

Tracking nickel-adaptive biomarkers in *Pisolithus albus* from New Caledonia using a transcriptomic approach

CLARISSE MAJOREL,* LAURE HANNIBAL,* MARIE-ESTELLE SOUPE,* FABIAN CARRICONDE,+ MARC DUCOUSSO,†‡ MICHEL LEBRUN§ and PHILIPPE JOURAND*

*IRD, UMR040 LSTM, Centre IRD, BPA5, Promenade Roger Laroque, 98848 Nouméa Cedex, New Caledonia, †Institut Agronomique néo-Calédonien (IAC), Axe 2 'Diversités biologique et fonctionnelle des écosystèmes terrestres', Nouméa IRD research centre – BPA5, 98800 Nouméa, New Caledonia, ‡CIRAD, UMR LSTM, TA A-82/J Campus International de Baillarguet, 34398 Montpellier Cedex 5, §Université Montpellier 2, UMR28 LSTM, TA A-82/J Campus International de Baillarguet, 34398 Montpellier Cedex 5, France

Abstract

The fungus *Pisolithus albus* forms ectomycorrhizal (ECM) associations with plants growing on extreme ultramafic soils, which are naturally rich in heavy metals such as nickel. Both nickel-tolerant and nickel-sensitive isolates of *P. albus* are found in ultramafic soils in New Caledonia, a biodiversity hotspot in the Southwest Pacific. The aim of this work was to monitor the expression of genes involved in the specific molecular response to nickel in a nickel-tolerant *P. albus* isolate. We used pyrosequencing and quantitative polymerase chain reaction (qPCR) approaches to investigate and compare the transcriptomes of the nickel-tolerant isolate MD06-337 in the presence and absence of nickel. A total of 1 071 375 sequencing reads were assembled to infer expression patterns of 19 518 putative genes. Comparison of expression levels revealed that 30% of the identified genes were modulated by nickel treatment. The genes, for which expression was induced most markedly by nickel, encoded products that were putatively involved in a variety of biological functions, such as the modification of cellular components (53%), regulation of biological processes (27%) and molecular functions (20%). The 10 genes that pyrosequencing analysis indicated were induced the most by nickel were characterized further by qPCR analysis of both nickel-tolerant and nickel-sensitive *P. albus* isolates. Five of these genes were expressed exclusively in nickel-tolerant isolates as well as in ECM samples *in situ*, which identified them as potential biomarkers for nickel tolerance in this species. These results clearly suggest a positive transcriptomic response of the fungus to nickel-rich environments. The presence of both nickel-tolerant and nickel-sensitive fungal phenotypes in ultramafic soils might reflect environment-dependent phenotypic responses to variations in the effective concentrations of nickel in heterogeneous ultramafic habitats.

Keywords: ectomycorrhiza, New Caledonia, nickel, *Pisolithus albus*, pyrosequencing

Received 24 July 2011; revision received 2 February 2012; accepted 6 February 2012

Introduction

The presence of high levels of heavy metals in terrestrial environments can result from natural or human-related factors, such as pollution. It may influence the ecology and evolution of plants, fungi, animals and

other organisms (Branco & Ree 2010; Colpaert *et al.* 2011). In the presence of high levels of heavy metals, ectomycorrhizal (ECM) fungal populations are differentiated into those that are highly tolerant of metal toxicity and those that are sensitive to such metals (Hartley *et al.* 1997).

In recent years, several studies have characterized and reviewed the various molecular mechanisms and cellular responses to stress that enable ECM fungi to

Correspondence: Clarisse Majorel, Fax: +687 26 43 26; E-mail: clarisse.majorel@ird.fr

tolerate heavy metals (Meharg 2003; Bellion *et al.* 2006). These mechanisms involve the uptake and subcellular sequestration of the metal (Blaudez *et al.* 2000a), intracellular binding of the metal (Jacob *et al.* 2004), responses to oxidative stress by the production of molecules that are involved in detoxification processes (Lanfranco 2007; Ramesh *et al.* 2009) and the synthesis of proteins involved in the transport and accumulation of metals. Additional mechanisms include antioxidative responses (Muller *et al.* 2007a; Ruytinx *et al.* 2011), extracellular chelation and binding of the metal to the cell wall (Bellion *et al.* 2006). The involvement of all of the above-mentioned mechanisms was confirmed recently using the complete genome and transcriptome data of the ECM fungal species *Tuber melanosporum* (Bolchi *et al.* 2011), which enabled the detection of genes related to metal homeostasis in this plant-symbiotic ascomycete. The genes that were identified were subdivided into three major functional classes, namely those involved in: (i) transport of metals, (ii) defence against the effects of oxidative stress and (iii) detoxification of metals (Bolchi *et al.* 2011). Such molecular processes could play a key role in enabling plants, fungi or microorganisms to persist in extreme habitats, such as serpentine soils, which are naturally rich in heavy metals (Branco 2009; Gonçalves *et al.* 2009; Rajkumar *et al.* 2009; Wright & Stanton 2011).

Serpentine soils are derived from ultramafic bedrock and are characterized by high concentrations of magnesium, iron and various heavy metals, such as chromium, cobalt, nickel and manganese. They are also extremely deficient in elements that are essential for plant nutrition, including nitrogen, phosphorus and potassium (Brooks 1987). Previous studies have shown that serpentine soils are characterized by a high biological diversity of plants and microorganisms that use various mechanisms to cope with the edaphic conditions (in particular, adaptation to toxic heavy metals, such as nickel) (Kazakou *et al.* 2008; Branco & Ree 2010). For example, it was shown recently that samples of the ECM fungus *Cenococcum geophilum* that were isolated from serpentine soils were highly tolerant of nickel, which suggests that the organism had adapted to its presence (Gonçalves *et al.* 2009). Recently, similar findings of fungal tolerance to nickel were observed in *Pisolithus albus* (Cooke & Masee) (Jourand *et al.* 2010a).

Pisolithus albus belongs to the genus *Pisolithus* Alb. & Schwein., a major ECM genus in the *Boletales* order, members of which are distributed globally and form ECM associations with a broad range of angiosperm and gymnosperm trees and shrubs (Marx 1977; Martin *et al.* 2002). *Pisolithus albus* grows on serpentine soils in New Caledonia, a tropical archipelago in the Southwest Pacific that is considered to be a hotspot of biodiversity

(Myers *et al.* 2000; Jourand *et al.* 2010a). Serpentine soils cover a third of the surface area of the main island (Perrier *et al.* 2006). In New Caledonia, isolates of *P. albus* from these soils cluster genetically into a single group that comprises both nickel-tolerant and nickel-sensitive isolates (Jourand *et al.* 2010a). It was suggested that the observed phenotypic variations in nickel tolerance might arise from variations in the concentrations of bioavailable nickel in these soils. In addition, the nickel-tolerant isolates of *P. albus* were found to improve the growth and tolerance of nickel in plant hosts that were exposed to toxic concentrations of the metal (Jourand *et al.* 2010b).

Given these findings, it is important to clarify the molecular mechanisms of ECM adaptation to serpentine soils and tolerance of nickel in particular. In the study reported here, we examined changes in the transcriptome upon exposure of a single nickel-tolerant *P. albus* isolate from the ultramafic soils of New Caledonia to nickel to identify biomarkers that are involved in the molecular responses to the metal. Data on the complete transcriptome were obtained using pyrosequencing. We identified genes for which expression was changed by the presence of nickel and verified their differential expression in nickel-tolerant and nickel-sensitive isolates by using real-time quantitative PCR (RT-qPCR). The use of these procedures enabled us to generate the first database of expressed sequence tags (ESTs) from a nickel-tolerant fungus and to identify five genes expressed exclusively in nickel-tolerant isolates of *P. albus*. The latter suggests that this species shows a molecular adaptive response to nickel-rich environments.

Materials and methods

Fungal material (in vitro and in situ)

The ECM fungal isolates used in the study were isolated from sporocarps collected from ultramafic soils in New Caledonia at collection sites 1, 2, 3, 4 and 5 described in Table 1 and Fig. S1 (Supporting information). Anatomical and morphological characterizations led to their identification as isolates of the fungal species *Pisolithus albus*. The Ni-tolerant isolate MD06-337, which was described previously (Jourand *et al.* 2010b), was also used in the study. The other isolates of *P. albus* that were included in the study were new samples and are described in Table 1. Whereas MD09-045 and MD09-001 are nickel-tolerant isolates, MD09-063 and MD09-078 are nickel-sensitive isolates. To confirm their identities at the genus and species levels, these new isolates were analysed phylogenetically on the basis of their internal transcribed spacer (ITS) rDNA sequences as described previously (Jourand *et al.* 2010a). The analysed ITS sequences were deposited in the EMBL Nucleotide

Table 1 Isolates of *Pisolithus albus* used in this study. In addition to description of the sites where isolates were sampled (map location and GPS coordinates), details regarding the accession number of ITS sequences and *in vitro* tolerance of nickel are provided for each isolate

Site description: ultramafic soils		<i>Pisolithus albus</i> isolates					
Site on map	Map location, GPS coordinates	Isolate code	DNA-ITS EMBL accession code	Nickel phenotype	*Ni EC ₅₀ μM MMN medium	*Ni EC ₅₀ μM UF-like medium	References
1	Trazy-Guerioum, Koniambo Massif (21°00'28" S; 164°49'50" E)	MD06-337	AM947121	Tolerant	569 ± 3	1800 ± 5	Jourand <i>et al.</i> 2010a
2	Pindjen Waterfall Road Koné (21°02'19" S; 164°46'26" E)	MD09-078	FR852890	Sensitive	42 ± 4	65 ± 1	This study
3	Pindai (21°19'49" S; 164°58'22" E)	MD09-063	FR852893	Sensitive	76 ± 2	29 ± 2	This study
4	Mont-Dore, Plum Road (22°15'17" S; 166°36'46" E)	MD09-045	FR852892	Tolerant	>1000	2600 ± 5	This study
5	Bois du Sud (22°10'18" S; 166°44'49" E)	MD09-001	FR852891	Tolerant	1194 ± 3	1500 ± 5	This study

EC₅₀, the effective concentration that inhibits growth by 50% (Blaudez *et al.* 2000b); ITS, internal transcribed spacer.

