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RESEARCH PAPER

The metal transporter PgIREG1 from the hyperaccumulator *Psychotria gabriellae* is a candidate gene for nickel tolerance and accumulation

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Abstract

Nickel is an economically important metal and phytotechnologies are being developed to limit the impact of nickel mining on the environment. More than 300 plant species are known to hyperaccumulate nickel. However, our knowledge of the mechanisms involved in nickel accumulation in plants is very limited because it has not yet been possible to study these hyperaccumulators at the genomic level. Here, we used next-generation sequencing technologies to sequence the transcriptome of the nickel hyperaccumulator *Psychotria gabriellae* of the Rubiaceae family, and used yeast and *Arabidopsis* as heterologous systems to study the activity of identified metal transporters. We characterized the activity of three metal transporters from the NRAMP and IREG/FPN families. In particular, we showed that *PgIREG1* is able to confer nickel tolerance when expressed in yeast and in transgenic plants, where it localizes in the tonoplast. In addition, *PgIREG1* shows higher expression in *P. gabriellae* than in the related non-accumulator species *Psychotria semperflorens*. Our results designate *PgIREG1* as a candidate gene for nickel tolerance and hyperaccumulation in *P. gabriellae*. These results also show how next-generation sequencing technologies can be used to access the transcriptome of non-model nickel hyperaccumulators to identify the underlying molecular mechanisms.

Key words: *De novo* sequencing, Ferroportin, hyperaccumulator, nickel, NRAMP, *Psychotria gabriellae.*

Introduction

Nickel is an important metal in the industry as it is used to make stainless steel, superalloys, and rechargeable batteries. However, nickel mining has a high impact on the environment because it generates metal pollution in soils or may require deforestation in tropical regions that are biodiversity hotspots such as Indonesia and New Caledonia (Mudd, 2010). To reduce the impact of mining on the environment, biotechnologies such as phytoremediation or phytomining, which use plants to extract metals from the soil, are being developed (Pilon-Smits, 2005; Chaney *et al.*, 2007; Rascio and Navari-Izzo, 2011; Losfeld *et al.*, 2012). The development of these technologies is particularly well adapted for nickel because more than 300 plant species, called hyperaccumulators, including *Nocceae caerulescens* (previously *Thlaspi*

Abbreviations: EST, expressed sequence tag; HBED, hydroxybenzyl ethylenediamine; FPN, Ferroportin; GFP, green fluorescent protein; MES, 4-morpholine-ethanesulphonic acid; NA, nicotianamine; NRAMP, Natural Resistance-Associated Macrophage Protein; RACE, rapid amplification of cDNA ends; WT, wild type.

caerulescens) and *Alyssum murale*, both of the Brassicaceae family, are able to accumulate more than 0.1% (dry biomass) nickel in their aboveground tissues. A better knowledge of the molecular mechanisms involved in nickel accumulation in these hyperaccumulators would favour the development of phytoextraction technologies (Verbruggen *et al.*, 2009; Krämer, 2010).

Nickel is an essential nutrient for plants because it is necessary for the activity of urease, which is involved in nitrogen metabolism. However, when present in excess, nickel competes with other essential divalent metals including iron and induces deleterious genotoxic and oxidative stresses (Seregin and Kozhevnikova, 2006; Yusuf *et al.*, 2011; Nishida *et al.*, 2012). Therefore, as for other toxic metals, all plants need to finely regulate nickel homeostasis by controlling uptake by root cells, chelation into inactive complexes, and sequestration in vacuoles. Genes involved in the basic mechanisms of metal homeostasis are actively transcribed in metal hyperaccumulator species. In addition these plants are able to very efficiently translocate metals from roots to shoots, where they accumulate (van de Mortel *et al.*, 2006; Roosens *et al.*, 2008; Verbruggen *et al.*, 2009; Rascio and Navari-Izzo, 2011).

Several organic molecules have been shown to bind nickel in plants (Callahan *et al.*, 2006). Nickel is chelated with low affinity by organic acids such as malate or citrate in vacuoles where it is sequestrated (Lee *et al.*, 1978; Kersten *et al.*, 1980; Krämer *et al.*, 2000; Agrawal *et al.*, 2012). The amino acid histidine binds to nickel with higher affinity and a high concentration of histidine was correlated with the ability to accumulate nickel in *Alyssum* species and *N. caerulescens* (Krämer *et al.*, 1996; Richau *et al.*, 2009). In addition, constitutive expression in *Arabidopsis thaliana* of ATP-phosphoribosyl transferase, catalysing the first rate-limiting step of histidine biosynthesis, increases both histidine concentration and nickel resistance (Wycisk *et al.*, 2004; Ingle *et al.*, 2005). Nicotianamine (NA) binds divalent cations including Ni^{2+} with a high association constant. NA is synthesized from *S*-adenosylmethionine by NA synthase (NAS), and expression in *Arabidopsis* of *N. caerulescens TcNAS1* increases nickel resistance and accumulation in leaves of transgenic plants (Pianelli *et al.*, 2005). A stable nickel–NA complex was detected in the xylem of *N. caerulescens* upon nickel treatment and was therefore proposed to be involved in root-to-shoot translocation (Mari *et al.*, 2006).

Only a few metal transporters have been shown to transport nickel in plants. *A. thaliana* AtIRT1, a metal transporter of the ZIP/IRT family that is required for iron uptake in roots, has a broad specificity for divalent metals and was recently shown to mediate nickel uptake in roots (Vert *et al.*, 2002; Schaaf *et al.*, 2006; Nishida *et al.*, 2011). In *Arabidopsis* root cells, nickel is then transported to the vacuole by AtIREG2/FPN2, a metal transporter of the IREG/Ferroportin (FPN) family that localizes in the tonoplast (Schaaf *et al.*, 2006; Morrissey *et al.*, 2009). Accordingly, the knockout mutant *ireg2-1* is hypersensitive to nickel for root growth and stores less nickel in roots (Schaaf *et al.*, 2006). AtIREG1/FPN1, a second member of this family, is located in the plasma membrane and is proposed to mediate loading of metals into xylem (Morrissey *et al.*, 2009). The *N. caerulescens* transporter TcYSL3 of the YSL/OPT family was shown to mediate uptake of nickel–NA complex when expressed in yeast. The expression of this transporter in vascular tissues suggests that it may be involved in the translocation of nickel from roots to shoots (Gendre *et al.*, 2007). Finally, the expression of *TjNRAMP4*, a gene coding for a metal transporter of the Natural Resistance-Associated Macrophage Protein (NRAMP) family from the nickel hyperaccumulator *Noccaea cochleariforme* (formerly *Thlaspi japonicum*), was shown to increase both nickel sensitivity and accumulation in yeast (Mizuno *et al.*, 2005). Inversely, the expression of *NRAMP4* from *N. caerulescens* increases nickel resistance and reduces nickel accumulation when expressed in yeast (Wei *et al.*, 2009). These results suggest that NRAMP transporters from nickel hyperaccumulators might have evolved to transport this metal; however, this was not demonstrated in plants.

Despite this knowledge, our understanding of the mechanisms involved in nickel homeostasis and accumulation is still scarce. In addition, this knowledge stems from studies on the Brassicaceae, whereas nickel hyperaccumulators have been described in more than 40 plant families (Verbruggen *et al.*, 2009; Kramer, 2010). It is therefore possible that distant plant families have evolved different mechanisms of nickel hyperaccumulation.

New Caledonia is an isolated island located in the south-west Pacific and is covered on one-third of its surface (5500 km^2) by ultramafic soils that are rich in nickel. As a consequence about 65 nickel hyperaccumulators, including *Pycnandra acuminata* (previously *Sebertia acuminata*), *Geissois pruinosa*, and *Psychotria gabriellae* (previously *Psychotria douarrei*), are endemic to this small territory (Brooks, 1998; Jaffré *et al.*, 2013). *P. gabriellae* is a pink-flowered shrub of the Rubiaceae family that grows only in rainforest on ultramafic soils. It is able to accumulate up to 4% nickel in its leaves, which is one of the highest concentrations of nickel measured in plants (Jaffré and Schmid, 1974; Baker *et al.*, 1985).

In this study we used next-generation sequencing technology to sequence the aboveground transcriptome of *P. gabriellae*. Using these original sequences we were able to clone and study the metal specificity of three transporters of the NRAMP and IREG/FPN families by heterologous expression in yeast. Further studies indicated that PgIREG1 is able to transport nickel in vacuoles of *Arabidopsis* cells and that *PgIREG1* is more expressed in *P. gabriellae* than in the related non-accumulator *Psychotria semperflorens*. Together, our results point to *PgIREG1* as a candidate gene for nickel tolerance and hyperaccumulation in *P. gabriellae*.

Materials and methods

Plant materials

Samples from *Psychotria* were collected in their natural environment according to the environment code of the South Province of New Caledonia (www.province-sud.nc/images/stories/pdf/environnement/code_oct2012.pdf). *Arabidopsis ireg2-1* mutant seeds corresponding to SALK_074442 line (Columbia ecotype) were obtained from N. von Wirén's laboratory (Schaaf *et al.*, 2006).

