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ORIGINAL ARTICLE

Evidence of prokaryote like protein associated with nickel resistance in higher plants: horizontal transfer of TonB-dependent receptor/protein in *Betula* genus or *de novo* mechanisms?

G Theriault[1] and KK Nkongolo[1,2]

Mechanisms of metal resistance have been reported in many plants but knowledge in woody species is scarce. The TonB-dependent receptors family (TBDTs) is a large group of proteins that facilitate the transport of molecules across the membrane of Gram-negative bacteria. Some evidence exists that TBDTs are involved in metal stress. The existence of a TonB-like mechanism in non-prokaryotes has not been established. The recent development of the *Betula papyrifera* (white birch) transcriptome has allowed the discovery of genes involved in plant adaptation to stress. The main objective of the present study was to identify novel genes associated with nickel resistance in *B. papyrifera*. Our results from next generation sequencing and RT-qPCR analyses show that genes involved in transport activities are upregulated in nickel-resistant genotypes compared with susceptible forms. Detailed analysis of gene expression and genome analysis shows for the first time the existence of a TonB-dependent receptor and TonB-like family protein in non-prokaryotes. In addition, we have found that these proteins are associated with nickel resistance in *B. papyrifera*. Our experiments suggest that the TonB-dependent receptor may be exclusive to the *Betula* genus, suggesting that *Betula* species may have acquired the gene via horizontal gene transfer from prokaryotes or fungi.
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INTRODUCTION

Metals are essential for proper homeostasis of all living organisms. They are involved in the stability of the tertiary structure of enzymes and are used as cofactors for enzymatic activities (Andreini *et al.*, 2008). If metal balance is disrupted, it may lead to deficiency or toxicity, often associated with oxidative stress. The production of reactive oxygen species usually results in deregulation of protein activity, organelle damage, membrane deterioration and nucleic acid damage (Yadav, 2010). Plants are often exposed to metals due to expansion of industries and their pollutants. For example, the Greater Sudbury Region in Northern Ontario, Canada is home to one of the largest nickel/copper deposits and extractive operations in the world. A decade of mining and smelting operations has left the surrounding land acidified and contaminated with high levels of metals (Hutchinson and Whitby, 1977; Freedman and Hutchinson, 1980; Gratton *et al.*, 2000). Years of exposure to toxic levels have led to development of metal-resistant plant population (Kirkey *et al.*, 2012).

Mechanisms of metal resistance have been reported in many plant species, but knowledge of how woody plants deal with metals is scarce (Erãnen *et al.*, 2009; Maron *et al.* 2013; Visioli *et al.* 2014). Resistance mechanisms differ from species to species and sometimes within the same species. Root exudation is one of the most commonly studied mechanisms of metal resistance. Plants using this strategy will lower the bioavailability of metals around the rhizosphere by secreting organic acids such as citrate and malate from their roots (Qin *et al.*, 2007; Ryan *et al.*, 2009; Maron *et al.*, 2013). Other plants species increase production of chelating molecules such as phytochelatins, metallothioneins and small molecules from metabolic processes to cope with metal contamination (Freeman *et al.*, 2005; Mari *et al.*, 2006; Picault *et al.*, 2006; Callahan *et al.*, 2008). These compounds chelate excess metals in cells to reduce toxicity. Some plant groups accumulate metals in their tissues. Most notable are hyperaccumulators that compartmentalize metals mostly in cell vacuoles to decrease their toxic effects elsewhere in the cells (Schaaf *et al.*, 2006; Merlot *et al.*, 2014).

De Silva *et al.* (2012) results showed that even a small amount of nickel at the bioavailable levels in the Greater Sudbury Region cause a decrease of stomatal density and chlorophyll content in *Acer rubrum*. They further showed that these effects are amplified when metal contaminations are combined with drought leading to reductions in hydraulic conductance, xylem-specific conductivity and leaf-specific conductivity.

Several studies have demonstrated that transporters play an important role in metal resistance in plants. In the hyperaccumulator *Psychotria gabriellae*, the overexpression of the PgIREG1 transporter leads to accumulation of nickel in the tonoplast (Merlot *et al.*, 2014). A similar mechanism has been reported in *Arabidopsis thaliana* where nickel is transported into the tonoplast via a IREG2/FPN transporter (Schaaf *et al.*, 2006; Nishida *et al.*, 2011). Transporters from the NRAMP family have been thought to play a role in nickel resistance (Wei *et al.*, 2009). This has been confirmed in a recent study showing

[1]Biomolecular Science Program, Laurentian University, Sudbury, Ontario, Canada and [2]Department of Biology, Laurentian University, Sudbury, Ontario, Canada
Correspondence: Dr KK Nkongolo, Biomolecular Science Program, Laurentian University, 935 Ramsey Lake Road, Sudbury, Ontario, Canada P3E 2C6.
E-mail: knkongolo@laurentian.ca
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a strong association between NRAMP transporters with nickel accumulation and resistance in *Betula papyrifera* (Theriault *et al.*, 2016c). TonB-dependent transports (TBDTs) have been reported to play a role in metal transport in bacteria. They are outer membrane proteins that bind and transport ferric chelates, nickel complexes and other compounds such as carbohydrates and vitamin B_{12} (Noinaj *et al.*, 2010). In some cases, genes associated with microbial proteins have been transferred to eukaryotes during their evolution. Such horizontal transfers with significant functional implications have been reported in higher plants and animals (Keeling and Palmer, 2008).

