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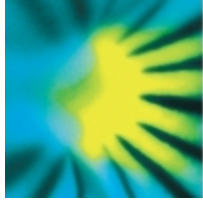
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# New Phytologist

## Wide Cross-species RNA-Seq Comparison Reveals Convergent Molecular Mechanisms Involved in Nickel Hyperaccumulation Across Dicotyledons

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1 **Wide Cross-species RNA-Seq Comparison Reveals Convergent Molecular**  
2 **Mechanisms Involved in Nickel Hyperaccumulation Across Dicotyledons**

3  
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40

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42 Convergence, *Noccaea caerulescens*.

## 44 **Summary**

- 45 • The Anthropocene epoch is associated with the spreading of metals in the  
46 environment increasing oxidative and genotoxic stress on organisms. Interestingly,  
47 about 500 plant species growing on metalliferous soils acquired the capacity to  
48 accumulate and tolerate tremendous amount of nickel in their shoots. The wide  
49 phylogenetic distribution of these species suggests that nickel hyperaccumulation  
50 evolved multiple times independently. However, the exact nature of these  
51 mechanisms and whether they have been recruited convergently in distant species  
52 is not known.
- 53 • To address these questions, we have developed a cross-species RNA-Seq approach  
54 combining differential gene expression analysis and cluster of orthologous group  
55 annotation to identify genes linked to nickel hyperaccumulation in distant plant  
56 families.
- 57 • Our analysis reveals candidate orthologous genes encoding convergent function  
58 involved in nickel hyperaccumulation, including the biosynthesis of specialized  
59 metabolites and cell wall organization. Our data also point out that the high  
60 expression of IREG/Ferroportin transporters recurrently emerged as a mechanism  
61 involved in nickel hyperaccumulation in plants. We further provide genetic evidence  
62 in the hyperaccumulator *Noccaea caerulescens* for the role of the NcIREG2  
63 transporter in nickel sequestration in vacuoles.
- 64 • Our results provide molecular tools to better understand the mechanisms of nickel  
65 hyperaccumulation and study their evolution in plants.

## 67 Introduction

68 Because of their chemical and biochemical properties, transition metals play  
69 fundamental roles in living organisms. However, when present in excess in the  
70 environment they induce adverse effects on biological systems producing oxidative and  
71 genotoxic stresses or causing secondary deficiency of essential elements because of  
72 competition (Waldron *et al.*, 2009; Andresen *et al.*, 2018). Even though they contain  
73 metal levels toxic to most plant species, metalliferous soils of geogenic or anthropogenic  
74 origin are colonized by specific floras adapted to these high concentrations of metals.  
75 While the majority of metallophyte species exclude metals from their tissues, over 700  
76 species have acquired the capacity to accumulate and tolerate tremendous amount of  
77 metals in their shoot (van der Ent *et al.*, 2013; Reeves *et al.*, 2018). Plants  
78 hyperaccumulating nickel (>1000 mg/kg shoot dry weight) represent, with around 520  
79 species, the vast majority of metal hyperaccumulators (Reeves *et al.*, 2018), likely  
80 because of the frequent occurrence of outcrops originating from ultramafic rocks,  
81 including serpentine soils, in regions of high biodiversity such as Cuba, New Caledonia  
82 or the Mediterranean basin. The large phylogenetic distribution of nickel  
83 hyperaccumulators in various plant families further suggest that nickel  
84 hyperaccumulation appeared independently several times during plant evolution  
85 (Kramer, 2010; Cappa & Pilon-Smits, 2014). However, whether the mechanisms  
86 allowing hyperaccumulation have been recruited convergently or are specific to different  
87 plant families is still poorly known.

88 As for other metals, the hyperaccumulation of nickel requires very efficient uptake by the  
89 roots, translocation of nickel to the shoot and its accumulation in leaf cells. The  
90 hyperaccumulation of nickel in leaves requires strong detoxification mechanisms  
91 involving antioxidant and metal-chelating molecules, as well as the sequestration of  
92 nickel in specific compartments (Verbruggen *et al.*, 2009; Kramer, 2010; Clemens,  
93 2019). In most of the cases, nickel is stored in the vacuoles of epidermal cells away from  
94 photosynthetic tissues (Küpper *et al.*, 2001; Broadhurst *et al.*, 2004; Bidwell *et al.*, 2004;  
95 Mesjasz-Przybylowicz *et al.*, 2016; van der Ent *et al.*, 2019), but nickel enrichment was  
96 also observed in the apoplast and cell walls surrounding epidermal cells (Krämer *et al.*,  
97 2000; Bidwell *et al.*, 2004; van der Ent *et al.*, 2019).

98 In the field of metal hyperaccumulation, most of the molecular studies have been focused  
99 on the deciphering of zinc and cadmium accumulation in Brassicaceae species related  
100 to *A. thaliana*. Genetic and transcriptomic studies performed on *Arabidopsis halleri* and  
101 *Noccaea caerulescens* indicated that metal hyperaccumulation is associated with high  
102 and constitutive expression of genes encoding metal transporters, enzymes involved in  
103 the biosynthesis of metal chelators and proteins involved in oxidative stress responses

104 (Becher *et al.*, 2004; Dräger *et al.*, 2004; Weber *et al.*, 2004; Hammond *et al.*, 2006;  
105 Shahzad *et al.*, 2010). In *A. halleri*, it was further shown that knocking down the  
106 expression of the highly expressed *HEAVY METAL ATPASE 4* transporter gene reduces  
107 zinc accumulation, thus demonstrating the essential role of *HMA4* in zinc  
108 hyperaccumulation (Hanikenne *et al.*, 2008). These data indicated that metal  
109 hyperaccumulation essentially evolved from a dysregulation of genes involved in basic  
110 mechanisms of metal homeostasis.

111 Despite the essential role of nickel in plants to sustain the activity of urease, our  
112 knowledge of the molecular mechanisms involved in nickel homeostasis is still scarce.  
113 In *Arabidopsis thaliana*, members of the IRT/ZIP and IREG/Ferroportin transporter  
114 families have been shown to play a role in nickel uptake and distribution (Schaaf *et al.*,  
115 2006; Morrissey *et al.*, 2009; Nishida *et al.*, 2011). Furthermore, genes encoding  
116 IREG/Ferroportin transporters have been shown to be expressed at higher levels in  
117 nickel hyperaccumulators compared to related non-accumulators from the  
118 Brassicaceae, Rubiaceae and Asteraceae families (Halimaa *et al.*, 2014; Merlot *et al.*,  
119 2014; Meier *et al.*, 2018). A recent transcriptomic study performed in roots of the non-  
120 accumulator *A. thaliana* revealed that high nickel treatment induces the downregulation  
121 of genes associated with cell walls, suggesting that the response to high nickel targets  
122 cell wall functions (Lešková *et al.*, 2019). In addition, several organic molecules,  
123 including histidine, nicotianamine and organic acids, have been shown to form  
124 complexes with nickel and have been proposed to play a role in hyperaccumulation  
125 (Krämer *et al.*, 1996; Vacchina *et al.*, 2003; Callahan *et al.*, 2012). However, whether  
126 these elements participate in the complex nickel hyperaccumulation trait still needs to be  
127 supported by functional and genetic evidences.

128 Here, we have developed an RNA-Seq approach combining differential gene expression  
129 analysis and cluster of orthologous group (COG) annotation to identify orthologous  
130 genes that have been convergently selected in plants to support nickel  
131 hyperaccumulation. Our cross-species comparative analysis performed on leaf  
132 transcriptomes of a wide diversity of dicotyledon families reveals several COGs  
133 associated with nickel hyperaccumulation including genes involved in metal transport,  
134 the biosynthesis of specialized metabolites and cell wall organization. We further provide  
135 genetic evidence in the nickel hyperaccumulator *Noccaea caerulea* that the  
136 IREG/Ferroportin transporter NcIREG2 is involved in nickel sequestration into vacuoles.

