Transcriptome and methylome analysis of Trembling Aspen (*Populus tremuloides*) under nickel stress.

By

Karolina M. Czajka[1]

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Faculty of Graduate Studies
Laurentian University
Sudbury, Ontario, Canada

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Thesis Examiners/Examinateurs de thèse:

Dr. Kabwe Nkongolo
(Supervisor/Directeur(trice) de thèse)

Dr. Abdelwahab Omri
(Committee member/Membre du comité)

Dr.. Nam-Soo Kim
(Committee member/Membre du comité)

Dr. Ewa Cholewa
(External Examiner/Examinateur externe)

Dr. Frank Mallory
(Internal Examiner/Examinateur interne)

Approved for the Office of Graduate Studies
Approuvé pour le Bureau des études supérieures
Tammy Eger, PhD
Vice-President Research (Office of Graduate Studies)
Vice-rectrice à la recherche (Bureau des études supérieures)
Laurentian University / Université Laurentienne

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Abstract:

Nickel is an essential micronutrient required at low concentrations for adequate plant growth and health. However, excessive amounts of bioavailable nickel ions in the surrounding soil can result in plant toxicity symptoms. Plants have evolved heavy metal tolerance mechanisms to adapt and cope with this abiotic stressor. The main objectives of the present research were to 1) further characterize the *P. tremuloides* transcriptome 2) compare gene expression dynamics between nickel-resistant and nickel-susceptible *P. tremuloides* genotypes with Whole Transcriptome (WT) sequencing, 3) determine the effects of different nickel concentrations on *P. tremuloides* gene expression and, 4) assess global methylation levels in *P. tremuloides* under nickel stress. Trembling aspen (*Populus tremuloides)* seedlings treated with varying concentrations of nickel nitrates (150 mg Ni / 1 kg of dry soil, 800 mg / kg, and 1, 600 mg / kg) showed phenotypic segregation of physical toxicity symptoms at the highest nickel dose of 1, 600 mg / kg. This study revealed that a metal transport protein (Potrs038704g29436 – ATOX1-related copper transport) was among the top upregulated genes in resistant genotypes when compared to susceptible plants. Other upregulated genes associated with abiotic stress were identified including a Dirigent Protein 10, GATA transcription factor, Zinc finger protein, Auxin response factor, Bidirectional sugar transporter, and thiamine thiazole synthase. Overall, an upregulation in ribosomal and translation activities was identified as the main response to Ni toxicity in the resistant plants. The results of the dosage analysis suggested that the 800 mg / kg nickel dose is the threshold at which an early abiotic stress response may be triggered as seen by the highly upregulated LEA protein and two calcium binding proteins when compared to water. The cluster of genes that had increased gene expression with increasing nickel dose also had multiple enriched GO terms related to heavy metal

and abiotic stress including metal ion transport, antioxidant activity, photosynthesis, and ribosomal activity.

Lastly, the initial screen for potential global methylation differences between nickel-resistant genotypes and water showed no significant difference in overall methylation levels. However, the potassium nitrate control for the 1,600 mg / kg dose did show significantl hypomethylation in comparison to the nickel-treated or water control samples. Future experiments could use target-specific methylation and gene expression assays to investigate the biological significance of the heavy metal stress candidate genes identified in this top-down study in trembling aspen. Understanding the heavy metal tolerance mechanisms and responses used by hardy species like trembling aspen is important for environment bioremediation and maintenance of healthy ecosystems.

Key Words: Trembling aspen (*Populus tremuloides*); Nickel toxicity; Transcriptome; Transcriptome analysis; Gene expression; RNA-seq; Abiotic stress; Heavy metal stress; Nickel stress; Global DNA Methylation, Hypomethylation.

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Chapter 1: Literature review

1.1. Metal toxicity in plants

Metal toxicity in plants has become an increasing problem seen in disturbed metal-contaminated environments due to mining industries, coal-burning, smelting and other anthropogenic activities. These processes often lead to metals such as zinc (Zn), cadmium (Cd), copper (Cu), lead (Pb), chromium (Cr), mercury (Hg), and nickel (Ni) leaking into the surrounding air, water, and soil and becoming major environmental pollutants (Winterhalder, 1996). Most plant species have developed mechanisms to uptake metals from the environment because small amounts of these elements are crucial for plant metabolism. However, when soil concentrations of metals in the bioavailable form exceed the thresholds levels that plants can tolerate, toxicity symptoms appear. Plants stressed by metal toxicity ultimately have altered metabolism, physiological, and biochemical processes, and biomass production (Mehes-Smith *et al.*2013). These effects can negatively impact the sustainability and health of the ecosystems in the vicinity, agricultural crops, and water supply.

Typically, soil metals are found in trace amounts, and this is normally sufficient for plants that require them for essential redox reactions and as an integral part of some enzymes. For instance, copper (Cu) has an integral role in photosynthetic proteins like plastocyanin in higher plants such as rice and cauliflower (Mahmood and Islam 2006; Chatterjee *et al.* 2000). It is a cofactor for oxidases, mono- and di-oxygenases, and other enzymes that are important for ROS defense (Demirevska-Kepova *et al.* 2004). Copper is also a micronutrient needed in ATP synthesis and CO_2 assimilation (Thomas *et al.* 1998). Despite its essential role in many plant processes, excessive Cu in the soil from industrial and mining activities has been shown to cause many metal toxicity symptoms in plants. Exposure to toxic levels of Cu can cause plant growth reductions, oxidative stress (Stadtman, 1993), and disturbances in metabolic pathways (Hegedus *et al.* 2001). When Cu and Cd are present in combination at toxic levels, this can cause further symptoms like inhibited germination and seedling growth such as seen in *Solanum melongena* (Neelima and Reddy 2002).

Zinc (Zn) is another metal that is an essential component of enzymes like superoxide dismutase and RNA polymerase. It is also essential for ribosome integrity and is a catalyst for oxidation reactions. Excess levels of Zn and Cd caused phytotoxicity symptoms like oxidative damage, impaired metabolism, and decreased growth in green bean (*Phaseolus vulgaris*) (Cakmak and Marshner 1993) and brown mustard plants (*Brassica juncea*) (Prasad *et al.* 1999). The catalytic efficiency may also be affected for the key enzymes that need Zn as a cofactor (Van Assche *et al.* 1998; Somasekharaiah *et al.* 1992; Romero-Puertas *et al.* 2004). Plants that are exposed to high Zn concentrations in soil can develop chlorosis in their leaves (Ebbs and Kochian 1997). This chlorosis effect may be caused in part by an induced Fe-deficiency because hydrated Zn^{2+} and Fe^{2+} ions compete for plant uptake through the roots (Marschner 1995). Manganese, copper, and phosphorus (P) deficiencies have also been observed in some plants exposed to excess Zn, each with their own consequences to plant metabolism (Lee *et al.* 1996; Ebbs and Kochian 1997).

Iron (Fe) is another significant metal that plants cannot survive without, because of its multiple biological roles in several processes. It is a major component of heme-containing proteins such as hemoglobin, catalase, peroxidase, and leghemoglobin (Marschner 1995). It is also a part of the heme proteins, cytochromes which are essential for photosynthesis, chlorophyll biosynthesis, and chloroplast development (Marschner 1995). Most mineral soils are iron-rich, but the majority of this metal is in an insoluble Fe^{3+} or insoluble Fe^{2+} form so plants are not adversely affected.

Excess amount of iron in its soluble form will lead to high Fe^{2+} uptake through the roots into the plant causing toxicity symptoms in species such as tobacco, canola, soybean, and water thyme (Sinha *et al.* 1997). A decrease in photosynthesis and an increase in oxidative stress due to Fe-induced free radical production are also seen in plants stressed by Fe toxicity (Arora *et al.* 2002; de Dorlodot *et al.* 2005; Sinha *et al.* 1997)

Lead (Pb) on the other hand, has no essential function in plants but it will still be absorbed through the roots when it is present in its phytoavailable form in soil water. For centuries, lead has been an important and useful metal in human activities. However, the inefficient and pollutive ore mining processes used to extract

lead have resulted in surrounding environments becoming contaminated with excess concentrations of this metal. Known to have toxic effects in other organisms like animals, lead can also be highly toxic to plants and may cause serious health problems if it enters the food chain in an ecosystem (Wierzbicka and Antosiewicz 1993). Germination inhibition after exposure to excess lead has been observed in plants such as *Spartiana alterniflora* (Morzck and Funicclli 1982) and *Pinus helipensis* (Nakos 1979). This may be caused by an inhibition of endospermic protease and amylase activities which are essential for mobilizing resources needed for germination as seen in rice seedlings (Mukherji and Maitra 1976). Lead toxicity can also inhibit seedling growth in soya bean (Huang *et al.* 2009), rice (Mukherji and Maitra 1976), maize (Miller *et al.* 1975), and other crops. Morphological abnormalities are commonly seen in many plants (Paivoke 1983) and other physical symptoms like chlorosis can appear (Hewilt 1953). Similar to other metals, excess lead in a plant system can induce oxidative stress (Reddy *et al.* 2005), inhibition of important enzymes in photosynthesis, and altered membrane permeability (Sharma and Dubey 2005).

Soluble proteins levels are often reduced due to metal stress because synthesis rates go down and the hydrolysis and breakdown of proteins may be accelerated. For instance, sugar beet plants exposed to Ni and Cd had a significant decrease in soluble protein content (Kevresan *et al*. 1998). However, in some cases, higher levels of soluble proteins have been reported in species such as rice sedge (*Cyperus difformis*) after exposure to Cd, Ni and Pb (Ewais 1997). Proteins can be directly damaged by reactive oxygen species arising from metal-induced stress (Baccouch et al 1998, 2001; Gajewska *et al.* 2006). This oxidative stress can be further increased as the concentration of low-molecular-weight proteins is depleted (Rao and Sresty 2000; Kukkola *et al.* 2000.). Metals can also bind to side protein groups. SH-groups are common and disrupt the protein function (Seregin and Kozhevnikova 2006). Furthermore, metal ions can directly damage proteins via oxidation of amino acid residues and free amino acids. The oxidative ability of metals often modifies amino acids like histidine, arginine, lysine, proline, methionine, and cysteine near the metal binding side of

a protein. This can impact protein function or unintentionally target some proteins for protease degradation (Roseman and Levine 1987).

Increased protein degradation can result in decreased protein concentrations and an accumulation of free amino acids within plant cells. A species-specific effect has been observed in terms of accumulation of free amino acids as a response to metal stress. This is seen particularly in hyperaccumulator or metal tolerant plants, which may use specific free amino acids as an important detox mechanism (Ahmad and Ashraf, 2011). For instance, asparagine has been reported to be an agent of chelating Cd, Pb, Ni, and Zn to form metal-ligand complexes that can immobilize the metal (Homer *et al.* 1997). Bhatia *et al.* (2005) studied the Ni hyperaccumulator species *Stackhousia tryonii* and found slight decreases in some amino acid levels such as glycine in plants exposed to Ni. However, concentrations of alanine, asparagine, and glutamine significantly increased in the xylem in response to the Ni stress suggesting that they may contribute to Ni complexation in these species. Proline is a prominent amino acid whose levels may increase in response to Ni stress and can potentially be used as a metabolic indicator for Ni tolerant plants. Furthermore, it may act as an osmoprotectant (Paleg *et al.* 1984), membrane stabilizer (Bandurska 2000; Matysik *et al.* 2002), metal chelator (Cobbett 2000), ROS scavenger (Alia *et al.* 2001), and enzyme protector (Sharma and Dietz 2006). Histidine has also been reported as potentially having the highest association to form metal-ligand complexes with Ni (Martell and Smith 1975).

An important part of the defense activated in response to metal stress are proteins and polypeptides (phytochelatins) with N-, O- or S-ligands (Van Assche and Clijsters 1990; Clemens 2001; Vacchina *et al.* 2003; Montarges-Pelletier *et al.* 2008) which bind Ni to immobilize metals from interfering in other reactions. Some proteins that can aid in complexing and binding Ni are permeases (Wolfram *et al.*1995; Eitginer and Mandrand-Berthelot 2000), metallothioneins (Schor-Fumbarov *et al.* 2005), and metallochaperones (Hausinger 1997; Olson et al 1997; Watt and Ludden 1998). These can all mitigate the oxidative stress caused by metal toxicity.

Metal toxicity can also cause genotoxic effects and plants can be damaged at the DNA level. Metal cations that have passed the nuclear membrane into the nucleus can readily interact with the negatively charged DNA and cause direct conformational changes leading to mutations, DNA strand breaks, rearrangements, and other abnormalities (Eichhorn *et al.* 1985; Duguid *et al.* 1993; Kasprzak 1995). Metals like iron and copper act as catalysts for oxygen reduction reactions which give rise to hydroxyl radicals at a rate higher than a plant's ROS defense can tolerate. ROS can cause direct DNA sequence alterations when they come in contact and remove or add H+ atoms to the nucleotides or the DNA backbone (Pryor, 1998). Metals such as Cr3+ in complexes can bind to the double helix and can impede access or deform the binding site for transcription factors that need to bind to DNA to initiate gene expression (Raja and Nair 2008). The increase in reactive oxygen species due to metal toxicity is well documented and they can also directly induce DNA damage and mutations (Cunningham 1997). Nickel particularly has been observed in model systems to outcompete magnesium ions for DNA binding and cause chromatin condensation in cells treated with excess Ni ions. Chromatin that is condensed and hypermethylated in normally coding regions of DNA results in the silencing of essential genes such as anti-oncogenic genes (Lee *et al.* 1995). Plants are not as susceptible to carcinogenicity because the plant cells remain contained within the cell wall and cannot proliferate and metastasize as seen in animal systems. However, the effects of metal toxicity on the plant's genetic material leads to an increased mutational load, increased DNA damage and gene silencing which will ultimately negatively affect plant survival and health.

Overall, there is a wide variety of negative effects seen in plants exposed to excess metals. Some toxicity symptoms can vary based on the metal, but in general, metal toxicity can inhibit growth, disrupt essential physiological processes, and lead to reduced plant survival.

1.2. Nickel toxicity in plants

Once absorbed by the root system, nickel and its compounds can be transported to the shoots and the leaves via the xylem (Peralta-Videa *et al.* 2002) (Krupa *et al.* 1993). In its bioavailable form, nickel can be

translocated within the plant quite easily because it is essential in plant processes. Some proteins can bind nickel and facilitate the transportation (Hausinger 1997; Colpas and Hausinger 2000). Metal-ligand complexes such as nicotianamine/ histidine also play a regulatory role (Vacchina *et al.* 2003; Haydon and Cobbett 2007). Normally, about 50% of nickel will be retained within the plant roots system (Cataldo *et al.* 1988). Some accumulator species (including *Populus tremuloides*) are hypothesized to retain a greater amount of nickel in the shoots and leaves in response to excess nickel concentrations to prevent its toxic effects from affecting the rest of the plant biomass.

Nickel can be found in fruits and seeds of some plants because it can be translocated via the phloem (McIlveen and Negustanti 1994; Welch 1995; Page e*t al.* 2006). As a result, some plants may develop coping strategies to limit the effects of nickel on subsequent seed germination. For example, nickel was found partitioned mainly in the pericarp (fruit wall) in seeds of *Stackhousia tryonii* and not in the endospermic and cotyledonary tissues. This suggests that this Ni hyperaccumulator species may have molecular processes that can limit nickel toxicity in the tissues that are essential for healthy germination and plant growth. (Bhatia *et al.* 2003)

Nickel's role as an essential plant nutrient in plant metabolic processes was first presented by Dixon *et al.* 1975). Their work with the Jack-bean showed that nickel is an integral component for proper functioning of the metalloenzyme urease. Since then, about 500 proteins and peptides have been discovered in living systems that are able to bind Ni, including other metalloenzymes. The major enzymes found in organisms that Ni is an integral component of are urease (Klucas *et al.* 1983), superoxide dismutase (Ragsdale *et al.* 2009), NiFe hydrogenases (Lubitz e*t al*. 2007), methyl coenzyme M reductase (Ragsdale 2003; Jaun and Thaeur 2007), carbon monoxide dehydrogenase (Lindahl and Graham 2007), acetyl coenzyme-A synthase (Doukov *et al.* 2008), other hydrogenases (Küpper and Kroneck 2007) and RNase-A (Ragsdale 2009).

Ni deficiency is uncommonly found in plants because they require only small amounts that are naturally present in most soils. A major consequence of Ni deficiency is the disturbance in urease function which

normally catalyzes the reaction of urea into ammonium in the N assimilation process. Thus, a buildup to toxic levels of urea may result as seen in Ni-deficient soybean leaf tips (Eskew *et al.* 1983). This can lead to plant toxicity symptoms such as chlorosis and necrosis.

Although nickel at low concentrations is a vital micronutrient needed for healthy plant function, many toxic effects can be seen when plants are exposed to excess concentrations of nickel. Some physical effects of nickel toxicity observed in plants are decreased shoot and root growth, branching system not fully formed, deformation of other plant parts such as irregular flower shape, decreased plant biomass, leaf spotting, chlorosis, and foliar necrosis (Nedhi *et al* 1990).

Roots are typically the first plant part to be in direct contact with soil, so this is often where symptoms of Ni toxicity may first appear. Roots will show reductions in growth and increased shape abnormalities (Wong and Bradshaw 1982; Yang *et al.* 1996; Kopittke *et al.* 2007). For instance, white birch (*Betula papyrifera)* and honey suckle (*Lonicera tatarica*) exposed to high Ni concentrations had a reduction in new root hairs forming and deformation of existing ones (Patterson and Olson 1983). Inhibited lateral expansion of root growth due to Ni toxicity has been seen in crops like maize (Seregin *et al.* 2003). It is hypothesized that this Ni-induced effect occurs because Ni can pass through the endodermal barrier with ease and accumulate at toxic concentrations in pericycle cells which are crucial for cell division and proliferation (Seregin *et al.* 2003**;** Seregin and Kozhevnikova 2006).

Plants cells undergoing excessive Ni stress can experience increased permeability of cell membrane due to the damage caused by Ni-induced oxidative stress. Reactive Oxygen Species arise in excess amounts and can degrade the membrane lipids and proteins through lipid peroxidation (Dat *et al.* 2000; Verma and Dubey 2003). Plants can utilize enzymes such as catalase (*CAT*), peroxidase (*POD*), superoxide dismutase (*SOD*), and glutathione reductase (*GR*) to minimize damage caused by ROS. Non-enzymatic compounds such as antioxidants, ascorbic acid, phenolics, tocopherols, reduced glutathione antioxidants can also be produced in plants to seek out and remove or neutralize ROS (Pandolfini *et al.* 1992; Noctor and Foyer 1998; Alscher

et al. 2002; Verma and Dubey 2003; Freeman *et al.* 2004; Maheshwari and Dubey 2009). At high levels of nickel exposure these mechanisms often fail to adequately deal with the increase in ROS and plant membrane damage occurs (Howlett and Avery 1997; Zhang *et al.* 2007; Wang *et al.* 2008). Increased membrane permeability can cause electrolyte leakage from cells and a loss of water which decreases cell turgor pressure (Wang *et al.* 2008; Llamas *et al.* 2008). Lipid peroxidation may also occur because some antioxidant enzymes cannot be activated with the appropriate metal cofactor such as Fe (i.e. catalase and peroxidase) (Ranieri *et al.* 2003) when high Ni levels in the soil are outcompeting these other elements for plant uptake. Ni also competes with metals that are needed for membrane stability like Ca and Zn (Valko *et al.* 2005; Taiz and Zeiger 2010).

Nickel toxicity affects photosynthesis and gas exchange processes in many ways resulting in an overall inhibition of photosynthesis (Nedhi *et al.* 1990; Bishnoi *et al.* 1993). A decreased photosynthetic rate (Sheoran *et al.* 1990; Bishnoi *et al.* 1993; Krupa and Baszynski 1995), transpiration rate (Bishnoi *et al.* 1993; Pandey and Sharma 2002), stomatal conductance (Heath *et al.* 1997) and efficiency of water-use (Bishnoi *et al.* 1993) have been observed in plants undergoing nickel stress. These effects are seen if Ni enters the plant system through uptake via roots or through direct application of Ni on isolated guard cell chloroplasts (Tripathy *et al.* 1981; Singh *et al.* 1989; Molas 1998; Boisvert *et al.* 2007).

Chloroplasts are sensitive to Ni toxicity and their structure can be damaged or deformed possibly due to the lipid peroxidation occurring from a Ni-induced increase in oxidative stress (Heath *et al*. 1997; Hermle *et al.* 2007). For instance, Molas (1998) observed alterations in the photosynthetic apparatus such as improperly formed grana and thylakoid membranes, smaller chloroplasts, and changed composition of the lipid membranes in wild cabbage (*Brassica oleracea*) that is exposed to excess Ni. As a result, photosynthesis reactions can be disrupted including the electron transport chain (Singh *et al.* 1989; Tripathy *et al.* 1981) and the Calvin Cycle via enzyme inhibition. The opening and closure of stomata may be affected as well and can lead to a CO_2 deficit which further inhibits photosynthesis (Sheoran *et al.* 1990). The electron transport chain

in plants is known to be inhibited by Ni during the light reaction through a similar mechanism as other metals (Mohanty *et al.* 1989; Krupa and Baszynski 1995). This starts in Photosystem II where nickel can cause the reduction of thylakoid membrane pigments including cyt b6-f and b559, ferredoxin and plastocyanin. The dark reactions may also be suppressed by nickel in some way considering there is evidence of other metals inhibiting key enzymes such as rubisco, 3-PGA kinase, F-1,6 biphosphotase, aldolase, and phosphoglyceraldehyde (Sheoran *et al.*1990**)**. In this case, an imbalance can be created, and the accumulation of light reaction products (ATP and NADPH) can ultimately create a pH gradient across the thylakoid membrane that is too high and impedes PSII activity (Krupa and Baszynski 1995).

1.3. Gene expression associated with metal contamination

Environmental stressors can cause changes in gene expression at the transcriptional level for plants to adapt. In particular, metals, heat shock, reactive oxygen species, and drought, have already been implicated in alterations of gene transcription (Matters and Scandalios 1986).

Changes in gene expression in response to excess metal concentrations are commonly seen in metal tolerant plants like accumulators. Genes that have been identified to possibly play a role in metal tolerance can be species-specific and metal-specific. However, it appears that this group of plants use genes that have a similar function and are commonly involved in metal transport.

For instance, *Thlaspi caerulescens* is well known to be able to hyperaccumulate Cd and Zn (Brown *et al.* 1995a,b) and some ecotypes may be able to accumulate Ni and Co as well (Baker, 1981; Baker and Brooks, 1989; Brown 1995b). Kochian *et al.* (2002) found that the *ZNT1* gene which codes for a zinc transporter protein is upregulated in *T. caerulescens* roots in the presence of Zn. The increase in *ZNT1* expression as the Zn^{2+} root uptake increased suggests that this gene is essential for Zn transport and hyperaccumulation in shoots. *ZNT1* has similarly been shown to be involved in Cd^{2+} uptake as well, suggesting that it is a Zn^{2+}/Cd^{2+} transporter (Lasat *et al.*1996).

The *ZNT1* gene is closely related to the *ZIP* (*ZRT/IRT*-like Protein) gene family, a group of metal transporter protein genes that can bind and transport metal cations including cadmium, iron, manganese, and zinc (Guerinot 2000). These genes are potential candidates for metal tolerance in accumulator plants such as *Arabidopsis halleri* (Eng *et al.*1998; Grotz *et al.* 1999). In fact, Kochian *et al.* (2002) found an increased expression of at least two other metal transporter genes in the *ZIP* family and an increased *NRAMP* expression, another metal transporter family, in *T.caerulescens* compared to the non-accumulating *T.arvense.* The low metal specificity of the *ZIP* and *NRAMP* genes suggests that some homologues may be involved in Ni-hyperaccumulation in a similar way as Zn (Mizuno *et al.* 2005). It is known that plants are not limited to accumulating only one type of metal and a gene that plays a role in tolerance to one metal may provide cross resistance to other metal ions. Considering that *Populus tremuloides* were able to accumulate high concentrations of Zn and Ni in the leaves (Kalubi *et al.* 2016), it is likely that they do so using similar genes and mechanisms.

The most current literature review considered both model and non-model plants and identified 11 genes associated with nickel resistance. These genes include 1-aminocyclopropane-1-carboxylic acid deaminase (*ACC*), high affinity nickel transporter family protein (*AT2G16800*), iron-regulated protein (*IREG*), glutathione reductase (*GR*), glutathione-s-transferase, Metal transporter (*NRAMP 1,2,3,4*), Nicotianamine synthase (*NAS3*), Putative transmembrane protein (*TMP*), Serine acetyltransferase (*SAT*), Thioredoxin family protein, Zn finger protein of *Arabidopsis thaliana* (*ZAT11*), and *MRP4* (Freeman *et al*. 2004; Lemaire *et al*. 2004; Stearns *et al.* 2005; Mizuno *et al*. 2005; Schaaf *et al*. 2006; Mari *et al.* 2006; Visioli *et al*. 2012; Liu *et al*. 2014; Theriault *et al.* 2016).

The *NRAMP* (Natural resistance associated macrophage protein) transporters is a family of genes whose main function is to bind and transport divalent metal ions. This is a highly conserved gene family during evolution and homologues have been identified in a large range of organisms including bacteria, yeast, mammals, and higher plants (Williams *et al.* 2000). The metal ions binding is dependent on the species and

the protein. Some divalent cations that can be bound by *NRAMP* metal ion transporters are Mn^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}, Cd^{2+}, Ni^{2+} and Co^{2+} (Supek *et al.* 1997; Liu *et al.* 1997; Chen *et al.* 1999, Nevo *et al.* 2004). Mizuno *et al.* (2005) were the first to identify the association of *NRAMP4* with Ni in a yeast study. They found that expression of Tj*NRAMP4* in yeast cells exposed to nickel increased Ni^{2+} sensitivity and Ni^{2+} concentration in the cell. In plants, Oomen *et al.* (2008) found that the metal hyperaccumulator *Thlaspi caerulescens* highly expressed *NRAMP3* and *NRAMP4* which can bind Fe, Mn, Cd and for both Zn for *NRAMP4*. Theriault *et al* (2016) further investigated the *NRAMP* genes and reported that they are implicated in Ni transport in white birch (*Betula papyrifera*). They also showed that plant resistance to excess nickel in white birch was associated with a downregulation of genes involved in binding and transporting activities like *NRAMP1-2*.

The Nicotianamine synthases genes (*NAS1, 3* and *4*) are a group of genes controlling enzymes that synthesize the metal chelator nicotianamine (NA). This nonproteinogenic amino acid can bind to some transition metals like Fe, Cu, Zn, Mn, and Ni and immobilize them for transport. NA has already been implicated in transporting Fe, Cu, and Zn for long distances within the plant (Wintz *et al.* 2003). An elevation in *NAS* gene expression has been observed in hyperaccumulator plants *Arabidopsis halleri* and *Noccaea caerulescens* (Deinlein *et al.* 2012; Visioli *et al.* 2014). It appears that an increase in nicotianamine contributes to metal tolerance in hyperaccumulator plants via metal chelation and facilitates metal translocation (Weber *et al*. 2004; Deinlein *et al*. 2012). In contrast, *NAS* genes seem to play a role in metal homeostasis (i.e. Fe, Cu, Zn and Mn) only in non-accumulating plants such as *Arabidopsis thaliana* (Curie *et al.* 2009).

The ATP Binding Cassette (*ABC*) transporters is a superfamily of genes that is represented in a wide range of organisms from archaea to humans (Higgins, 1992). The existence of some of these transporters in plants was first discovered in *Arabidopsis thaliana* with the identification of a multidrug resistance (*MDR*)-like gene (At*PGP1*) (Dudler and Hertig 1992). Since then, other *MDR* homologues have been found in *A. thaliana.* These membrane-associated proteins are Mg^{2+} ATPases and act as membrane pumps to mitigate the transport of molecules across extra- and intra-cellular membranes (Rea 1999). The *MRPs* are glutathione-

S conjugate pumps typically in vacuolar membranes and are important for the detoxification of harmful compounds within the cell and for metal vacuolar sequestration (Martinoia *et al.* 2002). Altered gene expression of *ABC* transporters genes has been observed in plants species exposed to different metal contamination. For example, Keinäen *et al.* (2007) observed an increased expression of an *ABC* transporter homologue *MRP4*, and *DnaJ*, a vacuolar sorting receptor-like protein, in Cu-tolerant birch (*Betula pendula)* compared to Cu-susceptible birch. Zientara *et al.* (2009) reported an increased expression of the At*MRP3* gene transformed into *Arabidopsis thaliana* when seedlings were exposed to excess concentrations of Cd, Ni, As, Co and Pb but not Zn and Fe. Thus, some members of this gene family are thought to facilitate metal tolerance via metal transport into sequestered areas like vacuoles.

Metal stress can affect the expression and activity of important antioxidant enzymes needed to deal with Reactive Oxygen Species (ROS). For instance, though nickel does not directly generate ROS because it is a redox-inactive metal (Boominathan and Doran, 2002), it has been reported to stimulate antioxidant enzymes such as superoxide dismutase (*SOD*), catalase (*CAT*), ascorbate peroxidase (*APX*), and glutathione s-transferase **(**Gabbrielli *et al.*1999**;** Rao and Sresty 2000**;** Baccouch *et al.* 2001**;** Gajewska and Sk*ł*odowka 2008**)** in plants. The activities of these genes appeared to be reduced based on a transcriptome analysis of white birch (*Betula papyrifera*). This result is possibly due to enzyme inactivation from direct binding of Ni^{2+} to a -SH group or histidine, Ni displacement of other metals in the active binding site, or indirectly (Theriault *et al.* 2016).

Kalubi *et al.* (2016) investigated how gene expression is affected by metal contamination in one Ni accumulator tree species, trembling aspen (*Populus tremuloides)* and one tree species red maple (*Acer rubrum*) that deals with excess Ni using the avoidance strategy. The transcript level was measured for target genes associated with nickel resistance including *NRAMP4, NAS3, AT2G16800* and *MRP4*. They found that *P. tremuloides* samples from both contaminated (Kelly Lake) and uncontaminated (St.Charles) areas in the Greater Sudbury Region had significantly higher gene expression than found in the *A. rubrum* samples.

Expression of *AT2G16800*, and *MRP4* was significantly increased in the *P. tremuloides* samples from the contaminated site compared to the uncontaminated. *NAS3* had a high upregulation in *P. tremuloides* for both sites compared to *A. rubrum* samples. Finally, *NRAMP4* expression was also higher in *P. tremuloides* samples from both sites compared to *A. rubrum* but this was the smallest difference. Though the downregulation of the *AT2G16800* and *MRP4* genes in *A. rubrum* samples from a metal-contaminated site was apparent, the direct environmental factors could not be established yet. It is hypothesized that *P. tremuloides* may be more sensitive to the abiotic stressors in the contaminated site which may trigger the change in the gene expression of these stress related genes to tolerate the excess nickel.

A gene expression study on northern red oak (*Quercus rubra)* conducted by Djeukam *et al.* (2019) found that other genes associated with metal-stress including ACC, SAT, and *NAS3* could be upregulated in response to high doses of nickel nitrate (800 mg/kg and 1,600 mg/kg). The red oak is another important species for the GSR because it is classified as a Ni/Zn accumulator and can store these metals in its leaves at high concentrations (Tran *et al.* 2014).

Overall, changes in gene expression induced by metal toxicity have been observed in many plants and there may be genes associated with metal resistance mechanisms used to tolerate excess metals.

1.4. Comparative Transcriptome analysis in plants

The development of next generation sequencing technologies and bioinformatic approaches has enabled researchers to analyze the whole genome (WG) or whole transcriptome (WT) of a sample population of cells. Whole transcriptome data reflects all the genes expressed at a given time and the number of transcripts associated with each gene indicates the level of gene expression. By looking at the entire mRNA population, one can characterize gene expression dynamics found in a sample, particularly focusing on biomolecular processes, molecular function, and cellular compartment. The transcriptomic profile in a particular tissue during a specific developmental/growth stage or during a stress response which requires changes in gene

expression can be analyzed. Data from this approach can be combined with other omics approaches like proteomics, metabolomics, and epigenomics to delineate and understand the dynamic mechanisms found in plants (Jamla *et al.* 2021).

Comparative transcriptomics analysis is a method that can be used to detect gene expression differences between sample groups that may have been induced by different experimental conditions. It has been useful for detecting gene expression changes in plants undergoing biotic or abiotic stress.

Biotic stressors are typically living organisms which can cause infections and disease in plants like insects, bacteria, fungi, and viruses. For example, transcriptomics was used to investigate the response of Arabidopsis plants to spider mite infections (Santamaria *et al.* 2021).

Abiotic stressors are non-living physical or chemical factors such as temperature, drought, salinity, and heavy metal stress, that can have a negative impact on plant health. To adapt to these environmental stressors, plants have evolved stress responses which are triggered via signaling pathways and a dynamic coordinated transcriptomic response can mitigate the potential damage (Singh *et al.* 2016). Transcriptomics can be used to reveal the regulatory networks that may be involved in the plant's general stress responses and identify candidate genes that are associated with a specific kind of abiotic stress. Furthermore, by varying the intensity of the stressor and comparing the resulting sample transcriptomes, it is possible to identify the thresholds where gene expression changes. For instance, numerous differentially expressed regulatory genes associated with stress response pathways were identified in drought-tolerant wheat when compared to drought-sensitive genotypes (Kumar *et al.* 2018). Similar methods have been carried out for other plant species such as sweet potato where more than 11 000 differentially expressed genes were found in drought-resistant genotypes (Yang *et al.* 2018). Also, key signalling pathways (Abscisic acid (ABA), ethylene (ETH), and jasmonic acid (JA)) and specific proteins (ABI-like protein and Ca2þ-ATPase) were identified in conferring drought resistance in sweet potato (Yang *et al.* 2018).

Additionally, considering that plants are often exposed to more than one abiotic environmental stressor at the same time, some transcriptomic studies have investigated the effects of multiple abiotic stressors. Rice (*Oryza sativa*) leaves were exposed to separate treatments inducing cold, iron or salt stresses and their transcriptional profiles were compared to determine how the complex stress responses may overlap or what unique genes may be involved in just one type of stress (do Amaral *et al.* 2016).

Excess heavy metals in the soil are a known type of abiotic stress in plants and many species have evolved specific heavy metal resistance mechanisms to cope and avoid or reduce toxicity symptoms. A comparative transcriptome analysis of *Brassica juncea* plants treated with varying concentrations of cadmium revealed more about its metal transport and tolerance mechanisms (Thakur *et al.* 2019). Lai *et al.* (2019) compared the transcriptomes of two plants species after treatment with cesium. They found a significant inhibition of specific K-transporter and K-channel proteins in the roots of the low-accumulating *Vicia faba* compared to the high-accumulating *Brassica juncea* plants.

Transcriptome analysis has been used to study the effects of nickel stress on ecologically important tree species found in the Greater Sudbury Region (GSR) in Canada. Theriault *et al.* (2016) compared resistant and susceptible white birch phenotypes that were identified amongst a sample population treated with the same 1600 mg/kg nickel dose. Through RNA-seq analysis, significant differences in gene expression were detected amongst genotypes. Gene expression patterns were identified including the downregulation of genes associated with ribosomal activities and translation in resistant genotypes. Furthermore, specific genes that were highly upregulated in nickel resistant birch genotypes compared to the susceptible samples were identified as good candidate genes for nickel resistance including two Nramp transporters, and Glutathione S-transferase (Theriault *et al.* 2016).

Alternatively, the maple study by Nkongolo *et al.* (2018) detected no significant differences amongs the RG and SG genotypes in the 1600 mg/kg nickel treatment group. However, the comparisons between the different nickel dose treatment groups and water controls did show significant differential gene expression.

Particularly, this effect was seen when comparing the high nickel dose (1600 mg/kg) to the lower nickel dose or control (150 mg/kg and 0 mg/kg). For example, there was an upregulation of transcripts associated with transport and translation in the high dose group. It is apparent from this study that a change in gene expression in response to high heavy metal concentrations can be triggered at a threshold dose between 800 mg / kg and 1600 mg / kg (Nkongolo *et al.*2018). The gene expression activities required to cope with the heavy metals are likely different from those in control plants or treated with a low concentration of 150 mg / kg corresponding to the bioavailable amouts of Ni at sites.

Overall, RNA-seq technologies provide the capabilities to investigate gene expression on a whole genome level. Alterations in gene expression can create the necessary changes at the morphological, physiological, molecular, and biochemical level needed for the plant to survive. This is a useful technique to further understand how plants adapt to the environment in real-time.

