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*...
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 Kozhevinikova, 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 Ni²⁺ with a high association constant. NA is synthesized from S-adenosylmethionine by NA synthase (NAS), and expression in Arabidopsis of N. caerulescens TcNASI 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 cochlleariforme (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 km²) by ultramafic soils that are rich in nickel. As a consequence about 65 nickel hyperaccumulators, including Pycnandra acuminata (previously Sebertia acuminata), Geissospermum 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. gabriellei 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 RNasy Plant Mini kit (Qiagen, Hilden, Germany; www.qiagen.com). The cDNA library and the sequencing were performed by Eurofins MWG Operon (Ebersberg, Germany; www.eurofin-sgenomics.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 ≤10⁻⁶. Phylogenetic analyses of metal transporters were performed with Mega5 as previously described (Migneon 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. gabriellei leaf cDNA using high-fidelity Phusion polymerase (Thermo Scientific, Waltham, MA, USA; www.thermoscientific.com) with primers PgIREG1-gfw and PgIREG1-gwr or PgIREG1-gwrs, PgNRAMP1.1-gfw and PgNRAMP1.1-gwrs, PgNRAMP2.1-gfw 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-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-AtrIREG2 (Schaal et al., 2006), pFL61-IRT1 (Eide et al., 1996), pDR195-AtrNRAMP1, and pDR195-AtrNRAMP4 (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-ethanesulfonic acid (MES; Sigma-Aldrich), pH 6.0/100 µM bathophenanthrolinedisulfonate (BPDS; Sigma-Aldrich) supplemented or not with 200 µM FeCl₃. 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 MnSO₄. Yeast sensitivity to nickel was scored from zrt1zrt2 transformants grown on SD-Ura containing 20 mM MES, pH 5.5/100 µM FeCl₃/1 mM ZnSO₄ and supplemented or not with NiCl₂ up to 400 µM. Metal accumulation was measured from zrt1zrt2 transformants grown to saturation in liquid SD-Ura containing 50 µM ZnSO₄. The precultures were diluted at OD₆₀₀ 0.4 in 50 mL of SD-Ura medium containing 10 mM MES, pH 5.5/10 µM MnSO₄/50 µM NiCl₂/50 µM ZnSO₄ 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 (Greifen 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 Bbel/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 SD-Ura and in 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 T₁ 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 NiCl₂. T₁ 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 NiCl₂ 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 NiCl₂. 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 NiCl₂ for 7 days. Roots and shoots were collected separately, washed twice with ice-cold 2 mM MES, pH 5.7/5.5 mM CaCl₂ 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% HNO₃ 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 T₁ 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* 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), *PgEF1a* (contig #21851, HE843072), and *PgGAPDH* (contig #6277, HE827507) in regions identical between the two species. *PgIREG1* and *PgEF1a* 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), *PgEF1a*-qF1/*PgEF1a*-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−ΔΔCt method (Livak and Schmittgen, 2001) using *PgEF1a* 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 596737 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 489134 reads were assembled into 34123 contigs longer than 150 bp, with an N50 contig size of 813 bp (Fig. 1A). These 34123 contigs represented 240400 bp 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 107603 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 (Schlesky et al., 2012). The sequence of the 34123 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 22619 contigs.
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 \textit{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, 1,436 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 \textit{PgIREG1}, \textit{PgNRAMP1.1}, and \textit{PgNRAMP2.1}, respectively. Phylogenic analyses revealed that \textit{PgIREG1} belongs to the same cluster as \textit{Arabidopsis} \textit{AtIREG1} and \textit{AtIREG2} (Fig. 2A; Supplementary Fig. S1, available at JXB online), which is distinct from a second cluster represented by \textit{AtIREG3/MAR1} located in chloroplasts and implicated in iron homeostasis (Schaff et al., 2006; Conte et al., 2009; Morrissey et al., 2009). \textit{PgNRAMP1.1} and \textit{PgNRAMP2.1} belong to two evolutionarily distinct NRAMP clusters represented by \textit{Arabidopsis} \textit{AtNRAMP1} and \textit{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 \textit{AtEIN2} involved in ethylene signaling (Migeon et al., 2010).

These results suggested that our transcriptome sequence data covered a significant fraction of the genes expressed in aboveground tissues of \textit{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.