*Ni EC₅₀ was determined in two media: Melin-Norkrans medium as reference medium (Blaudez *et al.* 2000b) and UF-like medium.

Sequence Database. To evaluate the expression of the nickel biomarkers in fungal material *in situ* further, samples from ECM root tips were collected at the study site where the nickel-tolerant isolate MD06-337 and the nickel-sensitive isolate MD09-078 were found. These fungal materials were stored in liquid nitrogen.

Growth conditions

To generate stock cultures from each sporocarp, a fresh piece of pileus trama was transferred aseptically to solid modified Melin-Norkrans (MMN) medium (Blaudez *et al.* 2000b). The MMN medium had the following components: KH₂PO₄ (0.5 g/L; K and P = 3.7 mM), (NH₄)₂HPO₄ (0.25 g/L; N = 3.78 mM and P = 3.67 mM), CaCl₂ (0.05 g/L; Ca = 0.34 mM), NaCl (0.025 g/L; NaCl = 0.43 mM), MgSO₄·7H₂O (0.15 g/L; Mg = 0.61 mM), thiamine hydrochloride (100 μg/L), FeCl₃·6H₂O (0.03 g/L), glucose (10 g/L), malt extract (3 g/L) and agar (14 g/L). The pH was adjusted to 5.6 using 1 M HCl to match the pH measured in ultramafic soils as reported by Perrier *et al.* (2006). The medium was autoclaved for 20 min at 120 °C. All fungal isolates were maintained as subcultures at 30 °C in this medium.

For further experiments, MMN medium was altered to mimic the extreme conditions of ultramafic soils. In the Koniambo Massif, where the *P. albus* isolate MD06-337 was sampled, the concentrations of chemical elements in the soil according to Jourand *et al.* (2010a) were as follows: N (3.6 g/kg, N = 257 mM), P

(0.07 g/kg, P = 2.25 mM), K (0.09 g/kg, K = 2.3 mM), Ca (0.26 g/kg, Ca = 6.5 mM) and Mg (4.1 g/kg, K = 170 mM), with an unbalanced molar ratio of Ca/Mg (1/26) and an excess of extractable Ni (Ni-diethylene triamine pentaacetic acid) (19 mg/kg, Ni = 327 μM). To mimic these conditions, the MMN medium was altered to generate what is henceforth referred to as UF-like medium, which contained the following components: KH₂PO₄ (0.25 g/L; P = 1.83 mM and K = 1.83 mM), (NH₄)₂HPO₄ (0.125 g/L; N = 1.8 mM and P = 0.9 mM), NH₄NO₃ (10 g/L; N = 250 mM, CaCl₂ (0.05 g/L; Ca = 0.34 mM, KCl (0.033 g/L; K = 0.44 mM, NaCl (0.025 g/L), MgSO₄·7H₂O (1.95 g/L; Mg = 7.9 mM, thiamine hydrochloride (100 μg/L), FeCl₃·6H₂O (0.03 g/L), glucose (1 g/L) and malt extract (0.3 g/L). The pH was adjusted to 5.6 using 1 M HCl to match the pH measured in ultramafic soils as reported by Perrier *et al.* (2006). The medium was autoclaved for 20 min at 120 °C. When needed, nickel was added after autoclaving under sterile conditions from a stock solution of NiCl₂ (1 M) to the required final concentrations. To check the nickel tolerance of all the isolates used in the study, the EC₅₀ of Ni was measured in both the reference MMN medium and the UF-like medium as described previously (Jourand *et al.* 2010a).

Extraction of total RNA

Total RNA was extracted from mycelium that had been cultivated *in vitro* as well as from frozen fungal

material that was collected *in situ*. A cetyltrimethyl ammonium bromide (CTAB)-based protocol was used as described by Salzman *et al.* (1999). Briefly, 1–5 g of frozen mycelium or ECM tissue was ground in liquid nitrogen using a mortar and pestle. Then, 0.6–1 mL of extraction buffer (2% CTAB, 2% polyvinylpyrrolidone PVP-K-40, 100 mM Tris-HCl, 25 mM EDTA and 2 M NaCl) was added to each tube, and the samples were incubated at 60 °C for 15 min. An equal volume of a mixture of chloroform:isoamyl alcohol (24:1) was added and mixed immediately for 2 min using a vortex mixer. The samples were then centrifuged at 10 000 g for 10 min at 4 °C. Supernatants solutions were transferred to a new tube, and a 1/3 volume of 8 M LiCl was added to each tube. The samples were mixed by inversion and stored at 4 °C overnight. Total RNA was purified using the silica membrane from an RNeasy Plant Mini kit (Qiagen, Courtaboeuf, France), in accordance with the manufacturer's instructions. The purified RNA was quantified using a NanoDrop1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) at wavelengths of 230, 260 and 280 nm. The integrity of the RNA was verified by separating sample components in 1.2% agarose gels, as described by Salzman *et al.* (1999).

Processing of 454 pyrosequencing data and assembly of cDNA sequences from the nickel-tolerant Pisolithus albus isolate (MD06-337)

To compare the gene expression in the nickel-tolerant *P. albus* isolate (MD06-337) in the presence and absence of nickel, two sets of total RNA were isolated using the CTAB-based protocol from 15-day-old liquid cultures of the isolate cultivated in UF-like medium with or without nickel (250 µM NiCl₂). The two sets of extracted RNA were sent to GATC Biotech (Constance, Germany), where the cDNA was pyrosequenced in accordance with the established procedures used by the company. The primer sequences were removed from the data. Sequence reads were then assembled into contigs using the Genomics Workbench software (CLC Bio, Aarhus, Denmark). Expression analysis was performed using the RPKM (reads per kilobase of exon model per million mapped reads) values (Mortazavi *et al.* 2008). The RPKM values, which are used to standardize sequence counts as a function of contig length and absolute size of the data set (i.e. mapped reads: assembled reads), were calculated as follows: (number of reads per contig) × (1000/length of contig) × (1 × 10⁶/total number of mapped reads in the assembly) (Mortazavi *et al.* 2008). On the basis of the ratio of the RPKM values obtained in the presence and absence of nickel (which represents the fold induction of gene expression in response to

nickel), we selected the genes for which expression was increased more than twofold above the background level for the pyrosequencing, as commonly performed in previous studies using comparative transcriptomic approaches (Chelaifa *et al.* 2010). This cut-off level for the induction of gene expression was used previously in another analysis of the transcriptomic response to a heavy metal in another ECM fungus (Jacob *et al.* 2004). The genes that presented both the highest RPKM values in the presence of nickel (RPKM ≥ 10) and the lowest RPKM values in the absence of nickel were selected as candidate biomarkers of nickel tolerance in *P. albus*. In addition, to identify genes, the contig sequences that were obtained by pyrosequencing were compared with sequences stored in the GenBank nonredundant database (nr), *Laccaria bicolor* database (<http://mycor.nancy.inra.fr/imgc/laccariagenome/>) and *Pisolithus microcarpus* database (free access was provided generously by the F. Martin (INRA laboratory, Nancy, France) using BLASTX with an *E*-value cut-off of 10⁻⁵, as described previously for other pyrosequencing transcriptome analyses (Schmid *et al.* 2010). All assembled contigs and their respective RPKM values sequences of the genes selected from the pyrosequencing data analysis were deposited in the EMBL Nucleotide Sequence Database and referenced under the accession numbers shown in Table 2.

Gene annotation using Gene Ontology (GO) terms

To identify the global functional categories of the unique contigs (with respect to the standard Gene Ontology (GO) categories of biological process, molecular function and cellular component), the two transcriptomic sequence banks and the pyrosequencing contig data obtained from *P. albus* MD06-337 in the presence or absence of nickel were analysed using the GO database. This analysis was based on the similarities of the sequences to those of known proteins in the GenBank nr protein database (*E*-value cut-off of 10⁻⁵), which had been annotated with GO terms using the software BLAST2GO V2.4.8 (Götz *et al.* 2008).