1.5. Methylation in plants in response to abiotic stressors

Epigenetics is the study of heritable change of gene expression that does not involve modification to the DNA sequence (Gallo-Franco *et al.* 2020). Epigenetics plays a pivotal role in regulating gene expression in development, in response to cellular stress or in disease states, in virtually all cell types (Lee *et al.* 2020). It is of natural occurrence but can be also the result of environmental stress response (Srikant and Drost 2021). Epigenetic mechanisms include DNA methylation and histone modifications, which consist of acetylation, methylation, phosphorylation, deamination, ubiquitylation, sumoylation, and ADP ribosylation (Gibney and Nolan 2010; Lee *et al.* 2020). This conformational change affects the accessibility of proteins involved in transcription which in turn plays a role in gene expression patterns as well as genome stability (Kumar and Mohapatra 2021). MicroRNAs (miRNAs) are short, non-coding RNA molecules that mediate RNA silencing and regulate gene expression. Because of this, miRNAs have also been identified as components of the epigenetic machinery (Lee *et al.* 2020).

Recent analysis of DNA modifications revealed that both hypermethylation and hypomethylation may contribute to the adaptation of plants to stress (Kim *et al.* 2016; Thiebaut *et al.* 2019). A review of research shows that methylation in plants is more complex and sophisticated than in microorganisms and animals (Chang *et al.* 2020; Bhadouriya *et al.* 2021). Cytosine methylation can be involved in the control of DNA replication, transcription, repair, and gene regulation (Vanyushin and Ashapkin 2011; Shell *et al.* 2013). To date, research in understanding the role of epigenetics in plant adaptations to environmental stressors is still in its infancy. There is strong evidence that DNA methylation in promoter regions silences genes, whereas DNA methylation in gene body sequences is positively correlated with increased gene expression in mammals and in some plant species (Liang *et al.* 2014). The main epigenetic events in plants are DNA methylation. Histone modification can also be induced by environmental stressors in plants (Asensi-Fabado *et al.* 2017; Chang *et al.* 2020; Bhadouriya *et al.* 2021). Chang *et al.* (2020) summarized epigenetic regulation in plant abiotic stress responses. This study discusses relevant literature on DNA methylation and histone modifications induced by drought, pH, metals, heat, cold temperatures, and salinity in plants.

Chromatin structure and modifications

Eukaryotic chromatin is packaged into nucleosomes where DNA is associated with histone proteins in the nucleus. The chromatin's conformation must undergo a modification from a transcriptionally inactive state to an active state for transcription to occur. This is known as chromatin remodeling which can be achieved by different processes such as DNA methylation and histone modification (Crusselle-Davis and Archer 2010). The change in rearrangement conformation between 'closed' to 'open' allows for the access of transcription factors and DNA binding proteins to regulate gene expression (Bhadouriya *et al.* 2021). Epigenetic modifications are essential for the normal development of organisms, the differentiation of cells and organs, reproduction, parental imprinting, acquired transgenerational trait inheritance, and stress adaptation (Konate *et al.* 2020)

Histone modifications

The modification of histones can change the chromatin conformation resulting in transcription activation or repression (Bannister and Kouzarides 2011; Bhadouriya *et al.* 2021). Many types of histone modification have been detected including histone methylation, acetylation, and phosphorylation (Gibney and Nolan 2010; Bannister and Kouzarides 2011). Specifically, if lysine 4 of histone 3 is methylated, this 'opens' the chromatin conformation and transcription can occur, whereas if lysine 9 of histone 3 is methylated, this 'closes' the chromatin conformation, and transcription is repressed (Bhadouriya *et al.* 2021). Further, the DNA-histone electrostatic interaction can be disrupted with the acetylation and phosphorylation of histones by reducing the histones positive charge leading to a less compact chromatin structure. This change in chromatin conformation can facilitate the access of proteins involved in transcription (Bannister and Kouzarides 2011).

The histones tails are also subject to post-translational modifications (PTMs) with the addition or removal of the chemical groups; methyl, acetyl and phosphate groups (Gibney and Nolan 2010). Methylation and demethylation of histone arginine is catalyzed by protein arginine methlyltranserase and deiminase respectively. The addition of a methyl group to a lysine of histones is accomplished by histone lysine methyltransferase. Acetylation and deacetylation of histones is catalyzed by histone acetyltransferases and histone deacetyl transferase. Further, phosphorylation of serine and threonine is due to the activity of histone kinases and phosphatases that remove phosphates (Gibney and Nolan 2010**).**

DNA methylation

DNA Methylation usually occurs at the gene promoter, and typically inhibits gene transcription. This change can be inherited through cell differentiation, and it is related to the pathogenesis of various diseases. DNA methylation constitutes three processes: *de novo* methylation, 5meC recognition, and active and passive demethylation. It was initially established that *de novo* DNA methylation occurs at symmetric CpG sites, starts with DNA methyltransferase (DNMT) 3A/3B, and is maintained by DNMT1 (Lee *et al.* 2020; Kumar and Mohapatra 2021). DNA methylation is presently the most documented epigenetic modification (Gallo-

Franco *et al.* 2020). It is a relatively stable and conserved epigenetic process that is crucial for organism development (Gallego-Bartolomé 2020; Gallo-Franco *et al.* 2020; Zhang et al, 2018). This mechanism plays an important role in the regulation of several cellular processes including regulation of gene expression, in the activity of transposable elements**,** genomic imprinting, genome stability, and cell differentiation, (Gallego-Bartolomé 2020; Gallo-Franco *et al.* 2020; Zhang *et al.*, 2018, 2020a; Phillips 2008).

Both DNA methylation and histone modification change the structure of the chromatin and DNA accessibility (Phillips 2008). Specifically, heterochromatic regions of plants and mammals is associated with high levels of DNA methylation. If a loss of heterochromatin maintenance occurs, genomic instability can arise causing phenotypic 'consequences' (Zhang *et al.*, 2020a). Thus, disruption in the process of DNA methylation can lead to developmental abnormalities in mammals and plants (Gallego-Bartolomé 2020). Consequences of DNA methylation disruption include human diseases, embryo lethality in mice and failure in tomato fruit ripening (Zhang *et al.* 2018, 2020a; Phillips 2008).

Mammals and plants differ in their DNA methylation patterns. In plants, DNA methylation is primarily found in the three following sequence contexts: CG, CHG and CHH (H can be A, C or T), and is mostly observed in heterochromatic transposable elements (TEs), while DNA methylation in mammals is seen in a CG context only (Gallo-Franco *et al.* 2020*;* Zhang *et al.* 2020b).

Studies determined that plants have the most methylated genomes of all eukaryotes, with up to 50% of their cytosine showing methylation (Moore *et al.*, 2013). In *Oryza sativa* L., DNA methylation profiles were determined and revealed that the most heavily methylated regions of DNA were transposable elements and repetitive sequences where methylation occurred in all three sequence contexts. Genes were also methylated but only in the CG context (He *et al.* 2010; Yan *et al.* 2010; Li *et al.* 2012). This was also seen in *Arabidopsis thaliana* where heavy methylated DNA also was found in the heterochromatin, where it is highly enriched with repetitive DNA sequences and transposable elements. Further, DNA methylation has also been detected in euchromatin within the interspersed transposon (Zhang *et al.* 2018, 2020a).

In mammals, about 45% of the genome contains transposable and viral elements where the majority are silenced due to methylation (Moore *et al.* 2013). These elements can become harmful if expressed because they could potentially replicate and insert into other areas of the genome leading to gene disruption and mutation of the DNA sequence (Moore *et al.* 2013). This indicates that a primary role of DNA methylation is to repress the expression of these genetic elements within the intergenic regions (Moore *et al.* 2013; Gibney and Nolan 2010).

Further, DNA methylation studies in plants determined that methylated DNA shows tissue specific patterns and sequence context (Niederhuth and Schmitz 2017). Studies of the *A. thaliana* DNA methylation patterns revealed tissue-specific methylation as well as differentially methylated sequence contexts. It was determined that about 6% of the cytosine in the immature floral tissue of *A. thaliana* was methylated. The most methylated sequence context in young plants was CG, followed by CHG and CHH at 24%, 6.7% and 1.7% respectively (Zhang *et al.* 2020b). Overall, DNA methylation is essential for maintaining stable and unique gene expression patterns in differentiated cell types (Gibney and Nolan 2010; Moore *et al.* 2013).

DNA methylation and demethylation

The gene expression regulation by DNA methylation is achieved by the recruitment of proteins involved in the repression of gene expression or by the inhibition of the binding of transcription factors to the DNA (Moore *et al.* 2013). The expression of certain genes during development is influenced by the methylation and demethylation of DNA (Gibney and Nolan 2010). This results in unique DNA methylation patterns that regulate tissue-specific transcription in differentiated cells (Moore *et al.* 2013; Bhadouriya *et al.* 2021). The DNA methyltransferases (METs) are the primary enzyme that methylate CG context sequence (CG methylases) whereas the chromomethyltransferases (CMTs), which are specific to plants, can methylate the CHH and CHG sequence contexts. The domain rearranged methyltransferases (DRMs) are responsible for the maintenance of non-CG methylation as well as de novo methylation for the CG, CHH and CHG sequence contexts (Gallo-Franco *et al.* 2020).

The demethylation of DNA has also been observed in plants and animals. In plants, the enzyme 5-methylcytosine glycosylases directly excise the 5-mC base, whereas in mammals DNA demethylation is accomplished by the oxidation and/or deamination of 5-methylcytosine (5-mC) (Gallo-Franco *et al.* 2020; Gibney and Nolan 2010). Research has determined the importance of the methylation and demethylation balance. Phillips (2008) demonstrated based on a mouse study that if a specific DNMT looses function, a reduction of DNA methylation occurs resulting in premature death of the animal.

It has been shown that chromatin remodeling proteins also play a crucial role in the proper maintenance of DNA methylation (Bhadouriya *et al.* 2021). There are several different mechanisms involved in the remodeling of chromatin (Crusselle-Davis and Archer 2010). Remodeling proteins can slide nucleosomes, removing or shifting histones, adding histone variants (Crusselle-Davis and Archer 2010). In absence of chromatin remodeling, nucleosomes prevent the access of DNA methyltransferases thus inhibiting DNA methylation (Bhadouriya *et al.* 2021). A nucleosome remodeler, decrease in DNA methylation 1 (DDM1) is involved in DNA methylation. Mutations in this nucleosome remodeler resulted in an overall decrease in methylation of all three sequence contexts, specifically in heterochromatin (especially in repeat regions). DNA methylation reductions were also seen in genes, but to a lesser extent (Zhang *et al.* 2020a, b). When the DDM1 gene is mutated, epigenetic variation occurs which lead to the steady transmission of morphological phenotypes throughout generations (Bhadouriya *et al.* 2021). It is therefore essential that chromatin homeostasis be maintained in order to achieve proper expression of gene patterns (Zhang *et al.* 2020a,b). Mechanisms of DNA methylation and demethylation in plants is described in detail in Kumar and Mohapatra (2021).

Effects of DNA Methylation and histone modifications on gene expression

The information contained in the DNA sequence is transcribed into short-lived mRNA which is translated into polypeptide chains or proteins which can then be subsequently modified. Even though all cells within

an organism effectively contain identical nucleotide sequences, different types exist, and their functions vary because of the quantitative and qualitative gene expression differences (Gibney and Nolan 2010).

Gene expression can be regulated by changes in the DNA sequence as well as changes in chromatin structure (Gibney and Nolan 2010). Even though the majority of cells within an organism contain the identical genome, the expression of genes vary by cell and tissue types, is plastic, and changes over time at different developmental stages and environments (Bhadouriya *et al.* 2021; Moore *et al.* 2013).

DNA methylation is involved in gene silencing or repression (Gibney and Nolan 2010). Patterns of gene expression can be influenced by the location of DNA methylation (Zhang *et al.* 2018). Studies revealed that methylation near promoter of genes is highly variable depending on the type of cell, where more methylated promoters resulted in low or no transcription (Phillips, 2008*).* In plants, DNA methylation has been observed in the promoter and within the transcribed gene body. When the promoter of genes is methylated, there is a repression in gene transcription (Zhang *et al.* 2018). This occurs because the methylated state of the promoter DNA prevents the binding of transcription activator or promotes the binding of transcription repressors (Phillips, 2008*).* In the model plant such as *A. thaliana*, about 5% of gene promoters are methylated. This indicates that transcription of most genes is not controlled by DNA methylation (Zhang *et al.* 2018).

Methylation within the genes has also been observed. This is called gene body methylation (gbM). The gene body is defined as the region following the first exon (Moore *et al.* 2013). The methylation of gene bodies is often seen in exons and lacking in the start and stop sequences (Zhang *et al.* 2020a; Zhang et al 2018). If the first exon is methylated, like promoter methylation, silencing of that gene occurs. It has been shown that the methylation of gene bodies can increase gene expression in dividing cells. This is not the case for nondividing cells such as in the brain (Moore *et al.* 2013).

In angiosperms, the genes with methylated DNA within their transcribed regions (with gbM) are usually longer compared to genes lacking methylated DNA (are unmethylated) and are often constitutively

expressed (Zhang *et al.* 2018*).* In addition, the location of methylated DNA in genes that are constitutively expressed is seen in the CG context (Zhang *et al.* 2020a).

Further, transposable elements are involved in gene expression. Gene**s** can be repressed when methylated transposons are found adjacent or within it (Gallo-Franco *et al.* 2020). DNA methylation at promoter regions of a gene is frequently influenced by adjacent transposons. Genes found in proximity of transposons and repeats are protected from transcriptional silencing because they are 'targeted by active DNA demethylation machineries' (Gallo-Franco *et al.* 2020).

Methylation and other epigenetic mechanisms are important for plant development and stress responses. It is a short-term way to alter gene expression to respond to the needs of different parts of a plant going through stages of development or in response to environmental stress (Bartels *et al.* 2018). In addition, once tissues are differentiated, the methylation levels and affected loci differ presumably to maintain the necessary gene expression profile specific to each tissue (Bartels *et al.* 2018). For instance, studies of the Chinese white poplar (*Populus tomentosa*) indicate methylation differences among various plant tissues like leaves, roots, xylem, and phloem buds (Du *et al.* 2012; Ma *et al.* 2012; Su *et al.* 2018). To respond to biotic and abiotic stressors in the environment, many signaling pathways are induced and short-term transcriptional changes are required. This can be modulated with epigenetic mechanisms to maintain elevated gene transcription or repression while the stressor is present and then to revert to a normal state (Boyko and Kovalchuk 2008). In other cases, some epigenetic marks can remain for the plant's life even be passed down to progeny to improve adaptation in anticipation of future adverse conditions (Whitelaw and Whitelaw 2006; Bender, 2004). This epigenetic memory is seen in plants more often than animals which coincides with the idea that due to their general immobility, plants require a variety of internal ways to adapt to their environment (Takeda and Paszkowski 2006).

Plants have more complex and widespread methylation patterns than mammals. Methylation primarily occurs in cytosine residues in three different sequence contexts: CG, CHG, CHH. Heritable methylation

changes in the form of epialleles were identified in rice exposed to different abiotic stressors including heavy metal toxicity and drought (Ou *et al.* 2012, Zheng *et al.* 2017). Methylation and demethylation can have different effects on gene expression depending on the plant species, tissue type, and location of methylation in relation to the gene (upstream, promoter, gene body, or downstream).

Gene expression can be repressed with transcriptional gene silencing (TGS) which is correlated with promoter sequence hypermethylation (Paszkowski and Whitham 2001). Alternatively post-transcriptional gene silencing (PTGS) can also occur, and it is more commonly associated with transcript or coding sequence hypermethylation (Paszkowski and Whitham 2001). This coincides with a general trend in previous studies where higher expression levels are seen in non-methylated genes and higher methylation levels are associated with suppression of gene transcription (Chan *et al.* 2005; Wang *et al.* 2016a,b). However, different results have been observed depending on the methylation context. For instance, rice (*Oryza sativa*) methylome and transcriptome data only conferred this pattern for genes with heavily methylated promoter regions, while methylation in the gene body correlated positively with gene expression (Zheng *et al.* 2017). A methylome and transcriptome analysis of drought-stressed apple (*Malus domestica*) showed the same trend of decreased promoter methylation resulting in higher gene expression, and increased gene body methylation correlates with higher expression (Xu *et al.* 2018). Weinhold *et al.* (2013) identified another example of promoter hypermethylation resulting in down-regulated gene expression in transgenic *Nicotiana attenuata* plants. Genome-wide methylation maps of *A. thaliana* showed evidence of high gene body methylation in constitutively active genes (Zhang *et al.* 2006).

High-throughput whole-genome bisulfite sequencing and single-base resolution methylome maps were used to analyze methylation changes and associated gene expression in *Populus trichocarpa* during tree trunk formation (Zhang *et al.* 2020b). The three different tissues involved in this process [(primary stems (PS), transitional stems (TS), secondary stems (SS)] were found to have significant differences in methylation and gene expression (Zhang *et al.* 2020b). An increase in stable methylated sites was observed in TS and SS

indicating that the poplars undergo clear methylome alterations once secondary growth is initiated (Zhang *et al.* 2020b). The reduced gene expression of demethylases (PtrDME-A, PtrDME-B, PtreDEMETER-LIKE 2-A, PtrDEMETER-LIKE 2B) and the increased expression of CHH methylases (PtrDRM1/2-B, PtrDRM1/2-C) correlate with this finding and may enable the higher methylation found in these tissues. Though the 5mC methylation of gene bodies appear globally correlated with transcript levels, the evidence for 5mC levels of nearby promoters correlating with transcript levels could not be established (Zhang *et al.* 2020b). This suggests that the relationship between methylation and gene expression is more complex than the idea that promoter methylation changes are the direct and only method to alter gene expression.

Epigenetic mechanisms that can modulate the gene transcription and expression of important heavy metal (HM) detoxification genes like metal chelators and transporters may enable the plant to adapt to this abiotic stress. DNA methylation within gene promoters or gene bodies may serve a major role in adapting to HM stress by controlling gene expression (Finnegan *et al.* 1998; Choi and Sano 2007). Abiotic stress can induce demethylation of stress response genes which results in transcriptional activation. One example of this trend is the aluminum-tolerance associated glycerophosphodiesterase-like protein (NtGPDL) which was upregulated in tobacco plants exposed to abiotic stress (Wada *et al.* 2004; Choi and Sano 2007). A study investigating the effects of cadmium stress in rice on the methylome and transcriptome supports the evidence that altered methylation is associated with subsequent differential gene expression (Feng *et al.* 2016). Genome-wide single-base resolution maps of methylated cytosines revealed 2320 differentially methylated regions and 2092 DNA methylation-modified genes (RNA sequencing) under Cd stress (Feng *et al.* 2016). Differentially methylated regions (DMRs) were identified for all sequence contexts throughout the genome. When considering all regions near and within genes including upstream, gene body, and downstream, more genes were found hypermethylated (Feng *et al.* 2016). RNA-seq revealed that these genes are involved in a range of pathways like stress response, metal transport and transcription factors, and their altered expression coincides with methylation changes (Feng *et al.* 2016).

Methylation does not have to strictly occur directly within a gene's promoter or gene body to alter its transcription. In fact, retrotransposons can be activated typically via demethylation and their methylation status can influence the expression of surrounding genes (Kashkush and Khasdan 2007). Plant studies show that these transposable elements are usually hypermethylated to maintain their inactivation and avoid disruption of proximal gene coding regions. (Bennetzen *et al.* 1994; Martienssen 1998; Rabinowicz *et al.* 2003). For *Oryza sativa* L. (cultivated rice), studies have shown high methylation levels for transposable elements and repetitive sequences in its genome (He *et al.* 2010; Yan *et al.* 2010). Methylation patterns of long terminal repeats (LTR) which flank the *Dasheng* retrotransposon were compared with the expression of adjacent genes in rice leaves (Kashkush and Khasdan 2007). Differential methylation was correlated with altered gene expression of nearby genes in a tissue-specific manner indicating the potential role of transposable elements in gene transcription regulation via methylation (Kashkush and Khasdan 2007).

Garg *et al* (2015) provided evidence suggesting that DNA methylation play an important role in abiotic stress adaptation /response by regulating expression of a set of stress-responsive genes in rice largely via methylation/demethylation of proximal TEs. Yang *et al.* (2015) examined the relationship between DNA methylation and floral gene expression on a genomic scale. They reported that the methylation and demethylation processes were correlated with expression change of > 3000 genes associated with normal flower development. Epigenetic regulation of heat stress in plants is complex. Various epigenetic mechanisms are involved in response to heat stress, a number of them are still unknown. Integrated analysis of the changes in the genome, transcriptome, and proteome by omic approaches should help to identify novel transcriptional, translational, and posttranslational regulation components and underlying mechanisms in plant heat responses. In fact, Lamelas *et al.* (2020) in a recent study used OMICs approaches to analyses the nuclear proteome in *Pinus radiata*. They quantified the expression of several genes and identified specific nuclear heat-responsive proteins. In addition to heat-shock proteins, several clusters involved in regulation

processes were discovered, such as epigenomic-driven gene regulation, some transcription factors, and a variety of RNA-associated functions.

Histones are frequently decorated with covalent modifications. These histone modifications are thought to be involved in various chromatin-dependent processes including transcription. Karlić *et al.* (2010) found that histone modification levels and gene expression are very well correlated and that only a small number of histone modifications are necessary to accurately predict gene expression. Different sets of histone modifications are necessary to predict gene expression driven by high CpG content promoters (HCPs) or low CpG content promoters (LCPs). Quantitative models involving H3K4me3 and H3K79me1 are the most predictive of the expression levels. Histone modifications that loosen DNA association with histones generally provide a permissive environment for transcription, whereas histone modifications that tightly package DNA and histones repress gene expression. Dnmts directly interact with enzymes that regulate histone modifications typically involved in gene repression. Both Dnmt1 and Dnmt3a are known to bind to the histone methyltransferase SUV39H1 that restricts gene expression by methylation on H3K9 (Moore *et al.* 2013). Furthermore, Dnmt1 and Dnmt3b can both bind to histone deacetylases that remove acetylation from histones to make DNA pack more tightly and restrict access for transcription (Moore *et al.* 2013).

Crosstalk of DNA Methylation and Other Epigenetic Mechanisms

It is established that methylation of DNA and of histone 3 at Lys 9 (H3K9) are highly correlated with gene silencing in eukaryotes (Du *et al.* 2015). Both epigenetic marks need to be established at specific regions of the genome and then maintained at these sites through cell division. Several lines of evidence confirm that different epigenetic marks interplay with each other rather than working independently in biological processes. Although each of the four main types of epigenetic marks that include, histone modifications, histone variants, DNA methylation and ncRNAs seems to have its own role in determining transcriptional outcomes, there is clearly a built-in connection among them (Wang *et al.* 2016a).

DNA methylation works with histone modifications and microRNA (miRNA) to regulate transcription (Zhang *et al.* 2020a). In eukaryotes, DNA is associated with histone proteins that help to package the long strands of DNA into the small nuclear compartment. Chemical modifications that include methylation, acetylation, ubiquitination, and phosphorylation are added to three specific amino acids on the N-terminal histone tails. These modifications influence not only how DNA strands are packaged but also their transcriptional activity (Moore *et al.* 2013).

Effects of Drought on methylation levels

Water is essential for plant growth and development and there are numerous dangerous effects on plant health when it is not available in adequate amounts (Guo and Tan 2013). Drought is a major and common stressor that plant species are subjected to and as a result abiotic stress responses evolved to adapt to water deficient conditions (Do *et al.* 2013). Many studies have been performed to identify drought-induced differential gene expression in plants to better characterize the role of general abiotic stress responses and specific drought-responsive genes (Wang *et al.* 2011; Wang *et al.* 2014; Zheng *et al.* 2017). This is of importance for the development of drought-resistant varieties of economically significant crops (Do *et al.*2013). Numerous physiological and molecular mechanisms have been identified in plant drought tolerance including increased antioxidant activity and physiological changes like reduced tiller growth (Ma *et al.* 2010; Zheng *et al.* 2017; Xu *et al.* 2021).

Wang *et al.* (2014) analyzed the changes in the drought tolerant *Citrullus colocynthis* transcriptome after exposure to drought conditions and found more than 2,500 significant differentially expressed genes. It is evident that a wide range of genes may have altered transcriptional levels to enable plants to adapt and survive a water deficient environment including transcription factors, dehydrins (dehydration-induced proteins), stress signaling factors, and detoxification genes (Reddy *et al.* 2004; Yamaguchi-Shinozaki and Shinozaki 2006; Wang *et al.* 2014). Epigenetics changes such as methylation likely play a role in a plant's ability to induce a wide ranged yet quick adaptive stress response. Evidence of increased expression of genes

involved in methylation was identified with transcriptome analysis and subsequently confirmed with RT-qPCR (Wang *et al.*2014). For example, the transcript Comp3048 which codes for myo-inositol-O-methyltransferase (*lmt1*) has consistently high expression levels in *C. colocynthis* plants exposed to drought stress (Wang *et al.* 2014). This enzyme catalyzes the methylation of the compound myo-inositol which is involved in numerous stress response and signal transduction pathways during abiotic stress like low temperature, drought and salinity (Valluru and Van den Ende 2012; Zhu *et al.* 2012; Papaefthimiou *et al.* 2012). It is apparent that overexpression of lmt1 can confer improved tolerance to abiotic stressors (Ahn *et al.* 2011).

Zheng *et al.* (2017) found that rice exposed to drought conditions had several stable methylation changes in stress-responsive genes which were passed on to progeny for multiple generations. All sequences contexts for methylation and all types of epimutations (DMPs, SMPs and DMRs) were considered in the high-throughput analysis and creation of single-base resolution maps (Zheng *et al.* 2017). From the rice varieties investigated emerged a drought-resistant type called Huhan3 which was compared to the drought-sensitive II-32B type (Zheng *et al.* 2013). The resistant variety had overall higher global methylation levels yet hypomethylation was seen in many gene coding regions (Zheng *et al.* 2013). Furthermore, the resistant type had a much higher percentage of methylation marks that were stably transmitted to the next generation (Zheng *et al.* 2013). Some site-specific transgenerational epi-mutations were consistently present through 11 successive water deficient generations and offspring were better able to tolerate and adapt to such adverse conditions (Zheng *et al.* 2017). Increased tolerance was defined by plants employing less of a survival response with decreased tiller amount and having better reproduction rates seen with higher seed sets (Zheng *et al.* 2017). This study further confirms evidence by Wang *et al.* (2011) that site-specific changes in methylation can be induced in plants to tolerate drought stress.

Wang *et al.* (2011) compared DNA methylation changes with MSAP for two rice cultivars (the drought-tolerant DK 151 and its drought-sensitive parent IR64) subjected to drought stress. The effects were

developmentally and tissue specific, but overall, about 12.1% of all the site-specific methylation differences were determined to be drought-induced (Wang *et al.* 2011). There was an overall global hypomethylation seen for both rice types particularly in roots, but they highly differed in locations of methylation/demethylation sites affected (Wang *et al.* 2011). Differences in gene expression caused by methylation alterations may explain the differences in drought tolerance. Some specific loci had increased DNA methylation which remained methylated even after drought conditions were no longer present, where about 70% of DNA methylation changes reverted to normal (Wang *et al.* 2011). It is yet to be determined if the loss of methylation state is a result of an active demethylation process or if methylation was lost passively (Wang *et al.* 2011). Other instances of DNA hypermethylation were observed in drought-exposed tobacco plants (Kovarik *et al.* 1997) and pea plants (Labra *et al.* 2002).

Histone modifications via methylation and acetylation are known to influence gene expression in plants during developmental processes (Sung and Amasino 2005; Dennis and Peacock 2007). Changes in chromatin arrangement have also been investigated for plants undergoing abiotic stress (Manzanero *et al.* 2002; Sokol *et al.* 2007). There were many histone modifications in H3K4me3 and H3K9ac levels seen in drought-associated genes for drought-exposed *A. thaliana* plants (Kim *et al.* 2008). One trend observed was that higher H3K4me3 and H3K9ac levels in the coding regions of the drought stress inducible genes (RD29A, RD29B, RD20, RAP2.4) correlated with their activation (Kim *et al.* 2008). After the stress was eliminated with irrigation, H3K9ac modifications were reversed (Kim *et al.* 2012). However, some H3K4me3 modifications were still present suggesting that this is one aspect of methylation that can be inherited to next generations as a type of "stress memory". (Kim *et al.* 2012; Liu *et al.* 2014). Recently, Van Dooren *et al.* (2020) performed a genome-wide analysis of DNA methylation and gene expression in *A. thaliana* and demonstrated that, mild drought induced changes in the DNA methylome of exposed plants.

Effects of pH on methylation levels

Studies on the effects of pH on DNA methylation are limited. The low pH of acidic soils can result in proton toxicity which has a detrimental effect on plants' ability to grow and survive in these environments (Kochain *et al.* 2004). Low pH increases the solubility of minerals and nutrients including contaminants like heavy metals that may be necessary for plants in small concentrations but are toxic to the organism at excessive levels. Plants growing in a high proton concentration are exposed to the resulting increased oxidative stress within tissues due to the accumulation of Reactive Oxygen Species (ROS) like superoxide radicals and hydrogen peroxide (Shi *et al.* 2006, Liu *et al.* 2011). This toxic effect is compounded by the oxidative stress induced by heavy metal contamination.

Kimatu *et al.* (2011) studied this combination of abiotic stress factors by determining the effects of low pH and aluminum toxicity on methylation in *Sorghum bicolor.* MSAP analysis indicated that treating plants with 150 uM Al3+ growing in low pH (4.0) conditions was enough to trigger changes in methylation levels. Some individual plants may possess various tolerance alleles which are thought to increase sorghum tolerance levels and their expression levels may be regulated via DNA cytosine methylation (Caniato *et al.* 2007; Kimatu *et al.* 2011). The large gene *AltSB* was identified in conferring aluminum tolerance in *Sorghum bicolor* and this region is homologous to the QTL region associated with aluminum tolerance in rice (Magalhães *et al.* 2004). In general, pH is an important factor to consider when studying abiotic stress in plants because of the potential toxic effects on its own and its interaction with other abiotic stressors that can enhance their toxic effects.

Effects of Heavy Metals on Plant Methylation

Elements that are considered heavy metals (HMs) have high atomic weight densities above 5g/cm3 and naturally occur as part of the Earth's crust (Gallo-Franco *et al.* 2020). In a plant physiological context, some HMs like iron (Fe), copper (Cu), nickel (Ni) and zinc (Zn) are essential elements needed in small concentrations for proper function of some enzymes (Gallo-Franco *et al.* 2020). Other HMs like cadmium (Cd), chromium (Cr), lead (Pb) and aluminum (Al) are non-essential elements with no known function in

the plant's physiology (Gallo-Franco *et al.* 2020). It is well documented that both types of HMs will have negative impacts on plant growth and development when they are present in soils at excess concentration (Gallo-Franco *et al.* 2020). For many metals, the cytotoxic, genotoxic, and mutagenic effects they have on plants are enhanced when they are present in excess in acidic soils (Gallo-Franco *et al.* 2020). These soil types referred to as ultisols or oxisols have a pH lower than 5.5 and they make up almost 30% of arable land worldwide (Gallo-Franco *et al.* 2020; Bojórquez-Quintal *et al.* 2017). Increased acidity in soil also increases the solubility of many heavy metals so the impact of these two environmental factors can compound the toxic effects on plant growth and development (Chuan *et al.* 1996).

After solubilization in the soils, the overabundance of heavy metal cations in their aqueous form including aluminum (Al3+), calcium (Ca2+), nickel (Ni2+) can have very damaging effects (Gallo-Franco *et al.* 2020). Epigenetics appear to be heavily involved in plant stress responses to adapt to and tolerate abiotic stressors like heavy metals (Boyko and Kovalchuk 2008). Though there are general stress response pathways that can be triggered to tolerate abiotic stressors, each type of abiotic stress can produce a partly specific epi-genetic response. The pattern of methylation changes in plants exposed to heavy metal stress has been investigated with multiple types of heavy metals.

Silencing RNAs (siRNAs) expressed during abiotic stress can signal epigenetic changes in plant tissues. For example, hemp (*Cannabis sativa* L) and clover (*Trifolium repens* L) exposed to heavy metals had specific hypomethylated genomic loci (Aina *et al.* 2004). DNA methylation changes under heavy metal stress were compared between metal-sensitive clover (*Trifolium repens* L.) and metal-tolerant hemp (*Cannabis sativa* L.) plants. The Slot-Blot immunolabeling technique was used to measure the levels of 5-methylcytosine(5mC) in the plant roots after growing in Ni2+, Cd2+ or Cr6+ (25 and 50 mg/kg potassiumdichromate, 25 and 100 mg/kg nickel chloride and 25 and 100 mg/kg cadmium sulphate) treated soil (Aina *et al.* 2004). In general, the control *C. sativa* plants had higher methylation levels when compared to clover DNA and methylation decreases for both species as the soil metal concentration increases. This

dose-dependent hypomethylation may be induced by the increased ROS and oxidative DNA damage (Aina *et al.* 2004). The methylation-sensitive amplification polymorphism (MSAP) technique was used to determine if any specific sequences had predominant changes in methylation and if these were linked to altered gene expression. The focus was on the 5'CCGG-3' sequence where its methylation patterns appear to be highly conserved within individual members of a species (Aina *et al.* 2004). After heavy metal treatment, changes indicating hypo- and hyper-methylation at certain sites were determined to be consistent within a sample group. This suggests that specific sequences are targeted for methylation changes which likely coincides with changes to gene expression activity as part of the plant defense response (Aina *et al.* 2004).

The heavy metal tolerance epigenetic mechanisms utilized by prominent plant species in the Greater Sudbury Region (GSR) are of interest because this is a known highly metal-contaminated environment (Mehes-Smith and Nkongolo 2015). Species like white birch (*Betula papyrifera)* have been able to adapt and colonize these areas despite the increased soil acidity and heavy metal soil contaminants resulting from mining industry activities (McCall *et al.* 1995, Theriault *et al.* 2013, 2014; Mehes-Smith and Nkongolo 2015). In addition to liming practices improving soil fertility, Theriault and Nkongolo (2016) postulated that non-heritable changes in cytosine methylation may be involved to enhance plant survivability. Considering there are *B. papyrifera* phenotypes that are more sensitive to oxidative stress than tolerant plants, suggests that some genotypic and/or epigenetic differences exist within species populations (Theriault and Nkongolo 2016). Significant differences in global methylation levels ([5mdC]/[dG]) were found when comparing *B. papyrifera* trees grown in Ni and/or Cu contaminated sites compared to uncontaminated sites and in some cases, there were differences between root and leaf tissues (Theriault and Nkongolo 2016). The primary trend seen was global cytosine hypomethylation in the metal-contaminated sites compared to uncontaminated sites. Methylation levels were measured with tandem mass spectrometry (MS/MS) coupled with LC (LC-MS/MS) method. (Theriault and Nkongolo 2016).

Another common species in the GSR is red maple (*Acer rubrum)* that has been found to have different coping mechanisms for heavy metal stress than *B. papyrifera* (Kalubi *et al.* 2015; 2016). Assessing the global methylation levels by the ratio of 5-methyldeoxycytidine (5mdC) to deoxyguanosine (dG) (MS/MS coupled with LC-MS/MS) in *A. rubrum* root samples from different contaminated and uncontaminated areas showed significant variation (Kalubi *et al.* 2017). In general, there was a strong negative correlation where the global cytosine methylation levels decrease when the bioavailable nickel/copper concentrations increase (Kalubi *et al.* 2017). This likely results in more total gene expression within the plant cells which can increase survivability and adaption to the HM stress. Considering that the sampled trees are all from the same natural gene pool within the area, these differences are likely in response to the heavy metal contamination (Kalubi *et al.* 2017). Kim *et al.* (2016) found a similar result when analysing *A. rubrum* samples from metal contaminated regions that showed an overall decrease in global methylation compared to populations from nearby uncontaminated regions. One group of samples from contaminated regions had few changes in methylation (Kim *et al.* 2016).

Aluminum

Methylation-sensitive amplification polymorphisms (MSAP) studies suggest that aluminum-tolerant and aluminum susceptible triticale [(*X Triticosecale*), a hybrid of wheat (*Triticum*) and rye (*Secale*)] both had induced demethylation when exposed to aluminum (Bednarek *et al.* 2017). The RP-HPLC technique used for a similar comparison of aluminum-treated tolerant and susceptible triticale plants showed increased methylation and decreased methylation respectively (Agnieszka 2018). Analysis of three wheat cultivars (cv. Haymana79, Kılçıksız, and Bezostaja1) under a low and high aluminum concentration treatment showed DNA hypomethylation at the low dose and DNA hypermethylation at a high dose (Pour *et al.* 2019). Alternatively, DNA hypermethylation appeared to be triggered by aluminum exposure in corn (*Zea mays* cv. RX9292) in addition to long terminal repeat retrotransposons (LTRs) mobilization (Taspinar *et al.* 2018).

