

1 **Title**

2 **Constitutively enhanced genome integrity maintenance and direct stress**
3 **mitigation characterize transcriptome of extreme stress-adapted *Arabidopsis***
4 ***halleri***

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27 **Running title**

28 Intra-species comparative transcriptomics

29

30 **Abstract**

31 Heavy metal-rich toxic soils and ordinary soils are both natural habitats of *Arabidopsis halleri*. The
32 molecular divergence underlying survival in sharply contrasting environments is unknown. Here we
33 comparatively address metal physiology and transcriptomes of *A. halleri* originating from the most
34 highly heavy metal-contaminated soil in Europe, Ponte Nossa (Noss/IT), and from non-metalliferous
35 (NM) soil. Noss exhibits enhanced hypertolerance and attenuated accumulation of cadmium (Cd),
36 and transcriptomic Cd responsiveness is decreased, compared to plants of NM soil origin. Among
37 the condition-independent transcriptome characteristics of Noss, the most highly overrepresented
38 functional class of “meiotic cell cycle” comprises 21 transcripts with elevated abundance in
39 vegetative tissues, in particular *Argonaute 9 (AGO9)* and the synaptonemal complex transverse
40 filament protein-encoding *ZYP1a/b*. Increased *AGO9* transcript levels in Noss are accompanied by
41 decreased long terminal repeat retrotransposon expression, and are shared by plants from milder
42 metalliferous sites in Poland and Germany. Expression of *Iron-regulated Transporter (IRT1)* is very
43 low and of *Heavy Metal ATPase 2 (HMA2)* strongly elevated in Noss, which can account for its
44 specific Cd handling. In plants adapted to the most extreme abiotic stress, broadly enhanced functions
45 comprise genes with likely roles in somatic genome integrity maintenance, accompanied by few
46 alterations in stress-specific functional networks.

47

48 **Keywords:** heavy metal, metal hyperaccumulation, edaphic adaptation, extremophile,
49 Brassicaceae, meiosis, transposable element, LTR-TE

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52 **Introduction**

53 Heavy metals, such as zinc (Zn), cadmium (Cd) and lead (Pb), are naturally omnipresent in the
54 biosphere at very low levels. Their concentrations can be regionally elevated and are extremely high
55 locally at rare sites as a result of geological anomalies. Human activities, for example ore mining,
56 metallurgical processing and sewage sludge deposition, contribute to anthropogenic contamination
57 of the biosphere with heavy metals, threatening environmental and human health (Nriagu and Pacyna,
58 1988, Lamas *et al.*, 2016). Heavy metal-enriched habitats often host characteristic ecological
59 communities, especially where human activities have introduced extremely high, toxic levels of
60 heavy metals only recently after the beginning of the industrial revolution (Ernst, 1974). Here we use
61 comparative transcriptomics to uncover the molecular basis of local adaptation to an extremely highly
62 heavy metal-contaminated soil in a land plant as a complex multi-cellular eukaryotic organism.

63 The Brassicaceae species *Arabidopsis halleri*, in the sister clade of the genetic model plant
64 *Arabidopsis thaliana*, is an emerging perennial model plant (Krämer, 2015, Honjo and Kudoh, 2019,
65 Nagano *et al.*, 2019). *A. halleri*, a perennial stoloniferous obligate outcrosser, has repeatedly
66 colonized highly heavy metal-contaminated, so-called metalliferous (M) soils (Ernst, 1974, Bert *et*
67 *al.*, 2000, Stein *et al.*, 2017). Natural populations of *A. halleri* are also found on non-contaminated
68 (non-metalliferous, NM) soils. Irrespective of its habitat soil type, *A. halleri* is a Zn and Cd
69 hyperaccumulator (Ernst, 1974, Bert *et al.*, 2000, Stein *et al.*, 2017). The about 750 known
70 hyperaccumulator plant species are land plants of which at least one individual was identified to
71 accumulate a metal or metalloid in its above-ground tissues to concentrations more than one order of
72 magnitude above critical toxicity thresholds of ordinary plants, at its natural site of growth in the field
73 (Reeves *et al.*, 2018). Hyperaccumulation in plants can act as an elemental defense against biotic
74 stress (Kazemi-Dinan *et al.*, 2014), and the associated species-wide heavy metal tolerance can
75 apparently facilitate the colonization of M soils by *A. halleri* (Pauwels *et al.*, 2005, Meyer *et al.*,
76 2010).

77 Cross-species comparative transcriptomics studies of *A. halleri* and *A. thaliana* established tens of
78 candidate metal homeostasis genes for roles in metal hyperaccumulation or metal hypertolerance,
79 which encode various transmembrane transporters of metal cations and isoforms of a metal chelator
80 biosynthetic enzyme (Becher *et al.*, 2004, Weber *et al.*, 2004, Talke *et al.*, 2006). Although the
81 generation of transgenic plants remains technically demanding and time-consuming in *A. halleri*,
82 subsequent work demonstrated functions in metal hyperaccumulation or hypertolerance for some of
83 these genes. The key locus making the largest known contribution to both metal hyperaccumulation
84 and hypertolerance in *A. halleri* is *Heavy Metal ATPase 4 (HMA4)*, which encodes a plasma
85 membrane P_{1B}-type metal ATPase mediating cellular export for xylem loading of Zn and Cd in the
86 root (Talke *et al.*, 2006, Hanikenne *et al.*, 2008). Transcript levels of *HMA4* are substantially higher

87 in *A. halleri* than in *A. thaliana* independently of cultivation conditions, as was also observed for tens
88 of other candidate genes. A combination of modified *cis*-regulation and gene copy number expansion
89 accounts for high expression of *HMA4* (Hanikenne *et al.*, 2008). Examples of other candidate genes
90 with experimentally demonstrated functions in *A. halleri* include *Nicotianamine Synthase 2* (*NAS2*)
91 (Deinlein *et al.*, 2012) contributing to Zn hyperaccumulation, *Metal Transport Protein 1* (*MTP1*)
92 acting in Zn tolerance (Dräger *et al.*, 2004) and *Ca²⁺/H⁺ exchanger 1* (*CAX1*) functioning to enhance
93 Cd tolerance (Baliardini *et al.*, 2015). While metal hyperaccumulation and hypertolerance are
94 species-wide traits in *A. halleri*, there is additionally a large extent of within-species variation in the
95 levels of metal accumulation, metal tolerance and gene expression (Meyer *et al.*, 2015, Stein *et al.*,
96 2017, Corso *et al.*, 2018, Schwartzman *et al.*, 2018).

97 Within-species transcriptomic differences between *A. halleri* populations originating from M and
98 NM soils can provide insights into the molecular and physiological alterations associated with the
99 natural colonization of M soils, but they have hardly been explored thus far. In order to address
100 adaptation to extremely high heavy metal levels in a metalliferous soil in *A. halleri*, we focused here
101 on the population at the most highly heavy metal-contaminated *A. halleri* site in Europe at Ponte
102 Nossa (Noss/IT) according to a large-scale field survey of 165 European populations (Stein *et al.*,
103 2017). Noss individuals were more Cd-tolerant and accumulated lower levels of Cd than both closely
104 related *A. halleri* from a population on NM soil in the vicinity, Paisco Loveno (Pais), and more
105 distantly related *A. halleri* from Wallenfels (Wall) on NM soil in Germany. Further contrasting with
106 the less Cd-tolerant plants of NM soil origin, transcriptomic Cd responses including the activation of
107 Fe deficiency responses were attenuated in the highly Cd-tolerant population. Instead, a large number
108 of transcripts differed in abundance in Noss (M soil origin) from both Pais and Wall plants (NM soil
109 origins) irrespective of cultivation conditions. Overrepresentation analysis indicated that differences
110 comprised primarily the global activation of meiosis- and genome integrity maintenance-related
111 functions in somatic tissues of Noss, accompanied by pronouncedly altered levels of few metal
112 homeostasis transcripts known for central roles in Cd accumulation and basal Cd tolerance of *A.*
113 *thaliana*. Our results suggest the possibility of roles in somatic tissues of *A. halleri* (Noss) for some
114 genes functions known to operate in meiosis of *A. thaliana*. This work provides insights into how
115 land plants cope with rapid anthropogenic environmental change and extreme abiotic stress levels.

116

117 **Materials and Methods**

118 For detailed Materials and Methods see Supplemental Information.

119 **Plant material and growth conditions**

120 Cuttings were made of *A. halleri* ssp. *halleri* O'Kane & Al-Shehbaz individuals originating from the
121 field (Stein *et al.*, 2017), followed by hydroponic pre-cultivation for 4 weeks to obtain rooted

122 vegetative clones. Experiments were conducted in 1x modified Hoagland's solution (Becher *et al.*,
123 2004) with weekly exchange of solutions. In Cd tolerance assays, plants were sequentially exposed
124 to step-wise increasing concentrations of CdSO₄ (0, 25, 50, 75, 100, 125, 150, 175, 200, 250, 300,
125 350, 400 and 450 μM) once per week in a growth chamber (10-h light at 90 μmol photons m⁻² s⁻¹,
126 22°C/ 14-h dark at 18°C, 65% constant relative humidity; *n* = 6 to 9 per population, with one to three
127 vegetative clones for each of three to six genotypes per population). Cd tolerance index was
128 quantified as EC₁₀₀, the effective Cd concentration causing 100% root growth inhibition (Schat and
129 Ten Bookum, 1992). For transcriptome sequencing and multi-element analysis (Table S4), 1x
130 modified Hoagland's solution was supplemented with either 2 or 0 (control) μM CdSO₄, with
131 cultivation in 16-h light (90 μmol photons m⁻² s⁻¹, 22°C)/ 8-h dark (18°C) cycles at 60% constant
132 relative humidity for 16 d.

133 **Multi-element analysis and transcriptome sequencing**

134 For multi-element analysis, apoplastically bound metal cations were desorbed from freshly harvested
135 roots (Cailliatte *et al.*, 2010)(see Supplemental Information). All tissues were washed twice in ddH₂O
136 before drying, digestion and multi-element analysis as described (Stein *et al.*, 2017). For
137 transcriptome sequencing, root and shoot tissues were harvested separately 7 h after the onset of the
138 light period, frozen immediately in liquid nitrogen, pooled from six replicate clones per genotype,
139 treatment and experiment, and stored at -80°C. Total RNA was extracted from aliquots of tissue
140 homogenates using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). One μg of total RNA was
141 used in NEBNext Ultra II RNA Library Prep Kit for Illumina (New England Biolabs, Frankfurt,
142 Germany), followed by sequencing to obtain 14 to 29 mio. 150-bp read pairs per sample that passed
143 quality filters (Novogene, Hongkong).