Transcriptome sequencing, de novo *assembly, annotation, and sequence analyses*

P. gabriellae samples were collected from several individuals in a population growing on ultramafic soil on Mont Mou (S 22° 4.430, E 166° 19.970, Païta, New Caledonia). Total RNA was extracted from flower buds and leaves with TRI Reagent (Sigma-Aldrich, St Louis, MO, USA; www.sigmaaldrich.com) and subsequently purified with RNeasy Plant Mini kit (Qiagen, Hilden, Germany; www.qiagen.com). The cDNA library and the sequencing were performed by Eurofins MWG Operon (Ebersberg, Germany; www.eurofinsgenomics.eu). The cDNA library was constructed by random priming of mRNA and normalized using a denaturation/renaturation protocol (cot curve). The library was then sequenced by Roche GS-FLX technology using titanium series chemistry on half a chip. Trimmed reads were assembled *de novo* into contigs using CLC Genomics Workbench 4.7 (CLC bio, www.clcbio.com) with default parameters (similarity 0.8, length fraction 0.5, insertion cost 3, deletion cost 3, mismatch cost 2). The functional annotation of the contigs was achieved with Blast2GO (Conesa *et al.*, 2005) using blastx interrogation to a non-redundant database with an expected *E* value of $\leq 10^{-6}$. Phylogenic analyses of metal transporters were performed with Mega5 as previously described (Migeon *et al.*, 2010).

Cloning of metal transporters

The coding sequences of *PgIREG1*, *PgNRAMP1.1*, and *PgNRAMP2.1* were extended from contigs #3856 (HE825086), #23116 (HE844336), and #5621 (HE826851), respectively, by 3′ rapid amplification of cDNA ends (RACE) (Scotto-Lavino *et al.*, 2006) and 5′ RACE with FirstChoice RLM-RACE kit (Ambion, Austin, TX, USA; www.invitrogen.com/ambion). The predicted full-length coding sequences were amplified from *P. gabriellae* leaf cDNA using high-fidelity Phusion polymerase (Thermo Scientific, Waltham, MA, USA; www.thermoscientific.com) with primers PgIREG1-gwf and PgIREG1-gwrs or PgIREG1-gwr, PgNRAMP1.1-gwf and PgNRAMP1.1-gwrs, and PgNRAMP2.1-gwf and PgNRAMP2.1-gwrs (Supplementary Table S1, available at *JXB* online). The resulting PCR products were sequenced on both strands to obtain the consensus sequence of the transporters found in leaf cDNA. The PCR fragments were reamplified with Phusion polymerase using universal Gateway AttB1 and AttB2 primers and recombined by BP reaction in pDONOR207 vector (Invitrogen, Carlsbad, CA, USA; www.invitrogen.com) to give pDON207-PgIREG1stop, pDON207-PgIREG1, pDON207-PgNRAMP1.1stop, and pDON207-PgNRAMP2.1stop. Constructs were sequenced on both strands to ensure that the sequence of the transporters conforms the consensus sequence identified from leaf cDNA.

Expression in yeast mutants and transporter activity characterization

The coding sequences of the transporters from pDON207-PgIREG1stop, pDON207-PgNRAMP1.1stop, and pDON207-PgNRAMP2.1stop were recombined into expression vector pDR195gtw (Oomen *et al.*, 2009) by Gateway LR reaction. pDR195-PgIREG1, pDR195-PgNRAMP1.1, and pDR195-PgNRAMP2.1 as well as pDR195-AtIREG2 (Schaaf *et al.*, 2006), pFL61-IRT1 (Eide *et al.*, 1996), pDR195-AtNRAMP1, and pDR195-AtNRAMP4 (Thomine *et al.*, 2000) were transformed in yeast *Saccharomyces cerevisiae* mutants *fet3fet4* (DEY1453; Eide *et al.*, 1996), *smf1* (Thomine *et al.*, 2000), and *zrt1zrt2* (ZHY3; Zhao and Eide, 1996) by the lithium acetate method.

Complementation of *fet3fet4* was scored by spotting series of diluted culture of individual transformants on SD-Ura containing 20 mM 4-morpholine-ethanesulphonic acid (MES; Sigma-Aldrich), pH 6.0/100 µM bathophenanthroline disulphonate (BPDS; Sigma-Aldrich) supplemented or not with 200 µM $FeCl_3$. Complementation of *smf1* was scored on SD-Ura containing 50 mM MES, pH 6.0/2 mM ethylene glycol tetra-acetic acid (EGTA; Sigma-Aldrich) supplemented or not with 200 µM $MnSO_4$. Yeast sensitivity to nickel was scored from *zrt1zrt2* transformants grown on SD-Ura containing 20 mM MES, pH 5.5/100 µM $FeCl_3$/1 mM $ZnSO_4$ and supplemented or not with $NiCl_2$ up to 400 µM. Metal accumulation was measured from *zrt1zrt2* transformants grown to saturation in liquid SD-Ura containing 50 µM $ZnSO_4$. The precultures were diluted at OD_{600} 0.4 in 50 ml of SD-Ura medium containing 10 mM MES, pH 5.5/10 µM $MnSO_4$/50 µM $NiCl_2$/50 µM $ZnSO_4$, and grown for 30 h at 28 °C with vigorous shaking. Yeast were harvested by centrifugation at 4 °C and pellets washed twice with ice-cold 20 mM MES, pH 5.5/10 mM EDTA and once with ice-cold ultrapure water. Yeast pellets were dried at 65 °C before measurement of metal content.

Expression of PgIREG1 *in* Arabidopsis ireg2-1 *mutant*

Arabidopsis Ubiquitin10 promoter was amplified by PCR from the pUB-Dest vector (Grefen *et al.*, 2010) using primers pUBI-for1 and pUBI-rev2 or pUBI-rev1 (Supplementary Table S1, available at *JXB* online). The resulting PCR products were cloned as KpnI/HindIII and BcuI/HindIII fragments respectively in pMDC43 and pMDC83 Gateway-compatible vectors (Curtis and Grossniklaus, 2003) to replace the 2×35S promoter and generate pMUBI43 and pMUBI83 vectors. The coding sequences of *PgIREG1* from pDON207-PgIREG1stop and pDON207-PgIREG1 were recombined in pMUBI43 [green fluorescent protein (GFP) N-terminal fusion] and pMUBI83 (GFP C-terminal fusion) vectors respectively to generate pMUBI43-PgIREG1 and pMUBI83-PgIREG1. Both constructs were transformed into the *Arabidopsis ireg2-1* mutant by floral dipping using the *Agrobacterium AGL0* strain (Clough and Bent, 1998). Transgenic lines were selected on half-strength Murashige and Skoog (MS) agar plates containing hygromycin B (15 µg·ml^{-1}). To analyse *ireg2-1* complementation, transgenic T_2 lines were grown vertically for 10–14 days with 16 h light at 21 °C on ABIS medium agar plates (Oomen *et al.*, 2009) containing 1% sucrose (w/v)/10 µM Fe-hydroxybenzyl ethylenediamine (HBED)/30 µM $NiCl_2$. T_3 lines showing a clear *ireg2-1* complementation and homozygous for the transgenes (100% resistance to hygromycin B) were used for further quantitative analysis. To measure $NiCl_2$ tolerance, plants were grown in the same conditions as for complementation assay for 14 days on a medium supplemented or not with up to 120 µM $NiCl_2$. Primary root length was measured from calibrated pictures of plates using ImageJ (Schneider *et al.*, 2012). To measure nickel accumulation, plants were grown vertically on ABIS medium agar plates containing 1% sucrose (w/v) and 10 µM Fe-HBED for 7 days and then transferred to the same medium supplemented with 25 µM Fe-HBED and 50 µM $NiCl_2$ for 7 days. Roots and shoots were collected separately, washed twice with ice-cold 2 mM MES, pH 5.7/5 mM $CaCl_2$ for 10 min and then once with ice-cold ultrapure water. Samples were dried at 65 °C before measurement of metal content.

Measurement of metal content in yeast and plant samples

The dry weight of yeast and plant samples were measured and samples were digested in 2 ml of 70% HNO_3 for a total of 3 h with temperature ramping from 80 to 120 °C. The metal content was measured with a Varian AA240FS atomic absorption spectrometer and concentration calculated by comparison with metal standards.

Confocal microscopy

Roots of *ireg2-1* transgenic T_2 lines transformed with pMUBI43-PgIREG1 and pMUBI83-PgIREG1 were stained with 10 µg/ml propidium iodide for 5 min. Roots were imaged at the Imagif platform (www.imagif.cnrs.fr) on a Leica SP2 inverted confocal microscope with laser excitation at 488 nm and collection of emitted light at 495–550 nm for GFP and 600–650 nm for propidium iodide.

Gene expression analysis

Young leaves of four independent plants from the *P. gabriellae* and *P. semperflorens* species were collected from ultramafic soil on Monts Koghis (S 22° 10.610, E 166° 30.460, Dumbea, New Caledonia) and immediately placed in RNAlater (Sigma-Aldrich) and conserved at 4 °C until RNA extraction. RNA from *P. gabriellae* samples was extracted and purified as described above. RNA from *P. semperflorens* samples was purified using NucleoSpin RNA Plant (Macherey-Nagel, Düren, Germany; www.mn-net.com). DNase-treated RNA (200 ng) was converted to cDNA using SuperScript III First-Strand (Invitrogen). For quantitative RT-PCR analyses, primers were designed from *PgIREG1* (contig #3856, HE825086, HF536479), *PgEF1α* (contig #21851, HE843072), and *PgGAPDH* (contig #6277, HE827507) in regions identical between the two species. *PgIREG1* and *PgEF1α* were amplified (two technical replicates) from the four independent cDNA samples from both *P. gabriellae* and *P. semperflorens* with primers (Supplementary Table S1, available at *JXB* online) PgIREG1-qF2/PgIREG1-qR2 (92–93% efficiency in both species), PgEF1α-qF1/PgEF1α-qR1 (97–100% efficiency), and PgGAPDH-qF1/PgGAPDH-qR1 (102–106% efficiency), respectively, using a Lightcycler 480II with SYBR Green I Master (Roche, Indianapolis, IN, USA; www.roche.com). Relative expression of *PgIREG1* in both *Psychotria* species was quantified with the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) using *PgEF1α* and *PgGAPDH* as reference genes.

