four
124 genes were significantly up-regulated in -Zn conditions (Figure S2), demonstrating that the
125 plants sensed and responded to the -Zn conditions.

126 In the panel of screened accessions, we observed broad phenotypic variation for root length
127 (Figure S3) that was highly heritable (broad sense heritability (H^2) ranging²⁵ from 0.36 to
128 0.44) (Table S3). We then conducted Genome Wide Association Studies (GWASs) using the
129 AMM method that corrects for population structure confounding²⁶, to identify loci that were
130 associated with root length under -Zn (Figure 1A, Figure 1B, Figure S4 and Figure S5). We
131 then corrected the association *P*-values for all SNPs for multiple testing using the Benjamini-
132 Hochberg-Yekutieli method²⁷. Due to the limited power of our association study and the

133 potentially high false negative rate due to population structure correction, and because we
134 aimed to test any major emerging candidate experimentally, we selected a relatively non-
135 conservative 10% false discovery rate (FDR) as our threshold for significant association (we
136 thus expect that 10% of the significant SNPs are false positives). Using this criterion, we
137 identified two chromosomal regions associated with root length in $-Zn$ conditions. On
138 chromosome 2, the significant peak (P -value = $3.27 \cdot 10^{-7}$; FDR $\approx 7\%$) was located in a region
139 with a cluster of similar genes encoding a Cysteine/Histidine-rich C1 domain family
140 (At2g21810). It was detected on the last day of the time course (day 7). These proteins require
141 Zn ions for their function²⁸ (pfam, PF00130). On chromosome 4, the significant peak (P -value
142 = $4.40 \cdot 10^{-7}$; FDR $\approx 6\%$) was located in a region that contains the lipid transfer protein
143 (LTP)-like *AZII* (At4g12470) gene and the 7 additional genes encoding for lipid transfer
144 proteins as a cluster (Figure 1B and Figure 1C). This peak was already detected early in the
145 duration of our time course (day 2) suggesting that this locus was relevant for Zn signalling
146 rather than being a consequence of low internal Zn levels. As *AZII* itself was known for being
147 involved in signalling: it mediates azelaic-acid-induced systemic immunity¹⁹; we
148 hypothesized that *AZII* was involved in mediating crosstalk between nutrient and immunity
149 signals. However, as the GWAS peak spanned multiple genes, we first tested whether the best
150 candidate in this region was indeed *AZII*. For this, we assessed all 8 genes in the genomic
151 region surrounding the association peak. Of these 8 genes, only *AZII* showed significant
152 transcript level alteration in response to $-Zn$ in Col-0 (Figure 1D), providing a strong hint that
153 it was involved in $-Zn$ dependent root growth regulation. To test this further, we determined
154 the root lengths of Arabidopsis Col-0 (WT), *azil* (T-DNA) mutant line and an *AZII*
155 overexpressing (OE *AZII*) line (35S::*AZII*) grown in $+Zn$ or $-Zn$ conditions over 7 days
156 (Figure S 6). In presence of Zn, no significant differences in root length could be observed
157 between the *azil* mutant and wild-type plants (day2, Figure 1E; 7 days Figure S 6). Grown

158 under -Zn, the root length of *azil* was significantly shorter than Col-0 and 35S::*AZII* plants
159 (day 2, Figure 1E, 7 days, Figure S6). Interestingly, starting from day 5 onwards, compared to
160 the Col-0 wildtype, roots of *azil* were significantly shorter and roots of 35S::*AZII* plants were
161 significantly longer respectively (Figure S6; day 5, Figure S7 A-B), which suggests that the
162 expression level of *AZII* is involved in controlling this trait (root growth). Day 5 was
163 therefore chosen as time point for further analysis. To assess whether this is a function of
164 *AZII* that is common to other micronutrients, we grew the same set of lines under low iron (-
165 Fe) conditions (Figure S7-C). There, no significant root length difference could be detected
166 for the 3 tested lines, which indicates that growth responses to comparable nutrient limitations
167 are not dependent on *AZII* and supports the notion of a rather specific *AZII* dependent
168 response to Zn. Taken together, these data show that *AZII*, previously described as a key
169 components of plant systemic immunity involved in priming defence^{19,29}, modulates root
170 growth in a Zn level dependent manner.

171

172 **Natural allelic variation of *AZII* determines root growth under zinc limiting conditions.**

173 While we had shown that *AZII* was involved in modulating root growth in a Zn level
174 dependent manner, this was no proof that the allelic variation of *AZII* is causal for the
175 observed root growth differences under -Zn. We therefore set out to test this. Sequence
176 analysis of the *AZII* genomic region (promoter and coding region) showed multiple
177 polymorphisms in the regulatory region as well as synonymous changes in the coding region
178 (Table S4) that were consistent different between contrasting groups of accessions with either
179 long or short roots on -Zn (Figure S8A and Figure S8B, table S1 and table S2). Consistent
180 with causal regulatory polymorphisms, *AZII* expression was significantly higher upon -Zn in
181 accessions with longer roots (Figure S8C and Figure S8D). For further analysis, we focussed
182 on two contrasting accessions, Col-0 and Sq-1, which were among the most contrasting
183 accessions regarding their root length on -Zn (Table S1 and Table S2) and each displayed the

184 variant of the marker SNP that was associated with long and short roots on $-Zn$ respectively.
185 To then experimentally test whether the difference in *AZII* expression level was due to the
186 natural allelic variation and whether this was also causal for the longer roots, we transformed
187 the *azil* mutant (Col-0 background) with constructs containing 1.6kbp of the promoter and the
188 coding region from either Col-0 (long roots in $-Zn$) or Sq-1 (short roots in $-Zn$), and an empty
189 vector (control). In five independent homozygous single insertion lines (Table S5)
190 complemented with the Col-0 pAZII:*AZII* the expression level of *AZII* under $-Zn$ was
191 significantly higher ($P < 0.01$) than that in plants transformed with the Sq-1 pAZII:*AZII*
192 construct (Figure 2A). Consequently, we tested these T3 lines for root length differences
193 under $-Zn$ and $+Zn$. Consistent with the hypothesis that our *AZII* variants determine root
194 growth specifically under $-Zn$, no difference in term of root length was observed between the
195 T3 lines grown on $+Zn$ (Figure S9), while under $-Zn$, we observed significantly longer roots
196 ($P < 0.05$) in the Col-0 pAZII:*AZII* plants compared to Sq-1 pAZII:*AZII* plants or *azil* plants
197 transformed with the empty vector (Figure 2B). Taken together, these data demonstrate that
198 allelic variation at the *AZII* locus causes variation of *AZII* expression levels and that this
199 leads to variation of PRG under $-Zn$. As there were multiple polymorphisms in the regulatory
200 region of the *AZII* gene (Col-0 and Sq-1 accessions, Figure S10), and only synonymous
201 changes in the coding sequence in these constructs, the data also suggest that this regulation is
202 mainly dependent on regulatory changes. We note, that we can't completely exclude the
203 additional involvement of other genes in the associated region in contributing to this response.

204

205 **Azelaic Acid Inhibits Arabidopsis Root Growth in a Zn-dependant manner.**

206 While *AZII* had not been implicated in any known process involving Zn, it is known to
207 mediate signal mobilization for systemic defence priming that can be triggered by AzA^{19,20}.
208 We therefore hypothesized that *AZII* would modulate growth and immunity programs
209 depending on Zn and AzA status. To test this hypothesis, we first established the effects of the

210 exogenous application of AzA on root growth. AzA affected root growth in a dose-dependent
211 manner starting with a relatively mild reduction of growth at 100 μ M to complete inhibition
212 of root growth at 200 μ M AzA (Figure 3A). We then determined whether this response is
213 dependent on *AZII*, and assessed root growth in Col-0 and *aziI* mutant at 100 μ M AzA and in
214 presence or absence of Zn after 5 d of treatment. While, AzA severely inhibited root growth
215 in Col-0 plants in presence of AzA and Zn (+AzA+Zn), the *aziI* mutant line was significantly
216 more resistant to the inhibitory effect of AzA (Figure 3B). This demonstrated that AzA
217 modulates root growth in an *AZII* dependent manner.

218 To test whether Zn modulates this response, we conducted the same assays under -Zn
219 conditions. Strikingly, low levels of Zn alleviated the growth inhibitory effect of AzA on Col-
220 0 to a large extent, and led to a further increased growth of the *aziI* mutant line (Figure 3B).
221 Taken together, these data show that AzA induced reduction of growth is modulated by Zn
222 levels, and that *AZII* is a key component for this modulation.