144 **Sequence data analysis**

145 Reads were mapped to the *A. halleri* ssp. *gemmifera* (Matsumura) O'Kane & Al-Shehbaz accession
146 Tada mine (W302) reference genome (Briskine *et al.*, 2017) using HISAT2 version 2.1.0 (Kim *et al.*,
147 2015). This reference genome contains a more complete set of *A. halleri* coding regions than the *A.*
148 *lyrata* reference genome, and its use resulted in considerably higher mapping rates. After multiple
149 mapping error correction according to COMEX 2.1 (Pietzenuk *et al.*, 2016), the number of fragments
150 per gene were determined using Qualimap2 (Okonechnikov *et al.*, 2016), followed by principal
151 component analysis (PCA) and differential gene expression analysis using the R package *DESeq2*
152 (Love *et al.*, 2014). Clustering was performed using the R package *pheatmap*. For gene ontology
153 (GO) term enrichment analyses *A. halleri* gene IDs were converted into *A. thaliana* TAIR10 AGI
154 codes, which were then used in hypergeometric distribution estimation through the function *g:GOST*
155 built in *g:Profiler* (Reimand *et al.*, 2016). Transposable element (TE) transcript levels were quantified
156 based on Reads Per Kilobase per Million reads (Mortazavi *et al.*, 2008) after mapping to the

157 *Arabidopsis lyrata* MN47 reference genome with TE annotations (Pietzenuk *et al.*, 2016). This was
158 necessary because the *A. halleri* ssp. *gemmaifera* reference genome lacks contiguity outside coding
159 sequences and in repetitive regions and its TE annotations are less thoroughly curated. Reads were
160 used to reconstruct cDNA variants with Integrated Genome Viewer (Robinson *et al.*, 2017). Multiple
161 comparisons of means were conducted using the *stats* and *agricolae*, with normality and
162 homoscedasticity tests in the *car*, packages in R (R_Core_Team, 2013).

163 **Validation by RT-qPCR and immunoblots**

164 We used aliquots of homogenized tissues as frozen during harvest. Real-time RT-qPCR reactions
165 were run in a 384-well LightCycler®480 II System (Roche Diagnostics, Mannheim, Germany) using
166 the GoTaq qPCR Mastermix (Promega, Walldorf, Germany) and cDNAs synthesized from DNase-
167 treated total RNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific,
168 Schwerte, Germany). Immunoblots were carried out following SDS-PAGE (Lämmli, 1970) using
169 anti-AtIRT1 (AS11 1780, Lot number 1203; Agrisera, Vännas, Sweden), HRP-conjugated secondary
170 antibodies (Thermo Fisher Scientific) and detection using the ECL Select Western Blotting Detection
171 Reagent (GE Healthcare, Little Chalfont, England) and a Fusion Fx7 GelDoc (Vilber Lourmat,
172 Eberhardzell, Germany).

173

174 **Results**

175 **Choice of populations and comparative physiological characterization**

176 In a field survey of 165 *Arabidopsis halleri* sites across Europe, average soil Cd concentrations were
177 by far the highest at Ponte Nossa/IT (Noss) in both the total and hydrochloric acid-extractable soil
178 fractions (900 and 203 mg Cd kg⁻¹ soil, respectively; second highest were soils of far lower
179 concentrations of 130 and 87 mg Cd kg⁻¹ soil, respectively)(Stein *et al.*, 2017). Therefore, we chose
180 the *A. halleri* population at this M site as the focal population in this comparative study, in
181 combination with two populations at NM sites for comparison, one from the geographically closest
182 site near Paisco Loveno (Pais/IT) approximately 38 km northeast of Noss, and one near Wallenfels
183 (Wall/DE) about 520 km to the North. Average soil total Cd concentrations in close proximity of the
184 *A. halleri* individuals studied here were 700-fold higher at Noss than at Pais (Table S1). Earlier
185 comparative transcriptomics studies of Zn/Cd hyperaccumulator plant species included individuals
186 originating from geographically distant M sites only, for example in Italy and Poland for *A. halleri*,
187 and they were not designed to compare between M and NM sites (Corso *et al.*, 2018, Schwartzman
188 *et al.*, 2018, Halimaa *et al.*, 2019).

189 In order to test for adaptation of the Noss population to locally extremely high soil Cd levels, we
190 quantified Cd tolerance of individuals collected at each of the three sites in hydroponic culture system
191 in a growth chamber. Based on the proportion of individuals maintaining root growth in a sequential

192 exposure test with stepwise increasing Cd concentrations, individuals from Noss were the most
193 tolerant of the three tested populations (Fig. 1a, Table S2, Fig. S1). Following a different approach
194 for quantifying metal tolerance (Schat and Ten Bookum, 1992), mean EC₁₀₀, the effective Cd
195 concentration causing 100% root growth inhibition in a given individual, was significantly higher for
196 Noss ($344 \pm 98 \mu\text{M Cd}$) than for Pais ($219 \pm 66 \mu\text{M Cd}$) and Wall ($221 \pm 56 \mu\text{M Cd}$) (Fig. 1b). In
197 line with species-wide Cd hypertolerance of *A. halleri*, 100% root growth inhibition was previously
198 reported to occur approximately at $30 \mu\text{M Cd}$ in *A. thaliana* (Becher, 2003).

199 In natural populations in the field, Cd concentrations accumulated in leaves of *A. halleri* at Noss were
200 at least 20-fold higher than at Pais and 3-fold higher than at Wall (Table S1)(Stein *et al.*, 2017). By
201 contrast, Zn concentrations in leaves of field-collected individuals were similar in all three
202 populations, despite vastly differing soil Zn levels (see Table S1)(Stein *et al.*, 2017). The leaf:soil
203 ratio of Cd concentrations (Cd accumulation efficiency) in the field was highest in the Wall
204 population from north of the Alps and lowest in Noss, suggesting attenuated leaf Cd accumulation at
205 Noss compared to both Pais and Wall (Fig. 1c). Upon exposure to a sub-toxic concentration of $2 \mu\text{M}$
206 CdSO_4 in hydroponic culture for 16 d, Cd concentrations in roots of Noss ($140 \pm 74 \mu\text{g Cd g}^{-1} \text{DW}$)
207 were only 43% and 40% of those in Pais and Wall, respectively (Fig. 1d, Table S3). Leaf Cd
208 concentrations were also lower in Noss ($160 \pm 74 \mu\text{g Cd g}^{-1} \text{DW}$) than in Wall (about 52% of Wall;
209 $P < 0.05$), and at about 76% of those in Pais (n.s.). We additionally observed between-population
210 differences in the accumulation of other nutrients (Fig. S2). Upon Cd exposure, Cu concentrations
211 were higher, and Mn concentrations were lower than under control conditions in roots of Pais and
212 Wall, but not in roots of Noss (Fig. S2a and d). Fe concentrations were higher in roots of Noss and
213 increased further following exposure to Cd, in contrast to the two populations from NM soils (Fig.
214 S2e). In shoots of Noss, Fe concentrations were about twice as high (*ca.* $75 \mu\text{g Fe g}^{-1} \text{DW}$) than in
215 the shoots of the other two populations under both control and Cd exposure conditions (Fig. S2e).
216 Taken together, these results showed that the Noss population of *A. halleri* is more tolerant to Cd
217 than both Pais and Wall, supporting adaptation to the composition of its local soil. This was
218 accompanied by attenuated Cd accumulation and a distinct profile of tissue concentrations of
219 micronutrient metals in Noss under standardized growth conditions in hydroponic culture.

220

221 **Comparative transcriptomics**

222 Transcriptome sequencing was carried out for a total of 72 samples, with three independent repeats
223 of each experiment comprising root and shoot tissues of two genotypes from each of the three
224 populations grown in control and Cd-amended medium for 16 d (0 and $2 \mu\text{M CdSO}_4$; Table S4; note
225 that this Cd concentration is far sub-toxic in all *A. halleri* populations under investigation and was
226 chosen to elicit possible responses without causing any toxicity symptoms). Principal component

227 analysis (PCA) suggested predominant differences between root and shoot transcriptomes (Fig. S3a).
228 In both roots and shoots, transcriptomes of the geographically neighboring populations Noss and Pais
229 grouped closer together in the first principal component by comparison to the geographically distant
230 Wall population (Fig. S3b and c). This suggested that individuals from Noss and Pais are genetically
231 more closely related to one another than either of these to individuals from Wall. Based on
232 genotyping-by-sequencing of more than 800 European *A. halleri* individuals, we established that
233 Noss and Pais are both in the Central Alpine clade of *A. halleri*, whereas Wall is in the distinct Central
234 European clade (Anderson *et al.*, manuscript in preparation).
235 Upon long-term exposure to a low level of Cd as applied here, transcriptomic differences compared
236 to controls untreated with Cd comprised a few hundred genes in both the Pais and the Wall population
237 originating from NM sites (Fig. 2a and b, Figs. S4 and S5, Dataset S1). By contrast, in the more Cd-
238 tolerant population Noss, no single transcript responded in abundance to Cd exposure under the
239 employed conditions in either roots or shoots. This observation is relevant because it argues against
240 the possible hypothesis that adaptation of *A. halleri* to high-Cd soils involves an enhanced sensitivity
241 of transcriptional responsiveness to Cd. In order to identify candidate transcripts that may contribute
242 to the ability of the Noss population to persist on extremely highly heavy metal-contaminated soil,
243 we next examined the genes showing differential expression between populations (Fig. 2c and d,
244 Dataset S2). As an initial set of candidate genes, we focused on those transcripts showing differential
245 abundance between Noss (M) and both of the populations from NM soils, Pais and Wall. This
246 comparison identified differential transcript levels for as many as 1,617 genes (801/599/217, higher
247 in Noss/ lower in Noss / intermediate in Noss) in roots and 1,638 genes (797/604/237) in shoots under
248 control conditions (under Cd exposure 848/697/236 and 1140/707/339 in roots and shoots,
249 respectively) (surrounded by a red line in Fig. 2c and d).

