Chapter 25

The ecophysiology, genetics, adaptive significance, and biotechnology of nickel hyperaccumulation in plants

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25.1 Introduction

Plants are challenged by various environmental stressors that interfere with their biochemical and physiological processes. Some heavy metals have critical physiological roles [1–3] in plants, but elevated concentrations of these essential heavy metals or exposure to other heavy metals, which lack any known physiological roles, may result in stress via interference with either the function of enzymes or information-coding DNA or RNA in cells [4–6]. Some plants are more metal-tolerant than others, and thus heavy metal tolerance has been central to understanding adaptive evolution in plants [7–12] as well as the development of biotechnological fields such as phytoremediation and phyto-mining [13–16].

Some soils, including serpentine soils [17], have naturally high levels of heavy metals, and plants that can withstand these metal-rich soils are known as metallophytes [18,19]. Metallophytes tolerate metals via exclusion or accumulation. Most metallophytes exclude metals by chelation, binding ions to organic acids or other ligands, or by sequestration, storing metals within vacuoles of root cells where they cannot interrupt key cellular processes [20]. Accumulators, on the other hand, concentrate metals in plant parts, especially the epidermal tissues of leaves, whereas excluders generally keep shoot metal levels consistently low over a wide range of soil metal concentrations. Some metallophytes, termed “hyperaccumulators” [21–23], take up and sequester considerably high concentrations of metals in their aboveground tissues, often beyond thresholds that would be lethal to most plants [3]. Among these metal-hyperaccumulating plants, those that take up nickel (Ni) have received much attention both in terms of basic and applied research [20,24–26].

Plants require Ni as a micronutrient for nitrogen (N) and plant antioxidant metabolism [27–29]. Urease (EC 3.5.1.5, urea amidohydrolase) is perhaps the most important of the several known Ni requiring enzymes in higher plants [30]. Nickel functions as a cofactor to enable urease to catalyze the conversion of urea into ammonium (NH₃), which plants can use as a source of N. Thus, urea conversion is impossible without Ni. Nickel-deficient plants develop leaf chlorosis and leaf tip necrosis [31], symptoms that can be prevented by Ni application, which increases leaf urease activity and prevents urea accumulation [32]. Thus, in generally N-poor soils such as serpentine [33], Ni may be particularly important for N acquisition and metabolism.

The effects of Ni on Ni-hyperaccumulating plants (i.e., those that take up >1000 µg Ni g⁻¹ dry leaf tissue) [22] have not received much attention beyond the well-studied phenomenon of the role of Ni in plant defense against pathogens and herbivores [34,35]. Enhanced growth [36–39] and increased flowering [40] of some metal hyperaccumulator plants in the presence of higher Ni concentrations have previously been reported, but no physiological mechanisms have been suggested. The growth-stimulating effect may stem from direct beneficial effects of Ni on N metabolism or from indirect effects resulting from a potential role of Ni-containing urease in supporting plant pathogen defense [41]. Plants that hyperaccumulate Ni are equipped with physiological mechanisms for both increased uptake and tolerance. For example, Ingle et al. [38] report constitutively high expression of the histidine biosynthetic pathway in the Ni-hyperaccumulating Alyssum lesbiacum (Brassicaceae). Nickel hyperaccumulation is a worldwide phenomenon...
spanning a vast diversity of higher plant families and taxa native to many metal-enriched ultramafic habitats [42–45] and is presumably mediated by the primary Fe\(^{2+}\) or Zn\(^{2+}\) transporters found within plant roots [46,47].

This chapter highlights current knowledge of the physiological mechanisms of Ni uptake, tolerance, and hyperaccumulation in plants and their underlying genetic bases and discusses the use of Ni-hyperaccumulating plants in biotechnological endeavors, especially in the use of plants to remediate Ni-contaminated soils (i.e., phytoremediation) and to mine Ni (i.e., phytomining/agromining).

25.2 Physiology: mechanisms of Ni uptake, translocation, chelation, and storage

Nickel is an essential micronutrient; however, it is toxic to most plants even at low concentrations [48–50]. The physiological mechanisms underlying metal accumulation are not fully understood, but recent studies have begun to tease apart the ways in which some species are able to tolerate and accumulate Ni [26,49]. Hyperaccumulation begins below ground in the rhizosphere, where Ni is taken up into roots from the soil. The bioavailability of soil Ni depends on the interaction of various chemical, physical, and biological processes in the soil. Low pH appears to be critical for making metals such as Ni more bioavailable [51,52] and some hyperaccumulating species secrete protons [53] to acidify the rhizosphere, thereby increasing Ni bioavailability [54]. Soil bacteria may also contribute to this process by releasing compounds that make metals more bioavailable for plant uptake [55,56]. Once Ni is bioavailable and is taken up into a root, it is quickly chelated by various amino acids and organic acids and translocated until it can be properly stored, all while moderating the ion’s toxic effects on cellular processes [20,26,49].