223
224 **Zn status strongly impacts immunity and modulates the response to AzA.**

225
226 Our data had not only shown that Zn levels and AzA modulate root growth, but also that the
227 root growth responses to these treatments strongly interact (Figure 3B). To test whether this
228 interaction is due to the modulation of molecular responses to AzA by Zn levels, we measured
229 the expression levels of 18 defence-related genes that had been shown a mild but significant
230 expression change upon AzA treatment ($P < 0.05$) in leaves of wild-type plants (Col-0)¹⁹, as
231 well as 2 additional genes regulating salicylic acid biosynthesis (*WRKY28* and *WRKY46*)³⁰,
232 ^{31,32}, *AZII*, and a marker gene frequently used as a reliable molecular **marker** for SA-
233 dependent SAR (*PRI*)²¹. Our q-RTPCR based gene expression analysis showed that almost
234 all (16) of these defence-related genes were upregulated in response to the application of the
235 application of AzA (Figure S11). Notably, the group of most strongly induced genes
236 contained genes involved in salicylic acid (SA) biosynthesis, such as *ISOCHORISMATE*

237 *SYNTHASE 1*, *WRKY28*, *WRKY46*, as well as the SA response marker *PRI*. AzA treatment of
238 plants grown on $-Zn$ medium ($-Zn+AzA$) resulted the upregulation of only 9 of the 16 genes
239 that were upregulated in $+Zn/+AzA$ (Figure S11). Notably, the SA response marker gene *PRI*
240 was not among these. Overall this suggests that interaction of $-Zn$ and AzA is not due to a
241 lack of induction of SA biosynthesis genes, but rather acts more downstream during SA
242 signalling. Furthermore, consistent with an effect of Zn levels on the expression of these
243 genes, plants grown on low Zn showed a down-regulation of 6 of the 16 defence-related genes
244 induced by AzA alone (Figure S11). Overall, these results demonstrate that Zn status impacts
245 the expression of defence-related genes and modulates the response to AzA in plants.

246

247 ***A. brasilense* Infection Inhibits Root Growth in Arabidopsis in a Zn-dependant manner.**

248 The link of Zn status and *AZII* to both, growth and defence prompted us to test whether
249 interaction of *AZII* and Zn status impacts growth responses to biotic stimuli. For this, we
250 chose to measure primary root responses upon infection with the bacterium *Azospirillum*
251 *brasilense* (Sp245) as it had been described that root growth as well as *AZII* expression is
252 changed in *A. thaliana* upon such infection³³. Consistently with³³ root growth of 5 d old WT
253 plants was reduced when grown on complete medium ($+Zn$) and inoculated with *A. brasilense*
254 (Figure 4A). When grown on $-Zn$, this *A. brasilense* induced root length reduction was
255 slightly less pronounced (Figure 3C). The bacterially induced growth inhibition under $+Zn$
256 was significantly less pronounced in the *azil* mutant, showing that *AZII* is involved in
257 regulating this growth response. Under $-Zn$, bacterial incubation resulted in further decrease
258 of root growth of *azil* mutant compared to $-Zn$ (Figure 3C). Overall, these data clearly show
259 that there is a complex interaction of Zn levels and the *AZII* gene that determines the balance
260 of growth and defence.

261

262 **Zn and AzA interaction is conserved in *Oryza sativa* root.**

263 The gene family to which *AZII* belongs, is strongly conserved throughout the *Viridiplantae*
264 (green plants)³⁴. This led us to hypothesize that the AzA mediated root growth regulation and
265 its modulation by Zn status is a conserved growth-immunity regulating pathway. We
266 therefore investigated the effect of AzA on root growth in presence and absence of Zn in the
267 monocot species rice (*Oryza sativa*) (Figure 4A and Figure 4B). Root growth increases in rice
268 grown in low Zn compared to +Zn condition (Figure 4A and Figure 4B). Interestingly, while
269 seedlings grown in medium that contained AzA (300 μ M) and Zn didn't develop any roots,
270 this growth inhibition was not observed when germinating the grains on -Zn medium
271 containing AzA (Figure 4, Figure S12). This demonstrated that AzA mediated growth
272 inhibition, as well as its regulation by Zn levels is an evolutionary conserved mechanism.

273

274 **Discussion**

275 Plants must sense changes in external and internal mineral nutrient concentrations and adjust
276 growth to match resource availability³⁵. Responses to nutrient limitations manifest very early
277 in plants life cycle, and root related processes are a major target of responses to nutrient
278 constraints in plants. Consequently, primary root growth responds early on and drastically to
279 nutrient limitations³⁶ and genome wide association mapping approaches are now being used
280 to understand the genetic and molecular factors that govern these early growth responses³⁷.
281 However, apart from these abiotic variables (e.g. nutrients and water levels), the root
282 continuously is exposed to changing biotic factors. Our study to identify genes and their
283 variants that determine early root growth responses to low Zn levels revealed a genetic and
284 molecular between root growth response to abiotic (Zn) and biotic (defence related signalling)
285 factors. In particular, our study revealed a significant association between the *AZII* locus and
286 primary root length in *Arabidopsis* accessions grown in -Zn condition, as well as an
287 intriguing *AZII* dependent interaction between Zn levels and the AzA pathway. One
288 interesting question is whether the *AZII* function that relates to abiotic factors are specific to

289 Zn or not. While there has been one report in which it was shown that ectopic expression of
290 *AZII* improved Col-0 seed germination under high salinity stress condition and *aziI* mutants
291 were overly sensitive¹⁷, many leads point in the direction of a largely Zn specific function:
292 Significant associations close to the the *AZII* locus were neither found in the GWAS for
293 accessions that were grown at the same time on +Zn (Figure S2, Figure S3, Figure S4 and
294 Figure S5), nor in other published root GWAS datasets for growth on MS medium³⁸, under
295 different nitrogen conditions³⁹, under NaCl stress⁴⁰, and under Fe deficiency³⁷. Moreover,
296 while loss of function of *AZII* caused decreased root growth of Arabidopsis plants grown
297 under -Zn conditions, and *AZII* overexpression caused the roots to be longer than WT (Figure
298 1), this was not observed when *aziI* or *35S:AZII* were grown under -Fe conditions (Figure
299 S6). Nevertheless, the level of exclusiveness or the extent of this specificity of *AZII* function
300 for Zn can however only be elucidated by further studies.

301
302 The responses that we have observed and studied occur early-on during plant development,
303 when Zn levels in the seedlings will not be significantly depleted. Moreover, it is likely that
304 traces of Zn will have remained in the washed agar that we used for these assays, generating
305 an environment very low in Zn, rather than a fully Zn depleted growth environment. Overall,
306 our findings thus exposed mechanisms that will relate more to sensing than to a Zn
307 starvation/depletion response, consistent with our observation that these low Zn levels
308 promote early root growth rather than to inhibit it. While we have identified a signalling
309 mechanism that contributes to this -Zn dependent increase in root growth, the Zn sensor still
310 remains elusive.

311 Our study proposes a possible mechanism for the regulation of root growth depending on the
312 environmental Zn level, in which *AZII* plays an important role, and probably in an interaction
313 with SA. *AZII*- and SA-related signals are known to interact²⁰, and possibly involved in a
314 self-amplifying feedback loop (for review^{41,42}). Our gene expression analysis revealed that Zn

315 status impacts the expression of *AZII*, as well as other immune- and SA-related genes in
316 Arabidopsis (Figure S11). Variation in SA concentration in leaves and roots of Arabidopsis
317 plants upon nutrient deficiency stresses has been reported⁴³, and appear to be nutrient
318 specific⁴³. For example, SA levels significantly increased in response to potassium deficiency
319 while low Fe caused a significant decrease of SA in roots⁴³. The involvement of
320 phytohormones in controlling root growth under different nutritional status has been
321 documented⁴⁴, and hormones accumulation “threshold” appeared to be critical for hormones
322 action⁴⁵. For example, in various plants species, treatment with low concentrations of SA led
323 to root growth promotion while treatment with high concentration of SA caused an inhibition
324 of root growth^{41,42}. Plants exposed to bacterial infection accumulate SA²⁰, AzA, and induce
325 the expression of *AZII*³³ presumably to promote defence response, often at the cost of growth.
326 In our gene expression analysis, a marker frequently used for SA accumulation, namely
327 *PRI*²¹, was induced by +Zn+AzA conditions, suggesting an accumulation of SA under these
328 conditions. Consistently, root growth of wild-type plants (Col-0) treated with (+Zn+AzA) was
329 severely reduced compared to control condition (+Zn-AzA). Similarly, plants (Col-0) treated
330 with Sp245 showed shorter root than the ones grown on control condition. The negative effect
331 of either AzA or Sp245 treatment on root growth was alleviated when these stresses were
332 combined with low Zn treatment, (-Zn+AzA or -Zn+Sp245). This was accompanied by an
333 absence of *PRI* induction, indicating that the otherwise root growth inhibitory SA response is
334 affected by low Zn levels at the molecular level. Our work exposed an interaction of Zn levels
335 and immunity and a common genetic and molecular basis for this. Interestingly it seems that
336 Arabidopsis can prioritize root growth over defence responses, if Zn levels are low during
337 early development. The data from rice point in the same direction and provide a hint that this
338 interaction is evolutionary conserved. It will be interesting to elucidate how this relates to the

339 molecular role of Zn in nutritional immunity in plants, perhaps somehow similar to the role of
340 Zn levels in infection sites in animal systems^{8,9,10}.
341 *AZII* belongs to a large family of pathogenesis-related proteins; LTPs. While some LTPs
342 were suggested to play a role in defence reaction in a root specific manner⁴⁶, the role for *AZII*
343 in defence responses was mainly investigated in aboveground tissues, and similar data for role
344 of *AZII* in roots are still absent. In addition to *AZII*, roles of *AzA* had been analysed on the
345 aboveground tissues, our data reveal an important role of this pathway in the root, which
346 extends the current models for underground defence priming. Importantly, the conserved
347 specific interaction of Zn and *AzA* that can be observed not only in the dicot species
348 *Arabidopsis* but also in the monocot species rice, is not only interesting from the view of
349 basic biology, but also harbours very interesting perspectives for an innovative
350 biotechnological application. This is because *AzA* is thought to directly mediate crop plant
351 responses to pathogens and herbivores or to mimic compounds that do⁴⁷ and is listed among
352 the natural compounds that induce resistance by a priming mechanism⁴⁸. To activate these
353 plant responses, *AzA* like others organic and inorganic chemicals can be applied as a foliar
354 spray, seed treatment, or soil drench⁴⁷. However, our work revealed that the soil-based
355 application of *AzA* might significantly impact root traits depending on Zn bioavailability
356 (*AzA* severely inhibited the root growth in +Zn), which could have an enormous and direct
357 impact on plant growth in the field.

