

Molecular analysis of *Betula papyrifera* populations from a mining reclaimed region: genetic and transcriptome characterization of metal resistant and susceptible genotypes

by

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Abstract

The objectives of the present study were to; 1) determine if there's an association between plant population diversity and genetic variation in white birch (*Betula papyrifera*) populations with soil metal contamination in the Greater Sudbury region (GSR), 2) assess if metal contamination and soil liming has an effect on global DNA methylation, 3) develop and characterize the transcriptome of *B. papyrifera* under nickel stress and, 4) assess gene expression dynamics in white birch in response to nickel stress. No association between plant population diversity and genetic variation with metal contamination was found. Liming increases plant population diversity but has no effect on genetic variation in the studied white birch populations. There was a decrease in root cytosine methylation in metal-contaminated sites compared to references. Treatment with the dose corresponding to total level of Ni or Cu (1,600 mg/kg Ni, 1,312 mg/kg Cu or combined) in sites of the GSR generated different responses within segregating populations analyzed. The main Ni resistance mechanism of white birch was associated with the prevention of translocation of Ni from root to shoot. We also observed lower ZAT11 and glutathione reductase expression in resistant genotypes compared to susceptible. The transcriptome of *B. papyrifera* was developed for the first time using Next Generation Sequencing. RNA from Ni resistant, moderately-susceptible, susceptible and water controls treatment was sequenced. A total of 209,802 trinity genes were identified and were assembled to 278,264 total trinity transcripts. In total, 215,700 transcripts were annotated and compared to the published *B. nana* genome. Overall, a genomic match for 61% transcripts with the reference genome was found. Expression profiles were generated and 62,587 genes were found to be significantly differentially expressed among the nickel resistant, susceptible, and untreated libraries. The main nickel resistance mechanism in *B. papyrifera* is a downregulation of genes associated with translation and cell

growth, and upregulation in genes involved in the plasma membrane. Seven candidate genes associated to nickel resistance were identified. They include Glutathione S-transferase, Thioredoxin, Putative transmembrane protein, Nramp transporter, TonB-like family protein and TonB-like dependent receptor. This TonB receptor was found to be exclusive to the *Betula* genus.

Keywords

White birch, *Betula papyrifera*, metal contamination, genetic variation, nickel resistance, transcriptome, gene expression, restoration, methylation, metal toxicity

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Chapter 1: Literature review

1.1. Soil pollution in terrestrial ecosystems

1.1.1. Metal contamination in the Greater Sudbury region

Since 1883, the Greater Sudbury region (GSR) located in Northern Ontario has been well known for its rich deposits in nickel (Ni) and copper (Cu) ores. Controversially, it is also known as one of Canada's most ecologically disturbed regions. The discovery of the Sudbury basin led to the rapid boom of the mining industry and the establishment of many mining companies within the region (1, 2). These include, but are not limited to, VALE (previously known as INCO) and Sudbury Integrated Nickel Operations. Initially, the primary method of extracting metals from the ores was by roast beds. Large quantities of ores were piled on beds of corewood, ignited, and allowed to burn for a long period. They would melt, allowing the metals to be concentrated and extracted. Roast beds dominated from 1888 to 1929 until they were banned Canada wide because of the toxic effects they exerted on ecosystems. Due to the high quantity of sulfides in the ore deposits, large amounts of sulfur dioxide (SO₂) and metal particles were released in the atmosphere in an uncontrolled manor. This caused metal contamination and acidification of soils surrounding roast beds (1). Furthermore, the deforestation to produce corewood for the roast beds led to soil erosion and nutrient-rich soil horizons were washed away (3).

Since the 1930's, smelters slowly became the predominant way of extracting metals. Advances in technology have helped reduce the impact of the mining industry on surrounding ecosystems. For example, the construction of the "Superstack" in 1972 helped reduce localized pollution (90%) due to filtration systems in the stack. However, smelters have and were still highly contributing to SO₂ and metal contamination in the GSR. It has been estimated that since the establishment of

the mining sector in the GSR, over 100 million tonnes of SO₂ and tens of thousands of tonnes of Co, Cu, Ni and Fe have been released by roast pits and smelters (4). High levels of metals and low soil pH have been found in soils surrounding smelters and roast pits (4–6). The combination of deforestation, soil acidification, and metal pollution has greatly affected vegetation. Soils surrounding smelters were completely barren of vegetation with only a few tree species remaining, white birch being one of the main ones (7). DeLestard (1967) reported that 64km² within the city center the GSR had been rendered completely barren of vegetation, 225km² only had a few species of shrubs and herbaceous cover, and 2735km² had been affected in some way (8). Several studies in the last four decades have shown that metal contamination and plant biomass decreases and soil pH increases as you move away from smelters (2, 9). Levels of Co, Cu, Fe and Ni in soils located in the vicinity of the GSR are still not within the limits set by Ontario Ministry of Environment and Energy (OMEE) (9).

1.1.2. Impact of the mining industry on soil chemistry

Sulfur dioxide and metal emissions from smelters can disrupt soil chemistry (1, 4, 5). The decrease in pH in contaminated sites is primarily caused by the production of acid rain that forms when the SO₂ released from smelters reacts with oxygen and water to generate sulfuric acid (10, 11). Sulfate and protons are then brought down to the soil with rain. Sulfuric acid is capable of releasing up to two hydrogen ions thus, acidifying the soil. In general, soil pH and metal bioavailability are inversely correlated. Hence, lower soil pH will increase metal solubility in the soil (12, 13). Metals such as Al and Fe can further decrease soil pH by hydrolyzing water molecules. When soil pH drops below 5.5, Al solubility increases dramatically. This leads to the hydrolysis of water and the production of Al(OH)₂⁺ and Al(OH)₃. Aluminum is found in silicates and clays. When soils are barren of vegetation, erosion and weathering of these minerals

increases the amount of aluminum (14). A similar phenomenon is seen with Fe. The increase in soil pH by acid mine drainage can be both spontaneous or with the help of microorganisms (acidophiles) through several chemical mechanisms (15, 16). This makes areas with high levels of Fe and Al susceptible to low pH (17).

Two classes of nutrients exist, macro and micronutrients. Both are required for the proper plant growth and reproduction. Macronutrients include Nitrogen (N), Phosphorus (P) Potassium (K), Calcium (Ca), Sulfur (S), and Magnesium (Mg) while Chloride (Cl), Iron (Fe), Boron (B), Manganese (Mn), Zinc (Zn), Copper (Cu), Molybdenum (Mo), and Nickel (Ni) are micronutrients. Macronutrients are required in large quantities and are found in basic cell metabolisms such as protein, DNA, and RNA synthesis. Micronutrients are needed only in trace amounts and generally play specific roles (ex: enzyme cofactor). The availability of these nutrients to plants is tightly linked to the composition and pH of the soil. In general terms, the solubility of micronutrients (ex: Fe, Cu and Ni) increases when the soil pH becomes acidic. In consequence, this affects the availability of the majority of macronutrients (macronutrients are precipitated and insolubilized).

Lower levels of soluble Mg, K, and N have been reported in areas surrounding smelters (18). Low soil pH will cause these nutrients to aggregate with others to form less soluble compounds thus, making them unavailable to plant roots. For example, when soil pH is high (>7.5), phosphate ions react with calcium and magnesium ions to form less soluble compounds. However, when soil pH is low, aluminum and iron become more soluble and react with phosphate ions to form less soluble compounds. Unlike other nutrients, nitrogen is not directly affected by soil pH. Nitrifying bacteria are responsible for oxidizing ammonium and making it available to plants. It has been reported that their growth and abundance is greatly affected at low

soil pH. A study conducted by Ste-Marie and Paré (1999) found no net nitrification was in the floor of Boreal forest stands below a soil pH of 4.5 (19). Other field studies have reported similar results (20–22). In addition, soil pH can also modify fungi abundance and composition (23–25). This could be a concern since the majority of soils in the GSR still have a soil pH below 4 (26).

Metals usually found in excess levels in mining regions include, Aluminum (Al), Copper (Cu), Nickel (Ni), Iron (Fe), Zinc (Zn), Cobalt (Co), Chromium (Cr), Lead (Pb), Arsenic (As), and Cadmium (Cd). Many of these are essential for plants however, they are only required in low concentrations (micronutrients). Metal solubility is affected by soil pH. Metal solubility increases when pH decreases. Their high solubility at low pH and the excess levels provided by pollution from the mining industry, has led to high metal bioavailability in soils (2, 26–29).

1.1.3. Impact of the mining industry on terrestrial ecosystem health

1.1.3.1. Plants

Soil pH, nutrient, and metal bioavailability are key factors that play an important role in the sustainability of an ecosystem. If one of the three is unbalanced, the ecosystem could potentially be affected. Different species require different soil conditions for their optimal growth. For example, conifers such as pines and spruces prefer slightly acidic soils ranging from pH 4.5 to 5.0. In contrast, California lilacs favor an alkaline slightly over 7.0 due to the high levels of lime-rich soils where the plant is native. Soil pH is not the only variable that plays a role in plant viability. The availability of macro and micronutrients in the soil must also be balanced for proper plant growth. At low soil pH, the increase in metal solubility can be potentially toxic to plants. Soil metal levels are positively correlated with plant metal uptake. For this reason,

vegetation growing in smelting communities show higher concentrations of metals in their tissues compared to undisturbed regions (9, 30–33).

An increase in metal levels in the plant's tissues leads to the production of reactive oxygen species (ROS) that can damage important cellular components (34). ROS are reactive molecules that possess oxygen with an unpaired electron (free radical). They include, but are not limited to, hydroxyl radicals (OH^\cdot), hydrogen peroxide (H_2O_2), and superoxide (O_2^\cdot). They are able to damage DNA, RNA, proteins, organelles, and membranes (35, 36). Plants growing in metal contaminated soil are susceptible to damage by ROS. Regions surrounding smelters are usually barren or semi-barren of vegetation due to soil infertility (2, 37–39). Most plants growing in metal contaminated soils have reduced biomass (40–42). This can be attributed to both metal toxicity and nutrient deficiency. For example, excess levels of phyto-available aluminum disrupts cell division at the apical region of the root by binding to phospholipids from the cell membrane (43–45). The result is a decrease in both root growth and strength (46). Despite its abundance in nature, aluminum is not a plant nutrient. When soil pH is below 5.0, manganese solubility increases and toxicity becomes an issue. Manganese toxicity causes brown spotting of leaves and/or elongated holes between leaf veins (47, 48). Nickel and copper toxicity leads to leaf chlorosis/necrosis due to deficiency in iron (49–51). High manganese, nickel and copper levels in areas surrounding smelters are not uncommon due to smelting emissions and the exposure of barren landscapes to erosion (4, 52, 53).

1.1.3.2. Humans and other vertebrates

Both SO_2 and metal contamination can negatively affect plants, animals, and humans. Many reports have shown that long-term exposure to SO_2 pollution could be linked to respiratory

diseases and cancer in humans (54–56). In fact, high levels of metals such as arsenic and mercury are dangerous to human and animal health because they are known to be carcinogenic (57, 58). Plants growing near smelters have higher levels of metals in their tissues (9, 30, 31). This is of concern mostly for organisms higher in the food chain. Grazing animals such as elk (*Cervus canadensis*), red deer (*Cervus elaphus*), and cattle (*Bos taurus*) living in metal polluted areas have been found to have high levels of metals in their tissues (59–61). Consumption of these plants or animals by humans can pose serious health issues. Therefore, the Canadian government has established strict guidelines to prevent soil, water, and air contamination.

1.1.3.3. Invertebrates and microorganisms

Plants and vertebrates are not the only organisms affected by metals. High levels of soluble metals also lead to toxicity in microorganisms. Wang *et al.* (2007) reported a lower microbial biomass in soil contaminated with Zn and Cu (62). Haimi and Mätäsniemi (2002) found similar results with various invertebrate decomposers in a metal contaminated coniferous forest (62). These organisms are responsible for recycling nutrients stored in organic matter, fix nitrogen from the atmosphere, and increase nutrient availability to plant roots. Therefore, toxicity in these organisms might have an indirect impact on plant health.

1.1.4. Emission reduction and the Sudbury Land Reclamation Program

In the past 40 years, emission control and re-greening projects have greatly contributed to the reestablishment of vegetation in contaminated areas. Emission levels in the Greater Sudbury region have been cut by approximately 90% because of recent advances in technology and emission caps. This can be partially attributed to VALE's new Clean AER (Atmospheric Emissions Reduction) program. Recycling plants were put in place to decrease sulfur dioxide

emissions by converting it to sulfuric acid. In addition, decreasing levels of metals in the Coniston area have been observed since the closure of the smelter (64).

In 1973, the Sudbury Technical Tree Planting Committee (now VETAC, Vegetation Enhancement Technical Advisory Committee) was formed. They help re-green the GSR by planting seedlings, fertilizing, and liming disturbed areas. To date, 12 million seedlings have been planted throughout the region. Due to their high tolerance to acidic soils, conifers (94.7%) were amongst the most planted. They included pines (*Pinus banksiana*, *P. resinosa* and *P. strobus*), spruces (*Picea glauca* and *P. mariana*), balsam fir (*Abies balsamea*) and eastern white cedar (*Thuja occidentalis*). Deciduous trees (4.5%), shrubs, and understory trees (0.8%) were also planted (65). The main hardwood species being planted included, maples (*Acer rubrum*, *A. saccharum* and *A. saccharinum*), oaks (*Quercus rubra*, *Q. alba* and *Q. macrocarpa*), and yellow birch (*Betula alleghaniensis*). Over 3,000 hectares of land was fertilized and limed. This was done either manually by hand or by air using planes. Fertilizers used were rich in phosphorus and contained legume seeds. Limes consisted of calcium carbonate and magnesium carbonate. They help increase soil pH and vegetation biomass in areas disturbed by mining activities (66). For example, lime-treated areas on the Sudbury Integrated Nickel Operations property and VALE tailings have shown a significant increase in pH (6). Current emission limits/reduction and the introduction of vegetation rehabilitation programs have significantly contributed to the rehabilitation of affected areas (67, 68).

1.1.5. Effects of liming on soil chemistry

Liming has been used in the last century to reduce soil acidity and to increase forest health after environmental pollution (69–71). Lime is usually generated from limestone, a rock primarily

composed of calcium carbonate (CaCO_3). Different grades of lime are available but the most used for restoration are calcium carbonate and magnesium carbonate. They are weak bases that allow the ongoing neutralization of hydronium ions in the soil. The effects of liming can last decades; therefore liming more than once is not needed (9).

Liming increases the recovery after environmental pollution. The first species to colonize well-drained limed soils are generally small grasses and shrubs followed by higher order species such as deciduous trees (72). Liming with magnesium carbonate or calcium carbonate has been shown to increase phyto-available calcium and magnesium, two macronutrients that are essential to plant growth (73–75). It has also been proven to increase the biomass of nitrifying bacteria (76). This leads to increasing levels of phyto-available nitrogen in the soil, an essential plant nutrient used for the production of proteins (76). Soil liming not only affects nutrient solubility, but it also decreases metal solubility (9). For example, it has been reported that liming decreases the phyto-availability and uptake of Cd and As in some plant species (77, 78). Other studies have shown that liming decreases the total amount of Cu, Ni, and Zn in soil close to smelters (74). A lower level of phyto-available metals in contaminated areas is important because it helped decrease their uptake by plants.

1.2. The genus *Betula*

The genus *Betula* belonging to the *Betulaceae* family, consists of roughly 60 species of deciduous trees. The taxonomy of the *Betula* genus is quite complicated however; five major groups have been identified (79–81). Birch are generally small to medium trees or shrubs. They are enwrapped in bark that is marked with horizontal lenticels and often separate into thin layers that resembles paper. The leaves are positioned alternate, singly or double serrate. They have a

petiolate or stipulate shape with a feather like venation. All birch are angiosperms; the male catkins form in the fall and do not bloom until spring, when the female catkins appear. The flowers are monoecious, meaning they contain both male and female reproduction organs. *Betula* species are anemophilous as a result they pollinate by wind, in the spring. Pollen grains are roughly 20 µm in diameter but size does vary from individuals and species (82). This is relatively small compared to conifers, which can have a pollen grain diameter of up to 100 µm (83). Birch pollen can stay in the air for few days and can be transported up to 1,000 km away from its original source (84). Most birch species are short-lived and are typically found in the Northern Hemisphere in temperate and Boreal climates (85). Birch can grow in a wide variety of environments but prefer well-drained lowlands with an acidic pH and a temperature of 18 – 22 °C (86). However, some species such as *Betula nana* are found in high altitudes. Most birch species are known as “pioneer species” due to their rapid growth and intolerance for shade. They are first to colonize open areas after disturbances such as forest fires and deforestation. Birch have also been known for being resistant to metal contamination and soil acidification (6, 87). Several studies have shown that some *Betula* species growing in metal contaminated soil displayed signs of metal resistance. These include white birch (*Betula papyrifera*), silver birch (*Betula pendula*), and mountain birch (*Betula pubescens* subsp. *czerepanovii*) (87–90). However, it is unclear whether resistance is shared throughout the *Betula* genus or if specific populations have adapted to metal contamination (87).

1.2.1. *Betula papyrifera*

White birch (*Betula papyrifera*) is an early successional of the Boreal forest of North America. According to Natural Resources Canada, it is found in every province of Canada, except Nunavut, as well as the northern United States (Figure 1). Several taxonomic studies have shown

that *B. papyrifera* shares a common ancestor with *B. nana* (dwarf birch) and *B. pubescens* (downy birch) (79–81). When young, the bark of white birch saplings is a reddish color but turns white as it matures. However, the branchlets remain reddish throughout the tree's life. White birch leaves are positioned alternately on the branch. The leaf position is alternate and its structure is as followed; ovate or triangular, acute tip, rounded base structure, doubly serrates margins with a length of 5-10 cm. White birch grows in a wide variety of soils and geographical regions, ranging from rocky hills to flat grounds. They prefer open areas with lots of sunshine and well-drained soils with an optimal pH of around 6.5. However, some studies have reported that white birch can grow in soil with a pH of as low as 3.5 (9, 87). White birch trees are capable of reaching heights of up to 35 m but this is dependent on the region and environmental conditions. It is considered a short-lived species and trees generally mature around 60-70 years, however some records show that they can live up to 140-200 years (91). White birch does not have a high economical value but their wood is often used to build furniture, flooring and provides a good source of firewood.

1.2.2. Effects of metal contamination and low soil pH on *B. papyrifera*

Little is known about the effects of environmental pollution on white birch. Several studies have reported that white birch is sensitive metal contamination (67, 87). Similar results have been reported in other birch species such as *B. populifolia* and *B. alleghaniensis* (92). In *B. papyrifera*, excess levels of metals in the soil are known to disrupt nutrient and water availability. Santala and Ryser (2009) found that nickel and copper spiked soil affected the response to water availability in white birch seedlings, even at metal concentrations that did not impact growth rate (93). Higher doses of Ni and Cu reduced total dry mass, stem diameter, radial

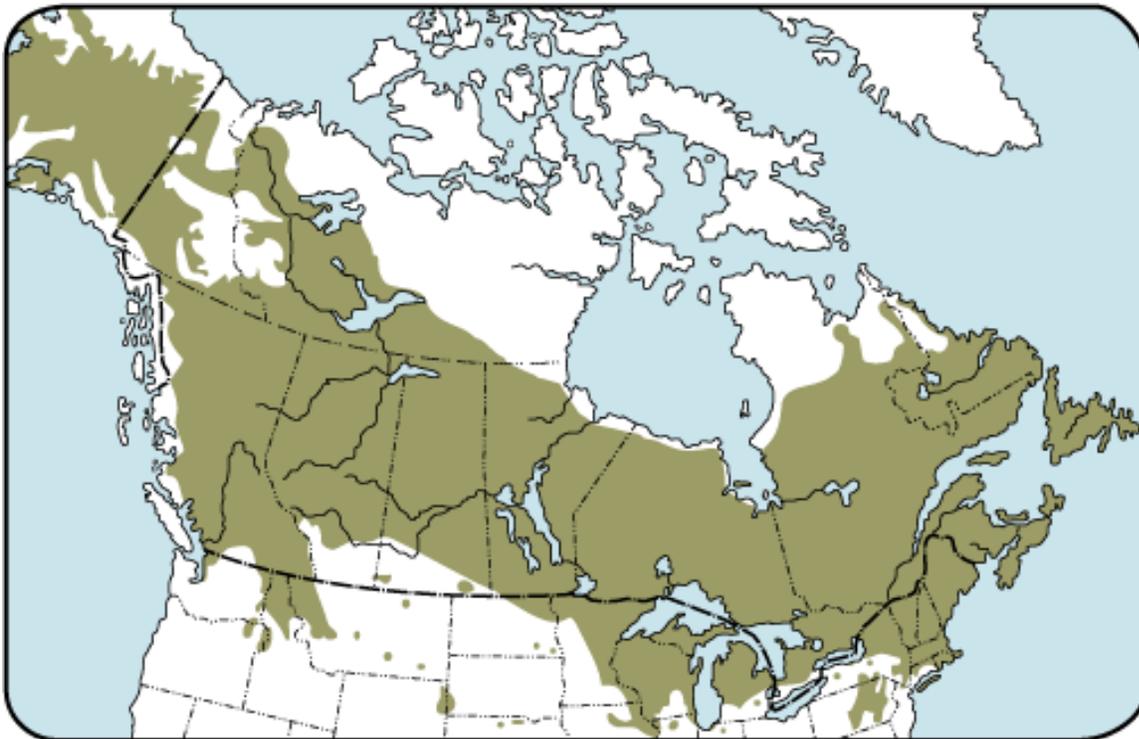


Figure 1. Distribution of white birch (*Betula papyrifera*) in North America

From Natural resources Canada (2015)

file length and cell size (93). However, other studies have shown that white birch seeds can grow without radical reduction in copper, nickel or cobalt concentration of up to 100 mg/L (94) and aluminum levels ranging from 80 mg/L to 120 mg/L (95, 96). White birch trees growing near smelting communities were found to accumulate higher concentrations of metals in their leaves compared to other tree species, especially zinc and cadmium (97, 98).

White birch populations from metal contaminated soils have shown to be more resistant to metals compared to trees growing in non-contaminated regions. For example, Kirkey *et al.* (2012) found that long-term exposure to metal contamination has led to resistant white birch populations in the Greater Sudbury area (Ontario) (87). Metal resistant population of other *Betula* species has also been documented (88, 99). Metals are not the only soil constituents that affect white birch health. White birch prefer a slight acidic pH of around 5 – 6.5. However, a study conducted by Davidson (1977) showed that 58 – 98% of white birch planted on acid coal spoils were able to survive on soils with a pH ranging from 3.0 to 4.0 (100). Most of the Greater Sudbury region is populated with white birch and a soil pH below 3 is not uncommon, specifically before land reclamation and emission reduction (6, 9).

A broad range in the level of metal resistance in white birch could be attributed to its high genetic variation (9). That allows white birch to adapt quickly to stressed environments. This would suggest that white birch could possibly be used to help re-green regions that have been metal disturbed.

1.3. Genetic variation

Genetic variation influences the sustainability of a population. The mutation in alleles of genes allows for the amelioration or declination of the quality of traits. Without this phenomenon,

evolution would never occur. Mutations happen at the molecular level in the DNA helix when at least one of the four nucleotides is exchanged for another, deleted or inserted. It is important to note that chromosomal translocations, deletions and insertion are also considered as mutations. Mutations can be deleterious; causing harmful effects, neutral; causing no effects or advantageous; being beneficial to the individual. There are two types of mutations. The first being natural mutations. They are due to the addition of the wrong nucleotide by the DNA polymerase during transcription. The error rate depends on the type of DNA polymerase (101). The second type of mutation is induced mutations. Environmental factors such as UV rays, ion radiation, and metal contamination have been known to cause these mutations (102).

In the event of an environmental disturbance or inter-species and intra-species competition, populations with higher genetic variation will have a greater chance of survival (103). This is because high genetic variation leads to a larger gene pool (104). As a result, there is a greater probability that some of the individuals in the population will possess genes that help resist against the stressor. If genetic variation is low, the gene pool will be reduced. Therefore, in the event of a stress, there might not be any resistant individual in the population. This could lead to its extermination or a population bottleneck (104). Inbreeding, a reduced population size, and ecological barriers have been known to lead to low genetic variation (105). When a population decreases in size or it is restricted by an ecological barrier, inbreeding becomes an issue and a population bottleneck could occur (106). This is because genetically related individuals are forced to breed with each other. Consequently, this increases the risk of diseases (especially recessive diseases) and lowers the population's fitness (107).

1.3.1. Effects of metal contamination on genetic variation

Metal contamination has been known to affect genetic variation both in plants (108–110) and in animal species (111, 112). In plants, studies have shown that genetic variation in herbaceous species is lowered by metal contamination. *Deschampsia cespitosa* populations growing in metal contaminated areas of the GSR and Cobalt (Ontario) were found to have lower genetic variation than populations growing in uncontaminated areas (110, 113). *Sedum alfredii* populations growing in lead/zinc mine spoils in China also showed low genetic variation compared to populations growing in undisturbed areas (108). However, in woody species such as *Picea mariana*, *P. glauca*, *Pinus banksiana*, *P. rubens*, *P. strobus*, and *B. papyrifera* growing in Northern Ontario, no link between genetic variation and metal contamination has been found (9, 53, 114, 115). It is possible that since woody species are long-lived, the effects of metal contamination on the genetic variation cannot yet be detected.

Metals are known to induce mutations therefore can also be beneficial by introducing new alleles in a population. High levels of heterozygosity in European beech (*Fagus sylvatica*) in Germany (116), scots pine (*Pinus sylvestris*) in Germany and Great Britain (117), trembling aspen (*Populus tremuloides*) and red maple (*Acer rubrum*) in the United States (118) has been observed. Higher level of heterozygosity indicates that these stands have a larger gene pool thus can more easily adapt to environmental changes.

1.4. Molecular markers

Since 1985, geneticists have been able to analyze genetic variation between and within populations due to advances in molecular marker technologies. Molecular markers are variable regions of DNA that provide important information in genetic linkage mapping, fingerprinting,

gene diversity, and more (119). They are also used for indirect selection of traits and in phylogenetic studies.

1.4.1. Restriction fragment length polymorphism (RFLP)

The first molecular marker system developed was RFLP (Restriction Fragment Length Polymorphism). The DNA samples are digested using restriction enzymes. Type II restriction enzymes are often used because of their selectivity. This would lead to fragments of various sizes being produced. The restriction fragments are then separated by their size using gel electrophoresis (120). Because of the genetic variations in the restriction sites, some individuals would show longer or shorter fragments (restriction enzyme is unable to recognize the site and cut it). This technique was most commonly used in DNA fingerprinting and genome mapping. The main disadvantages of RFLP are its low efficiency and the need for high concentrations of DNA for the digestion (121).

1.4.2. PCR based methods

Recent advances in Polymerase Chain Reaction (PCR) revolutionized molecular markers. With PCR, the target DNA sequence is amplified by using a polymerase and two short oligonucleotide sequences called primers. These are located upstream and downstream of the target DNA sequence. Because the primers are complimentary to the target strands, the sequence between them can be amplified. The main drawback is that the DNA sequence of the genome of the species you are working with is needed in order to design the primers (except for random priming). A wide variety of molecular markers were developed, including RAPD (122), AFLP (123), SSR (124), and ISSR (124). PCR-based marker systems have proven to be advantageous because only small amounts of DNA template are needed. They are also able to detect genetic variation

between individuals of the same population. Consequently, this has led scientists to step away from the use of restriction enzymes, which provided some difficulties such as throughput and sensitivity.

1.4.2.1. Amplified fragment length polymorphism (AFLP)

This technique uses a combination of RFLP and PCR. The DNA is digested with a restriction enzyme and then adaptor primers are ligated. The DNA is then amplified with PCR using a primer complementary to the adaptor sequence. Only fragments with the adaptor sequence will be amplified. The advantages of AFLP over RFLP are; high reliability and reproducibility, genome sequence not needed, highly polymorphic and co-migrating amplified fragments are usually homologous (123, 125). However, disadvantages include, a large amount of template needed, higher costs and lower throughput (more laborious) (122).

1.4.2.2. Random amplified polymorphic DNA (RAPD)

The random amplified polymorphic DNA (RAPD) technique uses short (roughly 10 bp) arbitrary primers with a GC content of 40 – 80 %. The primer sequences do not contain a palindrome sequence. RAPD primers will bind randomly in the genome and amplify the region in between. The main advantage of RAPD is that the sequence of the genome is not needed to design the primers. However, RAPD primers are very diverse and false positives are often an issue if there is any cross-contamination. Since RAPD primers are short, profiles are often difficult to reproduce if conditions are not identical (126, 127). In addition, RAPD is a dominant marker system therefore the presence or absence of a band on an electrophoresis gel does not distinguish dominant homozygous and heterozygous individuals (122, 128). For this reason, dominant markers provide less information than co-dominant markers such as SSR and SCAR. However,

dominant marker systems are more often used when working with plant species because the genome sequence is not needed.

1.4.2.3. Simple sequence repeat (SSR)

Microsatellites (SSR) are repeated regions of DNA. The nucleotide repeats are generally di, tri, tetra, penta or hexa and can be repeated 10 – 100 times (129). SSRs are found in every known eukaryote and are generally randomly distributed across the genome (130). However, some studies have reported that they are more commonly found in non-coding regions (131). SSR primers are designed by using regions flanking the microsatellite sequence. PCR is used to amplify the DNA and fragments of different sizes are obtained due to the variation in the length of microsatellite regions (132). The main advantages of SSR are; low amounts of DNA template needed, they are highly polymorphic, and the absence of amplification can lead to heterozygote individuals to be scored as homozygote (133). However, the main drawback is that the sequence of the genome is needed to design and produce the primers.

1.4.2.4. Inter simple sequence repeat (ISSR)

Inter Simple Sequence Repeat (ISSR) is a relatively new technique. It was developed in 1994 and since then has been the primary choice for analyzing genetic variation in non-model species. This can be attributed to its simplicity and low cost. It allows all the benefits of a regular genetic marker system but without the need of knowing the genome sequence of the species of interest (124). ISSR primers are designed by using repeated DNA sequences known as microsatellites (SSR). ISSR primers can be 5' or 3' anchored. This allows specific binding of the primer to the end or start of the SSR. Only one primer is used therefore the amplified sequence, known as the ISSR is found between two short inversely repeated SSRs. Like SSRs, ISSRs are also found in

every known eukaryote yet and are randomly dispersed throughout the genome (134). ISSR is a dominant marker system therefore the presence or absence of a band on an electrophoresis gel does not distinguish dominant homozygous and heterozygous individuals (135).

In order to visualize the profile, PCR products are separated by gel electrophoresis. The physical and chemical properties of porous gels like agarose and polyacrylamide make them ideal for DNA and RNA separation. DNA and RNA fragments are separated by their size and charge due their natural negative charge (migrates towards the cathode) and the structure of the agarose and polyacrylamide gels. DNA and RNA migrate towards the cathode because of their negatively charged phosphate backbone. Shorter fragments will move faster and longer fragments slower. There are two main types of buffers that can be used for agarose electrophoresis, Tris-Acetate-EDTA buffer (TAE) and Tris-Borate-EDTA (TBE). TAE is most commonly used when a DNA band needs to be purified (ex: cloning and sequencing experiments). The reason being, borate inhibits PCR. TAE gels also have a lower voltage capacity and will melt if ran for too long. TBE has the following advantages over TAE; it has a higher voltage capacity, obtains better resolution of smaller fragments, and allows faster migration (136). To visualize the DNA fragments, fluorescent dyes that intercalate into the DNA helix can be used. Ethidium bromide and SYBR green are amongst the most commonly used dyes. Ethidium bromide is a powerful mutagen that intercalates in the minor groove of the DNA helix (137). It is also capable of binding single stranded RNA (137). When bound to DNA, ethidium bromide can be excited with UV light at 254 nm, 302 nm or 366 nm and has an emission peak between 590 and 605 nm.

1.4.2.5. Sequence characterized amplified region marker (SCAR)

Michelmore *et al.* (1991) developed a PCR based technique that used RAPD generated fragments for the amplification of a specific locus (138). The RAPD fragment were first sequenced and then SCAR primers (15 – 30 bp) were designed with the end terminus. SCARs have a high reproducibility due to the greater length of the primers. They can also be developed from other PCR-based marker systems such as SRR and ISSR. SCARs provide additional information as opposed to random priming (ISSR and RAPD) because of its co-dominance. Recently SCAR markers have been used to identify and distinguish plants of the same species (139). They can also be used to identify particular traits or genes among species (140, 141). SCAR markers have also been useful in taxonomic and phylogenic studies (142, 143).

1.5. Species-specific and population-specific molecular markers

1.5.1. Species-specific molecular markers

Species-specific molecular markers are DNA bands that are found in one species and missing in others (144). They can be generated by both dominant and co-dominant marker systems. A mutation in the primer or anchoring base is generally the cause of species or population specific molecular markers. Conversion of RAPD, ISSR, and other PCR based markers to SCAR is usually required to confirm the specificity of the molecular markers. The markers can be used to help distinguish phenotypic or genotypic traits of different species. Species-specific molecular markers have been used to distinguish closely related species that have a similar morphology (145, 146). They have also been useful for detecting hybrids from different species (147, 148).

1.5.2. Population-specific molecular markers

Population-specific molecular markers follow the same concept as species-specific molecular markers but they are specific to a single population within the same species (144). They are also generated through PCR based dominant or co-dominant marker systems. In plants, they can be used to identify crop cultivars, as a diagnostic markers for certain diseases or to map the migration of a species (138, 149). Population-specific markers have never been identified in white birch. However, they have been discovered in other *Betula* species (150). Population-specific bands are usually not as common as species-specific markers as the genetic variation among species is generally greater.

1.6. Genomic and transcriptome analysis in plants

Recent advances in technologies have made sequencing the full genome or transcriptome of a species possible. Next Generation Sequencing (NGS) has revolutionized genomic and transcriptomics since it possesses many advantages over the traditional Sanger sequencing method, such as high throughput and lower costs. Before NGS, sequencing the genome of non-model organisms such as plants was almost impossible. This was due to high level of genome duplication, heterozygosity, ploidy, and repetitive sequences found in plant species (151, 152).

The study of transcriptomics includes cataloging and quantifying the RNA (mRNA, miRNA or non-coding RNA) in a given tissue. Before NGS, expression experiments were limited to qPCR and microarrays. In general, qPCR is highly quantitative and sensitive but it is not useful when analyzing more than a small set of genes. Microarrays are able to analyze large subsets of genes however; the detected genes are limited to what is printed on the array. Hence, the full transcriptome cannot be explored. The development of NGS has made sequencing the total

mRNA of a tissue possible (RNA-seq). The main advantage of RNA-seq over microarrays is that the whole transcriptome is sequenced. This has facilitated expression analyses in plants since scientists do not rely on homologous recombination (arrays in plants are difficult to produce). However, analysis and annotation of large RNA-seq datasets can be challenging, especially in non-model organisms.

RNA-seq is generally performed as an initial experiment to identify genes of interests in specific conditions or to distinguish expression differences between genotypes. They have been useful in unveiling drug mechanisms and pathways (153), cancer research (154, 155), and stress response in both animals (156, 157) and plants (158, 159). RNA-seq has made the analysis of hardwood transcriptomes possible. In the last decade, the full transcriptome of 20 hardwood species had been sequenced because of the Hardwood Genome Project (Institute of Agriculture, University of Tennessee). Very few studies have compared the transcriptome of hardwood species under environmental stresses such as drought, disease or metal stress (160–163). The transcriptome of American chestnut (*Castanea dentata*) and Chinese chestnut (*Castanea mollissima*) was sequenced to determine genes involved in resistance against chestnut blight fungus (*Cryphonectria parasitica*) (163). Such experiments can be useful in unveiling key genes involved in resistance mechanisms in plants.

1.7. Metal resistance mechanisms in terrestrial plants

The majority of plant species are not able to survive in areas where soil metal concentrations are high. However, plants with metal resistance genes can survive in these soils regardless of the toxic effects of metals.

Metal resistance in plants has been extensively reported in the literature. However, genes and mechanisms involved in plant adaptation to stress are still relatively unknown. Metal resistance mechanisms can be divided into two categories, avoidance and tolerance (164).

1.7.1. Avoidance mechanisms

Plants that possess avoidance mechanisms will restrict the amount of metals being absorbed by the root system. These plants are not able to cope with high cytoplasmic levels of metals. Thus, their resistance mechanisms must be efficient in reducing the toxicity of metals outside the plant.

1.7.1.1. Mycorrhizal symbiosis

Some avoidance mechanisms include forming a symbiosis between the plant's root system and mycorrhizal fungi. These symbiotic fungi are more resistant to high concentrations of metals than plants. They help decrease the bioavailability of metals around the rhizosphere by accumulating them or by secreting chelating compounds in the soil (165). Their large hypha protect the root system and facilitate the transfer of essential nutrients (166, 167). Arines *et al.* (1989) reported that *Trifolium pratense* (red clover) growing in acid soils had lower levels of manganese in their roots and shoots when they had formed a symbiosis with mycorrhizal fungi (168). It is likely that these oxidizing fungi were lowering Mn solubility around the plant's root system (168, 169). Other studies have reported similar results in *Betula papyrifera*, where *Scleroderma fiavidum* increased Ni resistance (170).

1.7.1.2. Root exudation

In order to reduce the bioavailability of metals around the rhizosphere in the absence of help from other organisms, some plants will secrete compounds from their roots (171–173). The secreted

molecules are generally negatively charged and have a high affinity for divalent cation metals. They render metals inactive (unable for uptake) by chelating them around the rhizosphere. Citrate, malate, and oxalate root exudation has been linked to metal resistance. This phenomenon has been observed in various plants ranging from crop species like *Zea maize* (174, 175) to trees such as *Populus tremula* (171).

1.7.1.3. Regulation of transport

Avoiders reduce the uptake of metals and limit their toxic effects. This can be achieved by down-regulating the expression of metal transporters in the apical region of the root (176). For example it has been reported that copper tolerant *Silene vulgaris*, were found to down-regulate Zn transporters in their roots when exposed to toxic levels (177).

1.7.2. Tolerance mechanisms

The second type of metal resistance mechanisms is tolerance. Tolerant plants will uptake metals and compartmentalize them in their parts where toxicity is reduced. There are three known types of plant tolerance mechanisms to metals; excluders, hyperaccumulators and indicators. Each differs in the quantity of metals accumulated in the plant as well as the type of tissue they are stored in.

1.7.2.1. Excluders

Excluders accumulate metals in their roots rather than in their shoots. To be classified as an excluder, metal content ratio between plant shoot/root must be less than one (164). Excluders tend to limit the root to shoot translocation of metals (178). Several lower (*Deschampsia*,

Oenothera, *Commelina*, *Silene* and *Agrostis*) and higher woody (*Populus*, *Pinus* and *Salix*) and plant species from different genera have been classified as excluders (179, 180).

1.7.2.2. *Hyperaccumulators/accumulators*

Hyperaccumulator plants accumulate high levels of metals in their shoots rather than their roots. They can store up to 100 times more metals than their non-resistant counterpart (181). Four criteria are used to include a plant in the hyperaccumulator group; 1) the metal content ratio between shoot/root must be greater than one, 2) the metal content ratio between shoot/soil must be greater than one 3) the plant must uptake 10 – 500 times more metals than a non-resistant plant growing in normal soil conditions, and 4) it must accumulate more than 100 mg/kg of cadmium, 1,000 g/kg of copper, lead, nickel, and chromium, and 10,000 mg/kg of zinc in its tissues (164). Roughly 1/3 of all known hyperaccumulators come from the *Brassicaceae* family. For instance, *Thlaspi goesingense* is able to hyperaccumulate over 1% of its dry shoot weight as nickel (182, 183).

1.7.2.3. *Indicators*

Indicators, like hyperaccumulators, will also accumulate metals in their aerial parts. However, metal concentrations in the plant are generally similar to the ones found in the soil (184). Indicators are important pollution markers since they will not survive if they continue to uptake metals (164).

1.8. Genes involved in metal resistance in plants

Metal resistant genes code for proteins that play a direct role in the detoxification of either metals or the reactive oxygen species produced by them. The majority of metal resistance genes can be classified into two distinct categories, chelators or metal transporters.

1.8.1. Chelators

Chelators are molecules that will sequester metals and render them inactive. They are generally small and low-carbon cost molecules that do not utilize much energy from the plant when produced. Many chelators are byproducts from the Krebs cycle or other metabolic pathways. For example, increased cytosolic levels in nicotianamine (185), citrate (172), malate (174), amino acids such as proline (186), histidine (187), and glutathione (188) have been reported to play a role in metal resistance in plants. Many hyperaccumulators depend on chelating substrates for proper metal storage. This is the case for nickel hyperaccumulator *Sebertia acuminata*, where nickel was only accumulated in the aerial tissues when bound to citrate (189).

Genes involved in the production of these metabolites are often upregulated in metal resistant plants. High levels of glutathione, Cys, and O-acetyl-l-serine (OAS) have been reported in the shoots of nickel hyperaccumulator *Thlaspi* spp. (188). Increased levels of these metabolites have been shown to be correlated with high expression levels of both serine acetyltransferase (SAT) and glutathione reductase, these enzymes play an important role in the production and turnover of glutathione (188). High levels of Mn, Fe, Cu, Ni, and Zn have also been correlated with increased expression of nicotianamine synthase (NAS) and nicotianamine production (190, 191). NAS is an enzyme that plays a role in the synthesis of nicotianamine by the trimerization of S-adenosylmethionine (192).

Larger molecules such as metallothioneins and phytochelatins have been extensively studied and known to play a role in metal detoxification and resistance (193). Metallothioneins (MT) are short cysteine-rich peptide that bind metals or reactive oxygen species through their thiol groups (194). MTs play a role in general metal homeostasis however; their involvement in metal resistance can't be overlooked (193). They can be found in organisms from all kingdoms. MTs can be divided into three classes based on their cysteine arrangement. Class I MTs are found in vertebrates and have highly conserved cysteine arrangements. While Class II MTs are less conserved and are primarily found in plants, fungi, and invertebrates. Class II MTs can be further divided into four more categories, ranging in what tissues they are found in (195). Class III MTs (phytochelatins) are exclusively found in plants (196). High expression of MTs have been correlated with resistance to Cu (197, 198) and Cd (199, 200) in plants.

Unlike methalothiones, genes do not directly encode phytochelatins. Their structure consists of large oligomers of glutathione. Like glutathione, PCs can bind and immobilize metals through their thiol groups (195). The PC pathway consists of three steps; 1) γ -glutamyl-Cys synthetase (GCS) catalyzes the dipeptide gamma-glutamylcysteine (γ -GC) from cysteine and glutamate 2) glutathione synthetase (GS) produces glutathione from γ -GC and an additional glutamate and, 3) phytochelatin synthase ligates multiple glutathione molecules to form long oligomer chains (195, 201). The *cad2-1 Arabidopsis* mutant is deficient in GCS (202). *Cad2-1* mutants have low levels of PCs and high sensitivity to cadmium. This shows the importance of PCs in metal homeostasis (202).

1.8.2. Transporters

The second class of metal resistant genes codes for large proteins called transporters. Transporters are involved in shuffling metals in different areas of the plant. In hyperaccumulators, excluders and indicators, transporters are responsible for compartmentalizing metals in specific tissues. The large vacuole (surrounded by a tonoplast) is often used to store metals. Once inside the vacuole, the toxic effects of metals on sensitive organelles are reduced (34, 203). Metal compartmentalization can also take place in the apoplast as well as specific cells of the epidermis and trichome (204). Transporters are often dependent on metal chaperons or other ions for proper functionality (193). For instance, antiporters pump both ions across the membrane in opposite directions in contrast to symporters that pump both ions in the same direction. Some metal transporters that have been known to play a role in metal resistance include NRAMP (natural resistance associated macrophage proteins), Metal ATPase (HMAs), Cation Diffusion Facilitator (CDF), ABC transporter (ATP-binding cassette), and the ZIP (ZRT, IRT-like proteins) family. They are responsible for maintaining proper metal homeostasis in bacteria, animals and plants (205). Many of these transporters are non-specific and are able to transport a class of metals that have similar physical properties. Transporters from the IRT1 family normally transport Fe but under deficient conditions can transport Zn, Ni, Cd, Co, and Mg (206). Low transport specificity is also seen in *A. thaliana*, where Ni and Zn compete for the AtZIP4/TcZNT1 transporter (207).

1.8.3. Evolution of metal resistance

What distinguishes a resistant and susceptible genotype is the level of gene expression. Therefore, resistance is mainly due to changes in expressions from mutations in the promoter

region or gene duplication (173, 208). High expression of some chelators and transporters has been linked to metal resistance in plants. Overexpression of AtATM3, an ATP-binding cassette transporter, is responsible for cadmium resistance in *Arabidopsis thaliana* (209).

It is important to note that metal resistance is not only associated to high or low expression. If a mutation occurs in a gene and leads to the relocation or modification of the native protein, it could have a direct impact on metal resistance/sensitivity. Relocation of metal transporters can be achieved when there are mutations in the N-terminal of the gene, where signaling peptides can be found. For instance, deletion of 9 amino acids from the N-terminus (putative signaling peptide) of the IRT1 transporter in *Arabidopsis thaliana* increases iron deficiency (210).

1.9. Nickel toxicity and resistance in terrestrial plants

1.9.1. Nickel homeostasis

Nickel is one of the least studied metals in plants. However, it is an essential micronutrient for plants that is required in minute concentrations (0.05 – 5 ppm in tissue). Only a handful of enzymes to date are known to use nickel as a co-factor. For instance, urease, an important enzyme that metabolizes urea into carbon dioxide and ammonia requires nickel for its proper function (211).