25.2.1 Uptake

The first step in the hyperaccumulation process is uptake of Ni from soil by roots. Nickel appears to be taken up primarily as Ni\(^{2+}\), and a significant decrease in Ni uptake by *Berkheya coddii* (Asteraceae; Fig. 25.1H) has been reported upon addition of chelating agents to soil that reduce bioavailable Ni\(^{2+}\) concentration [57]. Nickel uptake has been shown to increase under more acidic (low pH) conditions and decrease under more basic conditions [49], likely as a result of increased Ni solubility as H\(^+\) concentrations rise [52,58,59]. Uptake as Ni\(^{2+}\) is important, as soluble Ni can enter roots by coopting uptake mechanisms of cations of similar charge and size (such as Zn\(^{2+}\) or Fe\(^{2+}\)). For example, serpentine endemic Ni accumulators have been found to preferentially accumulate Zn\(^{2+}\) in the presence of both Ni and Zn [60,61]. This suggests that a high-affinity transport mechanism for Zn is also used to take up Ni into root cells as opposed to Ni-specific transport mechanisms. Similarly, Fe\(^{2+}\) transporters have been implied in Ni uptake [46,47]. In *Arabidopsis thaliana* (Brassicaceae), Ni is absorbed via the Fe uptake system, which is mediated by the iron-regulated transporter 1 (IRT1), the protein responsible for the primary Fe uptake mechanism in roots [47]. Excess Ni accumulation results in the induction of IRT1 expression, suggesting that the first stage of Ni accumulation may be facilitated by IRT1. Similarly, recent work on hyperaccumulators *Odontarrhena bracteata* and *O. inflata* (Brassicaceae) found that Ni uptake in *O. inflata* could be induced by Fe [62], suggesting a link between uptake of both metals. However, more work is necessary to fully understand the mechanisms of Ni uptake and the relationship between the accumulation of Ni and other divalent cations.

25.2.2 Chelation

After Ni is brought into the root, it must be quickly chelated and complexed in order to reduce its toxic effects [39,63,64]. This appears to be done by both amino acids and organic acids [20] with the former acting primarily in roots and shoots and the latter acting primarily in leaf and shoot tissues [65,66]. Numerous studies have established free histidine as a primary chelating agent for Ni in root tissue [38,50,67,68]. Free histidine concentration in roots was found to be greater in an accumulator species, *A. lesbiacum*, compared to that of a nonaccumulator species, *Brassica juncea* (Brassicaceae; [66]). In addition, an increase in free histidine concentration was positively correlated with increased uptake of Ni in *A. lesbiacum* [66]. Histidine was also found to increase the mobility of Ni in roots of *Noccaea caerulescens* (Brassicaceae; Fig. 25.1D) to make it more available for xylem loading and radial transport [50]. Histidine biosynthetic pathways were also found to be more active in the Ni hyperaccumulator *A. lesbiacum* than in a nonaccumulating congener *A. monatum* [38]. This strongly suggests an important role for histidine in the chelation and detoxification of Ni in roots. Similarly, nicotinamine, an amino acid similar in structure to histidine, may play a role in the process of Ni chelation in hyperaccumulators [69,70]. In *N. caerulescens* (Fig. 25.1D), Ni exposure triggers the accumulation of nicotinamine in roots and subsequent formation of Ni—nicotinamine complexes within the xylem sap, both of which did not occur when...
nonaccumulator *Thlaspi arvense* (Brassicaceae) was exposed to Ni, strongly suggesting that nicotinamine may act as a ligand in Ni chelation and transport in some hyperaccumulators [70]. Further, in *N. caerulescens* (Fig. 25.1D), a strong positive correlation between concentration of Ni and nicotinamine in leaf tissues was also observed, in addition to a negative correlation between foliar Ni and Fe concentrations, suggesting that Ni and Fe may compete for chelation by nicotinamine [71]. There is also evidence that organic acids are the primary complexing agents within leaf and stem tissue in some accumulators. Montarges-Pelletier et al. [63] used X-ray spectrography to demonstrate that across three hyperaccumulating species of Brassicaceae, *Leptopax emarginata, Odontarrhena chalcidica* (Brassicaceae; Fig. 25.2B), and *N. caerulescens* (Fig. 25.1D), malic acid was the main ligand for Ni in leaf tissue, while in stem tissue citric acid was the primary ligand. In the South African hyperaccumulator *Berkheya coddii* (Fig. 25.1H), chelidonic acid was found to be the major chelating agent of Ni [72]. Nickel is also often unchelated while moving through nonliving xylem cells; Alves et al. [73] found that the primary species of Ni found in xylem sap of the hyperaccumulator *Alyssum serpyllifolium* subsp. *lustianicum* (Brassicaceae) was hydrated Ni$^{2+}$. This shows that there is much variability in the form of Ni within a plant and along the steps of Ni translocation. Unlike hyperaccumulators of other metals, such as cadmium and zinc, Ni hyperaccumulators do not use peptides such as phytochelatins or metallothionines (see Ref. [74] for a review on phytochelatins and for metallothionines see Ref. [75]) to complex and detoxify Ni. This lack of specific peptide chelators, paired with Ni’s coopted, low-affinity import mechanism, illustrates how the Ni hyperaccumulation process may not be a Ni-specific gene-driven phenomenon [47,60,61].