Quantitative PCR analysis

To confirm the nickel-responsive differential expression revealed by pyrosequencing, we used RT-qPCR to verify the fold induction of the genes that had been selected as biomarkers of nickel tolerance. This technique is the most sensitive and reproducible method to quantify gene expression, as described recently in a study using the pyrosequencing approach (Chelaifa *et al.* 2010). For the RT-qPCR experiments, *P. albus* isolates were grown in UF-like medium that contained

Table 2 Candidate biomarker genes, shown by pyrosequencing analysis to be up-regulated by nickel, which show differential expression in nickel-tolerant *Pisolithus albus* isolate MD06-337. EMBL accession codes are provided for all DNA sequences, as are the closest homologs identified by BlastN analysis

Pyrosequencing analysis					Gene sequence and BlastN identification			
No. Contig	No. gene	Size (bp)	RPKM 0 μM Ni	RPKM 250 μM Ni	EMBL accession number	Species	E-value	% Identity
C10864	1	517	0	64	FR852894	<i>Pisolithus microcarpus</i>	2E ⁻³³	100%
C6406	2	411	0	65	FR852895	<i>P. microcarpus</i>	1E ⁻¹⁰⁵	96%
C9200	3	443	0	10	FR852896	<i>P. microcarpus</i>	3E ⁻⁹⁴	95%
C7318	4	411	0	90	FR852897	<i>P. microcarpus</i>	2E ⁻⁷⁹	95%
C10795	5	504	0	90	FR852898	<i>P. microcarpus</i>	1E ⁻¹³⁰	99%
C15327	6	235	0	17	FR852899	<i>P. microcarpus</i>	1E ⁻¹¹²	99%
C17653	7	1199	1	27	FR852900	<i>P. microcarpus</i>	9E ⁻⁴⁴	98%
C18133	8	1125	1	35	FR852901	<i>P. microcarpus</i>	1E ⁻¹⁵¹	99%
C10491	9	665	6	30	FR852902	<i>P. microcarpus</i>	9E ⁻⁹²	97%
C4552	10	1303	3	42	FR852903	<i>P. microcarpus</i>	1E ⁻¹⁵⁵	99%

bp, base pair; RPKM, reads per kilobase of exon model per million mapped reads (Mortazavi *et al.* 2008).

either 50 or 250 μM nickel provided as NiCl_2 . Flasks that contained 250 mL of UF-like medium were each inoculated with five plugs of mycelium from the MMN stock culture. The cultures were allowed to grow in the dark for 15 days, with continuous shaking at 130 rpm at 30 °C. The RNA was extracted from three biological replicates of the fungal isolate MD06-337, which had been cultivated *in vitro* as described above, and analysed by RT-qPCR. At the same time, we tested RNA samples extracted from ECM of the MD06-337 and MD09-078 isolates that had been isolated *in situ*. Furthermore, we analysed RNA samples extracted from other *P. albus* isolates (already described in Table 1). Aliquots of total RNA (1 μg) were treated with TurboDNase I (Applied Biosystems, Carlsbad, CA, USA) and used for cDNA synthesis, which was performed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) in accordance with the manufacturer's protocol. Real-time quantitative PCR was performed in duplicate in a volume of 20 μL , which contained 5 μL of diluted cDNA (1 ng of total RNA equivalent), 1 \times SYBR Green PCR Master Mix (Applied Biosystems) and 0.3 μM of each primer. Primers were designed from the *P. albus* contig data, which were obtained from the results of the pyrosequencing using the Primer Express software (Applied Biosystems). The primers amplified a gene fragment of 150 bp in length at an annealing temperature of 60 °C, as shown in Table S1 (Supporting information). PCR was applied to test the specificity of the primers and the efficiency of amplification (Table S1, Supporting information). The amplification profile consisted of an initial denaturation step at 95 °C for 15 min, then 40 cycles that each comprised a 95 °C step for 10 s, 59 °C for 30 s and 72 °C for

32 s, followed by a dissociation stage, in accordance with the manufacturer's instructions. Real-time PCR was performed using an ABI 7300 Sequence Detection System (Applied Biosystems) in the presence of SYBR Green. A melting curve analysis was performed at the end of each RT-qPCR by gradually increasing the temperature from 60 to 95 °C, while recording the change in fluorescence. A single peak at the melting temperature of the PCR product confirmed primer specificity, indicating that RT-qPCR had been performed with no contamination or formation of primer dimers.

Data and statistical analysis of qPCR

Data were analysed using two methods. The REST 2009 method, which is available at <http://www.gene-quantification.de/rest-2009.html> (Pfaffl *et al.* 2002), was used to determine the ratios of transcripts, whereas the $2^{-\Delta\Delta\text{Ct}}$ method was used to analyse mRNA accumulation (Livak & Schmittgen 2001). Each analysis was normalized with two reference genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and elongation factor 4 α (EF4 α). For each treatment, we performed three technical replicates for each of the three biological replicates per isolate and per nickel treatment, to evaluate the relative expression. In the REST method, the RT-qPCR results were analysed using the hypothesis test [$P(H1)$], which represents the probability that the alternate hypothesis differentiates between the sample and control groups only owing to chance. In the $2^{-\Delta\Delta\text{Ct}}$ method, one-way analysis of variance (ANOVA) was performed and the results were compared by Student's *t*-test ($P < 0.05$) using the XLSTAT software (Addinsoft 2005).

Results

Phylogenetic analysis of the fungal ITS sequence

To confirm that the fungal samples used in the study belong to the species *Pisolithus albus*, we analysed the ITS sequences (ITS1/5.8S rRNA gene/ITS2) of each isolate. The ITS sequence is a commonly used phylogenetic marker for Dikarya (Ascomycetes and Basidiomycetes) and some lower fungi. The sequences were deposited in the EMBL Database and referenced under the accession numbers reported in Table 1. The phylogenetic analysis was performed as described previously (Jourand *et al.* 2010a). The results, which are presented as supplementary data in Fig. S2 (Supporting information) confirmed that the isolates belong to *P. albus*.

DNA pyrosequencing, assembly and sequence analysis of the nickel-tolerant *Pisolithus albus* isolate MD06-337

We obtained two non-normalized cDNA libraries from the nickel-tolerant *P. albus* isolate MD06-337 grown in the presence and absence of nickel. Pyrosequencing of these libraries yielded 1 071 375 single reads, which comprised 542 680 trimmed reads from *P. albus* grown in medium that contained nickel and 528 695 trimmed reads from *P. albus* grown in the absence of nickel. The average read lengths were 238 and 236 bp, respectively (Table S2, Supporting information). We used a two-step assembly procedure to process these reads and generated two groups of contigs by excluding low-quality sequences (sequences shorter than the length threshold, i.e. <200 bp, were discarded). The two groups corresponded to 18 800 contigs from *P. albus* exposed to nickel and 16 636 contigs from *P. albus* that had not been exposed to nickel. These two groups of contigs were pooled and reassembled to highlight the genes that were expressed differently in response to nickel. A final group of 19 518 contiguous sequences (from 813 651 total read counts) from *P. albus* MD06-337 was obtained, with an average length of 698 bp. There were also 254 424 singleton reads (24%; Table S2, Supporting information). Hereafter, the contiguous sequences are referred to as genes. Of the 19 518 genes that were obtained through the *de novo* assembly of the sequence reads, the expression of 30% of the genes was modulated by nickel. Further analysis identified 4211 genes (21%) that were up-regulated by nickel and 1763 genes (9%) that were down-regulated by nickel. The global statistical distribution of these 19 518 genes is presented on a scatter plot in Fig. 1. To identify the greatest possible number of descriptive annotations for each sequence, global gene prediction was carried out on the basis of a

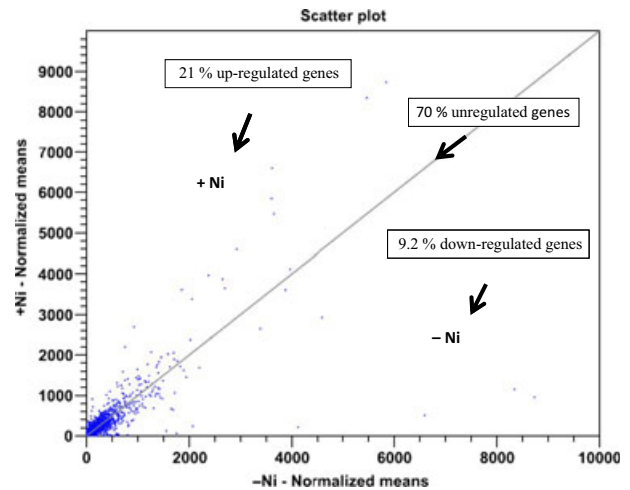


Fig. 1 Analysis of 454 pyrosequencing data. The scatter plot represents gene expression levels in a free-living mycelium of the nickel-tolerant *P. albus* isolate (MD06-337) grown without nickel or with nickel at 250 μ M. The expression levels of genes were normalized using a scale of 0–10 000. Each circle in the plot represents the level of expression of one gene.

set of BLASTX searches (Table S2, Supporting information). Among the 19 518 unique contigs, the global BLASTX analysis indicated that 11 489 contigs (59%) had significant matches in the GenBank database, whereas 10 926 contigs (56%) had matches in the *Laccaria bicolor* database and 15 150 contigs (77.6%) in the *Pisolithus microcarpus* database (Table S2b, Supporting information).

GO annotation of genes expressed differentially in *Pisolithus albus*

To determine the global variation in gene expression in the MD06-337 isolate of *P. albus* in the presence and absence of nickel, we assigned GO terms to the sequences identified in the two pyrosequencing transcriptomic databanks. The result showed that 23% and 25% of the sequences from the databanks for *P. albus* grown in the presence and absence of nickel, respectively, could be assigned a GO term (Fig. 2). The distribution of sequences among the following primary GO categories was analysed: cellular component, molecular function and biological process. This showed differences in distribution between the two conditions within the categories of biological process and cellular component (Fig. 2). To focus on the factors that underlie nickel tolerance in *P. albus*, we also performed a GO analysis of the 4211 contigs (21%) that corresponded to genes that were up-regulated by nickel (Fig. S3, Supporting information). The analysis indicated that only 12.6% of the up-regulated genes could be assigned to the three GO categories, among which 53% were assigned to cellular components, 27% were assigned to