Gallo-Franco *et al.* (2020) investigated the methylation and gene expression patterns of a 250 set of genes associated with rice aluminum tolerance for one highly Al tolerant cultivar (Nipponbare) and two susceptible types (IR64 and Pokkali). Rice has been a good species for investigating these mechanisms because it has a high aluminum tolerance, and it is an important staple crop (Famoso *et al.* 2010; Mustafa and Komatsu 2016). The number of methylated cytosines and gene expression values of these genes were measured before and after Al treatment. All genes showed significant changes in expression while Nipponbare had increased methylation and IR64 and Pokkali did not have significant differences in methylation. Some candidate genes that play a major role in aluminum tolerance include a Calmodulin binding protein (Loc_Os09g13890) and STAR1 (Loc_Os06g48060) (Zhang *et al.* 2016; Gallo-Franco *et al.* 2020). The nucleotide binding domain encoded by STAR1 associates with STAR2 and they are necessary for the functioning of an ABC transporter which works in roots to detoxify aluminum (Huang *et al.* 2009).

Methylation changes at specific loci encoding stress-response genes may be occurring. For instance, Borner *et al* (2003) found that tobacco (*Nicotiana tabacum*) plants exposed to Al stress had increased demethylation in the coding region of the glycerophophodiesterase-like protein gene (NtGPDL). The exact function of this gene is unknown, but it is hypothesized to be involved in stress responses. Furthermore, the gene insertion of the Al-tolerant plant derived *Andropogon virginicus* (AvSAMS1) into *A. thaliana* plants provided some increased Al tolerance (Borner *et al.* 2003). The AvSAMS1 gene encodes the main methyl group donor enzyme in plants and it can alter DNA methylation and histone H3 methylation in plants (Borner *et al.* 2003).

Cadmium

The effects of HM stress on nucleoid structure and methylation in hyperaccumulator *A. halleri (*I16 and PL22) populations were compared to the non-accumulator *A. thaliana* (Meyer *et al.* 2015, Corso *et al.* 2018). Plants were treated with zinc (Zn) or cadmium (Cd) at concentrations consistent with past literature ((100 uM ZnSO4, 50 uM CdSO4 for 2 weeks) so that the metal response is induced without exceeding toxicity thresholds (Li *et al.* 2015). With Cd treatment, the comet assay showed an increased DNA migration in *A.*

thaliana nuclei indicating high levels of DNA damage (Galati *et al.* 2021). There was no significant change observed for I16 *A. halleri* plants while the Cd-treated PL22 samples had a reverse trend with increased nucleoid condensation (Galati *et al.* 2021). The methyl-sens comet assay was further used to detect global methylation levels in response to Zn treatment. I16 nuclei showed a slight increase in CpG methylation while PL22 nuclei had decreased methylation (Galati *et al.* 2021). RT-qPCR indicated upregulated expression of symmetric methylation and histone deacetylation genes (*MET1, VIM, HDA8, DRM2, KYP, IBM1*) in PL22 plants. For I16 plants, there was decreased CpG DNA methylation and no significant expression changes of target genes when compared to the control (Galati *et al.*2021). Differences in the two hyperaccumulator populations reinforce the hypothesis that the environment and species-subtypes can cause differing metal detoxification strategies to evolve. It appears that one mechanism of PL22 plants to cope with heavy metal stress and avoid DNA damage from ROS and cadmium ions is with increased nucleoid condensation (Galati *et al.* 2021).

In another investigation, eight common wheat (*Triticum aestivum*) varieties were exposed to heavy metal stress and their phenotypes were compared. In particular, Pirsabak 2004 and Fakhar-e-sarhad types were considered Pb, Cd, and Zn resistant and sensitive, respectively (Shafiq *et al.* 2019). Measures of resistance were germination ability, primary root length, and epicotyl length (Shafiq *et al.* 2019). The sensitive variety had decreased antioxidant activity (SOD, POD CAT enzymes) though both types had accumulated comparable Pb, Cd, and Zn concentrations in their roots (Shafiq *et al.* 2019). To adapt to the heavy metal stress, the resistant strain had significant upregulation in the gene expression of the metal detoxification transporters *Heavy Metal ATPase 2 (TaHMA2)* and *ATP-Binding Cassette (TaABCC2/3/4)* compared to sensitive and control plants (Shafiq *et al.* 2019). There is evidence that TaHMA2 can transport Zn and Cd to the apoplast where it can be sequestered or exported out of the cell (Tan *et al.* 2013) while ABCC transporters detoxify heavy metals via vacuole sequestration (Szczypka *et al.* 1994, Ghosh *et al.* 1999). This increase in gene expression (TaHMA2, TaABCC2, TaABCC3, TaABCC4) was correlated with

hypomethylation in the promoters of these genes suggesting that DNA methylation is part of the epigenetic response for heavy metal stress tolerance in this wheat strain (Shafiq *et al.* 2019). It is important to note that these genes already have differing basal expression levels and TaABCC2, TaABCC3, TaABCC4 were higher in Pirsabak 2004 than Fakkar-e-sarhad. Genetic and epigenetic differences amongst resistant and susceptible plant varieties already exist and the phenotypic effects can be observed upon induction of stress responses from heavy metal exposure.

Fan *et al.* (2020) observed global hypermethylation in the roots of Col-0 *A. thaliana* plants after Cd exposure and this finding was correlated with repressed gene expression of three DNA demethylase genes *ROS1/DML2/DML3 (*RDD). The results were corroborated with *rdd* mutants that had a similar methylation profile to the original Cd-exposed plants (Fan *et al.* 2020**).** This response to heavy metal stress was confirmed to increase the Cd tolerance of the plants, in this case likely through better Fe nutrition (Fan *et al.* 2020). This study highlights that the epigenetic response to heavy metal stress involves the adjusted gene expression of several aspects of methylation mechanisms present in plants including the balance of DNA demethylation enzymes.

Ding *et al.* (2019) looked at how methylation can be altered in response to HM stress in the lesser studied endangered quillwort species (*Isoetes sinensis*). MSAP analysis indicated that though global methylation levels were similar for control plants and Cd-treated or Pb-treated plants, the methylation profiles were different (Ding *et al.* 2019). In general, there was less full-methylation (both strands) levels and more hemi-methylation in the heavy metal stressed samples when compared to the control (Ding *et al.* 2019). This may indicate that though overall levels may be the same, specific loci are still altered through demethylation, hypermethylation or re-methylation processes to adjust the necessary gene expression and cope with the HM stress (Ding *et al.* 2019).

The methylation changes in response to cadmium exposure of an endangered plant species from an aquatic environment called the Mediterranean tapeweed (*Posidonia oceanica)* were studied by Greco *et al.* (2012).

Aquatic plants can uptake aqueous heavy metals directly into their leaves or absorb it from sediments as seen in *Posidonia oceanica* (Greco *et al.* 2012; Maserti *et al.* 2005). They found that a 50 uM Cd treatment induced global DNA hypermethylation in this important metal bioindicator species (Lafabrie *et al.* 2007). This finding was consistent with increased gene expression of CMT (Chromomethylase which is primarily involved in maintenance of CHG methylation and de novo methylation) (Greco *et al.* 2012). In addition to these changes, long term Cd treatment also altered nuclear chromatin arrangements seen in a progressive heterochromatinization increase with high methylation levels and high density. (Greco *et al.* 2012). Analysis of methylation in plants indicates that despite variety in habitats and species, methylation patterns are a key component of abiotic stress responses like heavy metal contamination in plants.

Transcriptome analysis for roots of rice plants treated with 50 uM of cadmium for 24 hrs revealed upregulated gene expression of ROS-scavenging enzymes, metal chelators and transporters (Oono *et al.* 2014). Genes associated with other abiotic stresses like drought, high-salinity, and low temperature were also upregulated ((Oono *et al.* 2014). The identification of antioxidant activity genes suggests that the excess reactive oxygen species generated during drought or heavy metal exposure may act as signaling molecules to induce these stress response pathways (Oono *et al.* 2014). This study highlights the crossover between genes involved in stress response for heavy metals and drought suggesting some responses may be generalized to multiple abiotic stressors. These pathways are potentially regulated with methylation and demethylation mechanisms (Oono *et al.* 2014).

Lead and zinc

The genome-wide methylation levels of maize (*Zea mays*) plants were measured with methylated DNA immunoprecipitation-sequencing (MeDIP-seq) after exposure to lead (Pb) stress (Ding *et al.* 2014). A variety of characterized inbred maize lines with differing Pb root uptake ability and Pb tolerance were used (Zhang *et al.* 2012). In particular, line 9782 had gene expression changes in response to Pb treatment, it was lacking hyperaccumulating ability and had increased Pb stress tolerance (Ding *et al.* 2014). Model-based Analysis

for CHIP-Seq (MACS) was used to analyze the methylation-enriched gene regions (Ding *et al.* 2014). In general, there was higher promoter methylation than in gene bodies. Presence of hypermethylation in any area of the gene correlated with reduced gene expression (Ding *et al.* 2014). Multiple differentially methylated and expressed transcription factors were identified including AP2/ERF, bHLH, MYB, bZIP (Ding *et al.* 2014). Shafiq *et al.* (2019) identified DNA hypomethylation at the promoter region in *Triticum aestivum* lines treated with lead, zinc, and cadmium compared to control. Their results demonstrated that the DNA methylation difference between resistant Pirsabak 2004 and sensitive Fakhar-e-sarhad varieties in response to Pb, Cd and Zn contributes to the metal tolerance through the regulation of the expression of metal detoxification transporters.

Nickel and copper

Gulli *et al.* (2018) used the non-accumulating *A. thaliana* species to compare epigenetic changes to an hyperaccumulator species called alpine penny cress (*Noccaea caerulescens)*. Plants were treated with nickel to induce heavy metal stress. *N. caerulescens* genotypes that have adapted to a naturally stressed environment and heavy metal contaminants like nickel have been shown to accumulate them in their leaf tissues as a response (Gulli *et al.* 2018). The methylation levels were much higher in the Ni hyperaccumulator species *N. caerulescens* than Ni susceptible *A. thaliana* when these plants were grown in highly nickel-concentrated soil (Gulli *et al.* 2018). Specifically, differentially gene expression was observed for DNA methylation-related genes *MET1, DRM2, HDA8* between these two species. One purpose of increased methylation in response to HM stress is to protect DNA and avoid HM-induced damage like single-strand breaks or multi-copy transposition (Bender 1998). The alkaline comet assay showed high preservation of nuclei integrity within *N. caerulescens* leaf cells while *A. thaliana* nuclei had significant evidence of DNA damage (Gulli *et al.* 2018). Interestingly, the control *N. caerulescens* that were not exposed to nickel had in fact high integrity nuclei but they were structurally less compact suggesting that the compaction seen in the nickel treated samples was in response to the heavy metal (Gulli *et al.* 2018). Yagci *et al.*, 2019 demonstrated

that copper stress increased DNA methylation in *Lactuca sativa* compared to untreated plants. The field studies conducted by Kim *et al.* (2016), Kalubi *et al.* (2017), and Theriault *et al.* (2016) in areas with predominantly higher content of nickel and copper than any other metals suggest that these metals might induce hypomethylation of DNA in *A. rubrum* and *B. papyrifera*.

These results suggest that at high concentrations, DNA hypermethylation may provide a protective response for plants in a dose-dependent and species-specific manner. However, hypomethylation can additionally play a role at site-specific loci which can modulate gene expression in response to the HM stress.

Other abiotic stresses: cold, high temperature, salinity

Chang *et al.*, (2020) reported relevant knowledge on epigenetic regulation of plant responses to extreme temperatures, drought, salinity, the stress hormone abscisic acid, nutrient limitations and ultraviolet stress, and on epigenetic mechanisms of stress. Direct effects of abiotic stress on DNA methylation in plants was not discussed in detail. Liu *et al.* (2015) and recently Zhao *et al.* (2021) reviewed progress achieved on epigenetic regulation of heat responses, including DNA methylation, histone modifications, histone variants, ATP-dependent chromatin remodeling, histone chaperones, small RNAs, long non-coding RNAs and other epigenetic mechanisms. Correia *et al.* (2013) analyzed DNA methylation in *Quercus suber* by HPCE. The results of this investigation revealed an increase of DNA methylation at 55 ^{0}C, while protein Gel Blot showed the abundance index of AcH3 decreasing from 25 ^{0}C to 45 ^{u}C. This indicated that epigenetic mechanisms such as DNA methylation and histone H3 acetylation have opposite dynamics that can be crucial in the acclimation and survival of *Quercus suber* under severe heat such as 55 ^{0}C. Gao *et al.* (2014) using MSAP revealed DNA methylation/demethylation induced by heat stress in *Brassica napus*. The observed methylation patterns involved a wide range of genes.

Kumar *et al.* (2017) observed a genotype-and tissue-specific increase in cytosine methylation induced by NaCl stress that downregulated the expression of TaHKT2;1 and TaHKT2;3 in the wheat shoot and root tissues. Salt stress induces hypermethylation in *Nicotiana tabaccum* and *Triticum aestivum* and histone

modification in *N. tabaccum* and *A. thaliana* (Kovarik *et al.* 1997; Sokol *et al.* 2007; Kumar *et al.* 2017). The main demonstrated effect of cold stress in plants has been histone modifications (Manzanero *et al.* 2002; Zeng *et al.* 2019).

Exogenous substances that can alter methylation

Melatonin (MT) is a molecule created from the precursor Tryptophan and it aids in the stress response in plants exposed to heavy metals (Cai *et al.* 2017, Kanwar *et al.* 2020). Exogenous melatonin can be absorbed by plants, and it may detoxify toxic lead ions via a change in methylation which alters gene expression to improve antioxidant activity (Tang *et al.* 2021). Global methylation levels increased when radish plants were treated with Pb while there was hypomethylation seen for Cd-treated plants with melatonin added (Tang *et al.* 2021). Some genes that were differentially methylated between the two treatments include transition metal ion binding, cation binding, and antioxidant genes (RsAPX2, RsPOD52, RsGST) (Tang *et al.* 2021). Upregulated calcium-sensing genes for the MT + Cd treated plants including *CaM, CBL, CML, CPK, CDPK* play a role in the uptake, accumulation, and detoxification of heavy metals in plants (Ni *et al.* 2018, Jalmi *et al.* 2018). Antioxidant genes including APX2, POD52, POD64, and glutathione S-transferases (GSTs) were highly upregulated and this correlated with hypomethylation (Tang *et al.* 2021). A similar trend was observed for some metal transporter genes like RsYSL7, RsZIP11 and the ABC transporters RsABCF5, RsABCG14 (Tang *et al.* 2021).

Another example of exogenous substances that can aid in the plant heavy metal stress response are selenium and the polyamine (PA) spermine (Spm). Kumar *et al.* (2012) found that Cd-induced toxicity symptoms in red seaweed (*Gracilaria dura)* could be alleviated via altered methylation. This correlated with increased antioxidant gene expression when spermine or selenium were supplied. Cd exposure on its own can trigger the generation of reactive oxygen species (ROS), impair antioxidant activity, and increased demethylation (Kumar *et al.* 2012). Antioxidant enzymes particularly superoxide dismutase (*Mn-SOD*), glutathione

peroxidase (*GSH*), and glutathione reductase (*GR*) were differentially regulated which correlated with an increase in water soluble antioxidants (GSH, AsA NP-SH and PCs) (Kumar *et al.* 2012).

Transgenerational inheritance and somatic memory

Epigenetics changes in response to an environmental stressor can last for a short time and quickly revert to the gene's original state once the stress is no longer present. However, some epigenetic changes can be stable for the rest of the plant's lifetime with somatic memory or can even be passed on to future progeny as seen in transgenerational inheritance patterns (Gallo-Franco *et al.* 2020). Mechanisms like histone modifications may enable the stable preservation of methylation states for longer than one period of stress and produce stress-tolerant phenotypes. (Pecinka and Scheid 2012). (Gallo-Franco *et al.* 2020).

Whole-genome bisulfite sequencing was used by Feng *et al* (2016) to measure DNA methylation patterns in Cd-stress induced rice plants. They found differences in methylation for stress response genes involved in metal transport, metabolic processes, and transcriptional regulation. Some of these changes have been shown to be heritable and increase the ability of progeny to tolerate HM stress (Rahavi *et al.* 2011). Boyko *et al.* (2010) showed that exposure of *Arabidopsis* plants to stresses, including salt, UVC, cold, heat and flood, resulted in an increased global genome methylation, and higher tolerance to stress in the untreated progeny.

The heritability of epigenetic changes in rice was investigated by Cong *et al.* (2019) after identifying that epigenetically regulated stress responders, metal transporters and transcription factors could all be transcriptionally activated in rice leaf tissue in response to Cd-stress. Genome-wide single base resolution maps of methylated cytosines and transcript profiling aided in identifying relevant genes like Heavy Metal-transporting P-type ATPases (HMAs) (Feng *et al.* 2016). This gene family is well characterized in *A. thaliana* and each of the nine genes have some metal specificity and differences in expression, sub-cellular localization and regulation (Kim *et al.* 2009, Kobayashi *et al.* 2008, Abdel-Ghany *et al.* 2005). HMAs are important for the plant stress response when exposed to toxic heavy metal concentrations with a primary role in their uptake and movement in the cell for sequestration. The methylation status of HMAs and other

relevant target genes was measured when rice was exposed to two different concentrations of Cu, Cr, Cd, or Hg. Locus-specific CHG hypomethylations were observed at high Cd, Cr and Cu doses (20X the low dose) compared to low doses while methylation changes remained high at both Hg doses (Ou *et al.*2012). These hypomethylation events correlated with increased gene expression in 7 HMAs in at least one of the heavy metal treatments to cope with HM stress (Cong *et al.* 2019). The epigenetic and gene expression alterations of the HMA genes can be inherited to some extent in two subsequent generations with "transgenerational memory" for increased adaptability in anticipation of a heavy metal-contaminated environment, even if the metals are removed by that time (Cong *et al.* 2019).

A similar test was done by Ou *et al.* (2012) by self-propagating rice seedling-plants that were treated with the low Hg2+ dose and showed a high amount of hypomethylation. The resulting progeny had a variety of methylation patterns including "inheritance" of the parent methylation patterns, "new patterns" with further modification of the parent patterns or methylation reverted to original state before heavy metal treatment (Ou *et al.* 2012). Furthermore, the "new patterns" seen in F1 progeny continued in the same direction as parent progeny, where there was an enhanced hypomethylation increase (at CHG sites) (Ou *et al.* 2012). F1 progeny with methylation patterns classified as "new" were further propagated and the successive generation had stably inherited these methylation states (Ou *et al.* 2012). Plants in the third generation also showed the same result suggesting that methylation modification epialleles could be passed on to plants even without present-day heavy metal contamination (Ou *et al.* 2012). It is suggested that epigenetic transgenerational inheritance may more likely be incurred by mild heavy metal stress over very toxic concentrations (Ou *et al.* 2012).

Transposon methylation can also be inherited as seen in the transgenerational inheritance patterns of methylation at the Tos7 transposon in rice plants (Cong *et al*. 2019). In plants treated with heavy metals, the transposons Tos17 (and Ors42) were hypomethylated and as a result their expression was upregulated (Ou *et al.* 2012, Cong *et al.* 2019). However, the transposon remained silent for three generations and these

changes were not enough to induce transposon activation (Cong *et al.* 2019). An example of changes in gene expression occurring with transposable elements is seen in the study with barley by Kashino-Fujii *et al.* (2018). The multi-retrotransposon-like (MRL) insertion linked with demethylation increased the expression of the citrate efflux/aluminum detoxification gene when it was inserted upstream of the HvAACT1 coding region (Kashino-Fujii *et al.* 2018). In some cases, transposable elements near coding genes could be considered a type of epi-allele because they can act as a promoter and alter gene expression. (Slotkin and Martienssen 2007, Kashino-Fujii *et al.* 2018).

Other factors that improve heavy metal stress tolerance that can be passed down to other plant generations can be seen with homologous recombination frequency (HRF) (Rahavi *et al.* 2011). Arabidopsis plants exposed to Ni2+, Cd2+ and Cu2+ had increased homologous recombination rates and subsequent higher stress tolerance (Rahavi *et al.* 2011). The immediate progeny grown in similar conditions also had increased recombination but when these plants were propagated in the absence of stress the next generation reverted to normal HRF levels (Rahavi *et al.* 2011). The increase in HRF was metal- and dose-dependent and the 100 mM Cd2+ treatment had the highest recombination in progeny of stressed parent plants (Rahavi *et al.* 2011). When plants were consistently exposed to this heavy metal stress for five generations, progeny continued to have high HRF (Rahavi *et al.* 2011). The increased heavy metal tolerance (measured by root length) of progeny compared to parental plants suggest that this is a mechanism that enables plants to acclimate to the environment (Rahavi *et al.* 2011). These plants also had higher tolerance to salt (NaCl) stress and methyl methane sulfonate (MMS) which suggest that a general plant stress protective responses may be activated (Rahavi *et al.* 2011). Also, the tolerance increased with each generation where fifth generation plants were more tolerant than the stressed plants before them. (Rahavi *et al.* 2011). The patterns of stress response seen in these experiments suggest an underlying epigenetic mechanism, namely stress-induced signaling by small non-coding RNA, like siRNAs (Ito *et al.* 2011).

Van Dooren *et al.* (2020) showed that, changes induced by mild drought in *Arabidopsis thaliana* were not inherited. It remains to be determined whether transgenerational epigenetic variation in nature is caused by more dramatic environmental conditions than those tested so far in the laboratory, by combinations of several mild stressors, or by mutations in genes such as AtDDM1 that are involved in the epigenetic control of transposable elements (Quadrana and Colot 2016; Van Dooren *et al.* 2020).

Sun *et al.* (2021) summarized and discussed plant stress memory at the intergenerational and transgenerational levels, mechanisms involved in stress memory, exploitation of induced and natural epigenetic changes, and genome editing technologies with their future possible applications, in the breeding of crops for abiotic stress tolerance. Epigenetic modifications can lead to altered gene transcription and are an important mechanism for controlling gene expression during development in response to stimulation of the environment. This epigenetic information reflects the transcriptional memory associated with cell fate decisions, developmental changes, or stress responses: memory that is often required during reproduction to be deleted and reset (Sun *et al.* 2021).

1.6. Species of Interest: trembling aspen

The trembling aspen (*Populus tremuloides)* is a fast-growing species, and it is the most widely distributed tree in North America. It belongs to the Populus *genus* within the Salicaceae family and composed of 40 species of poplar, cottonwood, aspen, and hybrids (Ye *et al*. 2011). Many members of this genus, including *Populus tremuloides*, are commonly used in the forestry industry, particularly for pulp products like books and newsprint. Some species have been proposed as potential low-cost and low-maintenance bioenergy crops that could be used to produce biofuels (Ye *et al*. 2011). Trembling aspen also has an ecologically important role in forest ecosystems because it is a foundation species (Rai *et al.* 2013). Furthermore, it is considered a good model plant species for adaptive responses to climate change because it already thrives across many climatic zones with high adaptability (Rai *et al.* 2013).

P. tremuloides is a good candidate for land reclamation and phytoremediation for numerous reasons. It is dioecious; meaning male and female reproductive organs develop in separate individuals (Cox, 1988). Though it can sexually reproduce, the main mode of propagation is through root suckering. This is favorable for the species in disturbed soil environments because once one tree is successfully established, it can go on to propagate numerous clones through root suckering and populate an area (Elliott and Baker 2004).

Furthermore, there is evidence that *P. tremuloides* is a metal accumulator species. This means it can translocate excess metals away from the roots and store them in the shoots or leaves of the plant at high concentrations. Kalubi *et al.* (2015) found that nickel (Ni) and zinc (Zn) accumulated in the leaf tissues as opposed to the roots in *Populus tremuloides* samples growing in Northern Ontario. This means that this species can be useful in removing contaminants from soils, sediments, and water, through the biotransformation of metals into their inert forms and storing them at high concentrations in the plant leaves (Kalubi *et al.* 2015). The large biomass production of trembling aspen is also beneficial because larger sized trees have more area to store excess metals so they may be able to tolerate more heavily contaminated soils. Finally, the extensive root systems of *P. tremuloides* may enable them to survive in disturbed soils because their roots can reach deeper volumes of the soil where there is less contamination (Radwanski *et al*. 2017).

Overall, *Populus tremuloides* is a hardy and adaptable tree species that is important for the Greater Sudbury Region and across the continent North America.

1.7. Rationale and objectives

Knowledge about how plants respond to heavy metal stress and other abiotic stressors continues to increase. Relatively recent -omics technologies have enabled researchers to better understand the complex dynamics involved in heavy metal tolerance and resistance. Evidence shows that when plants experience environmental stress, it can trigger a general abiotic stress response by inducing a multitide of signaling pathways. In addition, some genes may have altered expression that are specific to mitigating the toxic

effects of the affecting stressor. The tolerance mechanisms used by the plant can also be dependent on species. For example, this study is focused on trembling aspen (*Populus tremuloides),* a common hardwood tree species found in the Greater Sudbury Region (GSR). It is an accumulator species that can absorb excess heavy metal ions from the soil via its roots and direct their transport into the leaves and shoots for sequestration. It is an important part of the GSR ecosystem which has been affected by mining activities that resulted in metal-contaminated (primarily Ni and Cu) and acidified soils. Many areas have been improved with liming practices and the establishment of hardy tree communities that include *P. tremuloides*. This study aimed to elucidate the *P. tremuloides* stress response when exposed to nickel toxicity using a global transcriptome approach with RNA-seq technologies. The experiments were designed to isolate for the specific effects of excess nickel ions on the dynamic gene expression and epigenetic patterns in *P. tremuloides* plants. This can help determine the sustainability of these populations in the GSR, better understand plant nickel resistance mechanisms, and contribute to bioremediation efforts with the genetic engineering of highly resistant plants for metal-contaminted soils.

The main objectives of the study were to: 1) Further characterize the *P. tremuloides* transcriptome, 2) Compare gene expression dynamics between nickel-resistant and nickel-susceptible *P. tremuloides* genotypes with Whole Transcriptome (WT) sequencing, 3) Assess the effects of different nickel concentrations on *P. tremuloides* gene expression and, 4) Assess global methylation levels in *P. tremuloides* under nickel stress.

Chapter 2: Transcriptome Analysis of Nickel - Resistant and Susceptible Trembing Aspen (*P. tremuloides)* genotypes

2.1. Introduction

The effects of heavy metal contamination on plants is an important topic of research particularly for areas that have been affected by anthropogenic mining industries. In the Greater Sudbury Region (GSR), decades of nickel and copper mining activities have increased the concentrations of these heavy metals in the surrounding soil and environment (Theriault *et al.* 2016a). Furthermore, the increased production of sulfur dioxide (SO_2) has resulted in soil acidification which consequently makes heavy metals more bioavailable for uptake from the soil by plants (Theriault *et al.* 2016a).

Populus tremuloides is an important founder species across North America and it is a key member of the GSR ecosystem. The ability of trembling aspen to accumulate excess heavy metals in its above-ground tissues via the translocation of metals absorbed in the roots to the shoots or leaves for storage may enable this species to better tolerate metal-contaminated-soils (Kalubi *et al.* 2015). Numerous other characteristics of this species like high genetic variation among populations can make them hardy and adaptable to different environments (Kirkey *et al.* 2012). This present study aims to assess potential gene expression differences between nickel- resistant and susceptible *P. tremuloides* genotypes based on growth chamber screening tests.

RNA-seq will help to characterize the gene expression profiles of these groups of genotypes at the whole transcriptome level. This could elucidate the nickel resistance/tolerance mechanisms that may be utilized by trembling aspen to cope with nickel stress and identify putative genes Ni transporter genes.

The specific objectives of this study are to 1) Assess nickel toxicity on *P. tremuloides* seedlings, 2) Develop and characterize the *P. tremuloides* transcriptome, 3) Analyze differential gene expression in nickel-resistant and susceptible *P. tremuloides* genotypes, and 4) Identify any novel candidate genes for nickel resistance.

2.2. Materials and Methods:

2.2.1. Assessment of nickel toxicity on trembling aspen (*P. tremuloides*) seedlings.

Populus tremuloides seeds were collected in Woodstock, NB (seedlot# 20001001) and provided by the Canadian Forest Services seed bank in Fredericton. They were stored at 4°C until further use.

Seeds were germinated in a Petawawa germination boxes and seedlings were grown in a deep tray with soil. Four-month-old seedlings were transplanted into pots containing a 50:50 sand/soil mixture and left to grow for an additional month and a half in a growth chamber. Plants were watered as needed and fertilized twice a week with equal amounts of nitrogen, phosphorus and potassium (20-20-20).

Ni toxicity was assessed by treating seedlings with an aqueous solution of nickel nitrate salt [$Ni(NO_3)_2$] at the following concentrations: 150 mg, 400 mg, 800 mg, and 1, 600 mg of nickel per 1 kg of dry soil. These doses represent the bioavailable fraction of total nickel available to biota, quarter total, half total, and total nickel, respectively found in metal-contaminated soils in the GSR. (Nkongolo *et al*. 2013**;** Kalubi *et al.* 2016). These levels correspond to 301.69 μmol, 150.85 μmol, 75.42 μmol, and 56.54 μmol of Ni, respectively. To control for any possible effects due to the increase in nitrate ions (NO_3) in the plants, an aqueous solution of commercial potassium nitrate (KNO_3) salts was used for controls in equal molar amounts to each dose of nickel salts. The nitrate controls for 1, 600 mg / kg, 800 mg / kg, 400 mg / kg and 150 mg / kg correspond to 603.38 μmol, 301.69 μmol, 150.85 μmol and 113.08 μmol of nitrate respectively. Salt-free water was used as a negative control (0 mg Ni per 1 kg of dry soil). The experimental design was a completely randomized block design with 12 replications per each nickel treatment, 11 for the water control, and 5 per nitrate control.

Damages to plants were assessed every two days based on a rating scale of 1 to 9, 1 representing no visible toxicity symptoms and 9 was dead plants as described in (Theriault *et al.* 2016a). Plants were rated individually and a genotype with a score of 1 to 3 was considered nickel resistant, 4 to 6 moderately

resistant/susceptible, and 7 to 9 susceptible. Plant height was recorded on the day of treatment and on the day 7 after treatment before harvest. On this day 7, roots and leaves of the plants were harvested from seedlings, frozen in liquid nitrogen and stored at -20°C. RNA was extracted from these samples and used for downstream transcriptome analyses in Chapter 2 and 3. DNA was extracted from these samples for the global methylation analysis in Chapter 4.

2.2.2. Statistical analysis for nickel toxicity assessment

Kruskal-Wallis and one way ANOVA (Post-hoc Dunnett's T3) statistical tests were performed using SPSS 20 for Windows to determine significant differences among means ($p \leq 0.05$) for average plant damage ratings.

2.2.3. RNA extraction

Total RNA was extracted using the Plant/Fungi Total RNA Purification kit from Norgen Biotek Corporation (Thorold, Canada). Samples were quantified with the Qubit® RNA BR Assay kit by Life Technologies (Carlsbad, United States). The RNA was run on a 1% agarose gel to verify its quality by confirming there were two sharp rRNA bands (28S and 18S) with no smearing/degradation.

2.2.4. RNA-seq and transcript alignment with a draft genome

RNA- seq libraries were created using the TruSeq RNA-Seq Sample Prep Kit and following manufacturer protocols (Illumina Inc., San Diego, CA). Messenger RNA (mRNA) was extracted from total RNA by selecting for RNA with poly-A tails. This fraction was chemically fragmented and then cDNA was synthesized in two steps (first strand and second strand). The cDNA ends were repaired, followed by the addition of adenosines to the 3' ends and then adapter ligation. Complementary DNA (cDNA) fragments (with sizes of 200 ± 25 bp) were gel-purified and enriched by PCR. The Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA) was used to quantify libraries and then sequencing was done on the Illumina Novaseq 6000 sequencing system (Illumina Inc.) at Seq Matic (Fremont California, USA). In total, 18

libraries were sequenced of *P. tremuloides* treated with varying concentrations of nickel salts : 6 total nickel-treated (3 resistant samples and 3 susceptible), 3 half-nickel treated, 3 bioavailable treated, 3 water-treated control, and 3 nitrate-treated controls (1 total-nitrate, 1 half-nitrate, 1 bioavailable nitrate).

STAR (Spliced Transcripts Alignment to a Reference) software was used for alignment and annotation of raw RNA-seq data. Reads were aligned to the draft *Populus tremuloides* genome published by Lin *et al.* (2018) using the two-pass method for increased sensitivity in detecting novel transcripts. All reads from the 18 sequenced samples were used for alignment and merged into one for input into the StringTie software to detect transcripts based on existing gene annotation, novel transcripts, and new isoforms. The CDS (coding sequences) of the transcripts were further delineated with the transdecoder program. The resulting peptide sequences of transcripts were mapped to protein sequences in the Uniprot database (http://www.uniprot.org/) using RAPsearch2. Annotation and gene ontology information was assigned to transcripts based on the best match.

2.2.5. Gene Expression and Sampling QC

Transcript alignment bam files were mapped to the updated annotation file of the draft genome and the FeatureCounts tool from Subread was used to determine raw gene count. Additional QC was performed to determine the number of genes expressed. Genes with at least 1, 2, 10, 50 or 100 counts were considered. Generally, the number of genes with two or more counts provided a rough estimate of genes expressed. Genes with only one read could be noise. The number of genes with 10 or more reads was an indication of how many genes had sufficient reads to be included in downstream statistical analysis. Another QC measure applied to the data was to verify that the top 100 genes with highest number of reads did not make up a disproportionately high percentage (>35%) of all RNA-seq reads. This could indicate a bottlenecking issue that occurred during library preparation where only a few genes were amplified many times.

For downstream QC analysis, genes that had a counts per million (CPM) value ≥ 1 for at least two samples were considered. The gene count data was processed with the TMM normalization method from the edgeR package. Samples were scaled with a normalization factor to remove differences between the RNA population composition of each sample. An extreme normalization factor (>1.5 or <0.66) for a sample could indicate an outlier or a large biological difference compared to other samples.

Boxplots summarizing gene expression distribution were created by log-2 transforming the normalized gene counts with the voom method from the R Limma package. This method indicates outliers or samples with large biological differences if the distribution is high or low compared to other samples.

Sample relationships were assessed with a multidimensional plot created using the R Limma package. Ideally, the biological replicates would cluster together, and different treatment samples separate from each other. Hierarchical clustering of samples was further assessed with a heatmap created with the made4 (multivariate analysis of microarrays data using ADE4) package from R Limma. It included the top 5000 genes with variable expression. Genes were chosen with a standard deviation (SD) >30% of the mean expression values. If there were more than 5000 variable genes, then genes with mean logCPM <1 were removed. Remaining genes were ranked by SD/Mean and the top 5000 were chosen. Gene expression levels were plotted for each sample to identify overall patterns of sample clustering. Scatterplots between pairs of samples indicated how similar they were to each other. Biological replicates or samples from the same treatment should have high correlation values. One total nickel resistant sample (TotNi.R3) was identified as a potential outlier because gene expression was more like susceptible samples. It was removed from this treatment group for downstream gene expression analysis. Principle component analysis (PCA) and an overview gene expression heatmap confirmed this conjecture.

2.2.6. Differentially Expressed Genes (DEG) analysis

DEG analysis was performed between sample treatments. All the genes in the expressed genes QC list that have ≥ 10 raw counts in at least three samples were tested. The gene expression values were calculated as Reads Per Kilobase per Million reads mapped (RPKM). First raw read counts were converted for each gene to counts per million (cpm) to normalize for sequencing depth. The cpm was divided by gene length in kb to normalize for transcript length. For paired-end data, two paired reads were treated as one fragment, and the FPKM value was determined using the same calculation method. Differentially expressed genes were filtered through two stringency levels. The high stringency condition is the recommended standard cut-off of a two-fold change with an FDR (False Discovery Rate) value ≤ 0.05 and logFC value ≥1 (upregulated) or ≤ -1 (downregulated). The low stringency cutoff of a two-fold change and p-value ≤ 0.01 was applied to treatment comparisons with ≤ 10 genes that met the standard cutoff. Gene set enrichment analysis (GSEA) analysis was performed for the upregulated and downregulated genes to identify top GO categories enriched in the data. Only gene sets with a minimum of 10 genes and a maximum of 1000 genes were included. The annotated gene set of all the DEGs identified for the nickel resistant vs. susceptible comparison was run through the Plant-Slim function and then GO category graphs were created with the OmicsBox BLAST2GO program. The percentage of differentially expressed transcripts distributed within each of the three GO function categories (Biological Process, Molecular Function and Cellular Component) was calculated. All the annotated genes from the water treatment were also run through Plant-Slim and GO category graphs were created to provide a summary of GO functions for the *Populus tremuloides* control.

2.3. Results

2.3.1. Assessment of nickel toxicity on *P. tremuloides* seedlings.

Significant differences were observed among the average damage ratings on day 6 after the treatments. Table 1 summarizes the damage ratings over time for plants treated with nickel. No significant damage was observed at the 150 mg/kg (bioavailable amount of Ni), 400 mg/kg, and 800 mg/kg doses. At the 1,600 mg/kg dose, significant damage was observed over time and some plants were dead or nearly dead by the end of the experiment (day 7). This treatment showed the highest variation in plant reactions to Ni salts where after day 7, there were three resistant, two moderately susceptible and seven susceptible plants. Plants treated with potassium nitrate salts used as control showed no significant damages.