The main objective of the present study was to identify novel genes associated with nickel resistance in *B. papyrifera*.

MATERIAL AND METHODS

This study is a comprehensive extension of the baseline transcriptome data described in Theriault *et al.* (2016c).

Nickel treatment

White birch (*B. papyrifera*) seeds were collected from a Laurentian University research field site located in the Greater Sudbury Region in Northern Ontario (Canada). The site has been contaminated with metals for >100 years. Details of seed germination and seedlings treatment with nickel nitrate are presented in Theriault *et al.* (2016c). Likewise, assessment of nickel toxicity is described in Theriault *et al.* (2016a, b). Gene expression in genotypes resistant and susceptible to a soil nickel concentration of 1600 mg kg^{-1} is analyzed in details in the present study.

De Novo Transcripts Assembly

Methods for extraction, RNA-seq libraries, new generation sequencing and *De Novo* Transcripts Assembly are described in Theriault *et al.* (2016c). The raw reads were mapped to Trinity assembled transcripts using bowtie (http://bowtie-bio.sourceforge.net/index.shtml), and RSEM (http://deweylab.biostat.wisc.edu/rsem) was used to quantify transcript and gene expression levels. Gene expression was calculated and expressed as reads per kilobase per million (RPKM) reads mapped. A differentially expressed gene analysis was performed between resistant and susceptible genotypes. The heatmap data describing the top 50 most expressed genes have be reported in Theriault *et al.* (2016c). The top 25 most upregulated genes based on Log2FC from the pairwise comparison were ranked in this study (Tables 1 and 2). Similar analysis was performed for the top 25 most downregulated genes. Baseline filtering of genes that likely are the effect of nitrate was conducted in order to make sure the selected candidate genes responded to nickel and not to nitrate (Theriault *et al.*, 2016c).

Validation of the expression of the TonB-dependent receptor using RT-qPCR

RT-qPCR was used to verify the transcriptome data. The RNA was treated with DNase1 (#EN0521) from Life Technologies (Carlsbad, CA, USA). PCR primers were designed using the transcriptome sequence. The cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit by Life Technologies.

Table 1 Top 10 most upregulated genes in nickel-resistant white birch (*Betula papyrifera*) compared with susceptible genotypes

Transcript ID	*FPKM*				*Log2FC*	*adj.P.Val*	*Description*
	Res 1	*Res 2*	*Sus 1*	*Sus 2*			
TR99219lc0_g1	88.837	261.961	0	0.54	11.54	1.97E-07	TonB-dependent receptor
TR91600lc2_g5	209.847	415.208	0	0	10.50	1.92E-18	TonB family protein
TR55738lc2_g6	49.072	24.947	0	0.048	10.14	3.40E-09	F-box/LRR-repeat protein
TR117076lc0_g1	75.234	281.904	0	0	10.02	3.56E-16	Calnexin
TR96264lc0_g1	2.728	3.487	0	0	9.46	4.57E-14	4-Hydroxyphenylacetate 3-monooxygenase oxygenase component
TR78530lc1_g1	44.645	71.609	0.115	0.072	9.44	3.09E-05	PREDICTED: protein PHLOEM PROTEIN 2-LIKE A1-like
TR86018lc0_g2	42.155	64.06	0	0	9.34	1.53E-13	Proline-rich protein PRP1
TR67414lc0_g5	48.434	40.922	0	0	8.90	5.98E-12	Protein induced upon tuberization
TR109068lc1_g1	10.829	25.767	0	0	8.88	1.27E-11	Putative transmembrane protein
TR100502lc2_g1	5.78	18.454	0	0	8.83	2.34E-11	NA

Abbreviations: adj.P.Val, adjusted *P*-values; FPKM, fragments per kilobase of transcript per million mapped reads; Log 2FC, fold change from log2.

Table 2 Top 10 most downregulated (fold change) genes in nickel-resistant white birch (*Betula papyrifera*) compared with susceptible genotypes

Transcript ID	*FPKM*				*Log2FC*	*adj.P.Val*	*Description*
	Res 1	*Res 2*	*Sus 1*	*Sus 2*			
TR16661lc0_g1	0	0	14.611	14.912	−11.94	2.78E-20	A protein
TR94889lc2_g1	0	0.028	232.706	100.675	−11.65	1.14E-14	mRNA cap guanine-N7 methyltransferase 2 isoform X2
TR53148lc0_g1	0	0	14.082	13.831	−10.85	3.07E-16	Replication-associated protein A
TR55969lc0_g1	0	0	26.541	55.026	−9.87	7.29E-13	Ankyrin repeat family protein
TR94980lc2_g4	0	0	65.692	57.535	−9.50	9.80E-12	Serine/threonine-protein kinase
TR61028lc0_g1	0	0	54.682	175.439	−9.19	1.07E-10	NA
TR56209lc7_g8	0	0	114.437	89.869	−9.14	1.56E-10	Putative disease resistance protein RGA3
TR35375lc2_g19	0	0	110.296	66.672	−8.93	6.51E-10	Chaperone protein dnaJ 8, chloroplastic isoform X2
TR79073lc0_g1	0	0	132.672	1.609	−8.90	8.14E-10	Putative ribonuclease H protein At1g65750-like
TR35437lc1_g1	0	0	44.098	88.2	−8.90	7.95E-10	NA

Abbreviations: adj.P.Val, adjusted *P*-values; FPKM, Fragments Per Kilobase of transcript per Million mapped reads; Log 2FC, Fold change from log2.