## 138 **Material and Methods**

### 139 *Plant material and sample collection*

140 Leaves of nickel hyperaccumulator plants belonging to 5 distinct plant families (Jestrow  
 141 *et al.*, 2012; Jaffré *et al.*, 2013; Borhidi *et al.*, 2016; Gonneau *et al.*, 2014), Brassicaceae  
 142 [*Noccaea caerulescens* *subsp. firmiensis* (*Ncfi*)], Cunoniaceae [*Geissois pruinosa*  
 143 (*Gpru*)], Euphorbiaceae [*Leucocroton havanensis* (*Lhav*)], Rubiaceae [*P. costivenia*  
 144 (*Pcos*), *P. gabriellae* (*Pgab*), *Psychotria grandis* (*Pgra*)], Salicaceae [*Homalium*  
 145 *kanaliense* (*Hkan*)], and leaves of related non-nickel hyperaccumulators, Brassicaceae  
 146 [*N. caerulescens* 'Viviez' (*Ncviv*), *N. montana* (*Nmon*)], Cunoniaceae [*G. racemosa*  
 147 (*Grac*)], Euphorbiaceae [*Lasiocroton microphyllus* (*Lmic*)], Rubiaceae [*P. revoluta*  
 148 (*Prev*), *P. semperflorens* (*Psem*)], Salicaceae [*H. betulifolium* (*Hbet*)], were collected on  
 149 individual plants growing in their natural environment and localized by GPS (Supporting  
 150 Information Table S1). We complied with local regulation for the access to these genetic  
 151 resources. For each sample, a fraction of leaves was washed with water and dried for  
 152 elemental analysis, and the other fraction fixed on site with liquid N<sub>2</sub> (*Gpru*, *Grac*, *Pgab*,  
 153 *Psem*) or with RNAlater (Sigma Aldrich) and stored at 4°C (*Ncfi*, *Ncviv*, *Nmon*, *Lhav*,  
 154 *Lmic*, *Pcos*, *Pgra*, *Prev*, *Hkan*, *Hbet*). RNAlater was removed in the laboratory and leaves  
 155 were immediately stored at -80°C before RNA extraction.

156 Additionally, to generate reference transcriptomes, *Ncfi* and *Nmon* were grown in  
 157 hydroponic condition in a climatic chamber (9 h light, 150 μE.m<sup>-2</sup>.s<sup>-1</sup>, 21 °C/17 °C  
 158 day/night, 70 % humidity) for 7 weeks using a modified Hoagland's solution (Lanquar *et*  
 159 *al.*, 2010), containing 20 μM Fe-HBED (Van Iperen International) and 37.5 μM NiCl<sub>2</sub>. *L.*  
 160 *havanensis* seeds were cultured *in vitro* on Murashige & Skoog Agar medium  
 161 supplemented with 3.2 mM NiSO<sub>4</sub>, as described (Gonzalez & Matrella, 2013).

162 A specimen of *Cunonia capensis* (*Ccap*) was obtained from a specialized plant nursery  
 163 (Ets. Railhet, France) and grown in a green-house on coconut coir supplemented with  
 164 fertilizer.

### 165 *Phylogenetic analysis*

166 Phylogenetic trees of nickel hyperaccumulator and non-accumulator species were  
 167 constructed with FigTree using the checklist of metal hyperaccumulators from (Reeves  
 168 *et al.*, 2018), the APG III classification (Chase & Reveal, 2009) and the plant time-  
 169 calibrated tree from (Magallón *et al.*, 2015). Phylogenetic analysis of IREG/FPN protein  
 170 family was performed with CLC Genomics Workbench v20 software (Qiagen).

### 171 *RNA sequencing*

172 Total RNA from leaves was extracted with RNeasy Plant Mini kit (Qiagen) for *Noccaea*  
 173 species, Qiagen hybrid method for woody plants for *Psychotria* species from Cuba



174 (Johnson *et al.*, 2012), CTAB-PVP method for *Geissois* and *Psychotria* species from  
175 New Caledonia (Johnson *et al.*, 2012), and TRI Reagent (Sigma-Aldrich) for  
176 *Leucocroton*, *Lasiocroton* and *Homalium* species. DNA was removed from all RNA  
177 samples by RNeasy Plant Mini kit on-column DNase I treatment.

178 RNA quality control, preparation of cDNA libraries, sequencing and raw reads processing  
179 were performed by the POPS transcriptomic platform (IPS2, Orsay, France). Libraries  
180 were prepared from 1 µg of total RNA using TruSeq Stranded mRNA kit (Illumina) and  
181 sequenced with an Illumina HiSeq2000 sequencing system in 100bp paired-end mode.  
182 Libraries meant to be directly compared were multiplexed and sequenced in a single run.  
183 Adaptors and low-quality pair-end sequences were removed from the raw reads and the  
184 ribosomal RNA was filtered using the SortMeRNA algorithm (Kopylova *et al.*, 2012). We  
185 obtained between 27 and 106 million reads per libraries (Supporting Information Table  
186 S2).

#### 187 *De novo transcriptome assembly and annotation*

188 The transcriptome sequences of *Ncfi*, *Nmon*, *Pgab*, *Psem*, *Pgra*, *Pcos*, *Prev*, *Gpru*, *Grac*,  
189 *Hkan*, *Hbet*, *Lhav* and *Lmic* were obtained independently by *de novo* assembly of paired-  
190 end reads using CLC Genomics Workbench v9 software (Qiagen). A single library per  
191 species was used to minimize genetic variability. Assembly parameters were set as  
192 default, except similarity (0.95), length fraction (0.75) and the word size was optimized  
193 for each sample (Supporting Information Table S2). The quality of *de novo* assemblies  
194 was assessed by Transrate v1.0.3 using trimmed read sequences (Smith-Unna *et al.*,  
195 2016) and BUSCO v4.0.4 using Viridiplantae odb10 lineage dataset (Simão *et al.*,  
196 2015). The sequences of the resulting contigs were blasted (Blastx, E-value of  $\leq 10E-6$ )  
197 against the Viridiplantae protein database (NCBI) and putative function annotated by  
198 Gene Ontology (cut-off = 55; GO weight = -5) using Blast2GO (Conesa *et al.*, 2005).  
199 Filtered contigs for length ( $\geq 200$  nt) and expression (TPKM  $> 1$ ) were translated for the  
200 longest Open Reading Frame. Translated sequences longer than 20 amino acids,  
201 together with *Arabidopsis thaliana* protein sequences (TAIR10, www.arabidopsis.org)  
202 were analyzed by OrthoFinder to annotate Clusters of Orthologous Group (COG) (Emms  
203 & Kelly, 2015).

#### 204 *Differential gene expression analysis*

205 For each pair of species, read count estimation was carried out using CLC Genomics  
206 Workbench v9 software by mapping sequencing reads of each sample replicate (default  
207 parameters, except similarity: 0.875 and length fraction: 0.75) to the transcriptome of the  
208 nickel hyperaccumulator species used as the reference. Statistical analyses to identify  
209 Differentially Expressed Genes (DEGs) were performed using the edgeR Bioconductor

210 package (Robinson *et al.*, 2010). To examine transcript abundance, reads per kilobase  
211 million (RPKM) were calculated. To evaluate the influence of the reference  
212 transcriptome, we additionally performed swapping analyses using the transcriptome of  
213 the non-accumulator species as reference (Supporting Information Table S2). The  
214 association of contigs between transcriptomes of a pair of species was established by  
215 reciprocal Blast.

### 216 *Multiple-testing analysis*

217 Following differential gene expression analyses, we recovered 25,313 COGs containing  
218 at least one DEG and collected for each of these COG a number of test statistics (i.e. p-  
219 value of all the contigs of this COG) per plant family. We further discarded COGs for  
220 which we had test statistics for less than 3 plant families (15,052 COGs), and finally kept  
221 10,261 COGs to perform the multiple-testing analysis using the Cherry R package  
222 version 0.6-12 (Goeman *et al.*, 2018). We first provided the complete list of the  
223 corresponding 121,133 p-values to Cherry and used the Simes inequality with the  
224 hommelFast function in R. Then for every COG and plant family, we identified contigs  
225 significantly up-regulated ( $FC > 2$ ) and down-regulated and used Cherry to get a lower  
226 bound on the number of H1 contigs (with a simultaneous 95% confidence using the  
227 pickSimes function in R). For every COG, we counted the number of plant families with  
228 at least one up-regulated contig (respectively down-regulated) identified by Cherry.  
229 Finally, COGs containing up-regulated or down-regulated contigs in hyperaccumulators  
230 species in at least 3 different plant families were selected as candidates COGs  
231 associated with nickel hyperaccumulation.

### 232 *Molecular cloning*

233 Predicted full-length coding region of *NcflIREG2*, *GpruIREG1* and *LhavIREG2* were  
234 amplified from leaf cDNAs of the corresponding species, using high-fidelity Phusion  
235 polymerase (Thermo Scientific) with gene specific primers containing AttB recombination  
236 sequences (Supporting Information Table S6). PCR products were first recombined into  
237 pDONOR207 (Invitrogen) and then in pDR195-GTW or pMDC83 for expression in yeast  
238 and *N. caerulescens* respectively (Curtis & Grossniklaus, 2003; Oomen *et al.*, 2008).

239 Artificial miRNA construct targeting *NcflIREG2* was designed using the WMD3-Web  
240 microRNA Designer (<http://wmd3.weigelworld.org>). The amiRNA was engineered as  
241 previously described (Schwab *et al.*, 2006), by PCR using the pRS300 backbone and  
242 specific primers (Supporting Information Table S6). The *NcflIREG2*-amiRNA precursor  
243 was recombined in pDONOR207 and then into the vector pK7GW2D (Karimi *et al.*,  
244 2002). All constructs were confirmed by restriction analysis and sequencing.