358

359 **Acknowledgments**

360

361 The authors are thankful to Prof. Peter Urwin (University of Leeds, UK) for providing seeds
362 of *azil* mutant and Dr. Youssef Belkhadir, Dr. Hironaka Tsukagoshi and Dr. Zaigham
363 Shahzad for helpful discussions and suggestions. We are also grateful to Bonnie Wohlrab,
364 Christian Göschl and Mushtak Kisko for technical assistance. This work was funded by the
365 “Institut National de la Recherche Agronomique – Montpellier - France” INRA (H.R.), and

366 supported by funds from the Austrian Academy of Science through the Gregor Mendel
367 Institute (GMI) and an Austrian Science Fund (FWF) stand-alone project (P27163-B22)
368 (W.B.).

369 **Author information**

370 **Affiliations**

371 - Biochimie et Physiologie Moléculaire des Plantes, Institut National de la Recherche
372 Agronomique, Centre National de la Recherche Scientifique, Université Montpellier 2,
373 Montpellier SupAgro. Bat 7, 2 place Viala, 34060 Montpellier cedex 2, France

374 Nadia Bouain, Chorpet Saenchai, Guilhem Desbrosses, Pierre Berthomieu and Hatem Rouached

375
376 - Plant Molecular And Cellular Biology Laboratory, Salk Institute for Biological Studies, La
377 Jolla, CA 92037, USA

378 Santosh B. Satbhai

379 - Gregor Mendel Institute (GMI), Austrian Academy of Sciences, Vienna Biocenter (VBC), Dr.
380 Bohr-Gasse 3, 1030 Vienna, Austria

381 - Plant Molecular And Cellular Biology Laboratory, Salk Institute for Biological Studies, La
382 Jolla, CA 92037, USA

383
384 Wolfgang Busch

385 386 **Contributions**

387
388 H.R and W.B. designed the research. H.R supervised this project. N.B, S.B.S and C.S.
389 performed most experiments. G.D. helped conduct bacterial infection experiments. P.B.
390 helped conducting the Zn root phenotyping data analysis. H.R and W.B. analyzed the data and
391 wrote the manuscript.

392 **Declaration of interest**

393
394 The authors declare no competing financial interests.

395 **Corresponding authors**

396 Correspondence to: Hatem Rouached or Wolfgang Busch

397

398 **Material and Methods**

399 **Plant materials and growth conditions**

400 We used 230 different genotype of *A. thaliana* from different geographic origins (Table S1,
401 S2, Figure S1A) and for each genotype grew 12 seedlings. The previously described⁴⁹ *azil*
402 insertion mutant (SALK_085727C) available in the Nottingham Arabidopsis Stock Centre
403 was provided by Peter Urwin (University of Leeds, UK). Lines overexpressing *AZII* under
404 35S promoter, expressing Col-0 or Sq-1 *AZII* locus under their native promoters were
405 generated in this *azil* mutant background. Plant phenotyping for GWAS was as described
406 previously³⁸. Briefly, for each growth condition all lines were grown side by side. For both Zn
407 conditions, GWAS assays were performed in the same growth chambers under the same
408 22°C long-day conditions (16 h light, 8 h dark). Seeds were placed for 1 h in opened
409 1.5 ml Eppendorf tubes in a sealed box containing chlorine gas generated from 130 ml of
410 10% sodium hypochlorite and 3.5 ml of 37% hydrochloric acid. For stratification, seeds
411 were imbibed in water and stratified in the dark at 4°C for 3 days to promote uniform
412 germination. On each plate, eight different accessions with three seeds per accession were
413 then germinated and grown in a vertical position on agar-solidified medium contained 0.5
414 mM KNO₃, 1 mM MgSO₄, 1 mM KH₂PO₄, 0.25 mM Ca(NO₃)₂, 100 μM NaFeEDTA, 30 μ
415 M H₃BO₃, 10 μM MnCl₂, 1 μM CuCl₂, 15 μM ZnSO₄, 0.1 μM (NH₄)₆Mo₇O₂₄, and 50
416 μM KCl, in presence of 1% (wt/vol) sucrose and 0.8% (wt/vol) agar. -Zn or -Fe medium was
417 made by not adding the only source of Zn (ZnSO₄) or FeEDTA to the medium, respectively.
418 For the assays involving azelaic acid treatments, AzA (246379 ALDRICH, Sigma) was added
419 in different concentration ranging from 25 to 200 μM. For the assay with rice (*Oryza sativa*
420 L.), Niponbare was used and seeds were soaked in deionized water over night in dark then
421 transferred in a controlled-environment chamber (light/dark cycle of 14/10 h, 200 μmol

422 photons $\text{m}^{-2}\text{s}^{-1}$, temperature of 28/25 °C and RH of 80%) to ¼ Yoshida media for 5 d^{50,51}:
423 0.36 mM NH_4NO_3 ; 0.41 mM MgSO_4 ; 0.19 mM CaCl_2 ; 0.13 mM K_2SO_4 ; 0.08 mM NaH_2PO_4 ;
424 4.72 μM H_3BO_3 ; 2.37 μM MnCl_2 ; 8.90 μM Fe-NaEDTA; 0.62 μM ZnSO_4 ; 0.04 μM CuSO_4 ;
425 0.02 μM $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$, adjust to pH 5.5. ZnSO_4 was removed in -Zn medium. For rice
426 treatments, azelaic acid (246379 ALDRICH, Sigma) was added in different concentration
427 ranging from 75 to 300 μM . For the control condition, rice plants were kept in nutrient
428 solution with the above-mentioned composition. Rice seedlings were grown in a growth
429 chamber under the following environmental conditions: light/dark cycle of 14/10 h,
430 temperature of 28/25 °C, and RH of 80 %.

431 GWAS root trait quantification was conducted using the BRAT software³⁸. Root length in
432 assays involving mutant and transgenic lines, as well as rice plants was measured using
433 ImageJ software, version 2.0.0 (<http://rsb.info.nih.gov/ij/>). Statistical differences between
434 genotypes were calculated using t-test analyses and ANOVA with subsequent post hoc tests
435 using Graphpad Prism (GraphPad Software Inc., San Diego, CA, USA) or Microsoft Excel
436 (Microsoft, USA).

437 **Gene expression analysis by quantitative RT-PCR**

438 Total RNA was extracted from roots of Arabidopsis wild type plants (different accessions)
439 grown in presence or absence of Zn, and with or without azelaic acid (246379 ALDRICH,
440 Sigma), using Plant RNeasy extraction kit (Qiagen) and RQ1 RNase-free DNase (Promega).
441 Two μg of total RNA were used to synthesize cDNA using poly-A oligos. Real-time
442 quantitative reverse-transcription PCR (RT-qPCR) was performed with a Light Cycler 480
443 Real-Time PCR System (Roche; Roche Diagnostics, Basel, Switzerland) using LightCycler
444 480 SYBR Green I Master mix (Roche, IN, USA). Primer list is provided in table S6. Gene
445 transcript accumulation quantification were performed in a final volume of 20 μL containing
446 optimal primer concentration 0,3 μmol , 10 μL of the SYBR Green I master mix, and 5 μL

447 of a 1:25 cDNA dilution. Real time-PCR conditions were as 95°C for 5 min, and followed by
448 40 cycles of 95°C for 10 s, 60°C for 10 s, 72 °C for 25 s, and finally one cycle 72 °C for 5
449 min. As a negative control, template cDNA was replaced by water. All PCR reactions were
450 performed in triplicates. For each sample, cycle threshold a (Ct) value was calculated from the
451 amplification curves. For each gene, the relative amount of calculated mRNA was normalized
452 to the level of the control gene *ubiquitin10* mRNA (*UBQ10*: At4g05320). For every sample,
453 the relative gene expression of each genes was expressed following normalization against the
454 CT values obtained for the gene used for standardization, for instance $\Delta CT_{AZII} = CT_{AZII}$
455 $- (CT_{UBQ10})$. Quantification of the relative transcript levels was performed as described
456 previously^{52,53}. Briefly, -Zn treatment was compared to +Zn , the relative expression of a each
457 gene was expressed as a $\Delta \Delta Ct$ value calculated as follows: $\Delta \Delta Ct = \Delta CT_{AZII}(-Zn) - \Delta$
458 $CT_{AZII}(+Zn)$. The fold change in relative gene expression was determined as $2^{-\Delta \Delta Ct}$.