250

251 **Candidate genes differentially expressed in Noss**

252 We subjected the genes expressed either at higher or lower levels in Noss compared to both Pais and
253 Wall (DEGs; 0 μ M Cd; Dataset S3) to a Gene Ontology Term (GO) enrichment analysis (Dataset
254 S4). Meiotic cell cycle (GO:0051321) was the most significantly enriched in the group of genes that
255 are more highly expressed in both root and shoot of Noss (Fig. 3a and b, Table 1). Among the genes
256 within the GO term meiotic cell cycle, transcript levels of *ARGONAUTE 9 (AGO9)* were substantially
257 higher in both root and shoot tissues of the Noss in comparison to both the Pais and Wall populations
258 (Fig. 3c and d, Table S5; Log₂FC = 6.6 and 4.4 to 4.6 in root and shoot, respectively; see Dataset S2).
259 By contrast, present knowledge suggests that the expression of *AGO9* in *A. thaliana* is restricted to
260 only a few cells during the early stages of meiotic megaspore formation (Duran-Figueroa and Vielle-
261 Calzada, 2010, Olmedo-Monfil *et al.*, 2010). Amino acid sequence alignment of AGO proteins in *A.*

262 *halleri* and *A. thaliana* (Fig. S6) confirmed that *AhAGO9* is the closest *A. halleri* homologue of
263 *AtAGO9* in clade 3 of ARGONAUTE proteins (AGO4, -6, -8 and -9)(Vaucheret, 2008). Sequence
264 read coverage for *AGO9*, obtained from gDNA of Noss and Pais, suggested that *AGO9* is a single-
265 copy gene (Table S6).

266 In the GO class meiotic cell cycle, transcript levels of the two gene copies of *ZIPper I* (*ZYP1a* and
267 *ZYP1b*) were also substantially higher in Noss than in both Pais and Wall (Fig. 3e and f, Tables S5
268 and S6). Amino acid sequences of *AtZYP1a* and *b* are highly similar and conserved in *AhZYP1a* and
269 *b* (Fig. S7). We validated sequencing-based transcriptomic data by reverse-transcription quantitative
270 real-time PCR for a representative set of genes including *AGO9* and *ZYP1a/b* (Table S8, Fig. S9).
271 An additional 15 genes in roots and 10 genes in shoots of the GO meiotic cell cycle showed elevated
272 transcript levels in Noss compared to both populations of NM soil (see Fig. 3a and b, Dataset S4).
273 The partly overlapping categories homologous recombination (GO:0035825), reciprocal meiotic
274 recombination (GO:0007131), meiosis I cell cycle process (GO:0061982) and condensed
275 chromosome (GO:0000793) were also enriched among transcripts of increased abundance in roots
276 of Noss. Among a total of 23 genes that were more highly expressed in either roots or shoots of Noss
277 compared to both Pais and Wall as well as grouped in over-represented GO terms related to meiotic
278 cell cycle and homologous/meiotic recombination, homologs of eight genes (35%) were previously
279 shown to have roles or implicated in somatic DNA repair in *A. thaliana* (Table 1). Furthermore, sulfur
280 compound metabolic process (GO:0006790), glucosinolate biosynthetic process (GO:0019761) and
281 plastid (GO:0009536) were enriched among transcripts of decreased abundance in shoot tissues of
282 Noss (see Dataset S4).

283

284 **Differential abundance of metal homeostasis-related transcripts between populations**

285 Next, we sought to identify metal homeostasis candidate genes contributing to the observed
286 differences in Cd tolerance and accumulation between Noss and the two other populations. For this
287 purpose, we intersected a manually curated list of metal homeostasis genes with the list of candidate
288 transcripts (see Fig. 3e-g, $|\text{Log}_2\text{FC}| > 1$, mean normalized counts across all samples > 2 ; Dataset S3,
289 Table S7). Transcript levels of *Heavy Metal ATPase 2* (*HMA2*) and two *ZRT/IRT-like Protein* genes,
290 *ZIP2* and *ZIP6*, were higher in roots of Noss than Pais and Wall (Fig. 3e). Root transcript levels of
291 *Iron-Regulated Transporter 1* (*IRT1*), *Plant Cadmium Resistance 9* (*PCR9*) and *ZIP10* were lower
292 in Noss (Fig. 3f). In shoots of Noss, transcript levels of *PCR8*, *HMA2* and *Cation/H⁺ Exchanger 2*
293 (*CHX2*) were higher than in both Pais and Wall (Fig. 3g).

294 The *IRT1* transmembrane transport protein is known as the high-affinity root iron (Fe^{2+}) uptake
295 system of dicotyledonous plants as well as the predominating path for inadvertent uptake of Cd^{2+} in
296 *Arabidopsis* (Vert *et al.*, 2002). Compared to Pais and Wall, an 84% to 92% lower expression of *IRT1*

297 in Noss (see Fig. 3f) would provide an intuitive adaptive route towards plant Cd tolerance *via*
298 exclusion (see Fig. 1d, Fig. S8). In *A. thaliana*, *IRT1* expression is under complex regulation at both
299 the transcriptional and post-transcriptional levels (Kerkeb *et al.*, 2008). To test whether reduced *IRT1*
300 protein levels accompany low *IRT1* transcript abundance in Noss, we carried out immunoblot
301 detection using an anti-*AtIRT1* antibody in subsamples of root tissues from the same experiments.
302 Note that amino acid sequences of *IRT1* proteins of *A. halleri* Noss, Pais and Wall individuals as
303 well as of *A. thaliana* are identical in the region corresponding to the peptide used to generate the
304 anti-*AtIRT1* antibody (Fig. S8, boxed). At the size corresponding to *IRT1* of Fe-deficient *A. thaliana*
305 (Fig. 3h, right lane), we observed weak bands in both Pais and Wall cultivated under both control
306 and 2 μ M Cd exposure conditions, but no band was visible in Noss, in agreement with our
307 observations at the transcript level (see also Fig. S9c for confirmation). Note that we loaded lower
308 total protein from Fe-deficient *A. thaliana* in order not to overload the detection with a very strong
309 signal and also because it served merely as a size marker. Note also that the extremely low levels of
310 *IRT1* protein under our non-Fe-deficient conditions can favor the detection of non-specific bands
311 which do not appear when *IRT1* protein levels are very high. Additional bands observed in *A. halleri*
312 at higher molecular masses are likely to constitute non-specific cross-reactions of the antibody (44
313 kDa). The sizes of bands at approximately 48 and 56 kDa are also consistent with multiply
314 ubiquitinated forms of *IRT1*, respectively, previously reported at distances of multiples of \sim 9 kDa
315 from *IRT1* (Fig. 3h)(Barberon *et al.*, 2011, Callis, 2014). In *A. thaliana*, the ubiquitination of *IRT1*
316 leads to its deactivation by removal from the plasma membrane through endocytosis (Barberon *et al.*,
317 2011). Taking these data together, the absence of a signal corresponding to the size of functional
318 *IRT1* in Noss on immunoblots, as well as low *IRT1* signals in Pais and Wall, with no changes upon
319 cultivation in 2 μ M Cd, were in full agreement with the between-population differences observed for
320 *IRT1* transcript levels using RNA-seq and RT-qPCR (see Fig. 3f, Fig. S9a).

321

322 **Cd exposure elicits transcriptional Fe deficiency responses in both populations from NM sites**

323 In the less Cd-tolerant populations, upon exposure to Cd, we found differential transcript abundance
324 for 210 genes (upregulated 53, downregulated 157) in shoots and of 428 genes (upregulated 143,
325 downregulated 285) in roots of Pais. A similar number of 197 (upregulated 65, downregulated 132)
326 in roots and 243 (upregulated 98, downregulated 145) in shoots responded to Cd in Wall (see Fig. 2a
327 and b, Dataset S5). Of these, the responses of 44 genes in roots and of 84 genes in shoots were shared
328 between both populations from NM soil, a larger number than expected by chance ($P < 10^{-50}$,
329 hypergeometric test). Transcriptomic Cd responses of both Pais and Wall were enriched in Fe
330 deficiency responses (Fig. 4, Dataset S6). The associated enriched GO categories included cellular
331 response to iron ion starvation (GO:0010106) among the upregulated transcripts, as well as

332 intracellular sequestering of iron ion (GO:0006880) and ferroxidase activity (GO:0004322) among
333 the downregulated transcripts in both roots and shoots.

334 Among transcripts known to increase in abundance in *A. thaliana* under iron deficiency, transcript
335 levels of *Popeye* (*PYE*), *Basic Helix-Loop-Helix 38/39/100/101* (*bHLH38/39/100/101*) transcription
336 factors, *Brutus* (*BTS*) and *Brutus-like 1* (*BTSL1*), as well as *Ferric Reduction Oxidases* (*FRO1* and
337 *FRO3*), *Nicotianamine Synthase 4* (*NAS4*), *Zinc Induced Facilitator 1* (*ZIF1*), *Oligopeptide*
338 *Transporter 3* (*OPT3*) and *Natural Resistance-Associated Macrophage Protein 3* (*NRAMP3*)
339 increased under Cd exposure (Table S9a). A number of genes have roles in iron storage/sequestration,
340 and their transcript levels are downregulated under iron deficiency in *A. thaliana*. Of these, *Ferritin*
341 (*FER1*, *FER3* and *FER4*) and *Vacuolar iron Transporter-Like protein* (*VTL1* and *VTL5*) transcript
342 levels were downregulated in Cd-exposed Pais and Wall plants (Table S9b).

343 Further GOs enriched among transcripts downregulated in abundance in response to Cd were
344 response to reactive oxygen species (GO:0000302), photosynthesis (GO:0015979), and chloroplast
345 thylakoid membrane (GO:0009535). The gene content of these, however, is less compelling in
346 implicating the respective biological processes (see Table S9b; Datasets S5 and S6). Chloroplast
347 thylakoid membrane is a child term of plastid (GO:0009536), which was enriched among transcripts
348 present at lower levels in Noss than in both Pais and Wall. Six genes were in common between these
349 (Table S10), of which only one, *Light Harvesting Complex of photosystem II* (*LHCB4.2*), appears to
350 function directly in photosynthesis. A more extensive search for transcripts that are Cd-responsive in
351 both Pais and Wall as well as differentially expressed in the Noss population identified *Bifunctional*
352 *inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein* in the root and *NAD(P)-*
353 *binding Rossmann-fold superfamily protein* in the shoot (Table S11). Finally, metal homeostasis
354 protein-encoding transcripts upregulated in abundance upon Cd exposure in Pais or Wall were
355 generally less abundant in Noss regardless of Cd treatment (Fig. S10, cluster R5 and S5). Conversely,
356 metal homeostasis protein-coding transcripts that were decreased in abundance under Cd exposure
357 in Pais or Wall were generally present at higher levels in Noss (Fig. S10, cluster R2 and S2). These
358 between-population differences primarily comprised genes associated with Fe deficiency responses
359 of *A. thaliana* as described above (see Fig. 4, Table S9), thus indicating against a constitutive
360 proactive activation in more Cd-tolerant Noss of the transcriptional Cd responses observed here in
361 the less Cd-tolerant Pais and Wall populations. These results suggested that transcriptional symptoms
362 elicited by Cd exposure were observed already at far-subtoxic Cd concentrations, which neither
363 caused growth impairment nor chlorosis, in the two less Cd-tolerant populations originating from
364 NM soils.