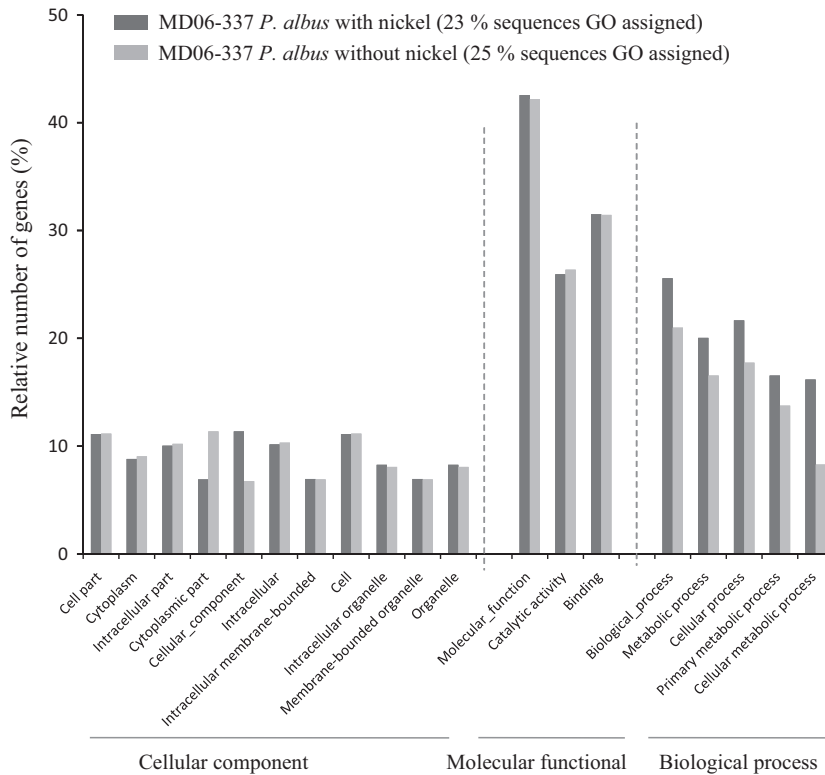


Fig. 2 Assignment of functional Gene Ontology terms and distribution of total sequences for two transcriptomes of the nickel-tolerant isolate MD06-337 of *P. albus* with (in the presence of 250 μM NiCl_2) and without nickel, in terms of the categories of biological process, molecular function and cellular component.

biological processes and 20% were assigned to molecular function (Fig. S3, Supporting information).

Tracking of nickel biomarkers in nickel-tolerant MD06-337 *Pisolithus albus*

Among the genes that were expressed differentially in response to nickel (4211 genes), we selected the 10 genes that showed the greatest variation on the basis of the RPKM values obtained in the pyrosequencing analysis, as shown in Table 2. The names of the sequences correspond to the contig numbers (No. contigs in Table 2). All nucleotide sequences have been deposited in the EMBL Database, and their accession number and lengths (in base pairs) are summarized in Table 2. These DNA sequences were analysed using BLASTN, and the sequences were found to be identical in approximately 95–100% of cases to sequences in the *Pisolithus microcarpus* database (currently not publicly available, personal communication from Martin F., INRA Nancy, France) (Table 2). In addition, the function of the genes was determined using BLASTX sequence alignments (with a threshold of $<1\text{E}^{-03}$). The results showed homologies to putative proteins from the following species: *Laccaria bicolor* (seven genes, No. 2, 3, 4, 6, 8, 9 and 10), *Coprinopsis cinerea* (two genes, No. 5 and 7) and *Saccharomyces cerevisiae* (gene No. 1). Full data from the BLASTX search are presented in Table 3. The analysis of gene

function showed that genes No. 1, 3, 5, 6, 7, 8, 9 and 10 encoded proteins with predicted functions, whereas genes No. 2 and 4 encoded proteins with no known function (Table 3). The genes with a predicted function encoded for (i) cell-wall components (genes No. 1 and 7), (ii) components involved in metabolic processes and amino acid transport (genes No. 3, 6, 8 and 9), (iii) components involved in RNA processing (gene No. 10) and (iv) components involved in metalloprotease processes (gene No. 5).

Validation of pyrosequencing results by RT-qPCR in nickel-tolerant MD06-337 *Pisolithus albus*

The 10 selected genes were analysed by RT-qPCR to confirm the differential expression that was indicated by the pyrosequencing data. For this purpose, specific primer sequences were designed from the DNA sequences obtained by pyrosequencing (Table S1, Supporting information). These primers were used in RT-qPCR to quantify gene expression in *P. albus* MD06-337 using two sets of cDNA. The first set corresponded to the cDNAs that were used for the pyrosequencing, whereas the second set corresponded to newly synthesized independent cDNAs from three independent mycelia. Both sets contained cDNAs that were obtained from *P. albus* grown in the presence of nickel and those of *P. albus* grown in the absence of nickel. The

Table 3 Functional description and assignment of GO terms for 10 candidate biomarker genes (No. 1–10), up-regulated following exposure to nickel, as selected from pyrosequencing analyses that revealed differential expression in nickel-tolerant *Pisolithus albus* isolate MD06-337

No. contig	No. gene	BLASTX functional identification					
		Best predicted function	Species	E-value	Blast hit	Functional classification	GO terms
C10864	1	GPI-anchored cell surface glycoprotein-like (AGA1)	<i>Saccharomyces cerevisiae</i>	5E ⁻⁰³	YNR044W	Cell-wall anchorage	No hits found
C6406	2	Unknown function	<i>Laccaria bicolor</i>	3E ⁻⁰⁸	XM_001881526.1	Unknown function	No hits found
C9200	3	Alpha-amino adipate reductase Lys1p (LbLYS1)	<i>L. bicolor</i>	8E ⁻⁶⁴	XM_001879583.1	Secondary metabolite biosynthesis	GO:0003824
C7318	4	Unknown function	<i>L. bicolor</i>	3E ⁻⁰⁷	XP_001878436.1	Unknown function	No hits found
C10795	5	Vacuolar protein sorting-associated protein 70	<i>Coprinopsis cinerea</i>	4E ⁻²⁰	XP_001837057.2	Metalloprotease activity	GO:0004180
C15327	6	S-adenosylmethionine-dependent methyltransferase	<i>L. bicolor</i>	2E ⁻²¹	XP_001874602.1	Methyl transferase	GO:0016740
C17653	7	Class III chitinase	<i>C. cinerea</i>	5E ⁻⁰⁸	XM_001828384.2	Chitin catabolic process	No hits found
C18133	8	Cytochrome P450 (CYP)	<i>L. bicolor</i>	4E ⁻⁴⁰	XP_001878510.1	Monoxygenase activity	GO:0009055
C10491	9	APC amino acid permease	<i>L. bicolor</i>	4E ⁻³⁹	XP_001875612.1	Amino acid transport	GO:0006560
C4552	10	bZip transcription factor	<i>L. bicolor</i>	9E ⁻¹⁰	XP_569310.1	RNA processing	GO:0009267

GO, Gene Ontology.

RT-qPCR results for the selected genes are presented in Table 4 and are given as the ratio of mRNA expression in the presence of nickel to mRNA expression in the absence of nickel (ratio +Ni/-Ni). The relative expression levels that were obtained from the cDNAs used in

the pyrosequencing analysis and from the new cDNA samples (means ± SD of the three replicates) are compared in Table 4. The data show that there were no significant differences between gene expression profiles in relation to the cDNA template used (pyrosequenced

Table 4 Validation of the pyrosequencing data by RT-qPCR. Relative induction of expression by nickel of the 10 selected genes (up-regulated by nickel: No. 1–10) was measured in the MD06-337 nickel-tolerant isolate, using GAPDH and EF4 α as reference genes

No. contigs/genes	C10864	C6406	C9200	C7318	C10795	C15327	C17653	C18133	C10491	C4552
Relative expression (cDNA 454)*	10***	10.4***	6.3	6.5	4.2***	8***	3.5	39***	17.3***	7.7***
(1) P(H1)	0.0001	0.0001	0.49	0.49	0.0001	0.0001	0.49	0.0001	0.0001	0.0001
†Relative expression (3n)	5.1**	4.1*	2.3	8***	5*	6.5**	6***	8.5	39.5**	5.2***
(2) P(H1)	0.004	0.05	0.175	0.0001	0.022	0.007	0.0001	0.066	0.005	0.0001

Relative expression values were determined by normalizing expression levels against those of the housekeeping genes that encode glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and elongation factor 4 α (EF4 α).

*Values in line (1) represent the relative expression levels of the 10 genes as determined using the cDNA used for the pyrosequencing analysis of MD06-337 isolate.

†Values in line (2) represent the means of three biological independent relative expressions qPCR on the MD06-337 isolate.

All statistical computation and analyses of qPCR data were carried out using the REST 2009 software (Pfaffl *et al.* 2002).

We used the hypothesis tests P(H1).

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Values indicate the mean ± SD ($n = 3$).

DNA or new cDNA; $P > 0.05$). The ratios of gene expression that were obtained using RT-qPCR confirmed the transcriptomic profile of nickel-tolerant *P. albus* MD06-337 that was obtained by pyrosequencing. We also used the REST method to compare the relative expression patterns of the 10 selected genes in the nickel-tolerant isolate MD06-337 at 50 μM , with expression patterns in the presence of 250 μM of nickel. The results are presented in Fig. S4 (Supporting information) and show similar profiles for the two concentrations of nickel tested. In addition, RT-qPCR was performed to verify the levels of mRNA expression of the 10 genes selected in nickel tolerant MD06-337 ECM root tips sampled in situ. The results (Table 5) showed that the selected genes were also expressed in symbiotic

ECM root tips sampled in situ, except for gene No.7, which was not detected in ECM root tips.