459

460 **Bacterial strains and growth conditions**

461 Wild-type strain of *Azospirillum brasilense* is used in this study⁵⁴. These bacteria strains were
462 cultivated and inoculated in plant culture medium as described previously⁵⁵. Statistical
463 differences between genotypes were calculated using t-test analyses and ANOVA with
464 subsequent post hoc tests using Graphpad Prism (GraphPad Software Inc., San Diego, CA,
465 USA).

466

467 **Plasmid construction and plant transformation**

468 The *AZII* locus from Col-0 and Sq-1 accessions were cloned with primers spanning the region
469 ranging from 1614 bp upstream of the *AZII* transcription start site to the stop codon of *AZII*
470 into the binary vector pCAMBIA1301 by restriction enzymes of *BamHI* and *PstI* using
471 primers listed in Supplementary table S3 and S4. The constructs were transformed into
472 *Agrobacterium tumefaciens* strain GV3101 and then used for Arabidopsis transformation by

473 the floral dip method⁵⁶. Transgenic plants were selected by antibiotic resistance, and only
474 homozygous descendants of heterozygous T2 plants segregating 1:3 for antibiotic resistance:
475 sensitivity were used for analysis.

476

477 **GWA mapping**

478 For GWAS, mean total root length values of 230 natural accessions were used (Table S1, S2).
479 The GWA analysis was performed in the GWAPP web interface using the mixed model
480 algorithm (AMM) that accounts for population structure²⁶ and using the SNP data from the
481 RegMap panel^{57,58,22}. Only SNPs with minor allele counts greater or equal to 10 were taken
482 into account. The significance of SNP associations was determined at 10% FDR threshold
483 computed by the Benjamini-Hochberg-Yekutieli method to correct for multiple testing²⁷.

484

485 **References**

486

- 487 1 Berg, J. M. & Shi, Y. The galvanization of biology: a growing appreciation for the
488 roles of zinc. *Science* **271**, 1081-1085 (1996).
- 489 2 Broadley, M. R., White, P. J., Hammond, J. P., Zelko, I. & Lux, A. Zinc in plants. *The*
490 *New phytologist* **173**, 677-702, doi:10.1111/j.1469-8137.2007.01996.x (2007).
- 491 3 Salgueiro, M. J. *et al.* Zinc status and immune system relationship: a review.
492 *Biological trace element research* **76**, 193-205, doi:10.1385/BTER:76:3:193
493 (2000).
- 494 4 Sinclair, S. A. & Krämer, U. The zinc homeostasis network of land plants.
495 *Biochimica et biophysica acta* **1823**, 1553-1567,
496 doi:10.1016/j.bbamcr.2012.05.016 (2012).
- 497 5 Vallee, B. L. & Auld, D. S. Zinc coordination, function, and structure of zinc
498 enzymes and other proteins. *Biochemistry* **29**, 5647-5659 (1990).
- 499 6 Fukada, T., Hojyo, S. & Furuichi, T. Zinc signal: a new player in osteobiology.
500 *Journal of bone and mineral metabolism* **31**, 129 (2013).
- 501 7 Haase, H. An element of life: competition for zinc in host-pathogen interaction.
502 *Immunity* **39**, 623-624 (2013).
- 503 8 Sohnle, P. G., Collins-Lech, C. & Wiessner, J. H. The zinc-reversible antimicrobial
504 activity of neutrophil lysates and abscess fluid supernatants. *Journal of Infectious*
505 *Diseases* **164**, 137-142 (1991).
- 506 9 Vignesh, K. S., Figueroa, J. A. L., Porollo, A., Caruso, J. A. & Deepe, G. S. Granulocyte
507 macrophage-colony stimulating factor induced Zn sequestration enhances
508 macrophage superoxide and limits intracellular pathogen survival. *Immunity* **39**,
509 697-710 (2013).
- 510 10 McDevitt, C. A. *et al.* A molecular mechanism for bacterial susceptibility to zinc.
511 *PLoS pathogens* **7**, e1002357 (2011).

- 512 11 Wodod *et al.* Response to zinc deficiency of two rice lines with contrasting
513 tolerance is determined by root growth maintenance and organic acid exudation
514 rates, and not by zinc - transporter activity. *New Phytologist* **186**, 400-414
515 (2010).
- 516 12 Dong, B., Rengel, Z. & Graham, R. D. Root morphology of wheat genotypes
517 differing in zinc efficiency. *Journal of Plant Nutrition* **18**, 2761-2773 (1995).
- 518 13 Shankar, A. H. & Prasad, A. S. Zinc and immune function: the biological basis of
519 altered resistance to infection. *The American journal of clinical nutrition* **68**, 447S-
520 463S (1998).
- 521 14 Brion, D. Zinc and Plant Diseases, . In book: *Mineral Nutrition and Plant Disease*,
522 *Publisher: APS Press, Editors: L.E. Datnoff, W.H. Elmer, D.M. Huber.*, 157-179
523 (2007).
- 524 15 Xu, D., Huang, X., Xu, Z.-Q. & Schläppi, M. The HyPRP gene EARL11 has an auxiliary
525 role for germinability and early seedling development under low temperature
526 and salt stress conditions in *Arabidopsis thaliana*. *Planta* **234**, 565-577 (2011).
- 527 16 Chai, Q., Li, B. & Xu, Z. Subcellular localization and resistance to *Gibberella*
528 *fujikuroi* of AtELHYPRP2 in transgenic tobacco. *Sheng wu gong cheng xue bao=*
529 *Chinese journal of biotechnology* **30**, 472-484 (2014).
- 530 17 Pitzschke, A., Datta, S. & Persak, H. Salt stress in *Arabidopsis*: lipid transfer
531 protein AZI1 and its control by mitogen-activated protein kinase MPK3.
532 *Molecular plant* **7**, 722-738 (2014).
- 533 18 Richards, K. D., Schott, E. J., Sharma, Y. K., Davis, K. R. & Gardner, R. C. Aluminum
534 induces oxidative stress genes in *Arabidopsis thaliana*. *Plant physiology* **116**, 409-
535 418 (1998).
- 536 19 Jung, H. W., Tschapinski, T. J., Wang, L., Glazebrook, J. & Greenberg, J. T. Priming
537 in systemic plant immunity. *Science* **324**, 89-91, doi:10.1126/science.1170025
538 (2009).
- 539 20 Yu, K. *et al.* A feedback regulatory loop between G3P and lipid transfer proteins
540 DIR1 and AZI1 mediates azelaic-acid-induced systemic immunity. *Cell Reports* **3**,
541 1266-1278 (2013).
- 542 21 Hammond-Kosack, K. E. & Jones, J. Resistance gene-dependent plant defense
543 responses. *The Plant Cell* **8**, 1773 (1996).
- 544 22 Horton, M. W. *et al.* Genome-wide patterns of genetic variation in worldwide
545 *Arabidopsis thaliana* accessions from the RegMap panel. *Nature genetics* **44**, 212-
546 216 (2012).
- 547 23 Jain, A., Sinilal, B., Dhandapani, G., Meagher, R. B. & Sahi, S. V. Effects of deficiency
548 and excess of zinc on morphophysiological traits and spatiotemporal regulation
549 of zinc-responsive genes reveal incidence of cross talk between micro-and
550 macronutrients. *Environmental science & technology* **47**, 5327-5335 (2013).
- 551 24 Khan, G. A. *et al.* Coordination between zinc and phosphate homeostasis involves
552 the transcription factor PHR1, the phosphate exporter PHO1, and its homologue
553 PHO1; H3 in *Arabidopsis*. *Journal of experimental botany* **65**, 871-884 (2014).
- 554 25 Lynch, M. & Walsh, B. *Genetics and analysis of quantitative traits*. Vol. 1 (Sinauer
555 Sunderland, MA, 1998).
- 556 26 Seren, Ü. *et al.* GWAPP: a web application for genome-wide association mapping
557 in *Arabidopsis*. *The Plant Cell* **24**, 4793-4805 (2012).
- 558 27 Benjamini, Y. & Yekutieli, D. The control of the false discovery rate in multiple
559 testing under dependency. *Annals of statistics*, 1165-1188 (2001).