365

366 **Lower transcript levels of long terminal repeat retrotransposons in Noss**

367 Comparative transcriptomics suggested that in particular, *AGO9* transcript levels were higher in Noss
368 compared to both Pais and Wall, with the largest quantitative difference within its functional context
369 (see Table 1, Fig. 3c and d). If this were indeed the case, then we would expect that long terminal
370 repeat retrotransposons (LTR-TEs), target loci known to be transcriptionally silenced dependent on
371 *AGO9* in *A. thaliana*, are less transcriptionally active in *A. halleri* from Noss than *A. halleri* from
372 Pais and Wall (Duran-Figueroa and Vielle-Calzada, 2010). In Noss, transcript levels derived from
373 LTR-TEs were indeed lower in shoot tissues by comparison to both Pais and Wall and also lower in
374 root tissues than in Pais (Fig. 5, Fig. S11, Dataset S7).

375

376 **Elevated *AGO9* transcript levels in *A. halleri* originating from European M sites**

377 In *A. halleri*, M sites were apparently colonized by individuals from neighboring NM sites (Pauwels
378 *et al.*, 2005). We identified two additional *A. halleri* population pairs combining a highly metal-
379 contaminated (M) site and a non-contaminated (NM) site in a joint geographic region (Stein *et al.*,
380 2017). For each of the three M-NM population pairs, clones of two individuals available in our
381 laboratory (Stein *et al.*, 2017) were cultivated hydroponically, and *AGO9* transcript levels were
382 quantified in leaves by RT-qPCR. Within each of the three pairs, *AGO9* transcript levels were
383 significantly higher in *A. halleri* of M soil origin than of NM soil origin (Fig. 6; see Fig. 3d, Dataset
384 S2). Among individuals from M sites, there was strong quantitative variation in *AGO9* transcript
385 abundance, but all means were higher than the means of individuals from the corresponding NM site
386 of the same population pair. Taking into account the spatial heterogeneity of M habitats and the
387 genetic diversity within outcrossing *A. halleri* (Krämer, 2018), we conclude that elevated expression
388 of *AGO9* is common among individuals from M site populations in several regions of Europe.

389

390 **Discussion**

391 **Divergent transcript levels between populations arise more abundantly than divergent** 392 **transcriptomic responses to Cd**

393 To globally identify molecular alterations associated with plant adaptation to extreme abiotic stress,
394 we conducted comparative transcriptomics of *A. halleri* populations from contrasting environments
395 of origin (Fig. 2). In line with their origin from the most highly Cd-contaminated *A. halleri* site known
396 in Europe, our results support environment-dependent phenotypic differentiation in *A. halleri* and
397 adaptation to extremely high soil Cd levels at Noss (Fig. 1). Transcript levels of a few hundred genes
398 differed between Cd-exposed and unexposed *A. halleri* plants from the two populations originating
399 from NM soils, Pais and Wall. Thus, these less Cd-tolerant plants attained a different transcriptomic
400 state in the presence of Cd through a process that must involve the initial interaction of Cd²⁺ with
401 primary Cd-sensing or -sensitive sites, of which the molecular identities are yet unknown. By contrast,

402 in *A. halleri* from the more Cd-tolerant Noss (M) population, there was no single gene of which
403 transcript levels differed between the Cd and the control treatment (Fig. 2). This implies that in Noss,
404 Cd²⁺ was either effectively kept away from all Cd-sensing and -sensitive sites (Krämer, 2018), which
405 is likely, or alternatively Cd²⁺ did not result in any transcriptomic adjustment within a timeframe
406 relevant for our harvest after 16 d of exposure. Given that Noss is more Cd-tolerant than both Pais
407 and Wall (see Fig. 1), the molecular mechanisms conferring tolerance must then either be active also
408 in the absence of Cd, or alternatively their implementation must operate entirely downstream of
409 transcript levels, for example at the translational or post-translational level.

410 Several thousand transcripts differed in abundance between the Noss (M) population of *A. halleri*
411 and either one of the two populations originating from NM sites irrespective of Cd exposure (Fig. 2;
412 see also Fig. S10). This suggests that between-population transcriptomic divergence is substantial
413 even among the geographically proximal and phylogenetically closely related *A. halleri* populations
414 Noss and Pais (Figs. S3, S11; Anderson *et al.*, manuscript in preparation), by comparison to between-
415 population differences in the responses to Cd exposure. Similarly, earlier cross-species comparative
416 transcriptomics studies identified responses to metal exposure in *A. thaliana*, a species with only
417 basal metal tolerance, whereas there were no remarkable additional species-specific responses among
418 metal homeostasis genes in the metal-hypertolerant species *A. halleri* (Talke *et al.*, 2006, Weber *et al.*,
419 2006). Instead, tens of genes with predicted roles in metal homeostasis were more highly
420 expressed in *A. halleri* compared to *A. thaliana* irrespective of the growth conditions.

421

422 **Constitutive global activation of meiosis-related genes**

423 Meiosis is essential for sexual reproduction in eukaryotes and occurs exclusively in male and female
424 gametophyte precursor cells during the reproductive phase of the life cycle (Mercier *et al.*, 2015).
425 Yet, in somatic tissues we observed an overrepresentation of the Gene Ontology Term (GO) “meiotic
426 cell cycle” among transcripts of higher abundance in Noss by comparison to both Pais and Wall (Fig.
427 3, Table 1, Dataset S4). This included genes functioning during entry into meiosis (*AGO9*, *SWI1*),
428 cohesion complex (*SMC3*), synaptonemal complex (*ZYP1a/b*), recombination and its control (*PRD3*,
429 *BRCA2B*, *MHF2*, *MSH5*, *FLIP*), for example, in *A. thaliana* (Mercier *et al.*, 2015). In addition to its
430 role during meiosis, DNA double-strand break repair (DSBR) through homologous recombination
431 (HR) functions to maintain genome integrity following DNA damage in somatic cells (Schuermann
432 *et al.*, 2005). Exposure of plants to an excess of aluminum, boron or heavy metal ions can cause DNA
433 damage (Kovalchuk *et al.*, 2001, Rounds and Larsen, 2008, Sakamoto *et al.*, 2011, Morales *et al.*,
434 2016). Of the meiosis-related genes identified here, there is evidence for an additional involvement
435 in somatic HR-mediated DSBR for *BRCA2* (Seeliger *et al.*, 2012), *EME1A/B* (Geuting *et al.*, 2009),
436 *HEB2* (Sakamoto *et al.*, 2011), *FLIP* (Bouyer *et al.*, 2018, Fernandes *et al.*, 2018), *RPA2B* (Liu *et al.*,

437 2017) and *ERCC1* (Dubest *et al.*, 2004) in *A. thaliana* (Table 1; Dataset S4). *ERCC1* was also
438 implicated in non-HR nucleotide excision repair (Hefner *et al.*, 2003).

439 The two most strongly differentially expressed meiosis-related genes, *AGO9* and *ZYP1a/b*, were so
440 far thought to function exclusively in reproductive development of *A. thaliana*. *AGO9* is required
441 pre-meiotically to prevent the formation of excessive gametophytic precursor cells and expressed in
442 a single layer of somatic companion cells surrounding the megaspore mother cell, as well as in pollen
443 in *A. thaliana* (Olmedo-Monfil *et al.*, 2010). *AtAGO9* was proposed to act in the silencing of
444 pericentromeric TEs, predominantly long terminal repeat retrotransposons (LTR-TEs), in the ovule
445 through the interaction with 24-nucleotide small RNAs (Duran-Figueroa and Vielle-Calzada, 2010).
446 Like other organisms, plants generally inactivate TEs in order to maintain genomic stability.
447 Exposure to abiotic stress, including also heavy metal exposure, can result in the expression and
448 transposition of some TEs, especially LTR-TEs, in plants (Grandbastien, 2015) and other organisms
449 (Horvath *et al.*, 2017). In *A. halleri* from Noss elevated expression of *AGO9* in somatic cells may
450 serve to counteract LTR-TE activation as an adaptation to their high-stress natural environment.
451 Consistent with this hypothesis, transcripts derived from LTR-TEs were present at overall lower
452 levels in both root and shoot tissues of Noss compared to Pais (Fig. 5).

453 We detected no global increase in the levels of transcripts derived from LTR-TEs or any other TEs
454 at 2 μ M Cd compared to control conditions. It is possible that an increase in TE expression is locus-
455 specific, or occurs at different times after the onset of Cd exposure or at higher levels of Cd exposure
456 which plants might encounter at the Noss site at least temporarily (Krämer, 2018), or in response to
457 other abiotic stresses or combined stress exposure, in nature (see Table S1). Published circumstantial
458 evidence has implicated *AGO9* in DNA damage repair in seedlings of *A. thaliana*, i.e. at the
459 vegetative stage (Oliver *et al.*, 2014). Available antibodies against *A. thaliana* *AGO9* and *ZYP1* did
460 not detect specific bands in immunoblots of floral buds in our hands, as established by comparing
461 wild-type *Arabidopsis* with the corresponding mutants (data not shown). Future work will address
462 the biological functions of *AGO9* in somatic tissues of *A. halleri*.

463 We propose that our results reflect a constitutive transcriptional activation of genome integrity
464 maintenance in vegetative tissues as a component of extreme abiotic stress adaptation of *A. halleri* at
465 Noss. The genes newly implicated here in somatic genome integrity maintenance (Table 1) may have
466 remained unidentified in the past because their functions in somatic cells are specific to *A. halleri* or
467 because extremely low general expression levels may be sufficient to accomplish a possible somatic
468 DNA repair function in *A. thaliana*. It should be noted that Cd is not the only abiotic stress factor at
469 the Noss site in the field, where soil concentrations of Zn, Pb and Cu are also extremely high (Stein
470 *et al.*, 2017).

471 Of the meiosis genes showing elevated transcript levels in *A. halleri* from Noss (see Table 1), the
472 four genes *ZYP1a/b*, *PRD3* and *SMC3* were among a total of eight meiosis genes identified to be
473 under selection in autotetraploid *Arabidopsis arenosa*, for which there is no information on transcript
474 levels to date (Yant *et al.*, 2013). The predicted amino acid substitutions reported as hallmarks of
475 tetraploidy in *A. arenosa*, when also divergent from diploid *A. thaliana* (Col), were all absent in *A.*
476 *halleri* individuals from both Noss and Pais (data not shown). Moreover, all known *A. halleri* are
477 diploid, including all populations of this study (Anderson *et al.*, manuscript in preparation). We
478 conclude that the alterations in meiosis gene expression in plants adapted to an extreme environment
479 reported here are unrelated to published work on the adaptation of meiosis to tetraploidy that was
480 initially conducted in *A. arenosa* (Bomblies *et al.*, 2015).