Comparison of expression patterns in nickel-tolerant (MD06-337) and nickel-sensitive (MD09-078) isolates of Pisolithus albus

We compared the expression profiles of the 10 selected genes that were up-regulated by nickel between the nickel-tolerant isolate (MD06-337) and a nickel-sensitive isolate (MD09-078) from the ultramafic soils. As shown in Fig. 3, the levels of expression of the genes analysed differed significantly between the nickel-sensitive isolate MD09-078 and the nickel-tolerant isolate MD09-337 ($P < 0.05$). Genes No. 1, 2, 5, 6 and 7 were expressed

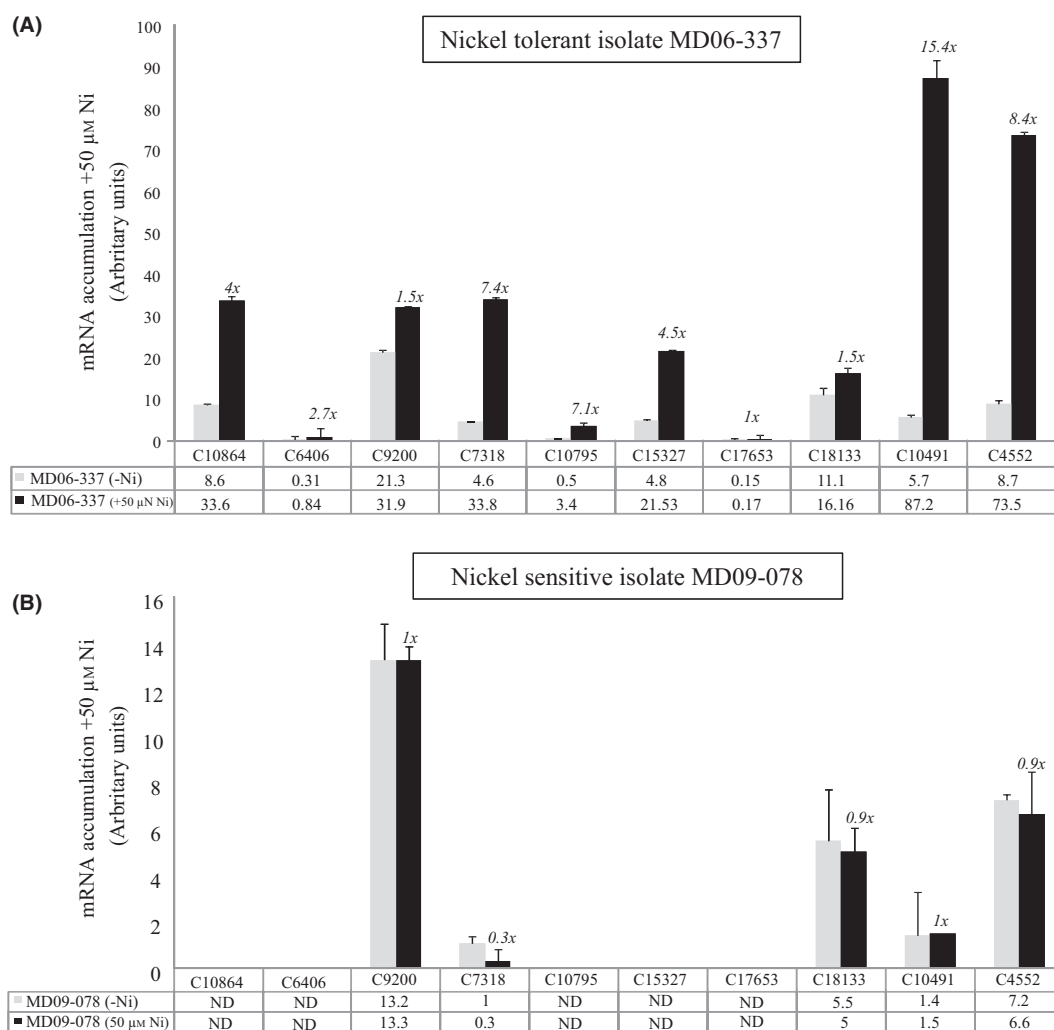


Fig. 3 Comparison of patterns of mRNA accumulation in *P. albus* isolates grown without nickel or with nickel (provided as 50 μM NiCl_2). Expression of the 10 genes up-regulated most by nickel, as determined by pyrosequencing, in nickel-tolerant (MD06-337) and nickel-sensitive (MD09-078) isolates. Transcript accumulation was quantified by RT-qPCR, using the $2^{-\Delta\Delta\text{Ct}}$ method with glyceraldehyde-3-phosphate dehydrogenase and EF4 α as reference genes for normalization, and expressed as arbitrary units. The data indicate mean values \pm SD, calculated from three technical replicates with triplicate biological samples. The fold induction by nickel is presented above the columns in italics. ND: mRNA nondetected (Ct values >37).

Table 5 *In situ* validation of the pyrosequencing data by RT-qPCR. Relative induction of expression by nickel of the 10 selected genes (up-regulated by nickel: No. 1–10) in ectomycorrhizal *Pisolithus albus* root tips (nickel-tolerant MD06-337 isolate and nickel-sensitive MD09-078 isolate) *in situ* was measured by comparison with the nickel-tolerant isolate MD06-337 and nickel-sensitive isolate MD09-078, *in vitro*. Transcript accumulation was quantified by RT-qPCR using the $2^{-\Delta\Delta ct}$ method with normalization using two reference genes, GAPDH and EF4 α

No. gene	No. 1 C10864	No. 2 C6406	No. 3 C9200	No. 4 C7318	No. 5 C10795	No. 6 C15327	No. 7 C17653	No. 8 C18133	No. 9 C10491	No. 10 C4552
mRNA accumulation										
ECM (MD06-337) <i>in situ</i>	11	0.4	8.4	5.1	1.2	1.3	nd	0.8	3	7
Nickel-tolerant isolate										
Control (<i>in vitro</i>)	10	10.4	6.3	6.5	4.2	8	3.5	39	17.3	7.7
MD06-337										
(UF-like + 250 μ M nickel)										
ECM (MD09-078) <i>in situ</i>	0.25	0.74	48.2	35.7	0.24	0.41	0.24	18.5	0.34	26.6
Nickel-sensitive isolate										
Control (<i>in vitro</i>)	nd	nd	22	3	0.30	0.24	0.21	8	1.5	3
MD09-078										
(UF-like + 50 μ M nickel)										

nd, not detected (CT > 37); ECM, ectomycorrhizal; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

very weakly or were not detectable (ND) in the MD09-078 isolate, whereas they were expressed strongly in MD06-337 isolate. As shown in Fig. 3, the normalized expression levels varied from 0.15 to 87 arbitrary units. The remaining genes, No. 3, 4, 8, 9 and 10, were expressed at substantial levels in both isolates. However, whereas in the nickel-sensitive isolate, the expression of these genes (No. 3, 4, 8, 9 and 10) was not affected by nickel (there was no significant induction), in the nickel-tolerant isolate, nickel induced an increase in expression of these genes of between 1.5- and 15-fold in the nickel-tolerant isolate. From this comparison of gene expression levels between nickel-sensitive and nickel-tolerant isolates, we identified genes No. 1, 2, 5, 6 and 7 as showing the most significant differences in terms of levels of expression. We concluded that they show the greatest promise as molecular markers (biomarkers) of tolerance to nickel in *P. albus* from the ultramafic soils of New Caledonia. We also included gene No. 9 in this set of promising biomarkers because it was induced strongly by nickel in the nickel-tolerant isolate. These six selected genes were used as biomarkers for the final analysis of nickel tolerance. In addition, RT-qPCR was performed to verify the levels of mRNA expression for the 10 selected genes in ECM samples from nickel-sensitive *P. albus* isolate MD09-078 sampled *in situ* (Table 5). The RT-qPCR results showed that the six genes that were selected as biomarkers were either not expressed, or only weakly expressed, in nickel-sensitive isolate from ECM root tips sampled *in situ*. These results are in agreement with those obtained by *in vitro* experiments conducted using the nickel-sensitive *P. albus* isolate MD09-078.

Comparison of biomarker expression between nickel-tolerant and nickel-sensitive *Pisolithus albus* isolates

We tested the expression levels of the six biomarkers in five isolates of *P. albus* from the ultramafic soils of New Caledonia. These included three nickel-tolerant isolates (MD06-337, MD09-045 and MD09-001) and two nickel-sensitive isolates (MD09-078 and MD09-063). The six biomarkers were expressed in all three nickel-tolerant isolates (MD06-337, MD09-045 and MD09-001), but they were not expressed in any of the nickel-sensitive isolates, as shown in Fig. 4. In the nickel-tolerant isolates, the presence of 50 μ M nickel increased the expression of the six genes by between one- and ninefold (Fig. 4).

Discussion

We have used a transcriptomic approach to characterize the expression of potential biomarkers of nickel tolerance in the ECM fungus *Pisolithus albus* from the ultramafic soils of New Caledonia. The demonstrations that 21% of all genes in *P. albus* are up-regulated in the presence of nickel and that six genes are expressed exclusively in nickel-tolerant isolates of *P. albus* together suggest the presence of a molecular transcriptomic adaptive response to nickel-rich environments in this species.