To date, only a few molecules have been found to chelate nickel in plants. Organic acids such as malate and citrate (212, 213), the amino acid histidine (212, 214), and nicotianamine (185, 215) have been reported to bind nickel with low to high affinity. Transporters that are involved in regulating nickel levels in plants include IRT1 from the ZIP/IRT family (216, 217), IREG1/2

from the IREG/Ferroportin family (216, 218, 219), YSL3 of the YSL/OPT family (220), and NRAMP4 from the Natural Resistance-Associated Macrophage Protein family (221, 222).

1.9.2. Nickel toxicity and deficiency

Plants deficient in nickel often accumulate urea in tissues, which leads to necrotic lesions on the leaf tips (211). Deficiency in nickel is very rare and almost unheard of since soil levels are generally higher than needed due to environmental pollution. However, excess levels can lead to toxicity. High levels of nickel in plants affects shoot/root growth and disrupt branch and flower development thus leads to a reduction in overall plant biomass (50). High nickel concentrations can also induce deficiency of other nutrients. For example, high levels of nickel has been correlated with iron deficiency, which leads to necrosis and chlorosis of leaves (50).

1.9.3. Nickel resistance

Only a handful of genes have been linked to nickel resistance in plants (188, 219, 223, 224). They include genes involved in the synthesis of glutathione (188, 208), nicotianamine (185, 215), histidine (225, 226), transporters from the IREG (219) and NRAMP family (222) as well as the transcription factor ZAT11 (224).

1.10. Copper toxicity and resistance in terrestrial plants

1.10.1. Copper homeostasis

Copper is an essential micronutrient with an average cellular level of 10 ppm (dry weight) (227). It is required for the activation of multiple enzymes (co-factor). It also plays an important role in photosynthesis and carbohydrate metabolism. Over 50% of the total copper in photosynthetic

cells is found bound to plastocyanin, an important protein that helps transfer electrons from Photosystem I to Photosystem II (228, 229).

Little is known about the transport of copper across plant membranes. In *Arabidopsis thaliana*, the COPT1 protein is a Cu transporter that plays a role in root elongation and pollen development (230, 231). P-type ATPases have been known to shuttle copper. HMA1 and HMA6 (PAA1) are involved in copper transport in the chloroplast and play a critical role in supplying copper to enzymes such as dismutase and plastocyanin (232, 233). Another P-type ATPase Cu transporter, HMA7 (RAN1) is responsible for shuttling Cu to the secretory pathway (234, 235). It is involved in ethylene signaling and is required for the proper formation of ethylene receptors (234, 235). Although these transporters play a key role in metal homeostasis, it is still unknown if there is a correlation with copper resistance.

1.10.2. Copper toxicity and deficiency

In excess levels, copper can be toxic to plants. Copper toxicity leads to interveinal foliar chlorosis and inhibition of roots and root hairs growth (236). High levels of copper can also lead to iron, molybdenum or zinc deficiency (236). In soils lacking bioavailable copper, deficiency can occur. Symptoms of copper deficiency are similar to nickel toxicity. It generally includes plant wilting and drooping as well as interveinal chlorosis of the leaves. Eventually leaves show necrotic spots, turn brown, and die. Therefore, proper mechanisms must be in place to insure that copper levels remain within healthy levels.

1.10.3. Copper resistance

Gene expression experiments revealed that 55 genes were upregulated more than 2 folds by the copper treatment in Cu-resistant compared to susceptible *Betula pendula* (237). The majority of differentially expressed genes were involved in cellular transport, regulation or cell rescue, and defense (237).

Mechanisms involved in the detoxification of copper include sequestration using chelators and compartmentalization by transporters. Studies have shown that circulating citrate and histidine in the xylem is responsible for chelating and transporting metals such as Cu and Ni throughout the plant (238). In addition, metallothioneins have been known to play a role in copper homeostasis and resistance (239). Copper-resistant populations of *Silene vulgaris* growing at a Cu mine have shown high levels of MT2b expression in roots and shoots, even when Cu was absent (240). Inversely, MT2a is primarily expressed in Cu-resistant *Arabidopsis thaliana* (241, 242).

1.11. Role of epigenetics in plant stress response

Epigenetics is the process in which the chemical structure of the nucleotides of the DNA helix is modified without actually changing the sequence itself. Epigenetic modifications can be inherited from daughter cells (or parents). There are many types of epigenetic modifications. They include acetylation, phosphorylation, methylation, ubiquitination, and sumoylation. For instance, chromatin condensation is partially controlled by the acetylation/deacetylation of histones. Acetylation of histones generally opens up tightly coiled chromatin and allows access and activations of genes (243). Methylation and sumoylation of histones is generally associated with repression of genes (244, 245) while ubiquitination has been shown to stimulate transcription (246). Phosphorylation of DNA plays a crucial role in the response to damaged chromatin (247).

Environmental conditions, lifestyle (diet, obesity, activity, smoking and drinking), and stress have been known to directly and indirectly modify epigenetic factors. Chemical toxins in the environment such as metals and hydrocarbons as well as temperature have been known to induce changes in epigenetics (248). In plants, a high level of methylation is generally seen in metal resistant genotypes. For instance, metal resistant hemp (*Cannabis sativa*) growing in various concentrations of Ni, Cd and Cr showed a 30% increase in 5-methylcytosine (5mC) in roots (249). Metal sensitive clover (*Trifolium repens* L) growing under the same conditions saw a 20 – 40% decrease in 5mC (249). Additional studies in radish (*Raphanus sativus* L.) (250), oilseed rape (*Brassica napus* L.) (251) and *Arabidopsis thaliana* (252) found similar results. It is believed that high methylation levels protect DNA from endonucleases and multi-copy transposons thus, increases resistance to metals (253, 254). However, lower level of global cytosine methylation was found in red maple (*Acer rubrum*) growing in metal contaminated areas compared to non-contaminated in the Greater Sudbury region (255). It is important to note that these studies only looked at global methylation patterns and that the areas methylated (and genes in those regions) are unknown.

1.12. Rationale

Mechanisms involved in metal stress and resistance in plants are still not well understood. The tools used to unveil complex mechanisms in plants have only been available in the last decade or so. These tools have made it possible to study non-model organisms. A century of mining activities in the Greater Sudbury region has left soils contaminated with high levels of metals (mainly Cu and Ni) and an acidic pH. Organisms living in these areas had to adapt or suffer to these extreme conditions. One of these species is white birch (*Betula papyrifera*). It is the predominant species of the Greater Sudbury region. In fact, it consists of over 50% of the all trees

found in the region. Hence, it is important to determine the sustainability of white birch populations in the GSR. Knowing the effects of metal stress and resistance in white birch can contribute to our understanding of how hardwood species cope with metal contamination, especially in mining regions. Moreover, understanding metal stress and resistance in genotypes can help in the development of highly resistant plants for greening or bioremediation of metal contaminated soils.

1.13. Objectives

The main objectives of this study were to; **1)** determine if there's an association between plant population diversity and genetic variation in white birch (*Betula papyrifera*) populations with soil metal contamination in the Greater Sudbury region (GSR), **2)** assess if metal contamination and soil liming has an effect on global DNA methylation, **3)** develop and characterize the transcriptome of *B. papyrifera* under nickel stress and, **4)** assess gene expression dynamics in white birch in response to nickel stress.

Chapter 2: Molecular and ecological characterization of plant populations from limed and metal-contaminated sites in Northern Ontario (Canada): ISSR analysis of white birch (*Betula papyrifera*) populations.

2.1. Abstract

The Greater Sudbury region in Northern Ontario is known as one of Canada's most ecologically disturbed regions because of the effects of metal pollution. The main objectives of the present study were to assess the effects of soil liming of sites contaminated with metals on species richness and abundance, population health in general, and to determine the level of genetic variability in white birch (*Betula papyrifera*) populations from Northern Ontario. Shannon-Wiener diversity index and tree species richness values were higher in populations from limed and reference sites compared to the non-limed areas. A significant improvement in forest community health (measured using a scale of 1 to 10) in limed sites over the non-limed areas was observed. Key results revealed no significant difference for metal content in white birch (*Betula papyrifera*) leaves from limed compared to non-limed sites. However, higher levels of Al, Ca, Mg, Mn, Ni, Sr, and Zn in leaves compared to the bioavailable amount in soil were observed. The levels of genetic variability in birch populations were moderate to high, ranging from 30 % to 79 % of polymorphic loci. A high level of genetic variability such as observed in the present study is usually associated with long-term sustainability in plant populations. No association was found between metal accumulation in soil or plants and the levels of genetic variation in white birch.

Key words: Metal contamination; white birch; species richness; Shannon index; ISSR markers; Northern Ontario

2.2. Introduction

The Greater Sudbury Region (GSR) located in Northern Ontario is known for its rich deposits of nickel, copper, and iron ores. However, it is also one of Canada's most ecologically disturbed regions because of the effects of metal pollution (1). It has been estimated that over 100 million tonnes of SO₂ and tens of thousands of tonnes of cobalt, copper, nickel and iron have been released by roast pits and smelters located in the Greater Sudbury region (4). The combination of deforestation, soil acidification and metal pollution greatly affected vegetation in the region. Soils surrounding smelters were completely barren soon after industrialization started with only a few tree species remaining (of which white birch was one of the commonest) (7). High metal concentrations and low soil pH have been found in the soil surrounding smelters (4, 5, 256). However recent studies show that soil metal concentrations of Cd, Co, Cu, Fe, Ni, and Zn near the Falconbridge smelter are within limits set by the Ontario Ministry of Environment and Energy (OMEE) guidelines (257). This suggests that recent reductions in emissions have allowed some recovery (68).

The reclamation of affected lands in the GSR has been conducted mainly through tree planting. Most of the seedlings that have been planted are conifers because these are highly tolerant to acidic soils (258). In addition to planting and seeding, over 3,400 hectares of land were limed with dolostone (magnesium limestone) by hand or air to neutralize acidic soils and boost revegetation. This lime consists of calcium carbonate and magnesium carbonate. Several studies have shown that adding lime to acidic areas will greatly increase the pH and allow revegetation (66). Effects of liming treatments on forest species richness and abundance as well as forest ecosystem health have not been investigated.

White birch (*Betula papyrifera*) is a major component of the Boreal forest of North America. It is considered a pioneer species because of its rapid growth and its intolerance for shade; therefore, it is the first to rapidly colonize open areas after deforestation. It can tolerate soil pH from 5 to 7.5 but prefer a slightly acidic pH. Furthermore, white birch can tolerate some soil metal contamination (87, 94–96). A high level of genetic variability within and among white birch populations is a prerequisite to their long-term sustainability.

The main objectives of the present study were 1) to compare tree species richness, abundance, and forest population health in reference (control), limed, and non-limed areas, and 2) to determine levels of genetic variation within Northern Ontario white birch populations.

2.3. Materials and methods

2.3.1. Sampling

Eleven forest populations in seven areas were selected for this study. Four areas were contaminated with metals and each had limed and non-limed counterparts; another three areas were metal uncontaminated and used as reference sites. Contaminated sites were located within the GSR near Daisy Lake, Wahnapiatae Hydro Dam, Kingsway Road, and Kelly Lake (Figure 2). Reference sites were at St-Charles, Onaping Falls, and Capreol. Contaminated sites were highly or moderately disturbed based on their proximity to smelters as described in (6).

Soil and leaf samples were collected from each site. Liming was performed up to 30 years ago through the Sudbury's Regional Land Reclamation Program using dolostone (259). For each area, 10 pedons were sampled, with soil samples being collected from the surface humus form (LFH), as well as from the underlying mineral horizons (namely the Ae, Bm, BC, and C, if present). Only the top organic layer samples were analyzed for this study. Soil samples were air-dried and stored in sealed plastic bags until analyzed. Leaves were collected randomly from 20 individual trees, dried, and stored for later analysis. A portion of the leaf samples were wrapped in aluminum foil, immersed in liquid nitrogen and stored at -20 °C until DNA extraction.

2.3.2. Study of plant species diversity, abundance, and population health

Contaminated (paired) sites were deemed highly or moderately disturbed based on their proximity to smelters. In each population, three transects of 10 m diameter each, were used to assess tree species richness and abundance. A health index was also assigned to each forest population ranging from 1 to 10 with 1 representing a population without trees or herbaceous

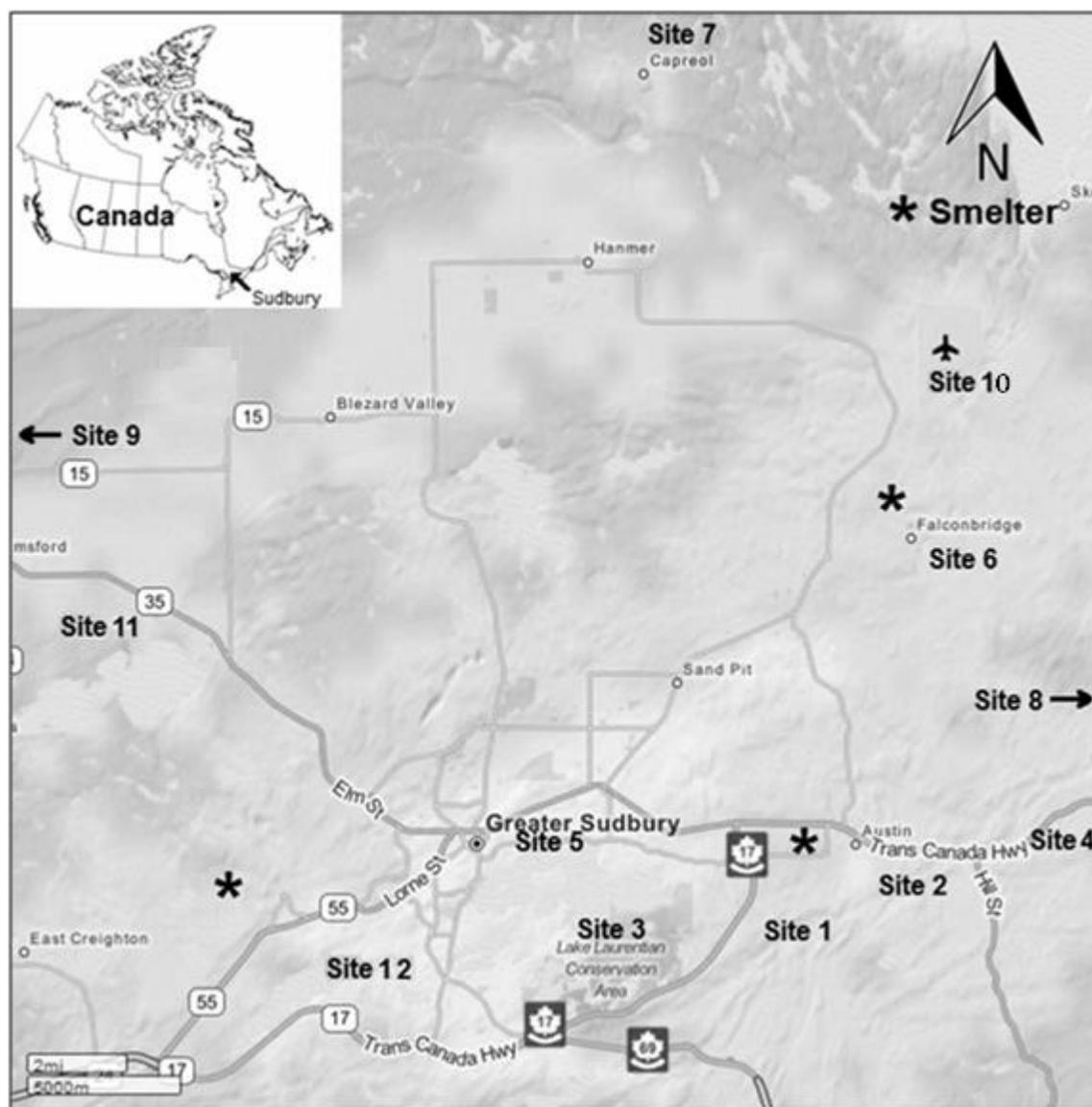


Figure 2. Location of white birch sampling sites in the Greater Sudbury region.

Site 1: Daisy Lake; Site 2: Wahnapiatae Hydro Dam; Site 3: Laurentian; Site 4: Kukagami; Site 5: Kingsway; Site 6: Falconbridge; Site 7: Capreol (reference); Site 8: St. Charles (reference); Site 9: Onaping Falls (reference); Site 10: Airport; Site 11: Azilda; Site 12: Kelly Lake.

layer (all vegetation below 50 cm in height) and 10 representing a population with maximum complexity with no apparent disturbance.

2.3.3. Metal analysis

Soil pH was measured in water and a neutral salt solution pH (0.1 M CaCl₂) (260). Total metal analyses were performed as described in (257), (260) and (53). Bioavailable metals were estimated by extracting 5 g of soil with 20 mL of 0.01M LiNO₃ in a 50 mL centrifuge tube in a shaker under ambient lighting conditions for 24 h at 20 °C (260, 261). The pH (LiNO₃) of the suspension was measured prior to centrifugation at 3000 rpm for 20 minutes, with filtration of the supernatant through a 0.45 µm filter into a 20 mL polyethylene tube and made to volume with deionized water. The filtrate was preserved at approximately 3 °C for analysis by ICP-MS. The quality control program was completed in an ISO 17025 accredited facility (Elliot Lake Research Field Station of Laurentian University). It included analysis of duplicates, Certified Reference Materials (CRM's), Internal Reference Materials (IRM's), procedural and calibration blanks, with continuous calibration verification and use of internal standards (Sc, Y, Bi) to correct for any mass bias. All concentrations were calculated in mass/mass dry soil basis. The data obtained for all elements of interest in analyzed CRM soil samples were within ± 12% of the certified level. Metal content in leaves was determined according to the protocol described in (257) and (260).

2.3.4. Statistical analysis

Metal levels in soil and tissue samples were analyzed using SPSS 7.5 for Windows, with all data being transformed using a log₁₀ transformation 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, were performed to determine significant differences ($p < 0.05$) among the sites. Data from limed and non-limed areas were compared using the Student's *t* test.

2.3.5. DNA extraction

Total DNA was extracted from fresh frozen leaf material using the cetyltrimethylammonium bromide (CTAB) extraction protocol as described in (262) and (263). The protocol used contained some modifications deviating from the Doyle and Doyle (264) procedure. The stock CTAB buffer (2% cetyltrimethylammonium bromide, 100 mM Tris HCl [pH 8.0], 1.4 M NaCl and 20 mM EDTA [pH 8.0]) was pre-heated each morning to 60 °C in a water bath until the salt was dissolved. A working stock was prepared everyday by adding 1% polyvinylpyrrolidone (PVP) and 0.2 % β -mercaptoethanol to 2X CTAB buffer. Exactly 20 mL of CTAB buffer was transferred into eight 50 mL centrifuge tubes and heated in a water bath at 60 °C. Leaves were ground using liquid nitrogen until a fine powder was obtained. The powder was then transferred into the centrifuge tubes containing buffer. The tubes were left in the water bath at 60 °C for 45 minutes and inverted each 10 minutes. An equal volume of chloroform/octanol mix (24:1) was added to the tubes. The tubes were then vigorously mixed by inversion for 5 minutes and centrifuged (12,500 rpm, 15 minutes, 25 °C). The aqueous phase (top) was transferred to a new set of tubes and washed twice more with equal volumes of 24:1 chloroform/octanol (12,500 rpm, 5 minutes, 25 °C). An equal amount of isopropanol was added to the tubes and gently inverted to allow the DNA to precipitate. The tubes were then stored overnight at -20 °C. The next day, the tubes were centrifuged (6,500 rpm, 5 minutes, 4 °C). Afterwards, they were decanted and the DNA pellet was washed with 5 mL of 70% ethanol. Once completely dry, the pellet was

dissolved in TE buffer (1:10 EDTA/Tris HCl), transferred into 1.5 mL properly labeled micro-centrifuge tubes and stored at -20 °C until further use.

2.3.6. ISSR analysis

A total of 15 ISSR primers were pre-screened for polymorphism and reproducibility. Of these, eight primers were identified. These included 17899A, 17899B, UBC 841, UBC 825, ISSR 5, 8, 9 and 10. Five of these eight primers (ISSR 5, ISSR 10, 17899A, UBC 825, UBC 827 and UBC 841) produced strong bands and were selected for the ISSR analysis.

PCR amplification was carried out as described in (263). The DNA amplification was done in 0.5 mL tubes. Each reaction contained 2 mM MgSO₄, 1X PCR Buffer, 0.5 μM of primer, 0.2 mM of dNTP stock (dATP, dTTP, dCTP and dGTP) 0.2 ng/μL of DNA, 0.625 Units of Taq polymerase (Bio Basic Inc.) and autoclaved distilled water to a final volume of 25 μL. All samples were covered with one drop of mineral oil to prevent evaporation and amplified with the Eppendorf Mastercycler gradient. The program was set to a hot start of 5 minutes at 95 °C followed by 2 minutes at 85°C which the *Taq* mix was added, then 42 cycles of 1.5 minutes at 95 °C, 2 minutes at 55 °C and one minute of 72 °C. A final extension of 7 minutes at 72 °C after which samples were removed from the thermocycler and placed in the -20 °C freezer until further analysis.

After the DNA samples were amplified, they were separated for analysis on a 2% agarose gel in 0.5x TBE with ethidium bromide. Five μL of 6x loading buffer were added to the PCR products and 10 μL of this solution was loaded into the wells of the gel. The gel was run at 3.16 V/cm, documented with the Bio-Rad ChemiDoc XRS system and analyzed with Image Lab Software.

The amplified ISSR bands produced were scored as either present (1) or absent (0) to determine the variations within and between populations. PopGene software version 1.32 was used to determine the percentage of polymorphic loci, observed and effective number of alleles, Nei's gene diversity and Shannon's information index. The genetic distances were calculated using Jaccard's similarity coefficients with Free Tree Program version 1.50. A neighbor-joining dendrogram was produced from the similarity coefficients, the method starts with a star-like tree with no hierarchical structure and in a stepwise fashion finds the two operational taxonomic units that minimize the total branch length at each cycle of clustering. The unrooted tree generated by the neighbor-joining method is constructed under the principle of minimum evolution (265).

2.4. Results

2.4.1. Tree species richness, abundance, and forest population health

White birch (*Betula papyrifera*) was the most abundant species representing 68% and 47% of the tree communities in non-limed and limed sites, respectively (Table 1). *Salix spp.*, *Pinus resinosa*, and *Populus tremuloides* were more abundant in limed compared to non-limed sites, whereas *Acer rubrum* and *Pinus strobus* were more prevalent in non-limed than in limed sites. The Shannon-Wiener diversity index was higher in limed and reference sites than in non-limed sites. Tree species richness was 4, 6, 7.7 for non-limed, limed and reference sites, respectively (Table 2). Overall, the mean forest population health index revealed a significant improvement in population health in limed sites compared to non-limed areas.

2.4.2. Soil metal analysis

Detectable bioavailable content ranged between 0.07 mg/kg for Sr and 169.1 mg/kg for Mg for limed sites. For non-limed areas, these values varied from 0.10 mg/kg for As to 82.3 mg/kg for Ca. The average concentration of bioavailable Cu and Ni, the main contaminants of interest in Sudbury, were 0.12 and 4.15 mg/kg, respectively. There were significant differences between limed and non-limed sites for bioavailable Al, Ca, Fe, Mg, Mn, and Sr with metals occurring in higher concentrations in non-limed sites except Ca and Mg (Table 3).

Except for manganese, there were no significant differences in the amount of metal content in leaves from limed compared to non-limed sites (Table 4). In general, we tend to see higher levels of Al, Ca, Mg, Mn, Ni, Sr, and Zn in birch leaves compared to the bioavailable amount in soil. This indicates that white birch trees accumulate these elements in their tissues.

Table 1. Tree Species diversity and abundance in limed and non-limed areas within the Greater Sudbury region.

Species	Non-limed sites (%)	Limed sites (%)
<i>Betula papyfera</i>	68.0	47.0
<i>Acer rubrum</i>	14.2	3.0
<i>Quercus rubra</i>	10.1	8.4
<i>Pinus resinosa</i>	1.0	4.2
<i>Salix spp.</i>	0.0	22.6
<i>Populus tremuloides</i>	0.0	10.3
<i>Pinus strobus</i>	5.0	0.0
<i>Pinus banksiana</i>	0.1	4.6
Other tree species	1.7	0.6

Limed sites: Daisy Lake, Hydro-Dam, Kingsway and Kelly Lake

Non-limed sites: Daisy Lake, Hydro-Dam, Kingsway and Kelly Lake

Table 2. Mean index values, species richness and forest population health of the non-limed, limed and reference sites of the Greater Sudbury region.

Type of site	Shannon-Wiener Index	Simpson's Index of Diversity	Species Richness	Population Health
Non-limed	0.76b	0.59a	4.00b	4.00 c
Limed	1.06a	0.45a	6.00a	7.67 b
Reference	0.97a	0.54a	7.67a	10.0 a

*Means in columns with a common subscript are not significantly different based on Tukey multiple comparison test ($p \geq 0.05$).

Non-limed and limed sites: Daisy lake, Dam, Kingsway and Kelly Lake. Reference sites: St. Charles, Onaping falls and Capre

Table 3. Mean concentration of bioavailable metals in the limed and non-limed organic surface horizons (LFH) of soils from the Greater Sudbury region sites (concentrations are in mg/kg, dry weight).

Sites	Elements ^a															
	Al*	As	Ca*	Cd	Co	Cu	Fe*	K	P	Pb	Mg*	Mn*	Na	Ni	Sr*	Zn
Limed	43.3	0.14	252.8	<DL	0.12	7.21	42.07	98.13	6.14	0.16	169.1	3.71	23.43	4.15	0.07	0.48
	±5.9	±0	±102	±0	±0	±3.38	±5.41	±22.3	±2.78	±0	±66.6	±2.6	±9.5	±1.8	±0	±0
Non-limed	77.8	0.10	82.3	<DL	0.31	12.04	108.6	129.22	6.05	0.22	39.78	13.95	13.66	6.85	0.33	1.48
	±12.5	±0	±5.3	±0	±0	±3.71	±17.4	±24.5	±2.25	±0	±5.28	±3.7	±3.5	±2.7	±0	±0.8

^a Results are expressed as mean values ± standard error based on three replicates (n = 3)

* Represents significant difference between treatments based on t-test (p < 0.05)

< DL indicates concentrations below detectable level.

Limed and No limed sites: Daisy Lake, Wahnapiatae Hydro Dam and Kingsway.

Table 4. Mean concentration of total metals in leaves of white birch (*Betula papyrifera*) from the Greater Sudbury region sites (concentrations are in mg/kg, dry weight).

Sites	Elements ^a											
	Al	As	Ca	Cd	Co	Cu	Mg	Mn	Ni	Pb	Sr	Zn
Non-limed	82.15a ±6.03	<DLa ±0	3702a ±383	0.30a ±0.09	1.02a ±0.1	11.9a ±3.4	1415a ±185	776a ±186	38.8a ±5.9	0.58a ±0.16	16.28ac ±1.76	96.95a ±20.5
Limed	63.75a ±12.9	<DLa ±0	5192a ±1255	0.22a ±0.05	0.49a ±0.2	5.17a ±2.6	2647a ±521	244b ±65	29.1a ±13	0.60a ±0.18	8.36a ±2.1	70.48a ±10.7
Reference	70.17a ±4.18	0.02a ±0.02	6826a ±763	0.51a ±0.12	0.37a ±0.1	12.9a ±5.9	2613a ±652	948a ±262	12.1a ±5.2	0.57a ±0.11	41.3bc ±13.6	152.2a ±37.3

^a Results are expressed as mean values ± standard error based on four replicates (n = 4) for non-limed and limed sites, and 3 replicates (n = 3) for reference.

*Means in columns with a common subscript are not significantly different based on Tukey multiple comparison test ($p \geq 0.05$).

< DL indicates concentrations below detectable level.

Limed and Non-limed sites: Daisy Lake, Wahnapiatae Hydro Dam, Kingsway and Kelly Lake. Reference sites: St. Charles, Onaping falls and Capreol.

In general, pH values in limed areas were significantly higher than the adjacent non-limed sites (Table 5). There were no significant differences in pH among non-limed sites, all values being within the expected range for the Canadian Shield soils. Significant differences were observed among limed sites with pH values ranging from 3.75 to 6.75 for the organic layer LFH1. On the other hand, the average pH measured in water was higher than pH (CaCl₂), only for the organic layer LFH1, but not for the mineral horizons where they were not significantly different. The mean values for pH (water) were higher in the organic layer compared to adjacent mineral horizons AE2. The opposite was observed for pH (CaCl₂) with lower values recorded for the LFH1 horizon compared to the mineral AE2.

2.4.3. Genetic diversity

The ISSR primers used in the present study are described in Table 6. Figure 3 depicts PCR products using ISSR primer UBC 841. The percentage of polymorphic loci, the observed number of alleles (Na), the effective number of alleles (Ne), Nei's gene diversity (h) and Shannon's information index (I) are described in Table 7. The level of inter-population polymorphism was 98.6 %. The total gene diversity (HT) and the mean gene diversity between populations (HS) were 0.20 and 0.14, respectively. The population differentiation (GST) value was 0.34 and the estimated gene flow (Nm) was 0.97. The levels of polymorphism per primer were 45.7, 63.2, 62.2, 37.7%, 60, and 66 percent for UBC 825, 17899A, UBC 827, ISSR 10, ISSR5, and UBC 841 primers, respectively.

The levels of genetic variation within populations were moderate to high. In fact the percentage of polymorphic loci (P) varied between 30.1% (Daisy Lake Limed) and 79 % (Wahnapitae Hydro Dam Limed). The observed number of alleles (Na) ranged from 1.3 (Daisy Lake Limed) to 1.8

Table 5. Soil pH for the first three soil horizons in limed and non-limed sites from the GSR.

		Horizon					
		LFH1		AE2		BM3	
Sampling sites	Type	pH H ₂ O	pH CaCl ₂	pH H ₂ O	pH CaCl ₂	pH H ₂ O	pH CaCl ₂
Daisy Lake	Non-limed	4.02	3.87	3.69	3.68	4.16	4.23
	Limed	4.12	4.05	3.90	4.18	4.25	4.38
Kingsway	Non-limed	3.87	2.35	3.92	3.70	4.37	4.23
	Limed	4.67	4.35	4.09	4.03	4.58	4.48
Dam	Non-limed	3.82	3.56	3.75	3.85	4.19	4.20
	Limed	6.75	6.34	5.88	6.41	5.50	5.79
Kelly Lake	Non-limed	3.75	3.37	4.18	3.83	4.51	4.14
	Limed	6.41	6.13	6.24	5.37	5.12	4.41
Capreol (reference)	Non-limed	3.92	3.43	4.11	3.74	4.70	4.54
	Means	4.45	3.99	4.19	4.20	4.50	4.55

pH values of the first three horizons from soils in the Greater Sudbury region using the H₂O and CaCl₂ methods.

Table 6. ISSR primers selected for white birch (*Betula papyrifera*) genetic analysis.

Primer	Sequence (5' - 3')	Fragment number	Fragment size (bp)
ISSR 5	(ACG) ₅ AC	30	280 - 1700
ISSR 10	(CTT) ₅ (CCT) ₆ CT	44	180 - 2010
ISSR 17899A	(CA) ₅ AG	40	302 - 1765
UBC 825	(AC) ₈ T	40	150 - 1650
UBC 827	(AC) ₈ G	31	220 - 1631
UBC 841	GAAG(GA) ₆ YC	24	200 - 1920

Note: Y stands for G or C

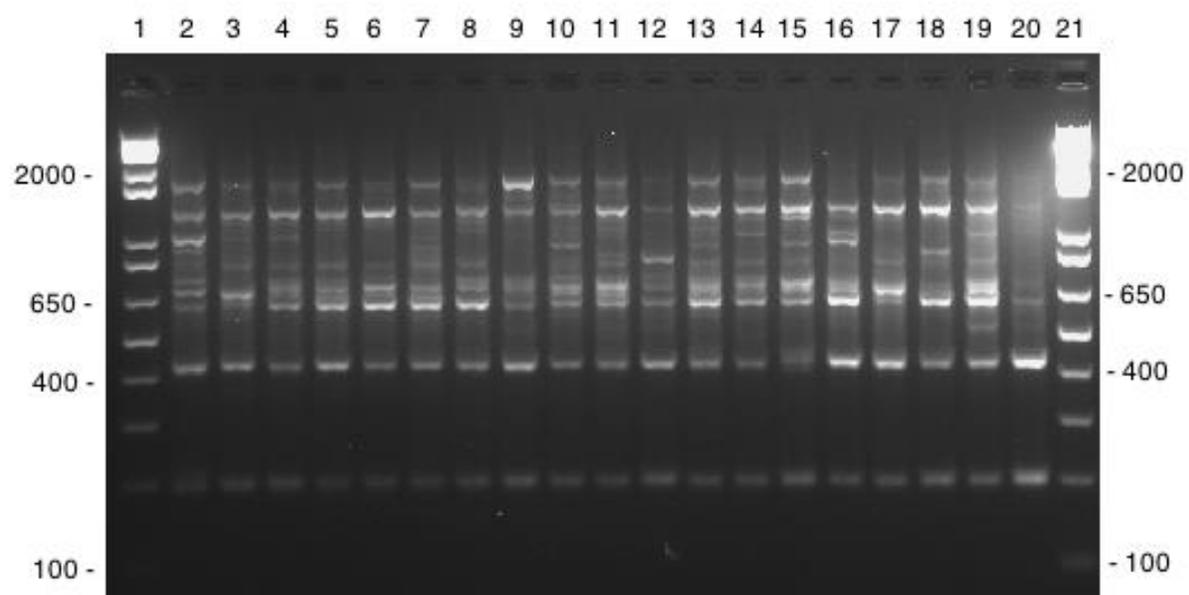


Figure 3. ISSR amplification of white birch DNA samples with UBC 841 primer. Lanes 1 and 21 contain 1-kb+ ladder; lanes 2-20 contain white birch DNA samples.

Table 7. Genetic parameters obtained by ISSR for 7 white birch (*Betula papyrifera*) populations from the Greater Sudbury region (Northern Ontario).

Population	Na	Ne	h	I	P (%)
Daisy Lake NL	1.4115	1.9222	0.1147	0.1766	45.15
Daisy Lake L	1.3014	1.1358	0.0822	0.1279	30.14
Dam NL	1.6316	1.3239	0.1920	0.2927	63.16
Dam L	1.7895	1.3470	0.2108	0.3290	78.95
Kingsway NL	1.3541	1.1764	0.1059	0.1623	35.41
Kingsway L	1.4067	1.1918	0.1180	0.1826	40.67
Capreol	1.4545	1.2088	0.129	0.1935	45.45

Na = Observed number of alleles

Ne = Effective number of alleles (266)

h = Nei's gene diversity (267)

I = Shannon's Information index (268)

P = Percent of polymorphic loci

(Wahnapitae Hydro Dam limed). Nei's gene diversity (h), ranged from 0.08 to 0.21. Shannon's information index (I) revealed a range between 0.18 and 0.33. Overall, the mean levels of polymorphism were 47% and 50% for limed and non-limed populations, respectively.

2.4.4. Genetic relationship

All genetic markers were scored based on the presence or absence of amplification products observed as bands on the agarose gels. A scale of 0 (identical) to 1 (different for all criteria) was used. A dendrogram was constructed based on distance matrix data (Figure 4). The present study revealed clearly that all the pairs of limed and non-limed populations were genetically closely related. Wahnapitae Hydro Dam limed and non-limed cluster together as well as Daisy Lake limed and non-limed. The reference site was separated from the three pairs with 100% degree of confidence.

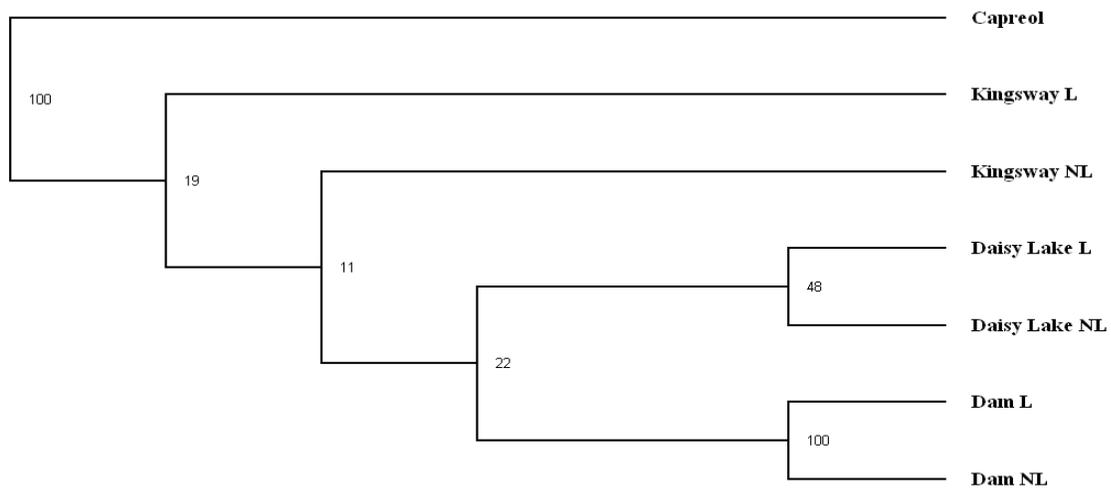


Figure 4. Dendrogram based on ISSR data for 7 white birch (*Betula papyrifera*) populations (Free Tree Program version 1.50).

2.5. Discussion

2.5.1. Forest population diversity, richness and health

Among the various indices used in ecological diversity assessment, the Shannon-Wiener index is the most widely used for comparing diversity between various habitats (269). Shannon's index is an entropy measure, and has mathematical properties useful for combining diversity measures (e.g. when calculating regional diversity). In the present study, Shannon-Wiener and Simpson indices were used to compare white birch growing in limed and non-limed sites. The Shannon-Wiener index is more sensitive to richness and less sensitive to evenness compared to Simpson diversity index (270). The two indices are more robust than species richness - the number of species in the community. Diversity and evenness can be positively or negatively related and evenness and diversity indices are not consistently regulated by richness (270, 271). In general, in both limed and non-limed sites, the diversity indices were small but the diversity and evenness in limed sites were higher than in non-limed sites. The distributions of individual trees in the populations were uneven in all the sites. The reference sites over 30 km of the metal contaminated areas also showed a limited level of diversity that was nevertheless significantly higher than limed and non-limed sites. The most significant differences between limed, non-limed and reference sites were observed in forest population health. There was a significant improvement of population growth quality in limed compared to non-limed sites. But there is still a significant gap between limed areas and reference sites in forest population health that can be reduced through further tree planting or seeding.

It should be pointed out that, there is no standardized method to measure forest population health. In recent studies, ecosystem response to human induced changes has been measured by a

functional trait approach, which classifies vegetation according to the plant characteristics that matter for ecosystem function and processes such as seed sizes and life cycle (272, 273). This method, an alternative to taxonomic classification allows for comparison between ecosystems even if they do not share the same species. The population assessment used in the present study is based on the same approach but it used different parameters - mainly tree canopy and damages and overall plant density. This approach is more appropriate for assessing site recovery following damages caused by air pollutants. The scale of 1 to 10 was easy to apply since both extremes had very damaged sites with no trees and limited or no vegetation to dense forests (used as reference sites) present in the targeted region of Northern Ontario. It should be pointed out that human induced changes of forest ecosystem may lead to a global loss of resilience and the increase of vulnerability of ecosystems to future disturbance (272, 274).

2.5.2. Metal analysis

The estimated levels of total metal concentrations in the soil samples for the different sites from the Greater Sudbury region have been described in previous studies (6, 257, 269). The data obtained for all elements of interest in analyzed certified reference soil samples were within the certification range. In the present study, we first measured the amount of bioavailable metals in soil and the total metal content in birch leaves to determine translocation factors of metals from soil to leaves. The US Environmental Protection Agency (EPA) has defined bioavailability as the level of a metal that can be absorbed by a living organism and induce physiological and/or toxicological response. The bioavailable metal level in soil analyzed was determined to be a critical aspect of phytotoxicity. Although total amounts of metal content in soil of certain sites were high based on previous reports, the percentage of bioavailable metal in the present study was much lower (5, 7, 257, 260). This indicates that the metal amount readily available to plants

is small and therefore the toxicity of such contaminated soils might be limited. There were significantly lower levels of bioavailable Al, Fe, Mn, and Sr in limed sites compared to non-limed areas. The amounts of bioavailable Ca and Mg are measurably higher in limed areas, which reflect the addition of calcitic and dolomitic lime.

The general trend shows that the amount of metals in white birch leaves is higher than the amount of bioavailable metal in soil. This indicates that white birch plants are able to accumulate metals in their tissues. Translocation of metals from soil or roots to above ground tissues is a crucial physiological process in an effective utilization of plant to remediate polluted sites (53, 275, 276). The accumulation of metals in leaves may suggest that this species that represents over 50% of all the tree species in the targeted regions plays a major role in phytostabilization (a component of phytoremediation) of restored forests, since it is able to grow and survive on heavily contaminated soil.

Soil pH is the most important parameter influencing metal-solution and soil-surface chemistry. Soil pH is commonly measured in water or 0.1 M CaCl₂. Soil pH in CaCl₂ is usually preferred as it is less affected by soil electrolyte concentration and provides a more consistent measurement (277). It is commonly used to compare data from different geographical areas under various soil conditions. In general, metal adsorption is relatively low at low pH values and it increases at intermediate pH from near zero to near complete adsorption (277, 278). If the pH of soil is too low, the availability of nutrients to plants may be disrupted as seen in the barren lands of the Sudbury region (66, 259). A low pH such as in the GSR is detrimental to plant growth, not because of the acidity itself, but because of imbalances in nutrient levels. For example, phosphate is poorly available, and Al and Mn may be available in toxic concentration. Increasing soil pH will increase the rate of organic matter mineralization. The pH (water) and pH (CaCl₂) of limed

areas were found to be measurably higher than non-limed areas in the various sites and specifically between adjacent areas. The pH values found in this present study shows that the liming of soils even as far as 30 years ago continues to produce long-term advantages. This may be the result of soil detoxification requiring roots to penetrate further into the soil and cycling calcium and magnesium from lower horizons to the surface layer. This phenomenon is referred to as a cation pump (259, 277). High pH values are also reflected on the metal content, as there were significantly lower bioavailable concentrations of aluminum, iron and strontium in limed areas.

2.5.3. ISSR analysis

Determining forest population sustainability is a critical process in monitoring land restoration. A decline in genetic variability may stunt the species gene pool and therefore decreases its chances of survival in environmental stresses. In the present study, white birch populations from limed and non-limed areas were analyzed using ISSR markers to determine the level of genetic variability. The levels of polymorphic loci were moderate to high ranging from 30% to 79%. No significant differences in polymorphisms were observed between populations from limed and non-limed areas. This lack of significant differences for genetic variability among populations despite difference in metal content in soil and white birch leaves is consistent with previous studies in conifers (53, 279, 280).

This is in contrast to data observed in herbaceous species such as *Deschampsia cespitosa* where the level of metal accumulation reduced significantly the level of genetic variation (257). Metals impose severe stress on plants, especially in the rooting zone, which has led to the evolution of metal-resistant ecotypes in several herbaceous species like *D. cespitosa* (115). Evidence of loss of genetic variation based on isoenzymatic analysis at the population level caused by metal pollution

has been demonstrated in some plant and animal species (114, 115, 281, 282). But, plants possess homeostatic cellular mechanisms to regulate the concentration of metal ions inside the cell to minimize the potential damage that could result from the exposure to nonessential metal ions. These mechanisms serve to control the uptake, accumulation and detoxification of metals (282, 283). This might be the case in white birch trees exposed to certain levels of metals.

The genetic distance matrix was calculated using the Jaccard's similarity coefficient analysis. Overall, the dendrogram generated from the genetic distance data revealed that white birch from limed and non-limed areas within the same site are genetically closely related. The white birch populations from different sites were relatively genetically distant. There is significant genetic variability within and among these populations to sustain environmental challenges within the Greater Sudbury region.

A detailed study to clearly establish how white birch plants cope with metal accumulation in soil along with physiological and genetic mechanisms involved in white birch tolerance to metal such as copper and nickel is highly recommended. This will contribute significantly to our understanding of white birch metal tolerance.

2.6. Conclusion

Lower levels of metals were found in soils of the Greater Sudbury region compared to previous studies. In fact, only small concentrations (> 1%) of the total measured amounts are actually in bioavailable forms. The liming that was performed over 30 years ago has significantly increased soil pH and vegetation in disturbed areas. There was no association between soil metal content and genetic variation in the studied white birch (*Betula papyrifera*) populations. It appears that current stands are genetically sustainable.