- 560 28 Sasaki, N., Park, J. W., Maule, A. J. & Nelson, R. S. The cysteine-histidine-rich region
561 of the movement protein of Cucumber mosaic virus contributes to plasmodesmal
562 targeting, zinc binding and pathogenesis. *Virology* **349**, 396-408,
563 doi:10.1016/j.virol.2006.02.035 (2006).
- 564 29 Cecchini, N. M., Steffes, K., Schläppi, M. R., Gifford, A. N. & Greenberg, J. T.
565 Arabidopsis AZI1 family proteins mediate signal mobilization for systemic
566 defence priming. *Nature communications* **6** (2015).
- 567 30 Wildermuth, M. C., Dewdney, J., Wu, G. & Ausubel, F. M. Isochorismate synthase is
568 required to synthesize salicylic acid for plant defence. *Nature* **414**, 562-565
569 (2001).
- 570 31 Vlot, A. C., Dempsey, D. M. A. & Klessig, D. F. Salicylic acid, a multifaceted hormone
571 to combat disease. *Annual review of phytopathology* **47**, 177-206 (2009).
- 572 32 van Verk, M. C., Bol, J. F. & Linthorst, H. J. WRKY transcription factors involved in
573 activation of SA biosynthesis genes. *BMC plant biology* **11**, 89, doi:10.1186/1471-
574 2229-11-89 (2011).
- 575 33 Spaepen, S., Bossuyt, S., Engelen, K., Marchal, K. & Vanderleyden, J. Phenotypical
576 and molecular responses of Arabidopsis thaliana roots as a result of inoculation
577 with the auxin-producing bacterium Azospirillum brasilense. *The New phytologist*
578 **201**, 850-861, doi:10.1111/nph.12590 (2014).
- 579 34 Silverstein, K. A. *et al.* Small cysteine-rich peptides resembling antimicrobial
580 peptides have been under-predicted in plants. *The Plant journal : for cell and*
581 *molecular biology* **51**, 262-280, doi:10.1111/j.1365-313X.2007.03136.x (2007).
- 582 35 Schachtman, D. P. & Shin, R. Nutrient sensing and signaling: NPKS. *Annu. Rev.*
583 *Plant Biol.* **58**, 47-69 (2007).
- 584 36 Gruber, B. D., Giehl, R. F., Friedel, S. & von Wirén, N. Plasticity of the Arabidopsis
585 root system under nutrient deficiencies. *Plant physiology* **163**, 161-179 (2013).
- 586 37 Satbhai, S. B. *et al.* Natural allelic variation of FRO2 modulates Arabidopsis root
587 growth under iron deficiency. *Nature communications* **8**, 15603 (2017).
- 588 38 Slovak, R. *et al.* A scalable open-source pipeline for large-scale root phenotyping
589 of Arabidopsis. *The Plant Cell* **26**, 2390-2403 (2014).
- 590 39 Gifford, M. L. *et al.* Plasticity regulators modulate specific root traits in discrete
591 nitrogen environments. *PLoS genetics* **9**, e1003760 (2013).
- 592 40 Kobayashi, Y. *et al.* Joint genetic and network analyses identify loci associated
593 with root growth under NaCl stress in Arabidopsis thaliana. *Plant, cell &*
594 *environment* **39**, 918-934 (2016).
- 595 41 Gutiérrez-Coronado, M. A., Trejo-López, C. & Larqué-Saavedra, A. Effects of
596 salicylic acid on the growth of roots and shoots in soybean. *Plant Physiology and*
597 *Biochemistry* **36**, 563-565 (1998).
- 598 42 Rivas-San Vicente, M. & Plasencia, J. Salicylic acid beyond defence: its role in plant
599 growth and development. *Journal of experimental botany* **62**, 3321-3338 (2011).
- 600 43 Forieri, I. *et al.* System analysis of metabolism and the transcriptome in
601 Arabidopsis thaliana roots reveals differential co - regulation upon iron, sulfur
602 and potassium deficiency. *Plant, cell & environment* **40**, 95-107 (2017).
- 603 44 Krouk, G. *et al.* A framework integrating plant growth with hormones and
604 nutrients. *Trends in Plant Science* **16**, 178-182 (2011).
- 605 45 Mouchel, C. F., Osmont, K. S. & Hardtke, C. S. BRX mediates feedback between
606 brassinosteroid levels and auxin signalling in root growth. *Nature* **443**, 458
607 (2006).

- 608 46 Jülke, S. & Ludwig-Müller, J. Response of *Arabidopsis thaliana* Roots with Altered
609 Lipid Transfer Protein (LTP) Gene Expression to the Clubroot Disease and Salt
610 Stress. *Plants* **5**, 2 (2015).
- 611 47 Gordy, J. W., Leonard, B. R., Blouin, D., Davis, J. A. & Stout, M. J. Comparative
612 effectiveness of potential elicitors of plant resistance against *spodoptera*
613 *frugiperda* (JE Smith)(Lepidoptera: Noctuidae) in four crop plants. *PloS one* **10**,
614 e0136689 (2015).
- 615 48 Aranega-Bou, P., de la O Leyva, M., Finiti, I., García-Agustín, P. & González-Bosch,
616 C. Priming of plant resistance by natural compounds. Hexanoic acid as a model.
617 *Frontiers in plant science* **5**, 488 (2014).
- 618 49 Atkinson, N. J., Lilley, C. J. & Urwin, P. E. Identification of genes involved in the
619 response of *Arabidopsis* to simultaneous biotic and abiotic stresses. *Plant*
620 *physiology* **162**, 2028-2041 (2013).
- 621 50 Yoshida, S., Forno, D., Cock, J. & Gomez, K. Routine procedure for growing rice
622 plants in culture solution. *Laboratory manual for physiological studies of rice*, 61-
623 66 (1976).
- 624 51 Saenchai, C., Bouain, N. & Kisko, M. The Involvement of OsPHO1; 1 in the
625 Regulation of Iron Transport Through Integration of Phosphate and Zinc
626 Deficiency Signaling. *Frontiers in plant science* **7** (2016).
- 627 52 Livak, K. J. & Schmittgen, T. D. Analysis of relative gene expression data using
628 real-time quantitative PCR and the 2⁻($\Delta\Delta C_T$) Method. *Methods* **25**, 402-
629 408, doi:10.1006/meth.2001.1262 (2001).
- 630 53 Rouached, H. *et al.* Differential regulation of the expression of two high-affinity
631 sulfate transporters, SULTR1.1 and SULTR1.2, in *Arabidopsis*. *Plant physiology*
632 **147**, 897-911, doi:10.1104/pp.108.118612 (2008).
- 633 54 Baldani, V., Alvarez, M. d. B., Baldani, J. & Döbereiner, J. Establishment of
634 inoculated *Azospirillum* spp. in the rhizosphere and in roots of field grown wheat
635 and sorghum. *Plant and Soil* **90**, 35-46 (1986).
- 636 55 Mantelin, S. *et al.* Nitrate-dependent control of root architecture and N nutrition
637 are altered by a plant growth-promoting *Phyllobacterium* sp. *Planta* **223**, 591-
638 603, doi:10.1007/s00425-005-0106-y (2006).
- 639 56 Clough, S. J. & Bent, A. F. Floral dip: a simplified method for *Agrobacterium*-
640 mediated transformation of *Arabidopsis thaliana*. *The Plant journal : for cell and*
641 *molecular biology* **16**, 735-743 (1998).
- 642 57 Atwell, S. *et al.* Genome-wide association study of 107 phenotypes in *Arabidopsis*
643 *thaliana* inbred lines. *Nature* **465**, 627-631 (2010).
- 644 58 Brachi, B. *et al.* Linkage and association mapping of *Arabidopsis thaliana*
645 flowering time in nature. *PLoS genetics* **6**, e1000940 (2010).
- 646 59 Scherf, U. *et al.* A gene expression database for the molecular pharmacology of
647 cancer. *Nature genetics* **24**, 236-244 (2000).

648 649 **Supplementary Tables**

650
651 **Supplementary Table 1.** Primary root growth of 230 natural accessions of *Arabidopsis*
652 *thaliana* grown on zinc sufficient (+Zn) medium over 7 days after germination.

653
654 **Supplementary Table 2.** Primary root growth of 230 natural accessions of *Arabidopsis*
655 *thaliana* grown on Zn limiting conditions (-Zn) over 7 days after germination.

656

657 **Supplementary Table 3.** Broad sense heritabilities for growth under $-Zn$ conditions.

658

659 **Supplementary Table 4.** Mean of primary root length of accessions measured at day2 and
660 *AZII* locus marker SNP-allele (Chr4 7400493) of all accessions used in this work.

661

662 **Supplementary Table 5.** List of primers used in this study.

663

664 **Figure legends**

665 **Figure 1. AZI1 controls root growth under zinc limiting conditions.** GWAS for mean root
666 length based on a set of 230 *A. thaliana* accessions grown under (A) zinc sufficiency (+Zn) or
667 (B) low zinc ($-Zn$) (day 2). The chromosomes are represented in different colors. The
668 horizontal dash-dot line corresponds to a FDR of 10% after Benjamini–Hochberg–Yekutieli
669 correction. The red box indicates the significant association. (C) Genomic region around *AZII*
670 locus (highlighted in blue). X-axis: genomic position. Y-axis: upper panel: Gene models.
671 Lower panel: LOD score from GWAS depicted in (B). (D) Expression changes (fold change)
672 of *AZII*, *EARLII*, *AZI3*, *At4g12500*, *AZI5*, *At4g12520*, *AZI7* and *At4g12540* in Col-0 grown
673 upon growth in $-Zn$ conditions compared to Col-0 plants grown under +Zn conditions. Every
674 data point was obtained from the analysis of roots collected from a pool of ten plants. Error
675 bars correspond to s.d.; three biological repeats. The *Ubiquitin* gene was used as an internal
676 reference. (E) Average primary root length (Day 2) of wild-type plants (Col-0 genotype), *azi1*
677 mutant and overexpressor line 35S::*AZII* (OE *AZII*) plants grown under +Zn or $-Zn$
678 respectively. Experiments were independently repeated three times, and data are represented
679 as mean \pm s.d.. $n = 10$. Letters a and b indicate significantly different values at $p < 0.05$
680 determined by one-way ANOVA and Tukey HSD.