Table 1: Damage rating of *Populus Tremuloides* treated with different nickel nitrate and potassium nitrate doses

Treatment	**Concentration (mg/kg)**	**Damage rating**		
		Days after treatment		
		Day 2	Day 4	Day 6
Water control	0	1	1	1**a**
Nitrate	150	1	1	1**a**
	400	1	1	1**a**
	800	1	1	1.5 ± 0.71**ab**
	1,600	1	1	1**a**
Nickel	150	1.11 ± 0.33	1.11 ± 0.33	1.11 ± 0.33**ab**
	400	1.11 ± 0.33	1.44 ± 0.53	1.44 ± 0.53**ab**
	800	1.22 ± 0.44	1.56 ± 0.68	1.89 ± 0.60**b**
	1,600	3.08 ± 2.25	5.75 ± 2.75	6.83 ± 2.32**c**

*Kruskal-Wallis test performed to determine if a difference exists across treatments on day 6 ($p<0.05$). Average ratings with different indices represent significant differences among means ($p \leq 0.05$) based on ANOVA-one way test (Post hoc Dunnett's T3) ($p < 0.05$ n= 49).

2.3.2. Transcript assembly and QC analysis

Sequencing generated 27-45 million 2X150 paired-end reads of raw data per sample. The alignment performed with StringTie Software added two groups of transcripts to the draft genome annotation. Group 1 contained 32,677 new isoforms that match to 17,254 genes. Group 2 contained 17,349 novel transcripts that represent 16,157 novel genes. The second group of novel transcripts representing 16,157 novel genes added to the draft annotation may contain mostly non-coding RNAs as indicated by the limited amount of long gene coding regions.

A total of 52,987 genes were identified and considered for downstream QC analysis. After normalization, samples generally met the QC test standards. The Ni susceptible plants have an overall smaller number of genes expressed. No potential outlier from the 18 samples was detected in the gene expression and sampling QC analysis. An MDS plot (multi-dimensional plot) made for sample clustering analysis did suggest that one nickel resistant sample (TotNi.3) appeared more like a nickel susceptible sample. Additional measures like the variable gene expression heat map were consistent with this observation. This sample was considered a treatment group outlier and was removed from the nickel resistant group for the resistant-susceptible genotype comparison and dosage analysis.

The entire *P. tremuloides* transcriptome dataset was deposited in the DDBJ/EMBL/GenBank and it is under review for the assignment of an accession and a project numbers.

2.3.3. Gene ontology classification of *Populus tremuloides*

All expressed annotated transcripts from each treatment were run through BLAST2GO. Gene ontology was assigned, and graphs were generated to display the distribution of expressed transcripts for biological function, molecular function, and cell compartmentalization. In general, the number of transcripts identified for each different group (control, resistant, susceptible) were similar. Gene ontology (GO) category graphs for all annotated genes expressed in the water control treatment plants are displayed in Fig. 1-3.

A total of 9,766 transcripts were assigned ontology for biological function. About 66.3% of all transcripts were involved in the transport, cellular component organization (CCO), carbohydrate metabolic process (CMP), response to stress, catabolic process, lipid metabolic process and response to chemical categories (Fig.1).

For molecular function, 9,953 transcripts were assigned ontology. About 37 % of transcripts code for proteins involved in nucleotide binding activities, 17.1 % for kinase activities, 15.7 % for DNA binding activities and 8.3% for transporter activities (Fig.2)

For cellular compartmentalization, 3,268 transcripts were assigned ontology. Overall, 12.5% of transcripts were localized to the ribosome, 12.2 % in chloroplast, 10.5 % in plasma membrane, 8.6 % in cytosol, 8.5% in mitochondria, 6.8% in Golgi apparatus, 5.5% in extracellular region, and 5.5% in cytoskeleton. (Fig 3).

Altogether, most of the expressed transcripts among these three principal gene ontologies were distributed into the categories of CCO, CMP, nucleotide binding, kinase and DNA binding activities, ribosome, chloroplast, cytosol, and mitochondria. These functional processes are likely majorly involved in typical *P. tremuloides* gene activities.

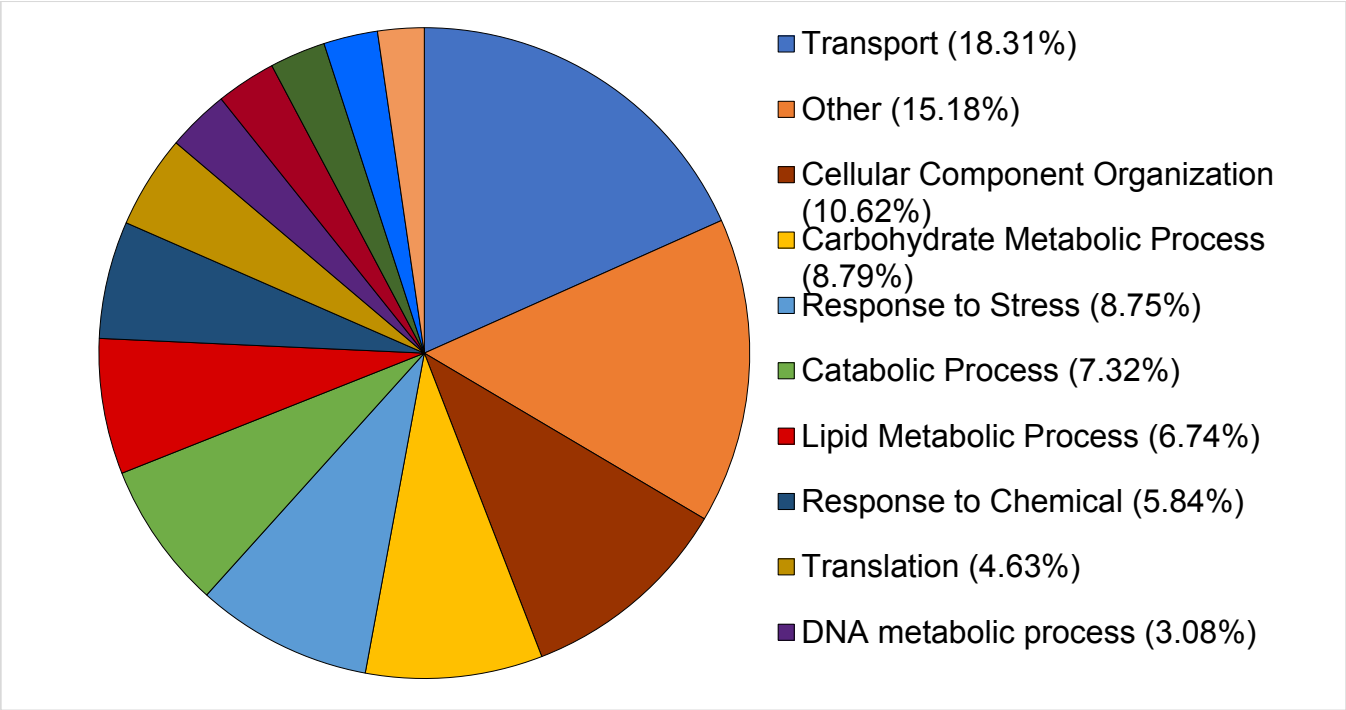

Fig 1. Percentage of transcripts in *Populus tremuloides* control samples. A total of 9766 transcripts were assigned ontology and grouped by biological processes using BLAST2GO. Categories under 2% were grouped together and classified as "other".

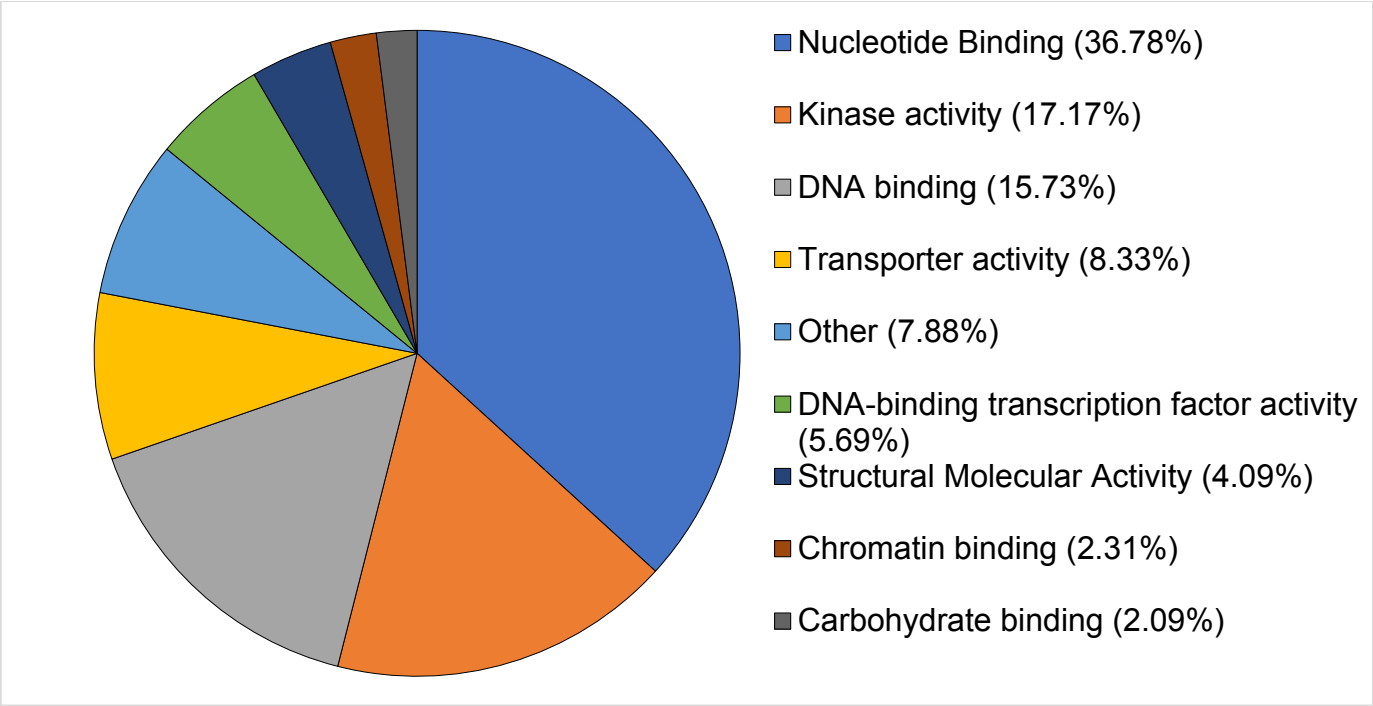

Fig 2. Percentage of transcripts in *Populus tremuloides* control samples. A total of 9953 transcripts were assigned ontology and grouped by molecular function used BLAST2GO. Categories under 2% were grouped together and classified as "other".

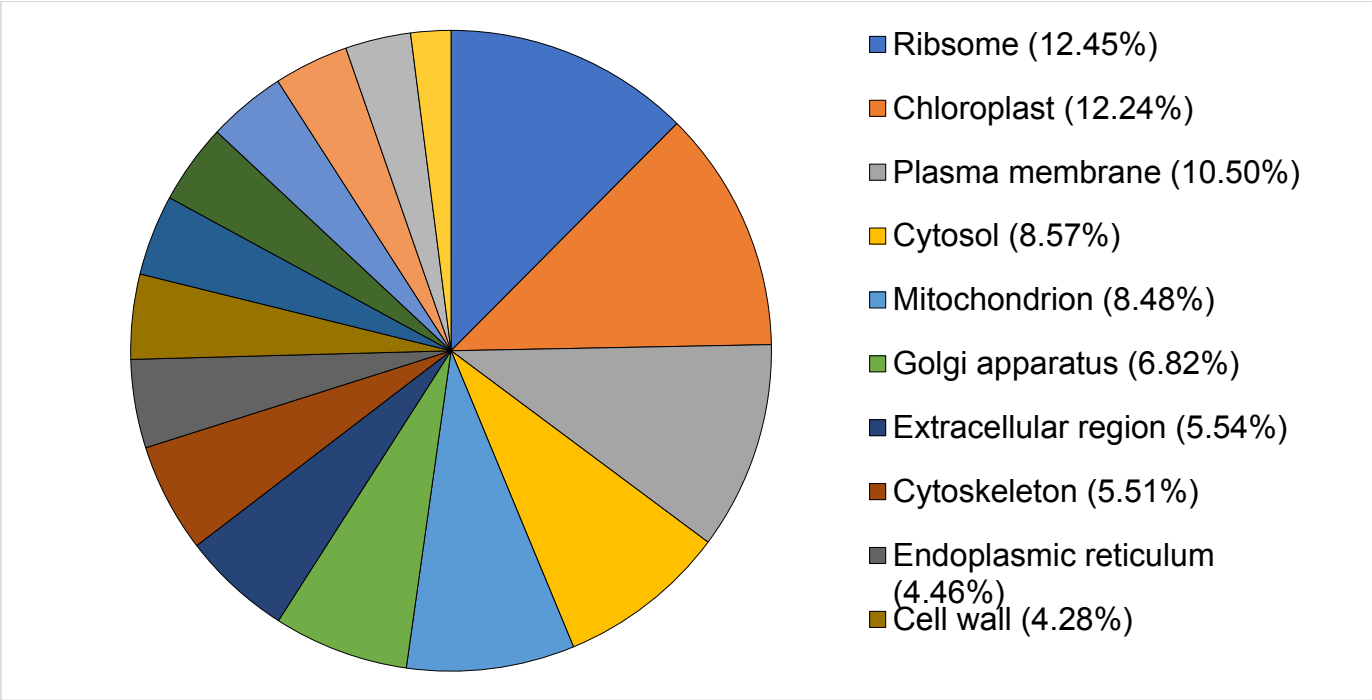

Fig 3. Percentage of transcripts in Populus tremuloides control samples. A total of 3268 transcripts were assigned ontology and grouped by cellular compartment using BLAST2GO. Categories under 2% were grouped together and classified as “other”.

2.3.4. Differentially Expressed Gene analysis

A total of 36,770 genes from the list of 52,987 genes were chosen as expressed for DEG analysis. These met the cut-off of ≥ 10 raw gene counts in at least three samples. The average expression values of these genes were compared in nickel resistant and nickel susceptible genotypes. With the high stringency (two-fold change, FDR value ≤ 0.05 and logFC value ≥1 (upregulated) or ≤ -1 (downregulated), 2,128 genes were upregulated, and 762 genes were downregulated in nickel resistant genotypes. With the low stringency filter (two-fold change and p-value ≤ 0.01), 3,070 genes were upregulated, and 1,186 genes were downregulated. GSEA analysis for these up- and down-regulated genes from the high stringency conditions was performed and 721 gene sets out of 3422 were used for analysis after filtering for gene set size. From these gene sets, 575 were upregulated and 146 were downregulated in nickel resistant phenotypes compared to susceptible genotypes.

Overall, the top upregulated GO functions in resistant samples were involved with the kinesin complex, microtubule-based movement, and cell wall modification (Supplementary material: Appendix C). The top down-regulated functions were DNA integration, regulation of growth and negative regulation of programmed cell death (Supplementary material: Appendix D).

2.3.5. Pairwise comparison of resistant and susceptible genotypes

There were no significant differentially expressed genes (DEGs) when nickel-resistant genotypes were compared to the water control using the high stringency cut-off. Using these same statistical requirements, the nickel-susceptible treatment group did have significant differences in gene expression when compared to nickel-resistant or the water control. The list of differentially regulated genes found in the pairwise comparison of nickel-resistant and susceptible transcriptomes was filtered through the three principle GO categories (Figs 4-6).

There was a higher number of total upregulated transcripts over downregulated for all three GO functions. Upregulation indicates increased expression of genes in RGs and downregulation indicates genes with increased expression in SGs.

For biological process (Fig. 4), the highest percentages of upregulated transcripts were in the categories of translation (11.8 %), carbohydrate metabolic process (11.8 %), transport (11.3 %), cellular protein modification process (10.4 %), and catabolic process (8.0%). The highest percentages of downregulated transcripts were in the categories of cellular protein modification process (16.7 %), transport (13.4%), carbohydrate metabolic process (9.0 %), response to stress (8.0 %) and signal transduction (7.3 %). When comparing between up and downregulation, translation had a much higher percentage of transcripts that were upregulated than downregulated. Carbohydrate metabolic process also had more upregulated genes in RS genotypes. Transport, cellular protein modification process, lipid metabolic process and signal transduction all had a noticeably increased percentage of downregulated transcripts over upregulated.

For molecular function (Fig. 5), the highest percentage of upregulated transcripts were in the categories of nucleotide binding (25.8 %), protein binding (21.6 %), kinase activity (12.3 %), structural molecule activity (12.3 %) and DNA binding (9.0 %). Most downregulated transcripts coded for proteins involved in nucleotide binding (26.6 %), protein binding (23.2 %), DNA binding (14.6 %) and kinase activity (13.2 %). Almost all differentially expressed transcripts for structural molecule activity were upregulated (12.3 %), with only 0.24% of downregulated transcripts. DNA binding transcripts were more downregulated (14.6 %) than upregulated (9.0%).

For cellular compartment localization (Fig 6), almost a third of upregulated transcripts were in the ribosome (32.7 %). The categories with the next highest percentage of upregulated transcripts are extracellular region (10.7 %), chloroplast (9%), plasma membrane (8.0 %) and cytosol (7.7 %). The cellular compartment associated with downregulated transcripts was more variably distributed across categories. The highest percentages of downregulated transcripts in RG localized to the chloroplast (20.0 %), mitochondrion (16.4

%), and cytosol (10.9 %). Most or all differentially expressed transcripts identified in the thylakoid, peroxisome, endosome, and nucleoplasm were downregulated in RGs.

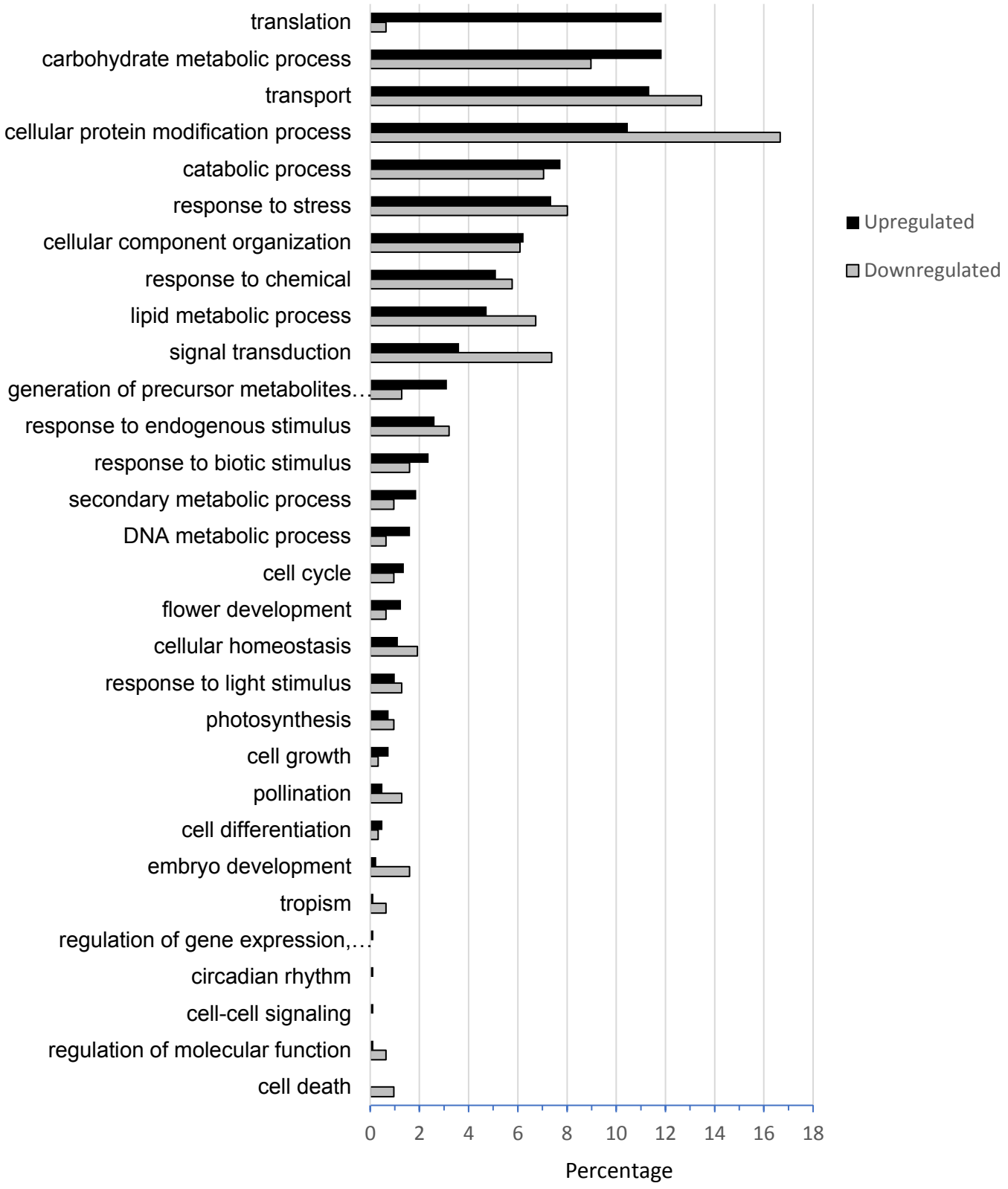

Fig 4. Percentage of upregulated (total number = 802) and downregulated (total number = 312) when nickel resistant *Populus tremuloides* genotypes were compared to nickel susceptible genotypes. Transcripts were assigned gene ontology and grouped by Biological Process using BLAST2GO (Omics Box).

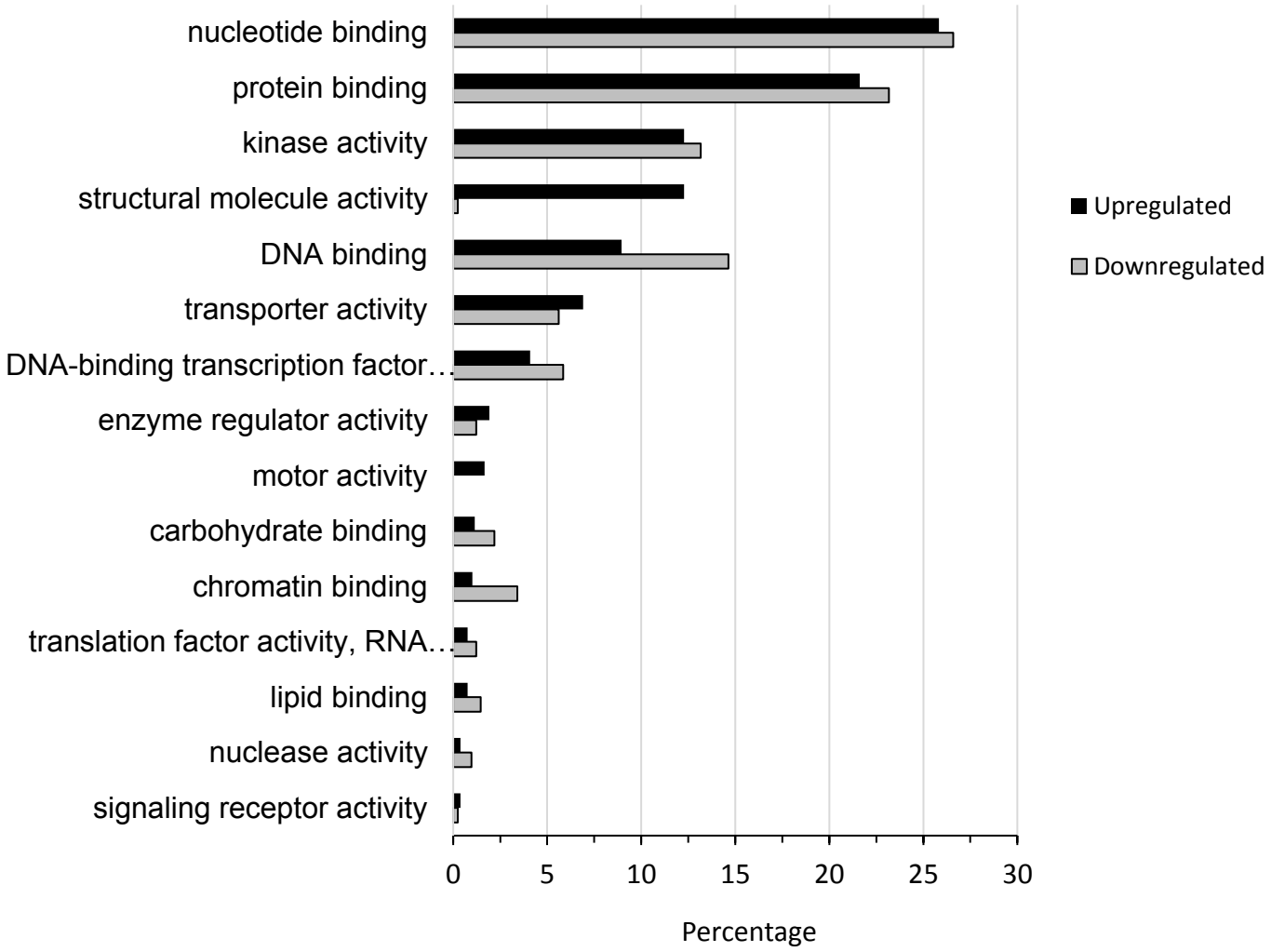

Fig 5. Percentage of upregulated (total number = 782) and downregulated (total number = 410) when nickel resistant *Populus tremuloides* genotypes were compared to nickel susceptible genotypes. Transcripts were assigned gene ontology and grouped by Molecular Function using BLAST2GO (Omics Box).

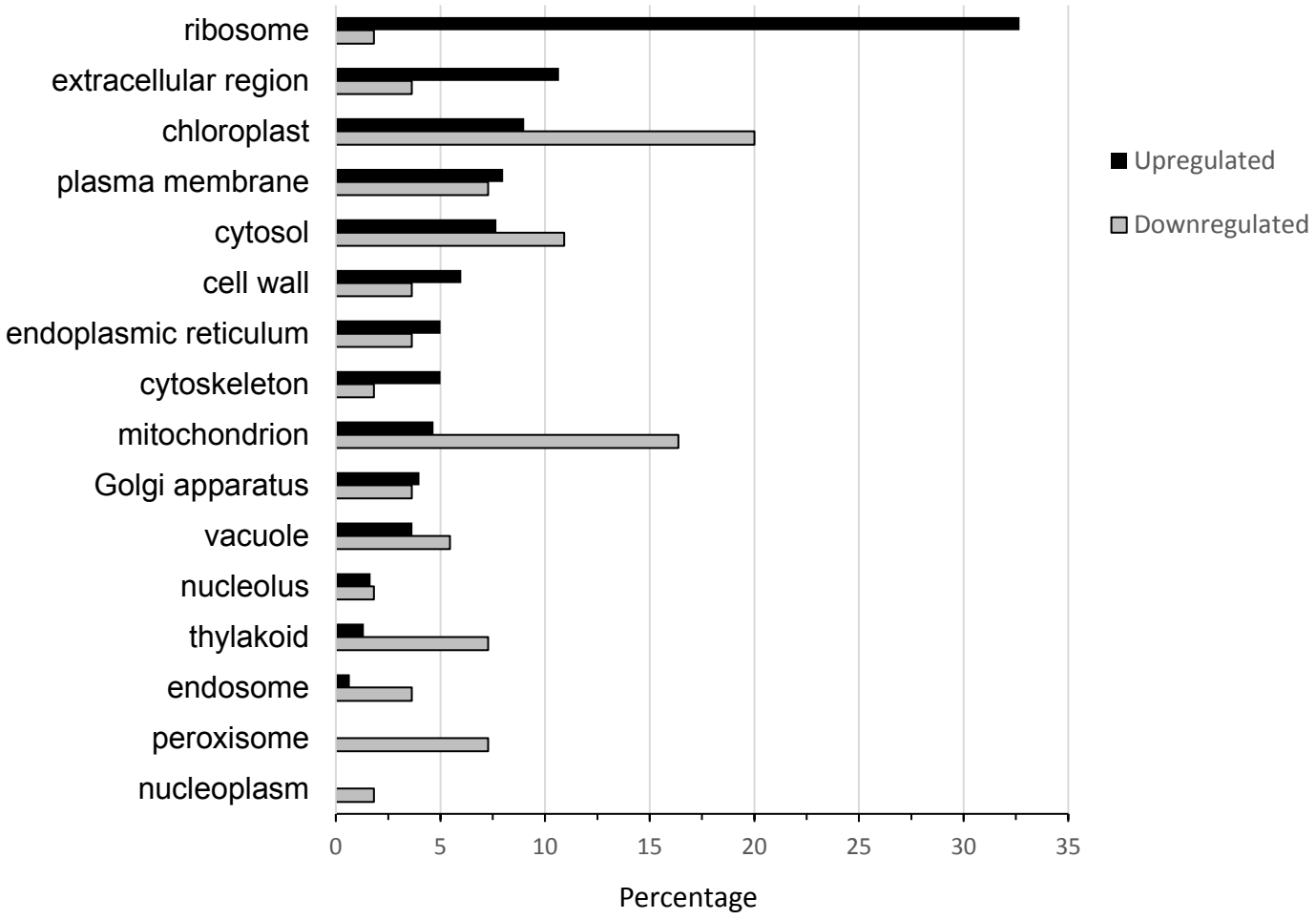

Fig 6. Percentage of upregulated (total number = 300) and downregulated (total number = 55) when nickel resistant *Populus tremuloides* genotypes were compared to nickel susceptible genotypes. Transcripts were assigned gene ontology and grouped by Cellular Component using BLAST2GO (Omics Box).

2.3.6. Characterization of highly differentially expressed genes

The heatmap in figure 7 displays the patterns of gene expression for the top 50 differentially expressed genes (DEGs) when resistant genotypes (RGs) were compared to susceptible genotypes (SGs). Genes were ranked based on LogFC expression values and the list of the top 50 DEGs is shown in Table 2. The majority of top DEGs were upregulated in RGs (n = 34). Most of the downregulated genes (n = 16) lack assigned gene ontology and description. This list of 50 genes was filtered through the three principal gene ontology categories to analyze the distribution of transcript functions.

For Biological process, most categories consisted of only upregulated or downregulated genes (Fig. 8). The exception is in biosynthetic process with three upregulated and two downregulated genes, and carbohydrate metabolic process with one upregulated and one downregulated gene. Other upregulated genes were associated with response to stress (3 genes), transport, response to chemical, signal transduction (2 genes each), response to endogenous stimulus, response to external stimulus, cellular component organization, DNA metabolic process and catabolic process (1 gene each). There was one downregulated gene each in secondary metabolic process, generation of precursor metabolites and energy, regulation of molecular function, lipid metabolic process and nucleobase-containing compound metabolic process.

For molecular function, the highest percentage of upregulated genes are involved in protein binding with four identified genes (Fig. 9). Hydrolase activity and DNA binding each had three upregulated genes. Nucleotide binding, enzyme regulator activity and DNA-binding transcription factor activity each have one upregulated gene. There were only four downregulated genes with annotations, and they were distributed evenly within protein binding, DNA binding, hydrolase activity and DNA-binding transcription factor activity.

There was limited gene ontology info for cellular component (Fig. 10). From the eight upregulated genes identified, three were localized to the nucleus, two to extracellular region, two to cell membrane and one to cell wall. There were no downregulated genes with cellular component data.

The Top 30 upregulated and downregulated genes identified in resistant genotypes are displayed in Table 3 and 4 respectively. Specifically, some of the highly upregulated genes in RG were characterized as Dirigent protein 10, GATA transcription factor, RuBisCO reductase (chloroplastic), Zinc finger protein, Cationic peroxidase 2, Probable mannitol dehydrogenase, Flavin-dependent oxidoreductase, Auxin response factor, Bidirectional sugar transporter, thiamine thiazole synthase, and DNA3-methyladenine.

One gene of interest is the copper transport protein found to be upregulated in resistant genotypes. Annotation information for the top downregulated genes in RG is limited but includes a DnaJ chaperon protein, phosphomethylpyrmidine synthase (chloroplastic), and leucine zipper protein.

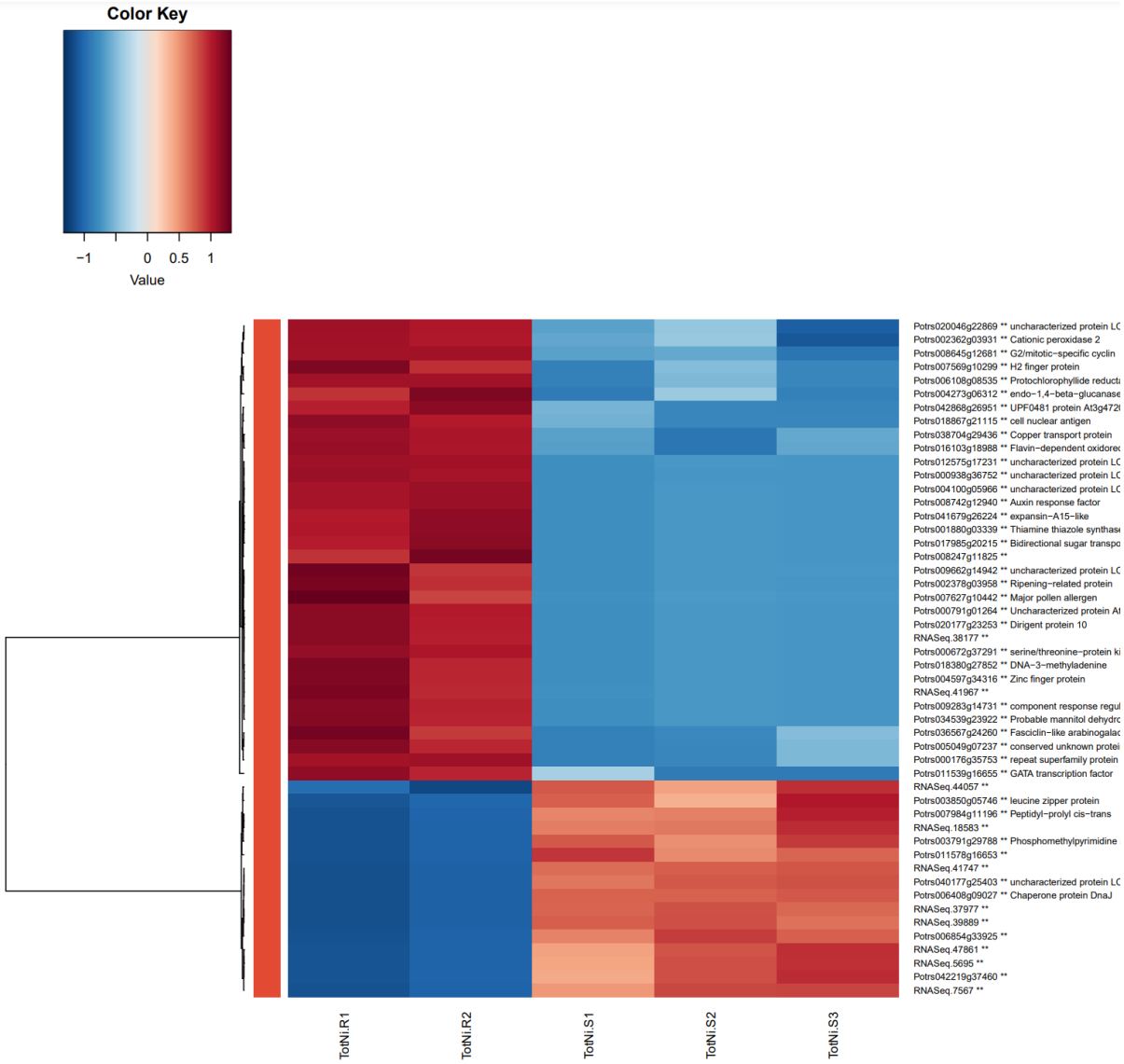

Figure 7. Top 50 differentially expressed genes between nickel-treated resistant and susceptible *Populus tremuloides* based on LogFC. The red colour represents an upregulation and downregulation is blue.