Accession numbers

P. gabriellae transcriptome raw sequencing reads were deposited in the Sequence Read Archive database under accession number ERP001334 (www.ebi.ac.uk/ena/data/view/ERP001334). *De novo* assembly of the reads was submitted to the Transcriptome Shotgun Assembly database and contigs received accession numbers ranging from HE821243 to HE855365. The coding sequences of *PgIREG1* (HF536479), *PgNRAMP1.1* (HF536480), and *PgNRAMP2.1* (HF536481) were submitted to the EMBL Nucleotide Sequence Database.

Results

Sequencing of P. gabriellae *transcriptome*

To get molecular insight into the mechanisms of nickel hyperaccumulation in the non-model plant *P. gabriellae*, we sequenced its transcriptome using Roche 454 pyrosequencing technology. We collected tissues from an isolated plant population growing in its natural environment on ultramafic soil. In pilot experiments we realized that it was extremely difficult to isolate roots *in situ* and to extract RNA from this tissue. In order to preserve this natural plant population, we decided not to include root RNA in this study. A cDNA library was constructed using RNA extracted from floral buds and leaves where nickel accumulates (Jaffré and Schmid, 1974). The cDNA library was normalized to increase the representation of rare transcripts and was sequenced on half a chip of a Roche GS-FLX using titanium series chemistry. We obtained 596 737 reads with an average size of 322 bases, which corresponds to the expected sequencing output using this technology. The reads were subsequently assembled *de novo* using CLC Genomic Workbench software. A total of 489 134 reads were assembled into 34 123 contigs longer than 150 bp, with an N50 contig size of 813 bp (Fig. 1A). These 34 123 contigs represented 24 040 kb of assembled sequences with a mean coverage of 20.3 reads/kb with 86% of the contigs covered by 2 to 10 reads/kb. The remaining 107 603 reads (18%) were left as singletons. Blastn analysis revealed that a few large contigs, #4149 (4567 bp), #3918 (4104 bp), and #20011 (4010 bp), shared strong homology with the chloroplast genome of *Coffea arabica* (Rubiaceae), and therefore are likely to correspond to the chloroplast genome of *P. gabriellae*. In total, 2880 reads were mapped to the *C. arabica* chloroplast genome (EF044213), indicating a minor contamination (0.5% of the reads) of the cDNA library by chloroplastic DNA that is AT-rich (Schliesky *et al.*, 2012). The sequence of the 34 123 contigs is available from the Transcriptome Shotgun Assembly (TSA) sequence database at the European Nucleotide Archive (ENA).

Functional annotation of P. gabriellae *contigs*

To identify the putative function of proteins encoded by the *P. gabriellae* contigs, we performed a Gene Ontology annotation using the Blast2GO software (Conesa *et al.*, 2005). Blastx interrogation of the non-redundant protein database at the US National Center for Biotechnology Information identified significant homologies (*E* value ≤10^{-6}) for 22 619

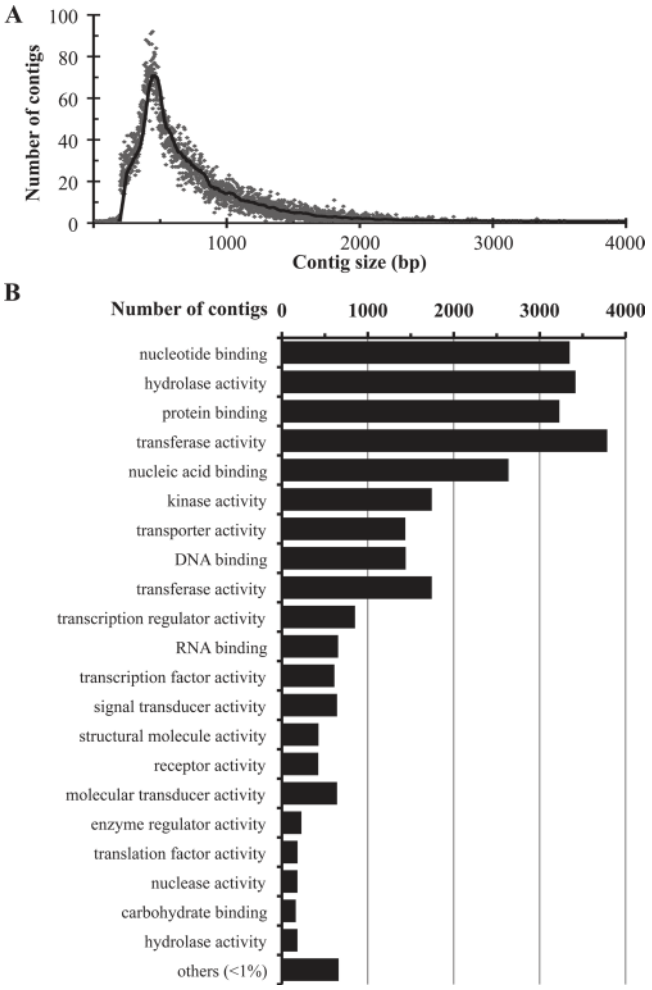

Fig. 1. *P. gabriellae* transcriptome *de novo* assembly and Gene Ontology annotation. (A) Size distribution of the 34 123 contigs generated by *de novo* assembly using CLC Genomics Workbench 4.7. (B) Functional distribution of coding sequences encoded by the 18 457 contigs that received Gene Ontology annotation using Blast2GO.

contigs and a Gene Ontology annotation was given to 18 457 of these contigs. The analysis of these results revealed that the genes encoded by *P. gabriellae* contigs covered a wide range of molecular functions including kinases and transcription factors that are generally transcribed at low levels (Fig. 1B). More interestingly in the context of metal hyperaccumulation, 1436 contigs were annotated with transporter activity.

We then sought to identify contigs encoding proteins potentially involved in nickel homeostasis and transport (Table 1). We identified contigs coding for NA synthase and ATP-phosphoribosyltransferase that correspond to key steps in the biosynthesis of the nickel ligands NA and histidine, respectively (Wycisk *et al.*, 2004; Ingle *et al.*, 2005; Kim *et al.*, 2005; Pianelli *et al.*, 2005). We also identified several contigs coding for members of the IREG/FPN, NRAMP, ZIP/IRT, and YSL/OPT metal transporter families. Subsequently, we focused our analyses on contigs coding for members of IREG/FPN (contig #3856) and NRAMP (contigs #23116 and #5621) families. The analysis of these contigs indicated that they did not cover the entire coding sequence of the transporters. We extended the sequence of contigs #3856, #23116, and #5621 by 5′ and 3′ RACE-PCR using leaf mRNA and obtained the predicted full-length coding sequences of *PgIREG1*, *PgNRAMP1.1*, and *PgNRAMP2.1*, respectively. Phylogenic analyses revealed that PgIREG1 belongs to the same cluster as *Arabidopsis* AtIREG1 and AtIREG2 (Fig. 2A; Supplementary Fig. S1, available at *JXB* online), which is distinct from a second cluster represented by AtIREG3/MAR1 located in chloroplasts and implicated in iron homeostasis (Schaaf *et al.*, 2006; Conte *et al.*, 2009; Morrissey *et al.*, 2009). PgNRAMP1.1 and PgNRAMP2.1 belong to two evolutionarily distinct NRAMP clusters represented by *Arabidopsis* AtNRAMP1 and AtNRAMP2 respectively (Fig. 2B; Supplementary Fig. S1, available at *JXB* online). These two clusters are strongly divergent from the clade containing the NRAMP-related protein AtEIN2 involved in ethylene signaling (Migeon *et al.*, 2010).

Table 1. *Contigs encoding putative proteins involved in nickel homeostasis*

Activity	Contig name	Size (bp)	Number of reads	Accession no.
Synthesis of Ni ligand				
Nicotianamine				
Nicotianamine synthase	21017	1251	31	HE842238
Histidine				
ATP-phosphoribosyltransferase	6251	738	55	HE827481
	1975	587	5	HE823208
Putative Ni transporters				
IREG/Ferroportin transporter family	**3856**	**1554**	**72**	**HE825086**
NRAMP transporter family	**23116**	**1495**	**62**	**HE844336**
	5621	**1405**	**21**	**HE826851**
	4257	1665	36	HE825487
	9717	1180	17	HE830947
ZIP/IRT transporter family	6191	1521	56	HE827421
	21621	1173	15	HE842842
	20706	1025	37	HE841927
	7536	1011	30	HE828766
	17195	827	9	HE838422
	3909	642	20	HE825139
	12118	432	31	HE833346
YSL/OPT transporter family	4003	2009	54	HE825233
	5104	1823	82	HE826334
	5727	1538	23	HE826957
	20418	1283	48	HE841639
	31566	439	26	HE852778

Bold text indicates the three genes that were studied (see text).

These results suggested that our transcriptome sequence data covered a significant fraction of the genes expressed in aboveground tissues of *P. gabriellae* when growing in its natural environment. Although the sequence of an important number of these contigs is probably partial, these sequences can be used to obtain full-length sequences and initiate molecular studies on genes of interest.