481

482 **Large alterations in only few metal homeostasis functions in *A. halleri* from Noss**

483 Attenuated Cd accumulation in *A. halleri* from Noss (see Fig. 1) observed here in hydroponics is
484 consistent with earlier results from the cultivation of Noss and Wall on an artificially Cd-
485 contaminated soil (Stein *et al.*, 2017) and with observations in hydroponic culture on accessions
486 collected nearby (Meyer *et al.*, 2015, Corso *et al.*, 2018). Among metal homeostasis genes, we
487 observed substantially lower *IRT1* expression in roots of Noss compared to both Pais and Wall at
488 both the transcript (Fig. 3f) and protein levels (Fig. 3h, Fig. S9c). This can account for both enhanced
489 Cd hypertolerance and attenuated Cd accumulation in *A. halleri* from Noss, considering that *IRT1* is
490 the primary route for non-specific uptake of toxic Cd²⁺ into *A. thaliana* (Vert *et al.*, 2002). Taking
491 our data together, comparably high tissue levels of iron (Fig. S2e) in Noss plants appear to arise
492 independently of *IRT1* (Fig. 3h), which warrants further study.

493 *ZIP2* is another member of the same protein family as *IRT1* and could mediate the influx of divalent
494 metals into the cytosol (Shanmugam *et al.*, 2013). In *A. thaliana*, transcript levels of *ZIP2* were
495 decreased under Mn and Fe deficiency (Milner *et al.*, 2013). Upon Cd exposure, concentrations of
496 Mn and Fe were lowered in roots of the both populations from NM sites compared to Noss (Fig. S2d,
497 e). Based on its enhanced transcript levels in Noss, *ZIP2* could be a potential candidate gene for a
498 role in root Fe and Mn uptake in Noss (Fig. 3e). In the Wall and Pais populations originating from
499 NM soils, Fe levels were comparable under both treatment conditions, which was probably a result
500 of compensation through Fe deficiency responses, by contrast to Mn concentrations which were
501 decreased under Cd exposure in both populations originating from NM sites.

502 Distinctly higher transcript levels of *HMA2* in both roots and shoots of Noss (Fig. 3e and g) when
503 compared to both the Pais and the Wall populations suggest that enhanced cellular export of Cd
504 contributes to enhanced Cd hypertolerance in this population. Alongside *HMA4*, the plasma
505 membrane efflux pump *HMA2* mediates root-to-shoot translocation of Zn in *A. thaliana* and can also

506 transport chemically similar Cd²⁺ ions (Hussain *et al.*, 2004). The *HMA2* transcript is present in roots
507 of Zn-deficient *A. thaliana*, in particular (Sinclair *et al.*, 2018). A possible role of *HMA2* in the
508 adaptation of *A. halleri* to local soil composition in Noss must involve functional alterations
509 compared to *A. thaliana*.

510

511 **Cd exposure induces Fe deficiency responses in both populations from NM sites**

512 In the less Cd-tolerant Wall and Pais populations originating from NM soils, we observed Fe
513 deficiency response-like transcriptomic changes upon exposure to 2 µM Cd for 16 d (Fig. 4; Table
514 S9). Plants that are non-hypertolerant to metals, for example *A. thaliana*, activate aspects of Fe
515 deficiency responses when exposed to an excess of heavy metals, for example Cd (Leskova *et al.*,
516 2017). Previously published results in *A. thaliana* were from heavy metal treatment conditions
517 causing a mix of possible toxicity symptoms and acclimation responses. By contrast, in this present
518 study the Cd treatment condition was far below toxicity thresholds of *A. halleri* that is known for
519 species-wide Cd hypertolerance (see Fig. 1). Our data suggest that even under exposure to very low
520 sub-toxic Cd concentrations, transcriptional responses of *A. halleri* reflect symptoms of nutrient
521 imbalances caused by Cd. However, these responses to Cd are not associated with evolutionary
522 adaptation to high-Cd soil in *A. halleri*.

523 A transcriptomics study reported Fe deficiency responses in *A. halleri* from the Polish M site PL22,
524 but not in the I16 population from a metalliferous site close to Noss (M), in response to high-Zn
525 exposure (Schvartzman *et al.*, 2018). A highly Cd-hyperaccumulating accession of *Noccaea*
526 *caerulescens* exhibited a Cd-induced Fe deficiency response, by contrast to a low Cd-accumulating
527 accession (Halimaa *et al.*, 2019). Both of these earlier studies were lacking the comparison with a
528 neighboring population from an NM soil environment that is likely to reflect the most recent
529 environment in evolutionary history, different from the work presented here. Consequently, central
530 aspects of the heavy metal responses relating to Fe acquisition systems and tissue Fe contents
531 according to this study cannot be directly compared to these earlier studies (Fig. S1, Table S9). A
532 study that has just appeared online compares different *A. halleri* individuals from around Noss with
533 *A. halleri* from a non-contaminated site around Lozio/IT (Stein *et al.*, 2017, Corso *et al.*, 2021). Corso
534 *et al.* (2021) independently support between-population differential transcript levels of *IRT1*, *ZIP9*,
535 *ZIP6*, *HMA2* and *FER4* (see Fig. 3 and S10). According to normalized transcript levels in the dataset
536 of Corso *et al.* (2021), shoot *AGO9* and *ZYP1b* transcript levels were on average 1.7-fold and 4.6-
537 fold higher, respectively, in *A. halleri* of M origin compared to NM origin.

538

539 In summary, this comparative study of *A. halleri* populations from different soil types of origin
540 identifies transcriptomic alterations in plants that are adapted to an extreme environment, highly
541 heavy metal-contaminated soil, which has arisen through rapid anthropogenic environmental change.
542

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556

557 **Author Contribution**

558 Designed research: UK, GL, JQ, JEA, BP; conducted experiments: GL, JQ, HA, VP, LS; analyzed
559 data: GL, UK, JQ, HA, NJ, BP, LS; wrote paper: GL, UK, with contributions from JQ, HA, BP,
560 VP.

561

562 **Figure Legends**

563 **Fig. 1.** Cadmium tolerance and accumulation in three populations of *A. halleri*. (a) Proportion of
564 individuals maintaining root growth (elongation) as a function of Cd concentration. Dashed vertical
565 lines indicate ED₅₀, the effective dose required to reach a proportion of 50% according to a dose-
566 response model (see Table S2, Fig. S1). (b) Cd tolerance index. Bars represent mean ± SD of EC₁₀₀
567 (effective Cd concentration causing 100% root growth inhibition) in a given plant individual (Schat
568 and Ten Bookum, 1992). Plants were sequentially exposed to stepwise increasing concentrations of
569 CdSO₄ in hydroponic culture every week ($n = 6$ to 9 per population, with one to three vegetative
570 clones of each three to six genotypes per population; (a, b)). (c) Cd accumulation efficiency at the
571 site of origin calculated from published field survey data (bars represent means ± SD of $n = 11$ to 12
572 individuals in the field; units employed, $\mu\text{g Cd g}^{-1}$ dry biomass in leaves, $\mu\text{g total Cd g}^{-1}$ dry mass in
573 soil; data from Stein et al. 2017). (d) Cd concentration in root and shoot tissues of hydroponically

574 cultivated plants. Shown are mean \pm SD ($n = 12$ to 20 clones per population comprising both
575 genotypes, from all three replicate experiments; see Table S1). Four-week-old vegetative clones were
576 exposed to 0 (-Cd) and $2 \mu\text{M}$ CdSO₄ (+Cd) in hydroponic culture for 16 d alongside the plants
577 cultivated for RNA-seq. Different characters denote statistically significant differences between
578 means based on two-way ANOVA, followed by Tukey's HSD test (Log-transformed data were used
579 in (c)) ($P < 0.05$; see Table S3, for nested ANOVA of genotypes within a population (D)).

580

581 **Fig. 2.** Between-population differences according to transcriptome sequencing. (a, b) Venn diagrams
582 show the numbers of genes exhibiting differential transcript abundances under exposure to Cd (+Cd)
583 compared to control conditions (-Cd) for the three populations Noss (N), Pais (P) and Wall (W) in
584 root (a) and shoot (b) tissues. Upregulation (red): $\text{Log}_2(\text{fold change } +\text{Cd vs. } -\text{Cd}) = \text{Log}_2\text{FC} > 0.5$;
585 downregulation (blue): $\text{Log}_2\text{FC} < -0.5$; all with adjusted P -value < 0.05 . (c, d) Venn diagrams show
586 the numbers of genes exhibiting differential transcript abundance between populations in root (c) and
587 shoot (d) tissues of plants cultivated under Cd exposure (violet) or control conditions (orange)
588 ($|\text{Log}_2\text{FC}| > 0.5$; adjusted P -value < 0.05). Red lines surround genes differentially expressed between
589 Noss and both populations of NM soils (Pais and Wall). Four-week-old vegetative clones were
590 exposed to $2 \mu\text{M}$ Cd or $0 \mu\text{M}$ Cd (controls) in hydroponic culture for 16 d before harvest. Data are
591 from three independent experiments (repeats), with two genotypes per population and material
592 pooled from six replicate vegetative clones (three hydroponic culture vessels) per genotype in each
593 experiment.