RT-qPCR was performed using the Dynamo HS SYBR Green qPCR Kit by Life Technologies according to the manufacturer's protocol. Each sample was amplified with the MJ Research PTC-200 Thermal Cycler in triplicates. The process included (1) initial denaturing at 95 °C for 15 min; (2) denaturing at 94 °C for 30 s; (3) 30 s annealing; (4) elongation at 72 °C for 30 s; (5) read; (6) repeat step 2–6 for 41 cycles; (7) final elongation at 72 °C for 7 min; (8) melting curve 72–95 °C, every 1 °C, hold for 10 s; and (9) final elongation at 72 °C for 3 min. The qPCR was run three separate times with each sample in triplicate, resulting in a total of nine data point for each sample. The data were analyzed using the MJ Opticon Monitor 3.1 by BioRad and delta $C(t)$ values were exported to Microsoft Excel. RNA concentrations were calculated using Delta $C(t)$ values and standard curves. Expression was first normalized against α-tubulin (housekeeping gene) then the relative expression was calculated by dividing the expression of resistant/susceptible genotypes over water controls. We compared the mean expression of the TonB-dependent receptor between resistant/susceptible genotypes and water controls. Data were analyzed using SPSS 20 for Windows, with all data being $\log_{10}$ transformed to achieve a normal distribution. Variance ratio test was performed with an assumption of data normality in the underlying population distributions of the data. ANOVA, followed by Tukey's HSD multiple comparison analysis, was performed to determine significant differences among means for qPCR ($P<0.05$).

TonB-dependent receptor in other species

PCR was performed using the cDNA or DNA template from *B. papyrifera* and other species (Table 3). Each PCR reaction included a forward primer (0.1 mmol), reverse primer (0.1 mmol), MgCl (2 mmol), dNTPs (0.2 mmol), 10× buffer and Taq Polymerase. The PCR was performed using the MJ Research PTC-200 Thermal Cycler: (1) initial denaturing at 95 °C for 15 min; (2) denaturing at 94 °C for 30 s; (3) 30 s annealing; (4) elongation at 72 °C or 30 s; (5) read; (6) repeat steps 2–6 for 41 cycles; and (7) final elongation at 72 °C for 7 min. Amplification products were run on agarose gels and the fragment sizes verified using the BioRad Doc system (Hercules, CA, USA).

A basic local alignment search (BLAST) using the dwarf birch (*Betula nana*) genome (http://birchgenome.org/) was performed to determine sequence similarity (Wang *et al.*, 2013).

RESULTS

Transcriptome

Differentially expressed genes between nickel-resistant, susceptible and water controls were identified. Broad data of the heatmap has been presented in Theriault *et al.* (2016c). The top upregulated and downregulated genes were ranked based on log 2FC. The study shows that nickel treatment triggers different regulation of several genes. In-depth analysis in the present study of molecular functions of the 25 most upregulated in resistant genotypes reveals that 32% were associated with catalytic activities, 12% with transport and 8% with binding (Figure 1). Among the top 25 most downregulated genes, 20, 0 and 8% were associated with catalytic, transport and binding activities, respectively (Figure 2). Detailed description of the top 10 most upregulated and downregulated is presented in Tables 1 and 2. Three of the 10 most upregulated genes in resistant genotypes were associated with transport (TR99219|c0_g1, TR91600|c2_g5, TR109068|c1_g1 and TR78530|c1_g1), three with binding (TR55738|c2_g6, TR117076|c0_g1 and TR96264|c0_g1), and four have unknown molecular function (TR86018|c0_g2, TR67414|c0_g5, and TR100502|c2_g1; Table 1). For downregulated genes, six were involved in binding (TR94889|c2_g1, TR53148|c0_g1, TR94980|c2_g4, TR56209|c7_g8, TR35375|c2_g19 and TR79073|c0_g1) and the other four (TR16661|c0_g1, TR55969|c0_g1, TR61028|c0_g1 and TR35437|c1_g1) had unknown molecular functions (Table 2).

The transcriptome assembly of *B. papyrifera* revealed that a TonB-dependent receptor and a TonB-like family protein transcript were the two most upregulated genes in resistant genotypes (RG) compared with susceptible (SG; Table 1). Their gene IDs in the *B. papyrifera* transcriptome are TR99219|c0_g1 and TR91600|c2_g5, respectively. This transcriptome has been deposited at DDBJ/EMBL/GenBank under the accession GEIC00000000 (Theriault *et al.*, 2016c). The sequence length of the TonB-dependent receptor and the TonB-like family protein transcript were 1604 and 258 bp, respectively (Figures 3 and 4).