Characterization of P. gabriellae *metal transporter specificities*

Members of the NRAMP and IREG/FPN transporter families from plants were shown to transport several divalent metals (Curie *et al.*, 2000; Thomine *et al.*, 2000; Schaaf *et al.*, 2006; Morrissey *et al.*, 2009). The coding sequence of *PgIREG1*, *PgNRAMP1.1*, and *PgNRAMP2.1* were cloned in a vector to express the corresponding proteins under the control of the strong PMA1 promoter in yeast mutants deficient in metal transport. The yeast mutant *smf1* is deficient in manganese uptake (Supek *et al.*, 1996). The expression of *PgNRAMP1.1* and *PgNRAMP2.1*, but not *PgIREG1*, was able to complement the growth of *smf1* when manganese is limited in the culture medium (Fig. 3A). This result indicated that both NRAMP transporters are expressed in yeast and mediate uptake of manganese. To confirm this result, we measured manganese accumulation in *zrt1zrt2* yeast cells expressing *PgNRAMP1.1* and *PgNRAMP2.1* (Fig. 4A). The result showed that cells expressing *PgNRAMP1.1* and *PgNRAMP2.1*, as well as the manganese transporter *AtNRAMP1* (Thomine *et al.*, 2000; Cailliatte *et al.*, 2010), accumulated more manganese than control cells or cells expressing *PgIREG1*, thus suggesting that PgNRAMP1.1 and PgNRAMP2.1 are able to transport manganese.

We then expressed *P. gabriellae* transporters in the *fet3-fet4* mutant defective in iron uptake (Dix *et al.*, 1994). *PgNRAMP1.1*, but not *PgNRAMP2.1*, complemented the phenotype of *fet3fet4* on a medium limited in iron (Fig. 3B), consistent with the possibility that PgNRAMP1.1 is an iron transporter. *PgIREG1* was also able to complement *fet3fet4*, which was more surprising because IREG/FPN proteins export metals from the cytoplasm (Ward and Kaplan, 2012). Accordingly, it was previously observed that *AtIREG2* complements the *Δccc1* mutant deficient in iron storage in the vacuole but not *fet3fet4* (Schaaf *et al.*, 2006; Morrissey *et al.*, 2009). However, in our assay conditions the expression of *AtIREG2* was also able to complement *fet3fet4* (Fig. 3B).

To study the zinc transport activity of PgIREG1, PgNRAMP1.1, and PgNRAMP2.1, we transformed the

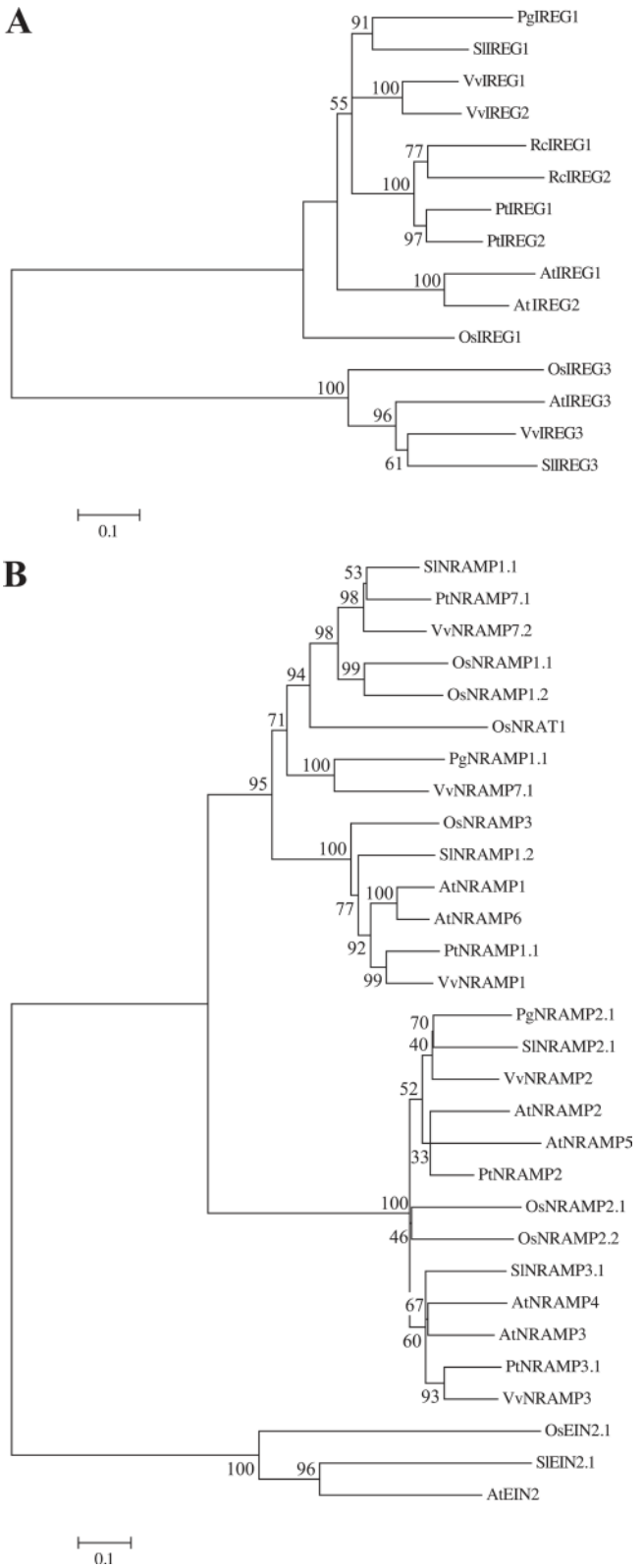

Fig. 2. Phylogenetic analyses of plant metal transporters from the IREG/FPN and NRAMP families. Neighbour-joining-based unrooted tree of IREG/FPN (A) and NRAMP (B) transporters from *P. gabriellae* (Pg) and selected species: *A. thaliana* (At), *Oryza sativa* (Os), *Populus trichocarpa* (Pt), *Ricinus communis* (Rc), *Solanum lycopersicum* (Sl), and *Vitis vinifera* (Vv). Bootstrap values are indicated (1000 replicates). The scale bar relates the length of a branch to the number of amino acid substitutions. The accession numbers for some transporters can be found in Migeon *et al.* (2010) and other accession numbers or gene loci references are as follows: PgIREG1 (HF536479), SlIREG1 (Solyc10g076280.1.1), SlIREG3 (Solyc01g100610.1.1), VvIREG1 (gi:225439578), VvIREG2 (gi:225439580), VvIREG3 (gi:225450573), RcIREG1 (gi:255571513), RcIREG2 (gi:255571511), PtIREG1 (POPTR_0386s00200.1), PtIREG2 (POPTR_0016s13630.1), AtIREG1 (gi:15224883), AtIREG2 (gi:42567622), AtIREG3 (gi:22327094), OsIREG1 (gi:115468536), OsIREG3 (gi:115489078), PgNRAMP1.1 (HF536480), PgNRAMP2.1 (HF536481), SlNRAMP1.1 (Solyc11g018530.1.1), SlNRAMP1.2 (Solyc03g116900.1.1), SlNRAMP2.1 (Solyc04g078250.1.1), SlNRAMP3.1 (Solyc02g092800.1.1), SlEIN2.1 (Solyc09g007870.1.1), and OsNRAT1 (gi: 115444029).

zinc-uptake mutant *zrt1zrt2* (Zhao and Eide, 1996) with plasmid constructs to express these transporters and directly measured zinc accumulation in yeast transformants (Fig. 4B). *PgNRAMP1.1*-expressing yeast accumulated five times more zinc than control *zrt1zrt2* or transformants expressing the other tested transporters. This result suggests that PgNRAMP1.1 has a zinc transporter activity.

Nickel sensitivity of yeast cells expressing P. gabriellae *transporters*

To investigate the ability of PgIREG1, PgNRAMP1.1, and PgNRAMP2.1 to transport nickel we tested the nickel sensitivity of *zrt1zrt2* yeast cells expressing these transporters (Fig. 5). The growth of *zrt1zrt2* cells was not affected by up to 200 µM $NiCl_2$ but was inhibited by 400 µM $NiCl_2$. The expression of either *PgNRAMP1.1* or *PgNRAMP2.1* increased nickel sensitivity of *zrt1zrt2* cells. On the contrary, cells expressing *PgIREG1* were resistant to 400 µM $NiCl_2$ in the culture medium. To better understand these effects on nickel sensitivity we measured nickel accumulation in the same transformants (Fig. 4C). The expression of *PgNRAMP1.1* and *PgNRAMP2.1* did not significantly affect the overall nickel accumulation in cells. In contrast, cells expressing *PgIREG1* accumulated half the concentration of nickel compared to control *zrt1zrt2* cells, consistent with a model in which PgIREG1 improves nickel resistance by transporting nickel out of yeast cells. These results suggest that PgIREG1 has a nickel exporter activity.