### 245 *Functional analyses of IREG/Ferroportin in Saccharomyces cerevisiae*

246 IREG/Ferroportin coding regions cloned into pDR195-GTW, as well as pDR195-  
247 AtIREG2 (Schaaf *et al.*, 2006), were transformed by the lithium acetate/PEG method into  
248 the *Saccharomyces cerevisiae* BY4741 strain complemented by a functional *HIS3* gene.  
249 Yeast sensitivity to nickel was scored by drop assay using serial dilution on histidine-free  
250 YNB agar medium containing 20 mM MES (pH5.5), supplemented or not with NiCl<sub>2</sub>.  
251 Empty pDR195 vector was used as control. Experiments were repeated twice with three  
252 independent transformants.

### 253 *Functional analysis of NcfiIREG2 in transgenic plants*

254 pK7GW2D-*NcfiIREG2-amiRNA* and pMDC83-*NcfiIREG2* were transformed into *N.*  
255 *caerulescens* subsp. *firmiensis* by *Rhizobium rhizogenes* (Arqua1 strain) mediated *in*  
256 *vitro* root transformation (Lin *et al.*, 2016). Transformed roots were selected using GFP  
257 fluorescence under a Leica MZ FLIII Fluorescence Stereo Microscope. Non-transformed  
258 roots were cut once a week until the whole root system was transgenic.

259 Independent lines transformed with pK7GW2D-*NcfiIREG2-amiRNA* were then  
260 transferred in hydroponic culture as described above for a week and then with the  
261 nutrient solution supplemented with 37.5 μM NiCl<sub>2</sub> for 4 weeks. For each transgenic line,  
262 the root system was divided in two samples for RT-qPCR and elemental analysis.

### 263 *Elemental analysis*

264 Dried environmental leaf samples were mineralized by HNO<sub>3</sub>. Multi-elemental analyses  
265 were performed using ICP-AES (LAMA laboratory, IRD, New Caledonia) or MP-AES.

266 Plant samples from hydroponic cultures were washed twice with ice-cold 10 mM  
267 Na<sub>2</sub>EDTA and twice with ice-cold ultrapure water. Samples were dried at 65 °C for 16  
268 hours, weighed and then digested with 70 % HNO<sub>3</sub> and H<sub>2</sub>O<sub>2</sub> for a total of 8 h with  
269 temperature ramping from 80 to 120 °C. Elemental analyses were performed using MP-  
270 AES (Agilent 4200, Agilent Technologies) and metal concentration was calculated by  
271 comparison with a metal standard solution.

### 272 *Confocal Imaging*

273 Roots transformed with pMDC83-*NcfiIREG2* were stained with 10 μg/ml propidium iodide  
274 (PI) and imaged on a Leica SP8X inverted confocal microscope (IMAGERIE-Gif  
275 platform) with laser excitation at 488 nm and collection of emitted light at 495–550 nm  
276 for GFP and 600–650 nm for PI.

### 277 *Quantitative RT-PCR analyses*

278 Total RNA from *Noccaea* species was extracted with RNeasy Plant Mini kit or with TRI  
279 Reagent for *Ncfi* transgenic roots. Total RNA from leaves of *Gpru* and *Ccap* was  
280 extracted with the CTAB-PVP method as described above. DNA was removed from all

281 RNA samples by RNeasy Plant Mini kit on-column DNase I treatment. RNA (1 µg) were  
282 converted to cDNA by random priming using SuperScript III or IV First-Strand reverse  
283 transcriptase (Invitrogen) according to the manufacturer's instructions. Quantitative PCR  
284 analysis were performed on a LightCycler 96 using LightCycler 480 SYBR Green I  
285 Master Mix (Roche) with the following conditions: Initial denaturation (95 °C, 300 s),  
286 followed by 40 cycles of amplification (95 °C, 15 s; 60 °C, 15 s; 72 °C, 15 s), and a  
287 melting curve (95 °C, 10 s; 65 °C, 30 s and 95 °C, 1s). Sequence information for Ccap  
288 (sample TIUZ) was obtained from the 1KP project (Matasci *et al.*, 2014). Reference  
289 genes for *Noccaea species* (*6-phosphogluconate dehydrogenase decarboxylating 3*,  
290 *Nc6PGDH*, Ncfi\_contig\_3009), and *Cunoniaceae species* (*Histone deacetylase 15*,  
291 *GpruHDAC15*, Gpru\_contig\_14406) were selected from our RNA-Seq analyses. Specific  
292 primers for *IREG/Ferroportin* and reference genes were designed to produce amplicons  
293 of 100-200 nt (Supporting Information Table S6). Relative gene expression was  
294 calculated using the primer efficiency correction method (Pfaffl, 2001). Two technical  
295 replicates were used for each sample.

#### 296 *General statistics*

297 Sample exploration and replication of sequencing data analysis were conducted using  
298 limma R package (Ritchie *et al.*, 2015), correlation analyses were conducted by linear  
299 regression using generic stats functions of R software and Heat-map clustering was  
300 carried out using heatmap.2 function of the gplots R package (R Development Core  
301 Team, 2015).

#### 302 *Data availability*

303 Raw sequence files, read count files and *de novo* assembled transcriptomes are publicly  
304 available in the NCBI's Gene Expression Omnibus under the SuperSeries accession  
305 number GSE116054 or SubSeries accession numbers GSE115411 (Brassicaceae),  
306 GSE116051 (Rubiaceae, New Caledonia), GSE116050 (Rubiaceae, Cuba),  
307 GSE116048 (Cunoniaceae), GSE116052 (Salicaceae) and GSE116049  
308 (Euphorbiaceae).

## 310 **Results**

### 311 *Sampling nickel hyperaccumulators from a wide diversity of plant families*

312 Nickel hyperaccumulators are found as herbaceous plants, shrubs or trees scattered in  
313 50 plants families almost exclusively in eudicots (45 families), in both Rosids and  
314 Asterids clades (Fig. 1). Some families such as Brassicaceae (87 taxons), Asteraceae  
315 (45 taxons) and families of the COM clade comprising Cunoniaceae (48 taxons),  
316 Euphorbiaceae (42 taxons) and Salicaceae (38 taxons) are enriched in nickel  
317 hyperaccumulators (Jaffré *et al.*, 2013; Reeves *et al.*, 2018). The distribution of nickel  
318 hyperaccumulators suggests that this complex trait appeared independently several  
319 times during plant evolution but also that some families may have a predisposition to  
320 develop this trait.

321 To cover this large diversity, we sampled leaves of 7 nickel hyperaccumulators from 5  
322 genera (i.e. *Noccaea*, *Psychotria*, *Geissois*, *Homalium*, *Leucocroton*) corresponding to  
323 Brassicaceae, Rubiaceae and the families of the COM clade Cunoniaceae, Salicaceae  
324 and Euphorbiaceae. These samples have been collected in their natural environment on  
325 ultramafic soils in France, New Caledonia and Cuba and the elemental analysis of these  
326 samples confirmed the nickel hyperaccumulator status of the corresponding species. For  
327 each hyperaccumulator, we also collected leaves of a closely related species or  
328 accession not hyperaccumulating nickel (Table 1; Supporting Information Fig. S1, Table  
329 S1). Both nickel hyperaccumulators and the corresponding non-hyperaccumulator  
330 species were collected in the same geographic area, and when it was possible non-  
331 hyperaccumulator species were collected on ultramafic soils (i.e. *N. montana*, *P.*  
332 *semperflorens* and *H. betulifolium*).

### 334 *Identification of genes differentially expressed between hyperaccumulators and related* 335 *non-accumulators*

336 To identify genes differentially expressed between nickel hyperaccumulator and closely  
337 related non-accumulator species, we performed RNA sequencing (RNA-Seq) using  
338 Illumina paired-end technology (Supporting Information Fig. S2). In absence of genomic  
339 reference sequences for these species, we assembled *de novo* the sequence of  
340 expressed genes for 13 selected species using short paired-end Illumina reads. These  
341 newly generated transcriptomes contain between 41,843 and 87,243 contigs for a total  
342 assembly size ranging from 35 Mbp to 49 Mbp and a N50 ranging from 692 to 1735 bp  
343 (Table 1, Supporting Information Table S2). With the exception of *N. montana*, these *de*  
344 *novo* assemblies received a TransRate score ranging from 0.31 to 0.49 and the analysis  
345 with BUSCO indicates that they contain from 61% to 89% of complete genes with 3% to

346 9% of missing genes (Supporting Information Table S2). These parameters indicate that  
347 the quality of these *de novo* assemblies is in the range of the assemblies usually obtained  
348 for plant transcriptomes with this sequencing technology. The significant higher number  
349 of contigs and the lower scores obtained for *Noccaea montana* is likely the result of the  
350 allogamous reproduction of *N. montana* increasing genetic diversity and thus affecting  
351 *de novo* assembly. Subsequent annotation of these transcriptomes using Blastx  
352 interrogation of Viridiplantae proteins database revealed significant homologies for 56 %  
353 of the contigs on average (E-value  $\leq 10E-6$ ). These annotated reference transcriptomes  
354 constitute a unique and comprehensive resource for molecular studies on plant groups  
355 of high interest.