Table 2: Top 50 differentially expressed genes (up or downregulated) in resistant trembling aspen (*Populus tremuloides*) genotypes compared to susceptible trembling aspen genotypes based on LogFC

		Plants (RPKM)							
Rank	**geneID**	**Res. 1**	**Res. 2**	**Sus. 1**	**Sus. 2**	**Sus. 3**	**logFC**	**adj.P.Val**	**Description**
1	RNASeq.41967	179.84	62.86	0.00	0.00	0.00	10.25	0.0205	
2	Potrs000938g36752	54.47	45.35	0.00	0.00	0.00	10.05	0.0205	uncharacterized protein LOC105142721 isoform
3	Potrs018867g21115	40.42	24.44	0.05	0.00	0.00	9.72	0.0205	cell nuclear antigen
4	Potrs020177g23253	21.77	12.05	0.00	0.00	0.00	9.65	0.0205	Dirigent protein 10
5	Potrs011539g16655	31.36	16.73	0.11	0.00	0.00	9.62	0.0208	GATA transcription factor
6	Potrs006108g08535	15.46	15.95	0.00	0.03	0.00	9.54	0.0205	Protochlorophyllide reductase, chloroplastic
7	Potrs004597g34316	13.50	5.75	0.00	0.00	0.00	9.51	0.0205	Zinc finger protein
8	Potrs000176g35753	9.08	9.96	0.00	0.00	0.02	9.49	0.0205	repeat superfamily protein isoform
9	Potrs002362g03931	50.73	41.35	0.08	0.19	0.00	9.48	0.0206	Cationic peroxidase 2
10	Potrs034539g23922	13.90	6.37	0.00	0.00	0.00	9.47	0.0205	Probable mannitol dehydrogenase
11	Potrs005049g07237	18.97	8.64	0.00	0.00	0.03	9.44	0.0205	conserved unknown protein
12	Potrs007627g10442	31.58	5.97	0.00	0.00	0.00	9.30	0.0216	Major pollen allergen
13	Potrs016103g18988	19.71	15.10	0.03	0.00	0.03	9.28	0.0205	Flavin-dependent oxidoreductase
14	RNASeq.38177	62.98	33.40	0.00	0.00	0.00	9.27	0.0205	
15	Potrs008742g12940	3.22	3.88	0.00	0.00	0.00	9.26	0.0205	Auxin response factor
16	Potrs004100g05966	12.75	15.25	0.00	0.00	0.00	9.25	0.0205	uncharacterized protein LOC105127813 isoform
17	Potrs000791g01264	11.80	7.13	0.00	0.00	0.00	9.25	0.0205	Uncharacterized protein At3g61260
18	Potrs017985g20215	8.34	15.81	0.00	0.00	0.00	9.19	0.0205	Bidirectional sugar transporter
19	Potrs001880g03339	6.54	11.01	0.00	0.00	0.00	9.19	0.0205	Thiamine thiazole synthase
20	Potrs008247g11825	12.45	43.60	0.00	0.00	0.00	9.18	0.0206	
21	Potrs041679g26224	7.36	12.51	0.00	0.00	0.00	9.18	0.0205	expansin-A15-like
22	Potrs036567g24260	28.65	7.94	0.00	0.00	0.04	9.18	0.0224	Fasciclin-like arabinogalactan protein
23	Potrs009283g14731	6.60	3.29	0.00	0.00	0.00	9.18	0.0205	component response regulator
24	Potrs038704g29436	10.28	8.62	0.01	0.00	0.02	9.15	0.0205	Copper transport protein
25	Potrs020046g22869	48.15	40.56	0.08	0.19	0.00	9.15	0.0205	uncharacterized protein LOC105122685
26	Potrs002378g03958	33.57	11.70	0.00	0.00	0.00	9.12	0.0205	Ripening-related protein
27	Potrs000672g37291	13.88	10.00	0.00	0.00	0.00	9.09	0.0205	serine/threonine-protein kinase yrzF
28	Potrs012575g17231	7.95	7.88	0.00	0.00	0.00	9.08	0.0205	uncharacterized protein LOC105142618
29	Potrs009662g14942	12.67	3.63	0.00	0.00	0.00	9.08	0.0205	uncharacterized protein LOC105136392
30	Potrs004273g06312	5.97	18.58	0.00	0.05	0.00	9.06	0.0244	endo-1,4-beta-glucanase
31	Potrs042868g26951	6.37	12.07	0.02	0.00	0.00	9.04	0.0205	UPF0481 protein At3g47200
32	Potrs007569g10299	54.17	20.11	0.00	0.10	0.00	9.03	0.0206	H2 finger protein
33	Potrs018380g27852	5.81	2.48	0.00	0.00	0.00	9.03	0.0205	DNA-3-methyladenine
34	Potrs008645g12681	10.10	10.89	0.02	0.02	0.00	9.00	0.0205	G2/mitotic-specific cyclin
35	RNASeq.7567	0.00	0.00	15.24	51.49	51.02	-8.99	0.0216	
36	RNASeq.41747	0.00	0.00	37.07	47.26	50.81	-9.01	0.0205	
37	Potrs006408g09027	0.00	0.00	25.59	25.82	28.22	-9.02	0.0205	Chaperone protein DnaJ
38	Potrs040177g25403	0.00	0.00	9.97	15.87	17.04	-9.03	0.0205	uncharacterized protein LOC105115473
39	Potrs007984g11196	0.00	0.00	7.02	7.41	29.68	-9.17	0.0231	Peptidyl-prolyl cis-trans
40	Potrs003791g29788	0.00	0.00	3.57	1.59	5.28	-9.30	0.0206	Phosphomethylpyrimidine synthase, chloroplastic
41	RNASeq.44057	0.12	0.00	86.30	28.90	157.76	-9.32	0.0407	
42	Potrs003850g05746	0.00	0.00	10.75	3.01	31.48	-9.33	0.0347	leucine zipper protein
43	Potrs006854g33925	0.00	0.00	29.96	86.88	48.55	-9.36	0.0205	
44	RNASeq.18583	0.00	0.00	37.90	41.03	125.39	-9.42	0.0215	
45	RNASeq.47861	0.00	0.00	24.27	86.94	146.15	-9.63	0.0321	
46	RNASeq.5695	0.00	0.00	5.71	22.67	34.63	-9.64	0.0335	
47	RNASeq.37977	0.00	0.00	42.63	57.91	39.58	-9.67	0.0205	
48	Potrs042219g37460	0.00	0.00	32.95	151.79	246.68	-9.74	0.0423	
49	RNASeq.39889	0.00	0.00	23.90	30.91	16.71	-10.12	0.0205	
50	Potrs011578g16653	0.00	0.00	55.19	14.19	25.08	-10.14	0.0231	

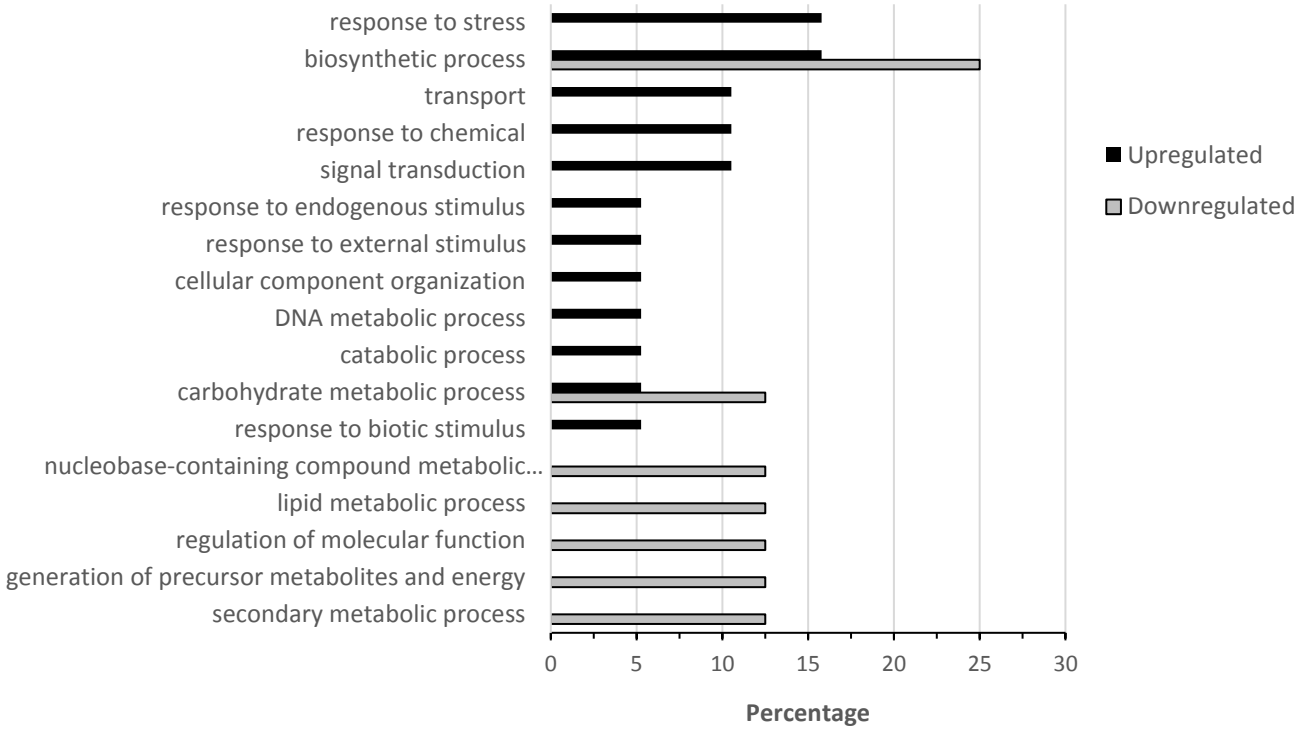

Fig 8. Percentage of upregulated (n= 19) and downregulated (n= 8) transcripts from the Top 50 differentially expressed genes (DEGs) when *Populus Tremuloides* with nickel resistant genotypes were compared to nickel susceptible genotypes. Only transcripts with assigned gene ontology were included and these were grouped by biological process using BLAST2GO (Omics Box).

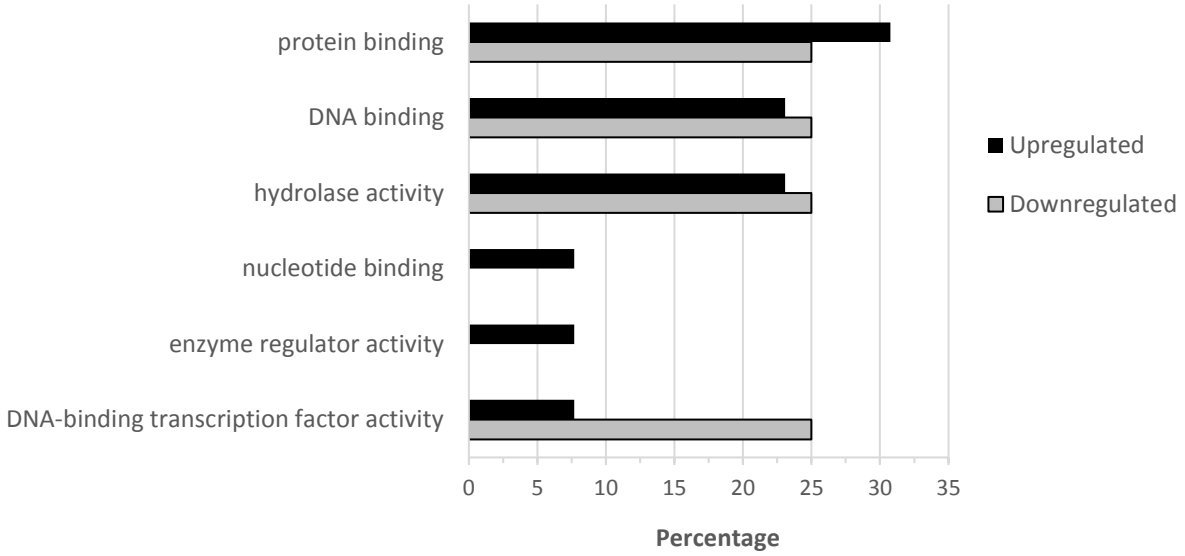

Fig 9. Percentage of upregulated (n= 13) and downregulated (n= 4) transcripts from the Top 50 differentially expressed genes (DEGs) when *Populus Tremuloides* nickel resistant genotypes were compared to nickel susceptible genotypes. Only transcripts with assigned gene ontology were included and these were grouped by molecular function using BLAST2GO (Omics Box).

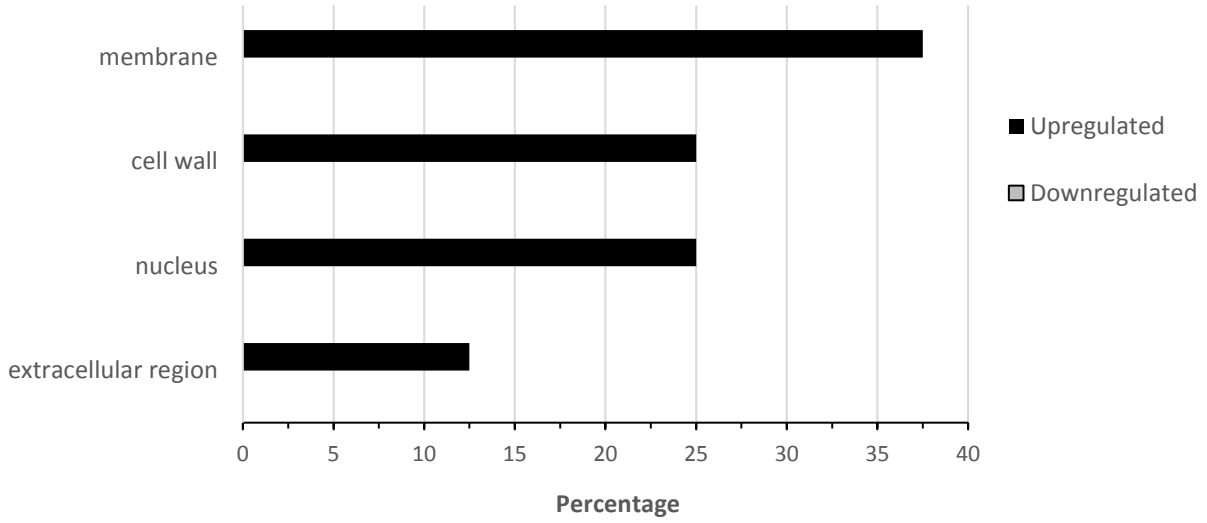

Fig 10. Percentage of upregulated (n= 8) and downregulated (n= 0) transcripts from the Top 50 differentially expressed genes (DEGs) when nickel resistant *Populus Tremuloides* genotypes were compared to nickel susceptible genotypes. Only transcripts with assigned gene ontology were included and these were grouped by cellular component using BLAST2GO (Omics Box).

Table 3: Top 30 upregulated transcripts in resistant trembling aspen (*Populus tremuloides*) genotypes compared to susceptible trembling aspen genotypes based on LogFC.

Rank	geneID	Plants (RPKM)					logFC	adj.P.Val	Description
		Res. 1	Res. 2	Sus. 1	Sus. 2	Sus. 3			
1	RNASeq.41967	179.84	62.86	0.00	0.00	0.00	10.25	0.0205	
2	Potrs000938g36752	54.47	45.35	0.00	0.00	0.00	10.05	0.0205	uncharacterized protein LOC105142721 isoform
3	Potrs018867g21115	40.42	24.44	0.05	0.00	0.00	9.72	0.0205	cell nuclear antigen
4	RNASeq.9446	87.68	11.05	0.00	0.00	0.00	9.72	0.0300	
5	Potrs020177g23253	21.77	12.05	0.00	0.00	0.00	9.65	0.0205	Dirigent protein 10
6	Potrs011539g16655	31.36	16.73	0.11	0.00	0.00	9.62	0.0208	GATA transcription factor
7	Potrs006108g08535	15.46	15.95	0.00	0.03	0.00	9.54	0.0205	Protochlorophyllide reductase, chloroplastic
8	Potrs004597g34316	13.50	5.75	0.00	0.00	0.00	9.51	0.0205	Zinc finger protein
9	Potrs000176g35753	9.08	9.96	0.00	0.00	0.02	9.49	0.0205	repeat superfamily protein isoform
10	Potrs002362g03931	50.73	41.35	0.08	0.19	0.00	9.48	0.0206	Cationic peroxidase 2
11	Potrs034539g23922	13.90	6.37	0.00	0.00	0.00	9.47	0.0205	Probable mannitol dehydrogenase
12	Potrs005049g07237	18.97	8.64	0.00	0.00	0.03	9.44	0.0205	conserved unknown protein
13	Potrs007627g10442	31.58	5.97	0.00	0.00	0.00	9.30	0.0216	Major pollen allergen
14	Potrs016103g18988	19.71	15.10	0.03	0.00	0.03	9.28	0.0205	Flavin-dependent oxidoreductase
15	RNASeq.38177	62.98	33.40	0.00	0.00	0.00	9.27	0.0205	
16	Potrs008742g12940	3.22	3.88	0.00	0.00	0.00	9.26	0.0205	Auxin response factor
17	Potrs004100g05966	12.75	15.25	0.00	0.00	0.00	9.25	0.0205	uncharacterized protein LOC105127813 isoform
18	Potrs000791g01264	11.80	7.13	0.00	0.00	0.00	9.25	0.0205	Uncharacterized protein At3g61260
19	Potrs017985g20215	8.34	15.81	0.00	0.00	0.00	9.19	0.0205	Bidirectional sugar transporter
20	Potrs001880g03339	6.54	11.01	0.00	0.00	0.00	9.19	0.0205	Thiamine thiazole synthase
21	Potrs008247g11825	12.45	43.60	0.00	0.00	0.00	9.18	0.0206	
22	Potrs041679g26224	7.36	12.51	0.00	0.00	0.00	9.18	0.0205	expansin-A15-like
23	Potrs036567g24260	28.65	7.94	0.00	0.00	0.04	9.18	0.0224	Fasciclin-like arabinogalactan protein
24	Potrs009283g14731	6.60	3.29	0.00	0.00	0.00	9.18	0.0205	component response regulator
25	Potrs038704g29436	10.28	8.62	0.01	0.00	0.02	9.15	0.0205	Copper transport protein
26	Potrs020046g22869	48.15	40.56	0.08	0.19	0.00	9.15	0.0205	uncharacterized protein LOC105122685
27	Potrs002378g03958	33.57	11.70	0.00	0.00	0.00	9.12	0.0205	Ripening-related protein
28	Potrs000672g37291	13.88	10.00	0.00	0.00	0.00	9.09	0.0205	serine/threonine-protein kinase yrzF
29	Potrs018426g20661	2.57	9.89	0.02	0.00	0.00	9.09	0.0320	ABC transporter G family member
30	Potrs012575g17231	7.95	7.88	0.00	0.00	0.00	9.08	0.0205	uncharacterized protein LOC105142618

Table 4: Top 30 downregulated transcripts in resistant trembling aspen (*Populus tremuloides*) genotypes compared to susceptible trembling aspen genotypes based on LogFC.

Rank	geneID	Plants (RPKM)					logFC	adj.P.Val	Description
		Res. 1	Res. 2	Sus. 1	Sus. 2	Sus. 3			
1	Potrs011578g16653	0.00	0.00	55.19	14.19	25.08	-10.14	0.0231	Probable carboxylesterase 13
2	RNASeq.39889	0.00	0.00	23.90	30.91	16.71	-10.12	0.0205	
3	Potrs042219g37460	0.00	0.00	32.95	151.79	246.68	-9.74	0.0423	
4	RNASeq.37977	0.00	0.00	42.63	57.91	39.58	-9.67	0.0205	
5	RNASeq.5695	0.00	0.00	5.71	22.67	34.63	-9.64	0.0335	
6	RNASeq.47861	0.00	0.00	24.27	86.94	146.15	-9.63	0.0321	
7	RNASeq.18583	0.00	0.00	37.90	41.03	125.39	-9.42	0.0215	
8	Potrs006854g33925	0.00	0.00	29.96	86.88	48.55	-9.36	0.0205	
9	Potrs003850g05746	0.00	0.00	10.75	3.01	31.48	-9.33	0.0347	leucine zipper protein
10	RNASeq.44057	0.12	0.00	86.30	28.90	157.76	-9.32	0.0407	
11	Potrs003791g29788	0.00	0.00	3.57	1.59	5.28	-9.30	0.0206	Phosphomethylpyrimidine synthase, chloroplastic
12	Potrs007984g11196	0.00	0.00	7.02	7.41	29.68	-9.17	0.0231	Peptidyl-prolyl cis-trans
13	Potrs040177g25403	0.00	0.00	9.97	15.87	17.04	-9.03	0.0205	uncharacterized protein LOC105115473
14	Potrs006408g09027	0.00	0.00	25.59	25.82	28.22	-9.02	0.0205	Chaperone protein DnaJ
15	RNASeq.41747	0.00	0.00	37.07	47.26	50.81	-9.01	0.0205	
16	RNASeq.7567	0.00	0.00	15.24	51.49	51.02	-8.99	0.0216	
17	Potrs020009g22834	0.00	0.00	10.82	8.76	16.94	-8.92	0.0205	Ethylene-responsive transcription
18	RNASeq.46539	0.00	0.00	27.28	41.15	16.84	-8.87	0.0205	
19	Potrs003649g29217	0.00	0.00	6.68	2.81	2.58	-8.81	0.0205	Serine carboxypeptidase-like
20	Potrs018399g35871	0.00	0.00	58.21	61.64	40.16	-8.75	0.0205	conserved unknown protein
21	RNASeq.32308	0.00	0.00	17.54	27.64	27.53	-8.74	0.0205	
22	RNASeq.16418	0.00	0.00	1.66	17.69	19.69	-8.65	0.0450	
23	Potrs035791g35223	0.00	0.00	25.72	34.68	33.41	-8.51	0.0205	CLAVATA3/ESR (CLE)-related
24	Potrs006080g29018	0.00	0.00	4.69	16.17	12.69	-8.47	0.0212	uncharacterized protein LOC105114046
25	RNASeq.10673	0.17	0.00	21.62	83.12	33.01	-8.43	0.0321	
26	Potrs031907g32627	0.00	0.00	9.25	16.15	13.90	-8.41	0.0205	protein N-like
27	Potrs004143g06038	0.00	0.00	55.80	111.96	23.44	-8.39	0.0216	
28	Potrs010001g15129	0.00	0.00	8.17	14.93	5.21	-8.38	0.0206	Uncharacterized protein At4g04980
29	RNASeq.44603	0.00	0.00	7.17	36.39	21.20	-8.33	0.0231	
30	RNASeq.40672	0.00	0.00	4.71	12.60	44.76	-8.33	0.0300	

2.4. Discussion

Effect of nickel toxicity to trembling aspen seedlings-

No visible physical damage was observed in seedlings treated with the nitrate solution controls at low and high doses or with water suggesting that excess nitrate is not impacting plant health (Table 1). Furthermore, the significant increase in plant damage seen at the highest nickel dose of 1,600 mg/kg is likely due to the excess of nickel and not nitrate because the corresponding nitrate control dose did not cause any significant damage. Slight changes in damage appeared when Ni doses was increased from 150 mg/kg to 800 mg/kg. It appears that 1,600 mg/kg is the threshold for nickel concentration in soil where significant nickel toxicity symptoms appear in trembling aspen.

The 1,600 mg/kg nickel treatment had the widest range of damage among the samples tested. There were two moderately susceptible and seven susceptible plants, while three of the genotypes were classified as resistant because they had a damage rating of 1 or 3 at the end of the experiment (Supplementary Material: Appendix C). This supports the hypothesis that high genetic variability exists within *P. tremuloides* trees from metal uncontaminated or contaminated areas (Hamrick 1979; Kirkey *et al.* 2012). This type of variability was seen in a similar analysis of *Betula papyrifera* populations (Theriault *et al.* 2016a). This species is widespread in Northern Ontario and can survive in metal contaminated areas in the GSR. Like *Populus tremuloides,* this species has been found to accumulate nickel at high concentrations in the aerial parts of the plant (Kalubi *et al.* 2016; Theriault *et al.* 2016a). It suggests that at this threshold dose of bioavailable nickel, plant phenotypes begin to segregate possibly due to genetic variation in metal tolerance ability. Epigenetic differences among the plant population may also exist which can affect gene expression in response to the heavy metal stress and there are varying levels of plant tolerance as a result.

Differentialy Expressed Gene (DEG) analysis

Overall, there were more upregulated transcripts than downregulated transcripts in RG. This is likely a reflection of overall decreased functioning of the susceptible plants as they are physically dying from the high dose of nickel treatment. This could result in less gene expression in SG as indicated by the low number of upregulated transcripts in these samples (considered downregulated in RG).

The differences between resistant and susceptible genotypes were investigated using several methods. Gene set enrichment analysis (GSEA) found two different sets of GO terms that were enriched in either RG or SG (Supplementary Material: Appendix D and E). In resistant genotypes, the upregulated GO functions related to the kinesin complex, microtubule movement and cell-wall modification. Multiple studies associated microtubule and other cytoskeleton elements with abiotic stress (Nick 2013). It is suggested that these components may be involved in early signaling responses after exposure to a stressor like toxic concentration of heavy metals as seen in nickel-stressed and copper-stressed seagrass (*Cymodocea nodosa*) (Malea *et al.* 2013).

The activities associated with DNA integration, regulation of growth and negative regulation of programmed cell death were downregulated in resistant genotypes compared to susceptible samples. These are fundamental processes that may be upregulated in the susceptible plants because of the high amount of damage experienced due to the nickel treatment. Regulating growth and programmed cell death may be pathways through which the plant is attempting to cope with the heavy metal stress.

Pairwise comparison of resistant and susceptible trembling aspen genotypes.

The biological process categories of carbohydrate metabolism, cellular protein modification and transport were highly differentially regulated between the two genotypes. These categories were among the highest percentages of associated genes for both upregulated and downregulated transcripts. This suggests that these processes are very dynamic in their part of the stress responses that are induced by nickel. Also, structural

molecular activity was highly upregulated in resistant genotypes and almost no downregulated transcripts were detected. This may be related to the increased cell wall modification found in GSEA analysis for these samples. Modifications within the cell structures may be required for responding to the increase in absorbed nickel ions from the treated soil.

In terms of cellular location, ribosomal genes were more upregulated in nickel-resistant *P. tremuloides* when compared to susceptible samples. This is validated by the increase in translation gene activities for biological function of the DEGs seen in RGs. Interestingly, this similar trend was identified for nickel-resistant *B. papyrifera* genotypes but only when compared to the water control. When these RG were compared with nickel susceptible samples there was a downregulation of ribosomal activity and a decreased expression of binding and transporter genes (Theriault *et al.* 2016a).

Top Differentially expressed genes from the pairwise comparison of RG vs SG

The annotated GO terms assigned to the top 50 differentially expressed genes (DEGs) were used to create GO category graphs. For biological function, multiple categories associated with stress response including response to stress, chemical, endogenous stimulus and external stimulus were upregulated. This validates that a stress response was induced in the poplar plants at this high dose of nickel.

Some highly upregulated genes of interest in RG from the Top 30 (Table 3) were characterized as a Dirigent protein 10, GATA transcription factor, Zinc finger protein, Auxin response factor, Bidirectional sugar transporter, thiamine thiazole synthase and copper transport protein.

One gene family found in the top differentially expressed genes are dirigent (DIR) proteins. These genes appear to be highly responsive to biotic and abiotic stresses in plants (Moura *et al.* 2010; Thamil Arasan *et al.* 2013). The main function of DIR proteins is to mediate the production of lignans and lignins from monolignol plant phenols (Burlat *et al.* 2001). Under different types of abiotic and biotic stress, the lignin composition and content in plant tissues is also known to undergo changes (Moura *et al.* 2010). In the case of heavy metals, one study comparing zinc-treated non-accumulating *Arabidopsis thaliana* and

accumulating *Thlaspi caerulescens* plants found high expression of genes associated with biosynthesis of lignin in both. This expression was noticeably higher for the zinc hyperaccumulator species *T. caerulescens*, along with other related genes including dirigent proteins (van de Mortel *et al.* 2006). Despite having similar functions in general, there is variation in when and where dirigent proteins are expressed depending on the plant tissue and environmental conditions (Thamil Arasan *et al.* 2013). The specific protein found upregulated in this present study in resistant trembling aspen genotypes is dirigent protein 10. This protein appears to be mainly expressed in plant roots as seen in *Arabidopsis* (Kim *et al.* 2012) and water-stressed Chinese cabbage (Thamil Arasan *et al.* 2013). The high expression of this protein in nickel-resistant *P. tremuloides* roots tissue also corroborates this expression pattern. Furthermore, there is clear evidence of the major role of DIR10 in the formation of the Casparian strip (CS) in *Arabidosis* (Hosmani *et al.* 2013). CSs are shaped like rings distributed throughout the plant root endodermis in vascular plants. These cell-wall modifications form a tight junction composed of lignin and they are an important part of many plant processes particularly selective water and nutrient uptake (Hosmani *et al.* 2013). Notably, the DIR protein's primary role in cell wall metabolism (Paniagua *et al.* 2017) may be related to the enrichment of genes associated with cell-wall modification found in the nickel-resistant trembling aspen genotypes in GSEA analysis.

A Zinc finger protein (ZFP) was also identified in the highly upregulated genes for RG. There are many types of ZFPs with varying functions but in general they act as transcription factors (TFs) and can modulate gene expression (Han *et al.* 2014). The Cys2/His-2-type (C2H2) ZFP evidently plays a large regulatory role in stress responses including oxidative stress in plants (Han *et al.* 2020; do Amaral *et al.* 2016). However, in addition to C2H2 ZFPs, a variety of ZFPs could be involved in these pathways as seen in a comparative transcriptomics study of salt stressed-rice plants (do Amaral *et al.* 2016). They found 10 upregulated and 12 downregulated zinc finger genes in saline-tolerant plants including the C2H2 and C3HC4 types (do Amaral *et al.* 2016).

A GATA transcription factor was identified, and it is another type of zinc finger protein (Guo 2021). Numerous GATA TFs have been cited in literature for abiotic stress tolerance in plants. One example was the observation that when *OsGATA16* is upregulated, it improves cold tolerance in rice (Zhang *et al.* 2021). Stress-responsive genes in plants are classified as either regulatory of functional genes (Tran *et al.* 2010). Regulatory genes like the ZFP and GATA transcription factors seen in this study are often part of a network which regulates the gene expression of functional genes needed to produce the compounds that confer heavy metal stress tolerance (Singh *et al.* 2016).

Thiamine thiazole synthase is another enzyme that has been linked to abiotic stress. Its major role in thiamine biosynthesis and mitochondrial DNA damage protection is established but recent studies also suggest it may be upregulated in plants as part of the abiotic stress response (Li *et al.* 2016). This trend has been seen for different stressors including salinity, drought, flooding, and sugar deprivation (Ribeiro *et al.*, 2005; Rapala-Kozik *et al.* 2008; Rapala-Kozik *et al.* 2012). These biosynthesis genes may be upregulated to produce more thiamine, an antioxidant which can mitigate the oxidative stress induced by most abiotic stressors (Tunc-Ozdemir *et al.* 2009; Rapala-Kozik *et al.* 2012). For example, an increase in total thiamine compounds was detected in maize seedling leaves under different stress treatments and this was most pronounced in the oxidative stress treatment (Rapala-Kozik *et al.* 2012).

The bidirectional sugar transporter found in the top upregulated genes could be a component of the plant abiotic stress response via control of carbohydrate metabolism (Gupta and Kaur, 2005). Yamada *et al.* (2018) described the different roles of sugar transporters (ST) in the plant stress response and how they regulate the accumulation of sugars under various environmental stress conditions. A transcriptomic study of nickel-treated *Acer rubrum* also reported an upregulation of a sugar transporter in plants treated with a high nickel dose and suggested that it may be involved in carbon distribution (Nkongolo *et al.* 2018b).

An auxin response factor was also among the highly upregulated genes in resistant genotypes. Auxin's important role in various developmental processes is well known but it may also be involved in abiotic and

biotic stress response signaling pathways (Ghanashyam and Jain 2009). Auxin could also play a role in heavy metal tolerance mechanisms like avoidance by inducing morphological changes in roots as suggested by Nkongolo (*et al.* 2018a).

The Potrs038704g29436 transcript is the 26th top DEG (Table 2) and it has been identified in *Populus tremuloides* and annotated as a coding gene for a metal ion binding protein that is involved in directing the movement of different metal ions. GO ontology predicted this transcript is a copper transport protein but it can also bind to other metals. Trembling aspen is a known accumulator species (Kalubi *et al.* 2015), so this transport protein may be upregulated in the nickel-resistant genotypes to transport and sequester the excess heavy metal. A comparison of nickel content in the roots and leaves of the resistant and susceptible plants could elucidate if excess nickel is being accumulated in the plant's above-ground leaf tissues. Furthermore, RT-qpcr could be utilized to confirm the gene expression differences.

There was limited annotation information for the top 30 downregulated genes found in the resistant genotypes when compared to susceptible. As a result, is it more difficult to infer about the gene expression dynamics that could be suppressed in resistant genotypes or upregulated in susceptible genotypes. Some genes of interest from this group include a probable carboxylesterase 13 (1. Potrs011578g16653), leucine zipper protein (9. Potrs003850g05746) and FKBP-type peptidyl-prolyl cis-trans isomerase (12. Potrs007984g11196).

Carboxylesterases are common enzymes found within plant cells and they are part of regular metabolic activities and the defense response (Cao *et al.* 2019). The leucine zipper protein is in the same gene family as the Arabidopsis gene AT5G65310 which is involved in regulating abscisic acid mediated signaling. Abscisic acid (ABA) is a key signaling hormone that is important for modulating the plant's defense pathways in response to variety of abiotic stressors including heavy metal stress (Vishvakarma *et al.* 2017). Finally, the identified FKBP-type isomerase enzyme is related to the Arabidopsis gene AT3G25230 which been linked to some abiotic stressors like heat and cadmium.

2.5. Conclusion

This study provided data to further characterize and add to the draft *Populus tremuloides* transcriptome. The high-throughput RNA-seq data generated 27-45 million paired-end reads per sample. After sequence alignment, two groups of transcripts consisting of 32,677 new isoforms and 17,349 novel transcripts respectively were added to the draft transcriptome. This improved sequencing data was used in the comparative transcriptomics analysis between nickel-resistant and nickel-susceptible trembling aspen genotypes. Overall, 36,770 genes were considered expressed among the samples and included in DEG analysis. From this list, 2,890 genes were found to be significantly differentially regulated between resistant and susceptible genotypes.

From a whole transcriptome level, an upregulation in ribosomal and translation activities was identified in the nickel-resistant plants. A gene from the top 50 differentially expressed group that encodes a metal binding transport protein was identified as a candidate gene for nickel resistance in trembling aspen. Other highly upregulated genes that are associated with many aspects of the abiotic stress response were identified in resistant genotypes including Dirigent protein 10, ZFP and GATA transcription factors, thiamine thiazole synthase, auxin response factor and a bidirectional sugar transporter.

Overall, next-gen RNA sequencing was a useful method to further elucidate the whole transcriptome in *Populus tremuloides* root tissues. It also enabled the comparison of resistant and susceptible heavy metal-stressed plants and identified new gene targets associated with the potential underlying nickel resistance and stress response mechanisms used by trembling aspen (*P. tremuloides*).

Chapter 3: Whole genome expression analysis of *Populus tremuloides* genotypes exposed to increasing doses of nickel.

3.1. Introduction

The effects of any stressor on an organism will vary based on many factors including the concentration of the substance. Consequently, different strategies may be used by the organism based on the parameters of the stressor. Since plants are immobile, they do not have the option of physically removing themselves from a threatening environment. Instead, they have evolved specific mechanisms and stress responses to cope with the different environmental stressors they may encounter. Stress response pathways can be induced by biotic or abiotic stress and trigger changes in gene expression to modulate the necessary production of proteins and other molecules that are part of the response. Prolonged chronic stress or very acute levels of stress will require different levels of adaptation than a stressor that may be minor or short-term. These adaptions could be achieved through natural genetic variation and selection or an ability to produce an adequate epigenetic response.

In the case of heavy metals like nickel, they are micronutrients that are essential in small concentrations for optimal plant health but in excess they can cause toxicity symptoms. Therefore, the gene expression required to utilize a small amount of absorbed nickel ions for the proper functioning of numerous proteins will likely be different than the mechanisms that are triggered due to nickel toxicity. For instance, trembling aspen can accumulate excess metal ions in their leaf tissues (Kalubi *et al.* 2015), so genes involved in metal transport and sequestration could be upregulated once a certain threshold of heavy metal stress is reached. This present study aims to 1) Assess gene expression dynamics in *P. tremuloides* seedlings treated with varying concentrations of nickel salts and, 2) Compare gene expression profiles among the different treatment groups to better characterize the plant's response to nickel at varying concentrations.

3.2. Materials and Methods

Populus tremuloides seedlings were grown in growth chambers as described in section 2.2.1. Different doses of nickel nitrate including 150 mg / kg, 800 mg / kg, and 1,600 mg / kg were used to treat six-month old seedlings. Potassium nitrate was used as a salt control and water was used as a baseline refrence. The experimental design was a completely randomized block with 11 replicates per nickel treatment and water control and 5 replicates per nitrate control.

RNA Extraction, RNA-seq and transcript alignment with a draft genome, Gene Expression and Sampling QC were similar to the description in sections 2.2.3, 2.2.4, and 2.2.5, respectively.

3.2.1. Differentially Expressed Genes (DEG) analysis

The workflow and statistical requirements used for DEG analysis has been described in section 2.2.6.