Expression of PgIREG1 *in the* Arabidopsis ireg2 *mutant deficient in nickel homeostasis*

To confirm the influence of PgIREG1 on nickel tolerance and accumulation *in planta*, we stably expressed *PgIREG1* in the *Arabidopsis ireg2-1* mutant, which is hypersensitive to nickel for root growth (Schaaf *et al.*, 2006). In these experiments *PgIREG1* was fused to *GFP* either at the N-terminus (*GFP-PgIREG1*) or at the C-terminus (*PgIREG1-GFP*) and expressed under the control of the constitutive *Arabidopsis Ubiquitin10* promoter (Grefen *et al.*, 2010). We first scored the complementation of the *ireg2-1* root growth phenotype on a medium containing 30 µM $NiCl_2$ (Table 2). Several transgenic lines in the T_2 generation (hereinafter referred to as T_2 lines) expressing either *GFP-PgIREG1* or *PgIREG1-GFP* showed an unambiguous complementation of *ireg2-1* root growth phenotype. Three *PgIREG1-GFP*-expressing T_2 lines did not

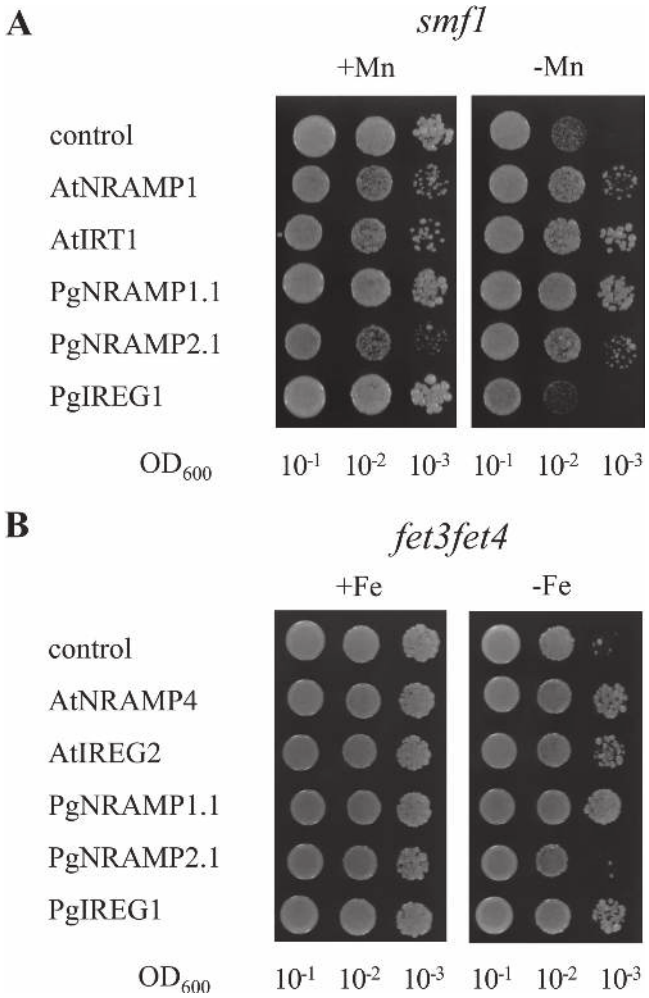

Fig. 3. *P. gabriellae* transporters activity in yeast. *PgIREG1*, *PgNRAMP1.1*, and *PgNRAMP2.1* were expressed in the *smf1* and *fet3fet4* mutants deficient in the uptake of manganese and iron respectively. (A) *smf1* complementation was scored by spotting serial dilutions of yeast transformants on a medium containing 2 mM ethylene glycol tetra-acetic acid (EGTA) without (–Mn) or with (+Mn) 200 µM $MnCl_2$. (B) *fet3fet4* complementation was scored on a medium containing 100 µM bathophenanthroline disulphonate (BPDS) without (–Fe) or with (+Fe) 200 µM $FeCl_3$. In these experiments, *AtNRAMP1*, *AtNRAMP4*, *AtIRT1*, and *AtIREG2* were used as positive controls and yeast mutants transformed with pDR195 empty vector were used as negative control. Experiments were repeated twice with three independent transformants.

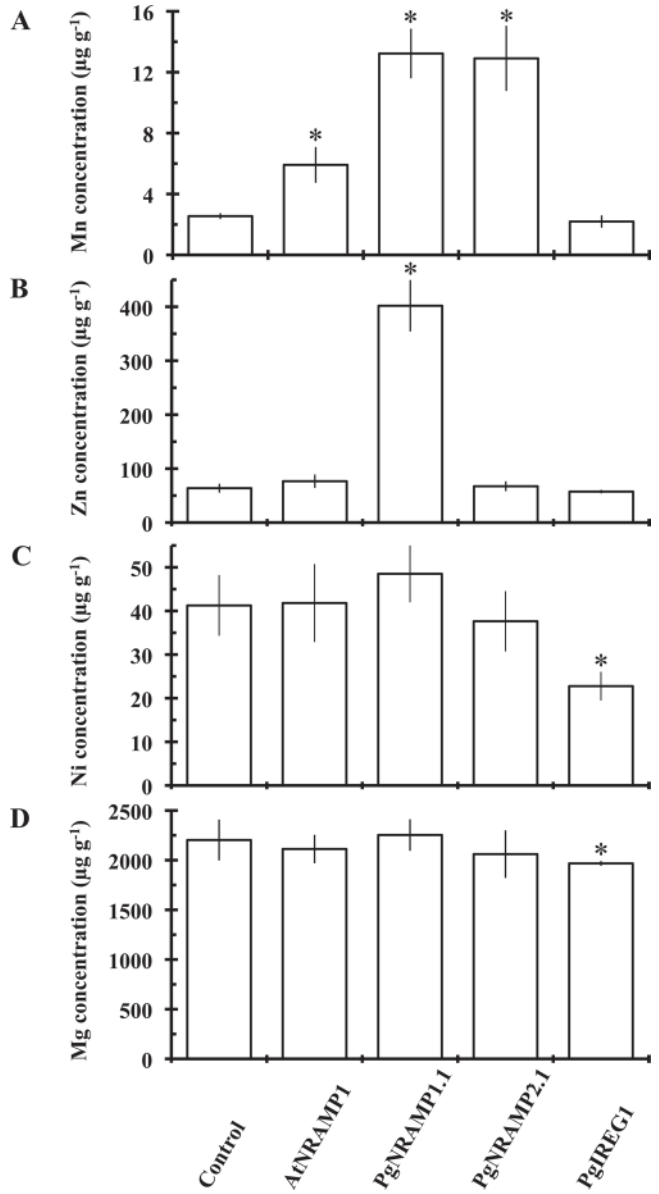

Fig. 4. Metal accumulation in yeast cells expressing *P. gabriellae* transporters. *PgIREG1*, *PgNRAMP1.1*, and *PgNRAMP2.1* as well as *Arabidopsis AtNRAMP1* were expressed in the *zrt1zrt2* double mutants deficient in zinc uptake. The control strain corresponds to *zrt1zrt2* transformed with the pDR195 empty vector. The accumulation of manganese (A), zinc (B), nickel (C), and magnesium (D) was measured by atomic absorption spectrometry. Magnesium, which is not transported by IREG and NRAMP proteins, was used as an internal control. Metal concentration is given as $\mu g \cdot g^{-1}$ of yeast dry weight. For each construct, the results are mean value ± SD ($n = 2$ independent transformants). An asterisk denotes a significant difference with control according to a Mann–Whitney test with $P \leq 0.001$. The experiment was repeated with two additional transformants and generated similar results.

show complementation of *ireg2-1* but displayed hygromycin resistance segregation lower than 75%, suggesting that the transgenes might have been silenced.

Several *ireg2-1* transgenic T_2 lines constitutively expressing *PgIREG1* fused to *GFP* had longer roots than wild-type (WT) Columbia plants when grown on 30 µM nickel (Table 2; Fig. 6A). To better characterize this phenotype, we measured root growth of two representative lines in the T_3 generation (hereinafter referred to as T_3 lines), homozygous for the *PgIREG1* transgene, on medium with increasing concentration of nickel (Fig. 6B). While the growth of *ireg2-1* and WT roots was virtually abrogated at 60 µM $NiCl_2$, roots of *ireg2-1* transgenic T_3 lines expressing *GFP-PgIREG1* and *PgIREG1-GFP* were still able to grow in the presence of 60 and 120 µM $NiCl_2$, respectively. In addition, the rosette leaves of the transgenic T_3 line expressing *PgIREG1-GFP* did not show visible symptoms of chlorosis in a medium containing 60 µM $NiCl_2$ (Supplementary Fig. S2, available at *JXB* online). We then measured nickel accumulation in both roots and shoots of Col, *ireg2-1*, and the homozygous T_3 line *ireg2-1*/*PgIREG1-GFP#I1* (Fig. 7). As previously observed, the *ireg2-1* mutation led to a significant decrease of nickel

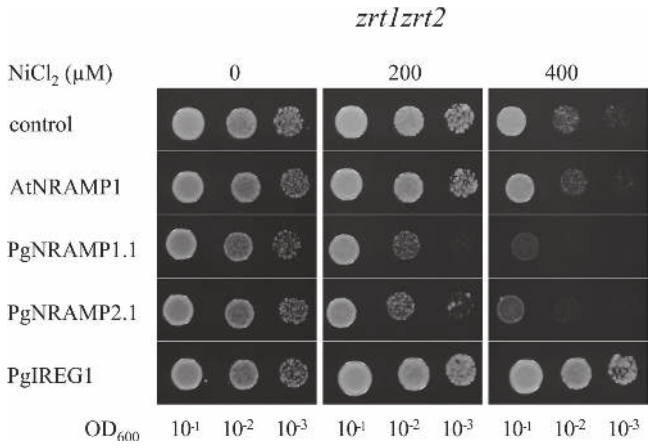

Fig. 5. Nickel sensitivity of yeast cells expressing *P. gabriellae* transporters. Yeast *zrt1zrt2* mutant cells transformed with *PgIREG1*, *PgNRAMP1.1*, or *PgNRAMP2.1*, and with pDR195 empty vector (control) and *AtNRAMP1* used as references, were spotted at serial dilutions on growth medium containing increasing concentrations of $NiCl_2$. Pictures were taken after 3 days of culture for the medium without $NiCl_2$ and 4 days for the media with $NiCl_2$. Experiments were repeated twice with three independent transformants.