356 The reads from each environmental sample replicates were then mapped to the nickel  
357 hyperaccumulator reference transcriptome to identify differentially expressed (DE)  
358 genes in 8 pairs of species (or accessions), each containing a hyperaccumulator and a  
359 related non-accumulator. The fraction of DE genes ranged from 2 % to 29 % (Fold  
360 Change  $\geq 2$ ,  $FDR \leq 0.05$ ), depending on the pair of species considered (Fig. 2a;  
361 Supporting Information Fig. S3, Table S4). To evaluate the influence of the choice of the  
362 reference transcriptome, we performed swapping analysis. To perform this analysis, we  
363 calculated the read count for each contig using the transcriptome of the non-accumulator  
364 species as reference and we performed the same transcriptomic comparison as before.  
365 We then compared the fold change of the contigs obtained with the hyperaccumulator  
366 and the non-accumulator transcriptomes successively used as references (Supporting  
367 Information Fig. S4). This analysis revealed that the results were strongly correlated in  
368 most pair of species ( $r = 0.81-0.85$ ; P-value  $< 0.05$ ). The lower correlation ( $r = 0.70$ )  
369 obtained when using the non-accumulator *N. montana* transcriptome as reference is  
370 likely the result of the lower quality of the transcriptome assembly for this species.  
371 Overall, the results indicated that the choice of the reference transcriptome when  
372 studying closely related species had only a marginal influence on the identification of DE  
373 genes.

374

### 375 *Identification of biological functions associated with nickel hyperaccumulation in distant* 376 *families*

377 To determine if dysregulated molecular and metabolic pathways are shared by nickel  
378 hyperaccumulators from distant plant families, it was necessary to compare their  
379 transcriptomes altogether with a functional emphasis. To this aim, we classified the gene  
380 products into Clusters of Orthologous Groups (COG). Combining the 13 reference  
381 transcriptomes, 443,400 contigs (66.5% of total) were assigned to 46,458 COGs



382 (Supporting Information Table S3), and 25,313 COGs included at least one DE gene in  
383 one plant family. Following the differential gene expression analysis in the different plant  
384 families, we performed a multiple-testing analysis at the COGs level using the R Cherry  
385 package (Goeman & Solari, 2011). We selected up-regulated and down-regulated COGs  
386 containing genes more expressed, and less expressed respectively, in  
387 hyperaccumulators from at least 3 different plant families. This analysis revealed 26  
388 groups, including 15 up-regulated COGs and 11 down-regulated COGs (Fig. **2b**;  
389 Supporting Information Table S5). The functional annotation of these COGs indicated  
390 that the most represented categories in up-regulated COGs (Fig. **2b, c**) correspond to  
391 class I transposable elements (TEs), known as retrotransposons and to genes involved  
392 in the biosynthesis and metabolism of specialized metabolites. In addition to organic  
393 acids and nitrogen/oxygen ligands (e.g. histidine and nicotianamine acid) that are known  
394 nickel ligands, more recent studies have highlighted the role of the phenylpropanoid  
395 compounds coumarins and flavonoids as potential metal ligands involved in metal  
396 transport, tolerance and accumulation in plants (Kasprzak *et al.*, 2015; Tsai & Schmidt,  
397 2017; Corso *et al.*, 2018). Therefore, the high expression of genes coding for enzymes  
398 involved in the biosynthesis of specific metabolites such as UDP-  
399 glucose:anthocyanidin/flavonol 3-O-glucosyltransferase (COG 35) or BAHD  
400 acyltransferase (COG 44) could therefore lead to an increased synthesis of metal ligands  
401 in nickel hyperaccumulators.

402 On the other hand, the most represented categories in down-regulated COGs  
403 correspond to genes involved in response to stress and in cell wall biogenesis or  
404 organization (Fig. **2d**). This latter category includes COGs corresponding to expansin  
405 (COG 75), xyloglucan endotransglucosylase/hydrolase (COG 109) and pectin  
406 acetyltransferase (COG 423). Because of their capacity to bind positively charged metals,  
407 cell walls may regulate the apoplastic mobility of metals or be a reservoir for metals in  
408 nickel hyperaccumulators (Krämer *et al.*, 2000; Krzesłowska, 2011; Le Gall *et al.*, 2015).  
409 However, it is difficult to predict the consequences of a reduced expression of these  
410 genes on cell wall properties regarding metals binding in nickel hyperaccumulators.

411

412 *The high expression of IREG/Ferroportin transporters in leaves has a conserved function*  
413 *in nickel hyperaccumulation*

414 Our analysis points to IREG/Ferroportin transporters (COG 1981) as some of the most  
415 robustly up-regulated function associated with nickel hyperaccumulation in several plant  
416 families (Fig. **2c**; Supporting Information Table S5). Phylogenetic analysis revealed that  
417 Brassicaceae species, including *N. caerulescens*, contain 2 gene clusters, represented

418 by *A. thaliana* IREG1 and IREG2, belonging to COG 1981 (Supporting Information Fig.  
419 S5). These transporters were shown to localize on different membranes and therefore  
420 proposed to play distinct functions in metal transport (Schaaf *et al.*, 2006; Morrissey *et*  
421 *al.*, 2009). In several species from the Euphorbiaceae, Salicaceae, Cunoniaceae and  
422 Rubiaceae families, we also observe a duplication of IREG/Ferroportin genes. However,  
423 these transporters do not cluster with AthaIREG1 or AthaIREG2 and it is therefore not  
424 possible to infer their specific function. Genes encoding IREG/Ferroportin transporters  
425 are significantly more expressed (from 4 to 800-fold increase) in several nickel  
426 hyperaccumulator species from the Brassicaceae, Rubiaceae and Euphorbiaceae  
427 families compared to their related non-accumulator species. Very recently, the high  
428 expression in leaves of an IREG/Ferroportin gene from the Asteraceae species *Senecio*  
429 *coronatus* was associated with the capacity to hyperaccumulate nickel (Meier *et al.*,  
430 2018). Together, these results strongly support the hypothesis that the high expression  
431 of *IREG/Ferroportin* in leaves may play an essential and conserved role in nickel  
432 hyperaccumulation in a wide diversity of plant families in both Asterid and Rosid clades.

433 In the selected pairs of species of the Salicaceae and Cunoniaceae families originating  
434 from New Caledonia, we did not detect differential expression of *IREG/Ferroportin* genes  
435 (Fig. 2c). However, the analysis of our RNA-Seq data indicates that *IREG/Ferroportin*  
436 genes are highly expressed in both hyperaccumulator and non-accumulator species  
437 from these families compared to non-accumulators from Brassicaceae and  
438 Euphorbiaceae families (Supporting Information Fig. S6a). Furthermore, the unique  
439 *IREG/Ferroportin* gene (COG 1981) detected in the hyperaccumulator *Geissois pruinosa*  
440 is 60-times more expressed than its orthologue in the non-accumulator Cunoniaceae  
441 species *Cunonia capensis* from South Africa (Supporting Information Fig. S6b). The high  
442 expression of *IREG/Ferroportin* genes in non-accumulator species endemic to New  
443 Caledonia might be a genetic footprint of nickel tolerance and hyperaccumulation caused  
444 by the recent (<35 Ma) colonization of this island then probably fully covered by an  
445 ultramafic rock layer rich in nickel (Pillon *et al.*, 2010, 2014). After adaptive radiation,  
446 some species that have colonized more recently exposed non-ultramafic areas might  
447 have lost some of the important functions necessary for the full expression of the nickel  
448 tolerance and hyperaccumulation traits while still expressing IREG/Ferroportin at high  
449 levels.

450

451 *IREG/Ferroportin* transporters have a conserved function in vacuolar sequestration of  
452 nickel in hyperaccumulators



453 To provide functional evidence for the conserved role of plant IREG/Ferroportin in nickel  
454 hyperaccumulation, we expressed in yeast IREG/Ferroportin orthologs cloned from 3  
455 nickel hyperaccumulators from the distant families Brassicaceae, Cunoniaceae and  
456 Euphorbiaceae (Fig. 3). The expression of these transporters increases yeast resistance  
457 to nickel. These results are consistent with a conserved function of plant  
458 IREG/Ferroportin as metal exporters, driving nickel out of the cytosol in the extracellular  
459 medium or in intracellular stores and thus reducing cytosolic toxicity.