Chapter 3: Genetic and metal analyses of fragmented populations of *Betula papyrifera* in a mining reclaimed region: identification of population-diagnostic molecular marker

3.1. Abstract

White birch (*Betula papyrifera*) is an open pollinated species that is dominant in the Northern Ontario after land reclamation. In fact, this species represents 65% of all trees in the Greater Sudbury region. We hypothesized that the exchange of genetic information between fragmented populations by range-wide paternal introgression is possible in wind-pollinated species such as *B. papyrifera*. On the other hand, the effects of metal contamination from mining activities on plant growth and population dynamics are well documented. The main objectives of the present study are 1) to assess the level of genetic variation, gene flow and population sustainability of *B. papyrifera* after land reclamation; and 2) to determine the level of phytoavailable metals in soil and their accumulation in trees. We found that *B. papyrifera* is a Ni and Zn accumulator with a translocation factor of 6.4 and 81, respectively and an indicator of Cu and Pb. The level of polymorphic loci, Shannon index, Nei's genetic diversity, observed number of alleles, and gene flow were determined for the fragmented populations within the targeted region. The percent of polymorphic loci ranged from 28 to 56 %; the gene flow was also low with a value of 0.89 and the population differentiation was very high with a value of 0.36. Two population-diagnostic ISSR markers were identified. They were cloned, sequenced and converted to SCAR markers. Overall, the fragmented populations of *B. papyrifera* in Northern Ontario are genetically sustainable based on the moderate level of intra-population variability.

Key words: *Betula papyrifera*; metal accumulation; population fragmentation; gene flow; Northern Ontario (Canada).

3.2. Introduction

The genus *Betula* belonging to the *Betulaceae* family, consists of roughly 60 species of hardwood. *B. papyrifera* (white birch), is a major component of the Boreal forest of North America. It is found in every province of Canada, except Nunavut, as well as the Northern United States. It is considered a pioneer species; therefore, it is the first to colonize open areas after disasters such as forest fires and deforestation. Little is known about the effects of environmental pollution on white birch because of its low economic value. Studies have shown that this species is sensitive to pH changes and metal contamination (67). However, others believe that long-term exposure to metal contamination has led to resistant *B. papyrifera* populations in the Northern Ontario (87).

Since 1883, the Greater Sudbury region located in Northern Ontario has been well known for its rich deposits in nickel, copper and iron ores. The discovery of the deposits led to the rapid boom of the mining industry and the establishment of many mining companies within the region. For this reason, it is also known as one of Canada's most ecologically disturbed regions. Large quantities of sulfur dioxide and metal particles were released from smelters and roast yards (4). This caused metal contamination and acidification of soils (1, 4, 5). In combination with deforestation, important soil nutrients were washed away by erosion (3). Sites surrounding smelters were completely barren of vegetation with only a few tree species remaining, with white birch being one of the main ones (7). Restoration efforts began in 1973 to reclaim the disturbed lands through planting seedlings, fertilization, and liming. This, along with emission reductions, has helped restore vegetation and nutrient-rich soil horizons (9).

White birch is an open pollinated species that is dominant in Northern Ontario after land reclamation. In fact, this species represents over 50 % of all trees in the region. Because of the long-distance pollen dispersal, gene flow among the fragmented populations is expected to be high. We hypothesized that the exchange of genetic information between fragmented populations by range-wide paternal introgression is possible in wind-pollinated species such as *B. papyrifera* and that the population differentiation will be low.

The main objectives of the present study are 1) to assess the level of genetic variation, gene flow and population sustainability of *B. papyrifera* (white birch) after land reclamation; and 2) to determine the level of phytoavailable metal in soil and accumulation in *B. papyrifera*.

3.3. Materials and methods

3.3.1. Diversity assessment

The distribution and percentage of each plant species was determined for every site. Three quadrants with a radius of 5 m were randomly selected across the twelve sites (Figure 5). They were 1 m apart. Tree species richness in each quadrant of each tree species was calculated based on direct counting. Individuals with trunks larger than 10 cm were counted as trees and those smaller as saplings.

3.3.2. Metal analysis

Soil samples were collected across the twelve sites. These sites were selected based on varying distances from the smelters. Only the first soil layer was analyzed for the present study. Total and phytoavailable metals were determined as described in (260) and (6). To measure total metals, soil or leaf samples were first digested in aqua regia. Roughly, 0.5 - 0.05 g of the sample was digested with 5 mL of concentrated HNO₃ and HCL using a MARS 5 microwave oven. The supernatant was transferred and brought up to 50 mL with deionized water. Metal levels were measured using an Inductively-Coupled Plasma-Optical Emission Spectrometry (ICP-AES), inductively - coupled Plasma-Mass Spectrometry (ICP-MS) and hydride generation atomic emission spectrometry (HG-AAS).

Bioavailable (phyto) metals were measured by adding 5 g of soil with 20 mL of 0.01 M LiNO₃ to 50 mL centrifuge tubes. Tubes were placed in a shaker under ambient lighting conditions for 24 hours at 20 °C (260). The pH (LiNO₃) of the suspension was measured prior to centrifugation at 3000 rpm for 20 minutes, with filtration of the supernatant through a 0.45 µm

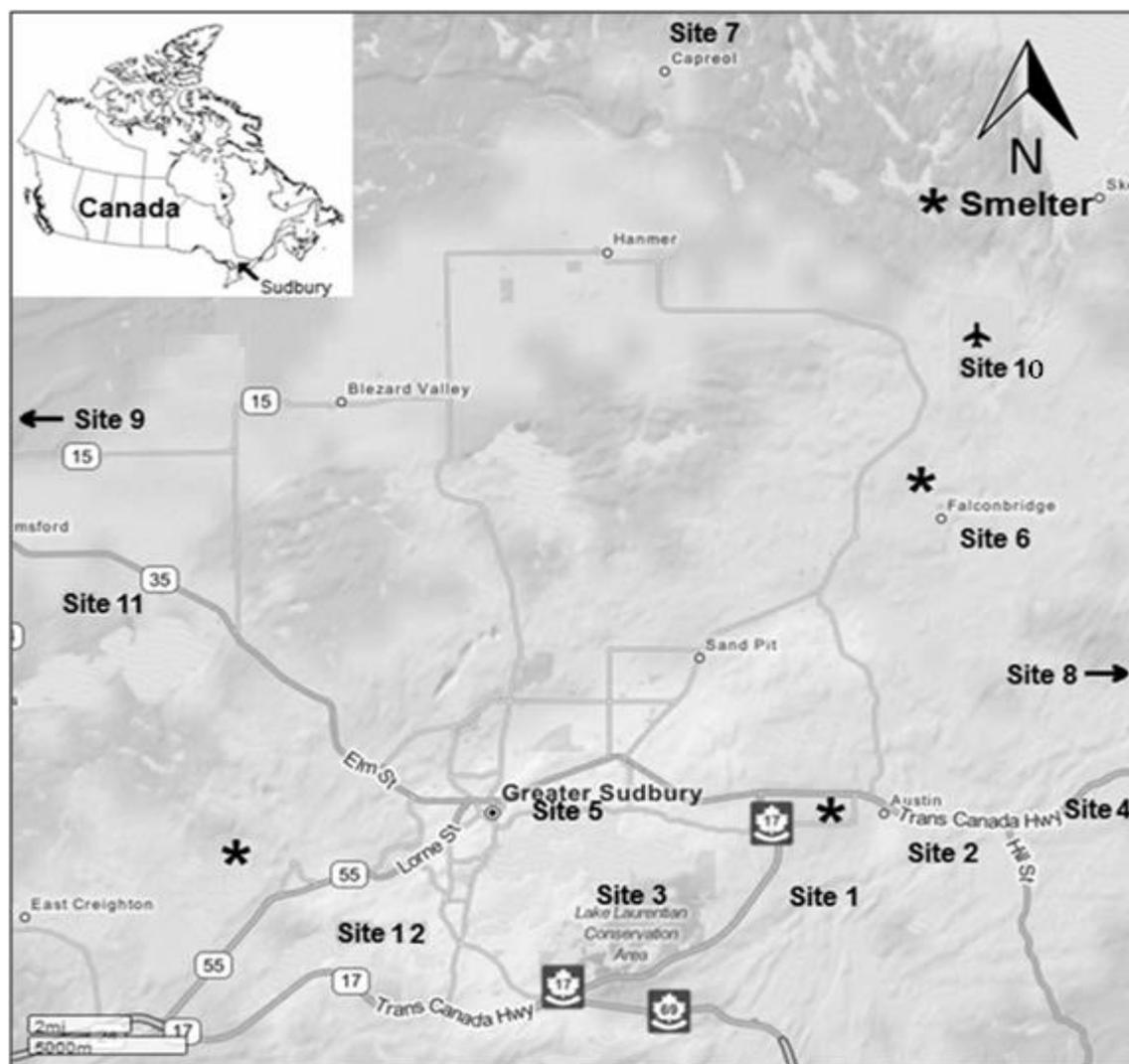


Figure 5. Location of white birch sampling sites in Northern Ontario within the Greater Sudbury region.

Site 1: Daisy Lake; Site 2: Wahnapiatae Hydro-Dam; Site 3: Laurentian; Site 4: Kukagami; Site 5: Kingsway; Site 6: Falconbridge; Site 7: Capreol (reference); Site 8: St. Charles (reference); Site 9: Onaping Falls (reference); Site 10: Airport; Site 11: Azilda; Site 12: Kelly Lake.

filter into a 20 mL polyethylene tube and made to volume with deionized water. The filtrate was preserved at approximately 3 °C for chemical analysis by ICP-MS. The quality control program completed in an ISO 17025 accredited facility (Elliot Lake Research Field Station of Laurentian University) included analysis of duplicates, Internal Reference Materials (IRM's), procedural and calibration blanks, with continuous calibration verification and use of internal standards (Sc, Y, Bi) to correct for any mass bias. All concentrations were calculated in mass/mass dry soil basis.

3.3.3. Statistical analysis

The metal data were analyzed using SPSS 20 for Windows, with all data being normalized. Variance-ratio test was done 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 ($p < 0.05$) among the sites. The translocation factors (TF) were determined according to the equations described in (284). It is estimated by the ratio of metal concentration in leaves over bioavailable metal content in soil.

3.3.4. DNA extraction

White birch leaves were collected from the twelve different locations across the Greater Sudbury region. Locations were selected based on soil contamination, previous soil treatments and wind patterns. Up to 40 white birch trees were sampled from each population. Green leaves were collected and flash frozen (duplicates) using liquid nitrogen and stored at -20 °C. The total DNA was extracted from each sample using the CTAB buffer method, established in (9).

3.3.5. ISSR analysis

Eighteen different ISSR primers synthesized by Invitrogen were used to screen a few samples from each white birch population. The DNA amplification was performed by PCR by methods described in (9).

Loading buffer was added to the PCR product and run on 2% agarose gels (3.16 V/cm). Seven primers that showed strong and reproducible amplification pattern were selected for the amplification of all samples. The gels were documented and bands were scored by their presence or absence using a 1 and 0 system.

Data were analyzed using the computer-based software, Popgene 32 and FreeTree program. Popgene 32 is able to calculate multiple genetic parameters when using dominant or co-dominant marker systems. The following parameters were estimated: Nei's gene diversity (h) Shannon's information index (i) and the percent of polymorphic loci per primer. The distance matrix was generated using the Jaccard's similarity coefficient analysis with the Free Tree Program.

3.4. Results

3.4.1. Tree species abundance

The number of trees and shrubs was measured for each of the 12 sites. The data was pooled together to give a general idea of the distribution of the different species found in the sites (Figure 6). The fragmented plant populations in the targeted northern Ontario region were mainly composed of hardwoods (over 90%), with white birch (*Betula papyrifera*) and red maple (*Acer rubrum*) being the predominant species. Conifers represent only a small percentage (<5%) of tree populations. Overall, *B. papyrifera* represents 53% of all trees and shrub species in the targeted region.

3.4.2. Metal analysis

Detailed data on total and bioavailable metals for the five elements of interest are presented in Table 8. As expected, total Cu, Ni, Fe, Pb and Zn were significantly higher in sites located around the smelters compared to reference sites. Only a very small amount of total metal was bioavailable (Table 8).

There were significantly higher levels of Zn and Ni in leaves compared to the bioavailable amount in soil. In fact, the translocation factors were 81 and 6.4 for Zn and Ni, respectively (Figure 7). These values were 0.5, 1.6, and 1.9 for Fe, Cu, and Pb, respectively.

3.4.3. ISSR analysis

Genetic diversity was measured across 12 white birch populations. The selected ISSR primers are described in Table 9. The observed number of alleles (Na), the effective number of alleles (Ne), Nei's gene diversity (h) and Shannon's information index (I) and the percentage of polymorphic

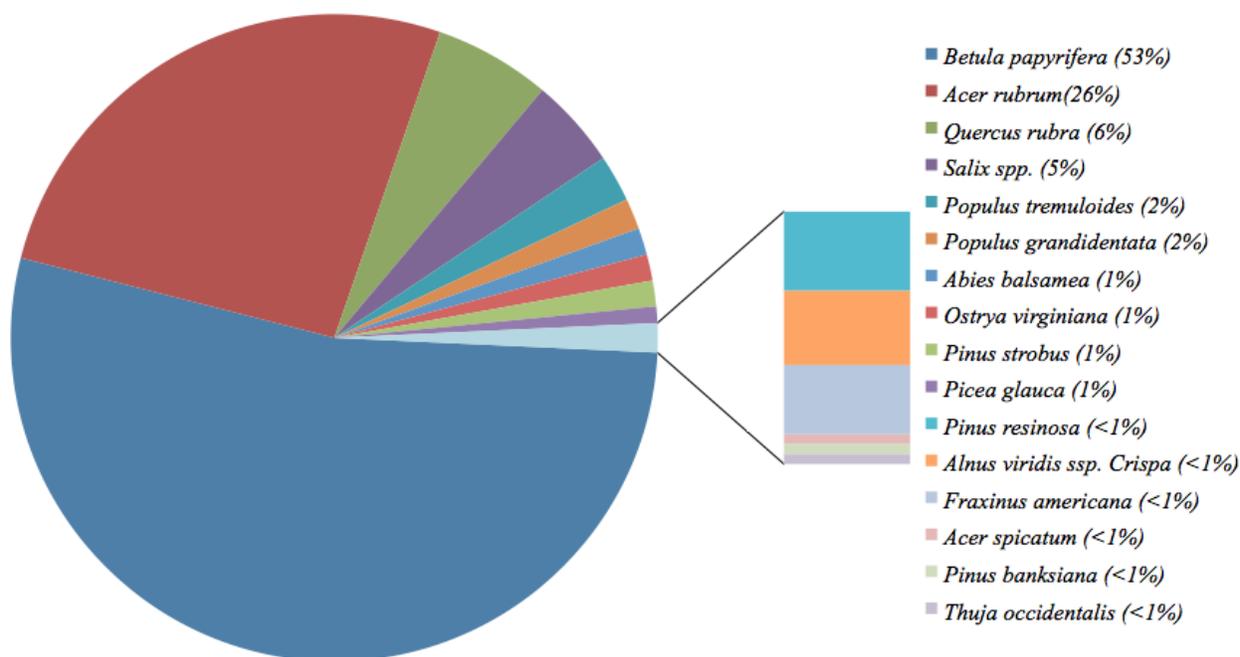


Figure 6. Distribution of tree and shrub species across 12 sites from the Greater Sudbury region.

Sites: Capreol, Onaping Falls, St. Charles, Daisy Lake, Wahnapiatae Hydro-Dam, Laurentian, Kingsway, Falconbridge, Kukagami, Azilda, Airport and Kelly Lake.

Table 8. Total and bioavailable concentration of metals in contaminated and reference soils from the Greater Sudbury region.

Metal (mg/kg)	Cu	Ni	Fe	Pb	Zn
<u>Total</u>					
Contaminated	991 (± 242)	1192 (± 336)	29200 (± 3453)	736 (± 141)	82 (± 13)
Reference	137.67 (± 25.21)	247 (± 57.98)	14367 (± 1625.15)	76.07 (± 10.93)	81.03 (± 10.75)
<u>Bioavailable</u>					
Contaminated	7.53 (± 2.44)	4.89 (± 1.57)	74.92 (± 22.00)	0.23 (± 0.07)	0.97 (± 0.26)
Reference	0.93 (± 0.61)	2.69 (± 1.62)	27.21 (± 11.34)	0.14 (± 0.14)	2.37 (± 1.09)

Contaminated sites include; Daisy Lake, Wahnapiatae Hydro-Dam, Laurentian, Kingsway, Falconbridge, Kukagami, Azilda, Airport and Kelly Lake. Reference sites include; St Charles, Capreol and Onaping Falls. Standard error is shown in parenthesis.

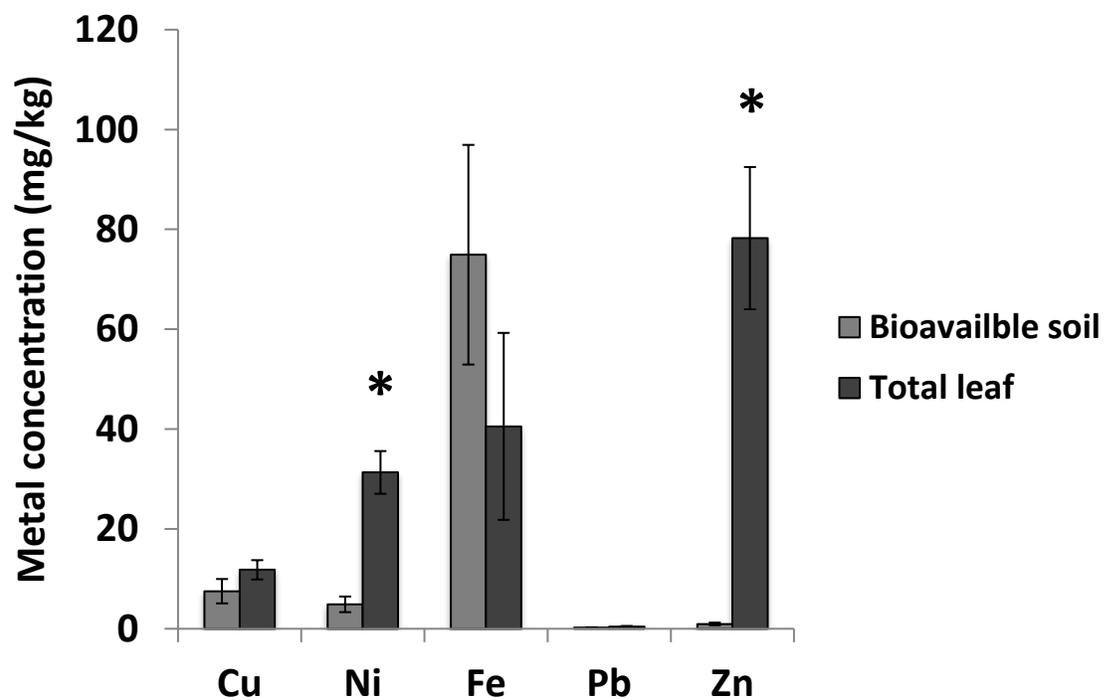


Figure 7. Bioavailable soil and total leaf concentration of Cu, Ni and Zn from contaminated sites.

Sites: Daisy Lake, Wahnapiatae Hydro- Dam, Laurentian, Kingsway, Falconbridge, Kukagami, Azilda, Airport and Kelly Lake. Significant differences were found among sites ($p < 0.05$). Error bars represent standard error.

Table 9. Sequence of selected ISSR primer used to amplify white birch (*Betula papyrifera*) DNA.

Primer	Sequence (5' - 3')	G/C content (%)	Fragment number	Fragment size (bp)	Polymorphism (%)
ISSR 5	(ACG) ₅ AC	65	40	280 – 1700	48.18
ISSR 10	(CTT) ₅ (CCT) ₆ CT	51	62	170 – 2000	33.58
ISSR 17899A	(CA) ₅ AG	35	49	170 – 1863	55.84
UBC 825	(AC) ₈ T	47	48	150 – 1751	48.30
UBC 827	(AC) ₈ G	53	44	220 – 1860	56.00
UBC 841	GAAG(GA) ₆ YC	56	38	200 – 1920	46.89
ECHT 5	(AGAC) ₂ GC	60	53	145 – 2000	47.17

note: Y stands for G or

loci (%), were also calculated (Table 10).

The number of observed alleles (N_a) ranged from 1.28 (Kingsway) to 1.56 (Falconbridge) with a mean of 1.44. The effective number of alleles (N_e) ranged from 1.13 (Kingsway) to 1.221 (Dam) with a mean of 1.18. Nei's gene diversity index (h) ranged from 0.08 (Kingsway) to 0.13 (Falconbridge) with a mean of 0.11. Shannon's information index (I) ranged from 0.13 (Kingsway) to 0.22 (Falconbridge) with a mean of 0.18. The percent of polymorphic loci (%) ranged from 28.4 (Kingsway) to 56.0 (Falconbridge) with a mean of 43.9. The gene flow was low across populations with a value of 0.89 and the population differentiation (G_{st}) was high with a value of 0.36. No significant difference was found between the contaminated and reference sites for all the genetic parameters estimated (Table 10).

The distance matrix was generated using Jaccard's similarity coefficient (Table 11). Overall, the genetic distances were high with values ranging from 0.31 (Kingsway – Wahnapiatae Hydro-Dam) to 0.71 (Kingsway - St. Charles) with a mean of 0.59. There were no associations between genetic and geographic distances.

3.4.4. Population diagnostic molecular markers

Two population diagnostic ISSR markers were identified in samples from the St. Charles site (Figure 8). These diagnostic bands of 469 and 399 base pairs were extracted, cloned and sequenced. Their consensus sequences are described in Figures 9 and 10.

Table 10. Genetic parameters generated from white birch ISSR data using Popgene 32.

Population	Na	Ne	h	I	P (%)
Metal - Contaminated					
Daisy Lake	1.3234	1.1474	0.0885	0.1369	32.34
Dam	1.4431	1.2211	0.1316	0.2016	44.31
Kingsway	1.2844	1.1311	0.0802	0.1245	28.44
Laurentian	1.5000	1.2059	0.1288	0.2035	50.00
Kukagami	1.5240	1.1981	0.1247	0.1986	52.40
Falconbridge	1.5599	1.2124	0.1345	0.2150	55.99
Airport	1.4012	1.1742	0.1066	0.1665	40.12
Azilda	1.5419	1.1982	0.1254	0.2005	54.19
References					
Capreol	1.3473	1.1374	0.0851	0.1349	34.73
St Charles	1.4551	1.1942	0.1175	0.1836	45.51
Onaping Falls	1.4491	1.1898	0.1168	0.1831	44.91

Primers are as followed; UBC 825, 17889A, UBC 827, ISSR 10, ISSR 5, UBC 841 and ECHT 5.

Genetic parameters are as followed; Observed number of alleles (Na), Effective number of alleles (Ne), Nei's gene diversity (h), Shannon's Information index (I) and Percent of polymorphic loci (P).

Table 11. Distance matrix of white birch populations from the Greater Sudbury region.

	Daisy Lake	Dam	Kingsway	Capreol	Laurentian	Kukagami	Falconbridge	St Charles	Onaping Falls	Airport	Azilda
Daisy Lake		0.6019	0.3098	0.3441	0.6383	0.6387	0.6468	0.6824	0.6303	0.6482	0.6118
Dam			0.5858	0.5952	0.5256	0.5021	0.5327	0.6750	0.5940	0.7016	0.6129
Kingsway				0.3422	0.6313	0.6255	0.6919	0.7142	0.6089	0.6615	0.6232
Capreol					0.6255	0.6379	0.6625	0.6744	0.6232	0.6536	0.6359
Laurentian						0.3805	0.4309	0.6270	0.6048	0.6302	0.6428
Kukagami							0.4008	0.6106	0.5943	0.6363	0.5686
Falconbridge								0.6299	0.6192	0.6653	0.6044
St Charles									0.5433	0.5962	0.5907
Onaping Falls										0.4951	0.5064
Airport											0.4597
Azilda											

Primers are as followed; UBC 825, 17889A, UBC 827, ISSR 10, ISSR 5, UBC 841 and ECHT 5. The distance matrix was generated using Jaccard's index (FreeTree Program).

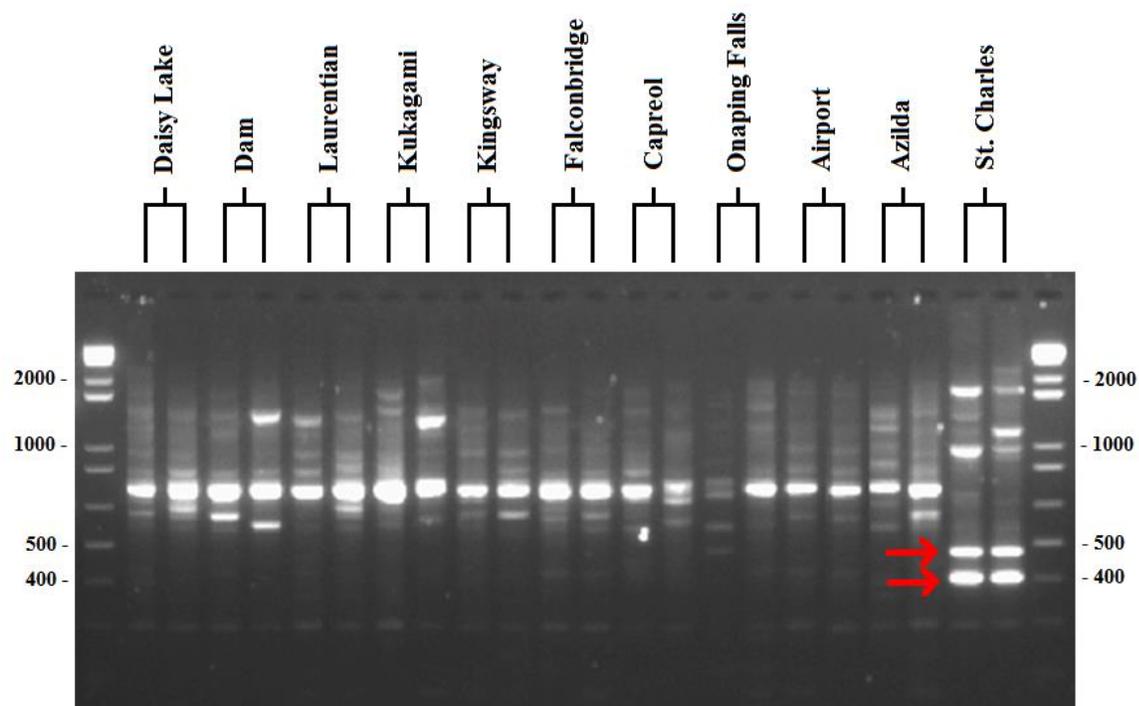


Figure 8. ISSR profile of 11 sites using primer ECHT 5.

* Population-diagnostic bands are marked by arrows.

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1  AGACAGACGC CCGGGCTAAA TGTGATCAG GAGTACATAA TGGGAACAAT ATTGCCAGGT
61 AGACCAATAG CCAATGTGTG CTTCAAGACA TATGGGTTTA TGAGCATGAG TCAAGCTATC
121 GCATTTCTTA GTGATTTCAA ATTGGGGCAC TACATGAAGA TCCCTCCAAG ATCAATGTTT
181 CTTGTACAGG TATCTTCACT ATTCTCTACA CTCGAACAAT TCACCAATAT TATATTTCTG
241 ACTTCTCATT ATATATACAG TAAAAATTGT AGTAAATCAA CCAATTATGT GATATTACAT
301 AATATTTTCA TGCATTCTA GTTCCTTGGG ACAATCATCG CAGGAACAAT CAATCTCAGC
361 GTGGCATGGT GGCTTTTGAC CACTGTCGGA AACATATGCC AACCAAACAT CTCTGAAAAC
421 AGTCCATGGA CATGTCCCAG CTCCCATGTC TTCTACGATG CGTCTGTCT

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Figure 9. Consensus sequence of a population-diagnostic ISSR marker (469 bp) from *Betula papyrifera* using primer Echt 5 (St. Charles).

* ISSR primers are underlined.

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1  AGACAGACGC TCTCGGATTC TGGTGTCCAA ATCAGAGGCA TGAGAATCGT GAGATGACAA
61  TGACGAATAA ACATCAGCCA CTTGAGCCT ACATTTGGGC TTCTCAAATC TGATTAAACT
121 ATCAATGGAC TTTAGAGCAG CTGATGGCAC GTGGATATCA CCATTTAGTT CCTCCCTAAC
181 GTAGGACCCA CCATTCCAAG CCTCACAACT TGGTGCTGCA GCACAGTAAT TGATGATTCC
241 CCAGTGATCA AGAAACCGAA CAATCCGAGC TAAATCTTCA TTATCAACGC CAACTACCAA
301 TTCTTGGCAC TCAGAGATCA TGAGCCTCTT TTCTGGATTT TCCATGTATT TGGCAACAAT
361 GTAGTTCCTA CATGCCATGT ATTTCTCTGG CGTCTGTCT

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Figure 10. Consensus sequence of a population-diagnostic ISSR marker (399 bp) from *Betula papyrifera* using primer Echt 5 (St. Charles).

* ISSR primers are underlined.

3.5. Discussion

3.5.1. Metal analysis

White birch is one of the most important tree species when it comes to the rehabilitation of disturbed regions in Ontario. It is important to understand the long-term effects of metal contamination on the sustainability of species populations. The number of studies analyzing the long-term effects of metal contamination on the sustainability of hardwoods from Northern Ontario is very small (9, 26).

In the present study, the level of metals was measured only in the organic-rich horizon (LFH) since previous studies have shown that this is where most of the airborne metal particulates reside (9, 26, 53, 257, 261). The metal data is consistent with previous studies where the bioavailable form of metals was found to be significantly lower than the total amount (9, 26). This indicates that only small fractions of metals are readily available to plants, thus toxicity should be much lower than previously thought.

The translocation factor was calculated using the bioavailable levels of metals since total levels do not represent forms that are readily available to plants (26). Results show that white birch accumulates Ni and Zn in their leaves (26). No significant accumulation of Cu, Fe, and Pb in leaves was detected. This is consistent with other studies, where white birch growing in metal contaminated areas accumulated higher concentrations of metals in their leaves (4, 98). Freedman and Hutchinson (1980) reported that leaves from white birch growing in close proximity of smelters had Ni and Cu concentrations of up to 170 mg/kg and 80 mg/kg, respectively (4). Our findings show that since then, emission control and the land reclamation program has helped lower accumulation in white birch leaves.

B. papyrifera (white birch) might therefore play a role in the phytoextraction of Zn and Ni. It can be considered as indicator of Cu and Pb since the amount in leaves was similar to the bioavailable content in soil. Since it represents up to 53% of all tree species in the targeted sites, it can contribute to the phytostabilization of restored forests, because it is able to grow and survive on heavily contaminated soil.

3.5.2. Molecular analysis

Genetic variation is one of key characteristics of forest sustainability. If genetic variation is low, the overall gene pool of a population will be small and could result in its extermination or a population bottleneck if a stress occurs (104). In this study, the genetic variation of 12 white birch populations was assessed using ISSR. This technique possesses many advantages compared to earlier molecular marker systems such as SSR and RFLP. With its high throughput and lack of need for genetic information, 220 samples were screened with 7 ISSR primers.

Dominant markers such as ISSR provide a cost effective approach of characterizing genetic parameters. However, the uncertainty about the underlying genotypes presents a problem for statistical analysis. The current version of Popgene (version 32) used in the present study is designed specifically for the analysis of co-dominant and dominant markers using haploid and diploid data.

The percent of polymorphic loci ranged from 28.44 to 56.0. This low to moderate genetic variation is smaller than the polymorphism observed in red oak, black spruce and jack pine populations growing in the same region (26, 114, 115). The means of Nei's gene diversity index (h) and Shannon's information index (I) were low (0.113 and 0.177) indicating a low allelic frequency and uneven distribution. In the present study the two generations were compared for

genetic variation and no significant differences in polymorphism was observed between the parental and the offspring generation. Likewise, no significant difference in genetic variation was found between white birch growing in metal contaminated and reference sites. This is consistent with other studies done on other tree species (26, 114, 115). These results suggest that the current level of metals and the limited number of generations are not sufficient to induce detectable selective effects on the targeted populations based on the neutral molecular markers used.

The gene flow among the fragmented populations was low despite the long-distance pollen dispersal of white birch. The exchange of genetic information between fragmented populations of this wind – pollinated species by range-wide paternal introgression appears to be limited. The genetic variation within populations was smaller than other hardwood species growing in the same areas such as red oak and red maples (26, 285). There was no evidence of population isolation, but in general, high values for interpopulation differentiation and low gene flow suggest a risk of population divergence overtime. The values of N_m of 0.88 and G_{st} of 0.36 may lead to the predominance of genetic drift and low genetic variation. It has been suggested that a value of G_{st} lying in the range 0 – 0.05 indicates little genetic differentiation; a value between 0.05 and 0.15, moderate differentiation; a value between 0.15 and 0.25, great differentiation; and values above 0.25, very great genetic differentiation (286–288). Taken together, the potentially limited capacity for dispersal among fragmented populations via both pollen and seeds may explain the high level of genetic differentiation found in white birch populations from Northern Ontario that were analyzed in the present study. This was confirmed by the data related to population relatedness. In fact, 85% of genetic distance values were above 0.50 with an average of 0.60 suggesting that the populations analyzed were not genetically closely related.

It is however well established that the bulk of pollen is deposited near the source plant (289). However, birch as well as the other species (*Alnus*, *Carpinus*, *Corylus*, *Ostrya*, *Fagus*, *Quercus*, *Castanea*) belonging to the order *Fagales* are wind-pollinated trees, generating vast amounts of pollen to ensure a sufficient level of fertilization of female flowers over receptor regions. Their pollen grains are quite small and light to facilitate the transportation of a substantial fraction (up to 1% for some plumes) of the released material over thousands of kilometers if weather conditions are suitable (84). The limited gene flow is likely caused by local conditions rather than species characteristics. It should be pointed out that the site selection was based on different wind directions. Therefore, any effect of wind direction on introgression of alleles between populations would have been detected.

The fact that only two population diagnostic markers were identified in one reference site suggests that the level of gene flow, although small, remain adequate to prevent population divergence. It should be pointed out that gene flow is an extremely complex phenomenon that can occur in many ways, and that can fluctuate over time. The lack of a generally applicable direct method for measuring gene flow hinders its evaluation as a potential cohesive force among con-specific populations (290). But, indirect methods used in several studies such this based upon the distribution of allelic frequencies permit the evaluation of gene flow parameters. The values on Nm described in the present study should be taken as general estimates of "effective gene flow".

The two population-diagnostic bands that were identified were sequenced and transformed to SCAR markers. The primers designed that flanked SCAR sequence amplified genomic DNA from all the populations, suggesting that the sequences were not truly population – specific. This

is an indication that the sequences are present in low copy number in all the populations, but in a higher frequency in the original population where it was identified with ISSR primers.

There exist, however other reasons that could explain why diagnostic marker once converted to a SCAR marker is not specific. The appearance of a band in one population or species and its absence in another could be the result of competition among DNA fragments during amplification (291). Amplified products which are complementary to each other are stabilized by internal base pairing that could prevent amplification by out-competing the binding of random primers (292); this is the most serious problem that leads to the incorrect interpretation of results. The formation of secondary structures including hairpin, by DNA fragments can also interfere with the final application outcome. Since the SCAR system is a more sensitive technique, the above problems will not interfere with the amplification of sequences even if they are present in a low copy number.

3.6. Conclusion

White birch represents over 50 % of the tree species in the targeted region following soil restoration. The total metal levels were found to be high, but the availability of these metals was much lower. Compared to previous data, metal levels are decreasing, possibly due to leaching and the phytoextraction by surrounding vegetation. White birch are accumulating Zn and Ni in their leaves and are indicators of Cu and Pb. The levels of genetic variation were low to moderate. Contrary to our hypothesis, the gene flow among the fragmented populations were low and population differentiation was relatively high in the reclaimed mining region. Populations within the targeted region were distantly related, and diagnostic markers were identified only for one of the 12 populations. No association between the levels of genetic variation in white birch

populations and metal content in the soil was established. The genetic analysis revealed that white birch populations from the Greater Sudbury region are genetically sustainable.

Chapter 4: Contrasting effects of metal contaminations and soil liming on cations exchange capacity and global DNA methylation in *Betula papyrifera* populations from a mining region

4.1. Abstract

The Greater Sudbury region in Northern Ontario (Canada) has been one of the most contaminated regions in the world. Soil liming with dolomitic limestone applications has decreased significantly the level of soil acidity resulting in forest regeneration. This reclamation process does not affect the level of soil metal contamination but results in metals availability decrement. The coping mechanisms of white birch (*Betula papyrifera*) to soil metal contaminations have been recently characterized. The objective of this study was to assess the effects of soil liming and metal contamination on cations exchange capacity (CEC) and whole DNA methylation in *B. papyrifera*. Cytosine and adenine methylations were measured using tandem mass spectrometry (MS/MS) coupled with LC (LC-MS/MS). The present study confirms that liming significantly increases soil pH even over 30 years after dolomitic applications. There was a decrease in the cation exchange capacity of metal contaminated sites (limed). CEC was significantly higher ($p < 0.05$) in limed and reference distal sites compared to non-limed areas. We also saw an increase in cytosine methylation (roots) in metal – contaminated sites compared uncontaminated sites. No significant difference in cytosine methylation level was observed between metal-contaminated limed and non-limed areas. This suggests that metal contamination mostly nickel and copper, might be associated with cytosine methylation.

Key Words: Metal contamination; Soil liming; Cytosine methylation; Cation exchange; Northern Ontario.

4.2. Introduction

The Greater Sudbury region (GSR) has been one of the most contaminated regions in the world. It has been estimated that over 100×10^6 t of SO₂ and several thousands of tonnes of metals including cobalt (Co), copper (Cu), nickel (Ni), and iron (Fe) were released by roast pits and smelters located in the GSR (Northern Ontario, Canada). Soils surrounding smelters were completely barren soon after industrialization started with only a few tree species remaining (*B. papyrifera* was one of the commonest) (7). To reclaim these degraded lands, soil liming and tree planting has been implemented since 1979. This has resulted in improved ecosystem landscapes. *Betula papyrifera* is a dominant tree species of the Boreal forest. It is a pioneer species and rapidly colonizes open areas and it represents over 50% of trees growing in the GSR. It has been reported that *Betula papyrifera* is sensitive to changes in soil acidity and soil metal contamination (9, 67, 293, 294).

Little is known about the adaptation of *B. papyrifera* to soil metal contamination even though it plays such a key role in forest sustainability. Soil liming with dolomitic limestone applications has decreased the level of soil acidity resulting in an improvement of plant population health. This reclamation process does not change the level of metal contamination but affects the availability of some metals (6, 26, 23). Its effects on cation exchange capacity (CEC) and the overall soil fertility is not clearly established. On the other hand, the ecological adaptation process of plants to stressed environments can be associated with cytosine modifications that are environmentally induced. This non-heritable methylations could influence preferential survival (295). Only limited studies on DNA modifications have been conducted under environmental conditions that plants experience in real ecosystems outside artificial laboratory.

The main objective of this study was to assess the effects of soil liming and metal contamination on CEC and whole DNA methylation.

4.3. Materials and methods

4.3.1. Metal and cation exchange capacity

Soil, root, and leaf samples were collected from nine locations throughout the GSR as described in (9) and (293). The sampling sites include four pairs of limed and non-limed areas close to smelters and contaminated with metals (Figure 11). Three distal sites were used as reference. The samples were flash frozen in liquid nitrogen and kept in aluminum foil at -80 °C for total cellular DNA extraction.

Soil pH was measured in de-ionized water and in a neutral salt solution pH (0.1 M CaCl₂) (296). The exchangeable cations (Al³⁺, Ca²⁺, Fe³⁺, K⁺, Mg²⁺, Mn²⁺, and Na⁺) were quantified by ICP-MS analysis of ammonium acetate (pH 7) extracts of soil samples, with the total exchange capacity being estimated by summation of the exchangeable cations (297). Metal analysis was performed as described in (260) and (6). Total concentration of metals was measured after digestion of 0.5 g of soil samples with 10 ml of 10:1 ratio of HF/HCl at 150°C. Total metals were detected using inductively coupled plasma mass spectrometry (ICP-MS).

4.3.2. Whole DNA methylation

The total cellular DNA from fresh leaves and roots was extracted using the CTAB extraction procedure described in (262) and (263). After extraction, this genomic DNA was stored in a freezer at -20 °C.

The general protocol for whole cytosine methylation is described in (298). Nucleoside quantification was determined using Tandem mass spectrometry (MS/MS) coupled

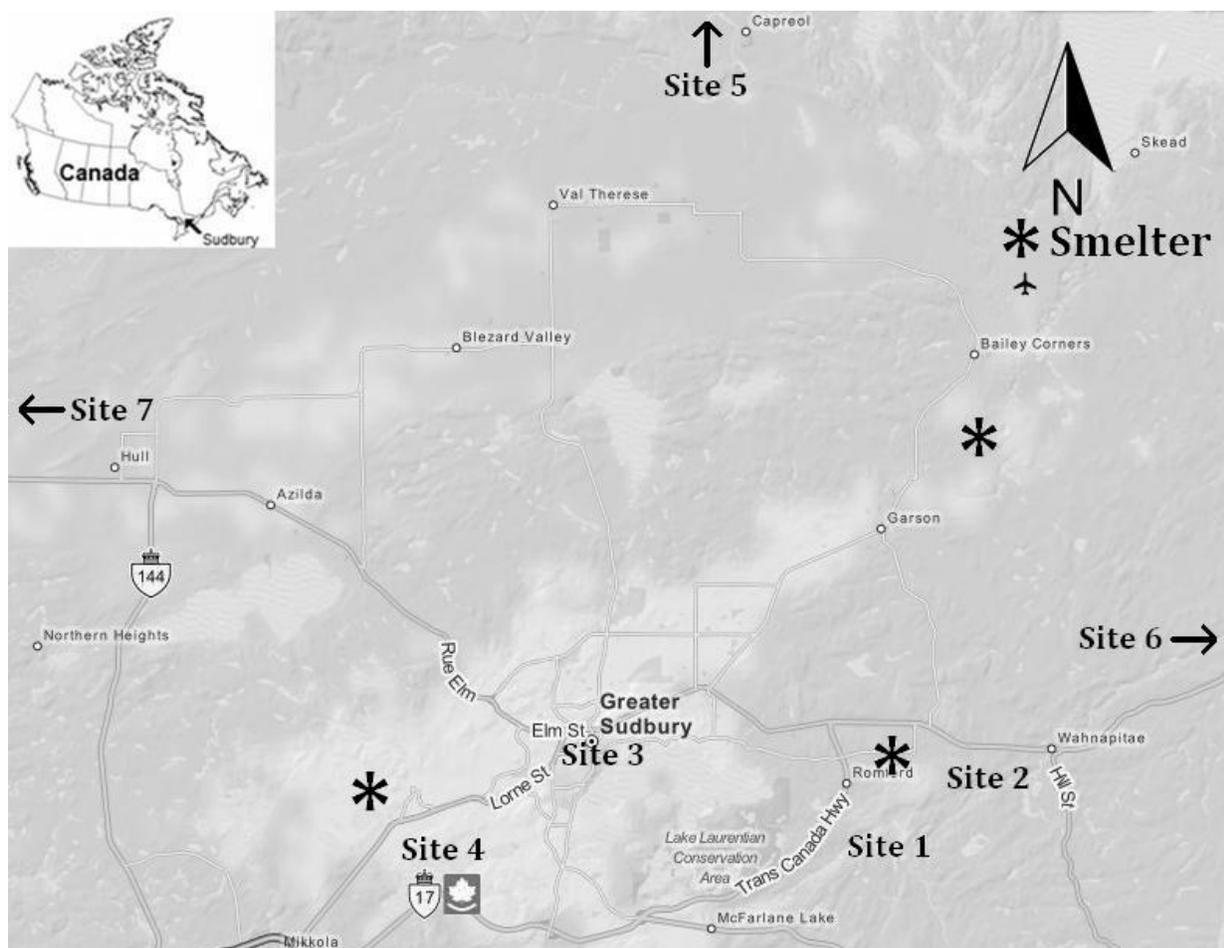


Figure 11. Locations of white birch (*Betula papyrifera*) sampling sites within the Greater Sudbury region.

Site 1: Daisy Lake; Site 2: Wahnapitae Hydro Dam; Site 3: Kingsway; Site 4: Kelly Lake; Site 5: Capreol (reference); Site 6: St. Charles (reference); Site 7: Onaping Falls (reference).

Total cellular DNA was digested with DNA Degradase Plus (ZYMO RESEARCH) following the procedure described by the manufacturer. LC separation was performed on a dC18 2.1x100 mm column at flow rate of 0.2 mL/min. The mobile phase was 15% CH₃OH, 85% H₂O with 1% formic acid and 10 mM ammonium formate. The injection volume was 15 uL. A Waters/Micromass Quattro Micro mass spectrometer was used for the detection of nucleosides. Electrospray ionization in positive ion mode was used to generate ions. Cytosine and adenine methylation levels are reported as [5mdC]/[dG], and [6N-mdA]/dT ratios, respectively.

4.3.3. Statistical analysis

All statistical analyses were performed using SPSS version 20 software (SPSS, Chicago, IL, USA). Data were transformed using log₁₀ transformation to achieve a normal distribution. Analysis of variance (ANOVA) was performed for total metal content, [5mdC]/[dG], and [6N-mdA]/dT values. This was followed by Tukey's HSD multiple comparison analysis to determine significant differences ($p < 0.05$) among means. Data from analysis of samples from metal-contaminated and uncontaminated sites and from limed and non-limed areas were compared using Student – T test ($p < 0.05$).