### 25.2.3 Transport

Another important aspect of the hyperaccumulation process is how the Ni is moved from roots through shoots to leaf epidermal cells. There is contrasting evidence for the relative importance of translocation in hyperaccumulation.
Some studies have found transport proteins to be among those consistently overexpressed in hyperaccumulators [77]. In contrast, research on *Thlaspi goesingense* (Brassicaceae) found that Ni translocation rates from roots to stem xylem were conserved between accumulators and nonaccumulators [77]. The process of Ni translocation between cell types appears to be another example of Ni coopting existing metal transport mechanisms, as there is evidence that Ni may be transported via the same efflux proteins used for Fe and Zn transport [78,79]. Some candidate transporters are in the Zrt/Irt-like proteins (ZIP) and NRAMP families, which are known to be low-specificity transport proteins for Zn and other transition metals [80]. There is evidence for root-to-shoot translocation of Ni being mediated by Fe-transporter in *Alyssum* species, as Fe deficiency appears to increase concentrations of Ni found in aboveground tissues [62]. The final step in Ni transport is moving the ion from the cytoplasm of a sink cell into the vacuole for storage. It has been shown in *Alyssum lesbiacum* that Ni may be pumped into the vacuole using secondary active transport through an H⁺/Ni²⁺ antiporter driven by a proton gradient created by V-ATPase proton pumps [81]. This could be an example of a Ni-specific transport mechanism, but the exact protein has yet to be characterized. Further research into the action and regulation of these proteins, and the genes that encode them, could reveal the nature of Ni transport, homeostasis, and detoxification [24].
25.2.4 Localization and storage

The final steps in the physiological processing of Ni by plants are localization to specific sink tissues and storage of Ni within these cells. There is evidence that during the localization process, hyperaccumulators transport Ni through phloem tissue, as visualized by X-ray analysis of *Senecio coronatus* (Asteraceae) roots [82] and phloem sap analysis of *N. caerulescens* (Fig. 25.1D), which shows bidirectional transport of Ni chelated with malate [83]. In addition, work on hyperaccumulating trees from Borneo, *Rinorea bengalensis* (Violaceae; Fig. 25.3) and *Phyllanthus balgooyi* (Phyllanthaceae; Fig. 25.4), also has shown Ni to be present in high concentrations in phloem sap (Figs. 25.3 and 25.4), 7.9% and 16.9%, respectively, and it likely is chelated with citrate [84,85,86]. Nickel mobility in phloem is also supported indirectly by research that shows the presence of Ni in flowers and seeds [87–90]. Reproductive tissues are major phloem sinks and the elevated Ni concentrations found there implicate the phloem as an important sink. Further, the ecological implications of Ni localization in leaves have led to numerous studies on multiple hyperaccumulating species. These studies have indicated that Ni is primarily localized in vacuoles and cell walls of leaf epidermal cells [82,91]. *Streptanthus polygaloides* (Brassicaceae [92]; Fig. 25.2C) and *O. chalcidica* (Fig. 25.2B) tissues were analyzed using scanning electron microscopy and energy-dispersive X-ray probing and were shown to have the greatest Ni concentration in leaf epidermal cell vacuoles, suggesting a role for Ni in herbivory or pathogen defense ([94]; see the following sections on adaptive significance of Ni hyperaccumulation). Similar studies on the hyperaccumulator *T. goesingense* have also provided evidence for Ni storage in leaf epidermal cell walls and to a lesser extent in vacuoles, complexed with citrate and histidine [84,85]. Similarly, energy-dispersive X-ray microanalysis of *Thlaspi montanum* var. *siskiyouensis* (Brassicaceae; now *Noccaea fendleri* subsp. *siskiyouensis*) shows Ni localization in subsidiary cells that surround guard cells, but not in guard cells or in other more elongated epidermal cells [91]. Cell walls, cuticles, and epidermal trichomes can also store high concentrations of Ni (e.g., [77,78,88,95,96–98]). Furthermore,

**FIGURE 25.3** Phloem tissue of *Rinorea bengalensis* (Violaceae; Malaysia) contains over 4% Ni. Plant (top left), leaves (top right), and piece of bark showing Ni-enriched phloem (bottom). Photo courtesy of Antony van der Ent.
Ni enrichment has been recorded in leaf tips, likely due to secretion of excess Ni via guttation [99], as well as in latex (e.g., *Pycnandra acuminata*, Sapotaceae [100] Fig. 25.4). Once Ni is stored in a manner that limits its toxic and oxidative effects, plants use Ni for a variety of biological functions [101], some of which may increase overall fitness as discussed in the following section on the adaptive significance of Ni hyperaccumulation.