460 We then wanted to investigate the biological function of IREG/Ferroportin in nickel  
461 hyperaccumulation *in planta* using *Noccaea caerulescens* as a model. We chose to  
462 target *NcIREG2*, the ortholog of *Arabidopsis thaliana* *IREG2*, because it shows the  
463 strongest expression in both shoot and roots of the nickel hyperaccumulator *N.*  
464 *caerulescens* subsp. *firmiensis* when compared with the non-accumulator *Noccaea*  
465 *caerulescens* 'Viviez' (Fig. 4; Supporting Information Table S4). We first used *Rhizobium*  
466 *rhizogenes* root transformation to express a C-terminal GFP tagged version of  
467 *NcfilIREG2* in *N. caerulescens* subsp. *firmiensis*. *NcfilIREG2*-GFP is able to complement  
468 the nickel hypersensitive phenotype of the *A. thaliana* *ireg2* mutant indicating that this  
469 fusion protein is functional (Supporting Information Fig. S7). Confocal imaging of *N.*  
470 *caerulescens* transgenic roots shows that *NcfilIREG2*-GFP localizes on the membrane  
471 of the vacuole suggesting a role of this transporter in nickel storage in this intracellular  
472 compartment (Fig. 5a). To support this hypothesis, we used the same transformation  
473 strategy to silence the expression of *NcfilIREG2* using artificial amiRNA technology. We  
474 generated 8 independent composite transgenic lines displaying different degrees of  
475 *NcfilIREG2* silencing (Fig. 5b). Elemental analysis of these transgenic lines revealed a  
476 decrease of nickel concentration in roots that strongly correlates ( $R^2 = 0.97$ , P-value <  
477 0.001) with *NcfilIREG2* expression (Fig. 5c). In contrast, the concentration of nickel in the  
478 shoots of these lines does not correlate with *NcfilIREG2* expression in roots ( $R^2 = 0.45$ ).  
479 Together, these results provide evidences that *NcfilIREG2* contributes to the  
480 accumulation of nickel in the roots of *Noccaea caerulescens* subsp. *firmiensis* by driving  
481 nickel sequestration in vacuoles. However, the expression of *NcIREG2* in roots does not  
482 seem to be a limiting factor for the transport of nickel to the shoot in *N. caerulescens*  
483 subsp. *firmiensis*.

## 485 Discussion

486 The development of RNA-Seq technologies has opened the possibility to study non-  
487 model species at the molecular level. Yet, comparative biology has not fully benefited  
488 from this revolution because of the difficulty to quantitatively compare transcriptomes  
489 from distant species (Roux *et al.*, 2015). In this study, we have used a combination of  
490 cross-species comparative transcriptomics analysis, COG annotation and multiple-  
491 testing analysis to identify genes associated with nickel hyperaccumulation in a wide  
492 diversity of plant families. While pairwise comparisons between a nickel  
493 hyperaccumulator and a related non-hyperaccumulator species identified a high number  
494 of candidate DE genes, it is expected that a large proportion of these candidate DE  
495 genes are not directly linked to the nickel hyperaccumulation trait but also to other traits  
496 differentiating these species. On the other hand, pairwise comparison of closely related  
497 species or accessions with a distinctive hyperaccumulation phenotype may also fail to  
498 reveal mechanisms involved in this multigenic trait as for example observed for IREG/  
499 Ferroportin genes in *Geissois* species from New Caledonia (Fig. 2c, Fig. S6). Thus, we  
500 focused our analyses on the identification of convergent mechanisms between families  
501 (Fig. 2a; Supporting Information Fig. S3). This strategy allowed the identification of a  
502 limited number of candidate DE genes playing a conserved function in nickel  
503 hyperaccumulation. This strategy may fail to identify molecular mechanisms specific to  
504 a group of plants (e.g. family, genus). However, a similar approach could likely be used  
505 to identify such specific mechanisms but would require to increase the number of species  
506 within this group.

507

508 The high expression of IREG/Ferroportin transporters was previously linked to nickel  
509 hyperaccumulation in Brassicaceae and Asteraceae species (Halimaa *et al.*, 2014; Meier  
510 *et al.*, 2018). Our results support these findings and further indicates that  
511 IREG/Ferroportin transporters have been recurrently recruited as a convergent  
512 mechanism for nickel hyperaccumulation in a wide diversity of plant species. It is also  
513 interesting to notice that we did not identify other families of metals transporters  
514 suggesting that, besides IREG/Ferroportin, diverse metal transporter families may have  
515 been recruited to support other important steps of nickel accumulation in leaves of distant  
516 hyperaccumulator species. Importantly, this result *a posteriori* validates our cross-  
517 species comparative approach to identify genes playing a convergent role in nickel  
518 hyperaccumulation.

519 Our results also point to a convergent role for several functions involved in the biogenesis  
520 and organization of cell walls and in the synthesis of specialized metabolites (Fig. 2c,d).

521 The high expression of several genes involved in the metabolism of phenylpropanoid  
522 compounds is linked to the hyperaccumulation trait in several families. Coumarins and  
523 flavonoids have the capacity to bind metals and have been previously linked to iron  
524 nutrition and cadmium accumulation (Kasprzak *et al.*, 2015; Tsai & Schmidt, 2017; Corso  
525 *et al.*, 2018). These results suggest that nickel hyperaccumulators synthesize  
526 specialized metabolites involved in the detoxification and transport of nickel. However,  
527 the functional annotation of the selected COGs does not allow to pinpoint specific  
528 pathways. Comparative metabolomic analyses of nickel hyperaccumulator and related  
529 non-accumulator species may identify specialized metabolites associated with nickel  
530 hyperaccumulation and therefore establish a direct link between identified COGs and  
531 specialized metabolites biosynthetic pathways. A recent report showed that the excess  
532 of nickel in *A. thaliana* leads to a reduced expression of genes associated with cell walls  
533 of root cells (Lešková *et al.*, 2019). Accordingly, we observed a lower expression of  
534 COGs associated with cell wall in several hyperaccumulator species. Interestingly, our  
535 analysis also revealed the up-regulation of COG 19, corresponding to the cell wall-  
536 associated kinase family (WAKL) in nickel hyperaccumulators (Fig. 2c). The expression  
537 of members of the WAKL family has been shown to be induced by metals in plants and  
538 the increased expression of *WAKL4* in *A. thaliana* increases tolerance to nickel (Hou *et*  
539 *al.*, 2005). Together, these results suggest that a modification of the cell wall structure,  
540 at least in specific tissues, could play a conserved role in nickel tolerance and  
541 hyperaccumulation in plants.

542 We also identified several COGs corresponding to Class I retrotransposon up-regulated  
543 in nickel hyperaccumulating species. The expression of these elements is known to be  
544 responsive to stress conditions and their transposition can generate several types of  
545 mutations that can result in the inactivation of gene function or in increased gene  
546 expression by cis-addition (Casacuberta & González, 2013; Makarevitch *et al.*, 2015). In  
547 the metal hyperaccumulator species *A. halleri*, Class I retrotransposons are linked to the  
548 triplication of the *HMA4* gene and may account for the high expression of this metal  
549 transporter essential for zinc hyperaccumulation (Hanikenne *et al.*, 2008). It is therefore  
550 tempting to speculate that transposable elements are involved in the evolution of the  
551 nickel hyperaccumulation trait by altering the activity of some important genes.

552

553 Although metal hyperaccumulators are not easily amenable to genetic engineering,  
554 molecular genetic studies are necessary to demonstrate the role of candidate genes in  
555 this complex trait (Hanikenne *et al.*, 2008). To support the essential role of  
556 IREG/Ferroportin in nickel accumulation, we performed molecular and genetic studies in  
557 roots of the hyperaccumulator *N. caerulescens subsp. firmiensis* (Fig. 5). These results

558 demonstrated that *NcIIREG2* localizes on the vacuole and that decreasing *NcIIREG2*  
559 expression in roots strongly affects nickel accumulation (Fig. 5b). These results provide  
560 a genetic evidence supporting a role of *NcIIREG2* in the storage of nickel in the vacuoles  
561 of *N. caerulescens* cells. Surprisingly, the silencing of *NcIIREG2* in roots did not lead to  
562 a significant increase in nickel accumulation in leaves (Fig. 5c). This result might appear  
563 as counterintuitive because the sequestration of metals in root cells has been proposed  
564 to be a factor limiting their translocation and therefore their accumulation in the shoot of  
565 hyperaccumulators. Our results rather suggest that the expression of *NcIIREG2* in roots  
566 of *N. caerulescens subsp. firmiensis* is not a factor limiting the hyperaccumulation of  
567 nickel in leaves. This conclusion is further supported by the parallel comparison of  
568 *NcIIREG2* expression and nickel accumulation in *N. caerulescens subsp. firmiensis* and  
569 *N. caerulescens* 'Viviez'. This analysis indicates that there is no negative correlation  
570 between the expression of *NcIIREG2* in roots and the capacity to accumulate nickel in  
571 shoots (Fig. 4). This conclusion does not rule out that the expression of *NcIIREG2* in roots  
572 may limit the translocation of nickel to shoots in other *Noccaea* accessions or species  
573 such as *N. montana* (Fig. 4) or *N. japonica* as very recently proposed (Nishida *et al.*,  
574 2020). In contrast, the high expression of *NcIIREG2* and the consecutive sequestration  
575 of nickel in shoots might create a sink effect favoring the transfer of nickel from roots to  
576 shoots in *N. caerulescens subsp. firmiensis*. To directly demonstrate the major role of  
577 *NcIIREG2* in nickel hyperaccumulation, it will be necessary to further develop  
578 transformation protocols of *N. caerulescens* to modify the expression of this gene in  
579 shoots.