The mean expression values for water controls, resistant and susceptible genotypes were significantly different based on ANOVA followed by the Tukey's HSD tests ($P<0.05$). In fact, the expressions of TonB-dependent receptor and TonB-like family protein transcripts were expressed 2978× and 1448× higher in RG compared with SG. We found also that the expression of TonB-dependent receptor transcript was 1082× higher in the RG compared with controls. There was no significant difference in the expression of the TonB-

Table 3 List of tree species tested for the presence of the TonB-dependent receptor sequence

Species	*Provenance*	*TonB receptor sequence*
Betula papyrifera	Daisy Lake, Sudbury, ON, CAN	Present
	Kingsway, Sudbury, ON, CAN	Present
	Skead, ON, CAN	Present
	Onaping, ON, CAN	Present
	Wahnapitae, ON, CAN	Present
	Azilda, ON, CAN	Present
	Capreol, ON, CAN	Present
	Gallants, NL, CAN	Present
	Prosser Brook, NB, CAN	Present
Betula alleghaniensis	Warren Lake NS, CAN	Present
	Sherbrooke QC, CAN	Present
	Richmond, PE CAN	Present
	Pembroke, ON, CAN	Present
Betula nana	Dundreggan, SCT	Present
Betula minor	Bay d'Espoir, NL, CAN	Present
Betula cordifolia	Ravine Big Gulch, CAN	Present
	St. Georges NL, CAN	Present
	Hawkes Bay, NL, CAN	Present
B. lenta	St Catharines, ON, CAN	Present
B. occidentalis	Adams Lake, BC, CAN	Present
B. populifera	Little Lake, NB, CAN	Present
	Foxley River, PE, CAN	Present
	Afton Road, PE, CAN	Present
Quercus rubra	Sudbury, ON, CAN	Absent
Acer rubrum	Sudbury, ON, CAN	Absent
Populus tremuloides	Sudbury, ON, CAN	Absent
Pinus strobus	ON, CAN	Absent
Pinus monticola	BC, CAN	Absent
Pinus nigra	Halle#50H Seed Orch, BEL	Absent
Pinus sylvestris	Hallestad District, SWE	Absent
Pinus contorta	CAN	Absent
Pinus banksiana	CAN	Absent
Pinus resinosa	CAN	Absent
Picea glauca	Sudbury, ON, CAN	Absent
Picea sitchensis	Cedarvale, BC, CAN	Absent
Picea wilsonii	A-PA-Tibetan, Sichuan, CHN	Absent
Picea jezoensis	Hokkaido Prefecture, JPN	Absent
Picea orientalis	Unknown	Absent
Picea pungens	Santa Fe, NM, USA	Absent

Abbreviations: BEL, Belgium; CAN, Canada; CHN, China; JPN, Japan; SCT, Scotland; SWE, Sweden; USA, United States of America.

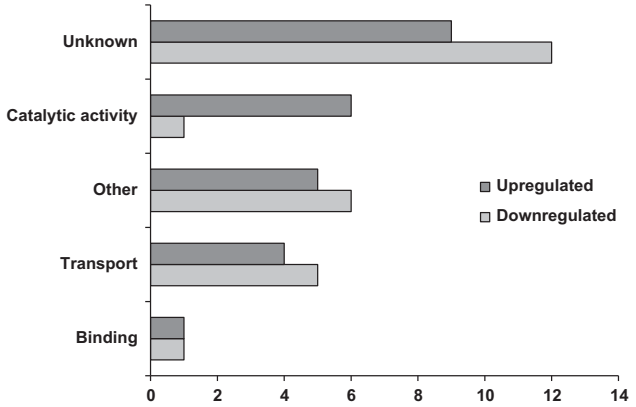

Figure 1 Top 25 upregulated and 25 downregulated transcripts when nickel-resistant *Betula papyrifera* genotypes were compared with control (nickel untreated genotypes). Transcripts were assigned gene ontology and grouped by molecular function using BLAST2GO.

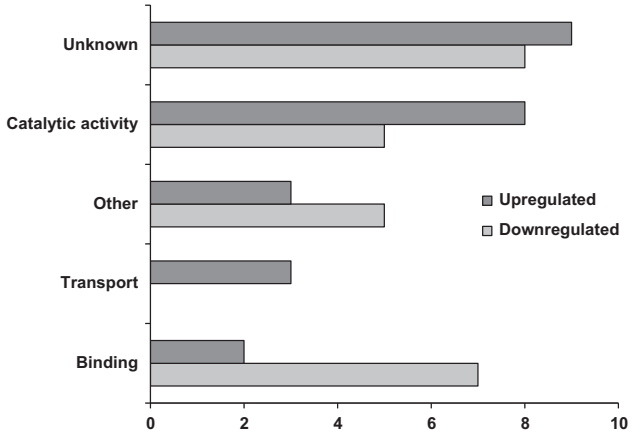

Figure 2 Top 25 upregulated and 25 downregulated transcripts when nickel-resistant *Betula papyrifera* genotypes were compared with nickel susceptible genotype. Transcripts were assigned gene ontology and grouped by molecular function using BLAST2GO.

dependent receptor transcript when the water control was compared with SG. But the expression of TonB-like family protein transcript was 2048× higher in the water controls compared with Ni-treated susceptible genotypes. No significant difference was found in the expression of the TonB-like family protein transcript when RG were compared with water controls.

RT-qPCR analysis

Overall, qPCR data showed significant differences among genotypes based on Tukey's HSD multiple comparison analysis ($P<0.05$). The expression of the TonB-dependent receptor was significantly higher in RG compared with SG or water controls (Figure 5).