25.3 Why hyperaccumulate nickel?

The ubiquity of Ni hyperaccumulation poses an interesting question about the evolutionary factors driving this unusual phenomenon. Since the “discovery” of Ni hyperaccumulation in 1948 [102], numerous hypotheses have been brought forth to explain the adaptive significance of Ni hyperaccumulation in plants, given that this process is energetically costly and that plants exhibiting this trait risk effects of Ni toxicity [103,104]. There is currently little consensus on the adaptive significance of Ni or other metal hyperaccumulation on a wide evolutionary scale; however, four main hypotheses have been presented with mixed experimental evidence, each stating that Ni hyperaccumulation plays a role in one or more of the following phenomena: elemental defense, nutrition, elemental allelopathy, and drought tolerance [34,105–107].

25.3.1 Elemental defense

Thus far, the prominent hypothesis for the adaptive significance of Ni (and other metal or metalloid) hyperaccumulation in plants is the elemental defense hypothesis, which states that intracellular storage of high concentrations of heavy metals can prevent, deter, or provide resistance against unadapted pests, pathogens, and herbivores, which may feed on hyperaccumulators [94,105,108]. There is experimental evidence demonstrating deterrence of herbivory by multiple hyperaccumulating taxa, notably *S. polygaloides* ([109] Fig. 25.2C), *Alyssum* spp. [110], and *Berkheya coddii* ([35,42,111] Fig. 25.1H). The
evolutionary and physiological mechanisms behind the utilization of Ni toxicity for defense against herbivory vary by taxon; however, two main explanations describe the relationship between Ni concentration and plant tissue toxicity. According to the defensive enhancement hypothesis, greater concentrations of Ni provide more effective defense from herbivory, which provides selective pressure to hyperaccumulate Ni in higher quantities \[94,108\]. Alternatively, as described by the joint effects hypothesis \[112\], some plants produce organic molecules that can enhance the effect of Ni toxicity in either additive or synergistic ways. For example, the presence of nicotine enhances the toxicity of Ni, resulting in higher deterrence than either of the substances alone \[113\]. Therefore, plants have two main strategies for increasing the toxicity of their tissues for purposes of elemental defense: accumulate more metals or synthesize chemicals with joint effects. Despite the potential toxic effects of heavy metals and other plant-produced chemicals, many specialist insects exist which feed on hyperaccumulating plants. One well-studied example is Melanotrichus boydi (Hemiptera: Miridae), a monophagous specialist herbivore on the Ni hyperaccumulator S. polygaloides, which, in host-choice studies, consistently prefers S. polygaloides over other nonhyperaccumulating plants found in its native range \[114\]. M. boydi sustains an elevated body Ni concentration of \(>700 \mu g \text{ Ni g}^{-1}\) \[115\]; however, there is little evidence that this trait offers the plant bug significant defense against its predators or pathogens. Boyd and Wall \[116\] demonstrated that Ni can be transferred up the food chain from high-Ni content insects to predators, resulting in moderate Ni accumulation in the third trophic level. However, predators varied in reaction to elevated body Ni concentration resulting from consumption of M. boydi, and only the crab spider Misumena vatia (Araneae: Thomisidae) showed adverse effects, resulting in significantly decreased survivorship when fed M. boydi, in comparison to Ni-absent, control diets. Thus, in this case, elevated body Ni concentration in a monophagous herbivore is most likely a consequence of consumption of high-Ni plant tissues, rather than a trait specifically selected for the purpose of defense \[117\]. Boyd et al. \[118\] demonstrated decreased susceptibility to two pathogens; a fungus (Alternaria brassicicola) and a bacterium (Xanthomonas campestris) in high-Ni-containing S. polygaloides, suggesting Ni can act as an antimicrobial agent in leaf tissues. In addition, Ni-treated Alyssum spp. show resistance to fungi Pythium mamillatum and P. ultimum, which cause damping-off disease of seedlings \[119\]. In both instances, plants grown on Ni-amended soil consistently outperformed those grown in the absence of Ni. Although these results are promising, more research is required to elucidate the relationship between Ni hyperaccumulation and resistance to pathogens commonly found in a given plant’s native range \[120\].

### 25.3.2 Nutritional demand

The nutritional demand hypothesis suggests that hyperaccumulating species have a physiological requirement for high levels of Ni, due to the extensive use of Ni in certain biochemical pathways. Nickel is required as a micronutrient in most plants \[101\] and Ni deficiency can result in leaf chlorosis and leaf tip necrosis \[31\]. Nickel-hyperaccumulating plants maintain tissue concentrations of the metal high above the sufficient amount for most plants, in quantities indicative of macronutritional demand. The presence of Ni has been demonstrated to enhance flowering of the hyperaccumulator Alyssum inflatum (Brassicaceae; \[40\]); however, the biochemical pathway responsible has yet to be elucidated. Nickel is used as a cofactor in multiple plant enzymes, most importantly in urease, which converts urea to ammonium, thereby making nitrogen (N) available to a plant \[30,41\]. Since serpentine soils that host these hyperaccumulator plants are generally N-poor \[33\], it is possible that the elevated Ni requirement is due to greater urease activity. Experiments on soybeans show that Ni supplementation improves the growth of urea-fed plants while having no apparent effect on nitrate-fed plants \[121\], suggesting that higher Ni concentration could be a result of greater levels of N fixation from urea. Nickel accumulation can also interfere with the uptake and homeostasis of other heavy metals such as copper and iron \[78\], because each of these ions have similar chemical properties and often share a transporter protein. Thus, due to its interaction with multiple micronutrients and macronutrients, Ni may be required in large quantities to regulate tissue concentrations of key elements found in serpentine soils.