4.4. Results

4.4.1. Cation exchanges, pH and soil metal contamination

The present study shows that the pH values of samples from areas limed with dolomitic stones over 30 years ago were significantly higher ($p < 0.05$) compared to those of non-limed samples. But the acidity level between metal-contaminated and uncontaminated site were not statistically different (Figure 12). Likewise, the level of cation exchange capacity was significantly higher in limed sites compared to non-limed areas. On the other hand, reference sites show a high level of Ca^{2+} , Fe^{2+} and Mg^{2+} cation exchange capacity compared to contaminated sites. Significant differences were observed for CEC values between metal-contaminated and distal reference sites. CEC values were also higher in limed areas compared to non-limed sites (Figure 13). Metal contamination levels are described in Table 12. No significant different was observed for soil metal content levels between limed and non-limed sites. The concentrations of cobalt (Co), copper (Cu), and nickel (Ni) in soil were higher in limed and non-limed areas compared to distal reference sites.

4.4.2. Whole DNA methylation

In the present study, significant differences ($p < 0.05$) for $[\text{5mdC}]/[\text{dG}]$ were observed between roots from metal-contaminated and uncontaminated sites (Figure 14a). These differences in methylations can be attributed to either higher Ni or Cu contamination or CEC, the two main factors distinguishing the two types of sites. Cytosine methylation levels varied also between roots and leaf samples in some sites (Figure 14b).

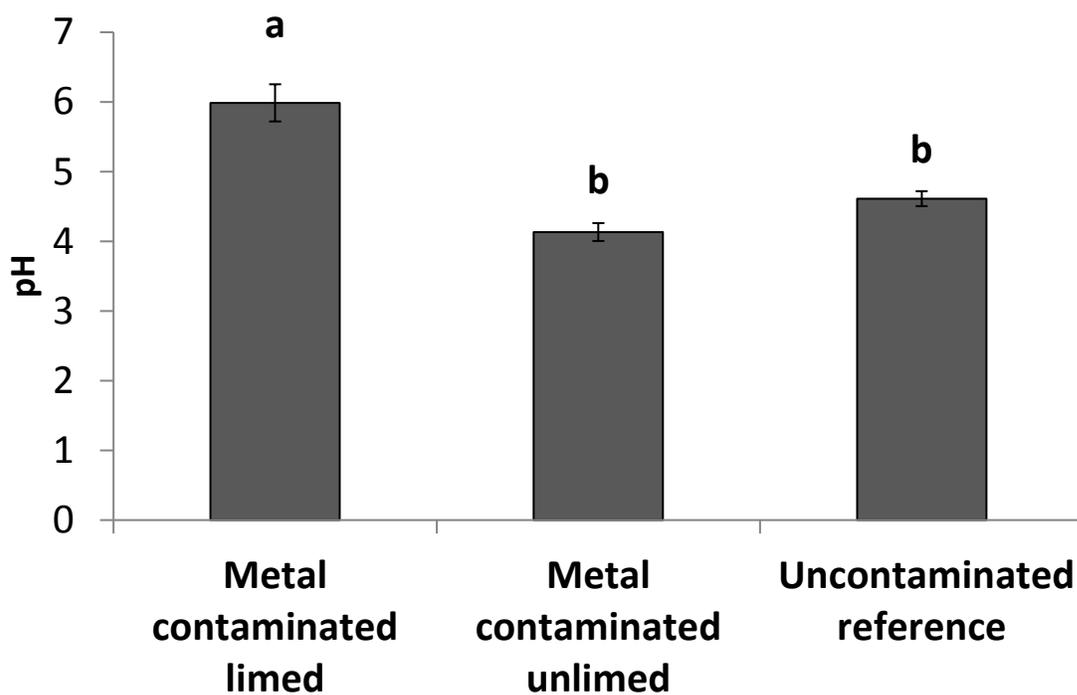


Figure 12. Soil pH in metal-contaminated limed, metal-contaminated non-limed, and metal-uncontaminated reference sites in the Greater Sudbury region.

Means with a common subscript are not significantly different based on Tukey's multiple comparison test ($p > 0.05$). Error bars represent standard error.

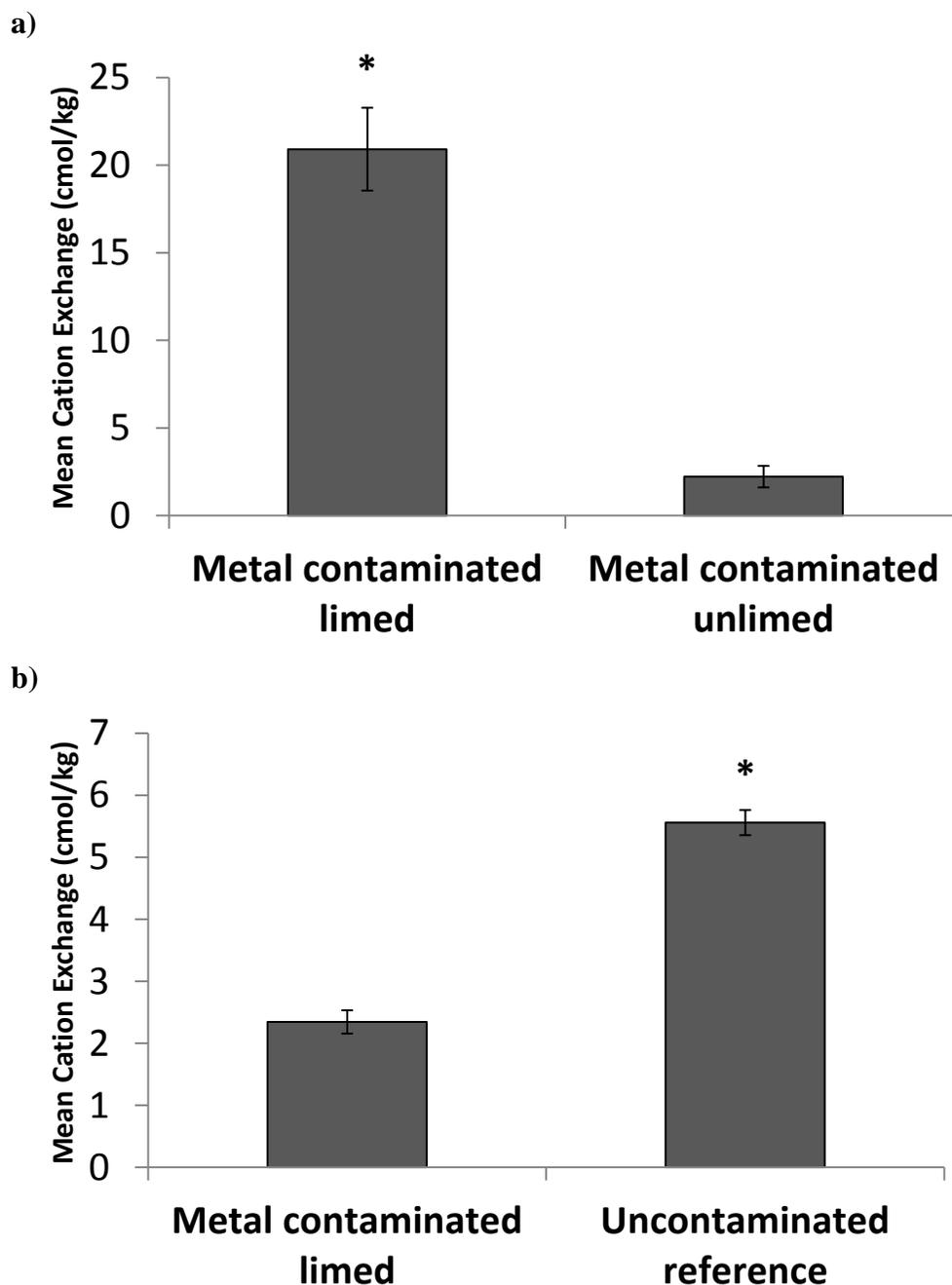


Figure 13. Mean cation exchange capacity based seven metals (Al, Fe, Ca, Mg, K, Mn and Na) for **a)** Metal-contaminated limed and Metal-contaminated non-limed sites **b)** Metal-contaminated and uncontaminated reference sites in the Greater Sudbury region. Error bars represent standard error.

* indicated significant differences based on the Student T-test ($p < 0.05$).

Table 12. Total concentration of nutrients and metals elements in the limed, non-limed and control organic surface horizons (LFH) of soils from the Sudbury region sites (concentrations are in mg/kg, dry weight).

Sites	As	Ca	Co	Cu	Pb	Mg	Ni	Sr	Zn
Limed	31.9a ±59	14526a ±5153	60.2a ±22	1304a ±491	135a ±61	3276a ±213	1552a ±642	75.5a ±3.6	83.1a ±24
Non-limed	45.5a ±20	5010b ±298	56.2a ±10	1255a ±359	141a ±44	2176b ±458	1363a ±392	76.5a ±10	76.6a ±18
Reference	3.46a ±2.2	5880b ±743	10.2b ±3.2	133b ±28	76.2a ±10	1576b ±126	205b ±92	83.5a ±8.4	77.2a ±10

* Results are expressed as mean values ± standard error.

Means in columns with a common subscript are not significantly different based on Tukey's multiple comparison test ($p > 0.05$).

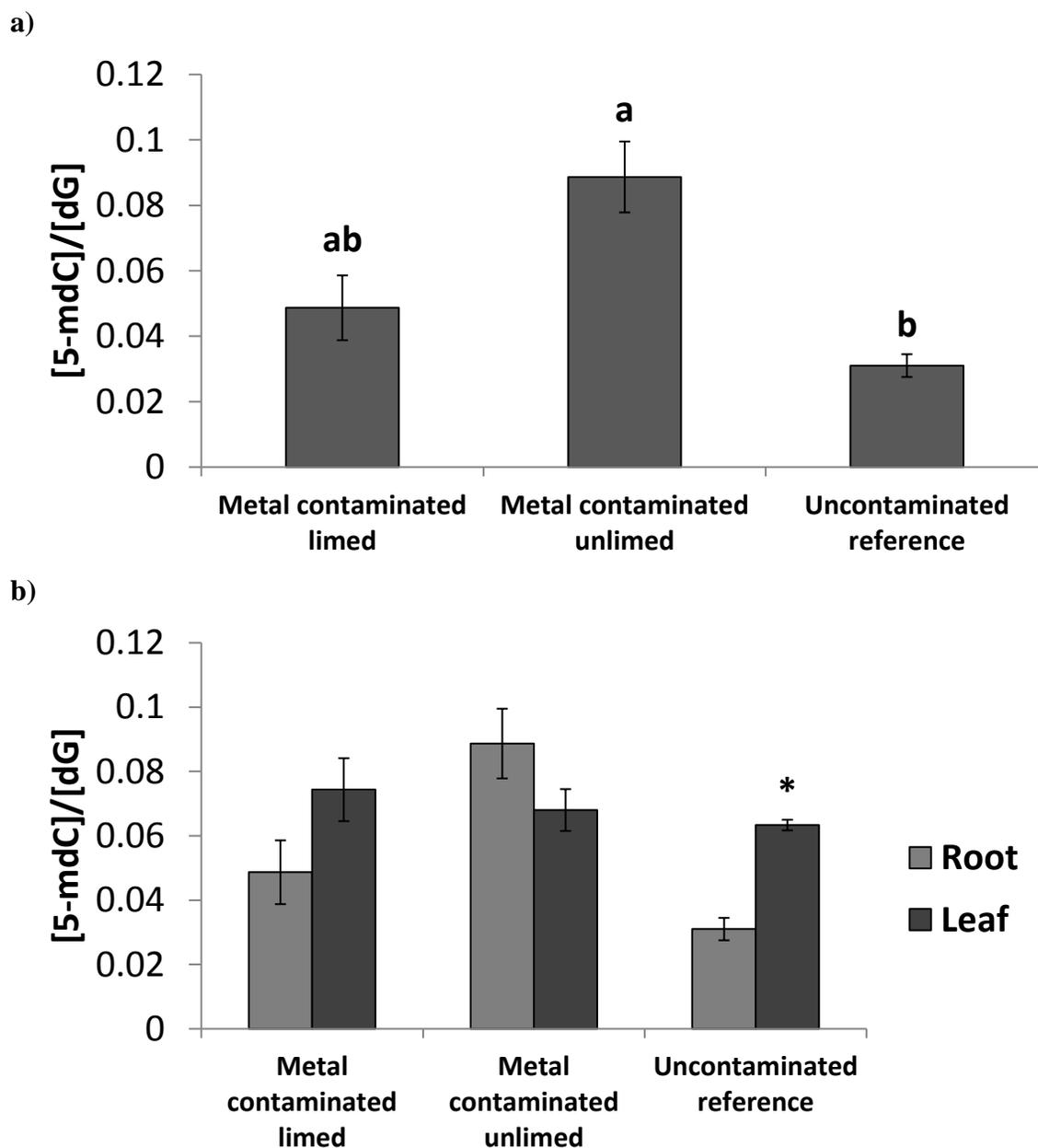


Figure 14. Methylation levels based on ratio of methylated cytosine to guanosine in **a)** roots and **b)** roots and leaves of white birch (*Betula papyrifera*) populations from limed and non-limed areas. Error bars represent standard error.

* indicated significant differences based on the Student T-test ($p < 0.05$).

No significant difference in adenine methylation was observed between limed vs. non-limed sites, metal-contaminated vs. uncontaminated sites, and leaves vs. roots. But the analysis of the chromatogram revealed two peaks that were resolved for the N6-mdA, one within the expected range at 5.65 and a second at 4.82 in 30% of the samples from both contaminated and uncontaminated sites (Appendix 1).

4.5. Discussion

Significant improvements of CEC in limed sites appeared to have contributed to an increased soil fertility and improved plant growth. It should be pointed out that the CEC is directly associated with soil pH. Low-pH stress (such as in non-limed areas) resulting to proton toxicity is considered to be one of the main factors inhibiting plant growth and development in acid soils (299). It also directly limits plant growth via a high hydrogen ion activity (300, 301). A high concentration of protons triggers oxidative stress by inducing an excessive accumulation of reactive oxygen species (ROS) in plant tissues, particularly superoxide radicals and hydrogen peroxide (302, 303). Reactive oxygen species (ROS) is the main toxicity mechanism involved in plant stresses including metal contamination. Toxic metal-induced oxidative stress is usually greater in sensitive plants than in tolerant ones such as *B. papyrifera*. In the present study, soil pH in reference uncontaminated sites was low (similar to metal contaminated non-limed sites) and consistent with the Canadian shields soil acidity. However, the CEC values were similar to limed sites with a high pH suggesting that the association between the two might be overridden by other factors such as organic matter content.

We also consider if epigenetics might be involved in the response of *B. papyrifera* to metal contamination and soil acidity and fertility. Analysis of DNA modifications caused by abiotic stresses have been shown to increase or decrease of hypermethylations, but few studies conducted on the effects of metals in plants reveal that hypomethylation is associated with a high level of metal contamination (249). These two phenomena could be involved in the adaptation of plants to stress (304).

A number of publications have provided convincing evidence that support the role of abiotic stresses including drought and high salinity in DNA methylation (305–308). Reports on the effects of metal contamination, pH, cation exchanges on DNA modifications are limited. The present study suggests that metal contaminations might be involved in DNA methylation

Tandem mass spectrometry (MS/MS) coupled with LC (LC-MS/MS) used in the present study to measure overall levels of DNA methylation is an established approach to nucleoside quantification specifically to measure global cytosine methylation (298, 309). In particular, it is a fast, sensitive, accurate and specific avenue for modified nucleoside quantification at trace (fmol) levels. Other procedures such as methylation-sensitive amplified polymorphism (MSAP) and methods based on bisulfite modifications of DNA that analyze the methylation status of specific sequences are also used in many studies. Each of these methods has its own peculiarities. MSAP approach was recently used to assess the effect of metals on cytosine methylation in *Acer rubrum* (red maple). This technique was sensitive enough to detect quantitative difference in DNA methylation between metal-contaminated and uncontaminated populations in *Acer rubrum* populations growing in the GSR (255). The use of bisulfite sequencing for cytosine methylation would be more informative in mapping the distribution of DNA modifications. But its wide application in plants epigenetic studies is cost prohibitive specifically for species whose genome has not been completely sequenced.

4.6. Conclusion

The present study confirms that nickel and copper are the main contaminants in targeted sites within the GSR and liming increases significantly soil pH even over 30 years after dolomitic applications. There was a decrease of cations exchange capacity in metal – contaminated sites

compared to uncontaminated sites. Cation exchange capacity was higher in limed and distal reference sites compared to non-limed sites. No significant difference in cytosine methylation level was observed between limed and non-limed areas with the same levels of metal contamination. However, we did observe an increase in cytosine methylation when metal contaminated sites were compared to controls (regardless of the lime treatment). This suggests that metal contamination (mostly nickel and copper) might be associated with cytosine methylation.

Chapter 5: Nickel and copper toxicity and plant response mechanisms in white birch (*Betula papyrifera*)

5.1. Abstract

Nickel (Ni) and copper (Cu) are the most prevalent metals found in the soils in the Greater Sudbury region (Canada) because of smelting emissions. The main objectives of the present study were to 1) determine the toxicity of nickel (Ni) and copper (Cu) at different doses in *Betula papyrifera* (white birch), and 2) assess segregating patterns for Ni and Cu resistance in *B. papyrifera* populations. This study revealed that *B. papyrifera* is resistant to Ni and Cu concentrations equivalent to the levels reported in metal-contaminated stands in the GSR. Resistant genotypes (RG) accumulate Ni in roots but not in leaves. Moderately susceptible (MSG) and susceptible genotypes (SG) show a high level of Ni translocation to leaves. Analysis of segregation patterns suggests that a single recessive gene controls resistance to Ni and Cu.

Key Words: *Betula papyrifera* (white birch); nickel and copper toxicity; metal contamination; metal translocation; genetic tolerance; gene expression.

5.2. Introduction

While nickel and copper are essential for plant growth at low levels, exposure to higher concentrations can impede metabolic processes and results in high toxicity (310, 311). Some species adopt an exclusion strategy to avoid excessive uptake and transport of metal ions (285, 294, 312). True excluders restrict metals from entering the plant. Other excluders accumulate metals in roots, but translocation to aerial parts is restricted (312, 313). In accumulator species, metals are concentrated in the above ground parts. Recent field studies showed that *Betula papyrifera* accumulates nickel in leaves, but does not store copper in its tissues (9, 293). Edaphic ecotypes tolerant to high levels of metal contamination have been reported around old mines and smelters (87). Genetic mechanisms involved in metal contaminations are not well defined.

The main objectives of the present study were to; 1) determine the toxicity of nickel and copper at different doses in *B. papyrifera*; and 2) assess segregating patterns for nickel and copper resistance in *B. papyrifera*.

5.3. Materials and methods

5.3.1. Growth conditions

Seeds were collected from the Laurentian University research field site in the Greater Sudbury region, Ontario (Canada) and stored at 4°C. They were germinated on wet filter paper in Petawawa boxes at 27°C. Seedlings were transplanted into pots containing a topsoil/peat moss mixture. They were left to grow for four months at 27°C being watered and fertilized as needed then transplanted into a 50:50 mix of quartz sand/peat moss and left to grow for another month.

5.3.2. Assessment of metal toxicity

The present study focuses only on toxicity of Ni and Cu since they were the most preponderant elements in contaminated sites compared to uncontaminated areas in the GSR (6, 293). To assess the toxicity of these two elements on *B. papyrifera*, plants were treated under controlled conditions in growth chambers. Commercial Ni(NO₃)₂ and Cu(SO₄) salts were used for Ni and Cu treatments, respectively. The experimental design was a completely randomized block with 15 replications per treatment. For nickel, plants were treated with a control (0 mg/kg) and three doses of Ni(NO₃)₂ equivalent to 5.6 mg/kg (5.6 mg of Ni per 1 kg of dry soil), 1,600 mg/kg (1,600 mg of Ni per 1 kg of dry soil), and 4,800 mg/kg (4,800 mg of Ni per 1 kg of dry soil). The dosage of 5.6 mg/kg Ni corresponds to the average of the published bioavailable amounts of nickel in contaminated sites while 1,600 mg/kg Ni is the level of total nickel in contaminated sites in the GSR as described in (6) and (293). For copper, the dosages included 9.16 mg/kg (9.16 mg of Cu per 1 kg of dry soil), 1,312 mg/kg (1,312 mg of Cu per 1 kg of dry soil), and 3,936 mg/kg (3,936 mg of Cu per 1 kg of dry soil) which are equivalent to bioavailable, total at site, and three times the amount of Cu in contaminated sites, respectively. Water treatment (0 mg of

Ni or Cu per 1 kg of dry soil) was used as a control. Potassium nitrate (KNO_3) and potassium sulfate (K_2SO_4) treatments were used to control for the nitrate and sulfate concentrations of the respective metal salts. The 4,800 mg/kg Ni (18 mM of Ni), 1600 mg/kg Ni (6 mM of Ni), and 5.56 mg/kg Ni (0.021 mM of Ni) contained 36 mM, 12 mM, and 0.042 mM of nitrate, respectively. The 3,936 mg/kg, 1,312 mg/kg, and 9.16 mg/kg of Cu corresponded to 30 mM, 10 mM, and 0.063 mM of Cu, respectively. The same molarity was used for K_2SO_4 salts.

Combined treatments including nickel and copper (5.60 mg/kg Ni + 9.16 mg/kg of Cu and 1,600 mg/kg Ni + 1,312 mg/kg of Cu) were also tested to mimic the contaminations levels in targeted sites. All the treatments were administrated in water using a pipette. Damage was assessed every two days on a scale of 1 to 9 described in Table 13. Genotypes with damage ratings of 1 to 3 were considered resistant (RG), 4 to 6, moderately susceptible (MSG), and 7 to 9 susceptible (SG). Figure 15 depicts RG, MSG, and SG.

5.3.3. Assessment of segregation patterns

This experiment was performed in conjunction with the toxicity studies. The pioneer population was composed of metal resistant trees that survived the most severe metal contamination of the GSR (259). To determine segregation pattern in the *B. papyrifera* population, about 400 seeds from over 60 trees representing the first generation families (M_1) from the pioneer trees were bulked. The resulting second-generation seedlings (M_2) were treated with 1,600 mg /kg of Ni or 1,312 mg/kg of Cu. RG, MSG, and SG were identified based on damage rating using the 1 to 9 scale described above.

Table 13. Damage rating scale and plant classification based on reaction to nickel and copper treatments.

% of Leaf area with chlorosis/necrosis	Damage Rating	Genotype Classification
0-10 10-20 20-30	1 2 3	Resistant (RG)
30-40 40-50 50-60	4 5 6	
60-70 70-80 > 80	7 8 9	



Figure 15. White birch (*Betula papyrifera*) plants at 8 days after the 1,600 mg/kg Ni treatment.

From left to right; susceptible genotype (score 9), moderately susceptible genotype (score 6), and resistant genotype (score 1). Damage rating score is on a scale of 1 to 9.

5.3.4. Metal accumulation in plants

Metal levels in soil and plant tissues were analyzed to determine translocation of metals in RG, MSG, and SG as described in (294). The detection of total Ni in soil and plant part digests was determined using inductively coupled plasma atomic emission spectrometry (ICP-AES). The quality control of the ISO 17025 accredited facility (The Forest Resources & Soil Testing (FoReST) Laboratory, Faculty of Natural Resources Management (Lakehead University Center for Analytical Services (LUCAS), Thunder Bay, Ontario, Canada), included analysis of duplicates, Certified Reference Materials (CRMs), Internal Reference Materials (IRMs), procedural and calibration blanks. Continuous calibration verification and use of internal standards (Sc, Y, Bi) was done to correct for any mass bias. The data obtained for all elements of interest in analyzed CRM soil samples were within $\pm 12\%$ of certified levels. Not enough tissues were available after the copper treatments to determine the translocation of this element in plants.

5.3.4. Statistical analysis

The data for damage rating and metal content were analyzed using SPSS 20 for Windows, with all data being transformed using a \log_{10} transformation to achieve normality. ANOVA, followed by Tukey's HSD multiple comparison analysis, were performed to determine significant differences among means ($p < 0.05$).

5.4. Results

Significant differences for damage rating were observed among nickel dosages after eight days of treatment based on ANOVA and Tukey mean comparison tests. Plant reactions to Ni and Cu treatments overtime are summarized in Table 14. There was no significant damage caused by nickel at 5.6 mg/kg dosage. At day 8 after nickel treatments, the mean damage ratings for 1,600 mg/kg and 4,800 mg/kg dosages were 5.7 and 9, respectively. The 4,800 mg/kg dose was extremely toxic as all the plants died within 48 hours of the treatment.

In general, the reaction of *B. papyrifera* to copper treatment follows the same pattern as nickel. However, copper toxicity appears to be more severe than that of nickel since plant treated with 1,312 mg/kg start dying four days after treatments. No toxicity was observed for the 9.16 mg/kg (amount of bioavailable Cu in the natural site) throughout the experiment (Table 14). Significant differences in plant damage were observed between the 9.16 mg/kg and 1,312 mg/kg dosages throughout the eight days of the trial. In fact, the average rating at day 8 for this latter dose was 7.8. This treatment represents the total Cu level in the Greater Sudbury region soils or 143 fold the amount of bioavailable Cu. The highest dose of 3,929 mg/kg resulted in severe damage on plants two days after the treatment and almost all the plants were dead within four days of Cu application.

No significant additive effects of nickel and copper toxicity were observed for the three Ni + Cu dosage combinations tested (Table 14). There were no symptoms observed on plants throughout the experiment for the 5.56 mg/kg Ni + 9.16 mg/kg Cu treatment. The treatment that corresponds to the total amount of nickel and copper (1,600 mg/kg Ni + 1,312 mg/kg Cu) in the sites was as toxic as the copper or nickel treatments alone during the eight days of the treatment.

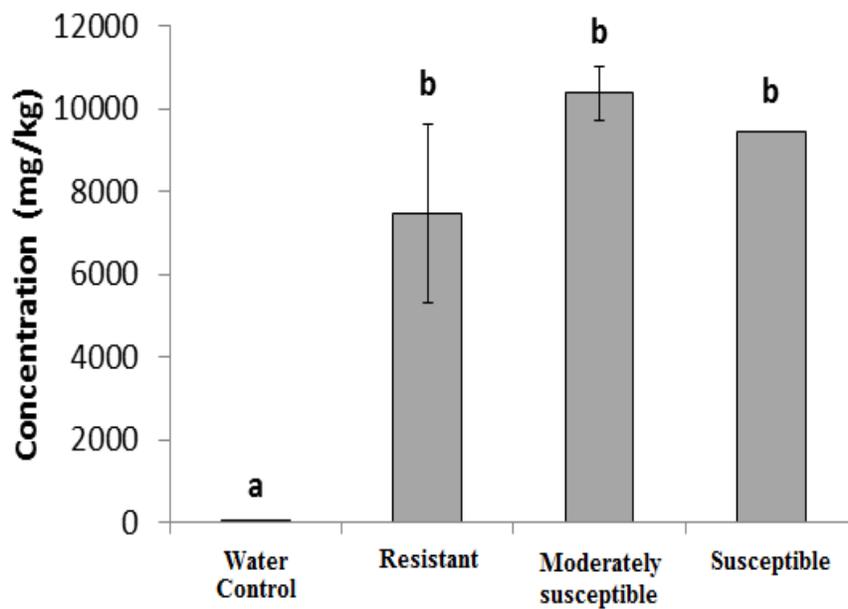
Table 14. Damage rating of *Betula papyrifera* treated with different doses of nickel and copper.

Treatment	Soil concentration (mg/kg)	Damage rating			
		Days after treatment			
		Day 2	Day 4	Day 6	Day 8
Ni	5.56	1.0 ± 0.0 a	1.0 ± 0.0 a	1.0 ± 0.0 a	1.0 ± 0.0 a
	1,600	2.8 ± 1.3 b	5.0 ± 2.3 b	5.3 ± 2.6 b	5.7 ± 2.9 b
	4,800	9.0 ± 0.0 c	9.0 ± 0.0 c	9.0 ± 0.0 c	9.0 ± 0.0 c
Copper	9.16	1.0 ± 0.0 a	1.0 ± 0.0 a	1.0 ± 0.0 a	1.0 ± 0.0 a
	1,312	2.6 ± 1.5 a	6.2 ± 2.2 b	7.3 ± 2.4 b	7.8 ± 2.1 b
	3,936	6.8 ± 2.1 b	8.1 ± 2.2 b	8.5 ± 1.6 b	8.5 ± 1.6 b
Nickel +	5.56 + 9.16	1.0 ± 0.0 a	1.0 ± 0.0 a	1.0 ± 0.0 a	1.0 ± 0.0 a
Copper	1,600 + 1,312	3.1 ± 1.9 b	7.2 ± 2.5 b	8.6 ± 0.9 b	8.8 ± 0.6 b

ANOVA, followed by Tukey's HSD multiple comparison analysis, were performed to determine significant differences among means. Means followed by different letters for each element (nickel, copper, and nickel +copper) are significantly different ($p < 0.05$; $n = 15$).

Based on damage ratings, resistant, moderately susceptible, and susceptible genotypes were identified. Metal analysis revealed that the RG, MSG, and SG classified based on screening with 1,600 mg/kg nickel dosage contains similar levels of Ni in their roots. But the RG contain significantly lower amount of Ni in their leaves compared to MSG (Figure 16).

a)



b)

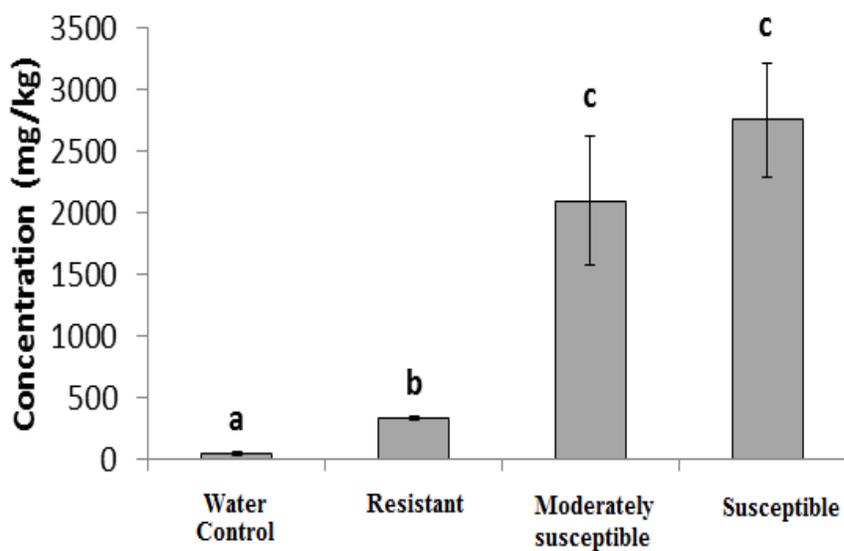


Figure 16. Total nickel content in a) roots and b) leaves of *Betula papyrifera* plants, eight days after treatment with 1,600 mg of Ni per kg of dry soil.

* Means followed by different letters are significantly different ($p < 0.05$; $n = 15$)

5.5. Discussion

In the present study, we focused on testing the toxicity of bioavailable and total Ni and Cu in soil. Bioavailable metals are the proportion of total metals in soil that are available for incorporation into biota (6, 260, 261). We found that resistant RG accumulate less nickel than MSG and SG in their leaves. Hence, tolerance is the main coping strategy involved in *B. papyrifera* resistance. Tolerant genotypes prevent translocation of Ni from roots to leaves while this process was observed in susceptible plants. The MSG showed a delay in symptom expression compared to SG. This variation in degree of tolerance might be controlled by gene modifiers (314). Recent data on metal translocation based on soil, root, and leaf samples collected directly from the field in the GSR indicate that *B. papyrifera* accumulates Ni in leaves (293). This lack of variation for Ni translocation in the field study suggested that all the trees analyzed earlier were either resistant to Ni or that soil nickel levels were insufficient to trigger the mechanism.

For reference purpose, a mean total Ni content of 26.8 mg/kg is considered representative of typical Ni concentration in background soil in Canada. This excludes areas of Ni enriched rocks and Ni bearing mineral occurrences (315). The average total copper concentration in Canada soil is estimated to be 20 mg/kg (316). Overall, this study indicates that *B. papyrifera* is tolerant to low doses of Ni and Cu similar to the bioavailable levels in contaminated sites. The treatments at high dose of 1,600 mg/kg of Ni and 1,912 mg/kg of Cu revealed segregation for reaction to these two metals in the populations analyzed. The grouping of the genotypes based on reaction to Ni for this treatment (1,600 mg/kg) consisted of 27 % tolerant, 28 % moderately susceptible and 35% susceptible. Based on metal content data, moderately susceptible and susceptible were included in the same group of susceptible. This resulted in the proportion of 27 % tolerant and 73% susceptible that is consistent with a ratio of 1 tolerant: 3 susceptible, a characteristic of a

single recessive gene control. A similar trend (ratio of 1 tolerant: 3 susceptible) was observed for copper. In fact, 22% of genotypes in segregating second-generation (M_2) populations were tolerant and 78% were susceptible, which is consistent also with single recessive gene control of resistance. Genetic studies of nickel tolerance are limited, but analysis of copper tolerance in *Mimulus guttatus* revealed a single major dominant gene inheritance (314, 317, 318).

It should be pointed out that the conclusion on segregation analysis for Ni and Cu tolerance is based on observations of the reactions of M_2 progenies representing half sib families from highly tolerant first generation trees growing as fragmented populations in nickel and copper contaminated sites for over 30 years. Previous molecular analysis revealed that all the fragmented populations in the areas are distinct and fertilization in the targeted pioneer population of female gametes with pollens from genotypes that were not resistant to nickel or copper was unlikely (9, 293). This segregation data reveals a trend rather than conclusive insights of the genetic mechanisms of Ni and Cu tolerance in *B. papyrifera*. This first assessment of genetic characterization of Ni and Cu tolerance in *B. papyrifera* needs to be confirmed with other population analyses. In depth analysis will require the production of progenies from self-and controlled pollinations. This is practically difficult and can take over 30 years since *B. papyrifera* is a long-lived open pollinated tree species.

Several studies have demonstrated that oxidative stress is involved in metal toxicity in plants, by either inducing oxygen free radical production or by decreasing enzymatic and non-enzymatic anti-oxidants (319–322). Physiological factors associated with plant defense to metal toxicity may play a role in metal accumulation and plant resistance to nickel in *B. papyrifera* genotypes that we analyzed. In fact, plants possess homeostatic cellular mechanisms to regulate the concentration of metal ions inside the cell to minimize the potential damage that could result

from the exposure to nonessential metal ions. The sensibility or resistance of plants to metals depends on physiological and molecular mechanisms that include uptake and accumulation of metals usually by binding to extracellular exudates and cell walls complexation of ions inside the cells. Some plants prevent metal ions from entering the cytosol through the action of plasma membrane (285, 293, 323). Overall, the scarcity of knowledge on mechanisms of Ni and Cu homeostasis, uptake, transport, and accumulation in *B. papyrifera* limits our understanding of relationship between metal toxicity and plant genetic response.

5.6. Conclusion

We found that the bioavailable levels of nickel and copper currently present in the soils of the Greater Sudbury region are not sufficient to induce toxicity in white birch (*B. papyrifera*) from the area. At higher doses, we start to see a segregation pattern resulting in resistant and susceptible genotypes. Metal analyses concluded that resistant genotypes limit root to shoot translocation of nickel, which is indicative of an excluder. A 3:1 segregating pattern suggests that a single recessive gene controls resistance in white birch. Further analyses are needed to confirm this hypothesis.

Chapter 6: Decrypting the regulation and mechanism of nickel resistance in white birch (*Betula papyrifera*) using cross-species metal-resistance genes.

6.1. Abstract

Recent studies have found that many transporters, metabolic products and chelators play a role in metal (HM) resistance in model plants. Knowledge of mechanisms involved in resistance to HM in higher plant species is limited. In the present study, the expression of four novel genes (AT2G16800, GR, ZAT11, and IREG1) in white birch (*Betula papyrifera*) growing in soil contaminated with different levels of nickel were investigated. *B. papyrifera* seedlings were treated with different doses of nickel including 5.56, 1,600, and 4,800 mg/kg in growth chamber screening trials. The expression of targeted genes in nickel resistant and susceptible genotypes was measured using RT-qPCR. Field trials were also conducted to assess the regulation of these genes in *B. papyrifera* growing in metal-contaminated and uncontaminated sites. The IREG1 transporter and glutathione reductase were the only genes affected (upregulation) by nickel at the low dose of 5.56 mg/kg. The expression of all the four genes was affected by the high dose of 1,600 mg/kg resulting in the upregulation of AT2G16800, GR, and ZAT11 and the downregulation of IREG1. ZAT11 and GR were differentially expressed in resistant genotypes. No differences in gene expression were found when samples from metal-contaminated and reference field sites were compared, but the expression of AT2G16800, IREG1 and GR was higher in roots compared to leaves. The findings of this study suggest that the bioavailable amounts of nickel that is usually found in highly metal-contaminated soils in mining regions cannot trigger a measurable genetic response in plants.

Keywords: Gene expression; Nickel resistance mechanism; *Betula papyrifera*; RT-qPCR

6.2. Introduction

Metal contamination in mining regions has become a growing concern worldwide. Metals can be toxic to virtually all-living organisms, but plants are among the most affected organisms because they are not mobile and depend on the soil they reside in for nutrients and water. HMs have been known to lower soil nutrient availability (complexion) (18, 324). However, they are most commonly associated with oxidative stress. Excess levels of metals can lead to membrane instability, protein disruption and nucleic acid damage, either by the metal itself or the production of reactive oxygen species (ROS) (34). This is especially troublesome for mining regions of the Canadian Boreal forest since the acidic nature of the soil further increases metal availability.

Areas barren due to mining activities are often colonized by pioneer species. For example, in Northern Ontario specifically in the Greater Sudbury region (GSR), an area known for its continued mining activities and the presence of smelters, *Betula papyrifera* trees represent over 50% of all the woody trees (293). The rapid growth, intolerance for shade and resistance to abiotic stresses of this species make it the ideal candidate for metal-contaminated soils (325).

High levels of genetic variation in plant populations are a prerequisite to ecosystem sustainability. Recent studies have revealed that most hard wood populations growing in Northern Ontario have sustainable levels of genetic variation (6, 26, 293). This high genetic variability has resulted in the development of plant genotypes resistant to high concentrations of metals (Hamrick et al 1979 ; Kirkey et al 2012). In fact, Kirkey et al. (2012) found that *B. papyrifera* populations growing in metal contaminated areas in the GSR were more resistant to metals than those growing in non-contaminated areas. Further investigations revealed that *B.*

papyrifera trees from this region accumulate nickel in aerial parts and have been found to be resistant to total nickel soil levels of up to 1,600 mg/kg (325).

Although resistance to HM has been reported in many plant species, little is known about the regulation mechanisms of genes involved in this process, especially in woody plants (173, 223, 327). Research on metal resistance in herbaceous species is much more extensive due to their lower level of complexity. HM resistant mechanism can vary from species to species. Some plants will change the bioavailability of metals around the rhizospheres by secreting organic acids from their roots (171–173). Others will form a symbiosis with mycorrhizal fungi that are able to tolerate higher concentrations of metals. Fungi will form large hyphae that will protect roots as well as transfer important nutrients (166). Other plant species will simply down regulate the expression of metal transporters in the apical region of the root to reduce metal influx and toxicity (176). Key genes associated with nickel tolerance in model and non – model plant species have been identified and characterized. They include some genes coding for transporters of the NRAMP and IREG family, metabolic proteins involved in glutathione synthesis and chelating peptides such as methalothiones (77, 188, 219, 223). There is also some evidence that transcription factors from the ZAT family play a role in the regulation of genes involved in nickel resistance (224). The main objective of the present study is to determine the level of regulation of these genes in white birch (*Betula papyrifera*) genotypes growing in soils with different nickel contamination levels.

6.3. Materials and methods

6.3.1. Growth chambers trials

Betula papyrifera seeds were collected from the Laurentian university research site (site 3) and stored at 4°C (Figure 17). They were then germinated on wet filter paper in Petawawa boxes at 27°C. Seedlings were transplanted into pots containing a topsoil/peat moss mixture. After a month they were fertilized (20:20:20) and left to grow for an additional four months at 27°C. Seedlings were then transplanted into a 50:50 mix of quartz sand/peat moss and left to grow for another month. To assess the toxicity of nickel, *B. papyrifera* seedlings were treated with different doses of Ni(NO₃)₂ leading to a final nickel concentration of 5.56, 1,600 or 4,800 mg/kg as previously described in (325). These concentrations correspond to the bioavailable, total and 3X total amount of nickel in contaminated sites in the mining region of Northern Ontario. Water treated plants were used as reference. To determine any effect of the excess nitrate on plant damage, KNO₃ was also used as an additional control. The experimental design was a completely randomized block with 15 replications.

Damage ratings were recorded every two days based on a scale of 1 to 9, 1 being no visible toxicity symptoms and 9 dead plants. Individual plants with score of 1 to 3 were considered nickel resistant, 4 to 6, moderately resistant and 7 to 9 susceptible.

6.3.2. Field experiments

For field samples, *B. papyrifera* leaves and roots were sampled from six locations in the Greater Sudbury region, three metal contaminated sites (Kelly Lake, Kingsway and Laurentian) and three references (Killarney, Capreol and Onaping Falls) (6, 293) (Figure 17).

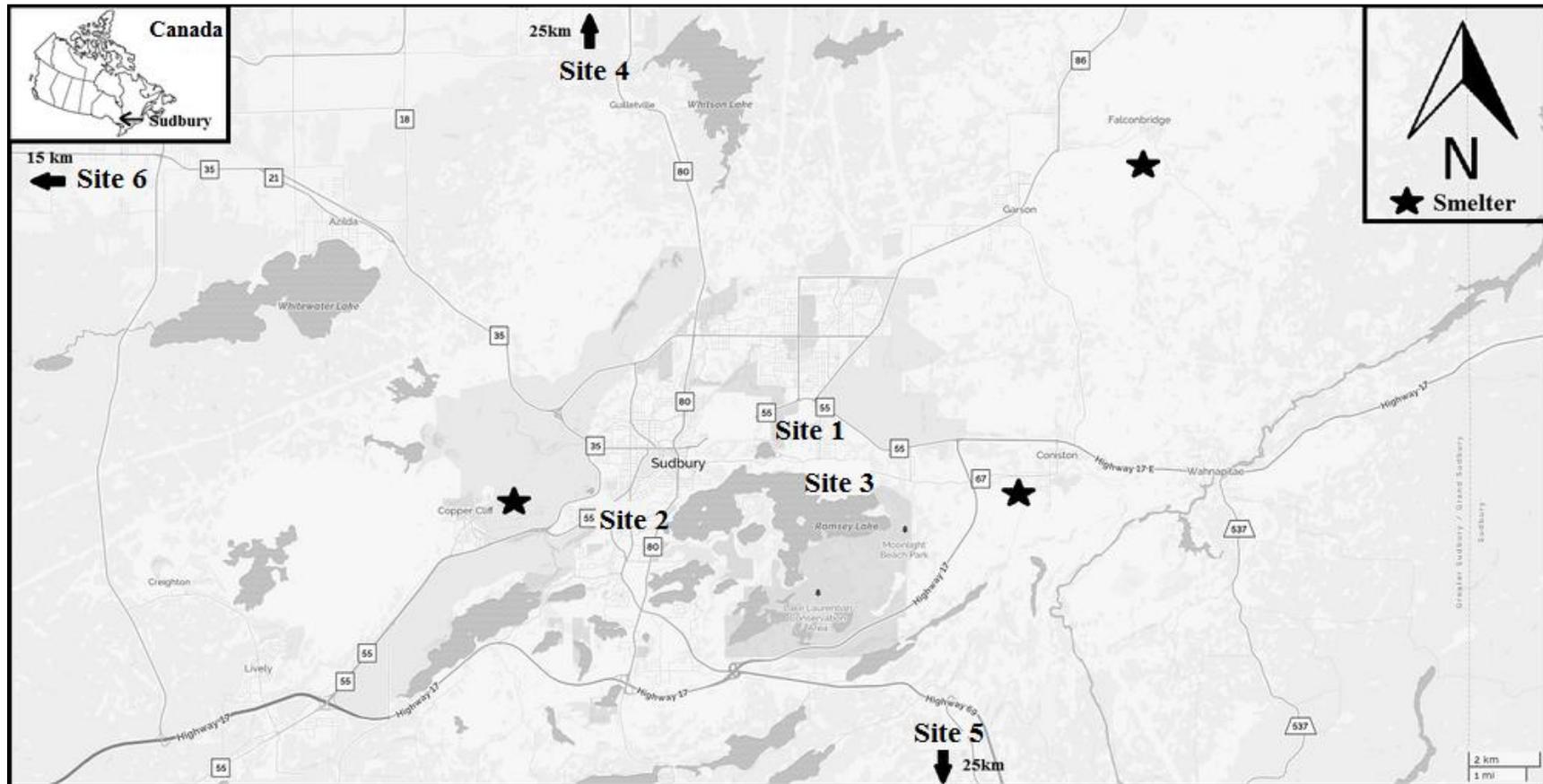


Figure 17. Location of sampling sites within the Greater Sudbury region.

Site 1: Kingsway; Site 2: Kelly Lake; Site 3: Laurentian; Site 4: Capreol; Site 5: Killarney and Site 6: Onaping Falls.

The levels of metal contamination in these sites have been reported in (6, 9, 293). Secondary roots were collected and washed. The samples were flash frozen and stored at -80°C until RNA extraction.