Transcriptomic database of nickel-tolerant *Pisolithus albus*

In previous studies, genes that are regulated by heavy metals in ECM fungi have been identified by

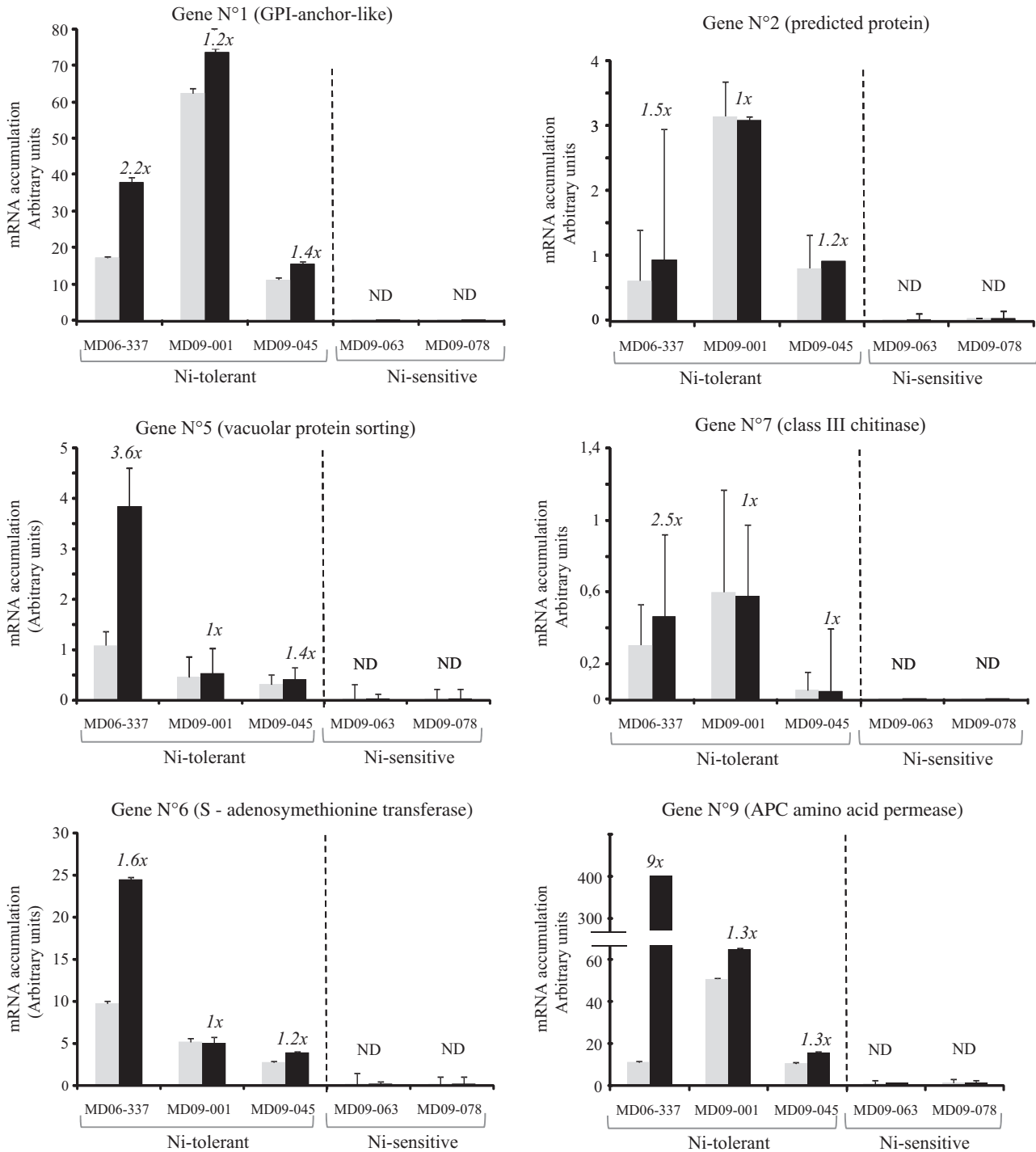


Fig. 4 Comparison of mRNA accumulation profiles for the six selected up-regulated genes in five *P. albus* isolates from ultramafic soil in the presence of nickel 50 μ M (black columns) and in the absence of nickel (grey columns). Three nickel-tolerant isolates (MD06-337, MD09-045 and MD07-001) and two nickel-sensitive isolates (MD09-078 and MD09-063) were compared. Transcript accumulation was quantified by qPCR using $2^{-\Delta\Delta CT}$ method with normalization to two reference genes, glyceraldehyde-3-phosphate dehydrogenase and EF4 α , and is expressed as arbitrary units. The data indicate mean values \pm SD values, calculated from three technical replicates with triplicate biological samples. The fold induction by nickel is presented above the black columns in italics. ND: mRNA nondetected (Ct values >37).

transcriptomic approaches, such as DNA microarray or cDNA-AFLP (amplified fragment length polymorphism) analysis (Jacob *et al.* 2004; Muller *et al.* 2007a). These studies were conducted mostly on ECM samples isolated from soils polluted with heavy metals as a result of human activities (Jacob *et al.* 2004; Muller *et al.* 2007b; Ruytinx *et al.* 2011). Here, we report a transcriptome database, generated using pyrosequencing, for an isolate (MD06-337) of the ECM fungus *P. albus*, which was isolated from extreme soils that naturally contain high concentrations of heavy metals, particularly nickel, and is tolerant of nickel. To the best of our knowledge, this is the first repository of its kind.

We also characterized molecular biomarkers that were associated with tolerance to nickel in *P. albus*. The reference database that we generated for *P. albus* comprised 19 518 contigs, of which 56% were also found in the *Laccaria bicolor* database (Martin *et al.* 2008) and 77.6% in the *Pisolithus microcarpus* database (F. Martin, *P. microcarpus* database version 1.0; INRA laboratory, Nancy, France). Moreover, the differential transcriptomic analyses that we conducted showed that, after growth for 15 days in the presence of nickel, 30% of all genes in the *P. albus* genome varied in their levels of expression relative to the expression levels in the absence of nickel. Analysis of the changes in the transcriptome of *P. albus* between these two conditions showed that of the differentially expressed genes, 21% were up-regulated following exposure to nickel and 9% were down-regulated following exposure to nickel. In a previous study, it was demonstrated that environmental changes could markedly affect gene expression profiles (Gibson 2008). We also identified the genes for which expression was affected the most by exposure to nickel. Our results suggest that, at a minimum, all of the 5855 genes that showed differential expression in the nickel-tolerant *P. albus* isolate in response to nickel contribute to tolerance of the fungus to the extreme ultramafic environment in New Caledonia. The experiments that were based on GO analysis and functional genetic tools suggest also the role of these genes as (i) biomarkers of tolerance to nickel and (ii) putative adaptive mechanisms of nickel tolerance in *P. albus*. Among the sequences obtained from the pyrosequencing analysis of *P. albus* grown in the presence and absence of nickel, only 25% and 23%, respectively, were assigned to one or more GO categories. Comparison of the results showed that the global distributions of GO terms for the two nickel conditions were similar for the category of 'molecular function', but not for the categories of 'biological process' and 'cellular component'. Moreover, a large number of orphan genes were present in the two databanks, which suggests that the accuracy of the functional annotation was limited. This limited accuracy

can probably be explained by the fact there is no reference transcriptome available for *P. albus*, and there is no transcriptome available for related species under similar conditions. Indeed, our database is the first to be based on the full transcriptome from an organism isolated from an environment that naturally contains high concentrations of a heavy metal.

Most previous studies that involved comparative transcriptomic approaches have focussed initially on genes up-regulated by environmental stimuli or developmental cues (Chelaifa *et al.* 2010). In our study, we focussed on the GO distribution of the genes for which expression was up-regulated by nickel. The majority of genes up-regulated by nickel belonged to the GO category 'cellular component'. Information on the annotations of these genes is valuable for the further investigation of gene functions, cellular structures and biological processes that might be involved in the tolerance of fungi to nickel via extracellular and intracellular mechanisms, as suggested by Bellion *et al.* (2006).

Predicted function of the specific biomarkers

In previous studies involving fungi, many genes involved in the response to stress induced by heavy metals were found to encode proteins that function as metal transporters or metal-binding proteins (Jacob *et al.* 2004; Bellion *et al.* 2006; Bolchi *et al.* 2011). However, in the present study, most of the nickel biomarkers did not belong to classes of proteins that are typically involved in response mechanisms to stress induced by heavy metals. This suggests that the mechanisms that underlie the tolerance to nickel in *P. albus* from ultramafic soils differ from those of other fungi.

Two of the biomarkers identified, namely levels of mRNA transcripts that encode chitinase-like and glycosylphosphatidylinositol (GPI) cell-wall structural proteins, are involved in extracellular processes and encode putative cell-wall proteins. Recently, it was suggested that modifications of structural elements of the cell wall, such as the rearrangement of chitin and biosynthesis of glucan- or galactosamine-containing polymers, might play a key role in modulating the integrity of the cell wall and its capacity to immobilize heavy metals. In this way, such modifications could confer tolerance to metals and affect the ability of fungi to survive in stressful environments (Meharg 2003; Bellion *et al.* 2006; Fuchs & Mylonakis 2009). Our findings regarding the expression of genes that are involved in the rearrangement of the cell wall in the presence of nickel suggest that these genes play a substantial role in modifying the chemical composition and hence the integrity of the cell wall in *P. albus*. This provides an effective barrier that can chelate the heavy metal.

The other biomarkers that were up-regulated in the presence of nickel were predicted to function in intracellular mechanisms such as resistance to oxidative stress, transcriptional regulation and subcellular compartmentalization. One biomarker encodes a putative S-adenosylmethionine-methyl (SAM) transferase. In the fungus *Podospira anserina*, overexpression of SAM transferase increases resistance to metal-induced oxidative stress by counteracting the pro-oxidant effect of phenolic compounds in the presence of heavy metal. This overexpression of SAM transferase leads to a decrease in the formation of reactive oxygen species (ROS) (Kunstmann & Osiewicz 2009), which are known to cause irreversible and lethal oxidative damage to cells (Gill & Tuteja 2010). Our results in *P. albus* suggest that SAM transferase is also overexpressed in the presence of nickel and thus might increase fungal resistance to metal-dependent oxidative stress by methylating phenolic compounds, which are known to accumulate in *P. albus*.

Another biomarker encodes a basic leucine zipper (bZIP) transcription factor. Previous studies on *Saccharomyces cerevisiae* have characterized AP-1 as a master regulator that orchestrates responses to metal/metalloid stress (Wysocki & Tamas 2010). Another study showed that a transcription factor related to AP-1-like (a member of the bZIP family) is up-regulated in the fungus *Paxillus involutus* upon exposure to cadmium. This suggests that Zip1-like proteins could play a critical role in the transcription of genes that are essential for protection against cadmium stress (Jacob *et al.* 2004). The overexpression of a bZip transcription factor in *P. albus* in the presence of nickel suggests that members of the bZIP family might act as regulators of genes involved in tolerance of metals.