580 Based on our wide cross-species transcriptomic analysis and functional evidences  
581 obtained in transgenic *N. caerulescens*, we propose that IREG/Ferroportin transporters  
582 play a conserved and essential role in the storage of nickel in the vacuole of leaf cells in  
583 a wide range of nickel hyperaccumulating species. This role was previously proposed for  
584 the transporter PgIREG1 from the nickel hyperaccumulator *P. gabriellae* (Merlot *et al.*,  
585 2014). However, we cannot completely exclude that some IREG/Ferroportin orthologues  
586 differentially expressed in hyperaccumulators might localize on the plasma membrane  
587 to transport nickel out of cells as this was proposed for the *A. thaliana* IREG1 transporter  
588 (Morrissey *et al.*, 2009). In the context of nickel hyperaccumulation, these transporters  
589 might facilitate cell-to-cell transport of nickel or transport nickel out of epidermal cells  
590 leading to its accumulation in the cell walls (Krämer *et al.*, 2000; van der Ent *et al.*, 2019).  
591 In the context of sustainable development, nickel hyperaccumulators are now viewed as  
592 crops to extract and recycle metals from large areas of metalliferous soils (Grison, 2015;  
593 Nkrumah *et al.*, 2016). As for other crops, we foresee that the molecular knowledge  
594 obtained in our study could become instrumental for marker-assisted selection of

595 cultivars or molecular monitoring of agricultural practices to improve nickel  
596 phytoextraction. This work provides a framework to identify additional key genes from  
597 root transcriptomes involved in the efficient uptake and translocation of nickel to the  
598 leaves. The identification of all molecular steps from uptake in roots to sequestration in  
599 leaves is necessary to fully understand this complex trait.

For Peer Review

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617

618 **Author contributions**

619 BF, VBS and SM designed the project; VSG, CML, DAG, BF, VBS and SM collected  
620 plant samples; VSG, CML, DAG, LB performed experiments; LST supervised RNA-Seq  
621 sequencing; VSG, GJR, YP, ST and SM analyzed the data; VSG, ST and SM wrote the  
622 manuscript; all authors commented and approved the content of the manuscript.

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823 nicotianamine synthase, a ZIP transporter and other genes as potential metal  
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826 **Figure legends**

827 **Figure 1: Phylogenetic distribution of nickel hyperaccumulators.** Eudicots Plant  
 828 families containing nickel hyperaccumulators are indicated in red on the phylogenetic  
 829 tree. The drawings illustrate the plant families and genera containing nickel  
 830 hyperaccumulators that we have sampled from France, New Caledonia and Cuba.

831 **Figure 2: Cross-family comparative transcriptomics and orthologous group**  
 832 **annotation of genes convergently associated with nickel hyperaccumulation.** (a)  
 833 Cross-species comparative transcriptomic reveals Differential Expressed (DE) genes  
 834 between nickel hyperaccumulator species (black) and related non-nickel accumulators  
 835 (grey). Genes (contigs) are plotted according to their mean level of expression (x-axis)  
 836 and their differential expression (y-axis) in the pair of species. The numbers of significant  
 837 DE (red and blue dots) and non-DE genes are indicated ( $\log_2FC > 1$ ,  $FDR < 0.05$ ). (b)  
 838 Distribution of the 26 selected Cluster of Orthologous Groups (COGs) associated with  
 839 nickel hyperaccumulation according to their predicted function and their level of  
 840 expression in hyperaccumulators: COGs containing DE genes more expressed in nickel  
 841 hyperaccumulators belonging to at least 3 distinct plant families (up-regulated COGs)  
 842 are in red and COGs containing DE genes less expressed in nickel hyperaccumulators  
 843 (down-regulated) are in blue. (c) Heat-maps of contigs associated with the selected  
 844 COGs up-regulated in nickel hyperaccumulators from 5 distinct families. COGs  
 845 corresponding to Class I transposable elements are not presented. (d) Heat-maps of  
 846 contigs associated with down-regulated COGs. The color scale represents the  
 847 expression Fold change of contigs in pairwise comparative analysis. The grey color  
 848 represents the absence of contigs. Abbreviations: *Ncfi* (*Noccaea caerulescens* subsp.  
 849 *firmiensis*), *Ncviv* (*N. caerulescens* 'Viviez'), *Nmon* (*N. montana*), *Pgra* (*Psychotria*  
 850 *grandis*), *Prev* (*P. revoluta*), *Pcos* (*P. costivenia*), *Pgab* (*P. gabriellae*), *Psem* (*P.*  
 851 *semperflorens*), *Gpru* (*Geissois pruinosa*), *Grac* (*G. racemosa*), *Hkan* (*Homalium*  
 852 *kanaliense*), *Hbet* (*H. betulifolium*), *Lhav* (*Leucocroton havanensis*), *Lmic* (*Lasiocroton*  
 853 *microphyllus*), IREG/FPN (Iron Regulated/ Ferroportin), UGT85A (UDP-  
 854 glucose:anthocyanidin/flavonol 3-O-glucosyltransferase), BAHD (BAHD  
 855 acyltransferase), CYP71 (Cytochrome P450 71), CYP704 (Cytochrome P450 704), FAR  
 856 (Fatty acyl-CoA reductase), SCPL (Serine carboxypeptidase), SSL (Strictosidine  
 857 synthase), WAKL (Wall associated receptor kinase like), GLP (Germin-like protein), COR  
 858 (Cold regulated gene), EXPA (Expansin), XTH (Xyloglucan  
 859 endotransglucosylase/hydrolase), PAE (Pectin acetyltransferase), CESA (Cellulose  
 860 synthase), MLP (Major latex protein), JMT (Jasmonic acid carboxyl methyltransferase),

861 GDSL (GDSL esterase/lipase), GULLO (L-gulonolactone oxidase), LTP (Bifunctional  
 862 inhibitor/lipid-transfer protein), DUF642 (DUF642 domain containing protein) and TPS  
 863 (Terpene synthase).

864 **Figure 3. Nickel sensitivity of yeast cells expressing plant IREG/Ferroportin**  
 865 **transporters.** Yeast cells expressing IREG/Ferroportin transporters cloned from *A.*  
 866 *thaliana*, *N. caerulescens subsp. firmiensis*, *G. pruinosa* and *L. havanensis* were plated  
 867 at different dilutions on a medium containing a toxic concentration of nickel for the control  
 868 line (transformed with pDR195 vector).

869 **Figure 4. Nickel accumulation and NcIREG2 expression in various accessions of**  
 870 **Noccea species.** All plants were grown in hydroponic condition for 8 weeks in presence  
 871 of 37.5  $\mu\text{M}$   $\text{NiCl}_2$ . (a) Nickel accumulation was measured in roots and shoots of *Noccea*  
 872 *caerulescens* 'Viviez' (*Ncviv*), *N. caerulescens subsp. firmiensis* (*Ncfi*) and *N. montana*  
 873 (*Nmon*). Results are mean value  $\pm$  SD ( $n = 3$  biological replicates). Letters denote  
 874 significant differences between accessions, with lowercase for roots and uppercase for  
 875 shoots (Tukey HSD test,  $p < 0.05$ ). (b) Quantitative RT-PCR analysis of *NcIREG2*  
 876 expression was performed on the same plants. *NcIREG2* expression was corrected  
 877 using *Nc6PGDH* as a reference gene and normalized to 1 for the expression in *Ncviv*.  
 878 Relative expression is displayed with a log<sub>2</sub> scale. Results are mean value  $\pm$  SD ( $n = 3$   
 879 biological replicates).