6.3.3. RNA extraction

The total RNA was extracted from samples from growth chamber and fields trials using the Plant/Fungi Total RNA Purification kit by Norgen Biotek Corporation (Thorold, Canada) or by methods previously described in (328). For growth chamber trials, only leaf RNA was extracted. For field experiments, root and leaf RNA from 15 individuals was extracted. The RNA was quantified using the Qubit RNA BR Assay kit from Life Technologies (Carlsbad, United States). The quality of the RNA was verified on a 1% agarose gel. One microgram of RNA from samples of the same treatment/site was pooled together and used for downstream processes. For field trials, samples from 10 individual trees per site were bulked together while for growth chamber experiments, samples from 4 to 6 individual plants for each treatment were bulked. Likewise, samples from six nickel resistant and six nickel susceptible plants were bulked separately.

6.3.4. RT-qPCR

The RNA was treated with DNase 1 (#EN0521) from Life Technologies. PCR primers were designed by matching gene sequences to the *Betula nana* (dwarf birch) genome (329). When possible, primers were designed to span the exon-exon border of the gene. Primers were then BLASTed using the *B. nana* genome. Primers were checked for hairpins, self and hetero-dimers using the OligoAnalyzer 3.1 by IDT (<https://www.idtdna.com/calc/analyzer>). The list of candidate genes with their associated primer pairs can be found in Table 15. The cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit by Life Technologies.

Regular PCR was performed on both *B. papyrifera* DNA and cDNA. The size of the amplicons derived from amplification with the primer pairs was verified on agarose gels. Only primers that showed a reproducible single band of the appropriate size were used for RT-qPCR. RT-qPCR was performed using the Dynamo HS SYBR Green 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 sec; (3) 30 sec annealing; (4) elongation at 72 °C for 30 sec; (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 sec; and (9) final elongation at 72 °C for 3 min. This qPCR, was run three separate times with each sample in triplicate. This resulted in a total of nine data point for each bulked sample.

The data was analyzed using the MJ Opticon Monitor 3.1 by BioRad and delta C(t) values were exported to excel. Delta C(t) values of samples from growth chamber assays were normalized to two separate housekeeping genes and the relative expression was calculated using the water control. For field samples, delta C(t) values were only normalized to the two housekeeping genes. Both housekeeping genes led to the same results therefore only one was used for simplicity.

6.3.5. Statistical analysis

The data was analyzed using SPSS 20 for Windows, with all data being transformed to achieve a normal distribution. Variance-ratio test was done with an assumption of data normality in the underlying population distributions of the data. ANOVA, followed by Tukey's HSD multiple

Table 15. Sequences of white birch (*Betula papyrifera*) primers used for RT-qPCR.

Target	Melting temp. (°C)	Primer	Expected Amplicon (bp)	PCR product
SAT	F: 66.01 R: 67.34	F: GTGGATATCCATCCGGGAGCTAAGA R: GACGGCCGCTCCTTCCCTC	326	none
GR	F: 64.57 R: 65.46	F: AGCGGTTATTGACGAATTCTGGGGT R: TGGCCAGGGATAGGGGGACG	169	169
NAS3	F: 65.03 R: 64.94	F: AGGCTCTGTGGGGAGGCAGA R: GAGAAAAGCCCGAGCCCCGT	320	320
NRAMP3	F: 62.90 R: 62.70	F: GTTTTGCCTCTCTGGGCTGG R: GTTGGGAGCAATCTTTCTTGACTGT	307	multiple
NRAMP4	F: 71.07 R: 71.49	F: TCCTCTCAGCCAGGGTCGGGGT R: AAGTTCCACGCCATTGGGCTTCGTT	350	multiple
ACC	F: 66.96 R: 66.44	F: TGGCAATCTCCTGGTTGAGCGT R: GGGATCATGGCTGAGTACATTGAAGG	315	none
AT2G16800	F: 64.68 R: 64.65	F: GAGCTCTCTGGGGGTGTGGC R: TGCCGGCACGACCATCATCA	335	335
IREG1	F: 64.82 R: 64.36	F: CAGAAGGCCATCCTCCAGAGAAGC R: CCAGAGTGCCAAAGCCACAGC	149	149
ZAT11	F: 64.61 R: 65.69	F: ACCGAGCCAGCCACAAGAGG R: CCGCCCAAAGCTTGCCCCATA	149	149
Housekeeping				
EF1 α	F: 65.10 R: 65.16	F: TGTTGACTGGTGCCCCACCG R: CACAAAGGCGCGCTTGGCAT	187	187
Cyclophilin	F: 64.78 R: 65.10	F: TGGGCGGATCGTGATGGAGC R: CACGACCTGGCCGAACACCA	370	370

comparison analysis, were performed to determine significant differences among means. The Student-T test was performed for field and pot experiments to compare means between sites and doses ($p < 0.05$).

6.4. Results

Primers that were used to amplify target genes are listed in Table 15. Five of the nine primer pairs were suitable for RT-qPCR. For growth chamber trials, four of the five candidate genes showed variation in expression across treatments. These include the High-affinity nickel-transport protein (AT2G16800), Glutathione reductase (GR), Zinc finger protein (ZAT11) and the Iron regulated transporter 1 (IREG1). All the plants treated with 4,800 mg/kg of nickel dose died within 24 h and were not suitable for RNA extraction. Figure 18 shows the resistant and susceptible genotypes treated with 1,600 mg/kg of nickel 7 days after the treatment.

IREG1 expression was significantly upregulated by both nitrate doses compared to water control (Figure 19a). At the low Ni dose (5.56 mg/kg), IREG1 expression levels were significantly higher than nitrate controls however, at the high dose (1,600 mg/kg), IREG1 expression levels were lower than nitrate controls (Figure 19b). When comparing the different genotypes, IREG1 expression was highest in susceptible plants and lowest in moderately susceptible plants (Figure 19c).

For field samples, IREG1 expression was downregulated in leaves compared to roots within each site (Figure 19d). Higher levels of IREG1 were found in leaves from control sites compared to leaves from contaminated sites (Figure 19d).

There was no significant difference in AT2G16800 (high-affinity nickel-transport family protein) expression between water and nitrate controls (Figure 20a). No significant difference was found between nickel and nitrate control for the low Ni dose, however, expression was higher for the high Ni treatment when compared to the nitrate control treatment (Figure 20b).



Figure 18. White birch (*Betula papyrifera*) treated with 1,600 mg/kg of nickel.

*Left plant is resistant and right plant is susceptible 7 days after treatment.

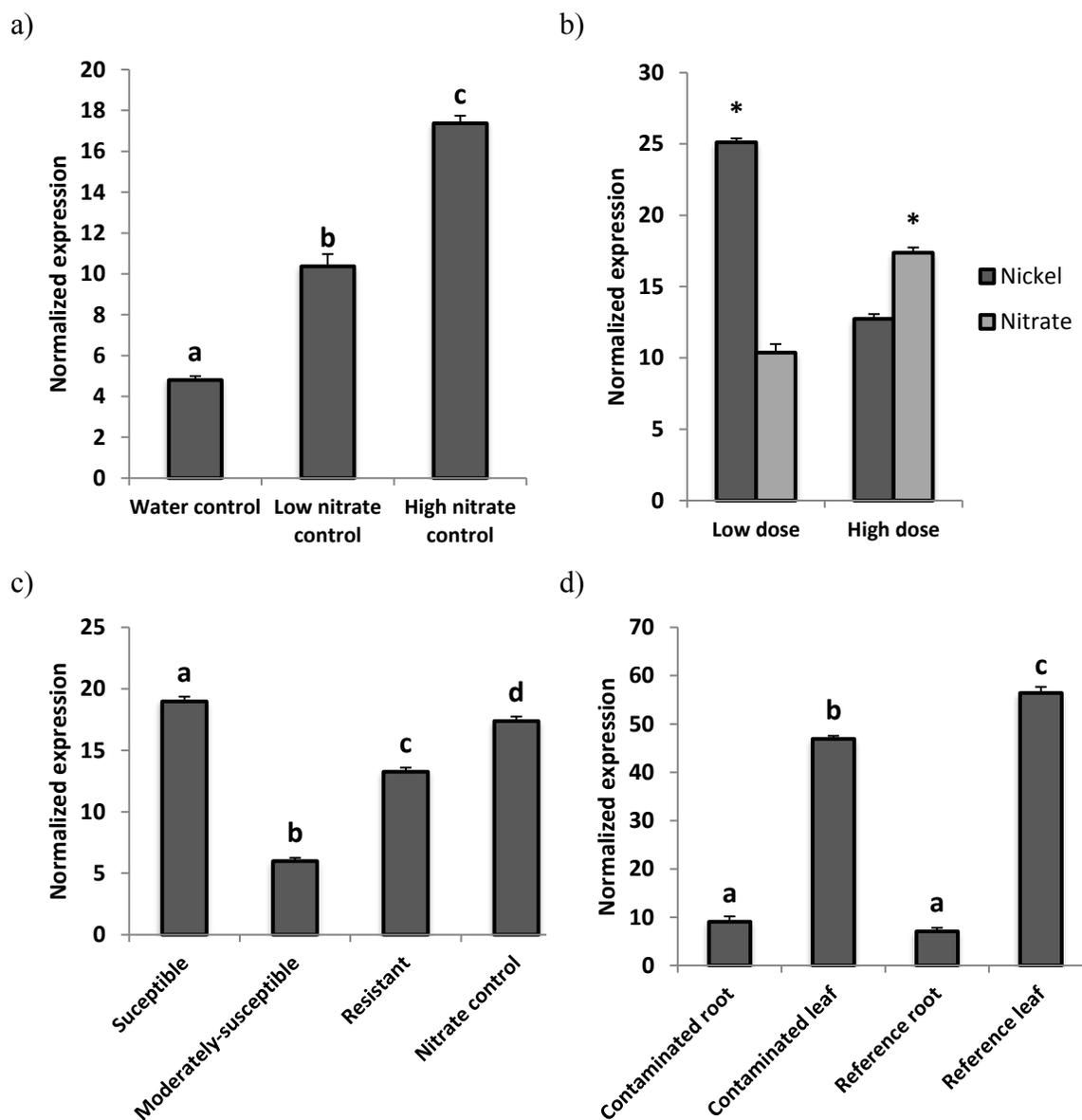


Figure 19. Expression of IREG1 (Iron regulated transporter 1) in white birch (*Betula papyrifera*).

a) water and nitrate controls; **b)** nickel treatments at low dose of 5.56 mg/kg and high dose of 1,600 mg/kg of nickel; **c)** susceptible, moderately susceptible, resistant plants treated at 1,600 mg/kg; **d)** field samples from metal-contaminated and reference root and leaves.

Expression of IREG1 was standardized based on the housekeeping gene *ef1 α* . Significant differences were found among groups using the Tukey's multiple comparison or t-test ($p < 0.05$).

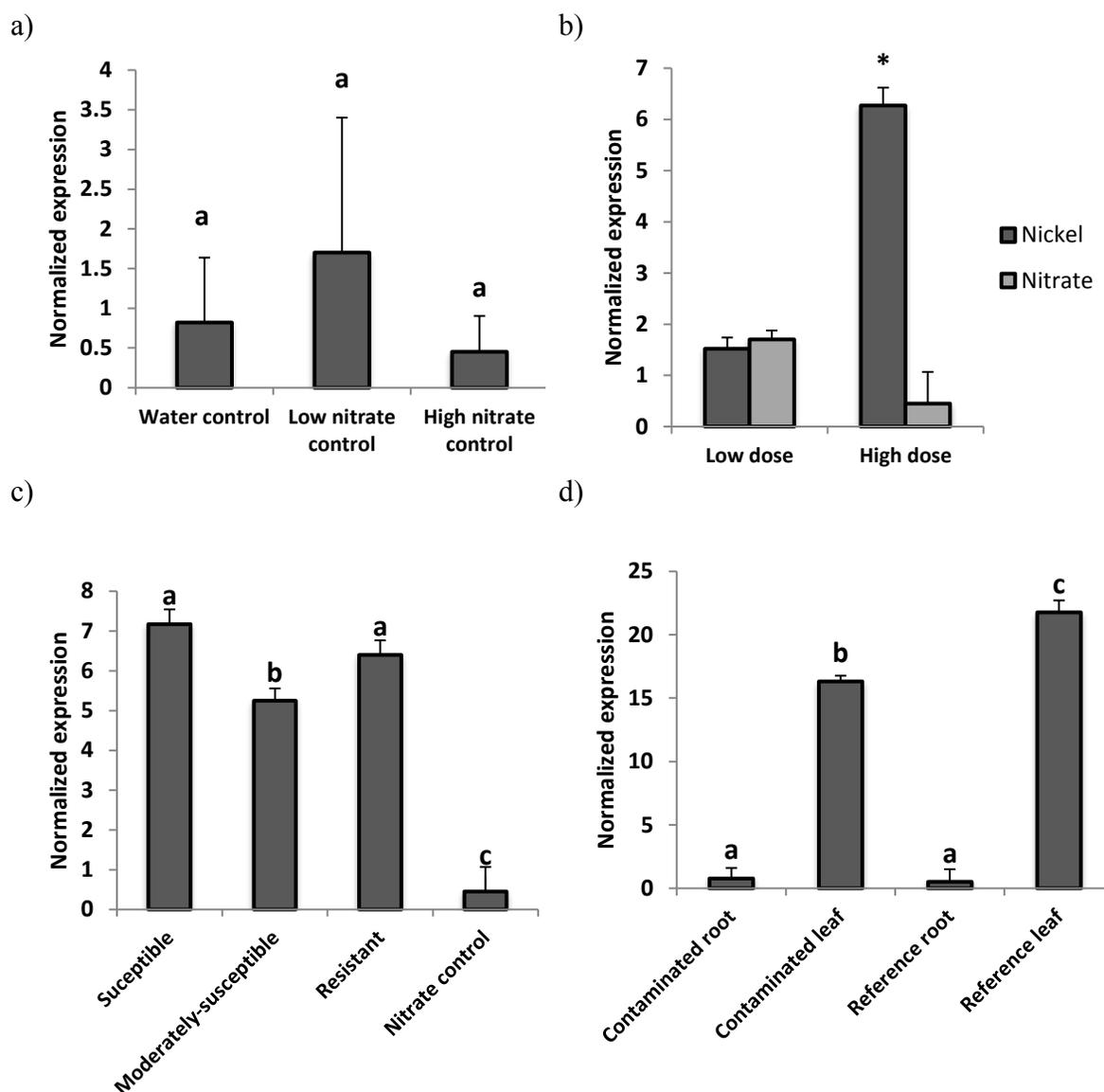


Figure 20. Expression of AT2G16800 (high-affinity nickel-transport family protein) in white birch (*Betula papyrifera*).

a) water and nitrate controls; **b)** nickel treatments at low dose of 5.56 mg/kg and high dose of 1,600 mg/kg of nickel; **c)** susceptible, moderately susceptible, resistant plants treated at 1,600 mg/kg; **d)** field samples from metal-contaminated and reference root and leaves. Expression of AT2G16800 was standardized based on the housekeeping gene *ef1a*. Significant differences were found among groups using the Tukey's multiple comparison or t-test ($p < 0.05$).

AT2G16800 expression was highest in the susceptible and resistant plants and lowest in moderately susceptible (Figure 20c). For field experiment, expression of this gene was significantly higher leaves from all sites (Figure 20d). Higher levels of AT2G16800 were found in leaves from reference sites compared to leaves from contaminated sites (Figure 20d).

No significant difference was found in ZAT11 expression between water and nitrate controls (Figure 21a). Nickel had no effect on expression at the low Ni dose but at the high dose, an increase in ZAT11 expression was observed (Figure 21b). ZAT11 expression was higher in susceptible and moderately susceptible genotypes compared to resistant and nitrate controls (Figure 21c). No significant difference was found between metal-contaminated and reference sites for roots however, ZAT11 expression was higher in leaves from reference sites compared to leaves from contaminated sites (Figure 21d).

The expression of the GR (glutathione reductase) was higher in nitrate controls compared to water control (Figure. 22a). Both nickel doses induced an increase in GR expression (Figure 22b). When comparing genotypes, GR expression was highest in moderately susceptible plants and lowest in water controls (Figure 22c). We found higher expression of GR in leaves compared to roots in both contaminated and reference sites (Figure 22d). No significant difference was found when comparing GR expression in roots from contaminated and reference sites (Fig. 22d). However, GR expression was higher in leaves from reference sites (Figure 22d).

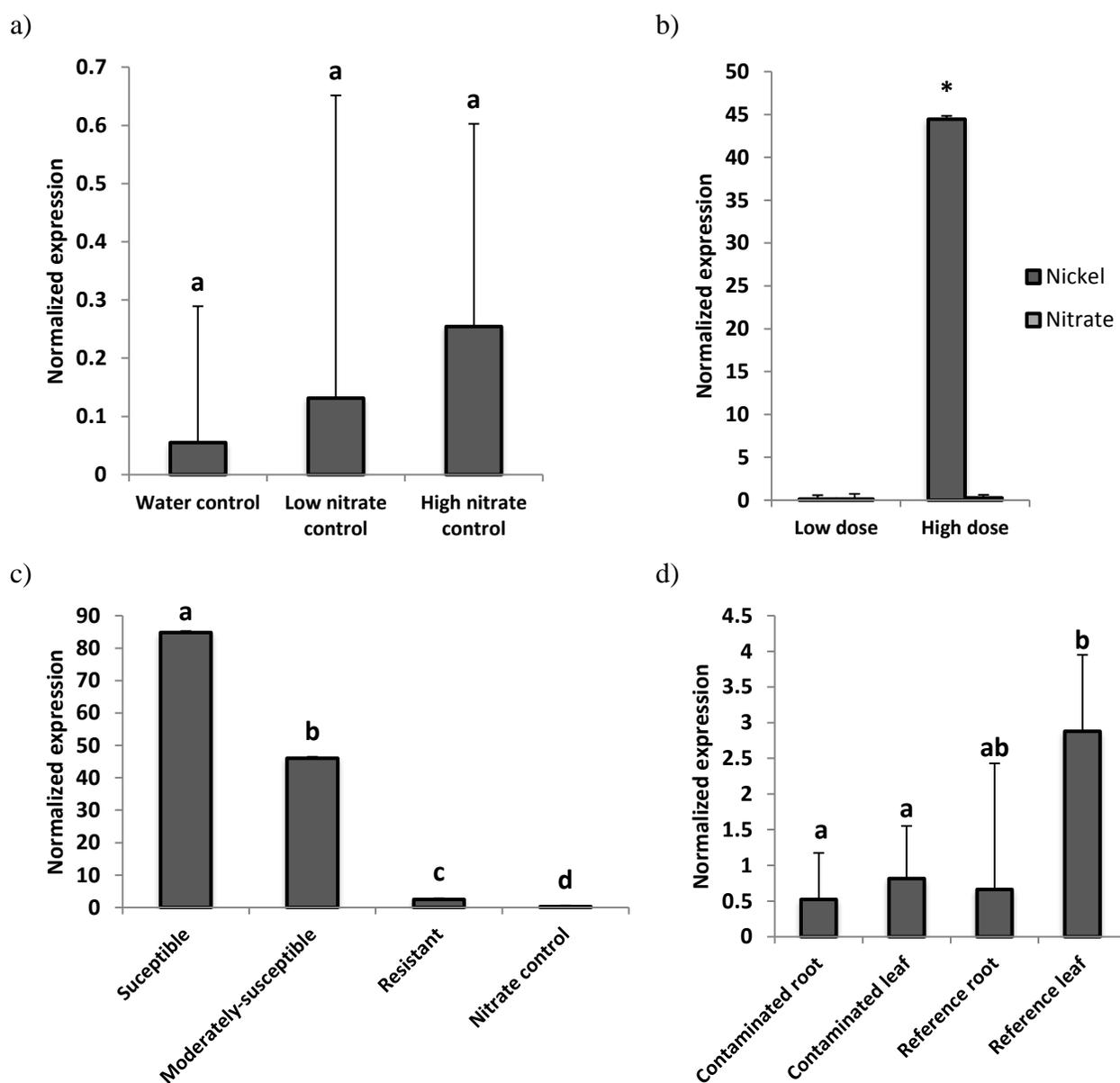


Figure 21. Expression of ZAT11 in white birch (*Betula papyrifera*).

a) water and nitrate controls; **b)** nickel treatments at low dose of 5.56 mg/kg and high dose of 1,600 mg/kg of nickel; **c)** susceptible, moderately susceptible, resistant plants treated at 1,600 mg/kg; **d)** field samples from metal-contaminated and reference root and leaves.

Expression of ZAT11 was standardized based on the housekeeping gene *e1 α* . Significant differences were found among groups using the Tukey's multiple comparison or t-test ($p < 0.05$).

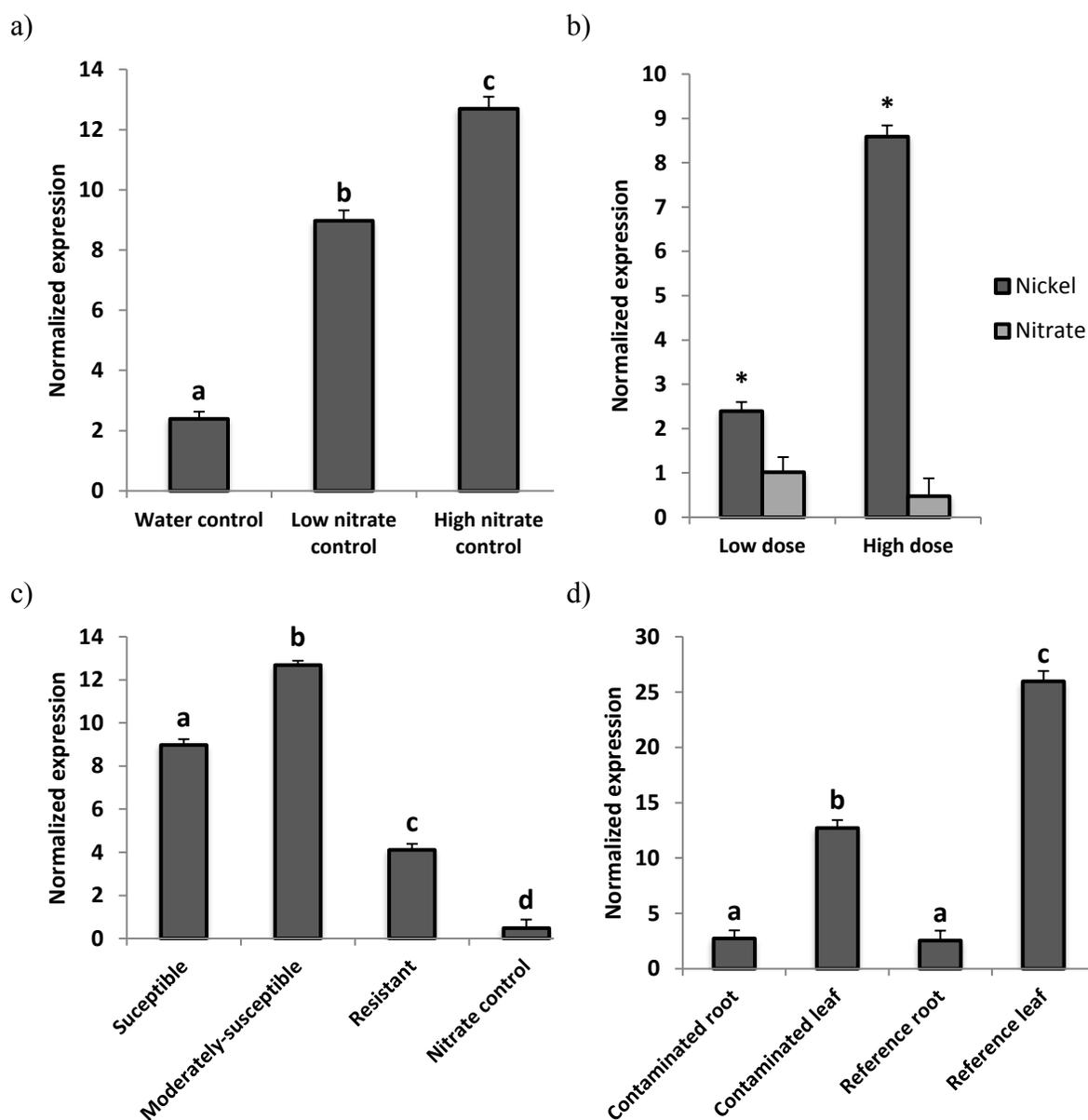


Figure 22. Expression of Glutathione reductase (GR) in white birch (*Betula papyrifera*).

a) water and nitrate controls; **b)** nickel treatments at low dose of 5.56 mg/kg and high dose of 1,600 mg/kg of nickel; **c)** susceptible, moderately susceptible, resistant plants treated at 1,600 mg/kg; **d)** field samples from metal-contaminated and reference root and leaves.

Expression of GR was standardized based on the housekeeping gene *ef1 α* . Significant differences were found among groups using the Tukey's multiple comparison or t-test ($p < 0.05$).

6.5. Discussion

6.5.1. Importance of treatment controls

Metals must be in their soluble and bioavailable form in order to be uptaken by the plant root system. In the majority of nickel experiments conducted in plants, nickel nitrate and nickel chloride salts are preferably used due to their high solubility and low cost. These salts ionize to $\text{Ni}^{2+}/\text{NO}_3^-$ and $\text{Ni}^{2+}/\text{Cl}^-$ in water. However, most analyses neglect the effects of anions when studying the effects of HM in plants (188, 219, 330). Both nitrate and chloride can be toxic to plants if soil concentrations exceed certain levels (331, 332). However, even at low and non-toxic levels, it is still important to assess if these compounds play a role in plant damage and gene expression. In fact, nitrate uptake for example has been associated with metal resistance (333). In the present study, the nitrate addition to nickel did have an impact on the expression of the target genes even at a low concentration, which suggests that the role of nickel in many studies using $\text{Ni}(\text{NO}_3)_2$ has to be taken with caution.

6.5.2. Effect of nickel on expression of target genes

Metal transporters are thought to be one of the major driving forces of metal resistance in plants. Only few metal transporters have been shown to transport nickel in plants. In *Arabidopsis*, AtIREG1 and AtIREG2 are two transporters from the IREG/Ferroportin family that mediate nickel transport in the tonoplast (216, 219). Both show striking similarities. However, AtIREG2 is only found in the roots while AtIREG1 is located in both roots and aerial parts of the plants (216, 219). Knockout studies of AtIREG1/2 have confirmed that mutants develop hypersensitivity to nickel and store less nickel in their roots (216, 219). It has been reported that overexpression of IREG1 in *Psychotria gabriellae* leads to nickel resistance and

hyperaccumulation in roots (219). In the current study, we investigated whether nickel transporters from other species might play similar roles in white birch (*Betula papyrifera*). IREG1 expression in *B. papyrifera* leaves was upregulated 1.5 fold by Ni at the low dose when compared to nitrate control. However, at the high Ni dose, expression was downregulated when compared to nitrate control. The decrease in expression at the higher Ni dose was driven by moderately susceptible and Ni resistant genotypes. This suggests that at low nickel concentrations, an increase in IREG1 could potentially play a role general metal detoxification in white birch since all plants at that dose were resistant. At higher Ni doses, only plants with lower levels of IREG1 survive. Similar results has been observed in *Arabidopsis thaliana* where an overexpression of the *AtNRAMP* transporter leads to cadmium hypersensitivity (334). This is consistent with our previous findings that showed that nickel-resistant *B. papyrifera* genotypes tend to accumulate nickel in their shoots rather than in leaves while susceptible plants cannot block the root to leaf translocation (335). This suggests that the lack of IREG1 expression is associated with nickel resistance. This mechanism has been observed in other metal accumulators, where plants will downregulate metal transporters to block translocation to a specific tissue (336–339).

AT2G16800 is part of the NiCoT protein family. Transporters from the NiCoT family uptakes nickel and/or cobalt using proton motive force (340). In prokaryotes, Ni/CoT have been found to be useful for nickel bioaccumulation and bioremediation (341). NiCoT transporters have been well characterized in prokaryotes but knowledge in plants is limited. We found that at the 5.56 mg/kg Ni dose, AT2G16800 expression in leaves was not directly affected by nickel. There was a 6 fold increase in AT2G16800 expression at the 1,600 mg/kg Ni dose suggesting Ni induces AT2G16800 expression but only at high Ni concentrations. No link between metal resistance and

susceptibility was found. It is possible that AT2G16800 plays a role in general metal homeostasis but these results indicate that it does not directly play a role in nickel resistance in *B. papyrifera*.

A Recent study has shown that the C₂H₂-type zinc finger protein ZAT11 positively regulates elongation of primary roots but reduces resistance to nickel (224). ZAT11 is thought to be a negative regulator of IREG2, however is it unclear whether it regulates directly or indirectly its expression (224). Our investigation on the expression of ZAT11 in *B. papyrifera* revealed that this gene is affected by nickel and it seems to play a role in nickel resistance. Only the high Ni dose had an impact on ZAT11 expression. There was a 44 fold increase in ZAT11 expression at the high Ni dose. The high expression can be attributed to the susceptible and moderately susceptible plants. This is the first study showing that ZAT11 can be expressed in aerial parts of plants. Furthermore, the results indicate that ZAT11 might also play a role in nickel resistance in *B. papyrifera* probably by modulating genes involved in the inhibition of oxidative stress or meristem development (224, 342).

Transporters are not the only genes involved in metal resistance. Genes from metabolic processes are often upregulated due to their ability to sequester ROS and chelate metals (185, 208, 343). Increased glutathione biosynthesis has been strongly correlated with the ability to resist and accumulate high levels of nickel in roots (188, 208). Production of glutathione is dependent of multiple proteins such as gamma-EC synthase (gamma-ECS), glutathione synthetase (GSH-S), acetyltransferase (SAT) and glutathione reductase (GR) (344). Some of these genes have been found to be activated by nickel suggesting that glutathione plays an important role in redox balance (345). Glutathione must be in its reduced form in order to neutralize ROS and metals. We have investigated whether glutathione reductase, the key enzyme that reduces glutathione, is upregulated by nickel in *B. papyrifera* leaves and if an increased expression contributed to nickel

resistance. GR expression was significantly upregulated by Ni. GR expression was 1 fold higher than controls at the low dose and 8 fold higher at the high dose. Higher levels of GR were found in susceptible and moderate susceptible genotypes compared to resistance. This indicates that glutathione does not seem to be the main mechanism for Ni resistance in *B. papyrifera*.

For the majority of the studied genes, we found no link with metal contamination in field. These results are expected for AT2G16800 and ZAT11 since pot experiments at the low Ni dose resulted in very little to no change in expression. The possibility remains that other molecules play a much stronger role in redox-balance and metal sequestration in *B. papyrifera*. In fact, chelating peptides like methalothiones (194, 346, 347), amino acids (226) and other metabolic compounds (185, 343) have been found to contribute to metal resistance.

Overall, the expression of the targeted genes in the present study was similar in susceptible and moderately susceptible genotypes. This suggests that the two groups are susceptible to nickel but the moderately susceptible genotypes show a delay in the expression of nickel damage. This is consistent with the metal and transcriptome analyses of the nickel treated genotypes (335).

6.6. Conclusion

In the present study, we investigated whether genes known to play a role in nickel resistance in other species are involved in *B. papyrifera* responses to soil nickel contamination. We found that out of the four genes analyzed (AT2G16800, GR, ZAT11, and IREG1), IREG1 and GR were the only ones affected (upregulated) by nickel at the low nickel dose of 5.56 mg/kg. The expression of AT2G16800, GR and ZAT11, was upregulated by the high dose of 1,600 mg/kg. We found variation in gene expression across all the studied genes but only ZAT11 and GR seem to be correlated with metal resistance. The findings of this study strengthen our theory that the

bioavailable amounts of nickel that is usually found in highly metal-contaminated soils in northern Ontario cannot trigger a genetic response in higher plant species.

Chapter 7: Comprehensive transcriptome analysis of response to nickel stress in white birch (*Betula papyrifera*)

7.1. Abstract

White birch (*Betula papyrifera*) is a dominant tree species of the Boreal forest. Recent studies have shown that it is fairly resistant to metal contamination, specifically to nickel. Knowledge of regulation of genes associated with metal resistance in higher plants is very sketchy. Availability and annotation of the dwarf birch (*B. nana*) genome enables the use of high throughput sequencing approaches to understanding responses to environmental challenges in other *Betula* species such as *B. papyrifera*. The main objectives of this study are to 1) develop and characterize the *B. papyrifera* transcriptome, 2) assess gene expression dynamics of *B. papyrifera* in response to nickel stress, and 3) describe gene function based on ontology. Nickel resistant and susceptible genotypes were selected and used for transcriptome analysis. A total of 208,058 trinity genes were identified and were assembled to 275,545 total trinity transcripts. The transcripts were mapped to protein sequences and based on best match; we annotated the *B. papyrifera* genes and assigned gene ontology. In total, 215,700 transcripts were annotated and were compared to the published *B. nana* genome. Overall, a genomic match for 61% of the transcripts with the reference genome was found. Expression profiles were generated and 62,587 genes were found to be significantly differentially expressed among the nickel resistant, susceptible, and untreated libraries. The main nickel resistance mechanism in *B. papyrifera* is a downregulation of genes associated with translation (in ribosome), overall cell growth, and an upregulation of genes involved with the plasma membrane. Five candidate genes associated to nickel resistance were identified. They include Glutathione S –transferase, thioredoxin family

protein, putative transmembrane protein and two Nramp transporters. These genes could be useful for genetic engineering of birch trees for metal resistance.

Key words: *Betula papyrifera* (white birch); transcriptome; nickel resistance; differential expression; candidate genes.

7.2. Introduction

Nickel is an essential micronutrient for plants with concentrations ranging from 0.01 to 5.00 mg/kg. A low level of bioavailable nickel in soil has a beneficial effect on plant growth, but excess accumulation of this metal in plants causes structural, metabolic, and physiological constraints that affects growth and development (348, 349).

White birch (*Betula papyrifera*) is a dominant tree species of the Boreal forest. It is a pioneer species and rapidly colonizes open areas (86). It is the predominant species in mining regions contaminated with metals in Northern Ontario, Canada (especially in the Greater Sudbury region), as it represents 50% of all tree species in many areas (293). This region is known as one of Canada's most ecologically disturbed areas. Over 100 million tonnes of sulfur dioxide and tens of thousands of tonnes of cobalt (Co), copper (Cu), nickel (Ni), and iron (Fe) have been released from roast pits and smelters (350). This caused localized metal pollution and acidification of surrounding ecosystems since industrial activities started over 100 years ago (259). Little is known about *B. papyrifera* adaptation to soil metal contamination even though it plays such a key role in forest sustainability. Recent studies have shown that this species is fairly resistant to metal contaminations specifically to nickel (7, 87).

Cellular mechanisms of nickel tolerance are unknown. In general, metal tolerance includes detoxification processes, complexation by phytochelatins, phylates, amino acids and organic acids, and compartmentation of toxic ions within the cell vacuole and apoplast (349). It is also known that toxic metal - induced oxidative stress is usually greater in sensitive plants than in tolerant plants, which shows reduced lipid peroxidation. In general, plants are rarely adapted to high concentrations of metals and enzyme activity is usually decreased or lost when they are

under metal stress. Studies suggest that glutathione (GSH) and its related metabolizing enzymes, proteins, and peptides play a pivotal role in metal tolerance by controlling reactive oxygen species (ROS) and methylglyoxal (MG) detoxification, metal (HM) uptake, translocation, chelation, and detoxification (351). Theriault *et al.*, (2014) showed that *B. papyrifera* is a nickel accumulator and it survives despite high concentrations of nickel in its aerial shoots (293). This is because accumulator plants biotransform contaminants into inert forms in their tissues. Analysis of segregating *B. papyrifera* populations exposed to a high dose of nickel in controlled environment suggested that this tolerance to nickel is controlled by a single recessive gene (335). In general, resistant plants develop specific mechanisms for uptake, translocation and storage of nutrients and toxic elements. It is very likely that these mechanisms are genetically closely regulated. Knowledge of regulation of genes associated with metals in higher plants is unclear. Genome sequence of only one birch species, dwarf birch (*B. nana*), has been completed (329). To date, transcriptome analysis in the genus *Betula* has not been investigated. Comparative transcriptome analysis is a tool for genetic characterization for stress resistance in plant populations (219, 352, 353). The main objectives of this study are to 1) develop and characterize white birch (*B. papyrifera*) transcriptome, 2) assess gene expression dynamics of *B. papyrifera* in response to nickel stress, and 3) describe gene function based on ontology.

7.3. Materials and methods

7.3.1. Nickel treatments

Nickel susceptible, moderately-resistant and resistant *Betula papyrifera* plants were obtained from a previous experiment (335) and used for the transcriptome analysis. The seeds were collected from the Laurentian University experimental site in the Greater Sudbury region (Northern Ontario) and stored at 4°C. They were then germinated on wet filter paper in Petawawa boxes at 27°C. Seedlings were transplanted into pots containing a topsoil/peat moss mixture. After a month, they were fertilized with equal amounts of nitrogen, phosphorus and potassium (20-20-20) and left to grow for four additional months at 27°C in a growth chamber. They were then transplanted into a 50:50 mix of quartz sand and peat moss and left to grow for another month. To assess the toxicity of nickel, segregating populations were treated with a single dose of Ni(NO₃)₂ salt which led to a final concentration of 1,600 mg /kg of nickel as previously described (335). This concentration corresponds to the total amount of nickel in contaminated sites in the mining region in Northern Ontario. Water treated plants were used as reference. To determine any effect of nitrate on plant damage, KNO₃ was also used as an additional control (nitrate control). The experimental design was a completely randomized block with 15 replications.

Damage rating was recorded every two days based on a scale of 1 to 9, 1 = no visible toxicity symptoms and 9 = dead plants. Individual plants with a score of 1 to 3 were considered nickel resistant, 4 to 6, moderately resistant/susceptible, and 7 to 9 susceptible. Figure 23 shows the differences between resistant and susceptible plants.



Figure 23. White birch (*Betula papyrifera*) from M2 populations treated with 1,600 mg/kg of nickel (Ni).

Left and right plants are resistant (damage rating score of 1) and susceptible (damage rating score of 9) seven days after Ni treatment, respectively.

7.3.2. RNA extraction

Total RNA was extracted using the Plant/Fungi Total RNA Purification kit from Norgen Biotek Corporation (Thorold, Canada). It was quantified using the Qubit RNA BR Assay kit provided by Life Technologies (Carlsbad, United States). The quality of the RNA was verified on a 1% agarose gel. The RNA was treated with DNase to remove any residual DNA traces.

7.3.3. De novo transcripts assembly

RNA-seq libraries were generated using the TruSeq RNA-Seq Sample Prep Kit according to the manufacturer's protocol (Illumina Inc. San Diego, CA). Poly-A RNA was isolated from total RNA and chemically fragmented. First and second strand cDNA syntheses were followed by end repair and adenosines were added to the 3' ends. Adapters were ligated to the cDNA and 200 ± 25 bp fragments were gel - purified and enriched by PCR. The library was quantified using Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA) and the sequencing was performed on the Illumina HiSeq 2000 sequencing system (Illumina Inc.) at Seq Matic (Fremont California, USA). The RNA sequence data from all the samples including 9 nickel-treated (3 resistant, 3 moderately resistant/susceptible, 3 susceptible), 3 water-treated (water control), and 3 nitrate-treated (nitrate control) were used as input to the Trinity program (<http://trinityrnaseq.github.io>) to assemble the transcripts. 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 (both gene counts and FPKM levels). Additional QC at gene level was performed, including number of genes detected,

percentage of reads belonging to the top genes, normalization for RNA composition, and grouping, and correlation between samples.

For the number of genes detected, we counted the number of genes that had at least 1, 2, 10, 50 or 100 counts. In general, the number of genes with two or more counts was used as a rough estimate of how many genes were expressed. Genes with only one read could be noise. In addition, the number of genes with 10 or more reads is a good indicator of how many genes have enough reads for the downstream statistical analysis. The genes were ranked by read counts, and the numbers of reads belonging to the top genes were computed (up to top 100). If the majority of the reads came from top genes, then the sample probably had bottlenecking issues where a few genes were amplified many times by PCR during library preparation.

Next, we removed genes that were not expressed. If a gene had a count per million (CPM) value ≥ 1 in at least two of the samples, we considered it expressed in the experiment and included it for downstream QC analysis. Gene expression was calculated and expressed as Reads Per Kilobase per Million reads mapped (RPKM) (354). The count per million (CPM) cutoff was 0.48 based on the average read count of all samples (20.9 million). This CPM cutoff roughly equaled to 10 raw reads in this experiment. A gene with a CPM value > 0.48 in at least two samples from the experiment was included for downstream analysis. The raw counts were normalized using the voom method from the R Limma package (<http://www.bioconductor.org/packages/release/bioc/html/limma.html>) (355). After normalization, most samples looked similar. In general, samples with high or low distribution may be outliers (or have large biological differences).

Multidimensional plots were created to view sample relationships. This was done using R Limma package. We also used the made4 (multivariate analysis of microarrays data using ADE4)

program to cluster samples and drew heatmaps (5,000 genes) based on genes that had variable expression across samples. These variable genes were chosen based on a standard deviation (SD) of expression values larger than 30 % of the mean expression value. Genes with a mean logCPM <1 were removed.

For differentially expressed genes, the normalized data was transformed to log₂CPM values using the voom method from the R Limma package. A linear model was built for each comparison using the R Limma package, and statistics for differential expression analysis were calculated. The statistical values included log fold change (logFC), p-value, and false discovery rate (FDR). An FDR of 0.05 was used as the standard cutoff (two fold change) to determine differentially expressed genes between treatments.

A flowchart summarizing all the steps and quality controls leading to candidate genes selection is described in Figure 24. Based on the data, three samples were marked as outliers and were removed from the analysis. All transcripts were mapped to protein sequences in the UniProt database (<http://www.uniprot.org/>) and the best match was used to annotate genes and assign gene ontology information. The annotated sequences were run through the GO-Slim function of the BLAST2GO program to provide a summary known gene functions(356). The ontology categories included biological process, cellular components, and molecular function.

7.3.4. Identification of genes associated with nickel resistance

For the identification of genes associated with nickel resistance, a pairwise comparison between resistant / moderately-resistant and resistant / susceptible which showed all the differentially expressed genes (DEGs) was performed. Top 50 genes based on Log₂FC from each pairwise comparison were ranked. Five candidate genes were selected based on the expression in resistant

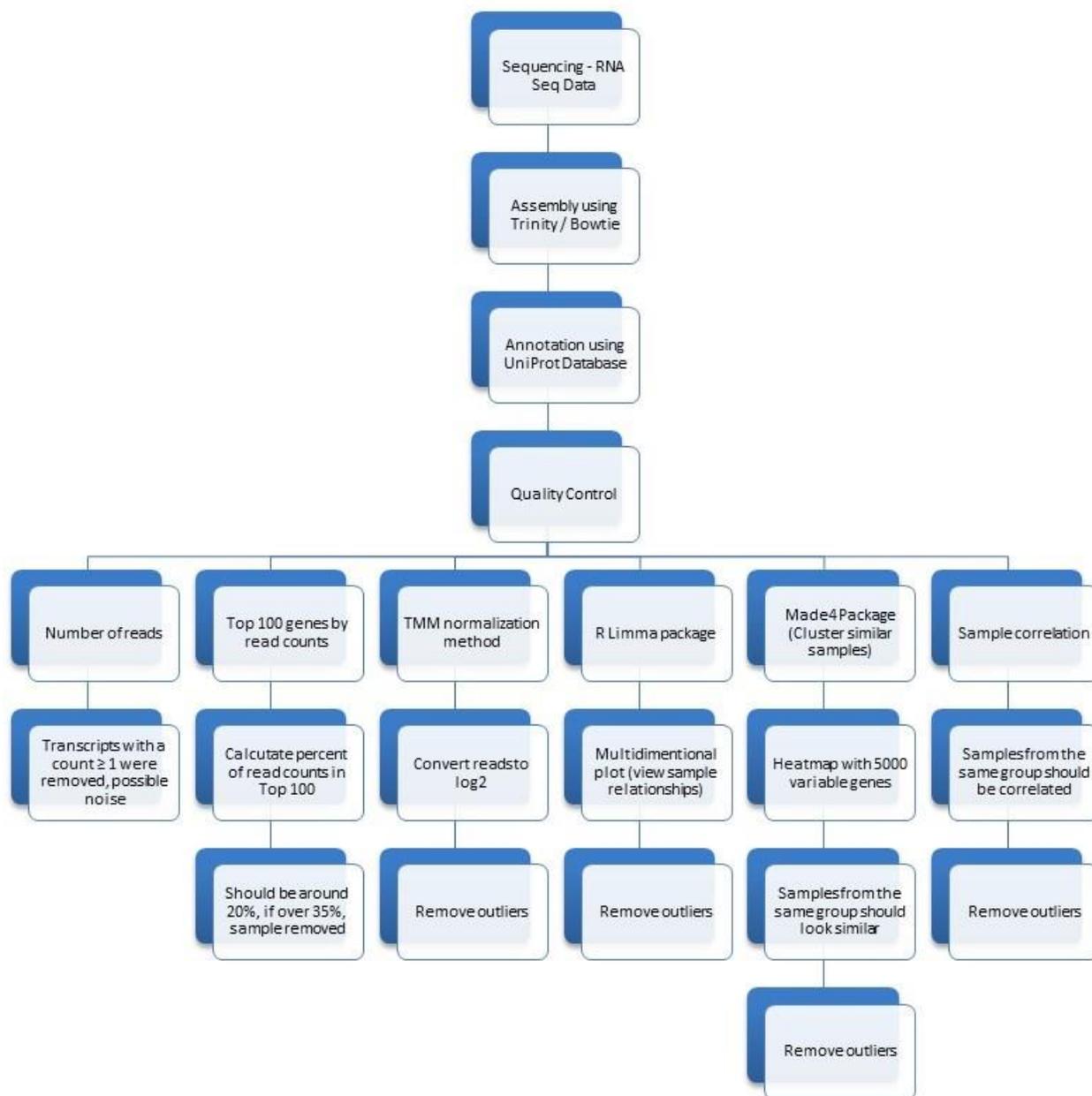


Figure 24. Flow chart of the *Betula papyrifera* transcriptome analysis.

genotypes compared to susceptible, on their function, and their characterization documented in existing literature.