594

595 **Fig. 3.** Candidate genes differentially expressed in Noss compared to both other populations Pais and
596 Wall. (a, b) Significantly enriched gene ontology (GO) categories for roots (a) and shoots (b) of plants
597 cultivated in $0 \mu\text{M}$ Cd (see Fig. 2c and d, Dataset S3). Genes differentially expressed between Noss
598 from M soil and both Pais and Wall from NM soils were subjected to a GO term enrichment analysis.
599 The number of genes in each over-represented category is shown inside bars (see Dataset S4 for all
600 genes). (c, d) Relative transcript levels of the top three genes (largest between-population differences)
601 taken from (a) and (b) in the over-represented GO category meiotic cell cycle, for roots (c) and shoots
602 (d). (e-g) Relative transcript levels of metal homeostasis genes of which transcript levels are high (e,
603 g) and low (f) in Noss (N) compared to both Pais (P) and Wall (W), for roots (e, f) and shoots (g)
604 (top three genes from Table S7). Bars show means \pm SD ($n = 6$) of normalized counts, which were
605 additionally normalized per kilobase of gene length (NCPK). Different characters represent
606 statistically significant groups based on two-way ANOVA, followed by Tukey's HSD test ($P < 0.05$,
607 see Table S5, for details). Data are from the same experiment as shown in Fig. 2. (h) Immunoblot of
608 IRT1. Total protein extracts ($40 \mu\text{g}$) from roots of Noss_05, Pais_09 and Wall_07 were separated on

609 a denaturing polyacrylamide gel, blotted, and detection was carried out using an anti-IRT1 antibody.
610 Total protein extract (10 µg) from roots of Fe- and Zn-deficient *A. thaliana* (*Ath*, right lane) served
611 as a positive control, with IRT1 detected as a single band at *ca.* 31 kDa. In *A. halleri*, additional bands
612 at *ca.* 44, 48 and 56 kDa are likely to constitute non-specific cross-reactions of the antibody.
613 Coomassie Blue (CB) stained membrane is shown as a loading control.

614

615 **Fig. 4.** Functional classification of the transcriptional Cd responses in less metal-tolerant populations
616 from NM sites. Significantly enriched GO categories among the transcriptional responses to Cd
617 common in both the Pais and Wall populations for roots (a) and shoots (b) (see Fig. 2a and b, Dataset
618 S6). The numbers of genes in each over-represented category are shown inside bars. Data are from
619 the same experiment as shown in Fig. 2.

620

621 **Fig. 5.** Genome-wide sum of transcript levels contributed by different types of transposable elements
622 in Noss, Pais and Wall. (a-d) Bars show means \pm SD ($n = 6$) of transcript levels of transposable
623 elements (TEs), corresponding to the sum total of RPKM for LTR (Long terminal repeat)
624 retrotransposons (a, b) and non-LTR TE (c, d) in root (a, c) and shoot (b, d), with RPKM ≥ 7 per
625 locus. Different characters represent statistically significant differences based on one-way ANOVA,
626 followed by Tukey's HSD test ($P < 0.05$).

627

628 **Figure 6.** *ARGONAUTE 9* (*AGO9*) transcript levels in *A. halleri* individuals originating from
629 geographically paired metalliferous and non-metalliferous sites in Italy, Poland and Germany.
630 Bargraphs show mean \pm SD ($n = 4$) of relative *AGO9* transcript levels quantified by RT-qPCR in
631 leaves of 5.5-week-old vegetative clones cultivated hydroponically (two independent PCR runs on
632 each of two independently synthesized cDNAs from a homogenized pool of six replicate clones; all
633 PCR reactions were conducted in hexuplicate). Data are shown relative to the constitutively
634 expressed gene *Helicase* (*HEL*). Asterisks represent statistically significant differences between M
635 and NM site of each pair, based on one-way ANOVA of Log-transformed data, followed by Tukey's
636 HSD test (***, $P < 0.001$). For details on collection sites and individuals see Stein et al. (2017).

637

638

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815 **Table 1.** List of genes in overrepresented meiosis- and homologous recombination-related GOs (see Fig. 3a, b).

Name	Description	AGI	Aha Id.	GO ^a	Somatic DNA repair in <i>A. thaliana</i>	log ₂ FC ^b	
						Max	Min
<i>AGO9</i>	Argonaute 9	AT5G21150	g12002	Meiosis	Oliver <i>et al.</i> , 2014	6.57 ***	3.88 ***
<i>ZYP1a</i>	Zipper-like 1a	AT1G22260	g13785	Meiosis/HR	n.i. ^c	4.84 ***	1.55 **
<i>ZYP1b</i>	Zipper-like 1b	AT1G22275	g13782	Meiosis/HR	n.i.	3.73 ***	2.44 ***
<i>EMB2656</i>	Embryo defective 2656	AT5G37630	g20599	HR	n.i.	3.31 ***	1.01 ***
<i>SMC2</i>	Structural maintenance of chromosomes 2	AT3G47460	g12815	Meiosis	n.i.	1.98 ***	1.12 *
<i>SW1</i>	SWITCH1	AT5G51330	g08416	Meiosis	n.i.	1.89 **	1.08 *
<i>ERCC1</i>	DNA excision repair protein ERCC-1	AT3G05210	g06284	Meiosis	Dubest <i>et al.</i> , 2004	1.63 ***	0.56 *
<i>MSH5</i>	MUTS-homologue 5	AT3G20475	g11121	Meiosis/HR	n.i.	1.60 ***	1.04 **
<i>PRD3</i>	Putative recombination initiation defects 3	AT1G01690	g01742	Meiosis	n.i.	1.60 ***	1.05 *
<i>EME1A</i>	Essential meiotic endonuclease 1A	AT2G21800	g23988	Meiosis	Geuting <i>et al.</i> , 2009	1.59 ***	0.72 *
<i>BRCA2B</i>	Breast cancer susceptibility 2 homolog B	AT5G01630	g27928	Meiosis/HR	Kumar <i>et al.</i> , 2019; Seeliger <i>et al.</i> , 2012	1.54 ***	-0.91 ***
NA	WEB family protein	AT3G02930	g31838	Meiosis/HR	n.i.	1.47 ***	0.55 ***
NA	Eisosome protein	AT1G04030	g02033	Meiosis	n.i.	1.39 ***	0.80 **
<i>RPA2B</i>	Replication protein A, subunit RPA32	AT3G02920	g02469	Meiosis/HR	Liu <i>et al.</i> , 2017	1.37 ***	0.98 ***
<i>MHF2</i>	MPH1-associated histone-fold protein 2	AT1G78790	g01439	Meiosis	n.i.	1.32 ***	0.58 *
<i>CAP-D3</i>	Condensin-2 complex subunit D3	AT4G15890	g20215	Meiosis/HR	n.i.	1.26 *	0.94 *
<i>MCM8</i>	Minichromosome maintenance 8	AT3G09660	g06091	Meiosis	n.i.	1.21 **	0.82 *
<i>ATK1</i>	<i>Arabidopsis thaliana</i> knotted-like 1	AT4G21270	g29731	Meiosis/HR	n.i.	1.15 ***	0.70 **
<i>SMC3</i>	Structural maintenance of chromosome 3	AT5G48600	g15955	Meiosis	n.i.	1.13 ***	0.46 *
<i>FLIP</i>	Fidgetin-like-1 interacting protein	AT1G04650	g02098	Meiosis	Fernandes <i>et al.</i> , 2018; Bouyer <i>et al.</i> , 2018	1.06 **	0.93 *
<i>HEB2</i>	Hypersensitive to excess boron 2	AT3G16730	g06531	HR	Sakamoto <i>et al.</i> , 2011	0.88 ***	0.40 *
<i>ESP</i>	Extra spindle poles	AT4G22970	g14375	Meiosis	n.i.	0.82 **	0.42 *
<i>EME1B</i>	Essential meiotic endonuclease 1B	AT2G22140	g28064	Meiosis	Geuting <i>et al.</i> , 2009	0.70 ***	0.35 **

816 ^aMeiosis, meiotic cell cycle or meiosis I cell cycle process; HR, homologous recombination (reciprocal meiotic recombination).

817 ^bMaximum and minimum of Log₂(fold change Noss vs. any population from an NM site) across all tissues and conditions (adjusted *P*-value < 0.001, ***; < 0.01, **; <
818 0.05, *). ^cNot identified.

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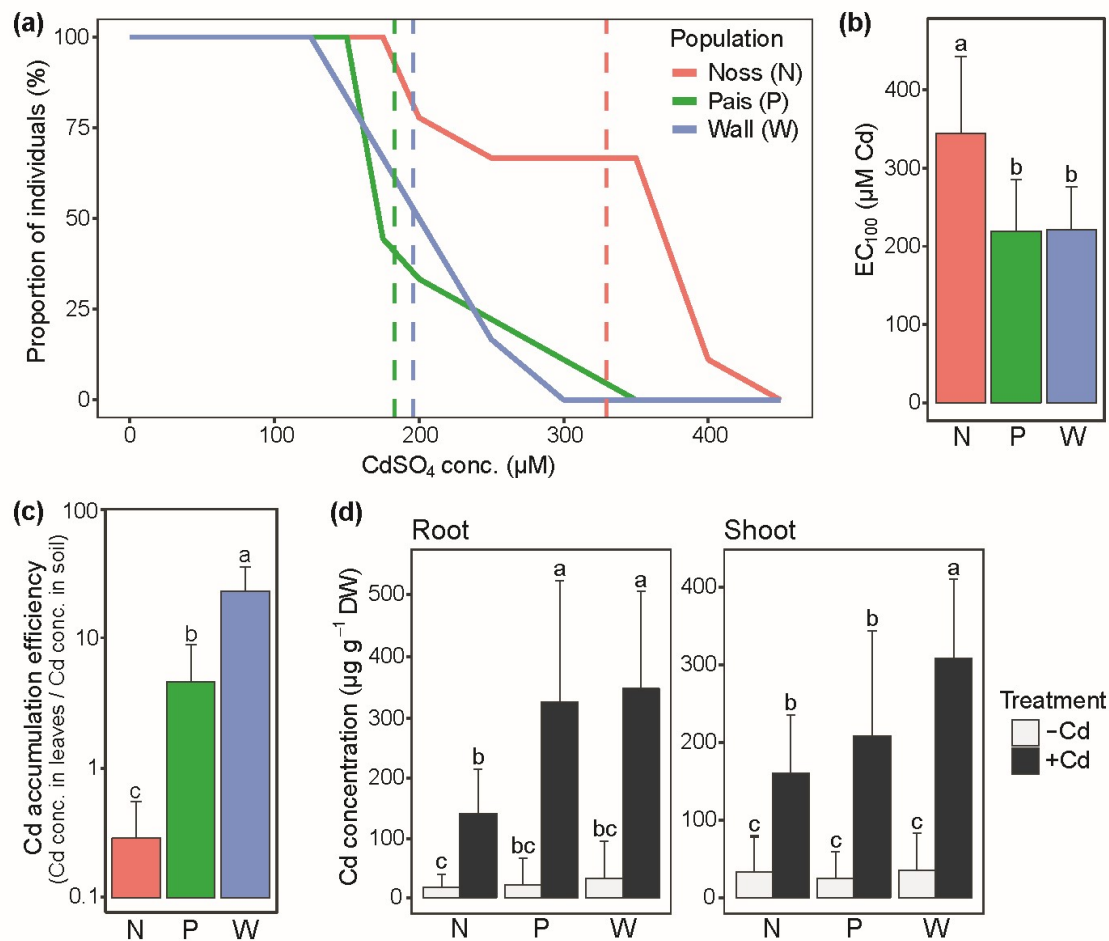