880 **Figure 5. Localization and silencing of the IREG/Ferroportin transporter NcfilREG2**  
 881 (a) *NcfilREG2* localizes on the vacuolar membrane in *N. caerulescens* cells. Confocal  
 882 picture of a transgenic line expressing *NcfilREG2*-GFP (green) in root cells. Cell wall was  
 883 stained with propidium iodide (magenta). The scale bar corresponds to 5 $\mu\text{m}$ . (b)  
 884 Silencing of the IREG/Ferroportin transporter *NcfilREG2* in roots of the nickel  
 885 hyperaccumulator *N. caerulescens subsp. firmiensis* reduces nickel accumulation. The  
 886 expression of *NcfilREG2* was quantified by RT-qPCR (black bars) in 8 amiRNA  
 887 transgenic lines and control lines transformed with the pK7WG2D vector ( $n=3$ ) growing  
 888 in presence of 37.5  $\mu\text{M}$   $\text{NiCl}_2$  for 4 weeks. *NcfilREG2* expression was corrected using  
 889 *Ncfi6PGDH* as reference gene and normalized to 1 for the expression in control lines.  
 890 Nickel concentration was measured in parallel in roots of the same lines by MP-AES  
 891 (green bars). (c) Correlation analysis between *NcfilREG2* expression and nickel  
 892 accumulation. The quadratic correlation model showed positive correlation ( $R^2 = 0.97^{**}$ ,  
 893  $P\text{-value} < 0.001$ ) between *NcfilREG2* expression and nickel accumulation in roots (black  
 894 dots) and non-significant correlation ( $R^2 = 0.45$ ) between *NcfilREG2* expression in roots  
 895 and nickel accumulation in shoots (grey dots).



897 **Tables**898 **Table 1. De novo assembled transcriptomes of nickel hyperaccumulators and**  
899 **related non-nickel accumulator species<sup>§</sup>**

Family/Species	[Ni] <sup>#</sup> (ppm)	Origin	Nbr. contigs	Assembly size (Mbp)	N50 (bp)
<b>Brassicaceae - Noccaea</b>					
<i>Noccaea caerulescens</i> <i>subsp. firmiensis</i> ( <i>Ncfi</i> )	7580	France	41843	43.6	1735
<i>N. caerulescens</i> 'Viviez' ( <i>Ncviv</i> )	239	France	na*	na*	na*
<i>N. montana</i> ( <i>Nmon</i> )	916	France	87243	48.8	692
<b>Rubiaceae - Psychotria</b>					
<i>Psychotria gabriellae</i> ( <i>Pgab</i> )	17618	New Caledonia	60899	45.5	1140
<i>P. semperflorens</i> ( <i>Psem</i> )	34	New Caledonia	66755	49.8	1181
<i>P. grandis</i> ( <i>Pgra</i> )	15176	Cuba	45143	36.8	1344
<i>P. costivenia</i> ( <i>Pcos</i> )	1251 <sup>&amp;</sup>	Cuba	46451	35.1	1240
<i>P. revoluta</i> ( <i>Prev</i> )	131	Cuba	56754	44.0	1245
<b>Cunoniaceae - Geissois</b>					
<i>Geissois pruinosa</i> ( <i>Gpru</i> )	6239	New Caledonia	54969	42.1	1243
<i>G. racemosa</i> ( <i>Grac</i> )	132	New Caledonia	58386	43.7	1021
<b>Salicaceae - Homalium</b>					
<i>Homalium kanaliense</i> ( <i>Hkan</i> )	6342	New Caledonia	52634	40.6	1259
<i>H. betulifolium</i> ( <i>Hbet</i> )	275	New Caledonia	49962	39.0	1294
<b>Euphorbiaceae – Adelleae tribe</b>					
<i>Leucocroton havanensis</i> ( <i>Lhav</i> )	14073	Cuba	58990	44.4	1206
<i>Lasiocroton microphyllus</i> ( <i>Lmic</i> )	43	Cuba	52421	39.8	1244

900 <sup>§</sup> Details are provided in Supporting Information Table S1 and S2901 <sup>#</sup> Mean nickel concentration measured in leaves (n=2, Table S1)902 <sup>\*</sup> not assembled: The transcriptome of *Ncfi* is used as a reference for *Ncviv*903 <sup>&</sup> Concentration likely underestimated because the samples were conserved in water-based RNA<sup>later</sup>

905 **The following Supporting Information is available for this article:**

906 **Figure S1:** Estimated time-divergence between species used in this study

907 **Figure S2:** Workflow used for RNA-Seq analyses

908 **Figure S3:** Additional MA-plot representation of cross-species comparative  
909 transcriptomic analyses

910 **Figure S4:** Effect of the choice of the reference transcriptome on the identification of  
911 Differentially Expressed genes

912 **Figure S5:** Molecular phylogeny of the plant IREG/Ferroportin family

913 **Figure S6:** Expression of *IREG/Ferroportin* orthologs in *Homalium* and *Geissois* species

914 **Figure S7:** The NcflIREG2-GFP fusion protein is functional in *A. thaliana*

915 **Table S1:** Metadata associated with plant samples

916 **Table S2:** RNA-Seq sequencing, assembly and mapping statistics

917 **Table S3:** Cluster of Orthologous Group analysis metrics

918 **Table S4:** Differentially Expressed Genes analysis

919 **Table S5:** List of Cluster of Orthologous Group associated with nickel hyperaccumulation

920 **Table S6:** List of primers.

921 **Notes S1:** References for Supporting Information

922



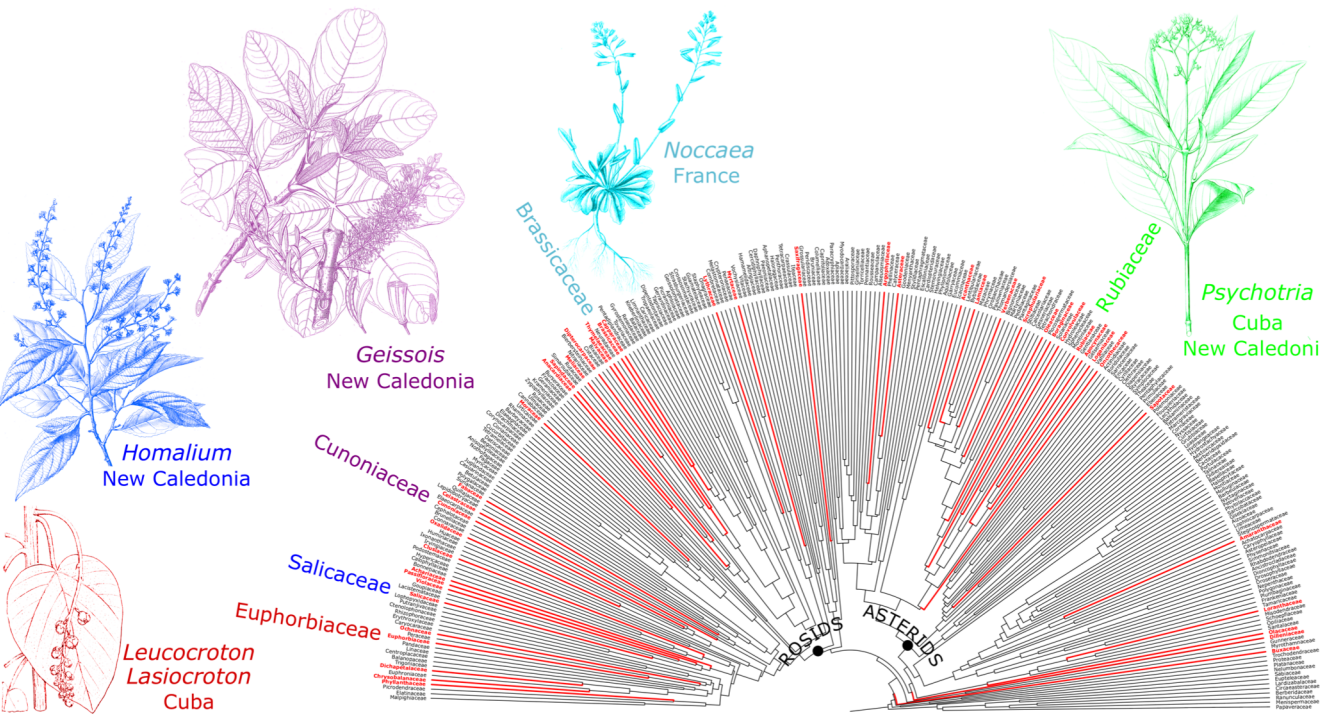
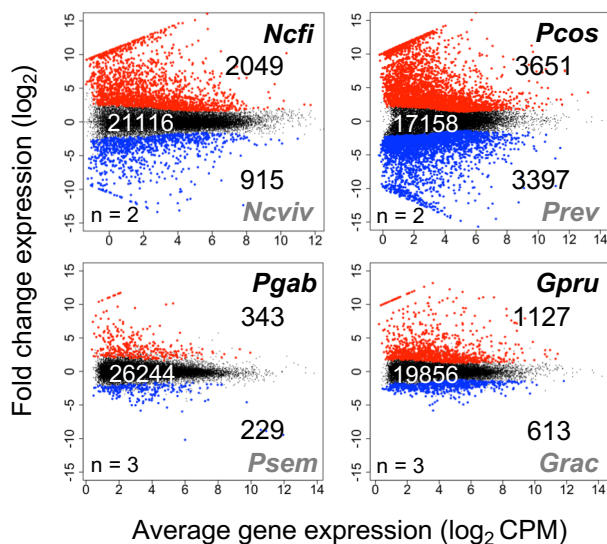
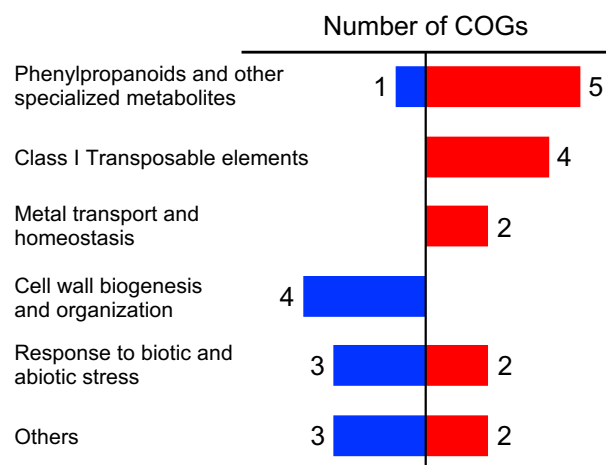