7.4. Results

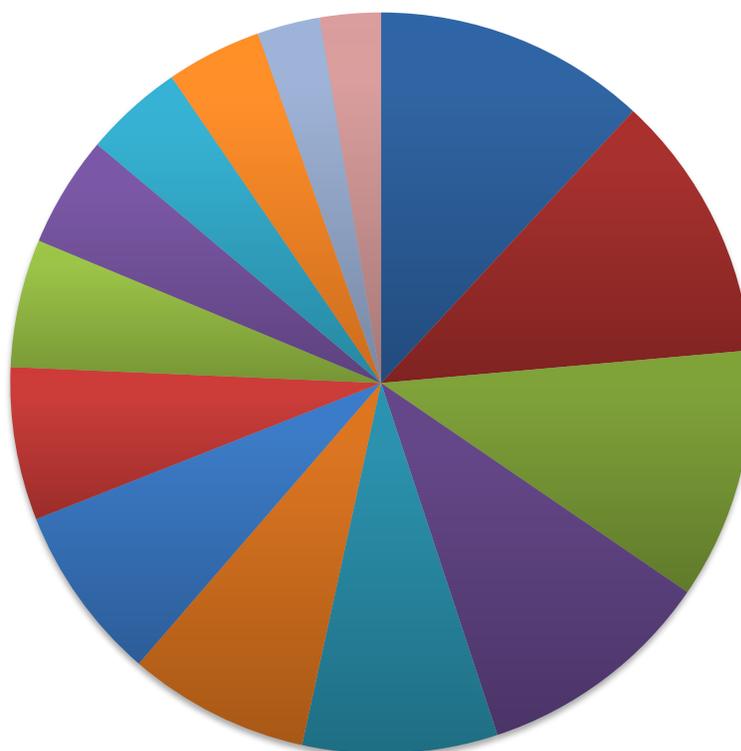
7.4.1. Transcriptome assembly and analysis

After trimming all the contaminations, a total of 208,058 trinity genes were identified and were assembled to 275,545 total trinity transcripts with an average length of 561.54. This represents a total of 154,728,281 bases. Overall, 215,700 transcripts were annotated and were compared to the published *B. nana* genome (<http://birchgenome.org>). A genomic match for 61% transcripts with the reference genome was found. This Transcriptome Shotgun Assembly project has been deposited at DDBJ/EMBL/GenBank under the accession GEIC00000000. The version described in this paper is the first version, GEIC01000000.

7.4.2. Gene ontology classification

Transcripts were assigned gene ontology and grouped by biological function, molecular functions, and cellular compartmentalization. Overall, the numbers of transcripts were similar among the different groups (control, resistant, and susceptible).

For biological function, 23,876 transcripts were assigned ontology. Roughly, 65% of all categories fall under cellular component organization (CCO), carbohydrate metabolic process (CMP), transport, catabolic process, response to stress, translation, and response to stimulus (Figure 25). For molecular functions, 35% transcripts code for proteins involved in binding activities, 13.7% for kinase activities, 12.7% for DNA binding, and 10.2% for transport activities (Figure 26).



- Other (12.09%)
- Cellular component organization (11.90%)
- Carbohydrate metabolic process (11.13%)
- Transport (10.56%)
- Catabolic process (8.64%)
- Response to stress (8.04%)
- Translation (7.81%)
- Response to stimulus (6.78%)
- Lipid metabolic process (5.68%)
- Signal transduction (4.93%)
- DNA metabolic process (4.39%)
- Cellular protein modification process (4.23%)
- Cell cycle (2.78%)
- Generation of precursor metabolites and energy (2.70%)

Figure 25. Percentage of transcripts in *Betula papyrifera* control samples grouped by biological function.

A total of 21,793 transcripts were assigned gene ontology using BLAST2GO. Categories under 2% were grouped together and classified as “other”.

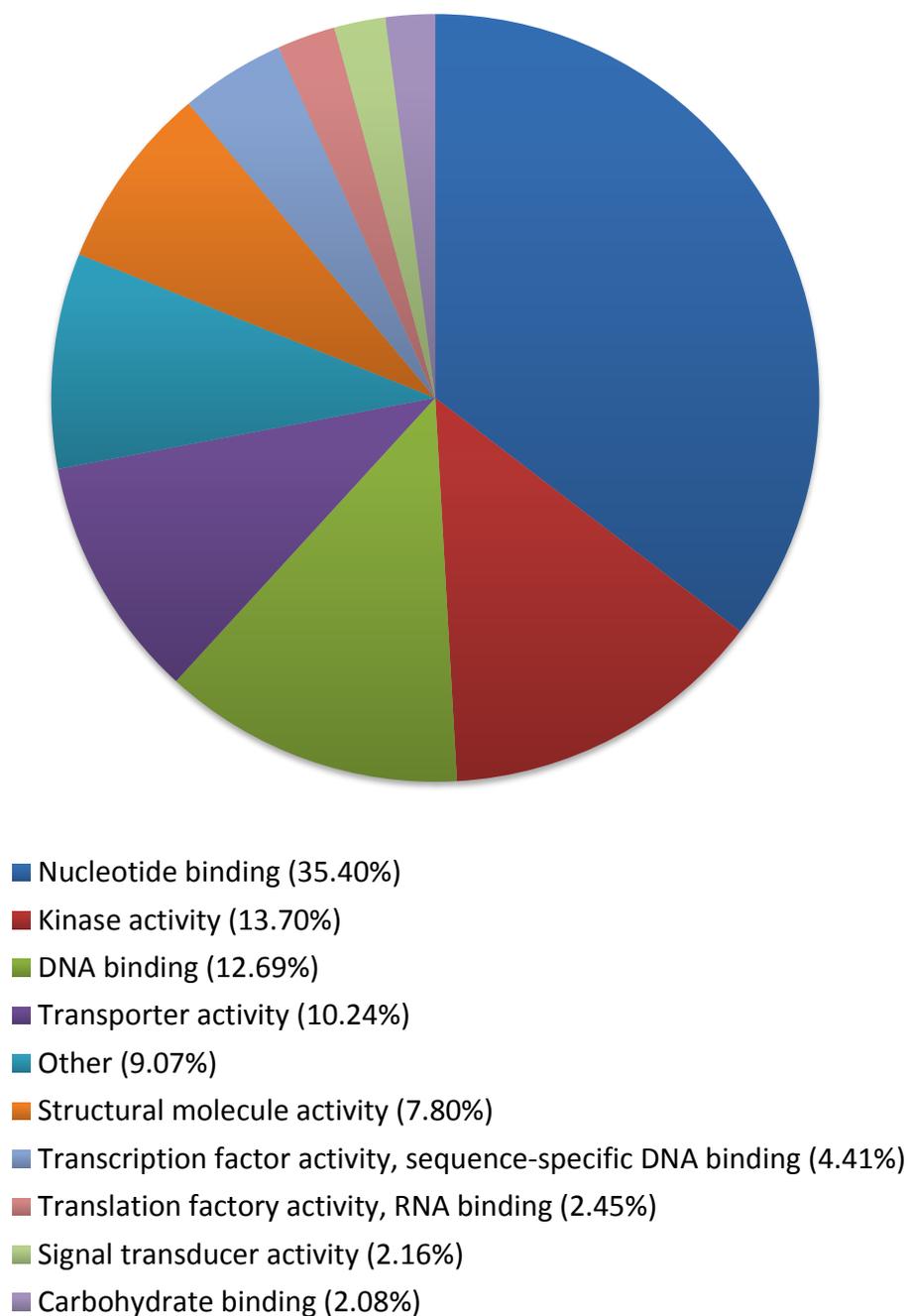


Figure 26. Percentage of transcripts in *Betula papyrifera* control samples grouped by molecular function.

A total of 23,876 transcripts were assigned gene ontology using BLAST2GO. Categories under 2% were grouped together and classified as “other”.

For cellular compartmentalization, 10,580 transcripts were assigned gene ontology. Overall, 16.4% of the transcripts were localized in ribosome, 15.1% in cytosol, 11% in plasma membrane, 11% in mitochondria, 9% in plastid, 6.8% in cytoskeleton, 6.2% in endoplasmic reticulum, and 5.9% in Golgi apparatus (Figure 27). Hence, among the three principal gene ontologies, most of the differentially expressed genes were classified into the terms CCO, CMP, transport, nucleotide binding, kinase activities, DNA binding, ribosome, cytosol, plasma membrane, and mitochondria suggesting that these functional processes play a major role in *B. papyrifera* gene activities.

7.4.3. Differential gene expression

Overall, 99,243 of the total 209,802 genes were expressed. After normalization, a total of 62,587 genes were selected as effectively expressed. Classification of differentially expressed genes was performed to examine their functional distribution characteristics. To determine the effect of nickel treatment, the whole transcriptome was analyzed and nickel – resistant and susceptible transcriptomes were compared to untreated samples. No significant difference in gene expression between moderately resistant and susceptible was found. Therefore only comparisons between susceptible and resistant genotypes were performed. In general, there was a higher number of upregulated than downregulated translation transcripts for biological functions in resistant plants. The same trend was observed for response to stress genes (Figure 28). Analysis of molecular functions reveals a high level of upregulation of structural molecule activities for resistant and susceptible genotypes compared to control (Figure 29). For cellular compartment, there was an upregulation of ribosome genes in both resistant and susceptible, with a higher increment of regulation in resistant samples. A down regulation of cytoskeleton and plasma membranes genes was observed in resistant genotypes (RG) and in plasma membrane genes in susceptible genotypes (SG). Plastid genes were downregulated in both RG and SG (Figure 30).

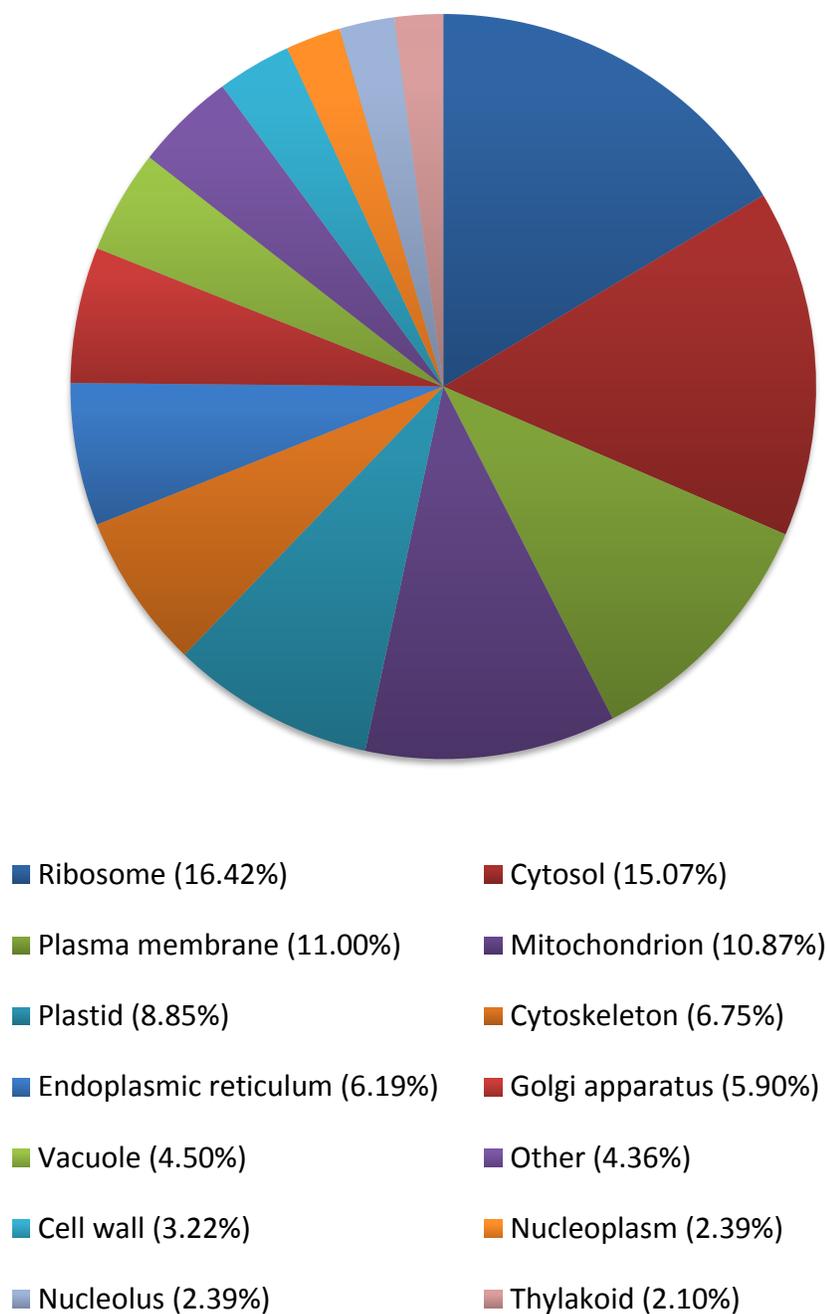


Figure 27. Percentage of transcripts in *Betula papyrifera* from the control grouped by cellular compartment.

A total of 10,580 transcripts were assigned gene ontology using BLAST2GO. Categories under 2% were grouped together and classified as “other”.

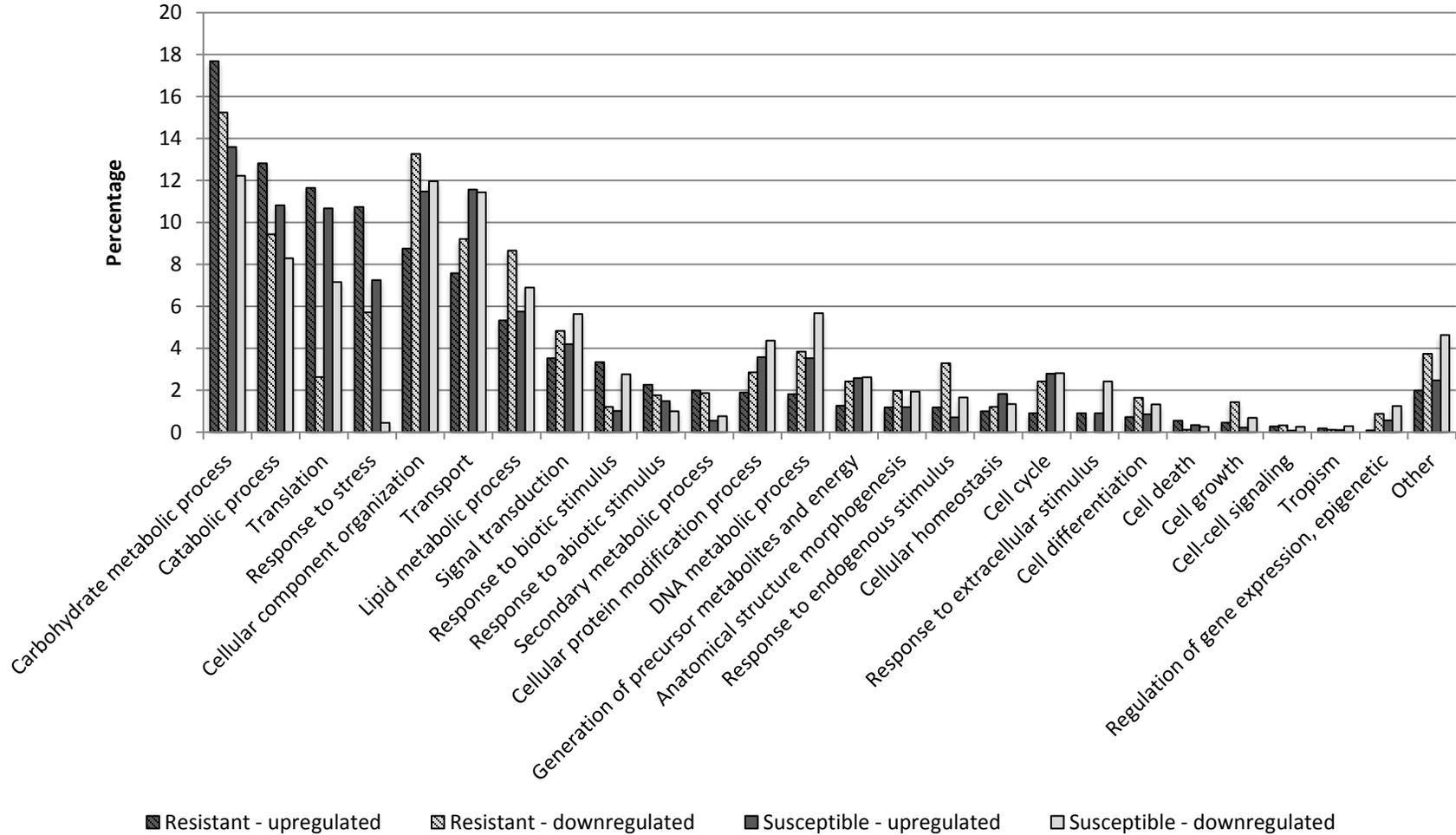


Figure 28. Percentage of upregulated and downregulated transcripts in the nickel- resistant and susceptible *Betula papyrifera* genotypes compared to control (nickel untreated samples).

Transcripts were assigned gene ontology and grouped by biological function using BLAST2GO.

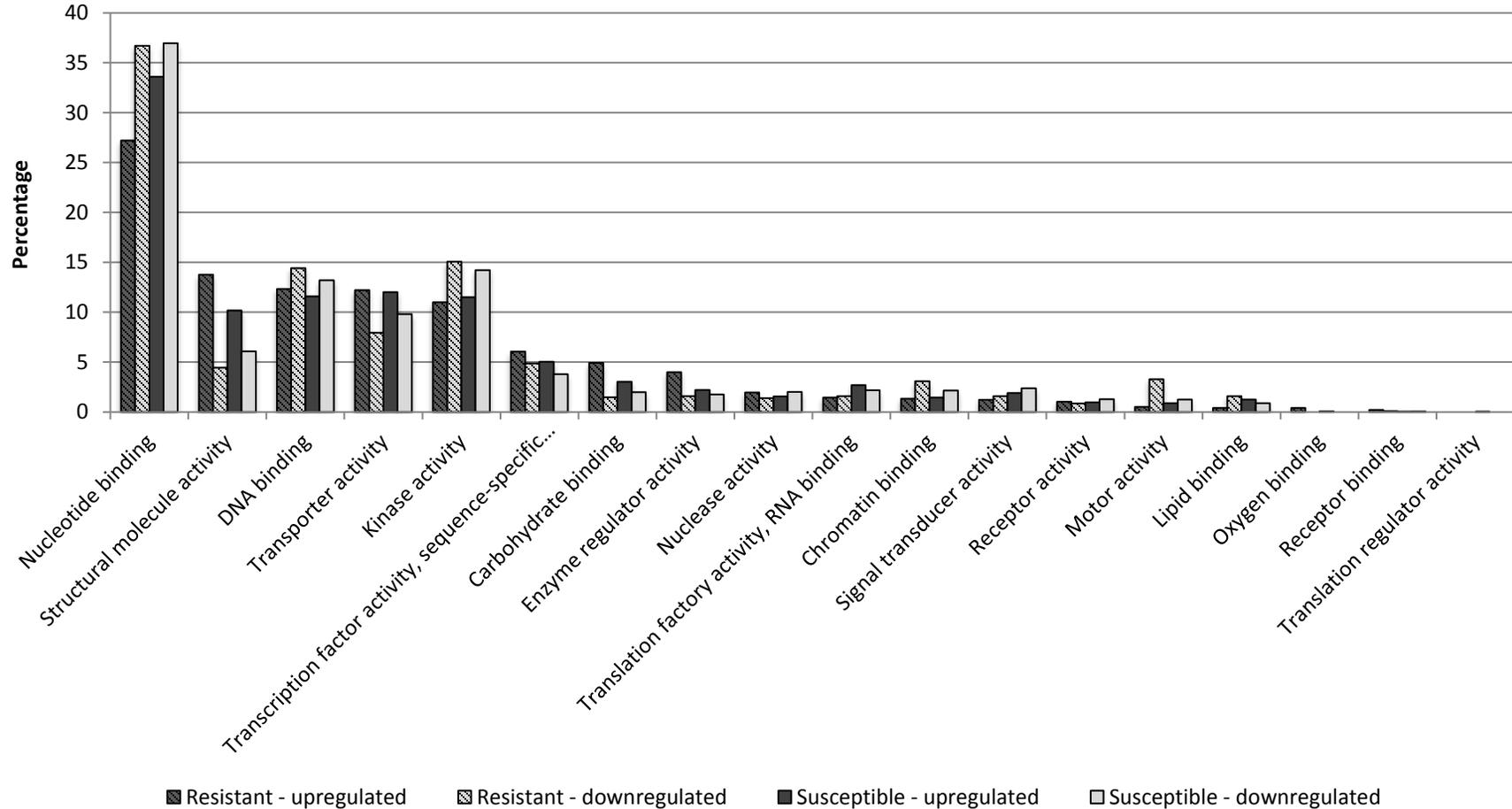


Figure 29. Percentage of upregulated and downregulated transcripts in the nickel- resistant and susceptible *Betula papyrifera* genotypes compared to control (nickel untreated samples).

Transcripts were assigned gene ontology and grouped by molecular function using BLAST2GO.

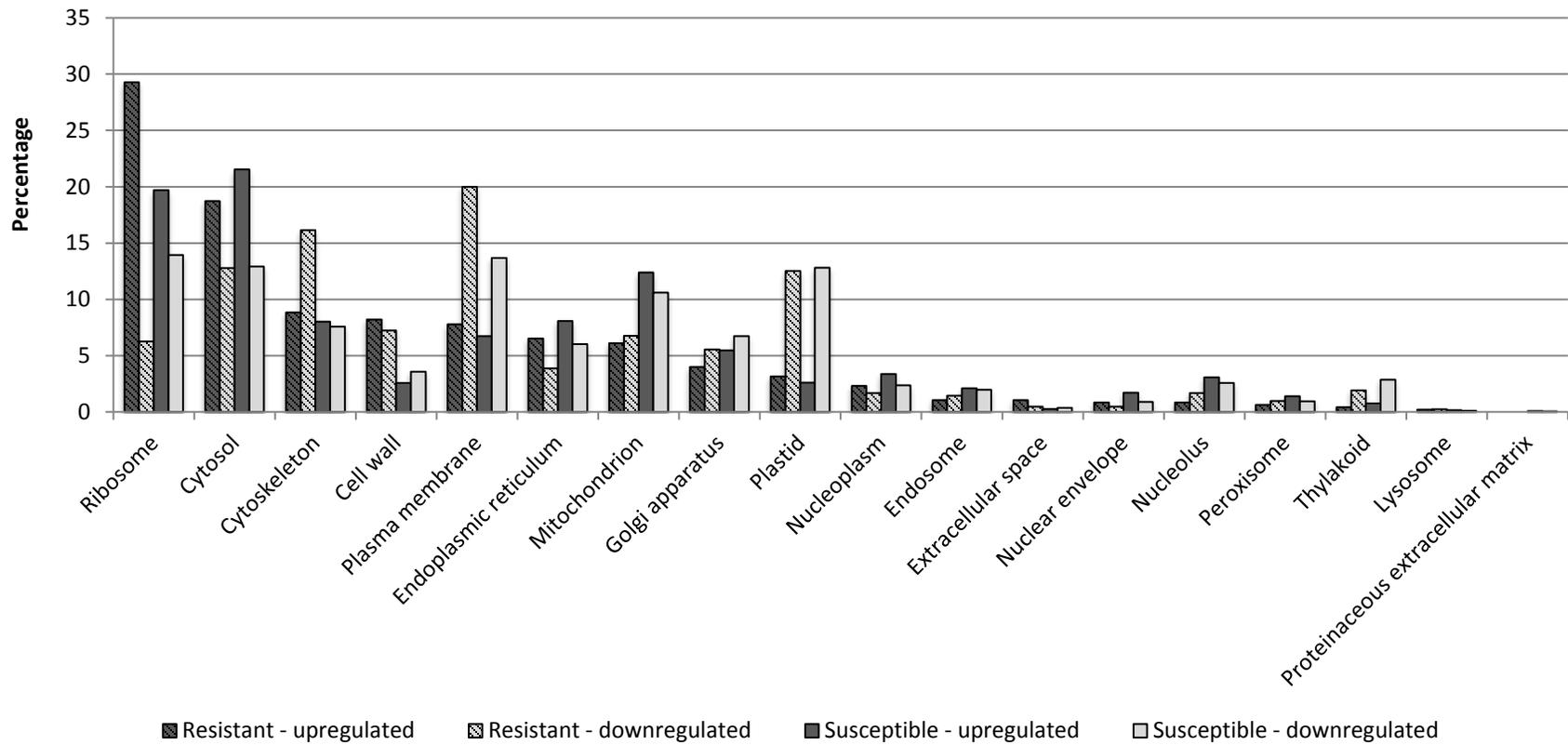


Figure 30. Percentage of upregulated and downregulated transcripts in the nickel- resistant and susceptible *Betula papyrifera* genotypes compared to control (nickel untreated samples).

Transcripts were assigned gene ontology and grouped by cellular compartment using BLAST2GO.

7.4.3.1. Pairwise comparison of resistant and susceptible genotypes

When highly resistant genotypes were compared to control, 3,225 genes were upregulated and 3,012 downregulated while 6,415 were upregulated and 2444 downregulated when susceptible genotypes were compared to control. On the other hand, we found 1,646 upregulated and 1,124 downregulated genes when susceptible genotypes were compared to resistant.

Figure 31 shows regulation of the top 50 genes when resistant genotypes (RG) were compared to susceptible; Appendix 2 when RGs were compared to control; and Appendix 3 when SGs were compared to control. Pairwise comparison for biological, molecular functions and cellular compartments between RG and SG are described in Figures 32 to 34. For biological functions, there were more upregulated than downregulated genes in RG compared to SG for response to stress, DNA metabolic process, generation of precursor metabolites, and response to biotic and abiotic stimuli (Figure 11). The opposite trend (more downregulated than upregulated genes) was observed for carbohydrate metabolite process, cellular component organization, translation, and cell cycle. For molecular function, the number of upregulated genes was higher than downregulated for nucleotide binding, signal transducer and receptor activities. The opposite was observed for structural molecular activity and carbohydrate binding (Figure 33). For cellular compartment, we observed 7x more upregulated genes than downregulated in plasma membrane but 2x more downregulated than upregulated in ribosomes, and 60% more downregulated genes than upregulated in cytosol (Figure 13). Comparative analysis of RGs and control, and SGs and control are described in the Appendix 4 to 9.

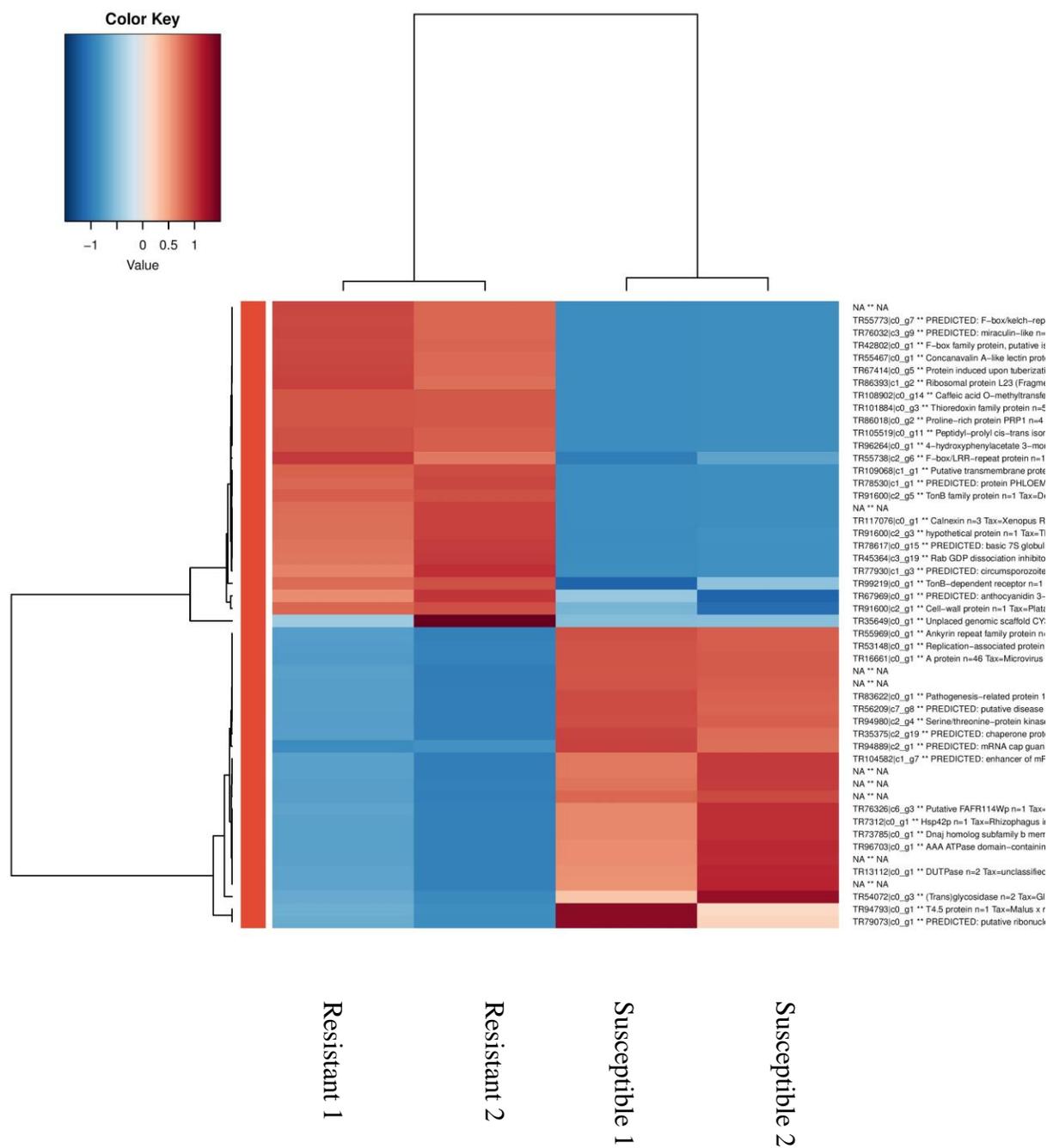


Figure 31. Top 50 differentially expressed genes between nickel-treated resistant and susceptible *Betula papyrifera* genotypes based on log₂FC.

The red colour represents an upregulation and blue downregulation.

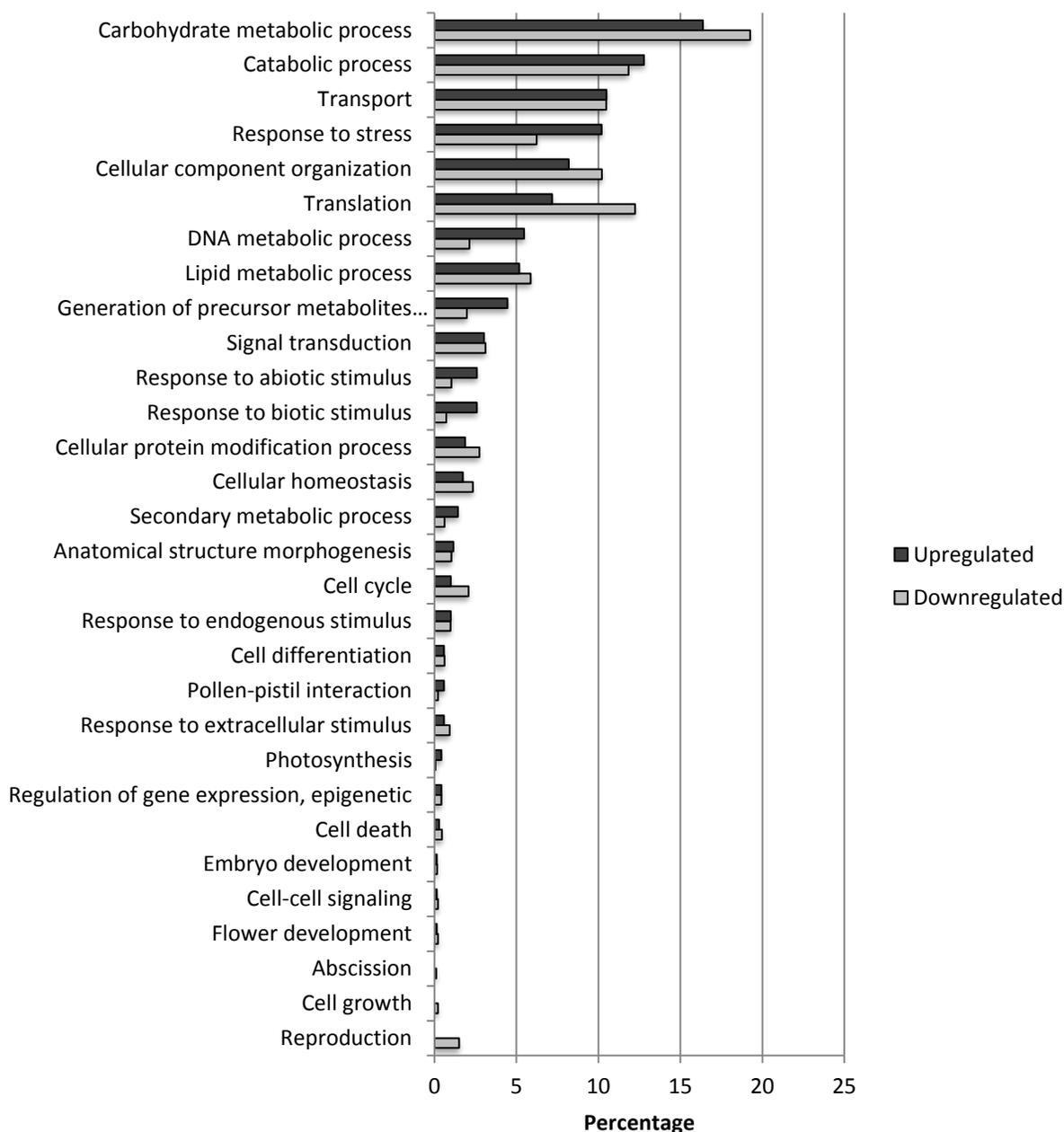


Figure 32. Percentage of upregulated (total number = 696) and downregulated (total number = 1,927) transcripts when nickel resistant *Betula papyrifera* genotypes were compared to nickel susceptible genotypes.

Transcripts were assigned gene ontology and grouped by biological function using BLAST2GO.

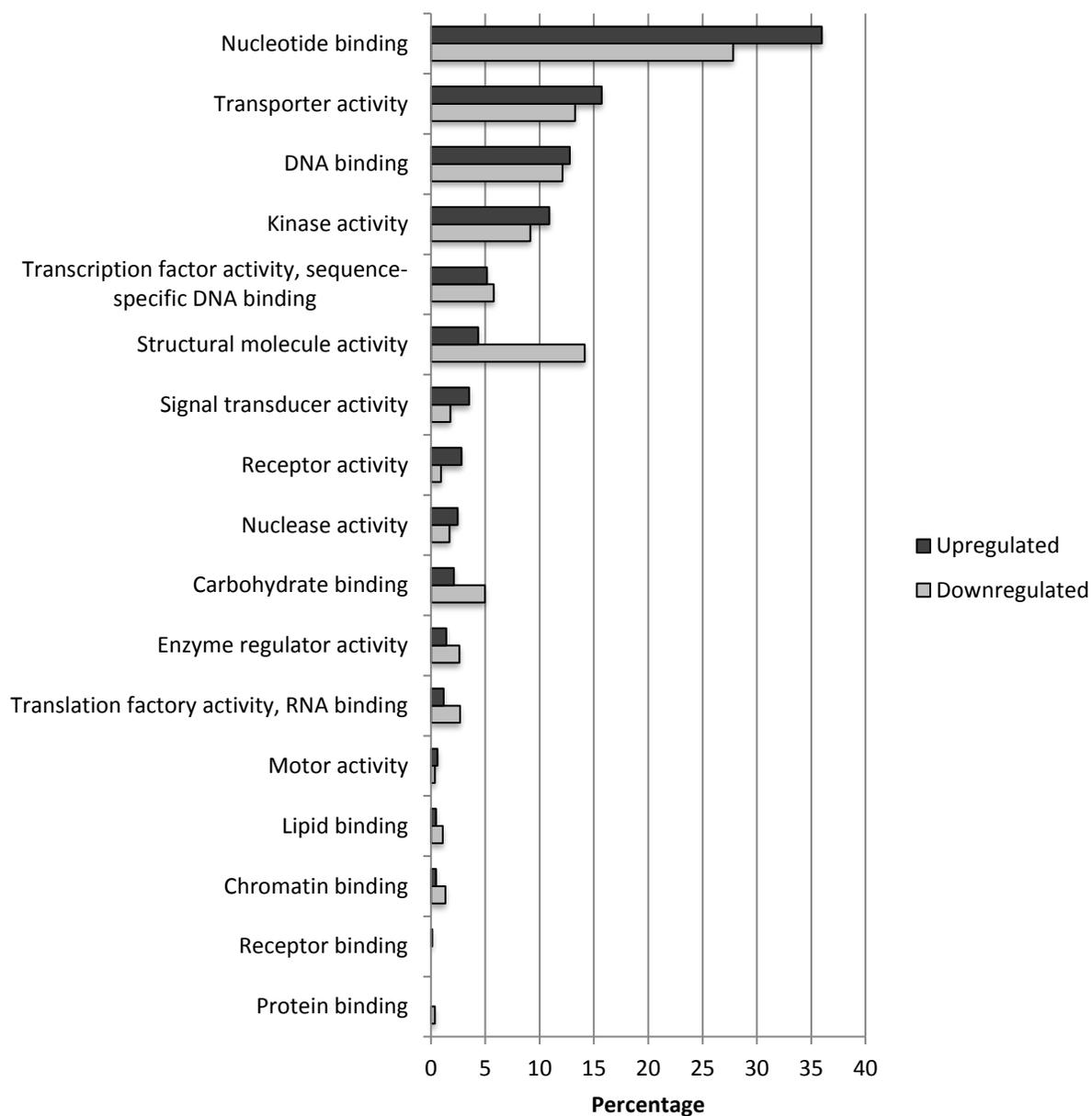


Figure 33. Percentage of upregulated (total number = 853) and downregulated (total number = 1,751) transcripts when nickel resistant *Betula papyrifera* genotypes were compared to nickel susceptible genotypes.

Transcripts were assigned gene ontology and grouped by molecular function using BLAST2GO.

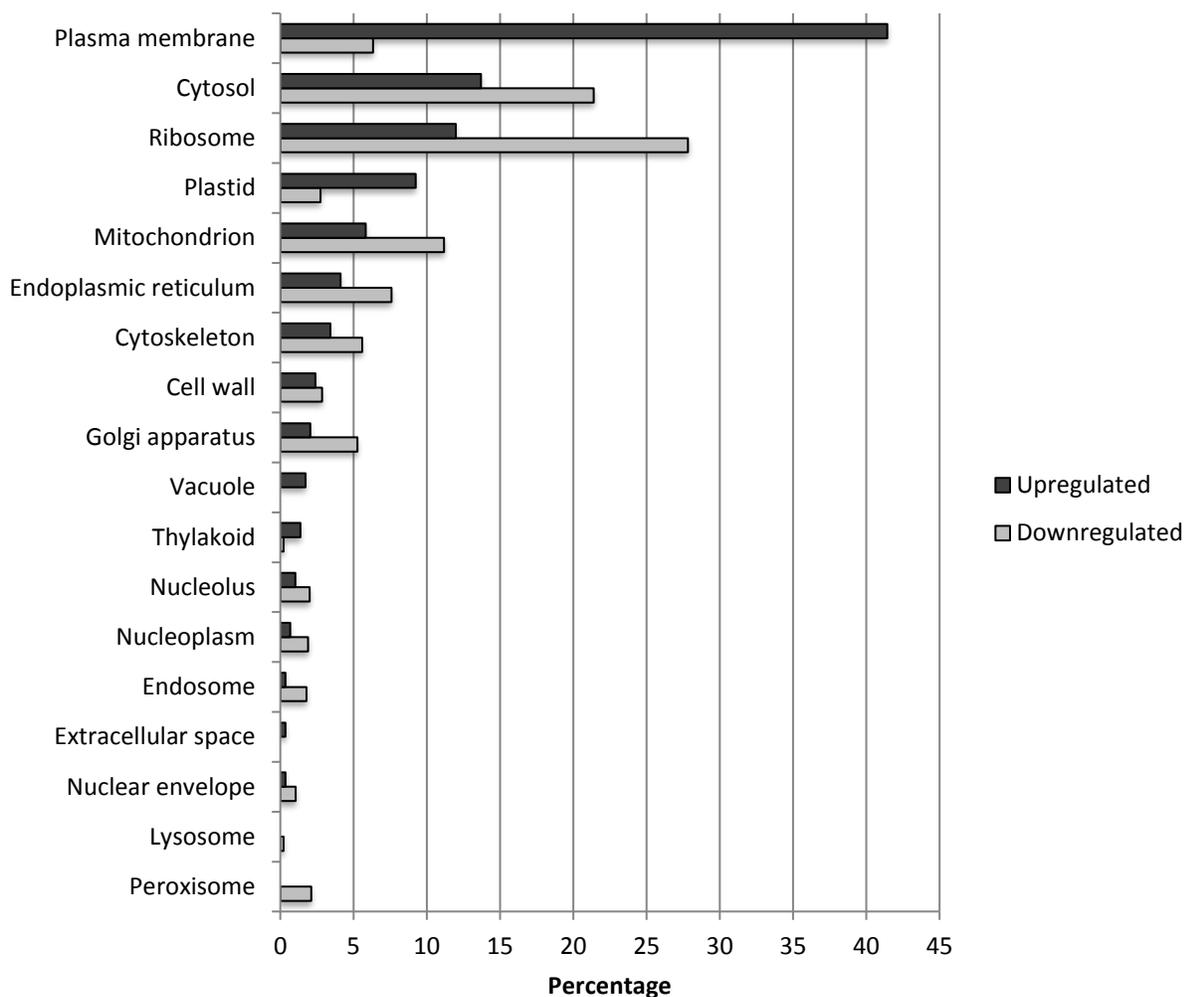


Figure 34. Percentage of upregulated (total number = 292) and downregulated (total number = 949) transcripts when nickel resistant *Betula papyrifera* genotypes were compared to nickel susceptible genotypes.

Transcripts were assigned gene ontology and grouped by cellular compartment using BLAST2GO.

7.4.3.2. Characterization of highly expressed genes

Molecular function data shows that among the top 50 transcripts/genes, 50% are involved in binding activity, 16% in catalytic activity, 6% in transport activity, 4% in nucleic acid binding transcription factor activity, 4% in other activities, and 20 % unknown activities when RG were compared to control (Figure 35). Overall, 80% of the genes involved in binding activity were downregulated and 20 % upregulated in nickel treated-resistant genotypes compared to nickel – untreated genotypes. The same trend was observed for genes involved in transporter activity since two of the three genes identified were upregulated in resistant genotypes. An opposite trend was observed for catalytic activity where 6 of the 10 genes were upregulated in resistant genotypes and 4 downregulated (Figure 35).

The specific genes that were upregulated in resistant genotypes include DNA, actin, calcium, and receptor binding; methyl transferase, isomerase/reduction, aconitate hydratase, cellulose synthase, and storage protein genes. The downregulated genes are involved in DNA, metal, GTPase, receptor, cellulose synthase/metal, and lipid bindings; and mRNA, hydrolase/reduction, reduction, storage protein, transporter, pectate lyase, metal binding reduction, cellulose synthase/metal binding, DNA binding nuclease, and DNA binding peptidase.

We found that 50% of the top 50 transcripts have unknown molecular functions based on gene ontology when RG and SG were compared. All the transport activity genes were upregulated in RG and all the genes for nucleic binding or transcription factor activity were downregulated (Figure 36). We discovered five genes that were expressed at significantly higher levels in RG compared to SG (from 300 to 500 fold). We further characterized these highly expressed transcripts in RG by querying against NCBI's 'nr' database and assigned GO terms in Blast2GO.