TonB-dependent receptor/-like protein in other species

Betula nana database revealed the presence of TonB-dependent receptor and TonB-like family protein. We performed a BLAST alignment of the *B. papyrifera* transcripts with the *Betula nana* (dwarf birch) genome (Supplementary Figures S1 and S2). The sequence similarity between both birch species for the TonB-dependent receptor and TonB-like family protein was 93 and 83%, respectively. No match was found in plant species when using the NCBI BLAST tool (Supplementary Figures S3 and S4). BLAST was also performed in additional databases (DDBJ/EMBL/GenBank, Poplar genome database, dendrome, Plantgdb, etc.) and no match in plants species was found outside the *Betula* genus. We performed a search in the UniProt database and it revealed the presence of TonB-dependent receptors/proteins in a number of bacteria and fungi species. We found that almost 100% of the 183 372 TonB protein matches were from bacterial and fungal species (Figure 6).

Primers were designed to confirm the presence of the gene in the *B. papyrifera* genome and transcriptome. PCR amplification using root and leaf cDNA revealed a single band of around 300 bp for the TonB-dependent family receptor (Figure 7a). Several *B. papyrifera* populations were screened and the same size band was observed. The presence of the gene was also confirmed in yellow birch (*B. alleghaniensis*) and other *Betula* species (Figure 7a). Figure 7b shows the absence of the band in other tree species. A complete list of all the species screened for the TonB-dependent receptor is presented in Table 3.

DISCUSSION

Recent general analysis of *B. papyrifera* transcriptome generated through shotgun methods revealed that the main mechanism involved in *B. papyrifera* resistance to nickel is downregulation of genes during translation (Theriault *et al.*, 2016a, b). In this study, we show that actually genes involved in transport activities are upregulated in resistant genotypes compared with susceptible forms. Those few genes are of particular importance in nickel resistance in many organisms.

TonB-dependent receptors and TonB protein family in prokaryotes

The TonB-dependent transporter (TBDT) family is a large group of proteins that facilitate the transport of molecules across the membrane of Gram-negative bacteria. To date, they have never been reported in plants. Their tertiary structure is a 22-stranded beta-barrel located in the outer membrane with a plug domain folded inside the barrel (Noinaj *et al.*, 2010). TBDTs are more commonly known for their role in iron regulation but have been associated with transport of vitamins, nickel and carbohydrates (Schauer *et al.*, 2008). In order to pass through the pore, two requirements must be met: the metal must be chelated to a siderophore and the inner membrane must provide energy. Transport through TBDT is dependent on the inner membrane protein complex TonB, ExbB and ExbD (Postle, 2007). The plug domain of TBDTs interacts with TonB to transfer energy via a proton motive force leading to the opening of the pore and passage of the sidosphore complex (Postle, 2007).

Some evidence exists that TBDTs are dealing with metal stress. In some species, TBDTs are repressed during heavy metal stress (Yoneyama and Nakae, 1996; Park and Ely, 2008) while in others, there is an induction (Hu *et al.*, 2005; Brown *et al.*, 2006). Hu *et al.* (2005) found that TBDTs were upregulated during heavy metal stress. However, their role in response to metal was unclear since TonB was downregulated (Hu *et al.*, 2005). Increase in TBDT expression has been linked to metal resistance. High expression of opdT and OmpC porins have been associated with copper resistance in *Pseudomonas aeruginosa* and *Escherichia coli* (Egler *et al.*, 2005; Teitzel *et al.*, 2006).

TonB-like mechanism in plants

The existence of a TonB-like mechanism other than in prokaryotes has not been established. Only the TonB box, a consensus sequence in bacterial TBDTs, has been found in plant transporters and G proteins (Assmann, 2002; Duy *et al.*, 2011). The role of these elements in eukaryotes is unknown. Plants, like bacteria and fungi, produce

siderophores. In iron-deficient plants, siderophores with a high affinity for iron (III) are secreted from the roots via a transporter (Walker and Connolly, 2008). The siderophores will chelate non-soluble iron (III) and so form an iron-siderophore complex (Walker and Connolly, 2008). The complex is then carried back into the plant's root system via a secondary transporter (Walker and Connolly, 2008). The presence of TonB-binding boxes and siderophore production in plants suggests that a mechanism involving a TonB receptor in plants is possible. Here we present the first data showing the presence of a TBDT in plants and its association with nickel tolerance in *B. papyrifera*.

Validating the presence of TonB-dependent receptor and TonB-like family protein

Expression of the TonB-dependent receptor transcript in resistant plants was much higher than susceptible genotypes and water control. Similar results were obtained by the confirmatory qPCR. This suggests that the expression of the TonB-dependent receptor is induced by nickel. The expression of the TonB-like family protein transcript was also significantly higher in resistant genotypes and water controls

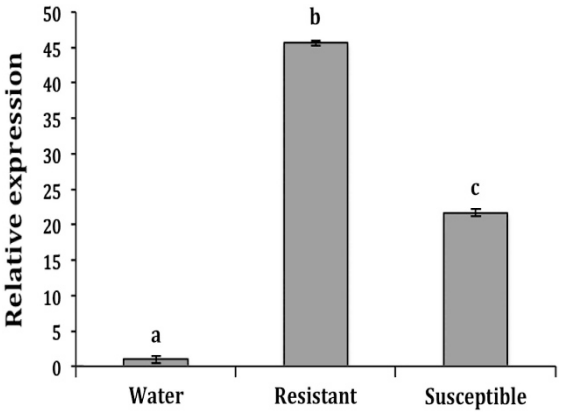

Figure 5 Quantitative qPCR measurement of the expression of TonB-dependent family receptor (TR99219lc0_g1) in nickel resistant and susceptible white birch (*Betula papyrifera*) treated with 1600 mg kg^{-1} of nickel. Expression was standardized using the housekeeping gene ef1a. Normalization was then performed against water controls (all values divided by water). *Significant differences were found using ANOVA (Tukey's HSD multiple comparison analysis, $P<0.05$).