Table 2. *Nickel-sensitive root growth of* ireg2-1 *T_2 transgenic lines expressing* GFP-PgIREG1 *and* PgIREG1-GFP

Lines	Root growth on 30 µM $NiCl_2$	Hygromycin resistance (%)
Wild-type (WT) Columbia	+	0
ireg2-1	–	0
ireg2-1/GFP-PgIREG1#A	++	76
ireg2-1/GFP-PgIREG1#B	±	77
ireg2-1/GFP-PgIREG1#C	±	75
ireg2-1/GFP-PgIREG1#D	±	n.d.
ireg2-1/GFP-PgIREG1#E	++	92
ireg2-1/GFP-PgIREG1#F	–	n.d.
ireg2-1/GFP-PgIREG1#G	±	n.d.
ireg2-1/GFP-PgIREG1#H	±	n.d.
ireg2-1/PgIREG1-GFP-#I	++	75
ireg2-1/PgIREG1-GFP-#J	±	78
ireg2-1/PgIREG1-GFP-#K	++	76
ireg2-1/PgIREG1-GFP-#L	–	27
ireg2-1/PgIREG1-GFP-#M	++	87
ireg2-1/PgIREG1-GFP-#O	–	7
ireg2-1/PgIREG1-GFP-#P	++	77
ireg2-1/PgIREG1-GFP-#Q	–	25

–, Root growth similar to *ireg2-1*; ±, root growth intermediate between *ireg2-1* and WT; +, root growth similar to WT; ++, root growth superior to WT; n.d., not determined.

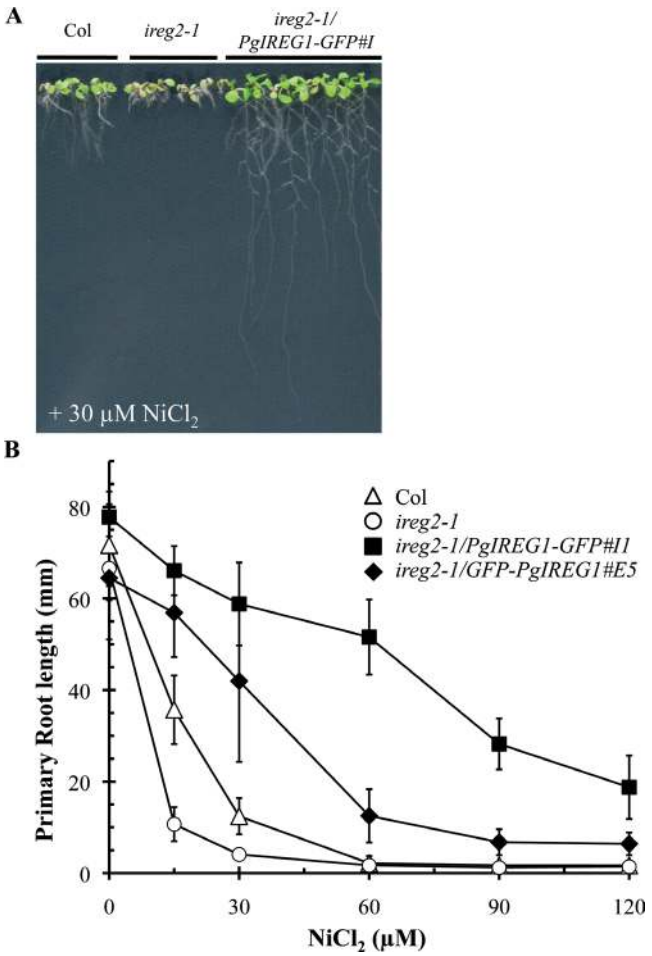

Fig. 6. Expression of PgIREG1 in *A. thaliana ireg2-1* mutant increases nickel resistance. *Arabidopsis* WT (Col), *ireg2-1*, and *ireg2-1* transgenic lines expressing *PgIREG1* were grown vertically on culture medium containing $NiCl_2$. (A) Picture of Col, *ireg2-1*, and *ireg2-1* transgenic T_2 line expressing *PgIREG1-GFP* growing for 12 days on 30 µM $NiCl_2$. (B) Root growth of Col, *ireg2-1*, and *ireg2-1* transgenic T_3 lines expressing *GFP-PgIREG1* or *PgIREG1-GFP* was measured after 14 days of cultivation on medium containing from 0 to 120 µM $NiCl_2$. Results are mean value ± SD (n = 12 roots on two replicate plates).

accumulation in roots (Fig. 7A; Schaaf *et al.*, 2006). In our experiments, we also observed that *ireg2-1* accumulated more nickel in shoots than WT (Fig. 7B). This effect of the *ireg2-1* mutation was not previously described; however, a single nucleotide insertion causing a frame shift in the coding sequence of *AtIREG2* was shown to be responsible, in several accessions of *A. thaliana*, for an increased shoot accumulation of cobalt that is also transported by AtIREG2 (Morrissey *et al.*, 2009). Expression of *PgIREG1-GFP* significantly increased nickel concentration in *ireg2-1* roots to a level similar to WT (Fig. 7A). In these assay conditions, iron and manganese accumulation were not significantly affected by the *ireg2-1* mutation or by the expression of *PgIREG1-GFP*. Interestingly, the accumulation of nickel in shoots of the T_3 line *ireg2-1/PgIREG1-GFP#I1* was similar to that in *ireg2-1* and significantly higher than that in the WT (Fig. 7B). Iron accumulation was reduced in shoots of *ireg2-1*, which is probably linked to the observed chlorosis of the *ireg2-1* mutant in these assay conditions. Expression of *PgIREG1-GFP* in the *ireg2-1* mutant restored iron accumulation to level similar to the WT. Together, these results show that the expression of *PgIREG1* complements the phenotype of *ireg2-1* and increases nickel tolerance, supporting the hypothesis that PgIREG1 is able to transport nickel in plants. These results also indicate that fusion to GFP did not significantly affect the nickel transport activity of PgIREG1.

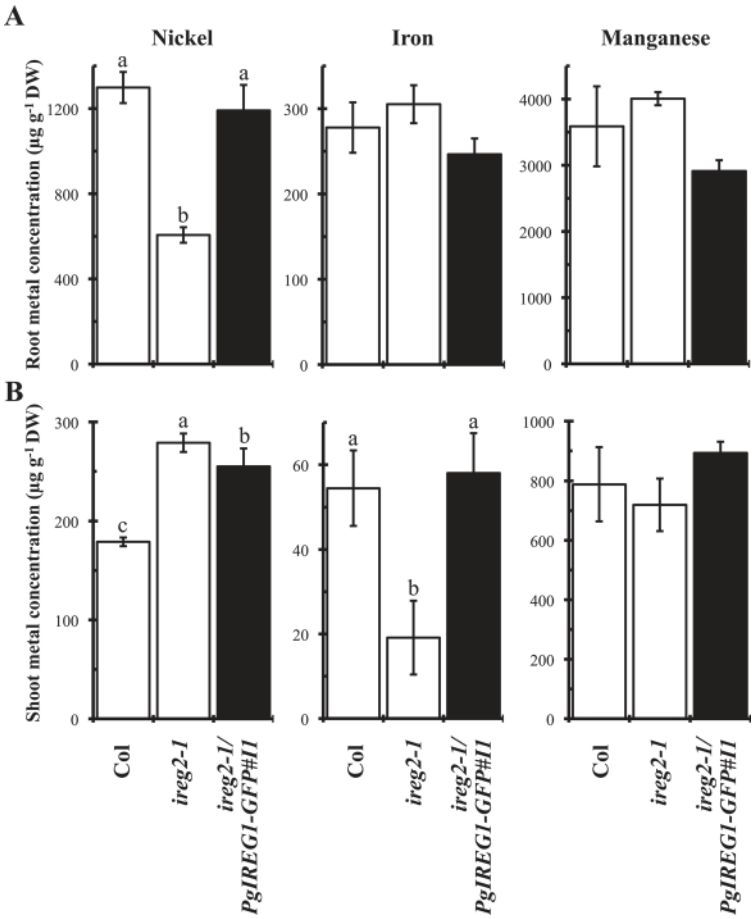

Fig. 7. Accumulation of metals in transgenic *ireg2-1* plants expressing PgIREG1. *Arabidopsis* WT (Col), *ireg2-1*, and *ireg2-1* transgenic T_3 line expressing *PgIREG1-GFP* were grown vertically for 7 days and then transferred on a medium containing 25 µM Fe-HBED/50 µM $NiCl_2$ for 7 days. Nickel, iron, and manganese accumulation was measured by atomic absorption spectrometry in roots (A) and shoots (B) from about 40 individuals. Results are mean value ± SD (n = 3 replicate plates). a, b, c, indicate significant differences according to a Kruskal–Wallis test ($P < 0.05$) followed by Tukey's range analysis.