Figure 1. Cadmium tolerance and accumulation in three populations of *A. halleri*. (a) Proportion of individuals maintaining root growth (elongation) as a function of Cd concentration. Dashed vertical lines indicate ED₅₀, the effective dose required to reach a proportion of 50% according to a dose-response model (see Table S2, Figure S1). (b) Cd tolerance index. Bars represent mean ± SD of EC₁₀₀ (effective Cd concentration causing 100% root growth inhibition) in a given plant individual (Schat and Ten Bookum, 1992). Plants were sequentially exposed to stepwise increasing concentrations of CdSO₄ in hydroponic culture every week ($n = 6$ to 9 per population, with one to three vegetative clones of each three to six genotypes per population; (a, b)). (c) Cd accumulation efficiency at the site of origin calculated from published field survey data (bars represent means ± SD of $n = 11$ to 12 individuals in the field; units employed, μg Cd g⁻¹ dry biomass in leaves, μg total Cd g⁻¹ dry mass in soil; data from Stein et al. 2017). (d) Cd concentration in root and shoot tissues of hydroponically cultivated plants. Shown are mean ± SD ($n = 12$ to 20 clones per population comprising both genotypes, from all three replicate experiments; see Supplemental Table 1). Four-week-old vegetative clones were exposed to 0 (-Cd) and 2 μM CdSO₄ (+Cd) in hydroponic culture for 16 d alongside the plants cultivated for RNA-seq. Different characters denote statistically significant differences between means based on two-way ANOVA, followed by Tukey's HSD test (Log-transformed data were used in (c)) ($P < 0.05$; see Supplemental Table 3, for nested ANOVA of genotypes within a population (d)).

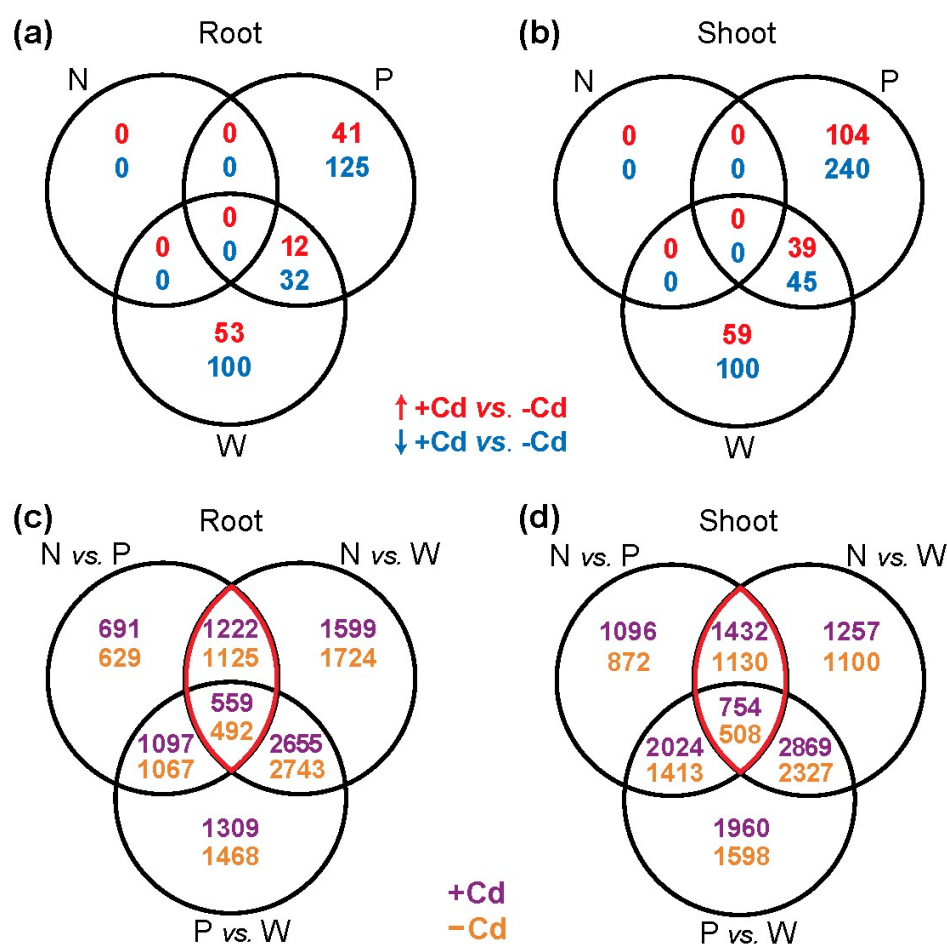


Figure 2. Between-population differences according to transcriptome sequencing. (a, b) Venn diagrams show the numbers of genes exhibiting differential transcript abundances under exposure to Cd (+Cd) compared to control conditions (-Cd) for the three populations Noss (N), Pais (P) and Wall (W) in root (a) and shoot (b) tissues. Upregulation (red): $\text{Log}_2(\text{fold change } +\text{Cd vs. } -\text{Cd}) = \text{Log}_2\text{FC} > 0.5$; downregulation (blue): $\text{Log}_2\text{FC} < -0.5$; all with adjusted P -value < 0.05 . (c, d) Venn diagrams show the numbers of genes exhibiting differential transcript abundance between populations in root (c) and shoot (d) tissues of plants cultivated under Cd exposure (violet) or control conditions (orange) ($|\text{Log}_2\text{FC}| > 0.5$; adjusted P -value < 0.05). Red lines surround genes differentially expressed between Noss and both populations of NM soils (Pais and Wall). Four-week-old vegetative clones were exposed to $2 \mu\text{M}$ Cd or $0 \mu\text{M}$ Cd (controls) in hydroponic culture for 16 d before harvest. Data are from three independent experiments (repeats), with two genotypes per population and material pooled from six replicate vegetative clones (three hydroponic culture vessels) per genotype in each experiment.

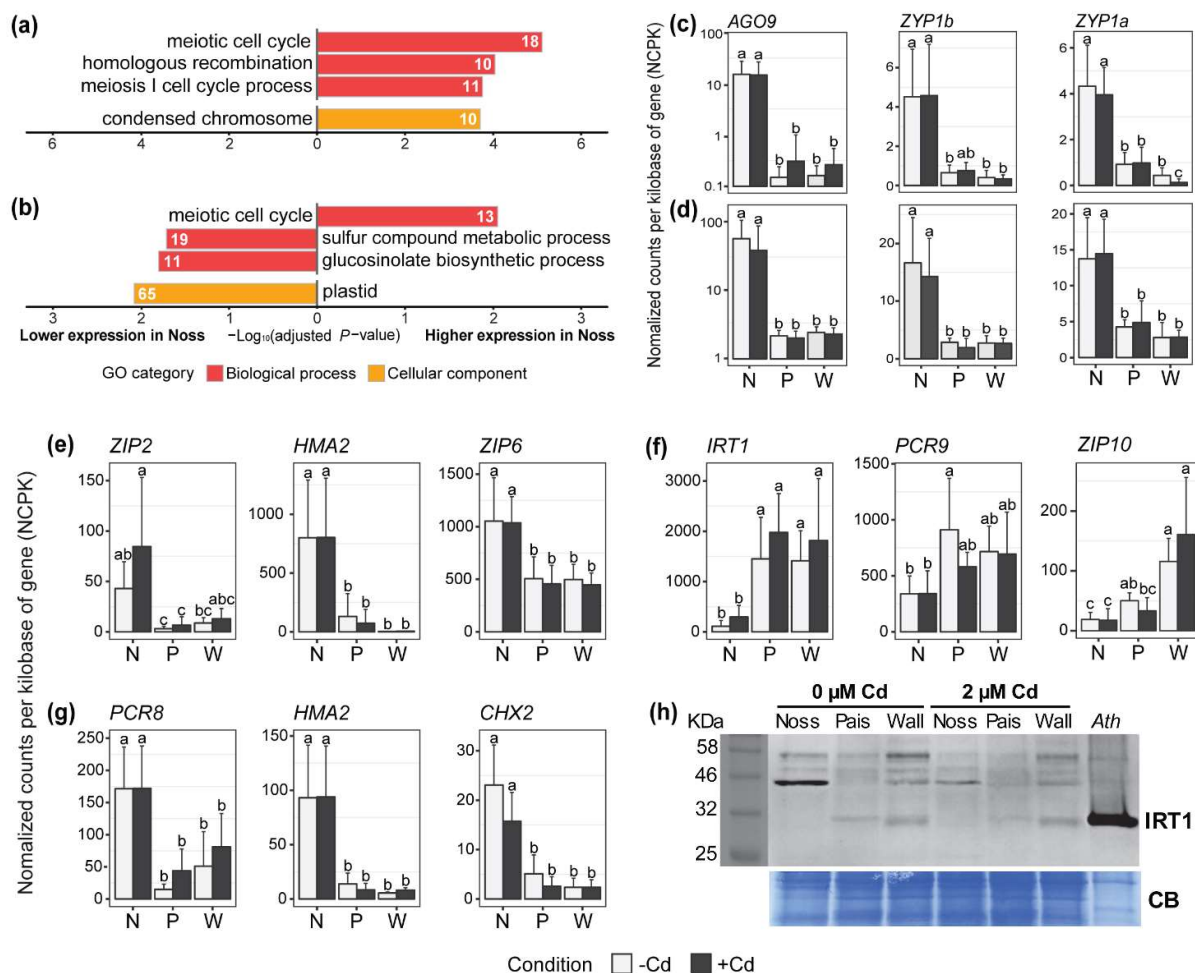


Figure 3. Candidate genes differentially expressed in Noss compared to both other populations Pais and Wall. (a, b) Significantly enriched gene ontology (GO) categories for roots (a) and shoots (b) of plants cultivated in 0 μ M Cd (see Figure 2c and d, Dataset S3). Genes differentially expressed between Noss from M soil and both Pais and Wall from NM soils were subjected to a GO term enrichment analysis. The number of genes in each over-represented category is shown inside bars (see Dataset S4 for all genes). (c, d) Relative transcript levels of the top three genes (largest between-population differences) taken from (a) and (b) in the over-represented GO category meiotic cell cycle, for roots (c) and shoots (d). (e-g) Relative transcript levels of metal homeostasis genes of which transcript levels are high (e, g) and low (f) in Noss (N) compared to both Pais (P) and Wall (W), for roots (e, f) and shoots (g) (top three genes from Table S8). Bars show means \pm SD ($n = 6$) of normalized counts, which were additionally normalized per kilobase of gene length (NCPK). Different characters represent statistically significant groups based on two-way ANOVA, followed by Tukey's HSD test ($P < 0.05$, see Table S6, for details). Data are from the same experiment as shown in Figure 2. (H) Immunoblot of IRT1. Total protein extracts (40 μ g) from roots of Noss_05, Pais_09 and Wall_07 were separated on a denaturing polyacrylamide gel, blotted, and detection was carried out using an anti-IRT1 antibody. Total protein extract (10 μ g) from roots of Fe- and Zn-deficient *A. thaliana* (*Ath*, right lane) served as a positive control, with IRT1 detected as a single band at ca. 31 kDa. In *A. halleri*, additional bands at ca. 44, 48 and 56 kDa are likely to constitute non-specific cross-reactions of the antibody. Coomassie Blue (CB) stained membrane is shown as a loading control.