### 25.3.3 Elemental allelopathy

Since the high shoot Ni concentrations common in hyperaccumulators are much higher than can be tolerated by most intolerant plants without experiencing Ni toxicity, it is possible that Ni deposition in surface soil could result in the deterrence or toxicity of intolerant plants, conferring a competitive advantage for hyperaccumulators. Zhang et al. \[122\] assessed the effects of high-Ni leaves shed by O. chalcidica by measuring both the change in soil Ni concentration over time and the germination rate of natural competitors in Ni-enriched soils. Results showed that, while Ni was quickly released from O. chalcidica biomass causing an increase of phytoavailable Ni in the soil, Ni concentration also rapidly decreased due to chelation by Fe and manganese (Mn) oxides and silicates, which are both commonly found in serpentine soils. Addition of O. chalcidica biomass, moreover, did not demonstrate any significant effect on seed germination
of eight different competing herbaceous species. Thus, these results do not support the hypothesis that elemental allelopathy via leaf litter provides a competitive advantage to hyperaccumulators via direct toxicity to competitors. It is possible that tissue deposition can result in allelopathy via phytoenrichment of the soil, which alters the microbial community and the resulting fitness of a competing plant species [123]. A study of *S. polygaloides* (Fig. 25.2C) and nonendemic serpentine congener *S. tortuosus* (Brassicaceae) showed that the latter experienced decreased germination rates and fruit development in high-Ni soil, while *S. polygaloides* had its greatest reproductive success in high-Ni soil [87]. Studies that aim to draw cause–effect relationships between leaf litter of hyperaccumulators and soil Ni phytoenrichment have produced mainly speculative results, however, because it is difficult to demonstrate that leaf litter is directly causing soil Ni enrichment, as opposed to plants being predisposed to grow in naturally Ni-enriched soils [124,125]. However, a recent study by Adamidis et al. [126] shows that Ni-resistant decomposers in serpentine soil contribute to the accelerated breakdown of high-Ni plant litter, suggesting a potential selective advantage for the metal-hyperaccumulating plants through litter decomposition on serpentine soils.

High-Ni concentration in floral tissues can cause Ni hyperaccumulators to host distinct floral visitor communities by selecting for specific pollinators (i.e., *elemental filter hypothesis* [127]). It is hypothesized that transfer of Ni to intolerant plants via pollinators carrying floral offerings, such as pollen, could lead to elemental allelopathy [128], as Ni is known to cause detrimental effects on plant reproductive systems [87]. However, thus far, there has been no support for elemental allelopathy via hetero-specific pollen transfer from Ni hyperaccumulators to Ni-intolerant plants [128].

### 25.3.4 Drought tolerance

Serpentine soil, by virtue of its shallow depth, poor soil structure, and high porosity can maintain exceptionally low water content at field capacity. Thus, serpentine soils in Mediterranean climates are subject to prolonged drought conditions, and there is a significant selective pressure for drought tolerance among serpentine-tolerant plants [129]. Since Ni often is stored in the leaf epidermis, including in guard cells of stomata [92], some researchers have hypothesized that Ni may act as an osmoticum for metal hyperaccumulators, adjusting the water potential of the plant so that it is able to maintain a favorable water potential gradient under dry conditions [105]. Studies of the Ni hyperaccumulator *Stackhousia tryonii* (Stackhousiaceae) showed enhanced Ni hyperaccumulation with increasing levels of drought stress, suggesting a possible role for Ni in osmotic adjustment [130]. A similar study investigating the effects of polyethylene glycol (PEG)-simulated drought on *Cleome heratensis* (Cleomaceae) reported that Ni exposure was necessary for optimum performance in drought conditions; however, it was unclear if this was an adaptation to Ni or drought [131]. Whiting et al. [132] stated that there was no strong evidence that Ni hyperaccumulation augmented drought tolerance for *O. chalcdica* (Fig. 25.2B) and that metal concentration had little effect on the rate of evapotranspiration or the osmolality of leaf-sap extracts obtained from the plant. Similarly, experiments on *Hybanthus floribundus* (Violaceae), a nickel-hyperaccumulating shrub violet, failed to elucidate a role of Ni in osmotic adjustment [133]. Investigations into the effects of other hyperaccumulated elements, such as zinc (Zn) or selenium (Se), on drought tolerance have failed to produce solid evidence that elemental hyperaccumulation results in a physiological advantage under dry conditions [134]. Zinc hyperaccumulation has no significant effect on drought tolerance in *N. caerulescens* at the whole-plant level, and most evidence shows that Zn accumulation is constitutively expressed, rather than activated as a stress response [132]. However, Hajiboland et al. [135] demonstrated that Se-treated wheat exhibited higher hydraulic conductivity than control groups, suggesting an improvement of water uptake capacity.