\section*{Characterization of \textit{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; Schaff et al., 2006; Morrissey et al., 2009). The coding sequence of \textit{PgIREG1}, \textit{PgNRAMP1.1}, and \textit{PgNRAMP2.1} were cloned in a vector to express the corresponding proteins under the control of the strong \textit{PMA1} promoter in yeast mutants deficient in metal transport. The yeast mutant \textit{smf1} is deficient in manganese uptake (Supek et al., 1996). The expression of \textit{PgNRAMP1.1} and \textit{PgNRAMP2.1}, but not \textit{PgIREG1}, was able to complement the growth of \textit{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 \textit{zrt1zrt2} yeast cells expressing \textit{PgNRAMP1.1} and \textit{PgNRAMP2.1} (Fig. 3A). The result showed that cells expressing \textit{PgNRAMP1.1} and \textit{PgNRAMP2.1}, as well as the manganese transporter \textit{AtNRAMP1} (Thomine et al., 2000; Cailliatte et al., 2010), accumulated more manganese than control cells or cells expressing \textit{PgIREG1}, thus suggesting that \textit{PgNRAMP1.1} and \textit{PgNRAMP2.1} are able to transport manganese.

We then expressed \textit{P. gabriellae} transporters in the \textit{fet3-fet4} mutant defective in iron uptake (Dix et al., 1994). \textit{PgNRAMP1.1}, but not \textit{PgNRAMP2.1}, complemented the phenotype of \textit{fet3-fet4} on a medium limited in iron (Fig. 3B), consistent with the possibility that \textit{PgNRAMP1.1} is an iron transporter. \textit{PgIREG1} was also able to complement \textit{fet3-fet4}, which was more surprising because IREG/FPN proteins export metals from the cytoplasm (Ward and Kaplan, 2012). Accordingly, it was previously observed that \textit{AtIREG2} complements the \textit{acc1} mutant deficient in iron storage in the vacuole but not \textit{fet3-fet4} (Schaff et al., 2006; Morrissey et al., 2009). However, in our assay conditions the expression of \textit{AtIREG2} was also able to complement \textit{fet3-fet4} (Fig. 3B).

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

\begin{table}[h]
\centering
\caption{Contigs encoding putative proteins involved in nickel homeostasis} \label{tab:1}
\begin{tabular}{lcccc}
\hline
Activity & Contig & Size & Number & Accession \\
 & name & (bp) & of reads & no. \\
\hline
Synthesis of Ni ligand & & & & \\
Nicotianamine & \#3856 & 1251 & 31 & HE842238 \\
nicotianamine synthase & \#23116 & 378 & 55 & HE827481 \\
Histidine & \#5621 & 587 & 5 & HE823208 \\
ATP-phosphoribosyltransferase & & & & \\
Putative Ni transporters & & & & \\
IREG/Ferroportin transporter family & \#3856 & 1554 & 72 & HE825086 \\
NRAMP transporter family & \#23116 & 1495 & 62 & HE844336 \\
 & \#5621 & 1405 & 21 & HE826951 \\
 & \#4257 & 1665 & 36 & HE825487 \\
 & \#9717 & 1180 & 17 & HE830947 \\
ZIP/IRT transporter family & \#6191 & 1521 & 56 & HE827421 \\
 & \#21621 & 1173 & 15 & HE824824 \\
 & \#20706 & 1025 & 37 & HE841927 \\
 & \#7356 & 1011 & 30 & HE828766 \\
 & \#17195 & 827 & 9 & HE838422 \\
 & \#3909 & 642 & 20 & HE825139 \\
 & \#12118 & 432 & 31 & HE833546 \\
YSL/OPT transporter family & \#4003 & 2009 & 54 & HE825233 \\
 & \#5104 & 1823 & 82 & HE826334 \\
 & \#5727 & 1586 & 23 & HE826957 \\
 & \#20418 & 1283 & 48 & HE841639 \\
 & \#31566 & 439 & 26 & HE852778 \\
\hline
\end{tabular}
\footnotesize{Bold text indicates the three genes that were studied (see text).}
\end{table}
zinc-uptake mutant \textit{zrt1zrt2} \cite{Zhao1996} with plasmid constructs to express these transporters and directly measured zinc accumulation in yeast transformants \citep{Fig4B}. \textit{PgNRAMP1.1}-expressing yeast accumulated five times more zinc than control \textit{zrt1zrt2} or transformants expressing the other tested transporters. This result suggests that \textit{PgNRAMP1.1} has a zinc transporter activity.