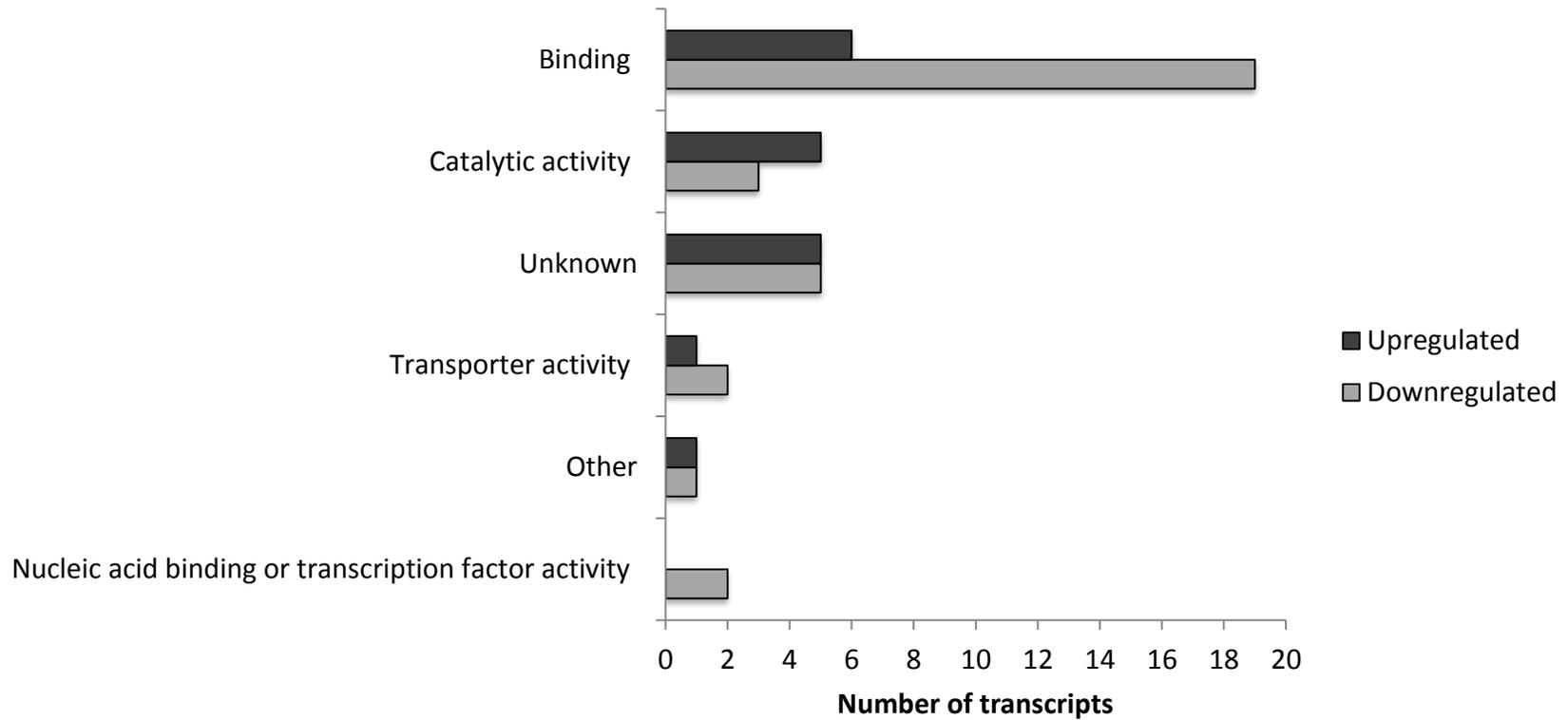


Figure 35. Top 50 transcripts when nickel resistant *Betula papyrifera* genotypes were compared to control (nickel untreated genotypes).

Transcripts were assigned gene ontology and grouped by molecular function using BLAST2GO.

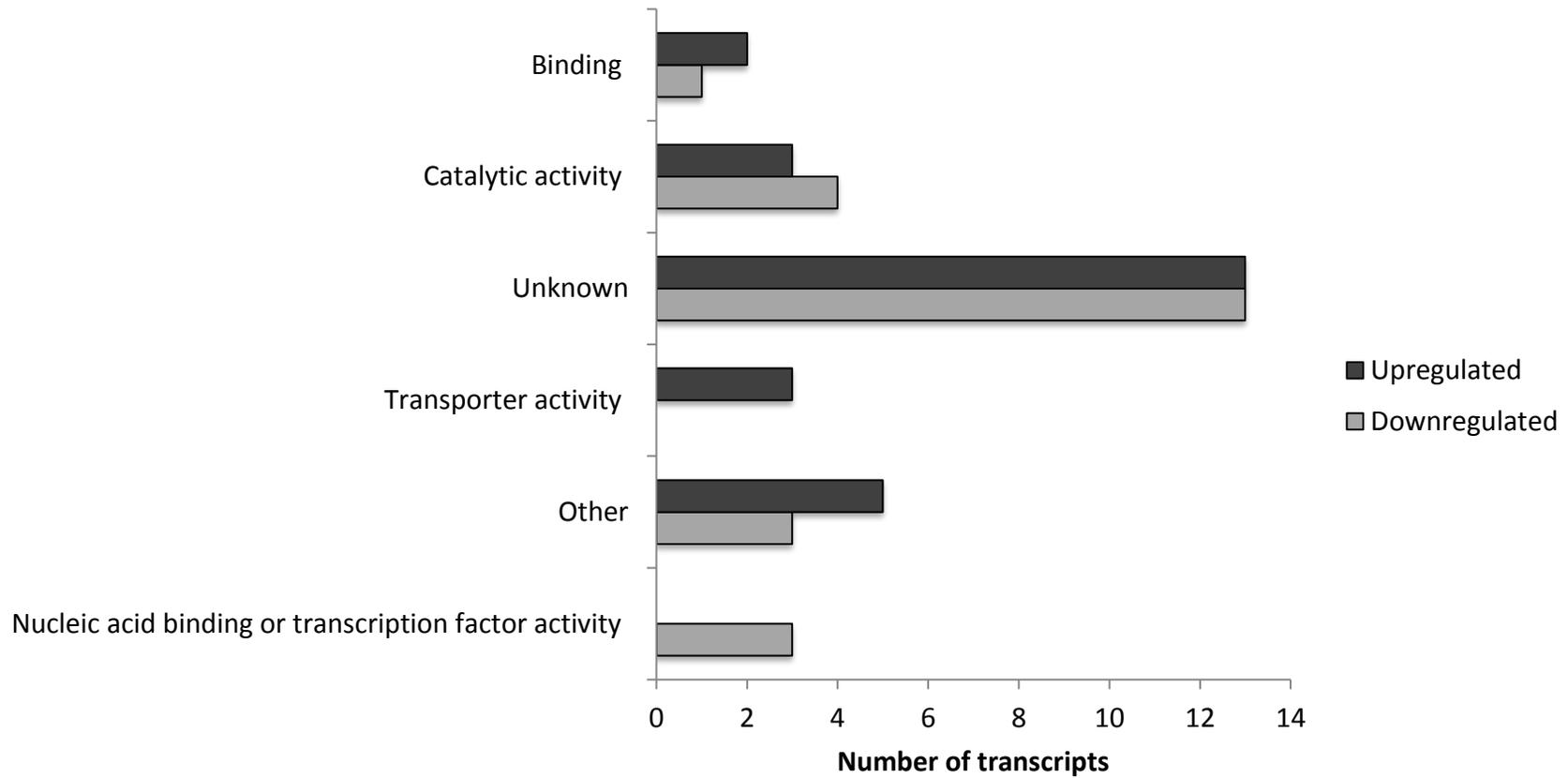


Figure 36. Top 50 transcripts when nickel resistant *Betula papyrifera* genotypes were compared to nickel susceptible genotype.

Transcripts were assigned gene ontology and grouped by molecular function using BLAST2GO.

These candidate genes involved in nickel resistance include Glutathione S-transferase (GST), thioredoxin family protein, putative transmembrane protein and Nramp transporters (Table 16). The expression of these genes was confirmed by q-PCR using designed primers described in Table 17.

Table 16. Candidate genes involved in nickel resistance of white birch (*Betula papyrifera*).

Transcript ID	Protein	Log2FC	Function	References
TR73973 c3_g5	Glutathione S-transferase	9.15	Detoxification of electrophilic xenobiotics using glutathione	(188, 357)
TR101884 c0_g3	Thioredoxin family protein	9.15	Defense against metal stress, metal binding	(358, 359)
TR109068 c1_g1	Putative transmembrane protein	9.10	Unknown	None
TR56135 c0_g6	Nramp transporter	8.62	Metal transport, found to play a role in Ni resistance and homeostasis in plants.	(221, 222)
TR56135 c0_g1	Nramp transporter	8.32	Metal transport, found to play a role in Ni resistance and homeostasis in plants.	(221, 222)

note: Log2FC is calculated based on susceptible white birch

Table 17. Primer used for real-time quantitative RT-qPCR verification.

Target gene	Forward primer (5'-3')	Reverse primer (5'-3')	Product size (bp)
Glutathione S-transferase	TCTGAAACTCAAGGGTGTTGATT	GACTGGAACCTTTTTGTGAACTG	100
Thioredoxin family protein	GAAAAGCTTCTTCAGATCTGGGT	GACTTGGCCTTTCTAAAACCTTGC	103
Putative transmembrane protein	TTCTAATAAGGTATTGTGCGCGT	GGAGGAAAAGATTCACCAAGAGT	221
Nramp transporter1	TACATTCTCGCCGTCATTTATCT	GTTGATGCCTTTGATCTTGAAAC	205
Nramp transporter2	CTAGCAAGATCAGAGAGATGGGA	GAAACTTTCTCCATCCTGGTTTC	201

7.5. Discussion

There were no differences in gene expression when moderately resistant/susceptible and susceptible genotypes were compared. In addition, one of our previous studies found no difference in leaf or root Ni accumulation between moderately resistant and susceptible genotypes, however resistant genotypes had much lower Ni levels in their leaves (335). This confirmed that the moderately resistant/susceptible genotypes are susceptible genotype with a delayed expression of damage when treated with a high dose of nickel.

Glutathione S-transferases (GSTs) are a superfamily of multifunctional enzymes that play a role in enzymatic detoxification of xenobiotics. Many GSTs can also act as glutathione peroxidases to scavenge toxic peroxides from cells. In plants, GSTs also provide protection against oxidative stress induced by abiotic stresses and oxidants (351, 360, 361). An increase in GST activity in both root and shoot tissues was observed in response to Ni stress in wheat (*Triticum aestivum*) and cadmium stress in *Arabidopsis thaliana* (362, 363). Freeman *et al.*, 2004 reported that elevated glutathione (GSH) concentrations are involved in conferring tolerance to Ni-induced oxidative stress in *Thlaspi spp.*, Ni hyperaccumulators (188). The role of GSH in metal homeostasis, antioxidative defense, and signal transduction under metal stress is discussed in details in (357).

Thioredoxins play a role in oxidative damage avoidance by supplying reducing power to reductases, detoxifying lipid hydroperoxides or repairing oxidized proteins. They could act as regulators of scavenging mechanisms and as components of signaling pathways in the plant antioxidant network (364). Thioredoxin (Trx), peroxiredoxin (Prx), and sulfiredoxin (Srx) in plant cells have been involved in the control of dithiol–disulfide exchanges of target proteins, which modulate redox signaling during plant adaptation to stress.

Nramp (Natural resistance-associated macrophage protein) defines a novel family of related proteins, which have been implicated in the transport of divalent metal ions. This gene family has been highly conserved during evolution and homologues have been found in a wide range of living organisms including bacteria, yeast, insects, mammals and also higher plant (365). Analyses of the expression of Nramp genes in *Arabidopsis* suggest that all these Nramp genes play roles in constitutive metal transport mostly iron and cadmium. Mizuno *et al.*, (2005) elucidated the role of Nramp metal-transporters for Ni²⁺- transport and homeostasis. They found that, the expression of TjNramp4 caused elevation of Ni²⁺ sensitivity and Ni²⁺ concentrations in yeast (221). The present study is the first to associate Nramp gene expression with both resistance to and accumulation of nickel. A putative transmembrane protein (TMP) associated with nickel resistance was also identified. The role of this TMP in metals transport in plants has not been investigated.

Recent studies have shown a strong association between metal tolerance and antioxidative and glyoxalase systems, in which plants with low antioxidant capacity exhibit susceptibility to HM toxicity (188, 351, 366). An increase of at least glutathione (GSH) biosynthesis plays an important role in nickel tolerance in *Thlaspi spp* (351). Moreover, in several plants, the Ni-induced changes in activity of reactive oxygen species (ROS)-scavenging enzymes, including superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) were detected (363, 367, 368). Plants usually respond to oxidative stress by elevating the activity of the antioxidant enzymes of the ascorbate-glutathione cycle such as catalase, peroxidase, superoxide dismutase, glutathione reductase, and ascorbate oxidase, which protect plant cells against free radicals (367, 369, 370). These activities appear to be reduced in Ni-accumulators plants to which *B. papyrifera* belongs (367). It is not clear if the changes in enzyme activities are triggered directly by Ni²⁺

effects including binding to SH-group or histidine or displacing the metals from metal-enzyme active centers, or indirectly when mediated by chain of reactions that affect the expression of the corresponding genes or exhaust their substrate pools (370).

The role of metal transporters has been investigated in many studies. However our knowledge of the transport processes for HM across plant membranes at the molecular levels is still very limited (219, 351, 365, 371–373). The present study showed a significantly upregulation of genes in ribosomes in samples treated with nickel compared to control. This was consistent with biological function profile that reveals a significant upregulation of genes associated with translation in nickel treated samples compared to control. When RG were compared to SG, the main nickel resistance mechanism revealed by the gene regulation analysis is a downregulation of genes associated with translation in ribosome and an upregulation of genes associated with the plasma membrane. As shown in the top 50 genes, significant downregulation of genes associated to binding is also a key characteristic of resistance to nickel in *B. papyrifera*. Three of the five candidate genes (putative transmembrane protein, Nramp transporters #1 and #2) identified are related to metal transport in plants.

7.6. Conclusion

In the present study, the *B. papyrifera* transcriptome was developed. A total of 208,058 trinity genes were identified and were assembled to 275,545 total trinity transcripts. Expression profiles were generated and 62,587 genes were found to be significantly differentially expressed among the nickel resistant, susceptible, and untreated libraries. The main nickel resistance mechanism in *B. papyrifera* is a down regulation of genes associated with translation (in ribosome) and binding, and an upregulation in genes involved with the plasma membrane. Five candidate genes

associated to nickel resistance were identified. They include Glutathione S –transferase (GST), thioedoxin family protein, putative transmembrane protein, and two Nramp transporters. These genes can be useful for genetic engineering of birch trees. The results of the present study also demonstrate that next-generation sequencing technologies can be used to access the transcriptome of higher accumulator plants and help identify underlying molecular mechanisms. Future work can be done to use the genomic sequence to improve the transcriptome (e.g. obtain exon/intron structure, merge fragments to get full length genes, identify promoter sequences etc.).

Chapter 8: 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?

8.1. Abstract

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 to 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.

Key word: TonB-dependent receptor and TonB-like family protein; Nickel resistance; *Betula papyrifera* transcriptome; Gene expression; Horizontal gene transfer.

8.2. 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 (374). If metal balance is disrupted, it may lead to deficiency or toxicity, often associated with oxidative stress. The production of reactive oxygen species (ROS) usually results in deregulation of protein activity, organelle damage, membrane deterioration, and nucleic acid damage (34). Plants are often exposed to metals due to expansion of industries and their pollutants. For example, the Greater Sudbury region (GSR) in Northern Ontario, Canada is home to one of the largest nickel/copper deposits and extractive operations in the world. A century of mining and smelting operations has left the surrounding land acidified and contaminated with high levels of metals (4, 5, 256). Years of exposure to toxic levels has led to development of metal resistant plant populations (87).

Mechanisms of metal resistance have been reported in many plant species but knowledge of how woody plants deal with metals is scarce (88, 173, 223). 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 (171–173). Other plants species increase production of chelating molecules such as phytochelatins (PCs), metallothioneins (MTs) and small molecules from metabolic processes to cope with metal contamination (185, 208, 343, 375). 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 cell (216, 219).

de Silva *et al.*, (2012) results showed that even small amount of nickel at the bioavailable levels in the GSR cause a decrease of stomatal density and chlorophyll content in *Acer rubrum* (376). 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 (219). A similar mechanism has been reported in *Arabidopsis thaliana* where nickel is transported into the tonoplast via a IREG2/FPN transporter (216, 217). Transporters from the NRAMP family have been thought to play a role in nickel resistance (222). This has been confirmed in a recent study showing a strong association between NRAMP transporters with nickel accumulation and resistance in *Betula papyrifera* (325). 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 B12 (377). 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 (378).

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

8.3. Materials and methods

This study is a comprehensive extension of the baseline transcriptome data described in (325).

8.3.1. Nickel treatment

White birch (*Betula 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 over 100 years. Details of seed germination, seedlings treatment with nickel nitrate and toxicity assessment are presented in (322, 325, 335). Gene expression in genotypes resistant and susceptible to a soil nickel concentration of 1,600 mg/kg is analyzed in details in the present study.

8.3.2. De novo transcripts assembly

Methods for extraction, RNA- seq libraries, new generation sequencing, and De Novo Transcripts Assembly are described in (325). 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 reads mapped (RPKM). A differentially expressed gene analysis was performed between resistant and susceptible genotypes. The heatmap data describing the top 50 most expressed genes have been reported in (325). The top 25 most upregulated genes based on Log2FC from the pairwise comparison were ranked in this study (Tables 18 and 19). Similar analysis was performed for the top 25 most downregulated genes. Baseline filtering of genes that likely are the effect

Table 18. Top 10 most upregulated genes in nickel-resistant white birch (*Betula papyrifera*) compared to susceptible genotypes.

Transcript ID	FPKM		Log2FC		adj.P.Val	Description	
	Res 1	Res 2	Sus 1	Sus 2			
TR99219 c0_g1	88.837	261.961	0	0.54	11.54	1.97E-07	TonB-dependent receptor
TR91600 c2_g5	209.847	415.208	0	0	10.50	1.92E-18	TonB family protein
TR55738 c2_g6	49.072	24.947	0	0.048	10.14	3.40E-09	F-box/LRR-repeat protein
TR117076 c0_g1	75.234	281.904	0	0	10.02	3.56E-16	Calnexin
TR96264 c0_g1	2.728	3.487	0	0	9.46	4.57E-14	4-hydroxyphenylacetate 3-monooxygenase oxygenase component
TR78530 c1_g1	44.645	71.609	0.115	0.072	9.44	3.09E-05	PREDICTED: protein PHLOEM
TR86018 c0_g2	42.155	64.06	0	0	9.34	1.53E-13	PROTEIN 2-LIKE A1-like
TR86018 c0_g2	42.155	64.06	0	0	9.34	1.53E-13	Proline-rich protein PRP1
TR67414 c0_g5	48.434	40.922	0	0	8.90	5.98E-12	Protein induced upon tuberization
TR109068 c1_g1	10.829	25.767	0	0	8.88	1.27E-11	Putative transmembrane protein
TR100502 c2_g1	5.78	18.454	0	0	8.83	2.34E-11	NA

FPKM: Fragments Per Kilobase of transcript per Million mapped reads

Log 2FC: Fold change from log2

adj.P.Val: Adjusted p values

Table 19. Top 10 most downregulated (fold change) genes in nickel-resistant white birch (*Betula papyrifera*) compared to susceptible genotypes.

Transcript ID	FPKM		Sus 1	Sus 2	Log2FC	adj.P.Val	Description
	Res 1	Res 2					
TR16661 c0_g1	0	0	14.611	14.912	- 11.94	2.78E-20	A protein
TR94889 c2_g1	0	0.028	232.706	100.675	- 11.65	1.14E-14	mRNA cap guanine-N7 methyltransferase 2 isoform X2
TR53148 c0_g1	0	0	14.082	13.831	- 10.85	3.07E-16	Replication-associated protein A
TR55969 c0_g1	0	0	26.541	55.026	- 9.87	7.29E-13	Ankyrin repeat family protein
TR94980 c2_g4	0	0	65.692	57.535	- 9.50	9.80E-12	Serine/threonine-protein kinase
TR61028 c0_g1	0	0	54.682	175.439	- 9.19	1.07E-10	NA
TR56209 c7_g8	0	0	114.437	89.869	- 9.14	1.56E-10	Putative disease resistance protein RGA3
TR35375 c2_g19	0	0	110.296	66.672	- 8.93	6.51E-10	Chaperone protein dnaJ 8, chloroplastic isoform X2
TR79073 c0_g1	0	0	132.672	1.609	- 8.90	8.14E-10	Putative ribonuclease H protein At1g65750-like
TR35437 c1_g1	0	0	44.098	88.2	- 8.90	7.95E-10	NA

FPKM: Fragments Per Kilobase of transcript per Million mapped reads

Log 2FC: Fold change from log2

adj.P.Val: Adjusted p values

of nitrate was conducted in order to make sure the selected candidate genes responded to nickel and not nitrate (325).

8.3.3. 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 DNase 1 (#EN0521) from Life Technologies. PCR primers were designed using the transcriptome sequence. The cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit by Life Technologies. 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 done 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 ($p < 0.05$).

8.3.4. TonB-dependent receptor in other species

Polymerase chain reaction (PCR) was performed using cDNA or DNA template from *B. papyrifera* and other species (Table 3). Each PCR reaction included a forward primer (0.2 μ M), reverse primer (0.2 μ M), MgCl (2 mM), dNTPs (0.2 mM), 1X buffer, 0.625 Units of Taq Polymerase and 50 ng of DNA/cDNA template. 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 30s; (3) 30s annealing; (4) elongation at 72°C or 30s; (5) read (6) repeat step 2–6 for 41 cycles; (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.

A basic local alignment search (BLAST) using the dwarf birch (*Betula nana*) genome (<http://birchgenome.org/>) was performed to determine sequence similarity (329) (Appendix 10 and 11).

Table 20. List of tree species tested for the presence of the TonB-dependent receptor sequence.

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

CAN = Canada; SCT = Scotland; BEL = Belgium; SWE = Sweden; CHN = China; JPN = Japan,

USA = United States of America.

8.4. Results

8.4.1. Transcriptome

Differentially expressed genes between nickel resistant, susceptible and water controls were identified. Broad data of the heat map has been presented in (325). The top upregulated and downregulated genes were ranked based on log₂FC. 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 37). Among the top 25 most downregulated genes, 20%, 0%, and 8% were associated with catalytic, transport and binding activities, respectively (Figure 38). 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 functions (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 to susceptible (SG) (Table 1). Their gene IDs in the *B. papyrifera* transcriptome are TR99219|c0_g1

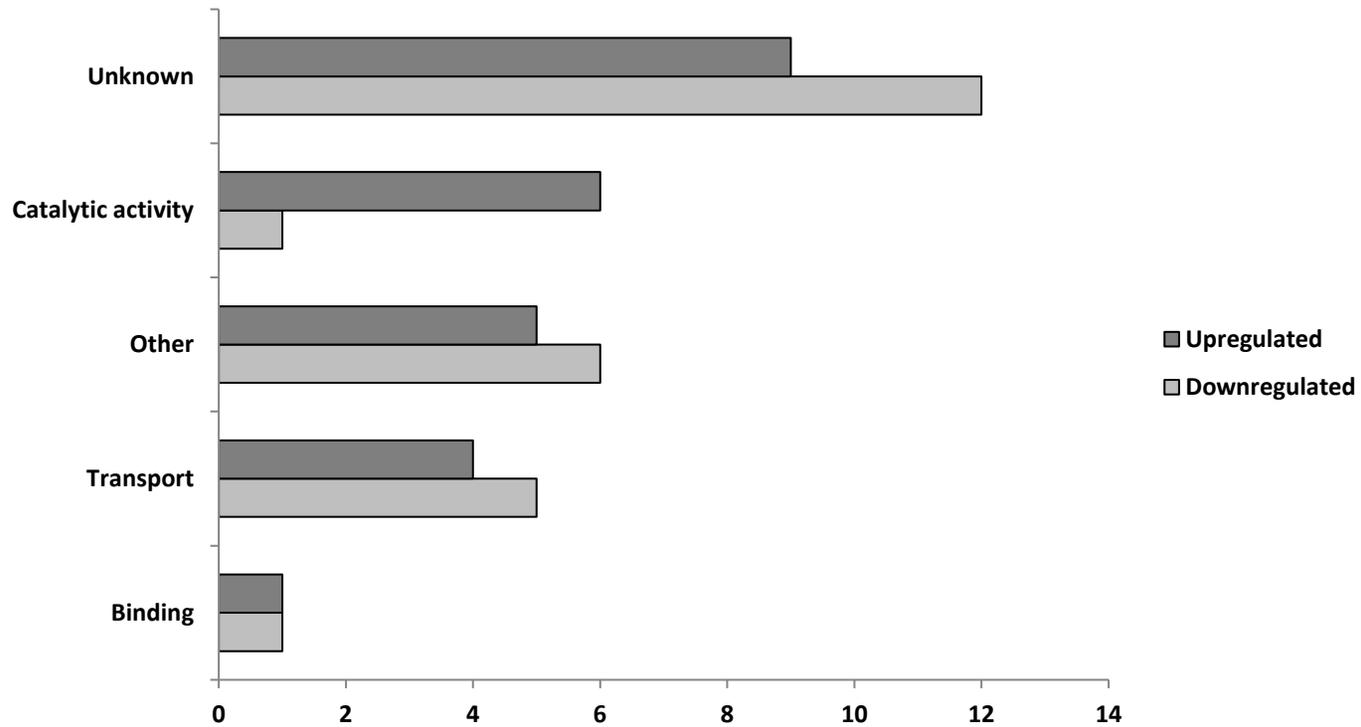


Figure 37. Top 25 upregulated and 25 downregulated transcripts when nickel resistant *Betula papyrifera* genotypes were compared to control (nickel untreated genotypes).

Transcripts were assigned gene ontology and grouped by molecular function using BLAST2GO.

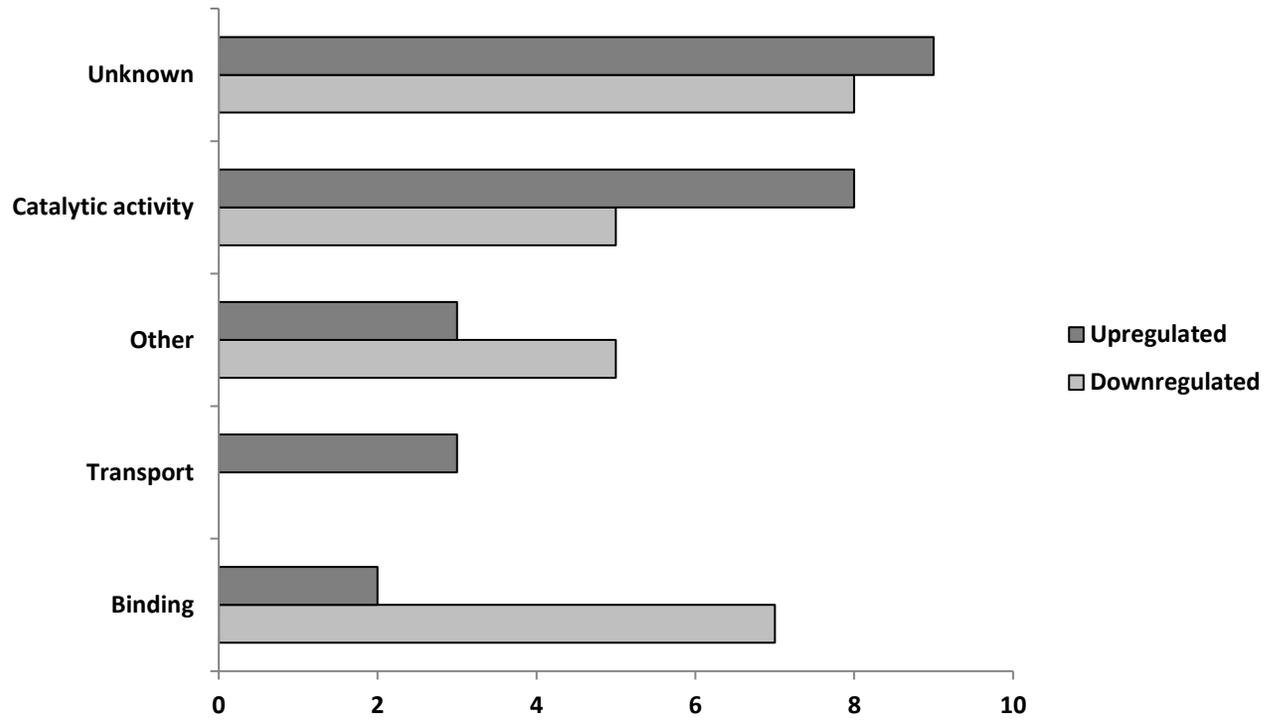


Figure 38. Top 25 upregulated and 25 downregulated transcripts when nickel resistant *Betula papyrifera* genotypes were compared to nickel susceptible genotype.

Transcripts were assigned gene ontology and grouped by molecular function using BLAST2GO.

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 bp and 258 bp, respectively (Figures 39 and 40).

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 2978x and 1448x higher in RG compared to SG. We also found that the expression of the TonB-dependent receptor transcript was 1082x higher in the RG compared to controls. There was no significant difference in the expression of the TonB-dependent receptor transcript when the water control was compared to SG. But the expression of TonB-like family protein transcript was 2048x higher in the water controls compared to Ni-treated susceptible genotypes. No significant difference was found in the expression of the TonB-like family protein transcript when RG were compared to water controls.

8.4.2. 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 to SG or water controls (Figure 41).

8.4.3. TonB-dependent receptor/-like protein in other species

The *Betula nana* database revealed the presence of the TonB dependent receptor and TonB-like family protein. We performed a BLAST alignment of the *B. papyrifera* transcripts with the

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
TCATGCCGAC	GGTCCC GGCA	AGGTCTCCCG	AAAACCGCTC	ATTCCCTTGT	GGGAAGGAAA	361
AGGTCCGCCAG	CTCGGTTGCT	TTGCAGCCCT	GGGCTCCTAC	ATCTACTATT	TTGGTGGGGC	421
GAGCCATACT	GGTTGGTTGC	GAGAGGGGCT	TCGCGACGGC	TACAAATTTT	AGGTACTCC	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	TAACTCTGCT	AGAGGATGAT	TATATGTACT	GTGTTATATT	1081
TGATGTTTCT	CGTATGCCCC	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	GTTCAATTTT	ATTTGACTTT	GTATTTGATT	TGCACTTTGC	1561
TTACATATGT	TTTGAATCAA	GAATCAATTT	CCGTCAGACT	TGAC		

Figure 39. Trinity assembled sequence of TonB-dependent family receptor (TR99219|c0_g1).

* Primer binding sites for PCR verification are shaded in grey.

CAGTGGCTCG	GCTTCTGGTG	CAGGTTCTGG	CTCTGCTTCT	GGGTCTGGTT	CTGGTTCTGC	61
TTCTGGGTCT	GGTCAAGGT	CTGATTCAGG	TTCTGAAGCA	GGCTCGTATG	CTGGGTCTCG	121
AGCTGGGTCA	GGTCAAAGG	GAAACCAAGA	ACGAGGAACA	GGGTCCGAGT	CAGGCCAAGG	181
CCGTGGGAGT	GGTCTGGTT	CAGGGTCTGG	TTCTGGTTCT	GGTCTGGAC	GTGGTGAGGG	241
TTCTGGTTCT	GGTCTGG					

Figure 40. Trinity assembled sequence of TonB-like family protein (TR91600|c2_g5).

* Primer binding sites for PCR verification are shaded in grey.

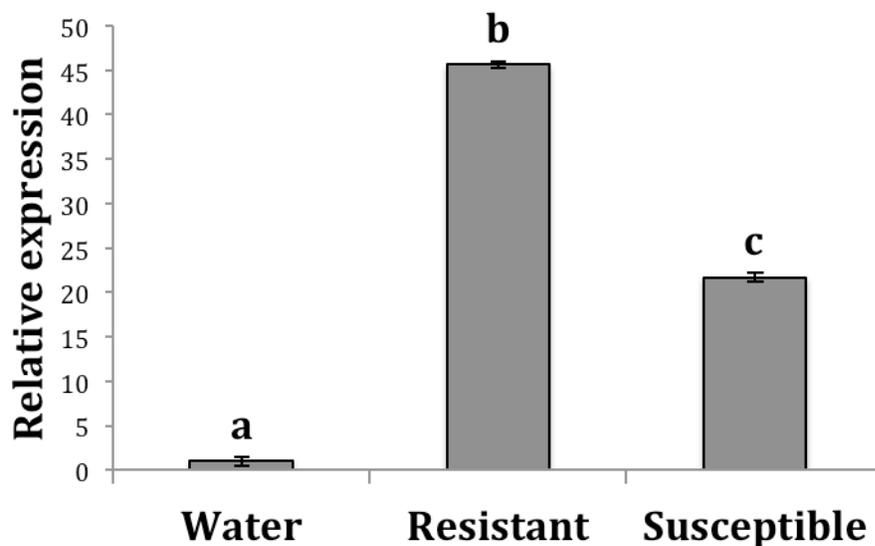


Figure 41. Quantitative qPCR measurement of the expression of TonB-dependent family receptor (TR99219|c0_g1) in nickel resistant and susceptible white birch (*Betula papyrifera*) treated with 1,600 mg/kg of nickel.

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, $p < 0.05$).

Betula nana (dwarf birch) genome (Appendix 10 and 11). The sequence similarity between both birch species for the TonB-dependent receptor and TonB-like family protein was 93 and 83percent, respectively. No match was found in plant species when using the NCBI BLAST tool. BLAST was also performed in additional databases (DDBJ/EMBL/GenBank, Poplar genome database, dendrome, Plantgdb, etc...). A match was only found in the EMBL database for *B. nana* and *B. platyphylla*. 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 42).

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 43). 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 43). Figure 44 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 20.

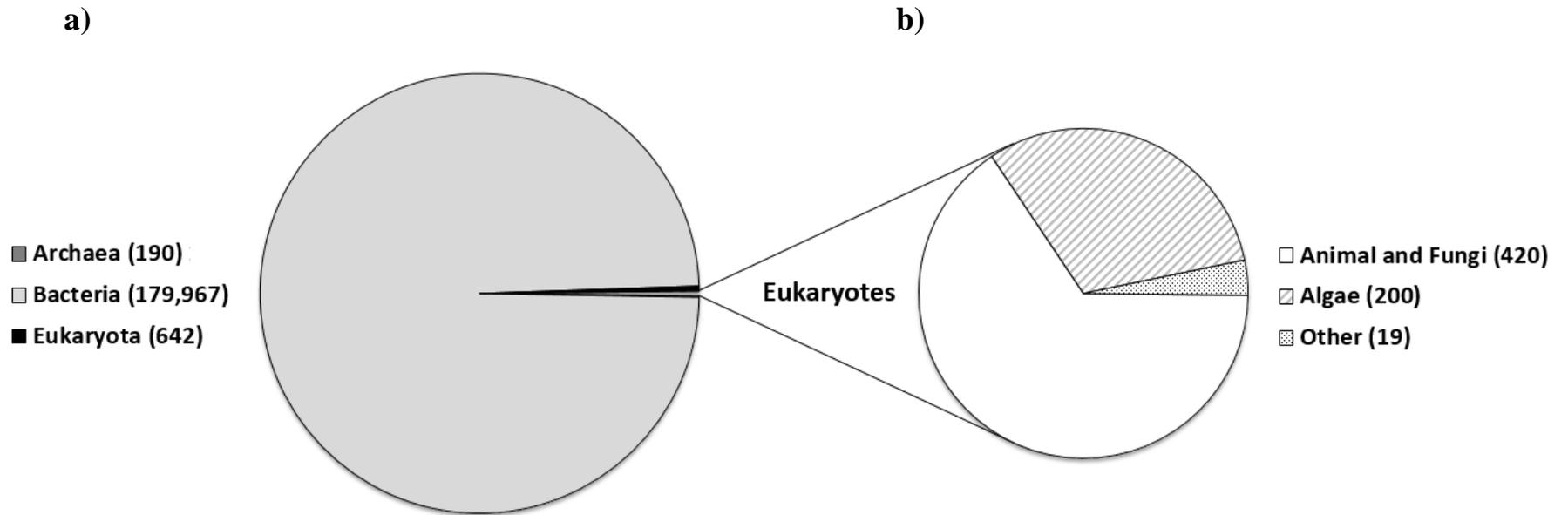


Figure 42. a) Distribution of TonB hits in Uniprot database classified by group of organisms (note that 99.5% hits are from bacteria);

b) Distribution of TonB hits in Uniprot database for Eukaryote.

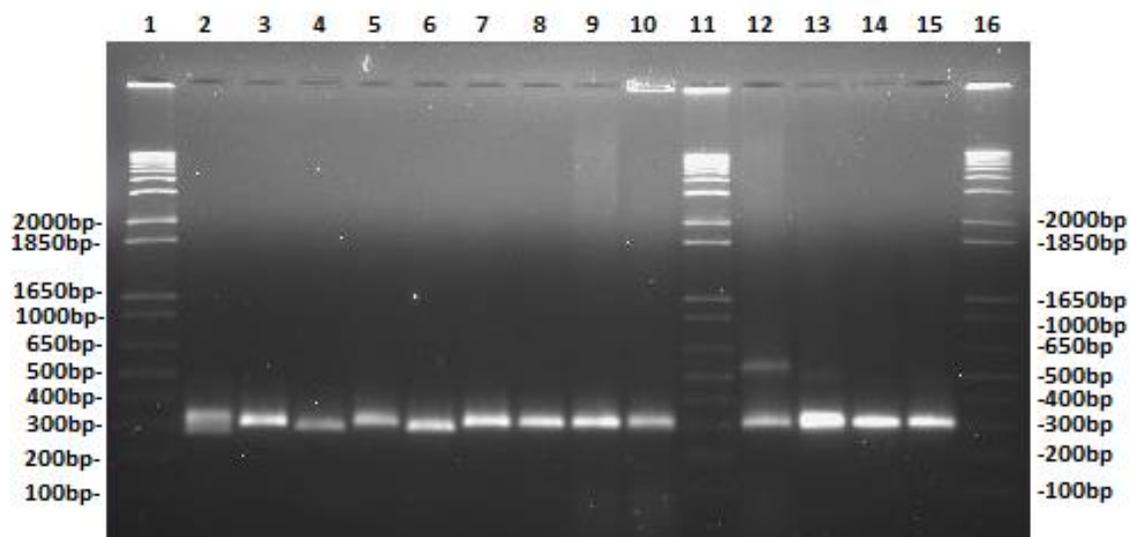


Figure 43. PCR amplification of *Betula* species with the designed TonB-dependent receptor primer pair.

A 300 bp band corresponds to the presence of the TonB-dependent receptor. Lanes 1, 11 and 16 are loaded with 1kb+ 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).

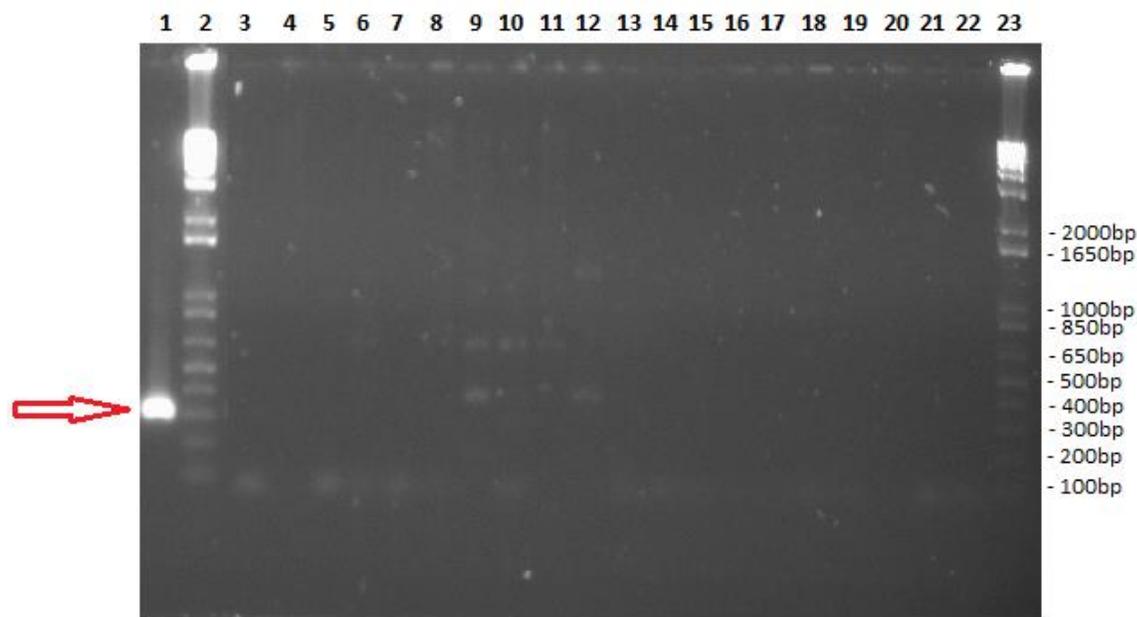


Figure 44. PCR amplification of other tree species with the designed TonB-dependent receptor primer pair.

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 1kb+ 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).

8.5. 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 a downregulation of genes during translation (325). In this study, we show that, actually genes involved in transport activities are upregulated in resistant genotypes compared to susceptible forms. Those few genes are of particular importance in nickel resistance in many organisms.

8.5.1. TonB-dependent receptors and TonB protein family in prokaryotes

The TonB-dependent transporter family (TBDTs) 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 (377). TBDTs are more commonly known for their role in iron regulation but have been associated with transport of vitamins, nickel and carbohydrates (379). 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 (380). 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 siderophore complex (380).

Some evidence exists that TBDTs are dealt with metal stress. In some species, TBDTs are repressed during metal stress (381, 382) while in others, there is an induction (383, 384). Hu *et al.*, (2005) found that TBDTs were upregulated during metal stress (383). However their role in response to metal was unclear since TonB was downregulated (383). 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* (385, 386).

8.5.2. 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 (387, 388). 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 (389). The siderophores will chelate non-soluble iron (III) and so form an iron-siderophore complex (389). The complex is then carried back into the plant's root system via a secondary transporter (389). 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*.

8.5.3. Validating the 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 compared to 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 aerial parts of the plant. Both analyses of *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 (390). Some live on the surface while others will enter the plant via lesions in the leaves or hydathodes (390). 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 *Betula 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 (80, 81). 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. papyrifera* 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 didn't 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 data bank of Japan), Plantgdb (Resources for Plant Comparative Genomics), Dendrome (forest trees genome database), *Populus* and *Betula 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 (391–396).

Yue *et al.*, (2012) identified 57 family of genes transferred from prokaryotes, fungi or viruses to moss *Physcomitrella patens* (394). Most of these genes were directly or indirectly related to plant defense and stress tolerance. Notably the glutamate-cysteine ligase (GCL) gene was acquired from bacteria by *P. patens*. GCL 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 metal detoxification. Interestingly, our initial analysis of *B. papyrifera* transcriptome revealed that glutathione is also upregulated in nickel resistant genotypes (322). 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.

8.6. 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 (*Betula 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 Ton-B dependent receptor and/or TonB protein family. Further studies including physical mapping of Ton-B 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 (*Acer rubrum*), red oak (*Quercus rubra*) and trembling aspen (*Populus tremuloides*) will be also investigated.

Chapter 9: General conclusions

The specific objectives of the present study were to; 1) determine if there's an association between plant population diversity and genetic variation in white birch (*Betula papyrifera*) populations with soil metal contamination in the Greater Sudbury region (GSR), 2) assess if metal contamination and soil liming has an effect on global DNA methylation, 3) develop and characterize the transcriptome of *B. papyrifera* under nickel stress and, 4) assess gene expression dynamics in white birch in response to nickel stress.

No association between plant population diversity and genetic variation with metal contamination was found. Liming increases plant population diversity but has no effect on genetic variation in the studied white birch populations. Detailed ISSR analysis showed that the level of polymorphic loci in the targeted populations (including limed) ranged from 28 – 56% with a mean of 44 %. Two population diagnostic markers were identified for the St. Charles reference population.

Total cytosine and adenine methylation levels were determined using tandem mass spectrometry (MS/MS) coupled with LC (LC-MS/MS). There was a decrease in cytosine methylation in metal-contaminated sites compared to uncontaminated sites. This suggests that metal contamination mostly nickel and copper, the main elements found in higher concentrations in contaminated sites might be associated with cytosine methylation. No significant difference in cytosine methylation level was observed between limed and non-limed sites contaminated with metals.

Growth chamber experiments under controlled temperature and humidity were conducted to assess the toxicity of Ni and Cu on white birch. We observed that the bioavailable (5.6 mg/kg Ni, 9.16 mg/kg Cu or combined) levels of Ni and Cu presently found in the GSR are not high enough

to induce a measurable physiological and genetic response in white birch. Treatment with the dose corresponding to total levels of Ni or Cu (1,600 mg/kg Ni, 1,312 mg/kg Cu or combined) generated different responses within segregating populations analyzed. Interestingly, white birch genotypes resistant, moderately resistant, and susceptible to nickel were identified. Metal analyses determined that Ni-resistant plants accumulated less Ni in their leaves compared to susceptible plants. No significant differences were found in Ni levels in roots between resistant and susceptible plants. Hence, the main Ni resistance mechanism of white birch was associated with translocation of these metals from root to shoot.

Expression of candidate genes associated with Ni resistance in other plant species were investigated in white birch. The expression of two Ni transporters (AT2G16800 and IREG1), a transcription factor (ZAT11) and an enzyme involved in the turnover of glutathione synthesis (Glutathione reductase) was measured in samples from growth chamber experiments and field. We found that Ni upregulated IREG1 and Glutathione reductase in white birch treated with the low Ni dose (5.6 mg/kg). Upregulation of AT2G16800, Glutathione reductase and ZAT11 and the downregulation of IREG1 were observed when white birch seedlings were treated with a high dose of Ni (1,600 mg/kg).