### 25.4 Genetics of nickel accumulation

Although many of the physiological mechanisms driving the process of Ni hyperaccumulation have been coopted from metal uptake and tolerance mechanisms known for metals such as Zn and Fe [46,78–80], the heritability and selection mechanisms for the hyperaccumulation trait also imply an underlying genetic basis. A number of techniques have been utilized to better understand the genetics of hyperaccumulation [102,136,137], including comparative population genome sequencing [138,139], identification of target genes from key proteins involved in hyperaccumulation ([80,136] Table 25.1), and phylogetic analyses [140–143]. In the future, continued experimentation, using RNA sequencing and mutated or knocked out target genes [77], will be extremely valuable in elucidating the ways in which certain genes and gene products contribute to the hyperaccumulation phenotype [24].
25.4.1 Identification of target genes involved in Ni hyperaccumulation by transporters

A primary area of research on the genetics underlying Ni hyperaccumulation involves the identification and characterization of target genes that directly or indirectly influence a plant’s ability to tolerate, transport, and accumulate Ni. One of the key distinctions between hyperaccumulators and metal-tolerant species is the tendency for hyperaccumulators to localize heavy metals in leaf epidermal cells, whereas metal-tolerant species tend to exclude metals from aboveground tissue [20]. This implies an important role for transport proteins in facilitating root-to-shoot translocation of heavy metals, as well as the characterization of the hyperaccumulation phenotype [144]. Membrane proteins such as IRT and ZIP have been shown to play important roles mediating Ni import and translocation [46,47]. Proliferation of next generation sequencing technologies and genetic manipulation tools is making it easier than ever for researchers to locate impactful genes. For example, a study of the genomes of *A. serpyllifolium* populations found on and off of serpentine soil shows high DNA polymorphism at loci encoding natural resistance-associated macrophage proteins (NRamp) and iron-regulated (IREG) transporter proteins, which are known to influence Ni tolerance [138]. This presents a potential example of local genetic adaptation, as opposed to phenotypic plasticity, resulting in Ni hyperaccumulation. A recent cross-species analysis of hyperaccumulator RNA sequences revealed that, across five genera, high IREG/ferroportin transporter expression was a conserved quality [77]. In addition, the study quantified the necessity of the *NcIREG2* gene for hyperaccumulation in *N. caerulescens* (Fig. 25.1D), as transgenic plants with reduced expression of *NcIREG2* had decreased Ni accumulation. The continued engineering of transgenic hyperaccumulator models will be a vital study tool in the future for understanding the genetic mechanisms of Ni hyperaccumulation.

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<th>Table 25.1 Known genes and gene products associated with Ni hyperaccumulation.</th>
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<td><strong>Gene</strong></td>
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<td>Tjznt1, Tjznt2</td>
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<td>TjNramp4</td>
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<td>PgIREG1</td>
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<td>NcIREG2</td>
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Most known genes that are vital to the process of Ni hyperaccumulation are either (1) ion transporters, which assist in the uptake of Ni from the environment or (2) involved in the biosynthesis and feedback pathways of Ni-chelating molecules.
Most genetic studies to date have utilized model organisms such as *Arabidopsis* and yeast to find influential DNA sequences or proteins and subsequently look for homologs and measure their expression activity in the hyperaccumulator in question. Alternatively, others have examined genes from accumulators and tested their expression patterns in model organisms. While a Ni-specific transport protein has yet to be discovered [8], most studies of Ni hyperaccumulation genetics have focused on integral membrane metal transporter proteins. For example, in *Thlaspi japonicum*, three genes presumed to be Ni transporters have been cloned [80]: ZIP transporters TjZnt1 and TjZnt2, and an NRamp transporter gene, all of which were homologous to genes known to influence metal transport in other species. When yeast were transformed with these genes and exposed to Ni\(^{2+}\), the yeast expressing ZIP transporters showed increased tolerance to Ni while those transformed with an NRamp transporter showed an increase in Ni concentration and sensitivity, suggesting a role for these proteins and genes in Ni accumulation [8]. Similar methods were used on *Psychotria gabriel-lae* (Rubiaceae) to determine if PgIREG1, which encodes an IREG family metal transporter protein that is thought to be responsible for sequestration of metal into vacuoles, was a candidate gene influential in Ni accumulation [136]. The authors observed a two and a half times higher rate of expression of PgIREG1 in *P. gabriellae* than in a nonaccumulating congener, *P. semperflorens*. In addition, a Yellow Stripe-Like (YSL) transporter family gene in *Thlaspi caerulescens* (syn. *N. caerulescens*; Fig. 25.1D), TcYSL3, increased Ni/Fe-nicotinamide chelate uptake in yeast, indicating TcYSL3 expression may serve as a key phenotype in metal uptake and accumulation in *T. caerulescens* [145]. A comparison of expression levels of many different genes across different ecotypes of the same species found a number of candidate genes that could be impacting accumulation [146], the most significant genes being those encoding HMA3 and HMA4 (both ATPase transporters) as well as the genes coding for IRT1 and ZIP10 proteins.