Another of the selected biomarkers encodes a putative vacuole targeting-related protein. A previous study on vacuolar-defective mutants of *S. cerevisiae* confirmed the essential role that the vacuole plays in the detoxification of metals as such as nickel, zinc, cobalt and manganese. It was hypothesized that compartmentalization of these toxic metals in the vacuole prevents them from harming the cell (Ramsay & Gadd 1997). In the present study, the overexpression of a vacuole targeting-related protein in the presence of nickel suggests that the vacuole may play a key role in metal detoxification in *P. albus*.

Regarding the other biomarkers, no known function related to nickel tolerance could be identified. Taken together, the results for predicted protein function agree with the results of the GO analyses, in which the genes that were up-regulated by nickel were classified into the established GO categories 'cellular components', 'molecular function' and 'biological processes'.

Characterization of biomarker expression in *Pisolithus albus*

Comparison of the levels of expression of the 10 genes that were up-regulated most strongly in response to nickel (Fig. 3) revealed marked differences between the nickel-tolerant and nickel-sensitive phenotypes of different *P. albus* isolates from ultramafic soils. It also enabled us to identify five genes that were expressed exclusively in nickel-tolerant isolates. The remaining five genes were not selected as candidate biomarkers because they were expressed in both phenotypes. However, these genes might also be of value in predicting nickel tolerance because their level of expression was higher in the nickel-tolerant isolate than in the nickel-sensitive one, and their induction by nickel was observed only in the nickel-tolerant isolate. To strengthen the robustness of the predictive value of these final five biomarkers, levels of the appropriate transcripts were analysed in additional nickel-tolerant and nickel-sensitive isolates of *P. albus*. The data (Fig. 4) confirmed that these five final selected biomarkers of *P. albus* are suitable for identifying nickel-tolerant fungal isolates.

Given that ECM symbioses are known to play a major role in the fitness of plants in the presence of heavy metals (Jentschke & Godbold 2000), these biomarkers should also be analysed in the context of symbiotic interactions between ECM fungi and plants. Preliminary results from *in vitro* assays of nickel-tolerant *P. albus* (MD06-337) and nickel-sensitive *P. albus* (MD09-078) in symbioses with two members of the *Myrtaceae*, the model species *Eucalyptus globulus* and *Tristaniopsis* sp., which is endemic to New Caledonia, show that these biomarkers are expressed exclusively in nickel-tolerant ECM symbioses (data not shown). This confirms their suitability for monitoring the adaptation of ECM symbioses to nickel both *in vitro* and *in situ*.

Such candidate gene approaches, which involve the identification of individual genes linked to nickel tolerance, provide a starting point for investigating the molecular basis of adaptation to heavy metal content in soils and contribute to understanding tolerance for ultramafic soils in natural fungal populations (Brady *et al.* 2005). Interestingly, for each gene, the level of expression that was induced by nickel varied among the isolates tested. This intraspecific variation in transcript profiles was also observed in cadmium-tolerant *Suillus luteus* isolates, which suggests that such variation helps fungal species to persist in novel environments (Ruytinx *et al.* 2011). Interestingly, for each gene, the level of expression that was induced by nickel varied among the isolates tested, for both nickel-tolerant and nickel-sensitive isolates of *P. albus*.

The presence of both nickel-tolerant and nickel-sensitive fungal phenotypes in the same ultramafic habitat could be explained by variation in the effective concentration of nickel in such soils. Recent studies have shown that the ecotoxicological risk of nickel to organisms in ultramafic soils depends on its availability and that nickel shows local concentration gradients and variation in metal mobility (Becquer *et al.* 2006; Echevarria *et al.* 2006; Wright & Stanton 2011). Edaphic variation could have induced a variety of responses to cope with nickel toxicity in ultramafic soils (Branco 2009). Alternatively, elevated levels of magnesium in the soils could explain the presence of the nickel-sensitive isolates. Indeed, high concentrations of magnesium were found exclusively in the soils at the sites where the two sensitive isolates MD09-078 and MD09-063 were sampled (Table S3, Supporting information). Previous studies have shown that in yeast, the magnesium transporter allows low-affinity uptake of other divalent cations such as nickel, manganese, zinc and cobalt and that the *GAI* mutant of the yeast *Schizosaccharomyces pombe*, which is defective in the transport of magnesium, is resistant to nickel and cobalt (Sarikaya *et al.* 2006). In addition, magnesium is known to be an antagonist of nickel (Sarikaya *et al.* 2006). These observations could indicate that magnesium prevents the nickel uptake, thereby alleviating nickel toxicity in ultramafic soils and allowing the growth of fungi with a nickel-sensitive phenotype. These fungal responses to nickel could result from environment-dependent phenotypic expression or from genetic adaptation (or specialization) to local biotic and abiotic conditions (Branco 2009; Wolfe *et al.* 2009). Selection in ultramafic environments is associated with multiple constraints in addition to nickel concentration. These include pH, metal abundance, shortage of macronutrients, cation exchange capacity and water deficit. These constraints might have favoured genetic differentiation of *P. albus* between ultramafic and non-ultramafic sites as previously described (Jourand *et al.* 2010a,b). Further experiments should be carried out to assess the existence of local adaptation or phenotypic plasticity of *P. albus* to nickel in ultramafic soils. These experiments should include comparison of fitness parameters for isolates of *P. albus* that have been collected from their native soils and cultivated under different nickel conditions, and analysis of the physiological and molecular responses to nickel in the presence of the other metals that are found in ultramafic soils.

Conclusion

We have described transcriptomic differences in the molecular response to nickel between nickel-tolerant and nickel-sensitive isolates of *P. albus* from ultramafic

soils. As a result, we have been able to identify specific biomarkers of nickel tolerance, which are expressed in nickel-tolerant isolates *in vitro* as well as *in situ* in ECM isolates. We suggest (i) that these results reflect a positive transcriptomic response of the fungus to nickel-rich environments and (ii) the presence of both nickel-tolerant and nickel-sensitive fungal phenotypes in ultramafic soils might reflect environment-dependent phenotypic responses to variations in the effective concentrations of nickel in heterogeneous ultramafic habitats. In addition, the results of our study suggest new directions for genetic research in terms of the selection of novel isolates that are tolerant to nickel or various other ultramafic conditions such as elevated levels of chromium, cobalt, or manganese, a low calcium:magnesium ratio, or low levels of essential macro-/micronutrients and drought stress.

Acknowledgements

This work was financed by the GIP CNRT 'Nickel and its Environment' [grant number GIPCNRT98]. We wish to thank Dr Francis Martin for help with the *Pisolithus microcarpus* database. We also thank Mr Pierrick Gailhbaud and Mr Antoine Leveau of Koniambo Nickel Society (KNS), Vavouto, Koné, New Caledonia, for their help. The qPCR and sequencing were carried out at the Regional Genomic Core Research Facilities for Life Science in New Caledonia (Plate-Forme du Vivant de Nouvelle-Calédonie: PFV-NC).

References

- Addinsoft (2005) XLSTAT software version 7.5.2. Addinsoft. <http://www.xlstat.com>.
- Becquer T, Quantin C, Rotte-Capet S, Ghanbaja J, Mustin C, Herbillion AJ (2006) Sources of trace metals in Ferralsols in New Caledonia. *European Journal of Soil Science*, **57**, 200–213.
- Bellion M, Courbot M, Jacob C, Blaudez D, Chalot M (2006) Extracellular and cellular mechanisms sustaining metal tolerance in ectomycorrhizal fungi. *FEMS Microbiology Letter*, **254**, 173–181.
- Blaudez D, Botton B, Chalot M (2000a) Cadmium uptake and subcellular compartmentation in the ectomycorrhizal fungus *Paxillus involutus*. *Microbiology*, **146**, 1109–1117.
- Blaudez D, Jacob C, Turnau K *et al.* (2000b) Differential responses of ectomycorrhizal fungi to heavy metals *in vitro*. *Mycological Research*, **104**, 1366–1371.
- Bolchi A, Ruotolo R, Marchini G *et al.* (2011) Genome-wide inventory of metal homeostasis-related gene products including a functional phytochelatin synthase in the hypogeous mycorrhizal fungus *Bolchi melanosporum*. *Fungal Genetics and Biology*, **48**, 573–584.
- Brady K.U., Kruckeberg A.R., Bradshaw H.D. Jr (2005) Evolutionary ecology of plant adaptation to serpentine soils. *Annual Review of Ecology, Evolution, and Systematics*, **36**, 243–266.
- Branco S. (2009) Are oaks locally adapted to serpentine soils. *Northeast Naturalist*, **16**(sp5), 329–340.