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1     TGATCTTGAT CGTCATCGTG ATCTTGATCT TGATCGTCAT CGTGATCTTG ATCTTGATCG
61    TCATCGTGAT CTTGATCTTG ATCGTCATCG TGATCGTCAT CGTCATCGTC ATCGTCATCT
121   TGATCGTGAT CGTGATCGTG ATCTTCGTTT TTCTCGTCGT TCTTACGACA TCATTTTCTT
181   TGTGCAGGAG GAGGATCCGG ATGAGGATTG GGATGGGGAT GAGGATGATG ATGTGGATGA
241   CAGTGACACT GACGACGATG GCAATTCGTG TCCGAATTGG AAGGGGTATA AAATTAGTAT
301   TCATGCGGAC GGTCCCGGCA AGGTCTCCCG AAAACCGCTC ATTCCTTTGT GGGAAGGAAA
361   AGGTCGCCAG CTCGGTTGCT TTGCAGCCCT GGGCTCCTAC ATCTACTATT TTGGTGGGGC
421   GAGCCATACT GGTTGGTTGC GAGAGGGGCT TCGCGACGGC TACAAATTTC AGGTTACTCC
481   CAGTTTCGCT AAGGAATGGT TCTCTCTTTC TCCCATGATT TGTAAGAGAG ATGAACCGTA
541   CGCTTCGGTT CTAGGCGGTA AGATATATGT TCGCAATCAT GAACCTCGAT GTCATACTGG
601   TAGTCATTGG GCTGAGGTTT TTGATCCCGC AAATGGAAAA TGGGAACCTT GCCCTAATCC
661   TCCAAATTAT GCTCCAAGAG GTGGGAAAAC TATAGTCATT TCTGCAGCTG TTGAGAATCC
721   AGACAGGATT ATCGTGGCTT ACCGTCTTGA TGATGATCAT GATGGCTCTC TTGATGATGA
781   TCATGATGAC TCTTTTGATG ATGACTATGA TGACGACTCT TATGCTACCT TCTATGCGTA
841   TAACGTGCAC TGTAGATCTT GGGATATGCT TGAGCCTGCT AAGCGCAAGC TCCACCGTAT
901   GTGTACTGAG GAGTGGGAAA CCGGTCTAAG TGTAGATTTG GATCTGTGGC TAGAAGGCTG
961   TCTAAAAGGT CTAGGAGATC TTCTTCCTCG TGGCGCACTT CCTTTCTTAT TCCATCTGGA
1021  GAAGCAGAGG TTCTGCCTCG TAACCTCTGC AGAGGATGAT TATATGTACT GTGTTATATT
1081  TGATGTTTCT CGTATGCCCG ACAAGAAGAC TTTAGCTATA TCGGTTGCGT GGGACCAACA
1141  ATATGCGATT GAACCAAGAA CACGTCGTGG ATTACCCGTT CTGTTACCAT ATTGCGCTAT
1201  ACTGCCTAAG TAGACCAAGC AAGAAGGAAG TGCAAGGAGG AAATTGGCTT AATAGATTAT
1261  AGTCTATATT AGGGGCAAGT AGATAGAGGA GAAATCAAGC AGGAAGGGTG TGCGTGGAGC
1321  AAAGAGTGTT TTTTGATGAG TGCAAAGAGA GGCTACAATG TCCAATCGTG CCAAAGGAAT
1381  ATGTTATCTT TTCTTATTTT TCTGAGATCT GGATACGTGC TTGAACTTAT GAATATTTTG
1441  GAGATCTATT ACAAAAGGTT TATATATTTT TTGTTTCATG AGCAAATTGT AATAGCGTTA
1501  GAGGTTTAAA AATTATGCAT GTTCATTTTT ATTTGACTTT GTATTTGATT TGCACTTTGC
1561  TTACATATGT TTTGAATCAA GAATCAATTT CCGTCAGACT TGAC
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Figure 3 Trinity assembled sequence of TonB-dependent family receptor (TR99219lc0_g1). Primer-binding sites for PCR verification are shaded in gray.

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1     CAGTGGCTCG GCTTCTGGTG CAGGTTCTGG CTCTGCTTCT GGGTCTGGTT CTGGTTCTGC
61    TTCTGGGTCT GGTTCAAGGT CTGATTCAGG TTCTGAAGCA GGCTCGTATG CTGGGTCTCG
121   AGCTGGGTCA GGTCCAAAGG GAAACCAAGA ACGAGGAACA GGGTCCGAGT CAGGCCAAGG
181   CCGTGGGAGT GGTTCTGGTT CAGGGTCTGG TTCTGGTTCT GGTTCTGGAC GTGGTGAGGG
241   TTCTGGTTCT GGTTCTGG
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Figure 4 Trinity assembled sequence of TonB-like family protein (TR91600lc2_g5).

compared with susceptible. However, no significant difference was found between resistant and water controls, suggesting that the transcript is constitutively expressed.