681

682 **Figure 2. Natural allelic variation of the AZII locus underlies phenotypic variation of**
683 **root length in zinc limiting conditions.** (A) Transcript level of *AZII* in *azi1* lines
684 complemented with pAZI1:*AZI* from either Col-0 or Sq-1 on $-Zn$ conditions and shown as

685 relative to +Zn condition (5 days). Five independent T3 lines were considered for this
686 analysis. Relative expression was quantified in three biological replicates using RT-qPCR.
687 The Ubiquitin gene was used as an internal reference. (B) Primary root length of *aziI* lines
688 complemented with pAZII:*AZII* from either Col-0 (n=50), Sq-1 (n=50) or the empty vector
689 (n=10) on -Zn conditions (5 days). For each genotype, three repeats each containing five
690 independent T3 lines. Box plots show analysis of the relative gene expression of *AZII* (A) and
691 primary root length (B) in of *aziI* lines complemented with pAZII:*AZI* from either Col-0, Sq-
692 1 or the empty vector on -Zn conditions. Center lines show the medians; box limits indicate
693 the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the
694 interquartile range from the 25th and 75th percentiles. Letters a, b and c indicate significantly
695 different values at $p < 0.05$ determined by one-way ANOVA and Tukey HSD.

696

697 **Figure 3. Azelaic acid, zinc level and bacterial presence interact to regulate root length**

698 **and in an *AZII* dependent fashion.** (A) Root lengths of wild-type seedlings treated with

699 different AzA concentrations on +Zn or -Zn medium for five days. Box and whisker plots

700 were generated using Prism (Graphpad), with the box represents the 25th to 75th percentiles

701 and the whiskers reaching to the lowest and highest values. The line in the box shows the

702 median. (B) Root lengths of 5-day-old Col-0 and *aziI* seedlings treated with 100 μ M AzA on

703 +Zn or -Zn medium. (C) Root lengths of Col-0 and *aziI* seedlings grown with *Azospirillum*

704 *brasiliense* (Sp245) on +Zn or -Zn medium for five days. Data are represented as mean \pm s.d..

705 (n = 10). Letters indicate significantly different values at $p < 0.05$ determined by one-way

706 ANOVA and Tukey HSD.

707

708 **Figure 4. Azelaic acid and zinc interact to regulate root growth in *Oryza sativa*.** A)

709 Images of representative Rice (*Oryza sativa*, Nipponbare) seedlings grown on Yoshida

710 medium supplemented with 300 μ M of AzA presence or absence of Zn for five days. Red

711 arrow indicates root. (B) Quantified primary root length of Rice seedlings (n=10). The data
712 are given as means \pm s.d.. Asterisk indicates statistical significance, $P < 0.05$. Double asterisk
713 indicates statistical significance, $P < 0.01$.

714

715 **Supplementary Figures**

716 **Figure S1. Genetic diversity and genotype by -Zn dependent root growth responses.** A)
717 Genetic diversity of accessions used in this study. Plotted are the two major principal
718 components (PCs) from²² that infer continuous axes of genetic variation. B, C) Mean root
719 lengths (pixels) of accessions grown in +Zn (x-axis) or -Zn (y-axis) growth conditions on the
720 days for which significant GWAS signals were found. Day 2 (B); Day 7(C).

721

722 **Figure S2. mRNA abundance of Zn-responsive genes ZIP3, ZIP5, ZIP12 and PHO1;H3**
723 **in roots of Col-0 plants exposed to different Zn availabilities.** Transcript levels of ZIP3
724 (At2g32270), ZIP5 (At1g05300), ZIP12 (At5g62160) and PHO1;H3 (At1g14040) in roots of
725 Arabidopsis (Col-0) seedlings grown on vertical agar plate in presence or absence of Zn (day
726 5) as determined by Real-time qPCR. Transcript levels of these genes are expressed relative
727 to the average transcript abundance of *UBQ10* (At4g05320) that was used as an internal
728 control, and relative to +Zn values that were set to 1. Every data point was obtained from the
729 analysis of roots collected from a pool of six plants. Data presented are means of three
730 biological replicates \pm SE. Asterisks indicate statistically significant differences compared to
731 the +Zn treatment for each gene ($P < 0.05$).

732

733 **Figure S3. Frequency distribution of mean primary root length of Arabidopsis**
734 **accessions grown on -Zn.** Histograms of the daily frequency distribution of mean primary
735 root length of 230 Arabidopsis accessions under -Zn conditions over a time course of seven
736 days.

737

738 **Figure S4. GWASs of mean root length grown on +Zn.** Manhattan Plots showing the
739 genome-wide associations of mean root length in a set of 230 *A. thaliana* accessions grown in
740 presence of zinc for seven days. The chromosomes are represented in different colours. The
741 horizontal blue dash-dot line corresponds to a nominal 0.1 significance threshold after
742 Benjamini–Hochberg–Yekutieli correction.

743
744 **Figure S5. GWASs of mean root length grown on –Zn.** Manhattan Plots showing the
745 genome-wide associations of mean root length in a set of 230 *A. thaliana* accessions grown
746 under Zn limiting conditions for seven days. The chromosomes are represented in different
747 colours. The horizontal blue dash-dot line corresponds to a nominal 0.1 significance threshold
748 after Benjamini–Hochberg–Yekutieli correction.

749
750 **Figure S6. Primary root length of *azi1* mutant, 35S *AZII* (OE *AZI*), Col-0 plants grown**
751 **on +Zn and -Zn.** Average primary root length of wild-type plants (Col-0 genotype), *azi1*
752 mutant and overexpressor line 35S::*AZII* (OE *AZII*) plants grown under +Zn or –Zn over a
753 time course of seven days. Experiments were independently repeated three times, and data are
754 represented as mean \pm s.d.. $n = 10$. Letters a, b and c indicate significantly different values at
755 $p < 0.05$ determined by one-way ANOVA and Tukey HSD.

756
757 **Figure S7. *AZII* is not involved in control of root growth under Zn or Fe limited**
758 **conditions.** (A) Representative root growth phenotypes of seedling (day 5) grown under +Zn
759 or –Zn conditions. Shown are wild-type plants (Col-0 genotype), *azi1* mutant and
760 overexpressor line (OE *AZII*) 35S::*AZII* plants. (B) Average primary root length of wild-type
761 plants (Col-0 genotype), *azi1* mutant and overexpressor line (OE *AZII*) 35S::*AZII* plants, day
762 5, grown under +Zn or –Zn respectively. (C) Average primary root length of wild-type (Col-
763 0), *azi1* mutant and overexpressor line 35S::*AZII* (OE *AZII*) plants, day 5, grown under +Fe
764 or –Fe, respectively. Experiments were independently repeated three times, and data are

765 represented as mean \pm s.d.. n = 10. Letters indicate significantly different values at p <0.05
766 determined by one-way ANOVA and Tukey HSD.

767

768 **Figure S8. Polymorphism patterns around the *AZII* locus in extreme accessions.** Gene

769 models and SNP polymorphisms among representative extreme accessions (4 accessions with

770 short root phenotype and 4 accessions with long root phenotype for the genomic region

771 surrounding the *AZII* gene. (A) Amino acid changes around *AZII* (At4g12470) locus. (B)

772 SNPs around *AZII* locus; Synonymous amino acid: green line, non- synonymous amino acid:

773 red line. Only genomes that were available in the SALK 1001 genomes browser

774 (<http://signal.salk.edu/atg1001/3.0/gebrowser.php>) as of August 2016 were considered. (C)

775 Representative images of contrasting PRG phenotype (day 5) of eight *Arabidopsis thaliana*

776 accessions grown in +Zn or -Zn conditions, scale bars: 1 cm. (D) Relative transcripts

777 accumulation of *AZII* gene in these eight accessions grown in -Zn conditions compared to

778 +Zn conditions.. *Arabidopsis Ubiquitin* gene was used as an internal reference. The data are

779 given as means \pm s.d.. n = 10.

780

781 **Figure S9. Natural allelic variation of the *AZII* locus is not relevant for root length**

782 **variation in Zn sufficient condition.** Primary root length of *azil* lines complemented with

783 pAZI1:*AZI* from either Col-0 (n=44), Sq-1 (n=44) or the empty vector (n=27) on +Zn

784 conditions for five days. For each genotype, three repeats each containing five independent

785 T3 lines. Box plots show analysis of the primary root length of *azil* lines complemented with

786 pAZI1:*AZI* from either Col-0, Sq-1 or the empty vector on +Zn conditions. Center lines show

787 the medians; box limits indicate the 25th and 75th percentiles as determined by R software;

788 whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles.

789

790 **Figure S10. Alignment of the promoters of Arabidopsis AZII genes from Col-0 and Sq-1**
791 **accessions.** Alignment was done using the MultAlign program ([http://bioinfo.genopole-](http://bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html)
792 [toulouse.prd.fr/multalin/multalin.html](http://bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html)).