Metal tolerance protein (MTP) family cation efflux transporters are also important in conferring Ni tolerance. In *T. goesingense*, enhanced expression of the TgMTP1 gene, producing the TgMTP cation efflux protein, was observed under hyperaccumulation and also was found to confer Ni tolerance in yeast, suggesting a role for the gene in Ni accumulation [147]. Further, two genes from *B. juncea* encoding MTP family proteins, BjCET3 and BjCET4, were found to contribute to Ni homeostasis in yeast cells, suggesting that cation efflux transporters (coded for by BjCET genes) may aid in Ni localization and tolerance [148]. Also, in *B. juncea*, the gene encoding another YSL family transporter protein (BjYSL7) was upregulated manyfold upon the addition of NiCl\(_2\) to the soil solution, implying a role for BjYSL7 in the import and storage of Ni. Taken together, these studies show that much of the genetic regulation for Ni hyperaccumulation relies on regulation of transport proteins. However, transport is only one part of the accumulation process, and additional genes and proteins influencing the process of hyperaccumulation have also been identified.

### 25.4.2 Identification of target genes involved in Ni hyperaccumulation by chelators

While much research has focused on identifying target genes for metal transport proteins, some research has been directed at elucidating the expression pathways for known chelators of Ni. For example, in *N. caerulescens* (Fig. 25.1D), the nicotinamide synthase gene (TcNAS) was important in Ni chelation to nicotinamide by producing nicotinamide in leaves and facilitating its transport to roots, although TcNAS expression did not change in response to Ni addition [70]. Studies on *Arabidopsis* to investigate the histidine biosynthetic pathway [149] identified eight different enzymes important in histidine synthesis, including HISN1, which is normally inhibited by histidine. However, transgenic *A. thaliana* mutants that constitutively produce HISN1 exhibit elevated levels of free histidine as well as significantly greater tolerance to Ni, suggesting an important role for HISN genes in regulation of Ni uptake and tolerance [38]. Although most studies on Ni hyperaccumulation have found little to no link with phytochelatins, in *Rauvolfia ser- pentina* (Apocynaceae) culture, expression of the short peptide and phytochelatin (y-Glu-Cys)\(_{2-4}\)Gly could be induced via introduction of Ni [150]. This suggests that Ni may directly influence phytochelation expression in some plants; however, more work needs to be done to firmly establish a broader importance of phytochelatins in Ni tolerance. Another interesting biochemical pathway involved in Ni hyperaccumulation is glutathione (GSH) synthesis. GSH is an antioxidant believed to be important in the process of decreasing the oxidative stress commonly induced by plant uptake of heavy metals [151,152]. In *T. goesingense* and transgenic *Arabidopsis*, GSH concentrations increased in response to addition of Ni [153]. Serine acetyl-transferase (SAT), which produces glutathione, was found to have greater activity in response to Ni, and plants overexpressing the gene encoding SAT and TgSAT-m had increased Ni tolerance, suggesting that there is an important genetic component to GSH-mediated Ni tolerance.

### 25.5 Phytoremediation and agromining

Due to their highly unusual physiology, metal hyperaccumulators hold the potential for exciting new methods of eco-friendly mining and bioremediation [154]. Ni-hyperaccumulating plants are being used for phytoextraction (for reviews
see Refs. [155–157]), which involves growing hyperaccumulators on metalliferous soils and subsequently removing the plant biomass from the site, either with the goal of harvesting valuable metals (i.e., phytomining or agromining) [154,158] or to clean metal-contaminated soils (i.e., phytoremediation) [157]. Nickel hyperaccumulators are excellent candidates for phytoremediation and agromining because Ni has relatively high monetary value in today’s market, it can be efficiently extracted from ash [159,160], and a number of Ni hyperaccumulators have sufficiently high tissue Ni concentrations to potentially support the economic viability of the practice [97,161,162].

It is hypothesized that long-term cropping of Ni hyperaccumulators can gradually detoxify mildly to moderately Ni-contaminated soils [163,164] and thus make such soils available for other agricultural uses. The time frame required for ample phytoextraction of heavy metals resulting in rehabilitation of contaminated soils is referred to as the “extraction period,” and varies largely depending on the concentration of metals in the soil and efficiency of extraction [165]. The main limitations to practical use of hyperaccumulators for phytoremediation relate to long extraction periods [166] and the costs of operation for these potentially multidecade endeavors. Successful long-term phytoremediation projects rely on income from biomass yield and element recovery to cover the cost of crop maintenance [167] and for this reason, soil remediation is often considered an advantageous by-product of agromining. Since both processes aim to extract toxic metals from the soil with high efficiency and most recent literature emphasizes the viability of commercial agromining with soil cleanup as a potential consequence, we will mostly focus on the logistics of agromining in this chapter.