**Nickel sensitivity of yeast cells expressing \textit{P. gabriellae} transporters**

To investigate the ability of \textit{PgIREG1}, \textit{PgNRAMP1.1}, and \textit{PgNRAMP2.1} to transport nickel we tested the nickel sensitivity of \textit{zrt1zrt2} yeast cells expressing these transporters \citep{Fig5}. The growth of \textit{zrt1zrt2} cells was not affected by up to 200 \(\mu\text{M} \text{NiCl}_2\) but was inhibited by 400 \(\mu\text{M} \text{NiCl}_2\). The expression of either \textit{PgNRAMP1.1} or \textit{PgNRAMP2.1} increased nickel sensitivity of \textit{zrt1zrt2} cells. On the contrary, cells expressing \textit{PgIREG1} were resistant to 400 \(\mu\text{M} \text{NiCl}_2\) in the culture medium. To better understand these effects on nickel sensitivity we measured nickel accumulation in the same transformants \citep{Fig4C}. The expression of \textit{PgNRAMP1.1} and \textit{PgNRAMP2.1} did not significantly affect the overall nickel accumulation in cells. In contrast, cells expressing \textit{PgIREG1} accumulated half the concentration of nickel compared to control \textit{zrt1zrt2} cells, consistent with a model in which \textit{PgIREG1} improves nickel resistance by transporting nickel out of yeast cells. These results suggest that \textit{PgIREG1} has a nickel exporter activity.

**Expression of PgIREG1 in the Arabidopsis \textit{ireg2} mutant deficient in nickel homeostasis**

To confirm the influence of \textit{PgIREG1} on nickel tolerance and accumulation in planta, we stably expressed \textit{PgIREG1} in the \textit{Arabidopsis \textit{ireg2}-1} mutant, which is hypersensitive to nickel for root growth \citep{Schaaf2006}. In these experiments \textit{PgIREG1} was fused to \textit{GFP} either at the N-terminus (\textit{GFP-PgIREG1}) or at the C-terminus (\textit{PgIREG1-GFP}) and expressed under the control of the constitutive \textit{Arabidopsis Ubiquitin10} promoter \citep{Grefen2010}. We first scored the complementation of the \textit{ireg2-1} root growth phenotype on a medium containing 30 \(\mu\text{M} \text{NiCl}_2\) (Table 2). Several transgenic lines in the \text{T}_2 generation (hereinafter referred to as \text{T}_2 lines) expressing either \textit{GFP-PgIREG1} or \textit{PgIREG1-GFP} showed an unambiguous complementation of \textit{ireg2-1} root growth phenotype. Three \textit{PgIREG1-GFP}-expressing \text{T}_2 lines did not as follows: \textit{PgIREG1} (HF536479), \textit{SiIREG1} (Solyc10g076280.1.1), \textit{SiIREG3} (Solyc01g100610.1.1), \textit{VvIREG1} (gi:225439578), \textit{VvIREG2} (gi:225439580), \textit{VvIREG3} (gi:225450573), \textit{RcIREG1} (gi:255571515), \textit{RcIREG2} (gi:255571511), \textit{PbIREG1} (POPTR_0386s00200.1), \textit{PbIREG2} (POPTR_0016s13630.1), \textit{AtIREG1} (gi:15224883), \textit{AtIREG2} (gi:42567622), \textit{AtIREG3} (gi:22327094), \textit{OsIREG1} (gi:115468536), \textit{OsIREG3} (gi:115468578), \textit{PgNRAMP1.1} (HF536480), \textit{PgNRAMP2.1} (HF536481), \textit{SnRAMP1.1} (Solyc11g018530.1.1), \textit{SnRAMP1.2} (Solyc03g116900.1.1), \textit{SnRAMP2.1} (Solyc04g078250.1.1), \textit{SnRAMP3.1} (Solyc02g092800.1.1), \textit{SiEIN2.1} (Solyc09g007870.1.1), and \textit{OsNRAT1} (gi:115444029).

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 \textit{P. gabriellae} (Pg) and selected species: \textit{A. thaliana} (At), \textit{Oryza sativa} (Os), \textit{Populus trichocarpa} (Pt), \textit{Ricinus communis} (Rc), \textit{Solanum lycopersicum} (Sl), and \textit{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 \textit{Migeon et al.} (2010) and other accession numbers or gene loci references are...
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$_3$ 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*#II (Fig. 7). As previously observed, the *ireg2-1* mutation led to a significant decrease of nickel accumulation in both roots and shoots of Col compared to WT, whereas the transgenic line *ireg2-1/PgIREG1-GFP*#II accumulated nickel to a similar extent as WT.
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 T3 line *ireg2-1/PgIREG1-GFP* 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 PglREG1 is able to transport nickel in plants. These results also indicate that fusion to GFP did not significantly affect the nickel transport activity of PglREG1.
Localization of PgIREG1 in Arabidopsis transgenic plants

To localize PgIREG1 in plant cells, we analysed by confocal microscopy the complemented *ireg2-1* transgenic T2 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 10241 ± 2158 µg Ni·g⁻¹ dry weight (mean ± SD, n = 4) and 55 ± 27 µg Ni·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*.
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; Eklblom 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.
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 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 might 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* (Schaaft 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 (Schaaft 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 *PgEF1a* 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 *AtEF1a* (*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 metaliferous 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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