793
794 **Figure S11. Expression levels of defence-related genes.** Transcripts accumulation of
795 At2g31890, At5g54250, At5g24150, At1g09070, At5g60890, At1g76850, At4g04210,
796 At3g54840, At5g66760, At2g37190, At1g14980, At1g10585, *WRKY46* (At2g46400),
797 At3g12580, *AZII* (At4g12470), *ICSI* (At1g74710), At5g47230, *WRKY28* (At4g18170),
798 At2g18210, At1g27730, At5g20030 and *PRI* (AT2G14610) in *Arabidopsis thaliana* Col-0
799 genotype grown in +Zn or -Zn conditions with or without 100µM AzA. The *Arabidopsis*
800 *Ubiquitin* gene was used as an internal reference. Experiments were independently repeated
801 three times. Data was normalized to +Zn/-AzA condition, log₂ transformed and visualized
802 using "clustered correlation" CIM (<http://discover.nci.nih.gov>)⁵⁹. Stars indicate genes
803 involved in the biosynthesis and response to salicylic acid accumulation.

804
805 **Figure S12. Conservation of the Zn/AzA interaction in rice.** Rice (*Oryza sativa*,
806 Nipponbare) seedling were grown for five days starting the day of imbibition on Yoshida
807 medium supplemented with 300 µM of AzA in presence or absence of Zn. Scans were taken
808 from 5-days old seedlings. Red arrows indicate roots.

Figure 1

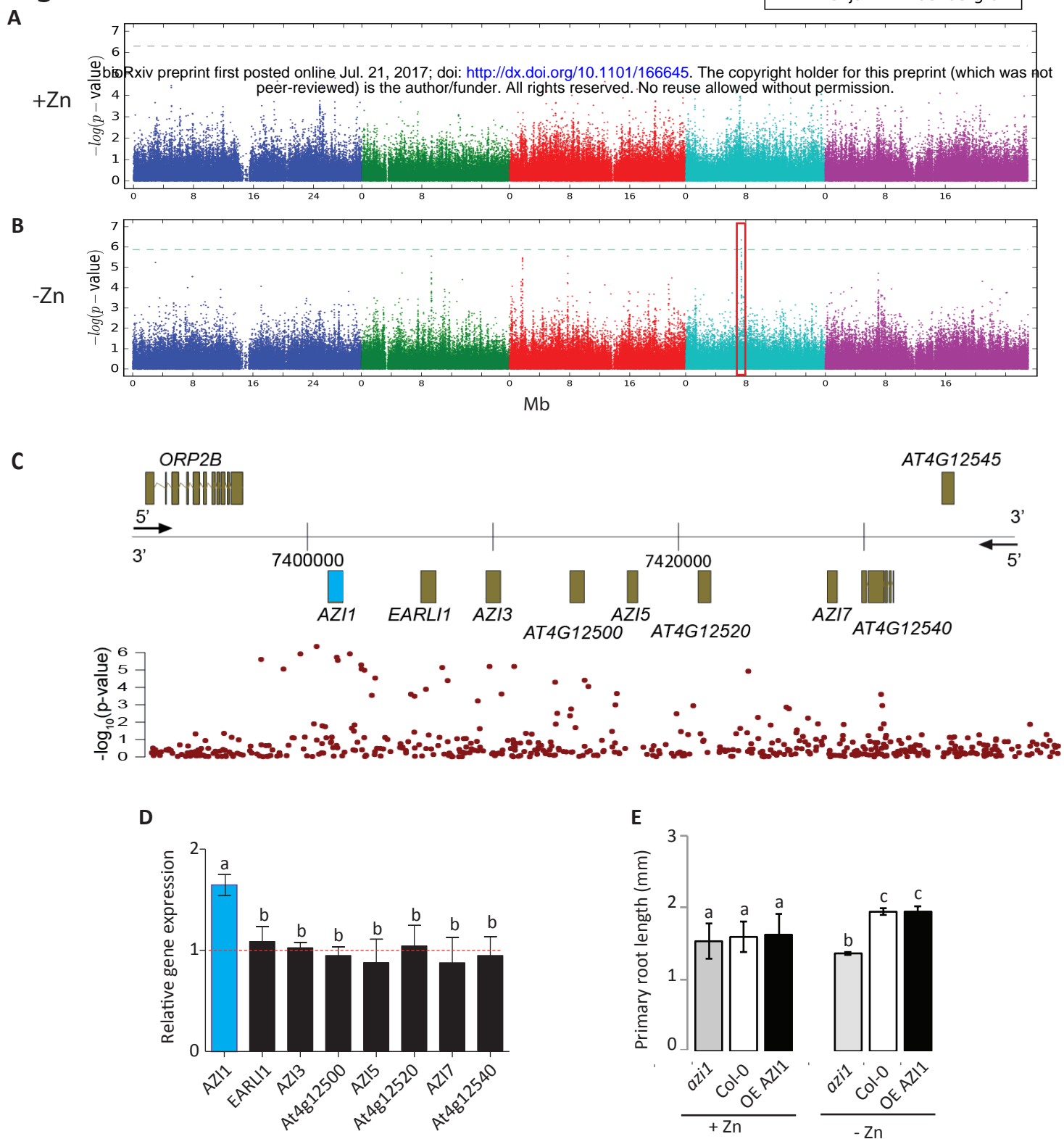


Figure 1. *AZI1* controls root growth under zinc limiting conditions. GWAS for mean root length based on a set of 230 *A. thaliana* accessions grown under (A) zinc sufficiency (+Zn) or (B) low zinc (-Zn) (day 2). The chromosomes are represented in different colors. The horizontal dash-dot line corresponds to a FDR of 10% after Benjamini-Hochberg-Yekutieli correction. The red box indicates the significant association. (C) Genomic region around *AZI1* locus (highlighted in blue). X-axis: genomic position. Y-axis: upper panel: Gene models. Lower panel: LOD score from GWAS depicted in (B). (D) Expression changes (fold change) of *AZI1*, *EARL11*, *AZI3*, *At4g12500*, *AZI5*, *At4g12520*, *AZI7* and *At4g12540* in Col-0 grown upon growth in -Zn conditions compared to Col-0 plants grown under +Zn conditions. Every data point was obtained from the analysis of roots collected from a pool of ten plants. Error bars correspond to s.d.; three biological repeats. The *Ubiquitin* gene was used as an internal reference. (E) Average primary root length of wild-type plants (Col-0 genotype), *azi1* mutant and overexpressor line 35S::*AZI1* (OE *AZI1*) plants grown under +Zn or -Zn respectively. Experiments were independently repeated three times, and data are represented as mean \pm s.d.. n = 10. Letters a and b indicate significantly different values at $p < 0.05$ determined by one-way ANOVA and Tukey HSD.

Figure 2

bioRxiv preprint first posted online Jul. 21, 2017; doi: <http://dx.doi.org/10.1101/166645>. The copyright holder for this preprint (which was not peer-reviewed) is the author/funder. All rights reserved. No reuse allowed without permission.

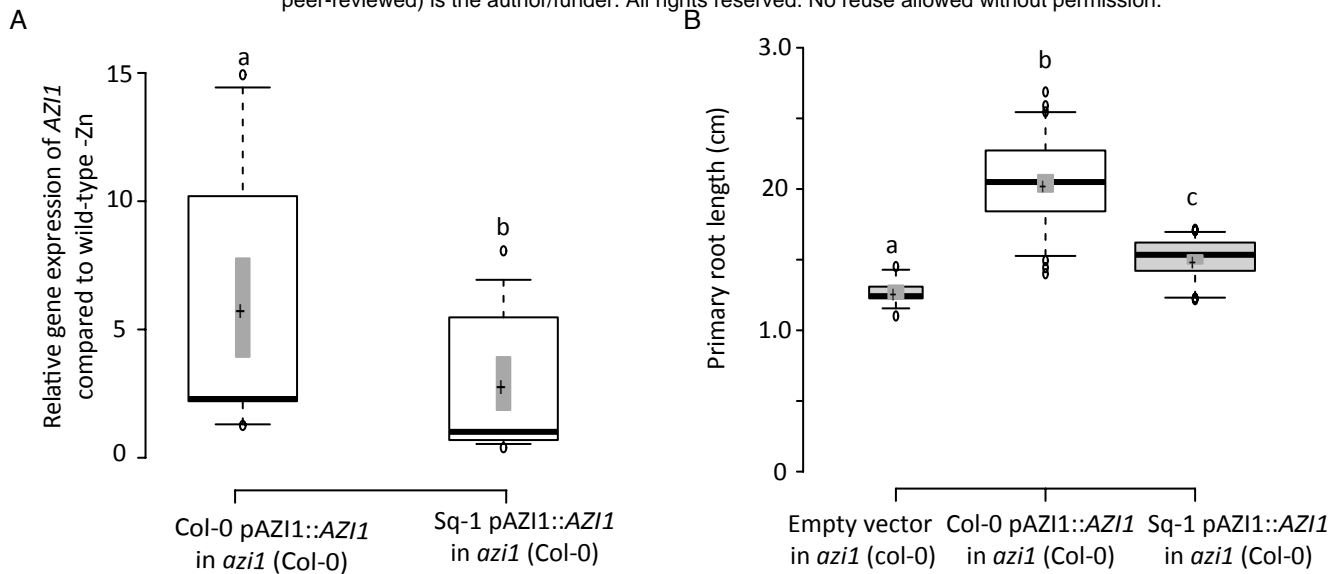


Figure 2. Natural allelic variation of the *AZI1* locus underlies phenotypic variation of root length in zinc limiting conditions. (A) Transcript level of *AZI1* in *azi1* lines complemented with pAZI1:*AZI1* from either Col-0 or Sq-1 on -Zn conditions and shown as relative to +Zn condition (5 DAG). Five independent T3 lines were considered for this analysis. Relative expression was quantified in three biological replicates using RT-qPCR. The Ubiquitin gene was used as an internal reference. (B) Primary root length of *azi1* lines complemented with pAZI1:*AZI1* from either Col-0 (n=50), Sq-1 (n=50) or the empty vector (n=10) on -Zn conditions (5 DAG). For each genotype, three repeats each containing five independent T3 lines. Box plots show analysis of the relative gene expression of *AZI1* (A) and primary root length (B) in of *azi1* lines complemented with pAZI1:*AZI1* from either Col-0, Sq-1 or the empty vector on -Zn conditions. Center lines show the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles. Letters a, b and c indicate significantly different values at $p < 0.05$ determined by one-way ANOVA and Tukey HSD.