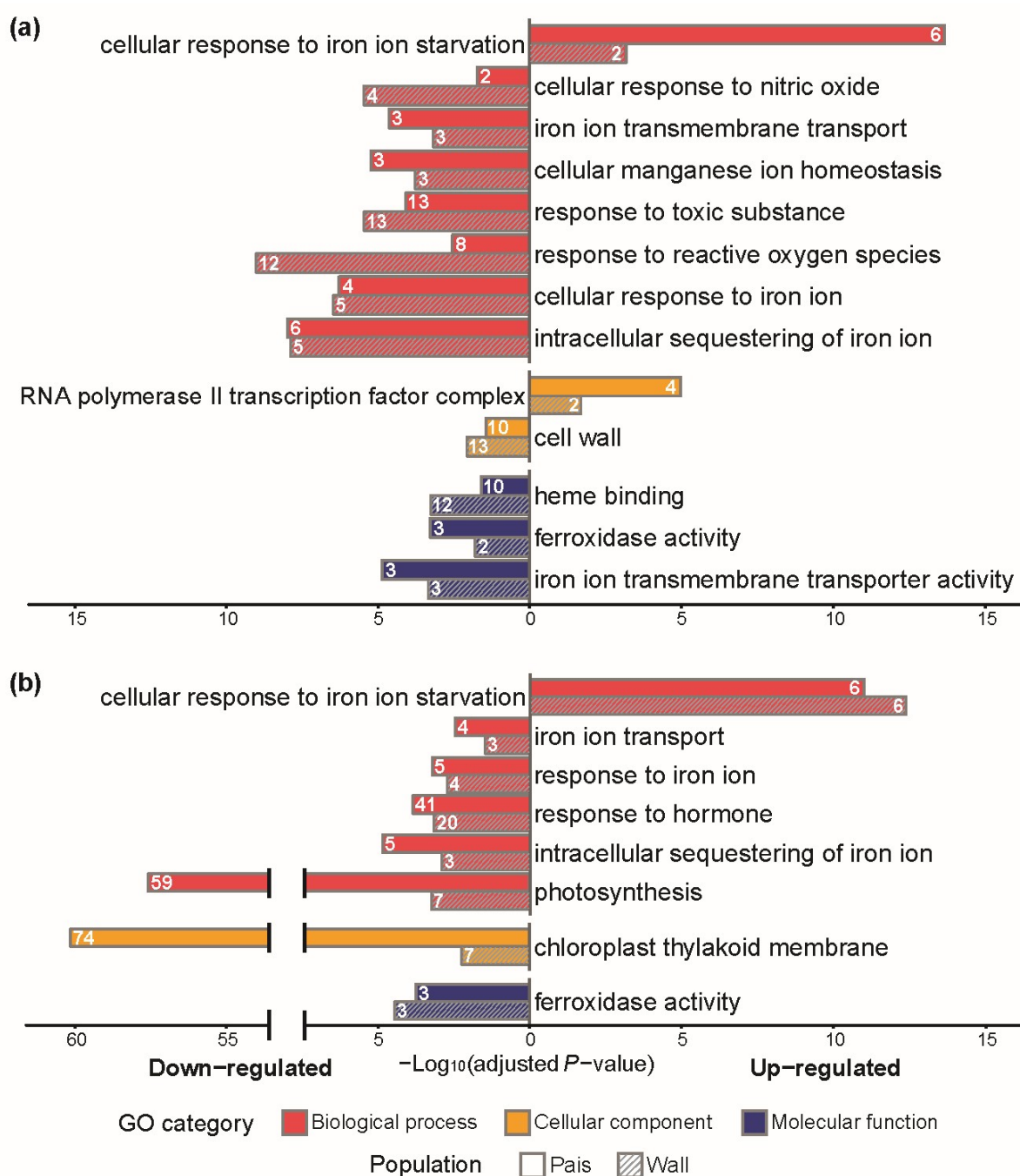


Figure 4. Functional classification of the transcriptional Cd responses in less metal-tolerant populations from NM sites. Significantly enriched GO categories among the transcriptional responses to Cd common in both the Pais and Wall populations for (a) roots and (b) shoots (see Figure 2a and b, Dataset S6). The numbers of genes in each over-represented category are shown inside bars. Data are from the same experiment as shown in Figure 2.

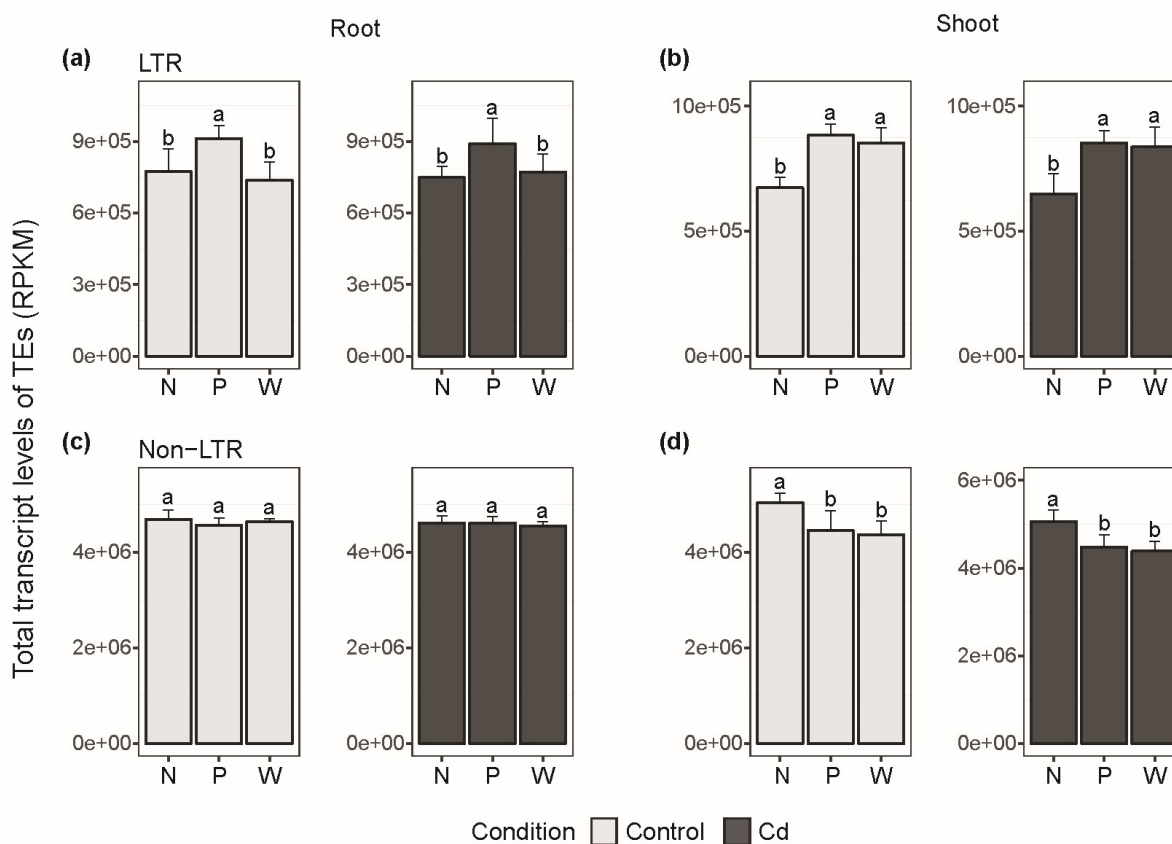


Figure 5. Genome-wide sum of transcript levels contributed by different types of transposable elements in Noss, Pais and Wall. (a-d) Bars show means \pm SD ($n = 6$) of transcript levels of transposable elements (TEs), corresponding to the sum total of RPKM for LTR (Long terminal repeat) retrotransposons (a, b) and non-LTR TEs (c, d) in root (a, c) and shoot (b, d), with RPKM ≥ 7 per locus. Different characters represent statistically significant differences based on one-way ANOVA, followed by Tukey's HSD test ($P < 0.05$).

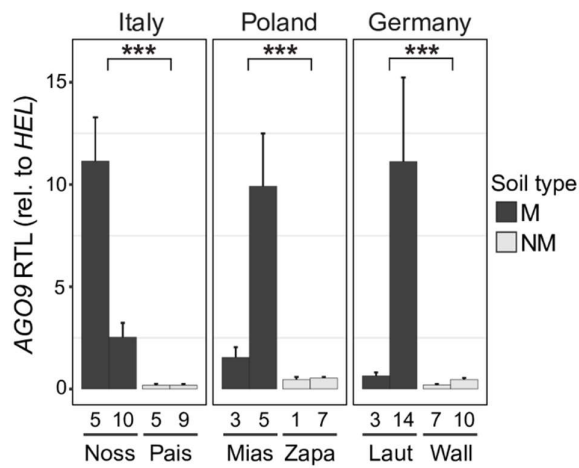


Figure 6. *ARGONAUTE 9* (*AGO9*) transcript levels in *A. halleri* individuals originating from geographically paired metalliferous and non-metalliferous sites in Italy, Poland and Germany. Bargraphs show mean \pm SD ($n = 4$) of relative *AGO9* transcript levels quantified by RT-qPCR in leaves of 5.5-week-old vegetative clones cultivated hydroponically (two independent PCR runs on each of two independently synthesized cDNAs from a homogenized pool of six replicate clones; all PCR reactions were conducted in hexuplicate). Data are shown relative to the constitutively expressed gene *Helicase* (*HEL*). Asterisks represent statistically significant differences between M and NM site of each pair, based on one-way ANOVA of Log-transformed data, followed by Tukey's HSD test (***, $P < 0.001$). For details on collection sites and individuals see Stein et al. (2017).