To exclude the possibility of any bacterial contamination of the roots analyzed in this study, we tested for the presence of the transcript/gene in the areal parts of the plant. Both analyses of

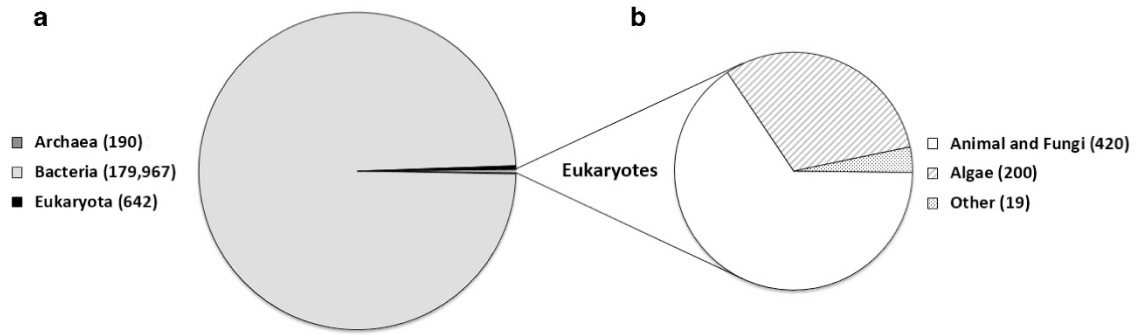

Figure 6 (**a**) Distribution of TonB hits in Uniprot database classified by group of organisms (note that 99.5% hits are from bacteria). (**b**) Distribution of TonB hits in Uniprot database for Eukaryote.

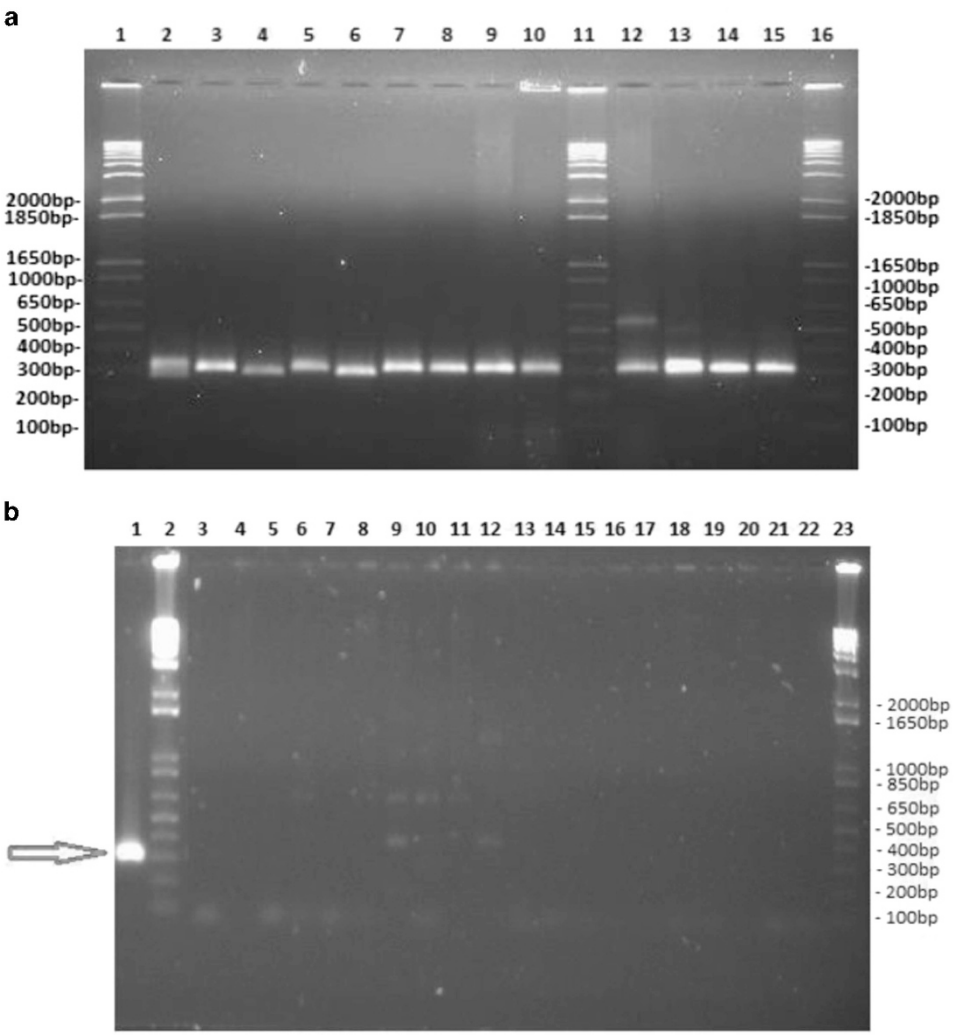

Figure 7 (**a**) PCR amplification products on 1% agarose gel. A 300 bp band corresponds to the presence of the TonB-dependent receptor. Lanes 1, 11 and 16 are loaded with 1 kb+ ladder; 2–10 with white birch (*Betula papyrifera*) from different populations (leaf DNA); 12 *Betula alleghaniensis* (seedling DNA); 13 *Betula minor* (seedling DNA); 14 and 15 white birch (root cDNA and leaf cDNA). (**b**) PCR amplification products on 1% agarose gel. A 300 bp band corresponds to the presence of the TonB-dependent receptor (red arrow). Lane 1: white birch (*Betula papyrifera*), 2 and 23 are loaded with 1 kb+ ladder, 3–22 are the following species in order: *Quercus rubra*, *Populus tremuloides*, *Acer rubrum*, *Pinus strobus*, *Pinus monticola*, *Pinus nigra*, *Pinus sylvestris*, *Pinus contorta*, *Pinus rigida*, *Pinus banksiana*, *Pinus resinosa*, *Pinus montana*, *Picea glauca*, *Picea sitchensis*, *Picea wilsonii*, *Picea jezoensis*, *Picea orientalis*, *Picea engelmannii,* and *Picea pungens* (pop. 1) *Picea pungens* (pop. 2). A full color version of this figure is available online at the *Heredity* website.