Localization of PgIREG1 in Arabidopsis *transgenic plants*

To localize PgIREG1 in plant cells, we analysed by confocal microscopy the complemented *ireg2-1* transgenic T_2 lines expressing *PgIREG1-GFP* and *GFP-PgIREG1* (Fig. 8). This analysis revealed that GFP-PgIREG1 was mainly localized in vesicular structures in the cytoplasm. However, a small fraction of the protein was localized in a large and continuous membrane system that detaches from the cell periphery and surrounds the nucleus (Fig. 8A, D). This latter localization is reminiscent of the tonoplast. In contrast, PgIREG1-GFP only visibly localized in the tonoplast (Fig. 8E, H). These results showed that the fusion of GFP at the N- or C-terminus has an impact on PgIREG1 localization or trafficking. However, the complementation of *ireg2-1* by expression of *PgIREG1-GFP* and *GFP-PgIREG1*, and the localization of the corresponding proteins, indicated that PgIREG1 is acting in the tonoplast and likely mediates nickel sequestration in vacuoles of transgenic *Arabidopsis* plants.

Comparison of PgIREG1 *expression in* P. gabriellae *and in the non-accumulator* P. semperflorens

To support the role of PgIREG1 in nickel accumulation we wanted to compare its expression in *P. gabriellae* and in the closely related non-accumulator *P. semperflorens* living in sympatry in rainforest on ultramafic soil (Fig. 9A; Baker *et al.*, 1985). These species belong to the *P. gabriellae* group in the Psychotria NC2 clade and diverged less than 3 million years ago (Barrabé, 2013; Barrabé *et al.*, 2014). Young leaves of *P. gabriellae* and *P. semperflorens* collected on ultramafic soil accumulated 10 241 ± 2158 µg $Ni \cdot g^{-1}$ dry weight (mean ± SD, n = 4) and 55 ± 27 µg $Ni \cdot g^{-1}$ dry weight (n = 4), respectively. To compare *PgIREG1* expression in both species by quantitative RT-PCR, we choose *PgEF1α* as a reference gene (Becher *et al.*, 2004) and designed primers for both genes in coding regions that are identical between the two species. We amplified *PgIREG1* and *PgEF1α* mRNA from four independent individuals of *P. gabriellae* and *P. semperflorens* and the relative expression of *PgIREG1* was compared in both species (Fig. 9B; Supplementary Fig. S3). These analyses revealed that *PgIREG1* was expressed in leaves of *P. gabriellae* at about half the level of *PgEF1α* (Supplementary Fig. S3A). *PgIREG1* was on average 2.5 times more highly expressed in *P. gabriellae* than in *P. semperflorens*. This result was further confirmed using the glyceraldehyde-3-phosphate dehydrogenase gene *PgGAPDH* as a reference (Supplementary Fig. S3B, C). These results are consistent with a link between *PgIREG1* expression and the nickel hyperaccumulation trait in *P. gabriellae*.

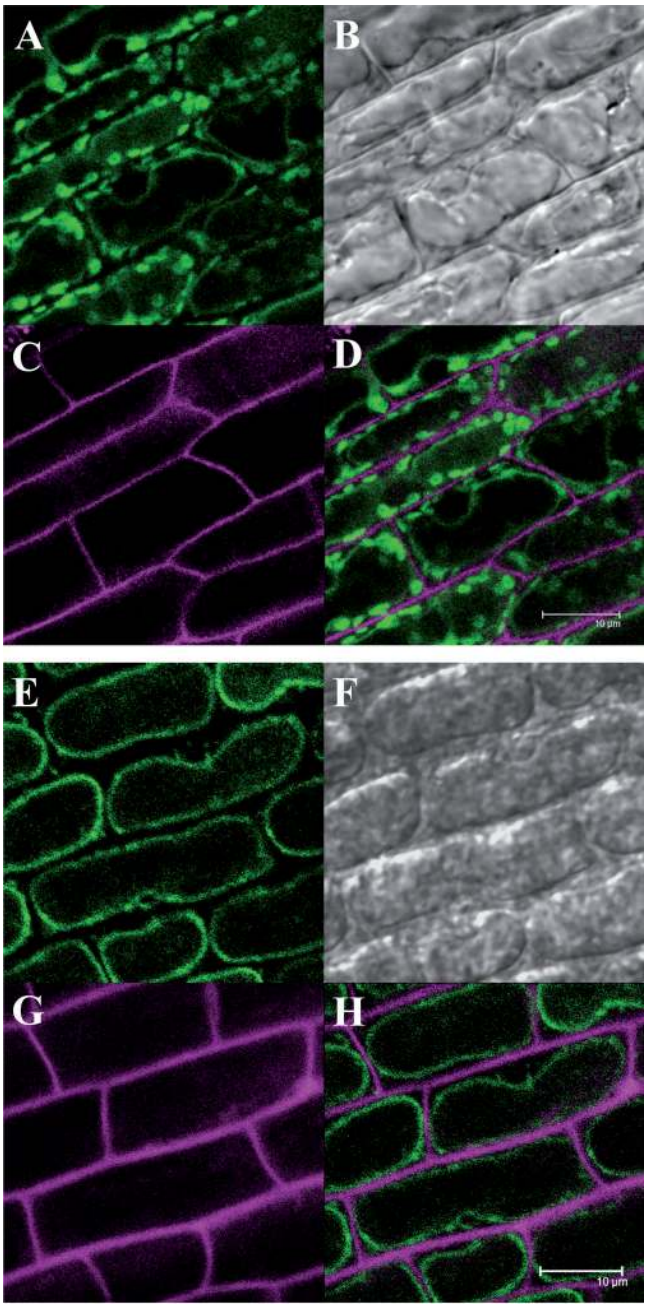

Fig. 8. Localization of PgIREG1 in *Arabidopsis ireg2-1* transgenic plants. GFP-PgIREG1 (A–D) and PgIREG1-GFP (E–H) were stably expressed in *Arabidopsis ireg2-1* mutant. Roots of 12-day-old T_2 transgenic plantlets were stained with propidium iodide and root epidermal cells close to the root tip were imaged by confocal microscopy to visualize GFP (A, E) and propidium iodide (C, G). Corresponding bright-field images (B, F) and overlay of GFP and propidium iodide staining (D, H) are also presented. Scale bar, 10 µM.

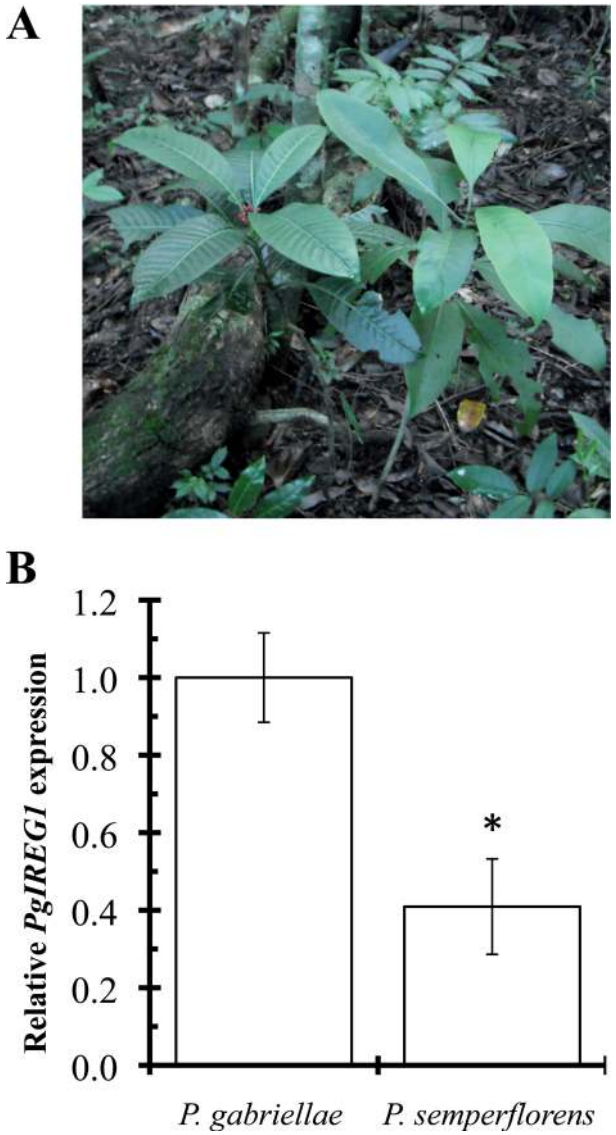

Fig. 9. *PgIREG1* expression in *P. gabriellae* and *P. semperflorens*. (A) Young leaves were collected from the nickel hyperaccumulator *P. gabriellae* (left) and the non-accumulator *P. semperflorens* (right) leaving in sympatry on ultramafic soil in rain forest (Monts Koghis, Dumbea, New Caledonia). (B) The relative expression of *PgIREG1* was compared by quantitative RT-PCR in both species using *PgEF1α* as a reference gene. Relative expression was normalized to 1 according to *PgIREG1* expression in *P. gabriellae*. Results are mean value ± SD (n = 4 independent samples). An asterisk denotes a significant difference according to a Mann–Whitney test ($P \leq 0.05$).