All expressed genes from all experimental samples were used as the background. Each nickel treatment (1,600 mg/kg, 800 mg /kg, and 150 mg /kg) was compared with the water control to assess the effects of increasing dosage on gene expression. The annotated lists of all DEGs identified for each comparison were run through the Plant-Slim function and GO category graphs were created with the OmicsBox BLAST2GO program. The percentage of differentially expressed transcripts distributed within each of the three GO function categories (Biological Process, Molecular Function and Cellular Component) was calculated. K-mean clustering was performed to further analyze the patterns in differential gene expression across the three nickel concentrations and water. Heatmaps were created using all samples (excluding TotNi.R3) to analyze expression patterns. The top 30 upregulated and downregulated genes were also identified for each treatment comparison to water.

3.3. Results

3.3.1. Differentially Expressed Genes (DEG) analysis

No differentially expressed genes were identified using the standard statistical cutoff (two-fold change with FDR<=0.05). When the low stringency cutoff (two-fold change and p-value <=0.01) was used, significant differences in gene expression among treatment groups were detected. In general, there was an increase in number of differentially expressed genes as the nickel concentration increased when compared to water, but the result was not strictly linear (Figure 11). In all three nickel-treated groups, the number of downregulated genes was relatively high (439-600) with a slight increase as nickel concentration was increased. There is a larger difference in the total number of upregulated genes per treatment (123-560) and this number increases with the nickel dose increase. There was limited overlap of genes from these three comparisons (only 24 genes were differentially expressed in all three treatments). The Top 30 upregulated and downregulated genes for each treatment when compared to the water control were ranked (Tables 5-10).

Three disease resistance proteins (3. Potrs001038g01787; 6. RNASeq.43432; 10. Potrs015365g18630) were found highly upregulated in the plants treated with the bioavailable nickel dose (Table 5). There were a few highly downregulated disease resistance proteins (23. RNASeq.42840; 30. RNASeq.26969) found in this treatment comparison as well (Table 6). A calcium-binding protein was also found to be upregulated (24. Potrs006099g08528).

Notable upregulated genes found in the half-nickel dose when it was compared to water include a Late Embryogensis Abundant (LEA) protein (1. Potrs007357g10204) and two calcium-binding proteins (8. Potrs006628g09162; 14. Potrs020191g23280) (Table 7). The calcium-binding Phospholipase D alpha protein (6. Potrs008635g12620) was downregulated for this comparison (Table 8).

For the highest (total) nickel dose, 7-deoxyloganetin glucosyltransferase was highly upregulated (19. Potrs019552g22004) (Table 9) while an auxin response factor (6. Potrs007626g10572), Flavonol 3-

sulfotransferase (7. Potrs031253g23611), and a predicted ABC transporter family protein (24. RNASeq.35112) were downregulated (Table 10).

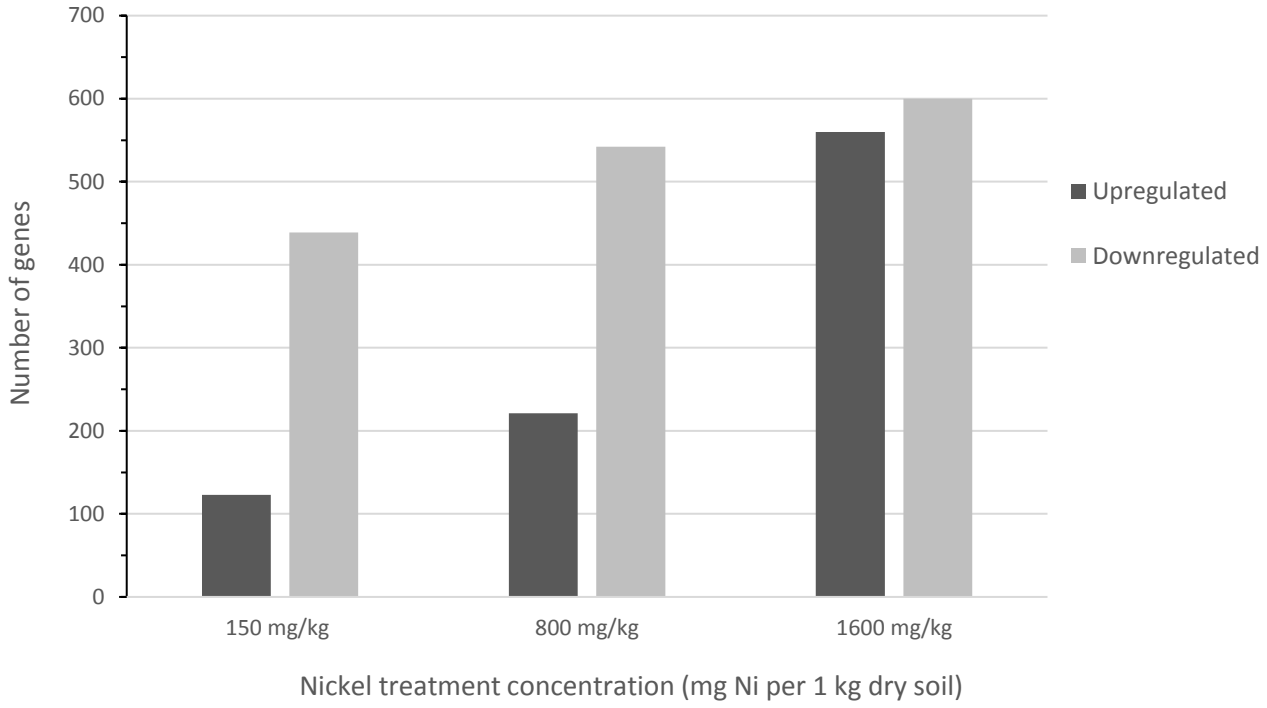

Figure 11: Number of differentially expressed genes in trembling aspen (*Populus tremuloides)* for each Nickel treatment concentration compared to water control. No differentially expressed genes were identified with the standard cutoff (two-fold change with FDR<=0.05). The graph values displayed are using a low stringent cutoff (two-fold change and p-value <=0.01).

Table 5: Top 30 upregulated in trembling aspen (*Populus tremuloides*) treated with the Bioavailable nickel dose (150 mg/kg) compared to the water control based on LogFC.

Rank	geneID	Plants (RPKM)						logFC	adj.P.Val	Description
		BioNi 1	BioNi 2	BioNi 3	Water 1	Water 2	Water 3			
1	Potrs008313g11887	22.93	78.48	132.67	0.00	0.00	0.00	7.90	0.3760	
2	RNASeq.27182	32.52	77.56	49.38	0.11	0.09	0.34	7.89	0.3760	
3	Potrs001038g01787	32.25	85.23	14.74	0.44	0.00	0.00	7.73	0.3760	Probable protein Pop3
4	Potrs009715g14974	4.60	2.60	12.50	0.03	0.00	0.02	7.68	0.3760	Dehydration-responsive element
5	Potrs041930g36215	2.28	1.97	11.99	0.00	0.00	0.00	7.54	0.3760	uncharacterized protein LOC105107450
6	RNASeq.43432	6.45	10.26	51.57	0.12	0.00	0.00	7.39	0.3807	TIR-NBS-LRR class disease resistance protein n=3 Tax=Populus RepID=A2I7Q5_9ROSI
7	Potrs042289g30386	1.47	1.38	5.35	0.00	0.00	0.00	7.32	0.3760	repeat-containing protein
8	Potrs149630g33024	3.06	8.24	6.75	0.00	0.00	0.00	7.11	0.3760	Cytochrome c biogenesis
9	Potrs035704g24090	4.61	6.44	5.05	0.00	0.00	0.00	7.01	0.3760	uncharacterized protein LOC105136599
10	Potrs015365g18630	2.86	1.13	1.68	0.00	0.00	0.00	6.98	0.3760	Disease resistance protein
11	Potrs018027g30293	11.72	2.93	10.23	0.37	0.02	0.01	6.95	0.5402	uncharacterized protein LOC105140158
12	RNASeq.18981	5.03	6.21	7.10	0.00	0.00	0.00	6.95	0.3760	
13	RNASeq.15485	2.88	6.60	3.05	0.00	0.04	0.00	6.92	0.3760	
14	RNASeq.14002	2.32	3.01	2.91	0.00	0.00	0.00	6.79	0.3760	
15	Potrs011062g16139	1.66	1.34	1.96	0.00	0.00	0.00	6.78	0.3760	Thylakoid membrane protein
16	RNASeq.30467	15.81	6.82	2.83	0.19	0.00	0.00	6.69	0.3815	
17	Potrs034354g23898	8.72	48.11	37.87	0.37	0.11	0.10	6.68	0.5168	Tropinone reductase homolog
18	RNASeq.45713	2.45	2.83	7.24	0.00	0.00	0.00	6.58	0.3760	
19	RNASeq.34645	7.29	9.87	6.68	0.36	0.00	0.07	6.46	0.3760	
20	RNASeq.1039	3.25	1.73	7.15	0.00	0.00	0.00	6.35	0.3760	
21	Potrs009100g34368	0.97	4.07	4.85	0.05	0.00	0.00	6.35	0.4181	Non-classical arabinogalactan protein
22	RNASeq.36670	6.46	10.62	18.51	0.00	0.09	0.25	6.31	0.3760	MATE efflux family protein n=4 Tax=Populus RepID=U7E321_POPTR
23	Potrs006099g08528	3.38	0.93	7.11	0.00	0.00	0.25	6.23	0.5022	Vacuolar cation/proton exchanger
24	Potrs011408g16550	1.96	4.74	5.54	0.00	0.00	0.13	6.23	0.4012	Prostaglandin reductase 1
25	Potrs038176g24656	6.23	8.61	4.01	1.86	0.00	0.00	6.20	0.5091	Aquaporin PIP1-1
26	RNASeq.31151	2.41	1.72	2.18	0.00	0.00	0.00	6.20	0.3760	
27	RNASeq.47353	1.33	5.91	4.41	0.00	0.00	0.23	6.17	0.4677	
28	Potrs012388g17095	0.29	1.01	0.88	0.00	0.00	0.00	6.12	0.4303	repeat-containing protein
29	Potrs164712g36901	37.06	75.50	10.37	0.51	0.44	0.00	6.12	0.4415	
30	RNASeq.3114	6.39	3.87	0.34	0.00	0.00	0.00	6.09	0.5104	Cyclic nucleotide-gated ion channel 20 family protein n=1 Tax=Populus trichocarpa RepID=B9I4X1_POPTR

Table 6: Top 30 downregulated in trembling aspen (*Populus tremuloides*) treated with the Bioavailable nickel dose (150 mg/kg) compared to the water control based on LogFC.

Rank	geneID	Plants (RPKM)						logFC	adj.P.Val	Description
		BioNi 1	BioNi 2	BioNi 3	Water 1	Water 2	Water 3			
1	RNASeq.36474	0.00	0.00	0.00	12.55	11.78	14.44	-8.34	0.3760	Mgatp-energized glutathione s-conjugate pump, putative n=1 Tax=Ricinus communis RepID=B9SKU1_RICCO
2	RNASeq.4577	0.00	0.00	0.00	6.02	5.56	4.03	-8.12	0.3760	
3	RNASeq.24901	0.00	0.13	0.14	31.68	24.60	37.22	-7.91	0.3760	
4	Potrs017183g19586	0.00	0.00	0.00	1.12	0.64	0.92	-7.79	0.3760	Long-chain-fatty
5	RNASeq.34079	0.00	0.00	0.00	5.67	16.63	28.91	-7.71	0.3760	
6	RNASeq.43161	0.00	0.00	0.00	10.90	19.80	12.65	-7.65	0.3760	
7	Potrs002847g04490	0.00	0.00	0.00	4.14	4.56	1.30	-7.56	0.3760	threonine-protein kinase
8	Potrs148340g27543	0.02	0.00	0.00	3.79	5.97	1.33	-7.56	0.3760	Anthranilate O-methyltransferase
9	RNASeq.21599	0.00	0.00	0.00	9.61	3.79	9.39	-7.51	0.3760	
10	Potrs147623g27327	0.00	0.00	0.00	2.26	2.85	2.15	-7.51	0.3760	Protein GAMETE EXPRESSED
11	RNASeq.44290	0.00	0.00	0.00	15.31	9.97	21.61	-7.48	0.3760	
12	Potrs008445g35684	0.00	0.00	0.00	14.88	19.58	6.01	-7.45	0.3760	related protein 45
13	Potrs039600g25163	0.00	0.05	0.00	1.42	3.46	5.54	-7.43	0.3760	uncharacterized protein LOC105133759 isoform
14	Potrs016831g34508	0.00	0.00	0.00	4.18	7.20	2.41	-7.42	0.3760	uncharacterized protein LOC105131492
15	RNASeq.42428	0.00	0.00	0.00	24.75	8.63	11.86	-7.40	0.3760	
16	RNASeq.37817	0.00	0.00	0.00	1.67	2.55	2.53	-7.37	0.3760	
17	Potrs004956g07144	0.00	0.00	0.00	1.07	1.13	0.93	-7.33	0.3760	Arf-GAP domain
18	Potrs002266g03830	0.00	0.00	0.00	0.60	8.70	5.34	-7.32	0.3760	uncharacterized protein LOC105107231 isoform
19	Potrs015656g34027	0.00	0.00	0.00	1.76	3.04	2.37	-7.31	0.3760	uncharacterized protein LOC105123045
20	Potrs014759g18336	0.02	0.00	0.00	1.45	4.10	4.02	-7.26	0.3760	Galactinol synthase 2
21	RNASeq.36742	0.00	7.21	0.00	56.90	31.03	19.62	-7.26	0.5104	
22	Potrs000382g00537	0.00	0.00	0.00	1.81	0.51	1.60	-7.21	0.3760	Copalyl diphosphate synthase
23	RNASeq.42840	0.00	0.00	0.00	10.96	16.54	3.38	-7.19	0.3760	Putative disease resistance gene NBS-LRR family protein n=1 Tax=Populus trichocarpa RepID=B9N945_POPTR
24	Potrs003578g05374	0.00	0.00	0.00	6.66	3.05	5.10	-7.18	0.3760	containing protein At2g01680-like isoform
25	Potrs015507g18688	0.00	0.00	0.00	1.33	7.55	2.72	-7.18	0.3760	uncharacterized protein LOC105124148
26	Potrs008906g36278	0.00	0.00	0.00	13.34	8.80	5.24	-7.17	0.3760	
27	RNASeq.45166	0.15	0.00	0.00	10.38	10.52	10.31	-7.09	0.3760	PREDICTED: LRR receptor-like serine/threonine-protein kinase GSO1 n=1 Tax=Populus euphratica RepID=UPI00057AC3DA
28	RNASeq.36836	0.00	0.00	0.05	3.30	4.20	4.51	-7.08	0.3760	
29	RNASeq.42663	0.00	0.00	0.00	9.24	19.95	2.15	-7.06	0.3760	PREDICTED: LOW QUALITY PROTEIN: protein SUPPRESSOR OF npr1-1, CONSTITUTIVE 1-like, partial n=1 Tax=Populus euphratica RepID=UPI00057B0870
30	RNASeq.26969	0.04	0.00	0.06	7.10	5.21	9.65	-7.06	0.3760	NBS-LRR type disease resistance protein n=1 Tax=Populus trichocarpa RepID=Q19PI8_POPTR

Table 7: Top 30 upregulated in trembling aspen (*Populus tremuloides*) treated with the Half-nickel dose (800 mg/kg) compared to the water control based on LogFC.

Rank	geneID	Plants (RPKM)						logFC	adj.P.Val	Description
		HalfNi 1	HalfNi 2	HalfNi 3	Water 1	Water 2	Water 3			
1	Potrs007357g10204	17.76	26.17	17.78	0.18	0.04	0.02	8.19	0.2491	embryogenesis abundant protein D-29
2	Potrs019743g22294	4.70	5.65	2.78	0.00	0.00	0.00	7.31	0.2491	Equilibrative nucleotide transporter
3	Potrs018638g20863	2.95	1.52	9.10	0.00	0.00	0.00	7.21	0.2813	uncharacterized protein LOC105113065
4	Potrs016761g19342	1.61	47.09	6.12	0.10	0.00	0.00	7.11	0.4099	
5	Potrs000442g00682	2.47	0.78	3.97	0.00	0.00	0.00	7.02	0.2813	Cyclin-dependent kinase
6	RNASeq.8094	2.46	7.57	4.59	0.00	0.00	0.00	6.98	0.2755	
7	RNASeq.20572	2.12	4.29	8.49	0.00	0.00	0.00	6.95	0.2755	
8	Potrs006628g09162	19.73	14.80	3.88	0.00	0.00	0.11	6.90	0.2911	Calcium-binding protein
9	RNASeq.45012	7.55	3.08	7.35	0.00	0.00	0.00	6.89	0.2755	
10	RNASeq.42242	7.68	6.32	31.38	0.15	0.00	0.00	6.84	0.3115	
11	Potrs042066g31207	7.24	13.57	19.69	0.00	0.00	0.14	6.82	0.2755	
12	RNASeq.42922	10.08	21.17	3.27	0.00	0.00	0.00	6.77	0.3069	
13	Potrs042066g31207	2.82	0.55	1.76	0.00	0.02	0.00	6.71	0.3115	Patatin-like protein
14	Potrs020191g23280	0.40	2.64	8.63	0.00	0.00	0.00	6.68	0.3600	sarcoplasmic reticulum histidine-rich calcium-binding protein
15	RNASeq.35744	3.08	5.66	3.33	0.00	0.33	0.00	6.66	0.3115	
16	Potrs003283g05031	2.08	0.38	1.75	0.00	0.00	0.01	6.66	0.3143	domain-containing membrane
17	Potrs013033g30694	0.94	0.32	2.83	0.00	0.00	0.00	6.64	0.3216	endo-1,3-beta
18	RNASeq.681	1.58	2.55	2.02	0.00	0.00	0.00	6.55	0.2806	
19	RNASeq.13962	7.18	11.59	13.97	0.18	0.00	0.00	6.54	0.2813	
20	RNASeq.44763	5.93	3.66	8.59	0.00	0.00	0.00	6.54	0.2882	
21	Potrs016434g19194	3.13	1.69	1.83	0.00	0.02	0.03	6.54	0.2755	Cytochrome P450 71B36
22	Potrs006895g29613	0.76	6.09	3.59	0.04	0.00	0.00	6.53	0.3451	Glutaredoxin-C4, chloroplastic
23	Potrs008849g13120	3.69	1.26	8.12	0.00	0.03	0.06	6.42	0.3533	methylene-furan-3-one reductase
24	Potrs149630g33024	4.24	1.23	6.15	0.00	0.00	0.00	6.37	0.3181	Cytochrome c biogenesis
25	Potrs040537g25588	0.72	1.66	2.92	0.00	0.00	0.02	6.35	0.3115	Galactinol synthase 2
26	RNASeq.7700	2.23	1.22	2.58	0.00	0.00	0.00	6.29	0.3115	
27	RNASeq.34630	0.65	1.03	5.47	0.00	0.00	0.00	6.27	0.3497	Concanavalin A-like lectin protein kinase family protein, putative n=1 Tax=Theobroma cacao RepID=A0A061E1Y7_THECC
28	Potrs010105g29188	5.68	3.46	11.80	0.00	0.00	0.00	6.25	0.3115	Uncharacterized protein At2g02148
29	RNASeq.28922	1.25	1.35	8.54	0.00	0.00	0.00	6.21	0.3497	PREDICTED: probably inactive leucine-rich repeat receptor-like protein kinase IMK2 isoform X1 n=1 Tax=Populus euphratica RepID=UPI000579E08F
30	Potrs019272g21579	2.44	2.25	3.28	0.00	0.00	0.12	6.21	0.3115	3-O-glucosyltransferase

Table 8: Top 30 downregulated in trembling aspen (*Populus tremuloides*) treated with the Half-nickel dose (800 mg/kg) compared to the water control based on LogFC.

Rank	geneID	Plants (RPKM)						logFC	adj.P.Val	Description
		HalfNi 1	HalfNi 2	HalfNi 3	Water 1	Water 2	Water 3			
1	Potrs010883g15885	0.00	0.00	0.03	12.10	13.10	5.09	-8.56	0.2491	Probable carboxylesterase 18
2	RNASeq.21791	0.00	0.00	0.00	19.87	15.75	11.34	-8.05	0.2491	
3	RNASeq.36370	0.00	0.00	0.00	22.32	5.30	17.47	-8.05	0.2491	PREDICTED: glycine-rich cell wall structural protein 1-like n=1 Tax=Populus euphratica RepID=UPI00057ADA65
4	Potrs000471g00757	0.00	0.00	0.00	0.97	6.84	2.72	-7.92	0.2755	GDSL esterase/lipase
5	RNASeq.37418	0.00	0.00	0.00	5.04	6.85	8.62	-7.84	0.2491	
6	Potrs008635g12620	0.00	0.00	0.00	2.85	1.12	1.04	-7.83	0.2491	Phospholipase D alpha
7	Potrs002847g04490	0.00	0.00	0.00	4.14	4.56	1.30	-7.75	0.2605	threonine-protein kinase
8	Potrs016200g19037	0.00	0.00	0.00	6.17	6.28	18.74	-7.68	0.2491	receptor-like protein
9	RNASeq.44290	0.00	0.00	0.00	15.31	9.97	21.61	-7.67	0.2491	
10	Potrs019521g21963	0.00	0.00	0.00	0.50	1.02	2.19	-7.62	0.2610	threonine-protein kinase
11	Potrs013736g30405	0.00	0.00	0.00	2.15	2.28	1.70	-7.59	0.2491	RuBisCO-associated protein
12	Potrs034098g23867	0.00	0.00	0.00	3.06	0.93	3.77	-7.45	0.2755	sericin 1-like
13	RNASeq.45927	0.00	0.00	0.00	5.54	3.80	3.11	-7.44	0.2491	Transporter associated with antigen processing protein 2 isoform 5 (Fragment) n=1 Tax=Theobroma cacao RepID=A0A061FYI3_THECC
14	Potrs008270g11884	0.00	0.00	0.00	0.62	1.48	0.99	-7.38	0.2491	receptor-like serine/threonine
15	Potrs011316g16416	0.00	1.15	0.00	21.40	9.26	5.22	-7.34	0.3967	Methionine aminopeptidase 2
16	Potrs015173g18492	0.00	0.00	0.00	1.79	1.38	0.47	-7.31	0.2755	Disease resistance protein
17	Potrs009541g33070	0.08	0.07	0.00	9.25	28.79	6.40	-7.23	0.2911	Disease resistance protein
18	Potrs009081g13927	0.00	0.00	0.01	2.30	1.05	1.18	-7.20	0.2610	IQ domain-containing protein
19	RNASeq.35696	0.00	0.06	0.00	7.63	6.18	6.23	-7.19	0.2491	
20	RNASeq.48125	0.00	0.00	0.00	12.52	7.57	6.56	-7.19	0.2610	
21	Potrs016204g19040	0.00	0.00	0.00	13.02	12.56	6.73	-7.18	0.2610	protein N-like
22	RNASeq.9581	1.72	0.00	0.05	24.39	18.76	21.40	-7.13	0.2911	
23	Potrs017342g19717	0.00	0.00	0.00	8.66	2.20	3.85	-7.13	0.2755	Abscisic acid receptor
24	Potrs003393g05173	0.00	0.00	0.00	3.55	0.68	1.31	-7.12	0.2755	repeat-containing protein
25	Potrs019946g22725	0.00	0.00	0.00	2.13	1.83	1.24	-7.11	0.2655	Myb-related protein
26	RNASeq.35995	0.00	0.00	0.00	5.59	4.15	4.25	-7.09	0.2610	
27	RNASeq.44896	0.00	0.00	0.00	3.15	10.99	2.29	-7.08	0.2806	
28	RNASeq.42428	0.00	0.13	0.00	24.75	8.63	11.86	-7.06	0.2755	
29	Potrs017902g20099	0.00	0.00	0.00	1.78	4.45	1.07	-7.06	0.2755	UPF0496 protein 1
30	RNASeq.44415	0.00	0.00	0.00	4.61	12.77	9.97	-7.03	0.2755	

Table 9: Top 30 upregulated in trembling aspen (*Populus tremuloides*) treated with the Total-nickel dose (1,600 mg/kg) nickel dose compared to the water control based on LogFC.

Rank	geneID	Plants (RPKM)					logFC	adj.P.Val	Description
		Res. 1	Res. 2	Water 1	Water 2	Water 3			
1	Potrs010519g15584	6.15	11.54	0.04	0.00	0.00	8.41	0.1951	cinnamyl alcohol dehydrogenase
2	RNASeq.29724	10.19	3.12	0.00	0.04	0.00	7.91	0.2285	
3	Potrs010058g35656	84.71	7.90	0.38	0.00	0.00	7.84	0.2748	
4	Potrs035148g35561	9.89	5.39	0.00	0.00	0.00	7.80	0.2061	uncharacterized protein LOC105128463
5	RNASeq.27182	42.57	50.76	0.11	0.09	0.34	7.80	0.1828	
6	Potrs041921g30331	1.93	6.82	0.00	0.00	0.00	7.68	0.2313	uncharacterized protein LOC105142639
7	RNASeq.23977	7.15	21.51	0.00	0.00	0.12	7.63	0.2400	
8	Potrs016761g19342	13.72	9.82	0.10	0.00	0.00	7.62	0.2061	
9	RNASeq.41340	18.18	23.26	0.00	0.14	0.00	7.57	0.2061	
10	RNASeq.48402	12.51	16.51	0.00	0.00	0.00	7.56	0.2061	
11	RNASeq.27779	5.64	7.91	0.00	0.00	0.00	7.48	0.2108	TIR/NBS/LRR protein n=1 Tax=Populus deltoides RepID=Q710T8_POPDE
12	Potrs012289g17008	1.95	7.39	0.06	0.00	0.00	7.24	0.2523	Dihydroflavonol-4-reductase
13	Potrs008313g11887	54.76	16.00	0.00	0.00	0.00	7.24	0.2400	
14	Potrs104342g32544	3.29	10.25	0.00	0.00	0.00	7.20	0.2381	Zinc finger BED
15	RNASeq.42000	43.20	31.24	0.20	0.17	0.16	7.17	0.2061	
16	Potrs009048g13665	16.08	19.92	0.08	0.07	0.25	7.16	0.2061	Adagio-like protein
17	Potrs002764g04419	6.33	1.48	0.00	0.00	0.00	7.16	0.2418	uncharacterized protein LOC105136599
18	RNASeq.45828	2.29	5.99	0.00	0.00	0.00	7.14	0.2365	Kinase superfamily protein, putative isoform 2 n=1 Tax=Theobroma cacao RepID=A0A061E617_THECC
19	Potrs019552g22004	3.66	2.16	0.00	0.00	0.00	7.11	0.2317	7-deoxyloganetin glucosyltransferase
20	RNASeq.11578	9.07	12.63	0.00	0.00	0.00	7.08	0.2285	
21	Potrs008006g11302	12.06	17.84	0.07	0.12	0.05	7.07	0.2132	NADPH:quinone oxidoreductase
22	RNASeq.47893	8.86	14.64	0.00	0.00	0.00	7.05	0.2317	
23	RNASeq.24446	7.02	8.73	0.07	0.00	0.05	6.98	0.2226	
24	RNASeq.21183	7.39	7.24	0.00	0.00	0.00	6.98	0.2347	
25	RNASeq.42878	6.94	6.96	0.00	0.00	0.00	6.87	0.2400	
26	Potrs014471g34514	30.82	11.19	2.08	0.00	0.00	6.85	0.2595	uncharacterized protein LOC105142118
27	Potrs020141g23238	4.40	2.49	0.00	0.00	0.00	6.84	0.2400	uncharacterized protein LOC107178155 isoform
28	RNASeq.42635	10.40	6.00	0.19	0.00	0.00	6.83	0.2400	
29	Potrs003555g31012	14.01	29.69	0.00	0.00	0.00	6.82	0.2400	
30	RNASeq.46489	28.24	17.22	1.79	0.08	0.00	6.82	0.2523	

Table 10: Top 30 downregulated in trembling aspen (*Populus tremuloides*) treated with the Total-nickel dose (1,600 mg/kg) nickel dose compared to the water control based on LogFC.

Rank	geneID	Plants (RPKM)					logFC	adj.P.Val	Description
		Res. 1	Res. 2	Water 1	Water 2	Water 3			
1	Potrs008093g34935	0.00	0.00	8.64	2.78	10.38	-9.58	0.2619	Protein trichome birefringence
2	RNASeq.38281	0.00	0.00	10.20	11.33	10.48	-9.50	0.1787	
3	RNASeq.25977	0.00	0.00	5.85	6.26	10.26	-8.82	0.2132	PREDICTED: probable serine/threonine-protein kinase GCN2 n=1 Tax=Populus euphratica RepID=UPI00057A58E9
4	RNASeq.21163	0.00	0.00	8.43	5.90	7.36	-8.80	0.2061	Fom-2 family protein n=1 Tax=Populus trichocarpa RepID=U5FQT4_POPTR
5	RNASeq.47829	0.00	0.00	38.34	40.89	31.28	-8.79	0.2061	
6	Potrs007626g10572	0.00	0.00	5.02	1.46	2.40	-8.56	0.2523	Auxin response factor
7	Potrs031253g23611	0.00	0.21	32.08	20.75	33.97	-8.55	0.2400	Flavonol 3-sulfotransferase
8	RNASeq.34882	0.00	0.00	7.46	13.90	23.03	-8.55	0.2403	
9	Potrs007958g29721	0.00	0.00	3.91	1.85	4.70	-8.55	0.2400	BURP domain-containing protein
10	Potrs007448g10154	0.00	0.00	0.77	5.21	3.31	-8.46	0.2686	uncharacterized protein LOC105138325
11	RNASeq.45909	0.00	0.05	9.41	8.08	25.05	-8.44	0.2603	Caspase, putative n=1 Tax=Ricinus communis RepID=B9SIN8_RICCO
12	RNASeq.45942	0.02	0.00	5.29	6.21	7.24	-8.33	0.2132	PREDICTED: putative ATP-dependent RNA helicase mfh2 n=1 Tax=Populus euphratica RepID=UPI00057A0689
13	Potrs036508g24269	0.00	0.00	3.00	1.87	2.00	-8.32	0.2226	threonine-protein kinase
14	Potrs000188g00184	0.00	0.00	0.73	4.52	2.33	-8.29	0.2610	receptor-like serine/threonine
15	Potrs006854g33925	0.00	0.00	27.31	23.96	18.88	-8.29	0.2190	
16	RNASeq.47472	0.00	0.00	7.21	13.91	12.69	-8.29	0.2285	PREDICTED: receptor-like protein kinase n=1 Tax=Jatropha curcas RepID=UPI0005FBA833
17	Potrs007674g10554	0.04	0.04	12.33	13.63	14.48	-8.27	0.2061	uncharacterized protein LOC105107893
18	Potrs016471g19220	0.00	0.00	4.23	1.28	1.11	-8.25	0.2545	threonine-protein kinase
19	Potrs148220g32308	0.00	0.00	79.81	63.70	98.23	-8.21	0.2226	
20	RNASeq.47861	0.00	0.00	26.71	24.37	19.71	-8.20	0.2209	
21	RNASeq.46577	0.00	0.00	13.37	5.27	18.58	-8.20	0.2529	Laccase n=11 Tax=Populus RepID=B9IG13_POPTR
22	RNASeq.16921	0.00	0.00	7.09	3.60	7.17	-8.09	0.2383	PREDICTED: LRR receptor-like serine/threonine-protein kinase GSO1 n=1 Tax=Populus euphratica RepID=UPI00057AC3DA
23	RNASeq.35112	0.00	0.00	8.75	2.83	6.43	-8.08	0.2523	ABC transporter family protein n=1 Tax=Populus trichocarpa RepID=B9GG01_POPTR
24	Potrs000144g00119	0.00	0.00	2.16	1.73	3.83	-8.02	0.2400	Probable transcription factor
25	RNASeq.24393	0.00	0.00	2.75	6.60	6.11	-7.96	0.2400	
26	RNASeq.44368	0.00	0.00	12.97	13.61	8.78	-7.94	0.2313	Serine/threonine-protein kinase n=5 Tax=Populus RepID=B9H1U8_POPTR
27	Potrs033493g27816	0.01	0.00	6.26	3.02	2.45	-7.93	0.2529	Disease resistance protein
28	RNASeq.37977	0.00	0.00	18.80	5.94	22.13	-7.92	0.2545	
29	RNASeq.21791	0.00	0.00	19.87	15.75	11.34	-7.92	0.2347	
30	RNASeq.36370	0.00	0.00	22.32	5.30	17.47	-7.92	0.2567	PREDICTED: glycine-rich cell wall structural protein 1-like n=1 Tax=Populus euphratica RepID=UPI00057ADA65

To further analyze gene expression patterns, K-mean clustering was performed using the average expression level of each DEG from all three comparisons. The heatmap in Figure 12 shows the genes grouped into six clusters based on the changes in expression as nickel concentration increased. Clusters 3 and 4 were the only two that followed a linear pattern, where the former increased in expression with higher nickel concentration and the latter decreased. For cluster 1, gene expression increased at the lowest bioavailable nickel concentration (150 mg /kg) and had a decreasing trend after at higher doses. In contrast, the genes in cluster 6 had decreased gene expression at the bioavailable concentration (150 mg /kg) and then slightly increased for the next two higher doses. Finally, Cluster 2 and 5 showed a pattern that changes after the medium half nickel dose (800 mg /kg). Gene expression increased until the half nickel dose (800 mg /kg) for cluster 2 and then decreased at the highest dose. For cluster 5, gene expression decreased for the first two concentrations and then increased for the highest nickel concentration.

Gene Set Enrichment Analysis (GSEA) was performed for each of the six clusters to identify top GO categories enriched in the data (Figures 13-18). Enriched GO terms for Cluster 3 (gene expression increases with each dose) include metal ion transport, metal ion binding (and zinc and calcium ion binding), oxidoreductase activity, regulation of transcription and translation (ribosome and structural constituent of ribosome), protein and nucleic acid binding (Figure 15). From cluster 4, enriched GO terms that decreased in expression with increasing nickel dose include ATP and ADP binding, protein kinase activity, integral component of membrane, protein phosphorylation and transmembrane transport (Figure 16). Next, Cluster 1 includes genes associated with metal ion binding, regulation of transcription, DNA binding transcription factor activity, sequence-specific DNA binding, and nucleus (Figure 13). Top terms from cluster 6 are involved in ATP and ADP binding, cell wall modification, membrane, protein kinase activity, and protein phosphorylation (Figure 18). Top enriched terms for Cluster 2 are regulation of transcription, nucleus, iron ion binding, catalytic activity, oxidoreductase activity and DNA binding (Figure 14). In cluster 5, highly

enriched genes are associated with oxidation-reduction process, integral component of membrane, ATP binding, protein phosphorylation, protein kinase activity, and peroxidase activity (Figure 17).

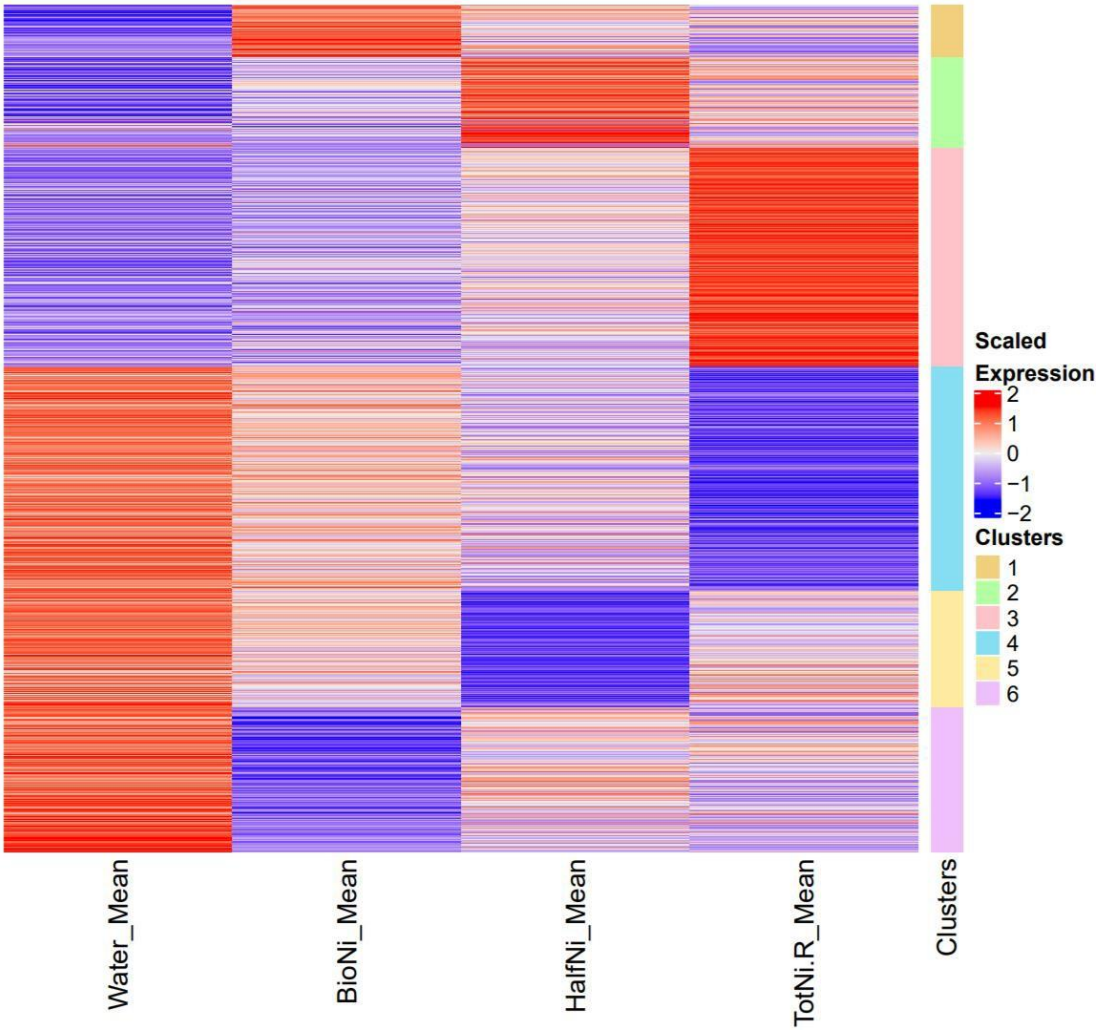

Figure 12: Heat-map of the six gene clusters identified from K-mean clustering analysis for the DEG data sets determined for three nickel treatment concentrations compared to the water control.