B. papyrifera leaf cDNA and genomic DNA confirmed the presence of the TonB-dependent receptor gene. While the contamination of foreign RNA is greatly diminished when using leaf tissue, bacteria can still colonize leaves. Phylobacteria are bacteria that survive on or inside plant leaves (Beattie and Lindow, 1999). Some live on the surface while others will enter the plant via lesions in the leaves or hydathodes (Beattie and Lindow, 1999). However, the fact that expression of this gene was found in both RG and SG exclude the possibility of contamination happening only in resistant plants.

Further analyses found that the TonB-dependent receptor and TonB-like family protein are also present in the *B. nana* (dwarf birch) transcriptome. The sequence alignment revealed a high degree of similarity between the *B. papyrifera* and *B. nana* genomes. This is expected since the two species (*B. papyrifera* and *B. nana*) are genetically closely related (Järvinen *et al.*, 2004; Li *et al.*, 2005). In addition, using PCR, we were able to confirm the presence of the TonB-dependent receptor in the genome of *Betula* species. We also screened several populations of *B. payrifera* from different locations in Northern Ontario and New Brunswick (Canada) and elsewhere and we confirmed the presence of the TonB-dependent receptor in all of them. When screening other species from different genera via BLAST or PCR, we found that the gene was exclusive to the *Betula* genus and was not found in any other tree species.

It should be noted that the lack of amplification with primer pairs targeting this gene in species outside the *Betula* genus could have been due to the absence of primer-binding sites or weak primer bindings. But such possibility is unlikely since the BLAST search of existing depositories did not report any match to gene for the TonB-dependent receptor or TonB-like family protein. In fact, NCBI (National Center for Biotechnology Information—USA), EMBL (European Molecular Biology Laboratory), DDP (DNA databank of Japan), Plantgdb (Resources for Plant Comparative Genomics), Dendrome (forest trees genome database), *Populus* and *B. nana* databases contain millions of sequences from different organisms but the BLAST search for the TonB-dependent receptor gene generated hits only for bacteria, fungi and *Betula* sequences.

It is possible that the gene for the TonB-dependent receptor or TonB-like family protein was transferred from bacteria to *Betula* through horizontal transfer during the evolution of the genus *Betula*. The notions of horizontal transfer between microorganisms and plants is a topic of great interest and has been discussed by many authors (Hanekamp *et al.*, 1997; Kim *et al.*, 2002; Nkongolo *et al.*, 2004; Richardson and Palmer, 2007; Yue *et al.*, 2012; Gao *et al.*, 2014).

Yue *et al.* (2012) identified 57 family of genes transferred from prokaryotes, fungi or viruses to moss *Physcomitrella patens*. Most of these genes were directly or indirectly related to plant defence and stress tolerance. Notably the glutamate–cysteine ligase gene was acquired from bacteria by *P. patens*. Glutamate–cysteine ligase is one of the two genes that catalyze the formation of glutamine. This compound is involved in plant disease resistance, photo-oxidative stress defense; and heavy metal detoxification. Interestingly, our initial analysis of *B. papyrifera* transcriptome revealed that glutathione is also upregulated in nickel-resistant genotype compared with susceptible (Theriault *et al.*, 2016c). The mechanism of possible transfer of TonB genes to *Betula* spp. is not established, but horizontal gene transfer is a widespread process involved in the evolution of multicellular eukaryotes.

CONCLUSION

This is the first study that shows and documents the existence of a TonB-dependent receptor and TonB-like family protein in plants. We found that they are associated with nickel resistance in white birch (*B. papyrifera*). A series of experiments showed that the TonB-dependent receptor could be exclusive to the *Betula* genus. This suggests that *Betula* species might have acquired the gene via a recent horizontal gene transfer from prokaryotes or fungi. Thanks to advances in next generation genome sequencing, gene depositories represent the main databases useful for identifying the presence of genes of interest in a species. Hence, it is possible that other plant genera or species whose sequences have not been deposited in a public gene bank might carry the TonB-dependent receptor and/or TonB protein family. Further studies including physical mapping of TonB-dependent receptor in *Betula* will be conducted using *in situ* hybridization. Transcriptional regulation by Ni on other plant species showing different resistance mechanisms to Ni toxicity such as red maple (*A. rubrum*), red oak (*Quercus rubra*) and trembling aspen (*Populus tremuloides*) will be also investigated.

DATA ARCHIVING

We have deposited data in the following depository: Repository/DataBank Accession: EmbL Accession ID: GEIC00000000 Databank URL: http://www.ebi.ac.uk/ena Repository/DataBank Accession: NCBI genbank Accession ID: GEIC00000000 Databank URL: http://www.ncbi.nlm.nih.gov/genbank

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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