Discussion

Use of next-generation sequencing technologies to study nickel hyperaccumulators

Despite the large number of nickel hyperaccumulators, our knowledge of the mechanisms involved in nickel accumulation in plants is still scarce because most of these species have not yet been subjected to genomic and molecular studies. The aim of this study was to initiate molecular studies on genes involved in nickel accumulation in the hyperaccumulator *P. gabriellae*, endemic to New Caledonia. When we started this work no nucleotide sequence was available for this species. The rapid development of next-generation sequencing technologies opened the possibility to sequence *de novo* the transcriptome of this non-model species (Vera *et al.*, 2008; Ekblom and Galindo, 2011; Ozsolak and Milos, 2011). We decided to use Roche GS-FLX titanium pyrosequencing technology because the longer sequence reads generated by this technology, compared to other available technologies at that time, was an advantage for *de novo* assembly of contigs

(Wang *et al.*, 2009; Martin and Wang, 2011; Schliesky *et al.*, 2012). We obtained about 600 000 reads with an average size of 322 nucleotides that were assembled *de novo* into 34 123 unique contigs using CLC Genomics Workbench (Fig. 1A). Similar results for both the number and the size distribution of the contigs were obtained using Mira 2.9.45x1 and DNA Star NSeq 2.0. Gene Ontology annotation was made for 18 457 contigs covering a large variety of molecular functions (Fig. 1B). The large number of unique contigs and the identification of contig coding for gene families that are generally expressed at low levels (e.g. kinases, transcription factors, metal transporters) suggested that this expressed sequence tag (EST) dataset covered a significant fraction of the genes expressed in aboveground tissues of *P. gabriellae* grown in natural conditions. The sequences of these contigs frequently displayed a few nucleotide ambiguities, which resulted from both sequencing errors in regions with low read coverage and also real sequence polymorphisms because of RNA having been extracted from several individuals. In addition, the analysis of contigs coding for metal transporters revealed that a significant fraction of these contigs did not cover the entire predicted coding sequence. Nevertheless, the cloning of *PgIREG1*, *PgNRAMP1.1*, and *PgNRAMP2.1* demonstrated that these sequences are of sufficient length and quality to clone full-length coding sequences using RACE-PCR. Further sequencing efforts using the latest development in next-generation sequencing technologies will be required to increase the proportion of full-length coding sequences and also to obtain sequences from roots that are essential in the context of nickel accumulation. This original EST dataset could be also very useful to initiate molecular studies on other nickel hyperaccumulators of the *Psychotria* genus found in Cuba, Indonesia, and Puerto Rico (Reeves, 2003).

Identification of metal transporters from P. gabriellae

We focused our functional studies on metal transporters because these proteins are important determinants of metal accumulation in plants (Verbruggen *et al.*, 2009; Krämer, 2010). We selected three candidate genes coding for metal transporters that belong to families that have been implicated in nickel homeostasis (Table 1). *PgNRAMP1.1* and *PgNRAMP2.1* are representatives of each of the two clades of the NRAMP transporters and *PgIREG1* is a member of the IREG/Ferroportin family (Fig. 2B). We characterized the activity of these transporters using heterologous expression in yeast mutants deficient in metal transport. Both PgNRAMP1.1 and PgNRAMP2.1 are able to transport manganese (Fig. 3A), which is general feature of NRAMP transporters from plants (Thomine *et al.*, 2000; Nevo and Nelson, 2006). PgNRAMP1.1 is also able to transport iron and zinc (Figs 3B, 4B). We observed that the expression of *PgNRAMP1.1* and *PgNRAMP2.1* increased nickel sensitivity in yeast (Fig. 5), as was observed with the related TjNRAMP4 transporter (Mizuno *et al.*, 2005). However, we were not able to measure a concomitant increase of nickel accumulation (Fig. 4C). Therefore, the increase in nickel sensitivity might result from an indirect effect of NRAMP activity toward other metals such as iron that is known to interfere with nickel sensitivity in yeast (Ruotolo *et al.*, 2008; Arita *et al.*, 2009). Alternatively, *PgNRAMP1.1* and *PgNRAMP2.1* expression might affect the distribution of nickel rather than its accumulation in yeast. In contrast, the expression of *PgIREG1* increases nickel resistance and reduces nickel accumulation in yeast (Figs 4C, 5), suggesting that PgIREG1 is an active nickel transporter, extruding nickel out of yeast cells. Surprisingly, we observed that *PgIREG1* and *AtIREG2* are able to complement the iron-uptake mutant *fet3fet4*, which is difficult to reconcile with the metal exporter activity of these proteins. Therefore, while we cannot exclude that PgIREG1 is able to transport iron, we favour the hypothesis that the complementation of the yeast mutant *fet3fet4* may result from an indirect effect of *PgIREG1* expression on metal homeostasis in yeast.

In addition, other transporters from the NRAMP, ZIP/IRT, and YSL/OPT families identified in our EST dataset (Table 1) might be involved in nickel transport in *P. gabriellae*. The study of YSL/OPT transporters would be particularly relevant since a nickel–NA complex has been recently detected in leaf extracts of *P. gabriellae* (Callahan *et al.*, 2012).

PgIREG1, *a candidate gene for involvement in nickel tolerance and hyperaccumulation in* P. gabriellae

The nickel transport activity of PgIREG1 was confirmed *in planta* by the expression of *PgIREG1* fused to *GFP* under the control of the constitutive Ubiquitin10 promoter in the *Arabidopsis ireg2-1* mutant. We showed that PgIREG1 fused to GFP localizes in the tonoplast (Fig. 8) as was previously observed for AtIREG2 (Schaaf *et al.*, 2006; Morrissey *et al.*, 2009). *PgIREG1* expression not only complements *ireg2-1* root hypersensitivity to nickel but also increases nickel resistance compared to the WT (Table 2; Fig. 6). *PgIREG1* expression in *ireg2-1* also restored nickel accumulation in roots to levels similar to the WT (Fig. 7A). These results strongly support that PgIREG1 behaves as a functional orthologue of *Arabidopsis* AtIREG2 at the cellular level, mediating the sequestration of nickel in vacuoles. Further immunolocalization studies would be required, however, to confirm the cellular localization of PgIREG1 in *P. gabriellae*.

Despite the functional similarity of PgIREG1 with AtIREG2, the expression pattern of *PgIREG1* is strikingly different. *AtIREG2* is specifically expressed in roots of *Arabidopsis* in response to iron starvation (Schaaf *et al.*, 2006; Morrissey *et al.*, 2009). In contrast, we obtained 72 sequencing reads covering the *PgIREG1* contig from RNA extracted from aerial parts of *P. gabriellae* grown in their natural environment (Table 1), indicative of a significant level of expression in aboveground tissues. Moreover, our quantitative RT-PCR analyses indicated that *PgIREG1* is expressed at levels comparable to those of the housekeeping genes *PgEF1α* and *PgGAPDH* in leaves of *P. gabriellae* (Fig. 9B; Supplementary Fig. S3). As a comparison in the nickel non-tolerant and non-accumulating species *A. thaliana, AtIREG2* (At5g03570) is expressed at about 300 times lower levels than *AtEF1α* (At1g07940) in leaves according to publicly available data on the Genevestigator website (www.genevestigator.com).

Interestingly, an *Arabidopsis ireg2-1* transgenic line constitutively expressing *PgIREG1* accumulates more nickel in shoots than the WT, but does not show symptoms of nickel toxicity, in contrast to *ireg2-1* (Figs 6A, 7). This result suggests that the constitutive expression of *PgIREG1* increases nickel storage capacity in vacuoles of leaf cells where the metal can be accumulated in non-toxic form, for example as organic acid chelates. This result supports the hypothesis that the constitutive and high expression of *PgIREG1* may contribute to nickel tolerance and hyperaccumulation in *P. gabriellae*.

Recent studies on *Arabidopsis halleri* and *N. caerulescens* revealed that zinc and cadmium tolerance and accumulation traits in Brassicaceae are linked to the high and constitutive expression of a few genes coding for metal transporters (Assunção *et al.*, 2001; Dräger *et al.*, 2004; Weber *et al.*, 2004; Hammond *et al.*, 2006; van de Mortel *et al.*, 2006; Hanikenne *et al.*, 2008; Ueno *et al.*, 2011; Craciun *et al.*, 2012). The Rubiaceae species *P. gabriellae* and *P. semperflorens* live in sympatry on ultramafic soil and are therefore both nickel-tolerant (Fig. 9A). Still, only *P. gabriellae* hyperaccumulates nickel. Using quantitative RT-PCR analyses we showed here that *PgIREG1* is 2.5 to 3 times more highly expressed in *P. gabriellae* leaves than in *P. semperflorens* when the species are growing side by side. This result is consistent with a link between *PgIREG1* expression and the nickel hyperaccumulation trait, and further supports that *PgIREG1* contributes to nickel accumulation in *P. gabriellae*. However, this result also suggests that other mechanisms are likely necessary to explain the large difference in nickel accumulation between these two species. To identify these mechanisms future studies will take advantage of RNA-Seq technologies to perform comparative transcriptomic analyses and therefore identify additional genes whose expression is linked to the nickel hyperaccumulation trait in *P. gabriellae*.

Natural variations in *AtIREG2* was previously linked to adaptation to serpentine soil in *Arabidopsis lyrata* (Turner *et al.*, 2010) and accumulation of cobalt in *A. thaliana* (Morrissey *et al.*, 2009). Together with the present study, these results suggest that IREG/Ferroportin transporters might play an important and conserved role in adaptation of plants to metalliferous soils such as serpentine or ultramafic soils through the transport of toxic metals such as cobalt and nickel. More generally, we believe that the study of nickel hyperaccumulation mechanisms in several evolutionarily distant hyperaccumulators such as *P. gabriellae* and *N. caerulescens* will broaden our understanding of the strategies used by plants to accumulate nickel and therefore identify more target genes to improve phytoremediation and phytomining biotechnologies.

Supplementary material

Supplementary material is available at *JXB* online.

Table S1. List of primers used in this study.

Fig. S1. Alignment of metal transporters from *P. gabriellae* and *A. thaliana.*

Fig. S2. Expression of *PgIREG1* in the *A. thaliana ireg2-1* mutant increases nickel resistance.

Fig. S3. Details of the quantitative RT-PCR analyses of *PgIREG1* expression in *P. gabriellae* and *P. semperflorens*.

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