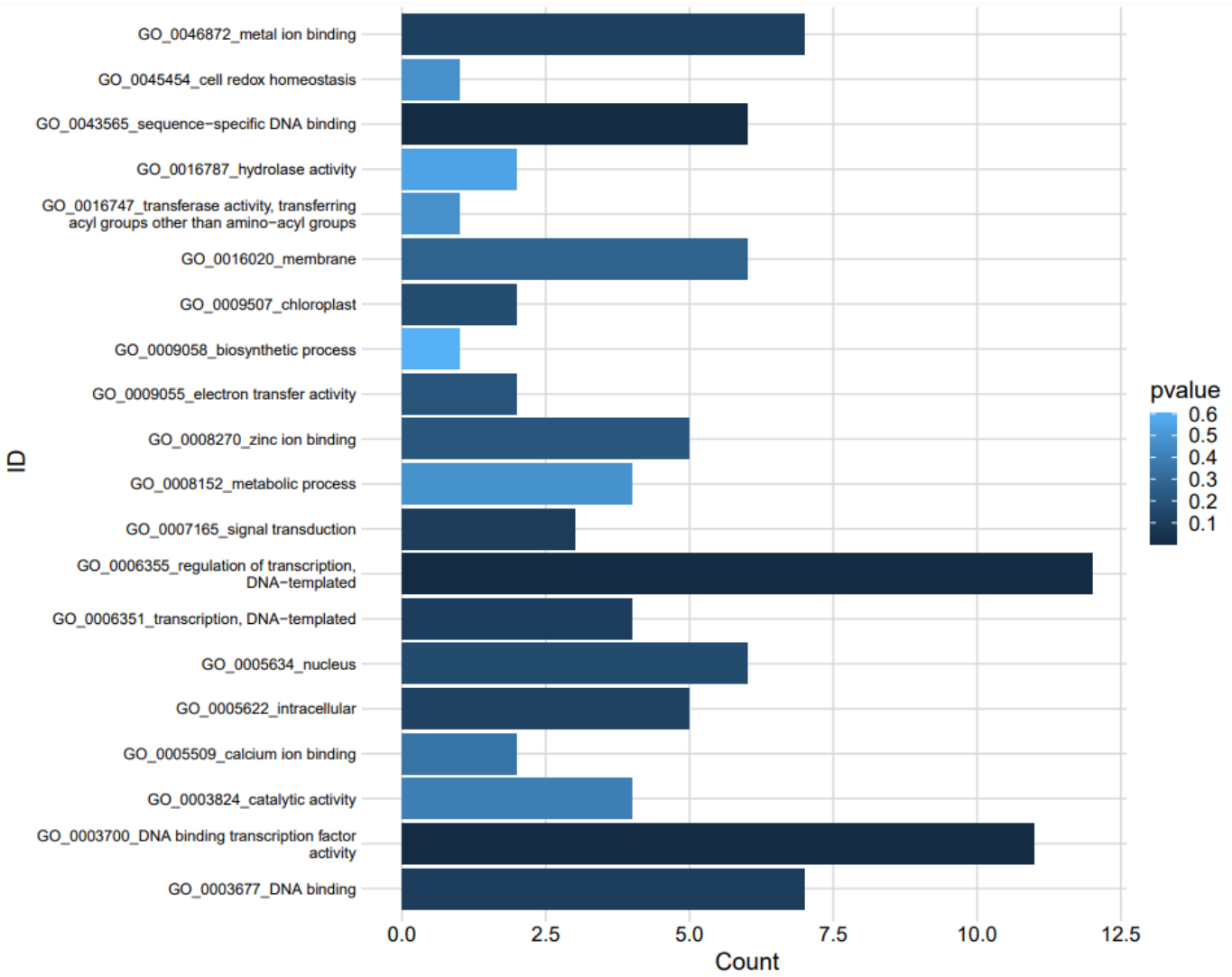

Figure 13: Top 20 enriched GO functions for Gene Cluster 1 (Increasing expression at Bio dose, then decreases) determined with GSEA.

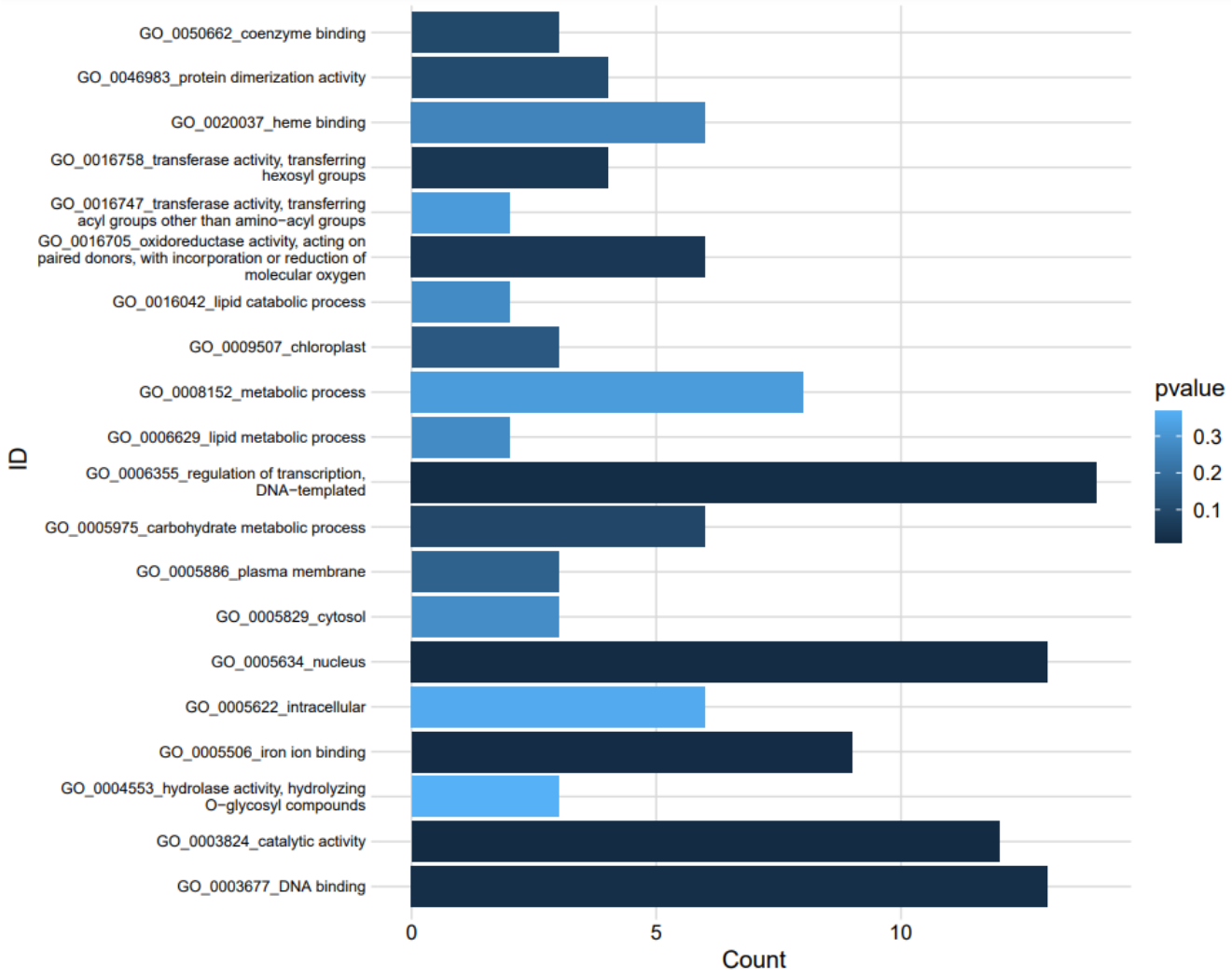

Figure 14: Top 20 enriched GO functions for Gene Cluster 2 (Increasing expression until half-nickel dose then decrease at total nickel) determined with GSEA.

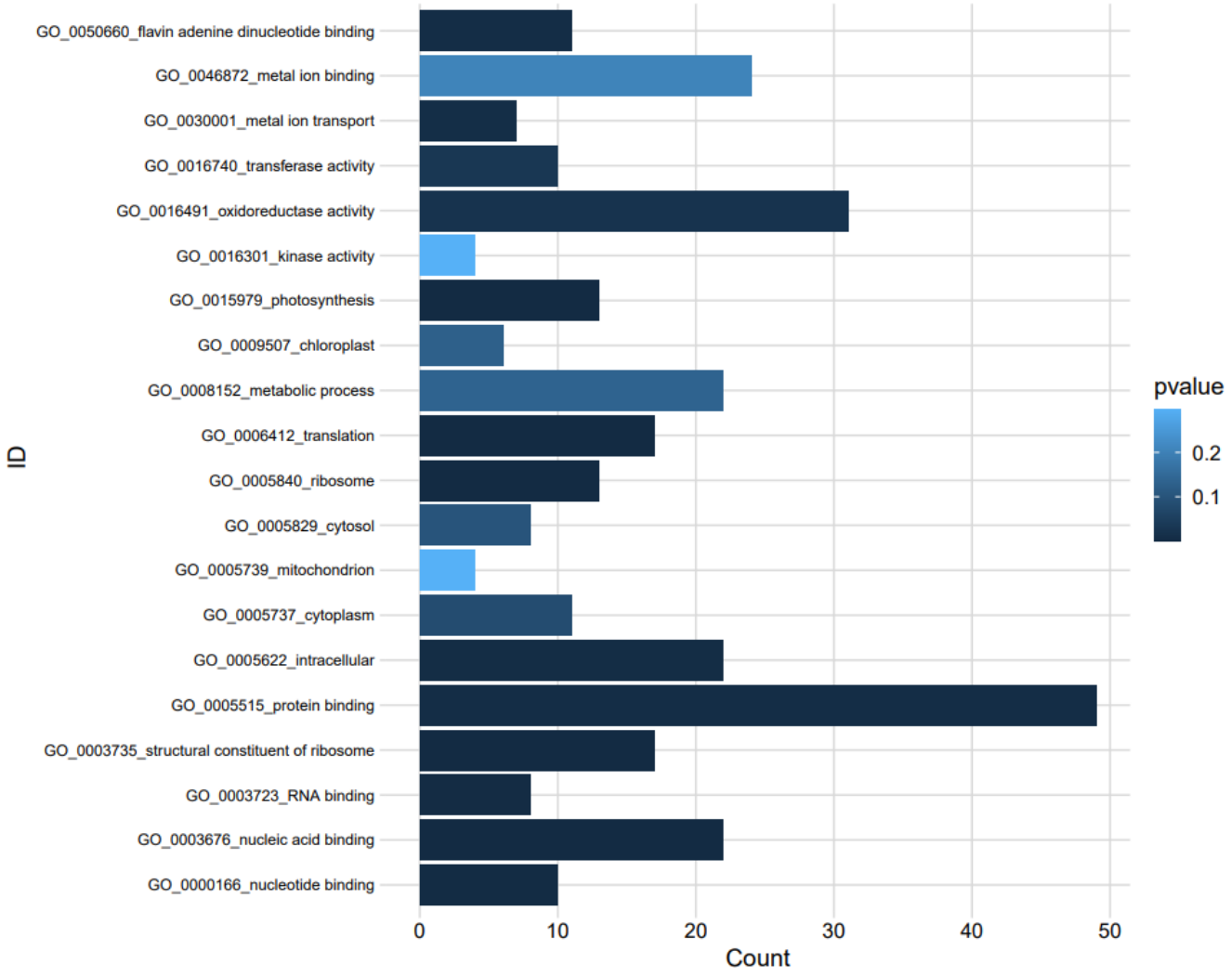

Figure 15: Top 20 enriched GO functions for Gene Cluster 3 (Increased expression with increasing nickel dose) determined with GSEA.

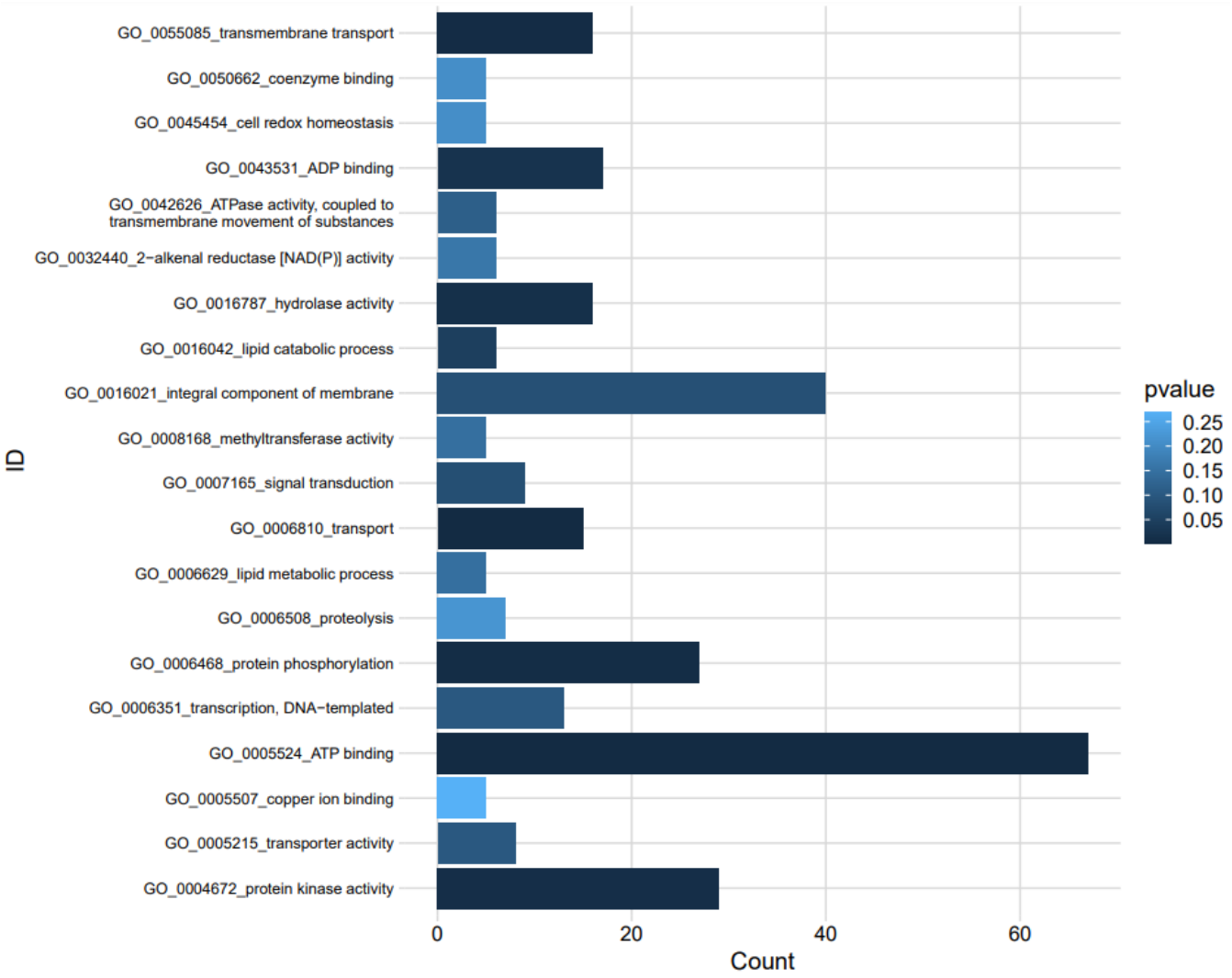

Figure 16: Top 20 enriched GO functions for Gene Cluster 4 (Decreased expression with increasing nickel dose) determined with GSEA.

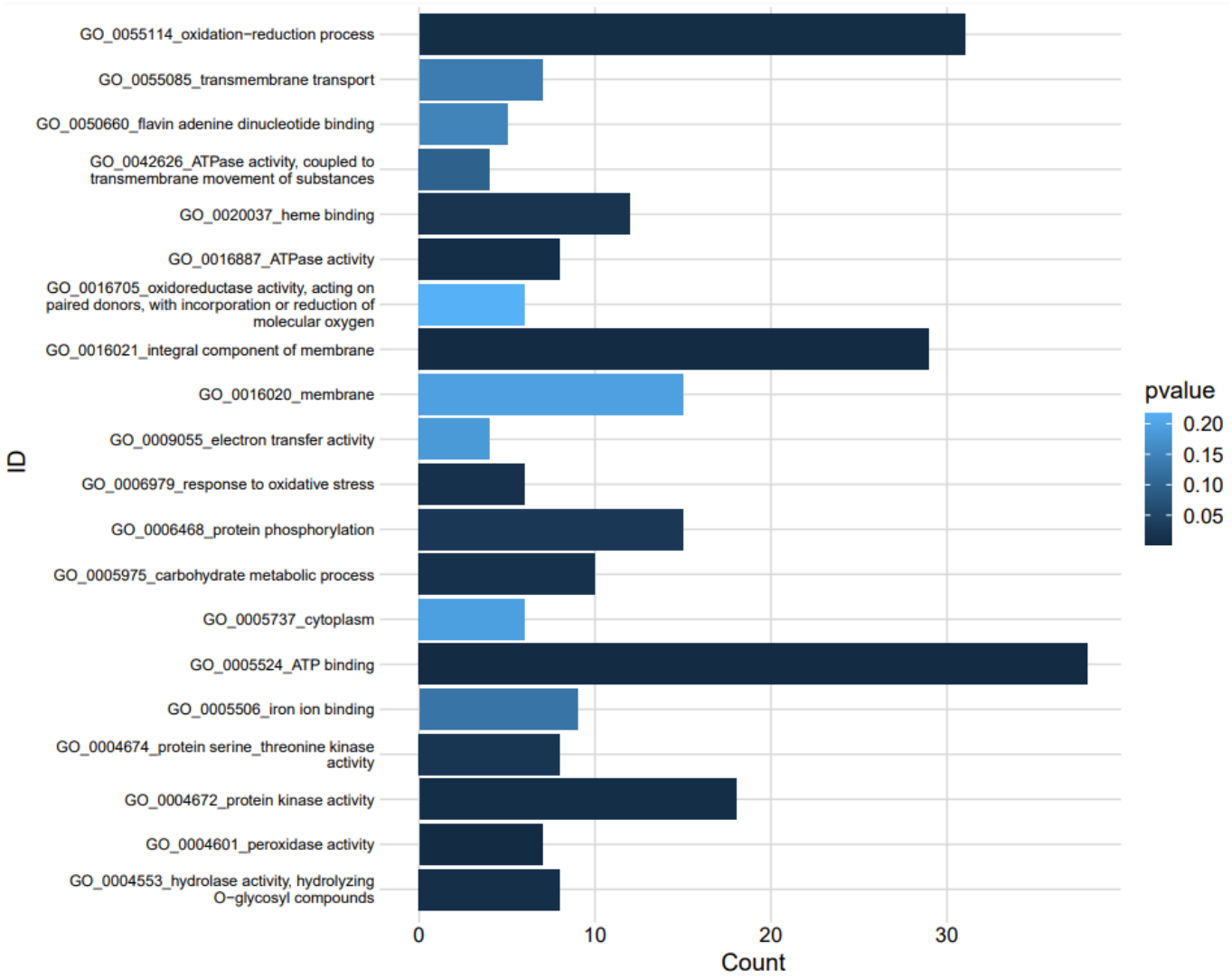

Figure 17: Top 20 enriched GO functions for Gene Cluster 5 (Decreased expression until half-nickel dose, then increase at total-nickel) determined with GSEA.

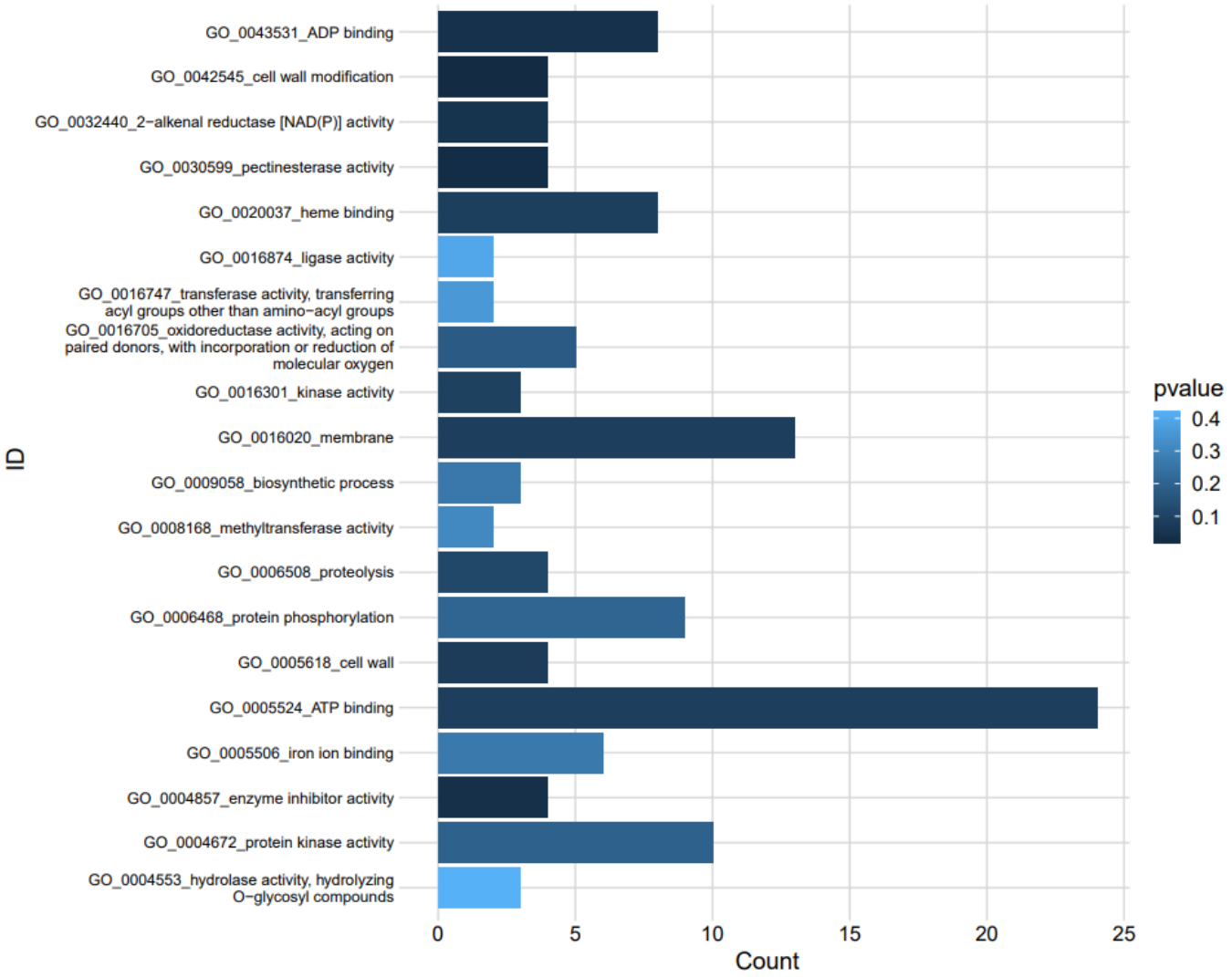

Figure 18: Top 20 enriched GO functions for Gene Cluster 6 (Decreased expression at Bio dose, then increases) determined with GSEA.

3.4. Discussion:

Statistical significance of the DEG data

The gene expression data from trembling aspen seedlings treated with varying doses of nickel nitrates generally clustered close together in the multidimensional plot created in sample relationship analysis. The exception was the sample group of nickel-susceptible samples from the 1600 mg/kg nickel dose treatment. As a result, only the nickel-resistant samples from the highest nickel dose were used for the dosage analysis. The similarities in overall gene expression among the water control, bioavailable Ni (150 mg /kg), half Ni (800 mg /kg), and total Ni (1,600 mg /kg) doses suggest that any changes associated with the nickel stress response across concentrations are more subtle than expected. This is reflected in the lack of significantly differentially expressed genes between treatments when using the high stringency cut-off.

However, there were hundreds of genes detected with differential expression levels among the treatments when using the low stringency cut-off. Enriched genes identified during the K-mean clustering analysis can indicate what type of genes are involved in the complex gene expression dynamics, but it is important to consider the statistical significance used. There is a higher chance of false positives among the gene set considering that no gene had an FDR value equal to or less than 0.05. To decrease the chance of this possibility, only enriched GO terms with a p-value equal to or less than 0.05 were considered for K-mean cluster analysis. Of the six, Clusters 3 and 5 have the most GO categories enriched that meet this requirement with 14 and 13 categories respectively. Furthermore, the Top 30 upregulated and downregulated gene tables for each nickel treatment vs. water comparison all have adjusted p values greater than 0.05. It is important to note this limited statistical significance when speculating on the biological significance of these genes in the *P. tremuloides* heavy metal stress response.

Disease resistance proteins

There are three defense response and disease resistance related proteins in the top 30 upregulated genes in the plants treated with the bio nickel dose. These are associated with a probable Pop3 protein (3. Potrs001038g01787), a TIR/NBS/LRR class protein (6. RNASeq.43432) and a CC/NBS/LRR class protein (10. Potrs015365g18630). There was an additional highly upregulated TIR/NBS/LRR protein identified in the total-nickel dose samples (11. RNASeq.27779). Alternately, both the bio-nickel and half-nickel treatment had two downregulated disease resistance proteins in the top 30. The associated transcripts are 23. RNASeq.42840; 30. RNASeq.26969 and 16. Potrs015173g18492, 17. Potrs009541g33070 respectively. The Pop3 protein has a stress-response A/B Barrel domain, and it is involved in plant defenses in response to biotic stressors like bacteria and fungi (Popgenie.org; Atgenie.org; Bingman *et al.* 2004). The NBS LRR protein family are an important part of the plant's immune system. They are involved in a host of signaling pathways that activate the necessary plant defense responses like oxidative bursts, calcium and ion fluxes, altered transcription of key genes, and the hypersensitive response (McHale *et al.* 2006).

Late Embryogenesis Abundant (LEA) Proteins.

The top upregulated gene in the half-nickel (800 mg/kg) treatment was a Late Embryogenesis Abundant (LEA) protein (Potrs007357g10204). Evidence has linked increased expression of LEA proteins in plants as part of the response to abiotic stress. In particular, numerous studies have characterized the roles of LEA family genes in conferring drought resistance (Liang *et al.* 2016, Magwanga *et al.* 2018). Other researchers are exploring how LEA proteins may be involved in other types of abiotic stress. For example, when OsLEA4 was overexpressed in rice, it enhanced tolerance to high salinity and heavy metal stress, in addition to drought (Hu *et al.* 2016).

Calcium-binding Proteins and calcium signaling.

Two calcium-binding proteins were also among the top upregulated genes in the half-nickel comparison. Studies have shown that calcium signaling is one of the first components of a plant's response that are activated by abiotic stress (Mohanta *et al.* 2018). A cytosolic influx of Ca2+ ions via selective calcium channels is often triggered when a stressor is first sensed by the plant. The calcium concentration in the cell needs to be strictly regulated for proper functioning and an elevated level of cytosolic Ca2+ triggers various down-streaming stress signaling pathways (Mohanta *et al.* 2018). The proceeding calcium signaling events are mainly mediated by various calcium-binding proteins like calmodulin (CaM) and calmodulin-like (CML) proteins. (Mohanta *et al.* 2017). The 8th highest upregulated transcript in the half-nickel treatment group was Potrs006628g09162 and it encodes for a calcium-ion binding calmodulin protein. This increased gene expression may indicate that an early abiotic stress response was already being induced through calcium signaling at this medium concentration nickel dose. The 14th top upregulated transcript (Potrs020191g23280) in this group is a sarcoplasmic reticulum histidine-rich calcium binding protein, and it may be involved in these processes as well. Alternatively, Phospholipase D alpha is a calcium ion-binding protein differentially expressed in this treatment comparison, but it was significantly downregulated in the half-nickel treated plants.

Another calcium-related gene was found to be highly upregulated in the Bioavailable nickel treatment group. Potrs006099g08528 is the 24th transcript with the highest upregulated expression in the nickel treatment when compared to water. This transcript codes for a vacuolar cation/proton exchanger, specifically sodium/calcium.

Additionally, three calcium ion binding proteins were identified among the enriched group of genes in Cluster 3 (increasing expression with increasing nickel dose) that are also associated with photosynthesis. They are in the Arabidopsis gene families that coincide with photosystem II subunit P-1

(Potrs001381g02438), photosystem II subunit O-2 (Potrs000878g01431) and photosystem II PsbR (Potrs007809g10746).

Highly differentially expressed genes in the Total Nickel dose treatment.

The 19th ranked upregulated transcript (Potrs019552g22004) in the total nickel group encodes a 7-deoxyloganetin glucosyltransferase. This protein has UDP-glucoronosyl and UDP-glucosyl transferase activity and is linked to carbohydrate metabolism, adjusting the cellular osmotic balance and chemical cellular homeostasis (Arisha *et al.* 2020; Vogt and Jones 2000). 7-deoxyloganetin glucosyltransferase has been implicated in abiotic stress studies for drought (Arisha et al. 2020), cold-tolerance (Qu et al. 2021), ethylene and salinity (Ma et al. 2021).

Genes identified in the total nickel group from the Top 30 downregulated include an auxin response factor (6. Potrs007626g10572), Flavonol 3-sulfotransferase (7. Potrs031253g23611) and a predicted ABC transporter family protein (24. RNASeq.35112).

Auxin is an important signaling hormone for many plant processes and a response factor was found to be upregulated in these total-nickel dose resistant genotypes when compared to the susceptible samples in Chapter 2 (Table 2: 16. Potrs008742g12940).

The flavonol 3-sulfotransferase is part of the sulfotransferase gene family which has been implicated in plant development and abiotic stress responses (Jin *et al.* 2019).

ABC transporter proteins are in a diverse gene family that can bind a variety of substrates like heavy metal chelates, peptides and sugars (Theodoulou 2000).

Metal ion transport and metal ion binding.

For Cluster 3, metal ion transport and metal ion binding were among the enriched terms although the latter has a higher p value. This correlation of increased gene expression of metal ion transport genes as nickel

dose increased may be related. Current literature shows the 7 transcripts associated with metal ion transport (Potrs018358g20582/ Potrs008070g11333/ Potrs004071g06058/ Potrs008930g13414/ Potrs009869g15061/ Potrs003082g04791/ Potrs002286g03846) are homologous to *P.trichocarpa* and *A.thaliana* genes in heavy metal transport, copper transport and detoxification proteins (popgenie.org). Specifically, four of these transcripts (Potrs018358g20582, Potrs008070g11333, Potrs004071g06058 and Potrs008930g13414) are related to the copper transport protein Antioxidant protein (ATOX1). This protein has been characterized in a variety of eukaryotes (Klomp *et al.* 1997; Valentine and Gralla 1997), and its primary role is to bind cytoplasmic copper ions and direct their transport to the vacuole for sequestration (Banci 2013). This metal ion binding action also has an antioxidant effect within the plant cell, and it can mitigate the generation of reactive oxygen species generated from heavy metal stress (Kim *et al.* 2015).

It was hypothesized that an accumulator species like *P. tremuloides* would likely have upregulated metal transport proteins to shuttle and sequester excess nickel ions in aboveground tissues. However, the main protein involved in nickel transport in trembling aspen has not yet been established. The expression of the nickel transport gene AT2G16800 has been measured in these nickel-treated samples before using RT-qPCR (Czajka *et al.* 2018). We found that this gene was moreso significantly repressed at higher concentrations of nickel nitrate treatment. It is possible that the genes pointed out here could be involved in nickel transport considering proteins can often bind more than one heavy metal but there is no definitive evidence. The copper transport protein identified in Chapter 2 isn't among these genes though they are all part of the same gene family.

Ribosome and translational activity.

Ribosomal-related activities were seen steadily up-regulated with increasing dose (Cluster 3). Associated enriched terms include translation, ribosome, protein binding, structural constitutent of ribosome and RNA binding. It is likely that increased RNA transcription and translation activities are required at higher nickel doses to modify the transcriptome enough to cope with the heavy metal stress. Considering that some of the

seedlings in the high nickel treatment group experienced severe damage or death after treatment, it suggests that the resistant genotypes did face nickel toxicity stress and changes in gene expression may have allowed them to cope.

Oxidoreductase activity and Oxidative stress.

Oxidoreductase activity was another highly enriched term with increasing expression as nickel concentration increased (Cluster 3). Oxidoreductases encompass a wide range of enzymes that catalyze re-dox reactions in many parts of the cell under different conditions. They are often implicated in biotic and abiotic stress responses, particularly as part of the oxidative stress response.

One major impact of heavy metal stress in plants is the increase in free radicals and reactive oxygen species (ROS) (Singh *et al.* 2016; Nkongolo *et al.* 2018a). Excess Nickel ions have been linked to increases in hydroxyl radicals, superoxide anions, nitric oxide, and hydrogen peroxide (Nkongolo *et al.* 2018a). Nickel does not induce free radical formation directly because it is not a redox-active metal but rather indirectly through the inhibition of antioxidant enzymes like ascorbate peroxidase (APX), catalase (CAT), glutathione peroxidase (GSH-Px), glutathione reductase (GR), guaiacol peroxidase GOPX), peroxidase (POD), and superoxide dismutase (SOD) (Nkongolo *et al.* 2018a).

Excess reactive oxygen species (ROS) left unchecked in the organism can cause cellular damage (Nkongolo *et al.* 2018a). Due to their instability and high reactivity, ROS can interact with numerous types of macromolecules like DNA, proteins and lipids which can cause many negative impacts and potentially generate more free radicals (Sharma and Dietz, 2009; Hossain *et al.* 2012; Foyer and Halliwell, 1976). Plants have an antioxidant defense system that can mitigate any potential damage caused by ROS by regulating ROS concentration in the cell (Singh *et al.* 2016). The system includes ROS scavenging enzymes including superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), ascorbate peroxidase (APX; EC 1.11.1.11), glutathione reductase (GR; EC 1.6.4.2), dehydroascorbate reductase (DHAR; EC 1.8.5.1),

glutathione peroxidase (GPX; EC 1.11.1.9), and glutathione-S-transferase (GST; EC 2.5.1.18) (Nkongolo *et al.* 2018a). There are also non-enzymatic components like the water-soluble antioxidant compounds ascorbate, and glutathione (Singh *et al.* 2016). Usually there is a redox balance state in the cell between ROS generation and antioxidant scavenging but when ROS exceed the scavenging potential then the collective damaging effects on plant processes is what is considered "oxidative stress" (Singh *et al.* 2016).

There is increasing evidence of the link between heavy metal tolerance and antioxidative systems in plants. Low antioxidant capacity is associated with increased susceptibility to HM toxicity (Theriault *et al.* 2016a). In this present study, it was hypothesized that antioxidant enzymes and related genes may be upregulated with increasing nickel dose in plants to mitigate any damage by a potential increase in ROS due to nickel. However, past studies suggest antioxidant activities may be repressed in nickel-accumulating plants as seen in those belonging to the *Alyssum* genus (Schickler and Caspi 1999). This may apply to other accumulator plants like *P. tremuloide*s and *B. papyrifera* (Theriault *et al.* 2016a).