- Branco S, Ree RH (2010) Serpentine soils do not limit Mycorrhizal fungal diversity. *PLoS One*, **5**, e11757.
- Brooks RR (1987) *Serpentine and its Vegetation: A Multidisciplinary Approach*. Dioscorides Press, Portland, OR.
- Chelaifa H, Mahé F, Ainouche M (2010) Transcriptome divergence between the hexaploid salt-marsh sister species *Spartina maritima* and *Spartina alterniflora* (Poaceae). *Molecular Ecology*, **19**, 2050–2063.
- Colpaert J, Wevers J, Krznanic E, Adriaensen K (2011) How metal-tolerant ecotypes of ectomycorrhizal fungi protect plants from heavy metal pollution. *Annals of Forest Science*, **68**, 17–24.
- Echevarria G, Massoura S, Sterckeman T, Becquer T, Schwartz C, Morel JL (2006) Assessment and control of the bioavailability of Ni in soils. *Environmental Toxicology and Chemistry*, **25**, 643–651.
- Fuchs BB, Mylonakis E (2009) Our paths might cross: the role of the fungal cell wall integrity pathway in stress response and cross talk with other stress response pathways. *Eukaryotic Cell*, **8**, 1616–1625.
- Gibson G (2008) The environmental contribution to gene expression profiles. *Nature Reviews Genetics*, **9**, 575–581.
- Gill SS, Tuteja N (2010) Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiology and Biochemistry*, **48**, 909–930.
- Gonçalves SC, Martins-Loução MA, Freitas H (2009) Evidence of adaptive tolerance to nickel in isolates of *Cenococcum geophilum* from serpentine soils. *Mycorrhiza*, **19**, 221–230.
- Götz S, García-Gómez JM, Terol J *et al.* (2008) High-throughput functional annotation and data mining with the Blast2GO suite. *Nucleic Acids Research*, **36**, 3420–3435.
- Hartley J, Cairney JWG, Meharg AA (1997) Do ectomycorrhizal fungi exhibit adaptive tolerance to potentially toxic metals in the environment? *Plant and Soil*, **189**, 303–319.
- Jacob C, Courbot M, Martin F, Brun A, Chalot M (2004) Transcriptomic responses to cadmium in the ectomycorrhizal fungus *Paxillus involutus*. *FEBS Letters*, **576**, 423–427.
- Jentschke G, Godbold DL (2000) Metal toxicity and ectomycorrhizas. *Physiologia Plantarum*, **109**, 107–116.
- Jourand P, Ducouso M, Loulergue-Majorel C *et al.* (2010a) Ultramafic soils from New Caledonia structure *Pisolithus albus* in ecotype. *FEMS Microbiology Ecology*, **72**, 238–249.
- Jourand P, Ducouso M, Reid R *et al.* (2010b) Nickel-tolerant ectomycorrhizal *Pisolithus albus* ultramafic ecotype isolated from nickel mines in New Caledonia strongly enhance growth of the host plant *Eucalyptus globulus* at toxic nickel concentrations. *Tree Physiology*, **30**, 1311–1319.
- Kazakou E, Dimitrakopoulos PG, Baker AJ, Reeves RD, Troumbis AY (2008) Hypotheses, mechanisms and trade-offs of tolerance and adaptation to serpentine soils: from species to ecosystem level. *Biological Reviews*, **83**, 495–508.
- Kunstmann B, Osiewacz HD (2009) The S-adenosylmethionine dependent O-methyltransferase PaMTH1: a longevity assurance factor protecting *Podospora anserina* against oxidative stress. *Aging*, **1**, 328–334.
- Lanfranco L (2007) The fine-tuning of heavy metals in mycorrhizal fungi. *New Phytologist*, **174**, 3–6.
- Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods*, **25**, 402–408.
- Martin F, Díez J, Dell B, Delaruelle C (2002) Phylogeography of the ectomycorrhizal *Pisolithus* species as inferred from nuclear ribosomal DNA ITS sequences. *New Phytologist*, **153**, 345–357.
- Martin F, Aerts A, Ahrén D *et al.* (2008) The genome of *Laccaria bicolor* provides insights into mycorrhizal symbiosis. *Nature*, **452**, 88–92.
- Marx DH (1977) Tree host range and world distribution of the ectomycorrhizal fungus *Pisolithus tinctorius*. *Canadian Journal of Microbiology*, **23**, 217–223.
- Meharg AA (2003) The mechanistic basis of interactions between mycorrhizal associations and toxic metal cations. *Mycological Research*, **107**, 1253–1265.
- Mortazavi A, Williams BA, McCue K, Schaeffer L, Wold B (2008) Mapping and quantifying mammalian transcriptomes by RNA-Seq. *Nature Methods*, **5**, 621–628.
- Muller LAH, Craciun AR, Ruytinx J *et al.* (2007a) Gene expression profiling of a Zn-tolerant and a Zn-sensitive *Suillus luteus* isolate exposed to increased external zinc concentrations. *Mycorrhiza*, **17**, 571–580.
- Muller LA, Vangronsveld J, Colpaert JV (2007b) Genetic structure of *Suillus luteus* populations in heavy metal polluted and nonpolluted habitats. *Molecular Ecology*, **16**, 4728–4737.
- Myers N, Mittermeier RA, Mittermeier CG, da Fonseca GA, Kent J (2000) Biodiversity hotspots for conservation priorities. *Nature*, **403**, 853–858.
- Perrier N, Amir H, Colin F (2006) Occurrence of mycorrhizal symbioses in the metal-rich lateritic soils of the Koniombo Massif, New Caledonia. *Mycorrhiza*, **16**, 449–458.
- Pfaffl MW, Horgan GW, Dempfle L (2002) Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Research*, **30**, e36.
- Rajkumar M, Ae N, Freitas H (2009) Endophytic bacterial and their potential to enhance heavy metal phytoextraction. *Chemosphere*, **77**, 153–160.
- Ramesh G, Podila GK, Gay G, Marmeisse R, Reddy MS (2009) Different patterns of regulation for the copper and cadmium metallothioneins of the ectomycorrhizal fungus *Hebeloma cylindrosporum*. *Applied Environmental Microbiology*, **75**, 2266–2274.
- Ramsay LM, Gadd GM (1997) Mutants of *Saccharomyces cerevisiae* defective in vacuolar function confirms a role for the vacuole in toxic metal ion detoxification. *FEMS Microbiology Letters*, **152**, 293–298.
- Ruytinx J, Craciun AR, Verstraelen K, Vangronsveld J, Colpaert JV, Verbruggen N (2011) Transcriptome analysis by cDNA-AFLP of *Suillus luteus* Cd-tolerant and Cd-sensitive isolates. *Mycorrhiza*, **21**, 145–154.
- Salzman RA, Fujita T, Salzman Z, Hasegawa PM, Bressan RA (1999) An improved RNA isolation method for plant tissues containing high levels of phenolic compounds or carbohydrates. *Plant Molecular Biology Reporter*, **17**, 11–17.
- Sarikaya A, Akman G, Temizkan G (2006) Nickel resistance in fission yeast associated with the magnesium transport system. *Molecular Biotechnology*, **32**, 139–145.
- Schmid J, Müller-Hagen D, Bekel T *et al.* (2010) Transcriptome sequencing and comparative transcriptome analysis of the scleroglucan producer *Sclerotium rolfsii*. *BMC Genomics*, **11**, 329.

- Wolfe BE, Parrent JL, Koch AM *et al.* (2009) Spatial heterogeneity in mycorrhizal populations and communities: scales and mechanisms. In: *Mycorrhizas – Functional Processes and Ecological Impact* (eds Azcón-Aguilar C, Barea JM, Gianinazzi S and Gianinazzi-Pearson V), pp. 167–186. Springer-Verlag Berlin Heidelberg, Germany.
- Wright JW, Stanton ML (2011) Local adaptation in heterogeneous landscapes. In: *Serpentine: the Evolution and Ecology of a Model System* (eds Harrison S and Rajakaruna N), pp. 155–181. The University of California Press, Berkeley and Los Angeles, CA.
- Wysocki R, Tamas MJ (2010) How *Saccharomyces cerevisiae* copes with toxic metals and metalloids. *FEMS Microbiology Reviews*, **34**, 925–951.

The main research interest of the authors focus on the role of ectomycorrhizal symbiosis in plant adaptation to extreme edaphic conditions as found in ultramafic soils (deficiency in major plant nutrients, unbalanced ratio Ca/Mg and high contents in heavy metals such as Co, Cr, Mn and Ni).

Data accessibility

Internal transcribed spacer DNA sequences: EMBL accessions FR852890, FR852891, FR852892, FR852893. The trimmed pyrosequencing contig sequences (Table 2) are available in the EMBL Database under the following accession numbers: contig C10864: FR852894; contig C6406: FR852895; contig C9200: FR852896; contig C7318: FR852897; contig C10795: FR852898; contig C15327: FR852899; contig C17653: FR852900; contig C18133: FR852901; contig C10491: FR852902; contig C4552: FR852903. mRNA accumulation data: The data used for Fig. 3 are included in the figure. The data used for Fig. 4 are available in Table S4 (Supporting information).

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 primer sequences (Primer express software), amplification size and efficiency of the selected ESTs and two reference genes (GAPDH and EF4) used for qPCR analysis.

Table S2 Pyrosequencing 454 data from *P. albus* MD06-337 isolate.

Table S3 Geochemical characteristics of rhizospheric soils site: major element content.

Table S4 Comparison of mRNA accumulation profiles for the six selected up-regulated genes in five *P. albus* isolates from ultramafic soil.

Fig. S1 General geographical map describing the New Caledonian archipelago with location of ultramafic massifs (in grey) and sites where *P. albus* isolates were collected (1, 2, 3, 4 & 5).

Fig. S2 Phylogenetic synthetic relationships among representative *Pisolithus* sp., in particular *P. albus* isolates from New Caledonia included in this study, i.e. MD06-337, MD09-078, MD09-045, MD09-063 and MD09-001 and worldwide reference isolates.

Fig. S3 Functional GO terms assignment and distribution of sequences GO terms of 4211 genes nickel up regulated from the differential transcriptome of MD06-337 nickel tolerant isolate from *P. albus* with +250 μM of nickel and without nickel, among Gene Ontology (GO) biological process, molecular function and cellular component.

Fig. S4 Relative expression patterns of 10 nickel selected genes from nickel tolerant *P. albus* MD06-337 isolate with two reference genes GAPDH and EIF4a. Panel A and B show respectively, relative expression patterns of 10 nickel with 250 or 50 μM of nickel, after 15 days of growth.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting information supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.