Figure 1

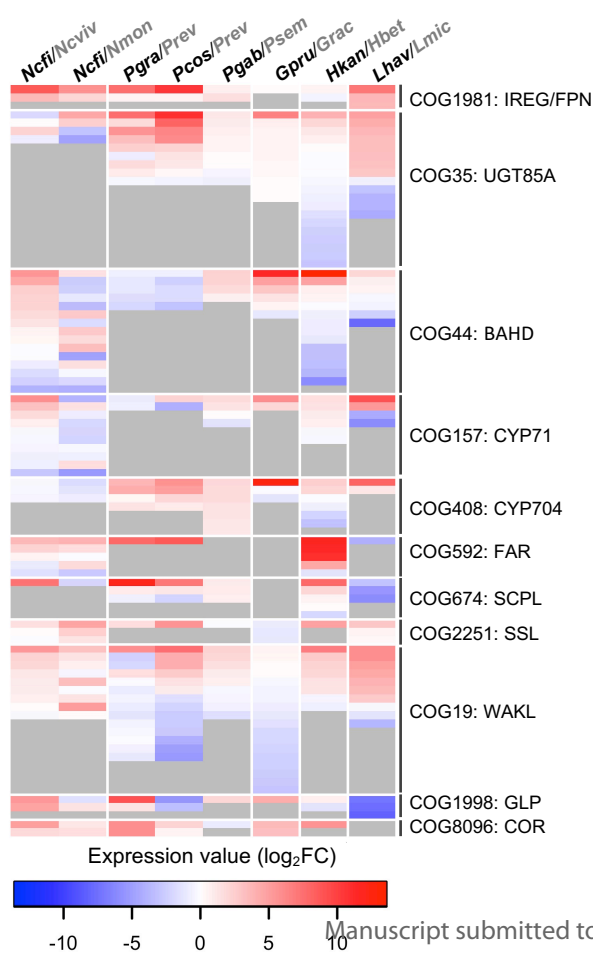
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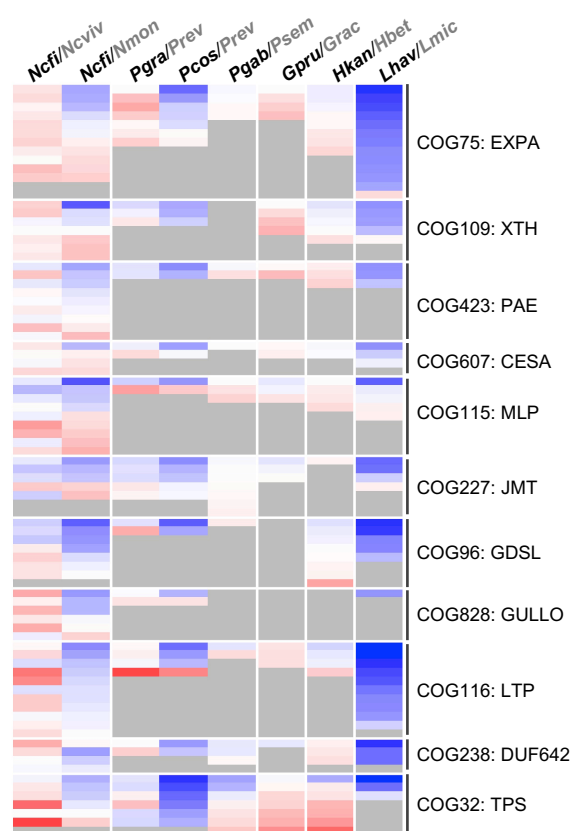
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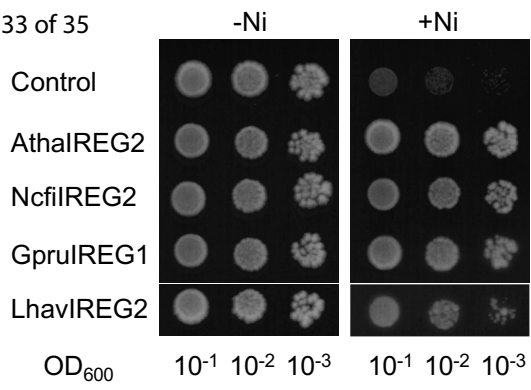


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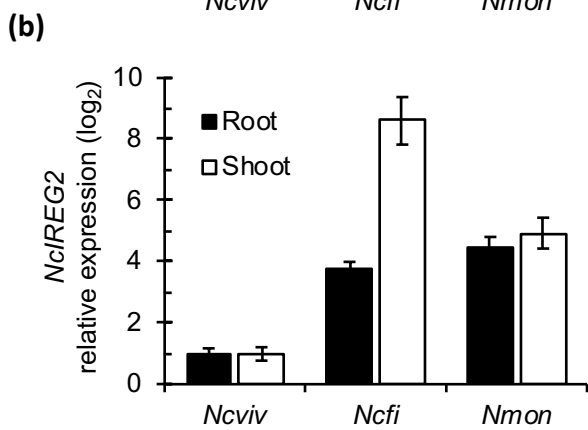
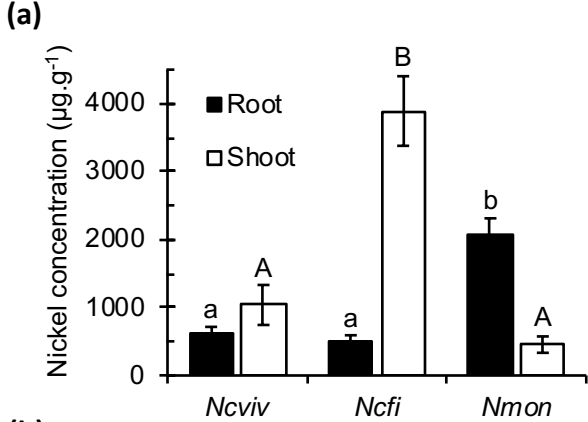


(d)





**Figure 3**



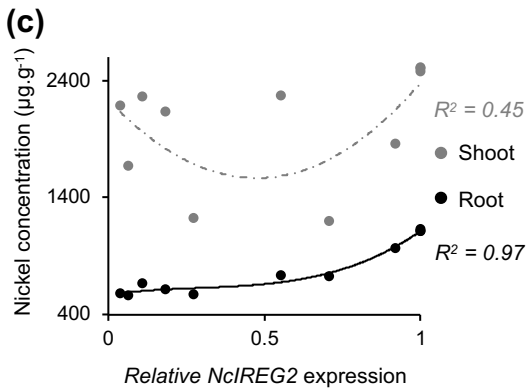
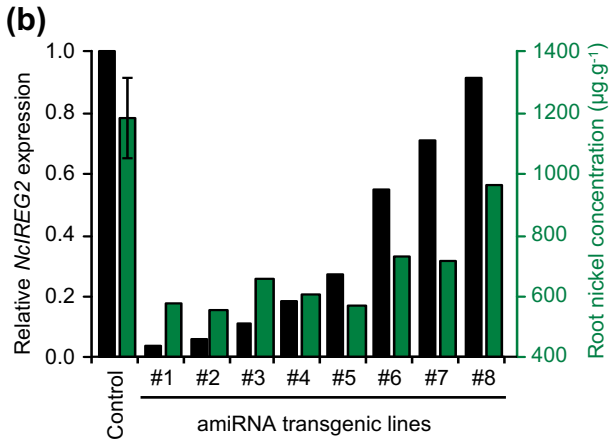
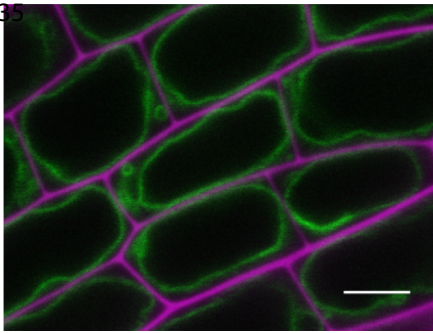


Figure 5