From the current results, multiple transcripts associated with oxidoreductase activity in Cluster 3 are also linked in some aspect with antioxidant activity. The major antioxidant enzymes (CAT, etc.) of the defense system described earlier may be repressed as suggested by Shickler and Caspi (1999) and Therieault *et al.* (2016a) but other antioxidant genes could play a role.

Some examples of the antioxidant-related transcripts are two peroxiredoxins (Potrs032946g23727, Potrs001304g02312), prostaglandin reductase 1 (Potrs146657g27265), protochlorophyllide reductase (Potrs006108g08535), lactate/malate dehydrogenase (Potrs019177g21465), aldehyde dehydrogenase (Potrs016855g19402) and NADPH: Quinone oxidoreductase (Potrs008006g11302).

Other transcripts or related homologues in other species have been linked to various abiotic stress responses like salinity response (Potrs008324g11913), Response to cadmium ion (Potrs009340g14710), and tropine dehydrogenase was associated with heavy metal stress (Potrs001088g01882).

Two enzymes (Potrs002151g03670 – ACC oxidase 1**,** Potrs013144g17512 – 1-carboxylate oxidase) involved in ethylene biosynthesis signaling were also in this group. Recent evidence suggests ethylene plays a role in cellular redox balance signaling and in regulating metal stress responses in plants (Houbon and Van de Poel 2019)

The general trend seen here is that other enzymes with antioxidant activities are increasingly upregulated as the concentration of nickel ions increases, perhaps due to an increase in the overall free radical load in the root cells triggering the defense system.

Interestingly, oxidation-reduction processes are highly enriched in cluster 5 where expression decreased until the highest nickel dose and increased in expression again. Many of the transcripts in this category are associated with components of the plant response to oxidative stress: Ascorbate oxidases (Potrs002761g043950, Potrs019602g22084) peroxidases (Potrs008002g11219, Potrs041172g25927, Potrs006457g36820, Potrs003697g05587, Potrs002362g03931), myo-inositol oxygenase (Potrs000125g00163), D-lactate dehydrogenase (Potrs040933g25796) and aldehyde dehydrogenase (Potrs005626g07937). It is unclear why these enzymes may have been steadily downregulated with the first two doses (150mg/kg and 800 mg/kg) but the increase again in expression at the highest dose suggests a possible mechanism may overcome this repression at very high nickel doses.

Overall, the antioxidant defense system response can be quite complex. Multiple signaling pathways, enzymes, and non-enzymatic components can be involved to address the excess reactive oxygen species often generated during abiotic stress. Also, many parts of the cell can be vulnerable to damage so it is important that the cell can regulate its antioxidant defense response at different levels of stress.

Photosynthesis

Genes associated with photosynthesis were enriched for having increased gene expression with increasing nickel dose. It is well documented that the photosynthetic apparatus and photosynthesis processes are easily

disrupted and vulnerable to abiotic stress (Nouri *et al.* 2015). Photosystem II proteins are particularly susceptible to damage (Sasi *et al.* 2018). Changes in gene expression for photosynthesis-related genes have been observed in plants exposed to various abiotic stressors as part of the plant defense response (Nouri *et al.* 2015). The set of genes in this analysis include multiple components from both photosystems:

Photosystem I (PSI): psaG / psaK (Potrs008665g12978), reaction centre subunit III (Potrs005749g08086) and reaction centre subunit N (PSAN or PSI-N)(Potrs000153g00259).

Photosytem II (PSII): subunit P-1 (Potrs001381g02438), subunit O-2 (Potrs000878g01431), PsbR (Potrs007809g10746), PsbW (Potrs008755g12889) and, PsbX (Potrs007161g09870).

The up or downregulation of a photosynthesis gene can vary with the species and type of abiotic stressor. For example, most photosynthesis genes were downregulated in drought-stressed rice leaf tissues (Wang *et al.* 2011). A downregulation of photosynthesis genes was observed in both chickpea roots and shoots under varying abiotic stresses as well (Garg *et al.* 2015). Another study of two rice cultivars exposed to multiple abiotic stresses (heat, drought and salinity) identified two photosynthesis related proteins (photosystem II 10 kDa polypeptide (PsbS) and photosystem II 22 kDa protein 2) that were upregulated in both rice types (Habibpourmehraban *et al.* 2022). It is important to note that the transcriptome of *P. tremuloides* roots are being analyzed in this present study and it is unclear if the photosynthesis gene upregulation serves a purpose considering plant root cells typically don't have any or high chloroplast activity. However, some studies indicate that under stressful conditions like shoot removal, roots do have the ability to increase chloroplast development (Kobayashi *et al.* 2017). In addition, 12.1 percent of total annotated transcripts in the water-control *P. tremuloides* samples were associated with the chloroplast (Figure 3) which clearly suggests some related-activities are occurring.

Conclusion

In conclusion, this comparative transcriptome approach enabled the characterization of gene expression dynamics in trembling aspen seedlings treated with different doses of nickel. Genes related to metal

transport, calcium signaling, antioxidant activity, defense responses and abiotic stress may be modulated in different ways in nickel-stressed plants.

Chapter 4: Global Methylation analysis of *Populus tremuloides* contaminated with a high concentration of nickel salts

4.1. Introduction

In plants, DNA Methylation is a common epigenetic mechanism that can cause gene expression changes that do not require direct modification of the DNA sequence (Gallo-Franco *et al.* 2020, Asensi-Fabado *et al.* 2017). Cytosine methylation can regulate gene expression in many ways throughout the plant's developmental stages, different cell types and in response to cellular or environmental stressors (Lee *et al.* 2020, Srikant and Drost 2021). It is also important in regulating other cellular processes like DNA replication, transcription, repair, transposable element activities, genomic imprinting and cell differentiation (Vanyushin and Ashapkin 2011, Gallego-Bartolomé 2020; Gallo-Franco *et al.* 2020; Zhang *et al.* 2018, 2020a; Phillips 2008).

Short-term alterations in gene expression are often required to respond to environmental stress induced in plants by the presence of biotic and abiotic stressors (Bartels *et al.* 2018). Stress response signaling pathways are activated and epigenetic mechanisms can work to upregulate or repress transcription of necessary genes needed to cope with the stressor. When the stress is eliminated from the environment, these transcriptional changes often return to their original state (Boyko and Kovalchuk 2008). However, in some cases these changes stably remain via the plant's somatic memory or they can even be trans-generationally inherited by future progeny (Gallo-Franco *et al.* 2020).

Heavy Metals (HMs) act as abiotic stressors to plants when they are present in excess concentrations in the environment, particularly in the soil. Epigenetic mechanisms like methylation can be induced when a toxic threshold is surpassed, and the plant can adapt by altering the gene expression of key HM detoxification genes including metal transporters and chelators. In particular, cytosine methylation and demethylation within the gene promoter or gene body can modulate the gene expression profile needed to eliminate or sequester the excess HM ions (Choi and Sano 2007; Wada *et al.* 2004).

There is evidence of increased hypermethylation in plant species with nickel tolerance adaptations like accumulation when compared to non-accumulating species during nickel stress (Gullì *et al.* 2018). Hypomethylation has also been observed in species like *Acer rubrum* and *Betula papyrifera* when samples from sites with known higher nickel and copper concentrations were tested (Kim *et al.* 2016, Kalubi *et al.* 2017; Theriault *et al.* 2016b). This study aims to assess any changes in global methylation dynamics in trembling aspen (*Populus tremuloides*) due to nickel stress.

Studies on the effects of abiotic stress on epigenetic modifications in plants are still scarce and limited to few species. The data reported in the literature on epigenetic changes might be underestimated since most authors used methods such as MSAP, HPLC and immunolabeling which are less sensitive compared to bisulfite sequencing and and single-base resolution methylome analysis. These later next generation sequencing techniques are associated with high costs that limit their use in ecological studies (Czajka *et al.* 2022). A critical analysis of the literature available shows that 44% of the epigenetic modifications induced by abiotic stressors in plants involved DNA hypomethylation, 40% DNA hypermethylation, and 16% histone modification (Czajka *et al.* 2022). Various epigenetic mechanisms are involved in response to heat stress, a number of them are still unknown. Integrated analysis of the changes in the genome by omic approaches should help to identify novel components underlying mechanisms involved in DNA methylation and histone modifications associated with plant response to abiotic stressors.

Effects of Ni toxicity on DNA methylation levels and distribution in *P. tremuloides* is lacking. The specific objective of this component was to assess variation in global methylation induced by different Ni and potassium nitrates in *P. tremuloides.*

4.2. Materials and Methods

Assessment of nickel toxicity was performed as described in section 2.2.1. Only the highest dose of salts, 1,600 mg/kg of nickel nitrate and potassium nitrates was used. Water was the reference control. Final damage ratings were taken 7 days after treatment before the plant tissues were harvested (Figure 19).

Figure 19: Image of two of the *P. tremuloides* seedlings on day 7 after treatment with either 1,600 mg / kg nickel dose (left – resistant plant) or water (right).

4.2.1. Methylation Assay

The ab233486 Global DNA Methylation Assay Kit (5 Methyl Cytosine, Colorimetric) by Abcam (Cambridge, UK) was used to assess the global levels of methylated cytosines within the treatment groups. All 17 samples were tested in duplicate with 100ng of isolated genomic DNA added to each well. Binding solution was added to the DNA samples, control, and standards to bind DNA in the wells. After a 1-hour incubation at 37C, the binding solution was removed, and the wells were washed with a wash buffer three times. 5-mC Detection Complex solution was added and after the 50-minute incubation at room temperature, the wells were washed another five times. Developer solution was added, and the reaction was stopped after

the solutions turned blue with the addition of the Stop solution. The absorbance at 450 nm for each well was read with a microplate reader.

4.2.2. Statistical Analysis

All treatments were assayed using a 96-well plate reader (Fluostar Optima) from BMG Technologies. Statistical analyses were conducted using the SPSS version 20 for windows (IBM, NY, USA). The Shapiro-Wilk test ($p \leq 0.05$) was performed to confirm normal data distribution. A Homogeneity of Variances test was performed ($P<0.05$) to confirm that the variance within each treatment group is similar to the other treatments. A one-way ANOVA test using the post-hoc LSD statistic ($P<0.05$) was used to determine if there were any significant differences between the average damage ratings of the different poplar treatment groups.

4.3. Results

Plants treated with the high nickel dose (1,600 mg/kg) had a significantly higher average damage rating when compared to the nitrate or water control (Figure 20.) This treatment group also had the most variation in damage where 3 nickel-resistant and 2 moderately susceptible plants were observed seven days after treatment.

The global methylation assay revealed significantly lower 5mC% in the potassium nitrate control samples when compared to water or the nickel treatment (Figure 21). No significant differences were observed between the water control and the nickel treatment.

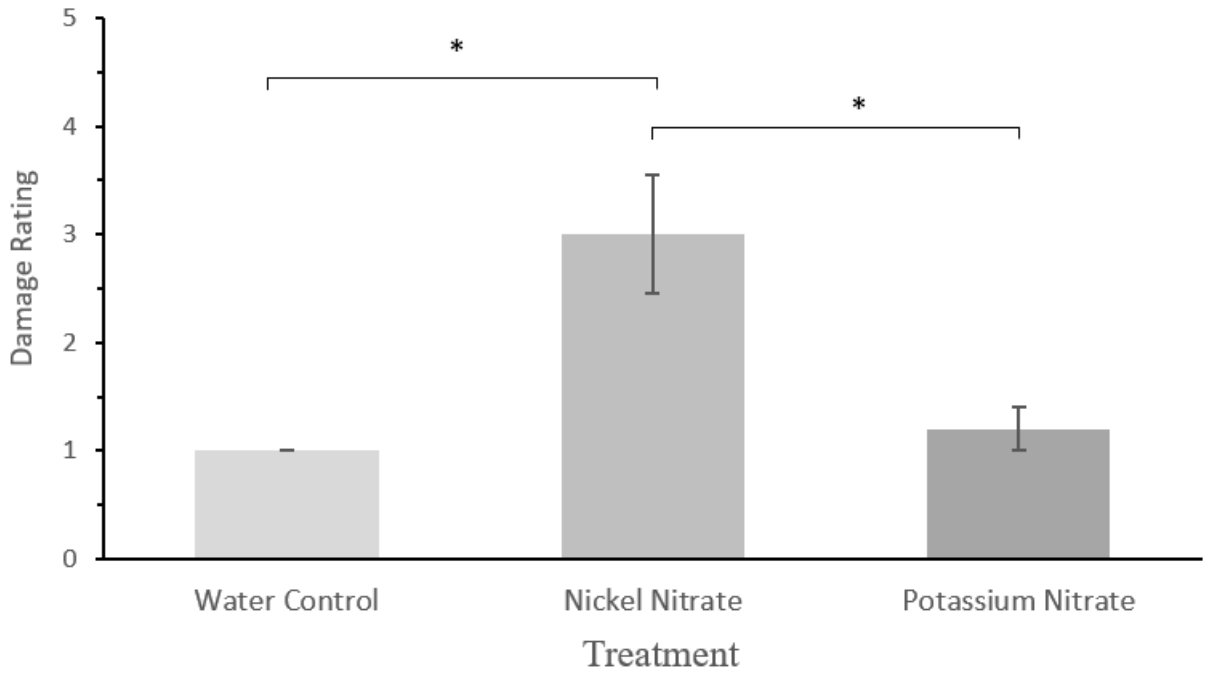

Figure 20. The phenotypic damage rating of trembling aspen (*Populus tremuloides*) seedlings treated with 1600 mg/kg of nickel nitrate or potassium nitrate control. Water was used as a negative control. The damage scores of 1 to 3 were considered healthy, 4 to 6 were moderately healthy, and 7 to 9 were damaged. Significant differences among the treatments are marked using * ($p \leq 0.05$). Error bars represent the standard error of the treatment.

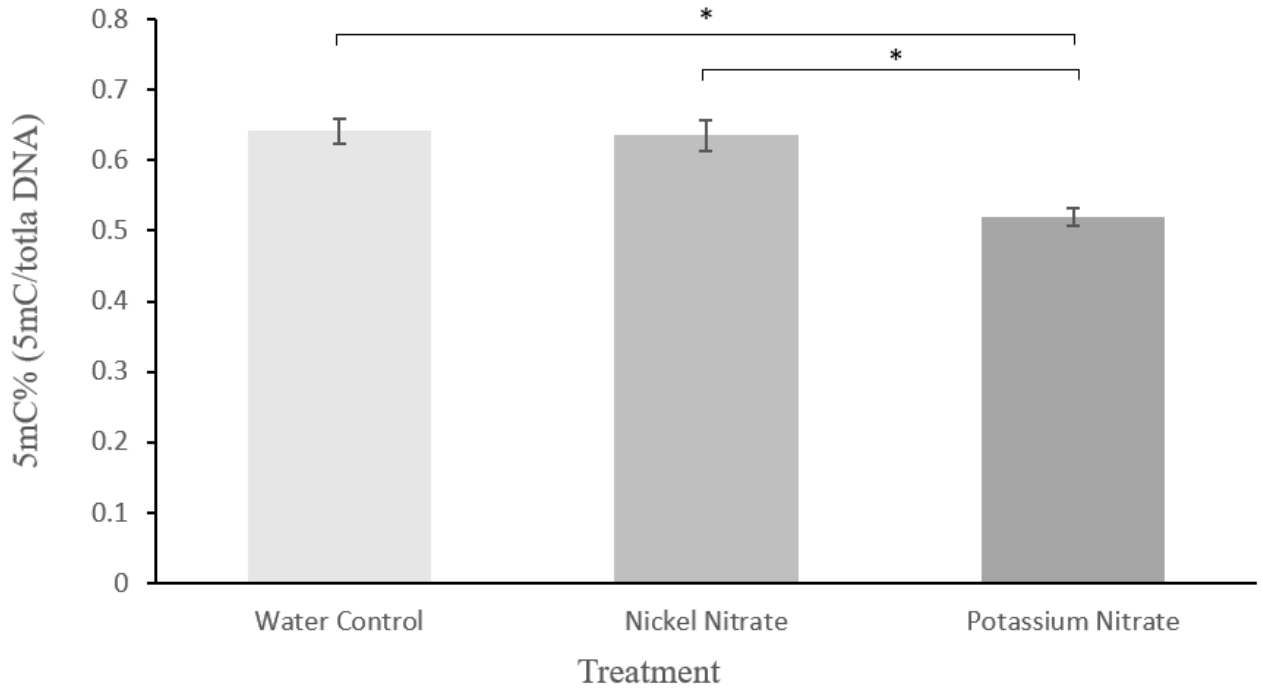

Figure 21. Average percentage of global 5-Methylcytosine (5-mC%) in trembling aspen (Populus tremuloides) roots ((Water control, n=7; Nickel Nitrate, n=5; Potassium Nitrate, n=5) treated with 1,600 mg/kg of nickel nitrate or the potassium nitrate control. Water was used as a negative control. Significant differences among the treatments are marked using * ($p \leq 0.05$). Error bars represent the standard error of the treatment.

4.4. Discussion

The 1, 600 mg/kg dose of nickel was used because at this concentration a segregation of phenotypes was observed among the poplar seedlings. This treatment group had the highest variation in physical damage seven days after treatment with the highest dose of nickel nitrate solution. The methylation assay included 3 resistant and 2 moderately susceptible genotypes. It was expected on average that the heavy metal nickel may induce a change in global methylation.

In the present study, the global DNA methylation (5-mC) profile was quantitated using the indirect ELISA (enzyme-linked immunosorbent assay) method. The antigen is the DNA of interest (5-methylcytosine DNA—5-mC or 5-hydroxymethylcytosine DNA—5-hmC) and the antibody has affinity for methylated or hydroxymethylated CpG sites (anti-5-mC or anti-5-hmC). Results are expressed as percentage of methylation/hydroxymethylation compared to fully methylated/hydroxymethylated control DNA (100%) (de Oliveira *et al*. 2020).

This methylation assay indicated there were no significant changes in the overall percentage of methylated cytosines when the nickel nitrate treated samples were compared with water. However, a significant hypomethylation was observed in the potassium nitrate control when compared with water and nickel nitrate. A similar result was seen for *Picea glauca* when treated with potassium sulfate suggesting that potassium ions are involved in this hypomethylation.

Another study conducted to assess the effects of nickel on trembling aspen seedlings using nickel nitrate and nitrate control salt solutions looked at the gene expression of nickel resistant target genes *NAS3* and *NRAMP4.* A qPCR assay for these genes showed a significant increase in gene expression in the potassium nitrate treatments which served as controls for the 800 mg / kg and 1, 600 mg / kg nickel nitrate treatments (Czajka *et al.* 2019). In this case, it was suggested that the nitrate concentration induced such an effect in the control samples while the nickel in the nickel nitrate treatments may have suppressed this increased

expression. However, potassium could still be the affecting variable. In this methylation assay, the overall decrease in methylation seen in the potassium nitrate control treatment may indicate the apparent effects of potassium on demethylation in the plant. Global hypomethylation, in turn is often associated with an increase in gene transcription and expression, particularly if the demethylation occurs within the gene promoter.

A global demethylation in the system could be accomplished by the increased expression of demethylation machinery. In plants, demethylation typically occurs through the active and direct excision of the 5-mC base by 5-methylcytosine glycosylases (Gallo-Franco *et al.* 2020). Some members of this family of enzymes include Demeter (DME), Repressor of silencing 1 (ROS1), Demeter-like 2 (DML2), and Demeter-like 3 (DML3) (Penterman *et al.* 2007). A future RT-qPCR assay to measure the expression of these genes in the potassium nitrate samples and compare with other treatments could elucidate the potential role of increased expression of active demethylation enzymes resulting in the observed hypomethylation.

The lack of significant differences in global methylation in the nickel nitrate samples compared to water, does not discount that methylation changes may still have occurred at site-specific loci which in turn altered gene expression. The methylation status of a few target promoters and genes associated with nickel/heavy metal resistance could be tested next. It is important to specify the location within the gene being assessed because it can have different effects on methylation. For instance, current evidence from mammals and certain plant species suggests a trend of inhibited gene expression when the promoter is methylated while gene body methylation correlates with an increase in gene expression (Zhang *et al.* 2018; Liang *et al.* 2014).

In animal models, hypermethylation caused by exposure to nickel compounds has been observed in a hamster G12 cell line and in nickel-induced tumors in wild type C57BL/6 mice (Lee *et al*. 1995; Govindarajan *et al.* 2002; Kowara *et al.* 2004). It has been suggested that the ability of Ni to substitute for magnesium in the phosphate backbone of DNA might be the main mechanism leading to hypermethylation. Ni might be better at increasing chromatin condensation and triggering *de novo* DNA methylation of critical genes that can be incorporated into heterochromatin in animal models (Arita and Costa 2009).

There are a wide range of techniques that can be used to characterize methylcytosine in a genome (Schrey *et al.* 2013; Alonso *et al*. 2016; Herrera *et al.* 2016; Kim *et al.*, 2016). Tandem mass spectrometry (MS/MS) coupled with LC (LC-MS/MS) is an established approach to nucleoside quantification specifically to measure global cytosine methylation (Hu *et al*. 2013; Tsuji *et al.* 2014). It is a fast, sensitive, accurate and specific avenue for modified nucleoside quantification at trace (fmol) levels (Alonso *et al.* 2016). Quantitative data revealed significant decrease in cytosine methylation in metal contaminated sites in *Acer rubrum* (Kim *et al.* 2016).

For quantification of genome-wide cytosine methylation, the chemical method (LC-MS/MS) is recommended because of its global assessment, accuracy, and reproducibility (Fraga and Esteller 2002; Lisanti *et al.* 2013; Alonso *et al.* 2016). MSAP is a robust, highly reproducible, and chip-based molecular tool useful to detect variations in the DNA methylation status. MSAP has been used to determine the differentiation in cytosine methylation in targeted populations. Although the scoring of MSAP markers is not straightforward, the technique does not require a sequenced reference genome and provides many anonymous loci randomly distributed over the genome for which the methylation status can be ascertained (Alonso *et al.* 2016). Moreover, methylation detected by MSAP is greater than genome wide estimates obtained by HPLC (Alonso *et al.* 2016). MSAP analysis revealed no significant differences between uncontaminated and metal-contaminated *Acer rubrum* populations (Kim *et al*. 2016) suggesting that nickel or other metals have no significant impact on cytosine methylation. Aina *et al.* (2004) reported hypomethlylation of DNA induced by heavy metals while Greco *et al.* (2012) observed a hypermethylation induced by cadmium in *Posidonia oceanica* based on MASP. Using the same technique, Cicatelli *et al.* (2014) showed an extensive DNA hypermethylation in leaves when mycorrizal poplar (*Populus alba*) plants were grown in the presence of 950 mg / kg of total Zn and 1, 300 mg / kg of total Cu compared to control. One of the shortcomings of this method is that some methylated states can be missed because the banding pattern observed when MsPI and HpaII fail to cut can be caused by genetic or epigenetic process. Moreover,

there is no consensus on the interpretation and scoring of the four possible outcomes that can be obtained from the combined MSAP banding patterns (Fulnecek and Kovarik 2014).

More recent efforts have concentrated on the analysis of the methylation status of specific DNA sequences in plants (Kim *et al*. 2016; Czajka *et al*. 2022). Optimization of the bisulfite-based DNA modification methods facilitates the analysis of limited CpGs in restriction enzyme sites, the overall characterization of differential methylation and allows very specific patterns of methylation to be revealed (bisulfite DNA sequencing) (Shen and Waterland 2007). However, the application of this method in ecological studies is cost-prohibitive.

In the present study, the ELISA method detects specific antigen-antibody binding via enzymatic reactions that are observable through color change of the reaction medium, and results can be read spectrophotometrically. This technique is fast, cost-effective, and yields accurate results since anti-5-mC/anti-5-hmC are highly specific and present no cross-reactivity with unmethylated DNA. Although the global methylation profile is associated to various contexts, this technique is an initial screening and does not provide information on isolated CpG sites. Therefore, gene-specific methylation analysis should not be overlooked (de Oliveira *et al*. 2020) in future experiments.

Chapter 5: General Conclusions

The main objectives of the present research were to 1) further characterize the *P. tremuloides* transcriptome 2) compare gene expression dynamics between nickel-resistant and nickel-susceptible *P. tremuloides* genotypes with Whole Transcriptome (WT) sequencing, 3) assess the effects of different nickel concentrations on *P. tremuloides* gene expression, and 4) assess global methylation levels in *P. tremuloides* under nickel stress.

P. tremuloides treated with varying concentration of nickel nitrate salts showed a significant variation in damage at the highest dose of 1,600 mg Ni per kg of dry soil. Extracted RNA from these resistant and susceptible genotypes was compared on a whole transcriptome level using RNA-seq technologies and significant differences in gene expression were observed. This pairwise comparison identified highly upregulated and downregulated genes in resistant genotypes. Gene ontology (GO) analysis characterized what biological process, molecular function and cellular component was associated with each differentially expressed gene. A copper transport protein was highly upregulated in resistant genotypes, and it may have binding ability for other heavy metal ions like nickel. In addition to the candidate metal transport protein, a variety of other genes were characterized in the top differentially regulated gene data set that have been implicated in the general plant stress response to abiotic stressors. These include a Dirigent Protein 10, GATA transcription factor, Zinc finger protein, Auxin response factor, Bidirectional sugar transporter and thiamine thiazole synthase. Overall, this analysis validated that differential gene expression may enable some *P. tremuloides* genotypes to better tolerate and cope with heavy metal stress when compared to those that are susceptible. Changes in the complex gene expression dynamics can occur in many parts of the cell and a general increase in ribosomal and translation activities in resistant genotypes was observed.

The dosage analysis revealed that potential differences in gene expression between *P. tremuloides* treated with varying concentration of nickel nitrate salts and a baseline water control were more subtle than expected. However, when a lower stringency statistical cut off (two-fold change and p-value ≤ 0.1) was used,

hundreds of differentially expressed genes were identified for each treatment comparison (150 mg / kg, 800 mg / kg or 1, 600 mg / kg compared to water). The top upregulated and downregulated genes found in each comparison and the K-mean cluster analysis revealed that multiple components of the abiotic stress response may have been induced at higher nickel doses. The highly upregulated LEA protein and two calcium-binding proteins found in the 800 mg / kg comparison suggest that this nickel concentration may be the threshold for when changes in gene expression related to the abiotic stress response begin to appear. The cluster of genes that had increased gene expression with increasing nickel dose also had multiple enriched GO terms related to heavy metal and abiotic stress including metal ion transport, antioxidant activity, photosynthesis, and ribosomal activity.

No differences were found in global methylation levels between nickel-resistant genotypes treated with 1,600 mg / kg of nickel and the water control plants. It is still possible that the methylation status of specific loci could still be altered to modulate gene expression of target genes. Candidate genes identified in the transcriptome analyses could be used for a methylation assay that can determine the methylation status for a specific chosen DNA sequence. This methylation assay did show a significant global hypomethylation in the nitrate control samples.

In conclusion, the transcriptome and methylome data used in these studies aided in characterizing *P. tremuloides* under control conditions and under nickel-stress. This top-down approach identified numerous genes associated with heavy metal stress and future studies could use target-specific assays to investigate gene expression and methylation status of each gene. Understanding the heavy metal tolerance mechanisms and responses used by hardy species like trembling aspen is important for environment bioremediation and maintenance of healthy ecosystems.

Future studies

The proposed future stuadies include methylation mapping using bisulfite sequencing of candidate genes identified in the trasnscriptome analyses. This might reveal the location and the level of DNA methylation along the candidate genes.

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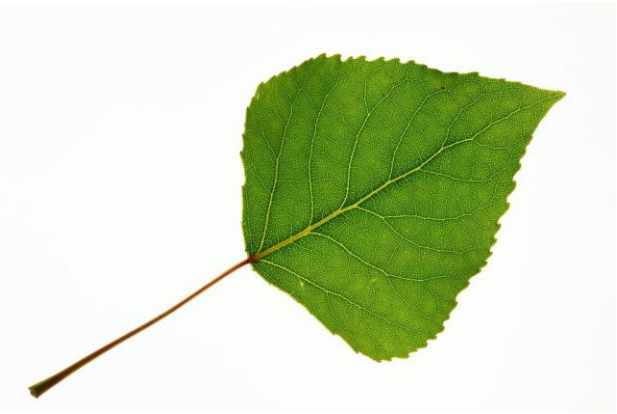
Nicotiana tabacum plants is increased by cadmium, nickel, arsenic, cobalt and lead but not by zinc and iron. *Journal of Biotechnology*, *139*(3). https://doi.org/10.1016/j.jbiotec.2008.12.001

Appendices

Appendix A: Trembling Aspen (*Populus tremuloides)*



Appendix B: Six month old *P. tremuloides* seedlings in the growth chamber prior to nickel treatments.

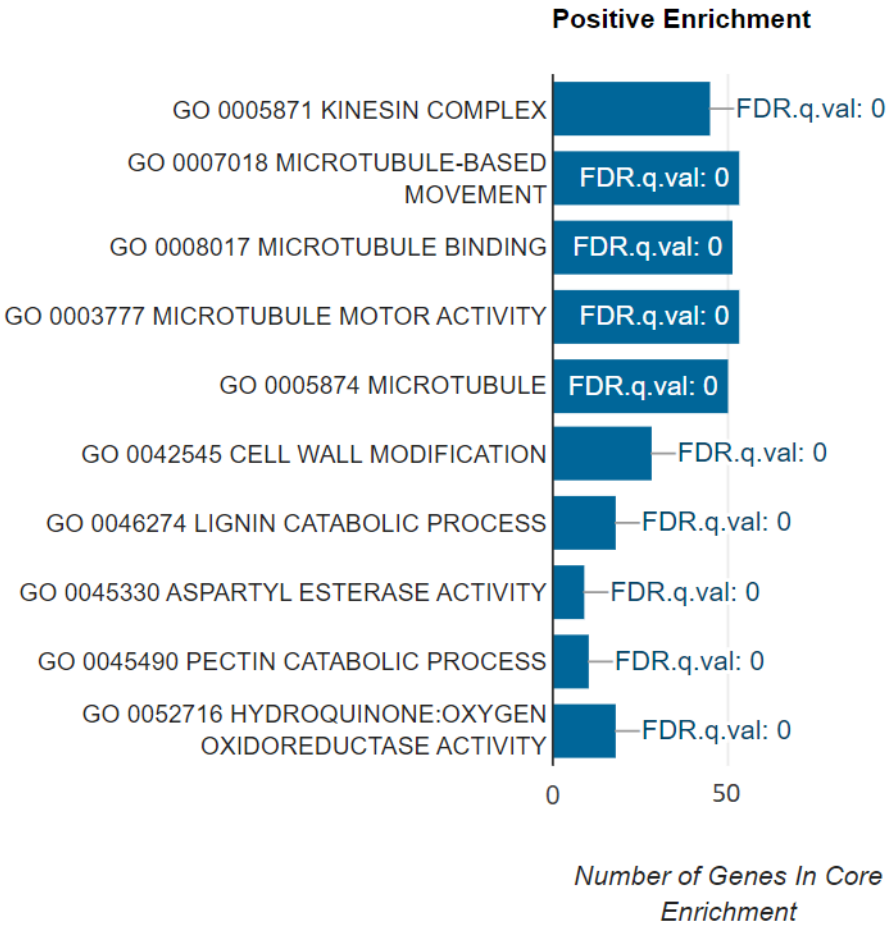
Appendix C: A nickel-resistant (left), moderately susceptible (middle) and nickel-susceptible (right) *P. tremuloides* seedling on day 7 after treatment with 1,600 mg/kg nickel nitrate.

Damage Rating : **1**
No visibile toxicity symptoms

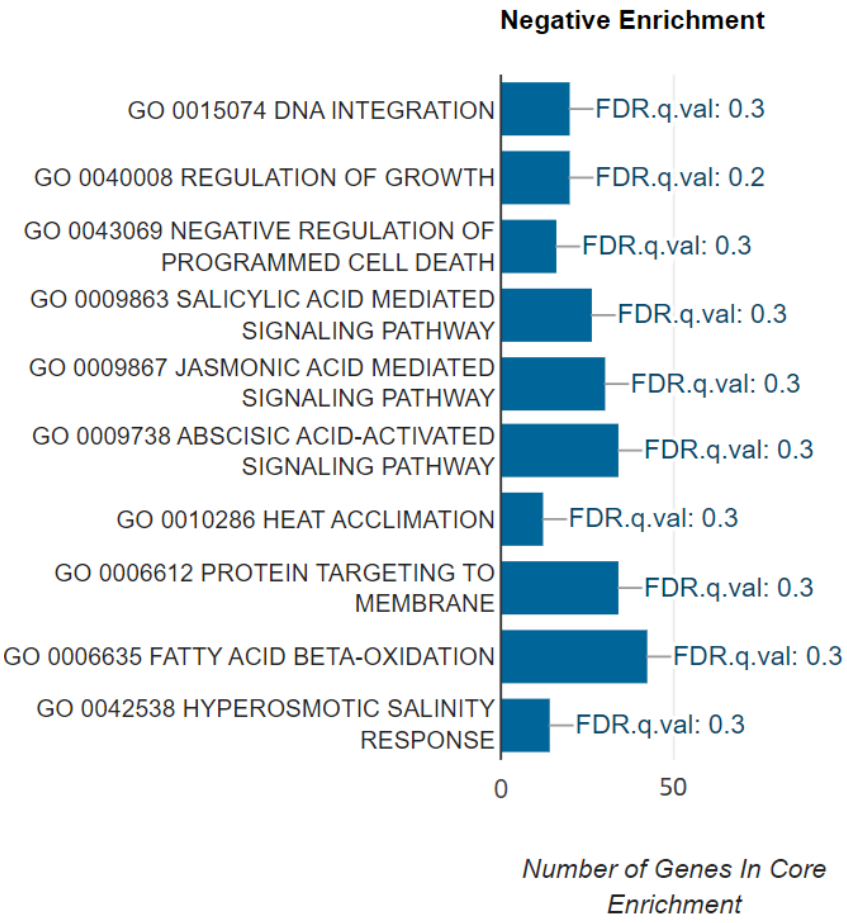
Damage Rating : **4**

Damage Rating : **9**
Death

Appendix D: Top enriched GO terms from GSEA for upregulated (positive) genes in nickel resistant genotypes when compared with nickel susceptible genotypes.



Appendix E: Top enriched GO terms from GSEA for downregulated (negative) genes in nickel resistant genotypes when compared with nickel susceptible genotypes.



List of Publications

1) Czajka, K. M., Michael, P., & Nkongolo, K. (2019). Differential effects of nickel dosages on in vitro and in vivo seed germination and expression of a high affinity nickel-transport family protein (AT2G16800) in trembling aspen (Populus tremuloides). *Ecotoxicology, 28*(1). https://doi.org/10.1007/s10646-018-2003-8

2) Czajka, K. M., & Nkongolo, K. (2018). High level of nicotianamine synthase (NAS3) and natural resistance associated macrophage protein (NRAMP4) gene transcription induced by potassium nitrate in trembling aspen (populus tremuloides). *American Journal of Biochemistry and Biotechnology, 14*(3). https://doi.org/10.3844/ajbbsp.2018.183.190

3) Czajka, K., Mehes-Smith, M., & Nkongolo, K. (2022). DNA methylation and histone modifications induced by abiotic stressors in plants. *Genes and Genomics* (Vol. 44, Issue 3). https://doi.org/10.1007/s13258-021-01191-z

4) Czajka, K. and Nkongolo, K. (2022). Transcriptome Analysis of Nickel - Resistant and Susceptible Trembling Aspen (P. tremuloides) genotypes. *Ecology and Evolution*. Under Review.

5) Czajka, K. and Nkongolo, K. (2022). Whole genome expression analysis of Populus tremuloides genotypes exposed to increasing doses of nickel. *Plant Gene*. Under Review.

6) Czajka, K. and Nkongolo, K. (2022). Potassium ions induce DNA hypomethylation in Populus tremuloides. *Applied Genetics*. Under Review.

Conferences/ Poster Presentations

1) Czajka, K., & Nkongolo, K. (2017). Response of trembling aspen (Populus tremuloides) to nickel toxicity: analysis of gene expression. Presented at the American Society of Agronomy, Crop Sciences Society of America, and Soil Sciences Society of America, Annual Meeting held in Tampa, Florida, October 22 -25. Abstract # 105622

2) Czajka, K., Michael, P., & Nkongolo, K. (2019). Response of trembling aspen (Populus tremuloides) to nickel toxicity: analysis of seed germination and gene expression. Presented at the Poster Session at the Mining and the Environment Conference. Meeting held in Sudbury, Ontario, June 24-26.

3) Czajka, K., & Nkongolo, K. (2021). Whole-Genome Expression Analysis of Trembling Aspen (Populus tremuloides) Exposed to Nickel Stress. Presented at the American Society of Agronomy, Crop Sciences Society of America, and Soil Sciences Society of America, Annual Meeting held in, Salt Lake City, Utah, November 7-10. Poster Board # 1286.