Expression of the same four genes was also measured in nickel resistant, moderately resistant and susceptible genotypes that were treated with 1,600 mg/kg of nickel. ZAT11 and Glutathione reductase were differentially expressed in resistant genotypes. A Lower Glutathione reductase level in the resistant genotypes indicates that Ni toxicity has been reduced in these plants. In addition, it demonstrates that glutathione might not be involved in Ni resistance in white birch. Further experiments would need to be conducted to confirm these results, either by directly measuring glutathione levels or by measuring the expression of other genes in the glutathione

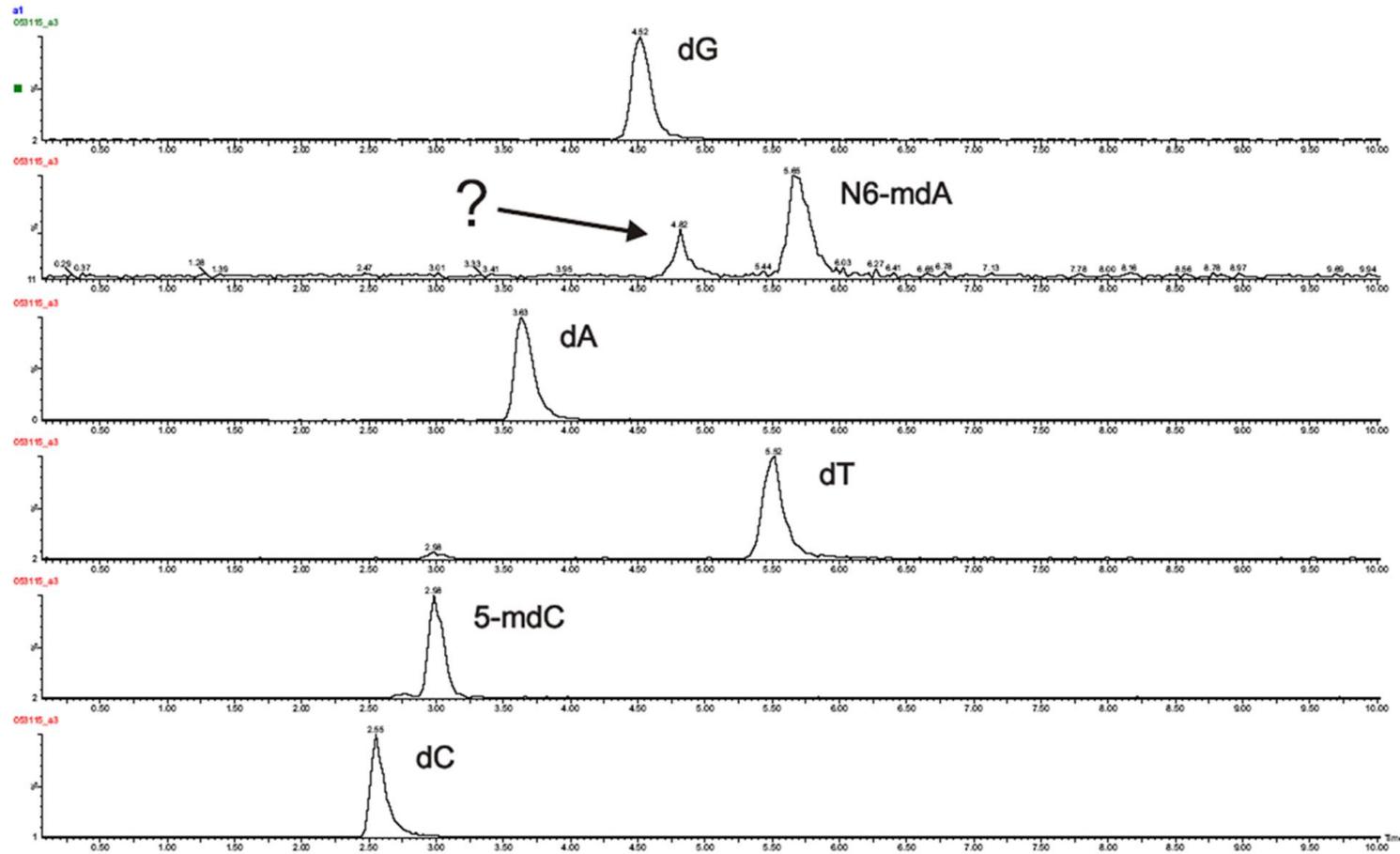
pathway. We also observed lower ZAT11 expression in resistant genotypes. ZAT11 is a transcription factor that modulates genes involved in root elongation and it was found to be negatively correlated with Ni resistance (224, 397). We have reason to believe that ZAT11 plays a similar role in leaves, where it helps modulate genes involved in meristem development. Thus, the decrease in ZAT11 due to Ni, could lead to the downregulation of genes involved in meristem development and overall growth. In fact, plant growth in resistant genotypes was significantly reduced after the Ni treatment. A slowdown in growth could allow the plant to “turn on” Ni resistance mechanism in order to reduce toxicity.

No differences in gene expression were found when samples from metal-contaminated and reference sites were compared. But the expression of AT2G16800, IREG1 and Glutathione reductase was higher in roots compared to leaves.

The transcriptome of *B. papyrifera* was developed for the first time using Next Generation Sequencing. RNA from Ni resistant, moderately resistant, susceptible and water controls treatment were sequenced. Expression profiles were generated and 62,587 genes were found to be significantly differentially expressed among the nickel resistant, susceptible, and untreated libraries. Genes were assigned ontology and we found downregulation of genes associated with translation (in ribosome), overall cell growth and an upregulation of genes involved with the plasma membrane. Seven candidate genes associated to nickel resistance were identified among the most highly differentially expressed genes. They include Glutathione S-transferase, a Thioredoxin family protein, a Putative transmembrane protein, two Nramp transporters, a TonB-like family protein and a TonB-like dependent receptor.

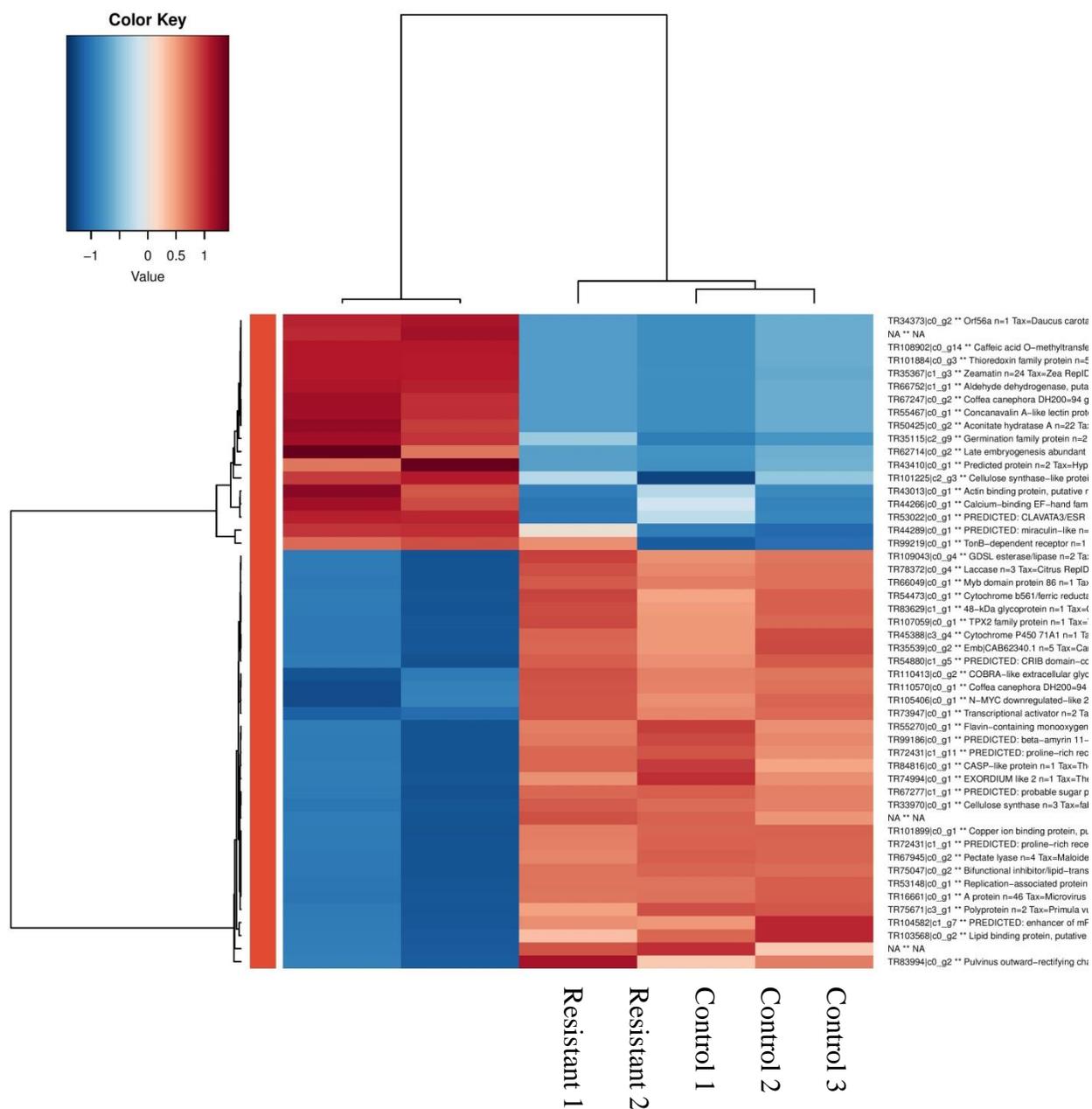
Since the presence of a TonB mechanism outside prokaryotes has never been reported, further analyses were conducted. Quantitative RT-PCR experiments confirmed that the TonB-dependent receptor was highly expressed in resistant genotypes. This TonB receptor was found to be exclusive to the *Betula* genus. Transcriptional regulation by Ni on other plant species showing different resistance mechanisms to Ni toxicity such as red maple (*Acer rubrum*), red oak (*Quercus rubra*) and trembling aspen (*Populus tremuloides*) need to be investigated.

Appendices



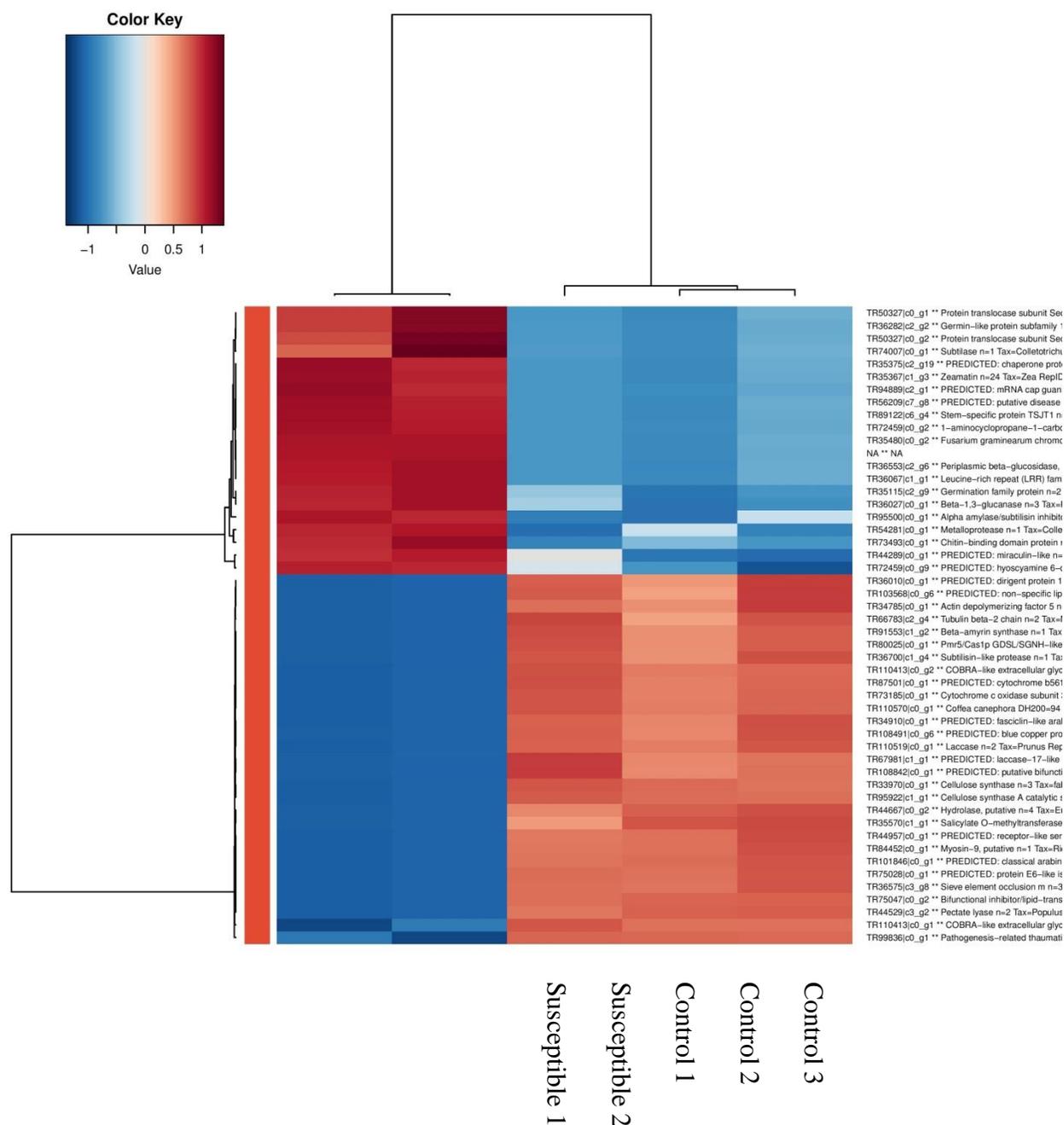
Appendix 1. Integrated LC-MS/MS chromatograms for dG, N6-mdA, dA, dT, 5-mdC, and C.

* The arrow indicates unusual second peak for N6-mDA in *Betula papyrifera* DNA samples.



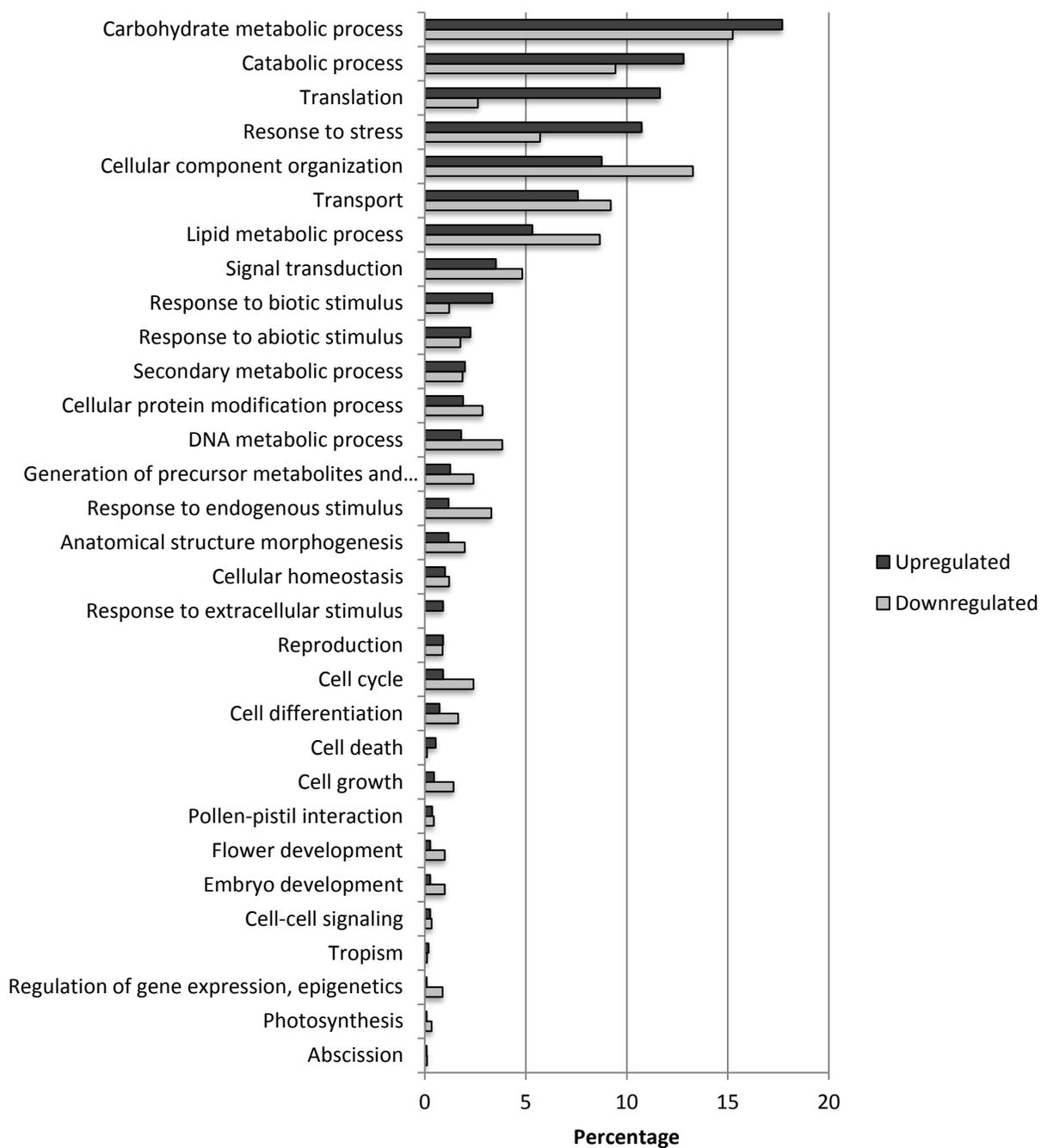
Appendix 2. Top 50 differentially expressed genes between nickel – treated *Betula papyrifera* resistant genotypes and control (nickel-untreated) based on log₂FC.

The colour red represents an upregulation and blue downregulation.

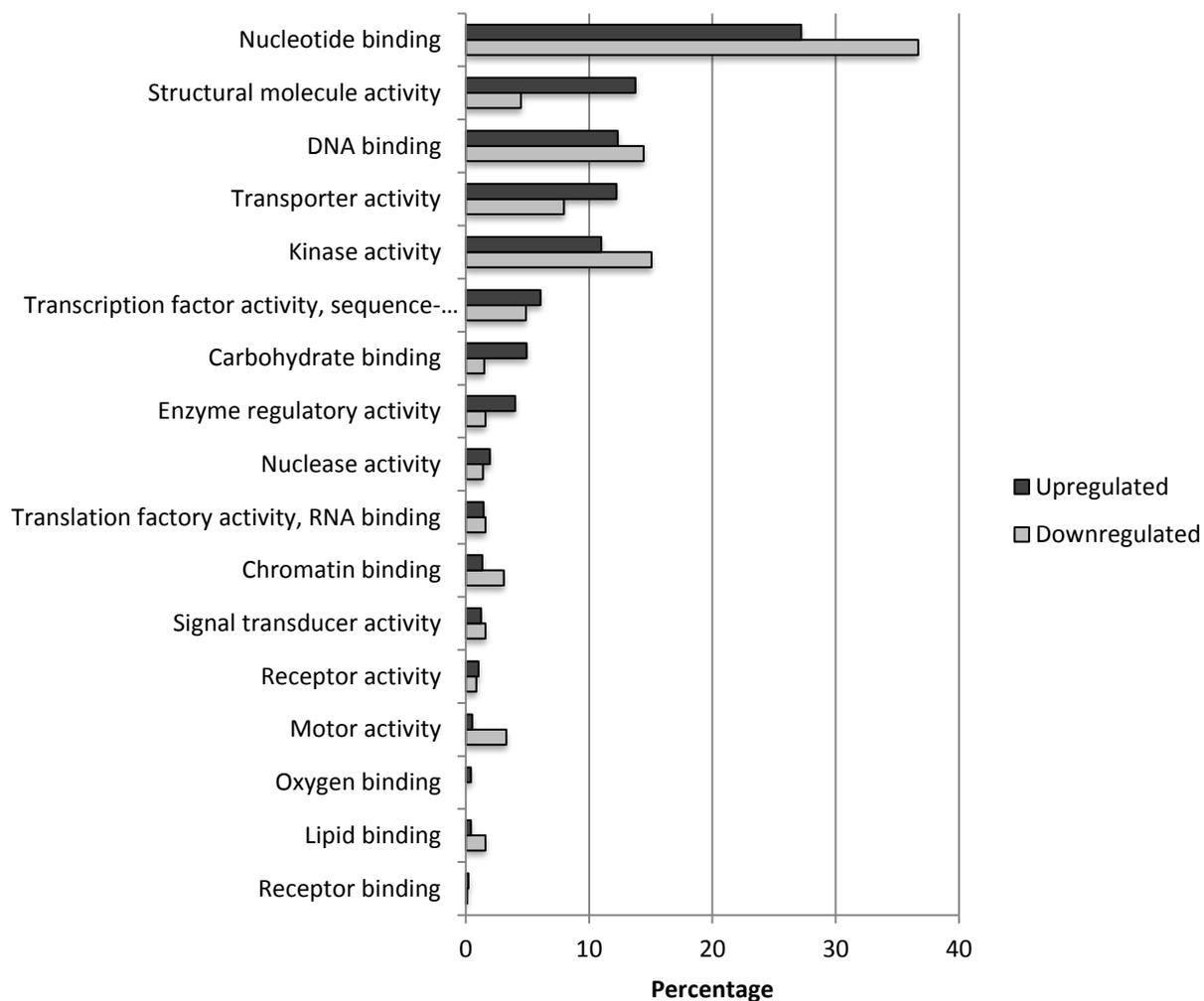


Appendix 3. Top 50 differentially expressed genes between nickel – treated *Betula papyrifera* susceptible genotypes and control (nickel-untreated) based on log₂FC.

The colour red represents an upregulation and blue downregulation.

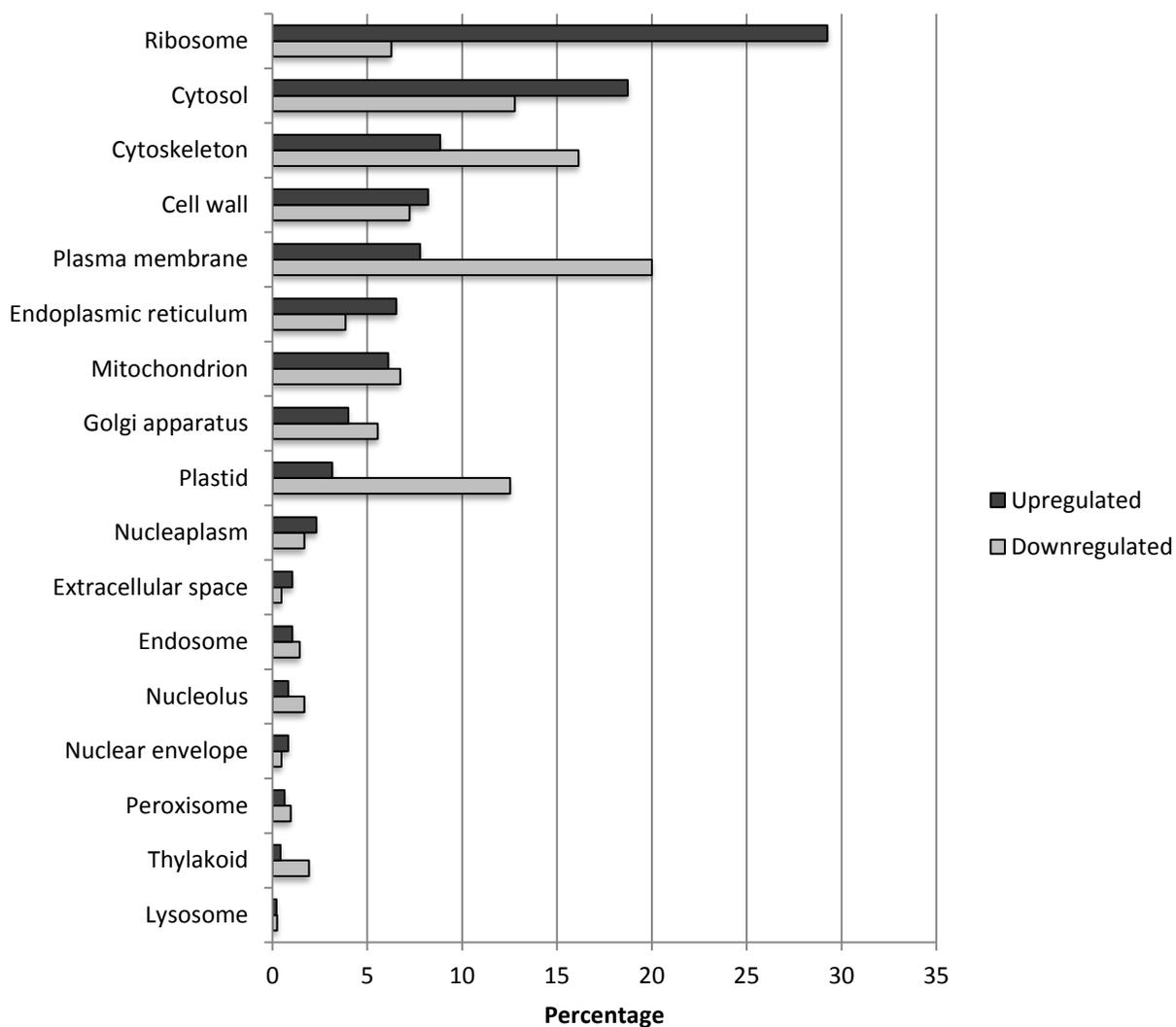


Appendix 4. Percentage of upregulated (total number = 1,108) and downregulated (total number = 912) transcripts when nickel – resistant *Betula papyrifera* genotypes were compared to control (nickel –untreated).



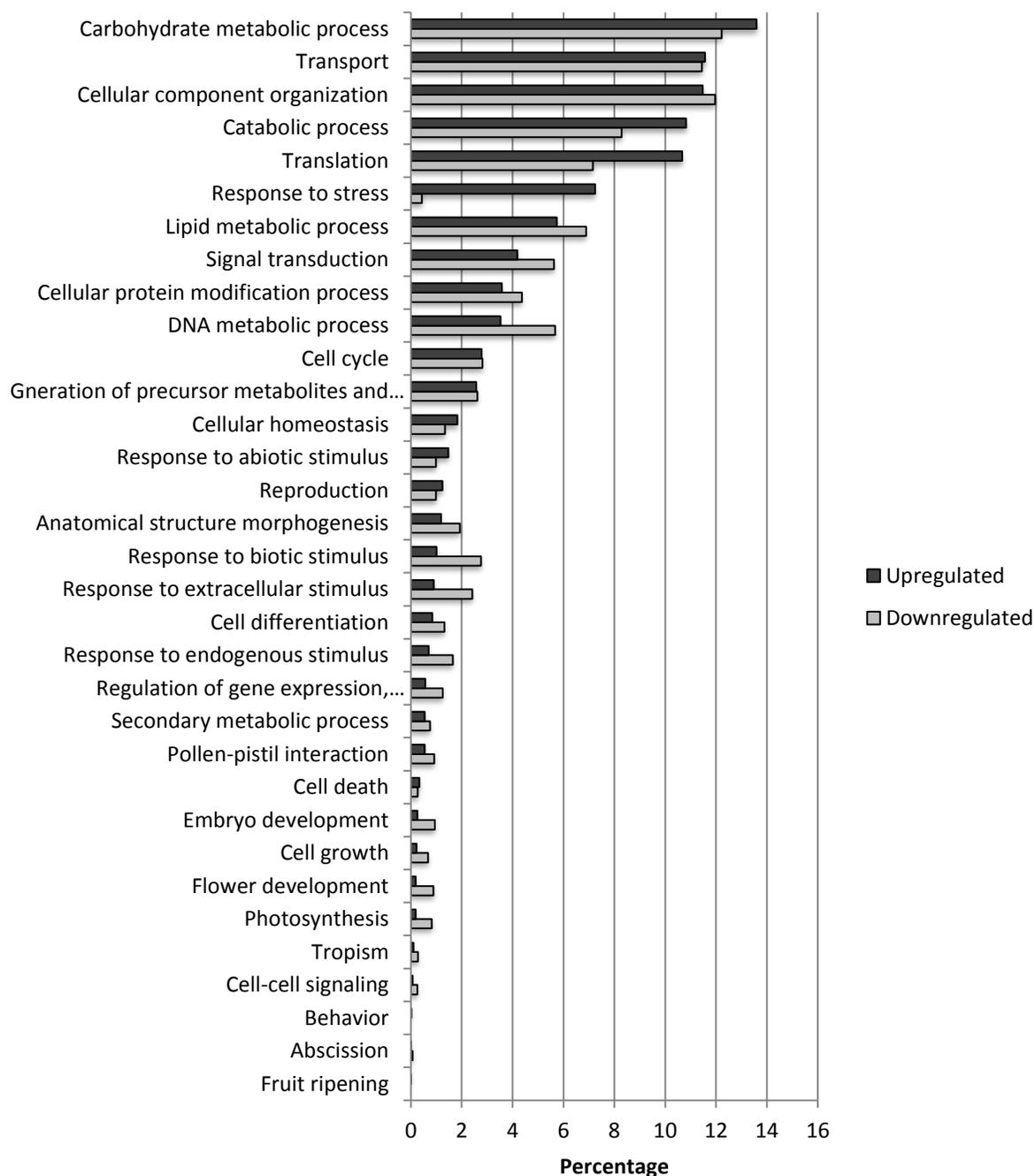
Appendix 5. Percentage of upregulated (total number = 974) and downregulated (total number = 943) when nickel – resistant *Betula papyrifera* genotypes were compared to control (nickel – untreated).

Transcripts were assigned gene ontology and grouped by molecular function using BLAST2GO.



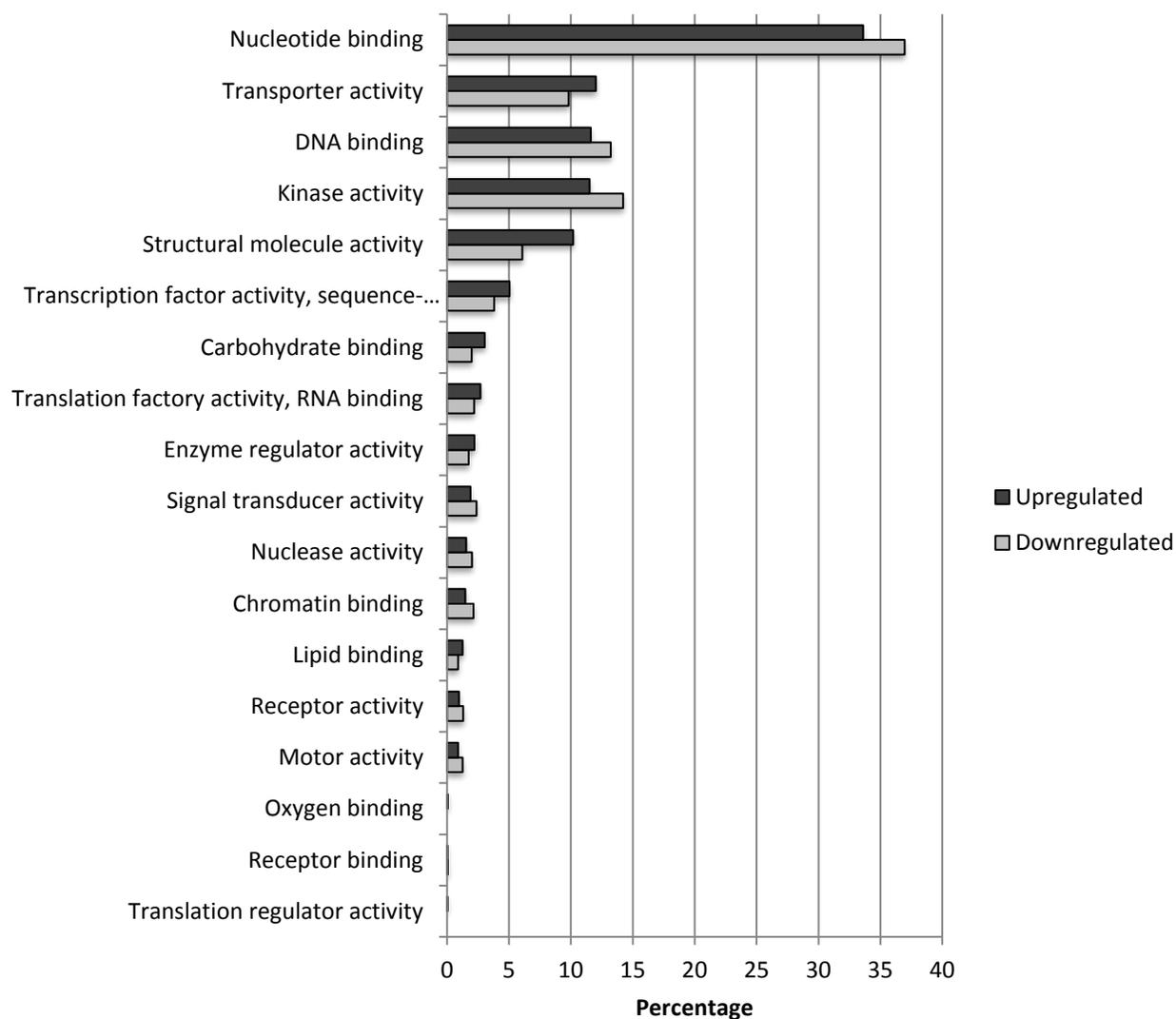
Appendix 6. Percentage of upregulated (total number = 475) and downregulated (total number = 415) transcripts when nickel – resistant *Betula papyrifera* genotypes were compared to control (nickel – untreated).

Transcripts were assigned gene ontology and grouped by cellular compartment using BLAST2GO.



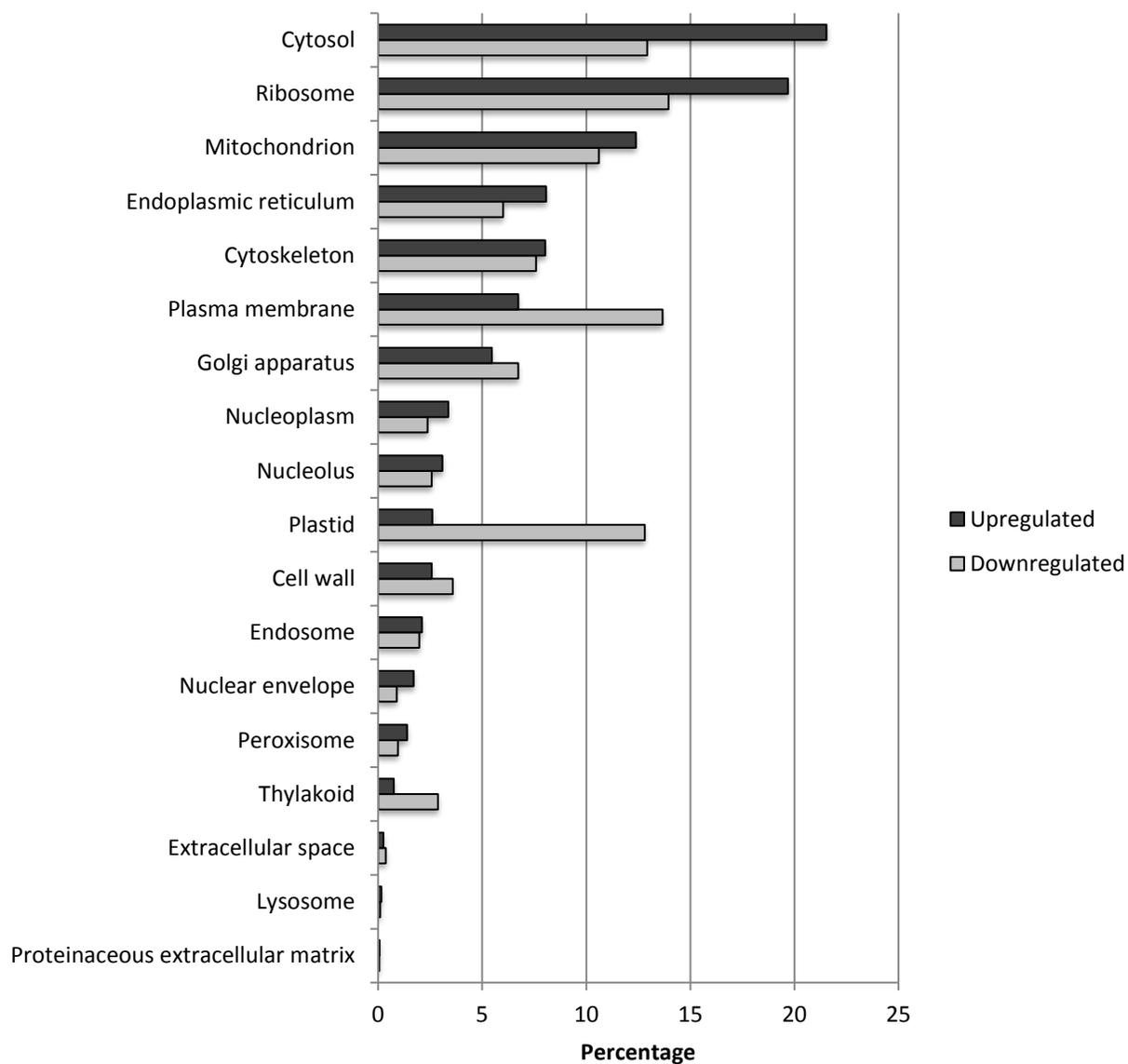
Appendix 7. Percentage of upregulated (total number = 15,380) and downregulated (total number = 11,032) transcripts when nickel – susceptible *Betula papyrifera* genotypes were compared to control (nickel – untreated).

Transcripts were assigned gene ontology and grouped by biological function using BLAST2GO.



Appendix 8. Percentage of upregulated (total number = 16,652) and downregulated (total number = 13,480) when nickel – susceptible *Betula papyrifera* genotypes were compared to control (nickel – untreated).

Transcripts were assigned gene ontology and grouped by molecular function using BLAST2GO.



Appendix 9. Percentage of upregulated (total number = 8,718) and downregulated (total number = 5,472) transcripts when nickel – susceptible *Betula papyrifera* genotypes were compared to control (nickel – untreated).

Transcripts were assigned gene ontology and grouped by cellular compartment using BLAST2GO.

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1. Score	E value	Identities	Gaps	Strand
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Subject 30597	TCGTCATCTTGATCGTGATCGTCGTTTTGATCGTCATCGTCATCGTGATAGTCATAGTCA			30656
Query 64	T---CGTGATCTTGATCTTGATCGTCATCGTGATCGTCATCGTCATCGTCATCT			120
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Subject 30774	CTTTGTGGAGGAGGAGGATCCTGATGAGGATTGGGATGGGGATGAGGATGATGATGTGGA			30833
Query 238	TGACAGTGACACTGACGACGATGGCAATTCTGTCGGAATTGGAAGGGGTATAAAATTAG			297
Subject 30834	TGACAGTGACACTGACGACGATGGCAATTCTGTCGGAATTGGAAGGGGTATAAAATTAG			30893
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Subject 30954	AAAAGGTCGCCAGCTCGGTTGCTTTGACGCCCTGGGCTCCTACATCTACTATTTTGGTGG			31013
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Subject 31134	GTACGCTTCGGTTCTAGGCGGTAAGATATATGTTTCGCAATCATGAACCTCGATGTCATAC			31193
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Query 658	TCCTCCAAATTATGCTCCAAGAGGTGGGAAAACATAGTCATTTCTGCAGCTGTTGAGAA			717
Subject 31254	TCCTCCAAATTATGCTCCAAGAGGTGGGAAAACATAGTCATTTCTGCAGCTGTTGAGAA			31313
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Query 823	TGCTACCTTCTATGCGTATAACGTGCACTGTAGATCTTGGGATATGCTTGAGCCTGCTAA			882
Subject 31434	TGCTACCTTCTATGCGTATAACGTGCACTGTAGATCTTGGGATAT-----GCCTGCTAA			31487
Query 883	GCGCAAGCTCCACCGTATGTGTACTGAGGAGTGGGAAAACCGGTCTAAGTGTAG 935			
Subject 31488	GCGCAAGCTCCACCGTATGTGTACTGAGGAGTGGGAAAACCGGTCTAAGTGTAG 31540			

Appendix 10. Sequence similarity between *Betula papyrifera* and *B. nana* for the TonB-dependent receptor.

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1. Score	E value	Identities	Gaps	Strand
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Query 43	GTCTGGTTCTGGTTCTGCTTCTGGGTCTGGTTCAAGGTCTGATTCAAGTTCTGAAGCAGG			102
Subject 2866	GTCTGGTTCAAGTTCTGCTTCTGGGTCTGGTTCAAGGTCTGATTCAAGTTCTGAAGCAGG			2807
Query 103	CTCGTATGCTGGGTCTCGAGCTGGGTCAAGTCCAAAGGGAAACCAAGAACGAGGAACAGG			162
Subject 2806	CTCGTATGCTGGGTCTCGAGCTGGGTCAAGTCCAAAGGGAAACCAAGAACGAGGAACAGG			2747
Query 163	GTCCGAGTCAGGCCAAGGCCGTGGGAGTGGTTCTGGTTCAAGGTCTGGTTCTGGTTCTGG			222
Subject 2746	GTCCGAGTCAGGCCAAGGCCGTGGGAGTGGTTCTGGTTCAAGG-----TCTGG			2699
Query 223	TTCTGGACGTGGTGAGGGTTCTGGTTCTGGTTCTGG		258	
Subject 2698	TTCTGGACGTGGTGAGGGTTCTGGTTCTGGTTCTGG		2663	

Appendix 11. Sequence similarity between *Betula papyrifera* and *B. nana* for the TonB-like protein.

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Publications related to thesis

1. **Theriault**, G., and Nkongolo, K. K. (2016) Nickel and Copper Toxicity and Plant Response Mechanisms in White Birch (*Betula papyrifera*). *Bull. Environ. Contam. Toxicol.* **97**, 171–176
2. **Theriault**, G., and Nkongolo, K. K. (2016) Contrasting Effects of Metal Contaminations and Soil Liming on Cations Exchange Capacity and Global DNA Methylation in *Betula papyrifera* Populations from a Mining Region. *Am. J. Environ. Sci.* **12**, 55–62
3. **Theriault**, G., Michael, P., and Nkongolo, K. K. (2016) Decrypting the regulation and mechanism of nickel resistance in white birch (*Betula papyrifera*) using cross-species metal-resistance genes. *Genes Genom.* **38**, 341–350
4. **Theriault**, G., Michael, P., and Nkongolo, K. K. (2016) Comprehensive Transcriptome Analysis of Response to Nickel Stress in White Birch (*Betula papyrifera*). *PLoS one* **11**, e0153762
5. **Theriault**, G., and Nkongolo, K. K. (2016) Evidence of prokaryote like protein associated with nickel resistance in higher plants: horizontal transfer of Ton –B-dependant receptor/protein in *Betula* genus or de novo mechanisms? *Heredity*
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11. **Theriault, G.**, Nkongolo, K. K., Narendrula, R., and Beckett, B. (2013) Molecular and ecological characterization of plant populations from limed and metal-contaminated sites in Northern Ontario (Canada): ISSR analysis of white birch (*Betula papyrifera*) populations. *Chem. Ecol.* **29**, 573–585

Published abstracts

1. **Theriault, G.**, Michael P. and Nkongolo, K. K. (2015) Comprehensive transcriptome analysis of response to nickel stress in white birch (*Betula papyrifera*). Paper presented at the Annual Meeting of the American Society of Agronomy, Crop Sciences Society of America, and Soil Sciences Society of America. Minneapolis, Minnesota, USA, November 15-18, 2015
2. **Theriault, G.**, Michael P. and Nkongolo, K. K. (2015) Analysis of nickel tolerance and whole- genome methylation of white birch (*Betula papyrifera*) populations from a mining region. Paper presented at the Sudbury Mining Conference and Environment. Sudbury, Ontario, Canada, June 20- 25, 2015
3. **Theriault, G.**, and Nkongolo, K. K. (2014) Identification and characterization of population- diagnostic molecular markers in fragmented populations of *Betula papyrifera* (Marsh.) from Northern Ontario. Paper presented at the 9th Canadian Plant Biotechnology Conference. Montreal, Quebec, Canada, May 12-15, 2014
4. **Theriault, G.**, and Nkongolo, K. K. (2014) Genetic analysis of *Betula papyrifera* (Marsh) populations from a mining reclaimed region: expression of Ni and Cu tolerance genes. Paper presented at the Annual Meeting of the American Society of Agronomy, Crop Sciences Society of America, and Soil Sciences Society of America. Long Beach Bay, California, USA, November 2-5, 2014
5. **Theriault, G.**, and Nkongolo, K. K. (2013) Molecular analysis of white birch (*Betula papyrifera* Marsh.) populations from Northern Ontario. Paper presented at the Annual Meeting of the American Society of Agronomy, Crop Sciences Society of America, and Soil Sciences Society of America. Tampa Bay, Florida, USA, November 3-6, 2013

6. **Therriault, G., and Nkongolo, K. K.** (2012) Molecular Analysis of White Birch (*Betula papyrifera*) Populations From Limed and Metal Contaminated Sites in the Greater Sudbury (Ontario) Region. Paper presented at the Annual Meeting of the American Society of Agronomy, Crop Sciences Society of America, and Soil Sciences Society of America. Cincinnati, Ohio, USA, October 21-24, 2012