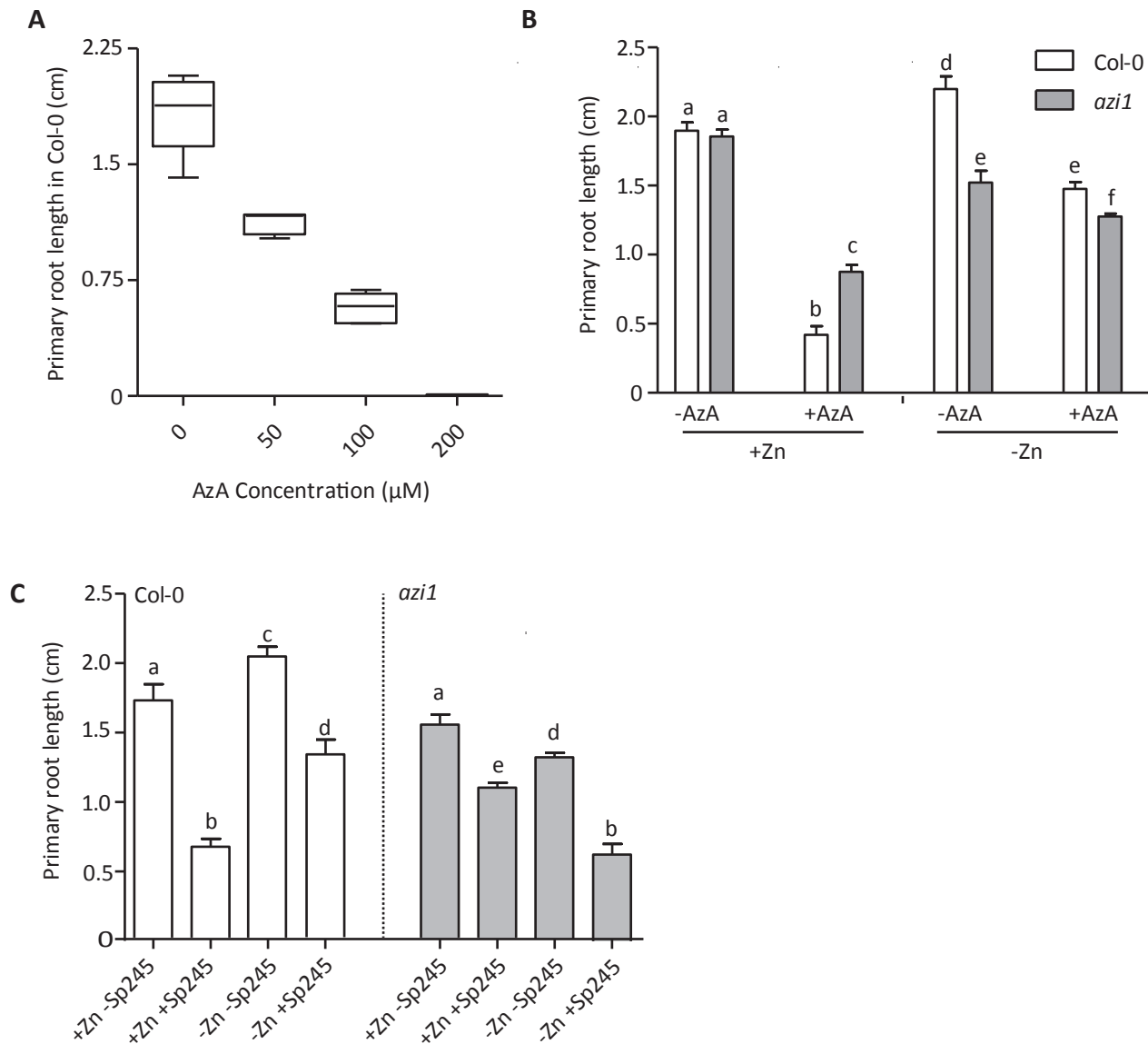


Figure 3. Azelaic acid, zinc level and bacterial presence interact to regulate root length and in an AZI1 dependent fashion. (A) Root lengths of wild-type seedlings treated with different AzA concentrations on +Zn or -Zn medium for five days. Box and whisker plots were generated using Prism (Graphpad), with the box represents the 25th to 75th percentiles and the whiskers reaching to the lowest and highest values. The line in the box shows the median. (B) Root lengths of 5-day-old Col-0 and *azi1* seedlings treated with 100μM AzA on +Zn or -Zn medium. (C) Root lengths of Col-0 and *azi1* seedlings grown with *Azospirillum brasilense* (Sp245) on +Zn or -Zn medium for five days. Data are represented as mean ± s.d.. (n = 10). Letters indicate significantly different values at p < 0.05 determined by one-way ANOVA and Tukey HSD.

Figure 4

bioRxiv preprint first posted online Jul. 21, 2017; doi: <http://dx.doi.org/10.1101/166645>. The copyright holder for this preprint (which was not peer-reviewed) is the author/funder. All rights reserved. No reuse allowed without permission.

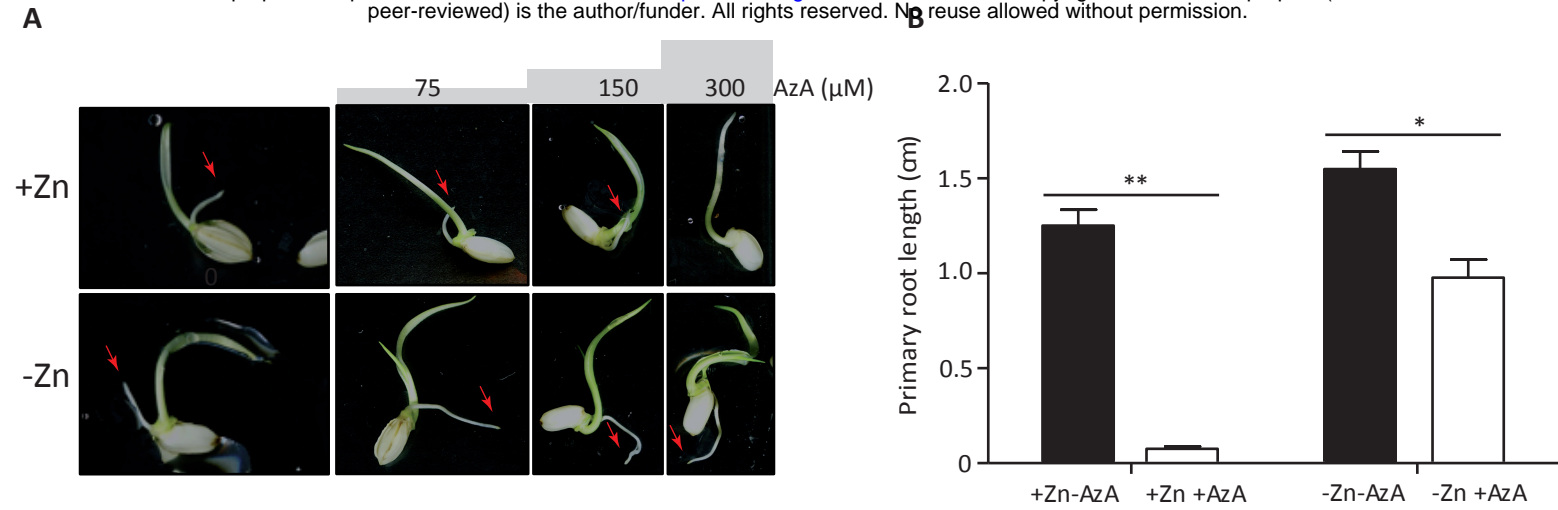


Figure 4. Azelaic acid and zinc interact to regulate root growth in *Oryza sativa*. A) Images of representative Rice (*Oryza sativa*, Nipponbare) seedlings grown on Yoshida medium supplemented with 300 μ M of AzA presence or absence of Zn for five days. Red arrow indicates root. (B) Quantified primary root length of Rice seedlings (n=10). The data are given as means \pm s.d.. Asterisk indicates statistical significance, $P < 0.05$. Double asterisk indicates statistical significance, $P < 0.01$.