In conventional mining practices, ores are extracted from the earth and then smelted to yield pure metals. Since this is an energetically and monetarily expensive operation, for the practice to be economically viable the ore must contain a minimum level of target metals, termed the “mineralogical barrier” (sensu [168]). Estimating the threshold for economic viability of Ni extraction via traditional strip-mining processes is complicated because (1) Ni is often produced as a by-product while mining for other metals, such as copper, and (2) differing mineralization processes of Ni indicate varying thresholds for economic viability. The proposed mineralogical barrier for laterite, one of the most common rock types mined for Ni, is approximately 3000 mg kg$^{-1}$ [169], which means that this is the lowest Ni concentration for which conventional mining practices may produce an adequate yield of Ni to support ongoing extraction. Many Ni-hyperaccumulating plants grow naturally on serpentine soils with Ni concentrations well below 1500 mg kg$^{-1}$, and multiple field trials for phytoextraction have been performed on these relatively low-Ni soils [170,171], which means that they could be potentially acceptable for agromining on a wide scale. Critically high-concentration ore deposits are limited to small areas and many of these deposits are being depleted due to continuous mining [169], while subeconomic ultramafic soils are more widespread and largely unused for direct human economic benefits [14]. Thus, low-Ni concentration soils inherently lend themselves better to agromining than strip-mining.

The ideal soils for commercial agromining have the highest possible phytoavailable Ni while still maintaining a moderate pH range (between 4 and 7); have an acceptable level of soil structure, depth, and water-holding capacity; and are found on relatively flat land [165]. Such soils are found throughout the Mediterranean, as well as in subtropical areas such as New Caledonia and Sabah, Malaysia [161,172,173]. Nkrumah et al. [169] estimated the gross monetary yield of an extensive agromining practice to be approximately US$1980 ha$^{-1}$, which is higher than the estimated monetary yields for common crops on fertile soils, such as corn in the United States, which yields approximately US $1400 ha$^{-1}$ in today’s market at the current price of US$3.68 per bushel [175]. In addition, these crops would not displace any preexisting farms, except for those on metalliferous soils, which would naturally yield a suboptimum harvest of most food crops. The economic viability of agromining on disturbed serpentine soils, therefore, is noteworthy, especially in areas dominated by ultramafic soils that offer little agricultural potential.

Increasing the efficiency and monetary yield of agromining has become a hot topic in applied ecology in recent years, prompting investigations into various techniques to improve shoot Ni concentration or biomass of hyperaccumulating plants [176]. Basic regimens have been established for irrigation, pest control, and fertilization in agromining practices [174], which vary by the species being used. All these practices aim at increasing plant survivorship and biomass; however, greater shoot biomass in the plant is usually accompanied by lower shoot Ni concentrations [177]. In contrast, soil enrichment with excess nitrate can enhance IRT1 expression in the roots of Arabidopsis spp., thereby increasing the rate of Ni import [178]. In addition, soil amendment with Ca is useful, as foliar Ca and Ni concentrations are positively correlated in many instances [86], and soil Ca levels commonly become depleted due to periodic removal from the field when harvesting bioore [174]. Utilization of select rhizobacterial inoculants could increase Ni yield by enhancing shoot biomass [179]; however, there is little information available concerning the specific physiology of advantageous soil symbionts. Nitrogen fertilization promotes the growth of a healthy bacterial community in the rhizosphere of O. chalcicola (Brassicaceae; syn. O. chalcedica; Fig. 25.2B) by alleviating the deleterious effects of Ni toxicity on the microbes, resulting in an increase in both shoot Ni concentration and biomass [180]. Cocropping of multiple hyperaccumulator species inoculated with naturally occurring rhizobacteria
results in significant increase of Ni uptake by the plants and an increase in diversity of the microbial community, highlighting the importance of using multispecies covers in agromining operations [181]. An excellent strategy to improve levels of soil nitrogen and microbial enzymatic activity at the same time involves cocropping Ni hyperaccumulators with legumes, as demonstrated with *O. chalcidica* grown in conjunction with *Lupinus albus* (Fabaceae), which proves beneficial to both species and provides an opportunity to produce food and Ni simultaneously [182]. Further investigations of legumes tolerant of ultramafic soils are necessary to optimize this regimen for practical agromining [183].

Genetic engineering provides a cutting-edge option to optimize agromining by producing modified hyperaccumulators designed specifically to take up maximum amounts of Ni. Upregulation or overexpression of key genes important for Ni tolerance and accumulation is the common strategy for producing strains ideal for agromining [184]. Generally, target genes are selected based on their presumed role in ion transport, metal chelation, or metal detoxification [185]. Although some novel genetic modifications have been made to enhance metal tolerance and uptake in some plant species [20], no instances of genetic manipulation directly aimed at increasing Ni hyperaccumulation for the purposes of commercial agromining have been described to date.

One excellent case study on implementation of agromining at an economically viable level is that of *O. chalcidica* grown on ultramafic vertisols in Albania (Fig. 25.2B [177]), resulting from a series of field experiments performed between 2005 and 2014, optimizing procedures for Ni phytoextraction. The steps followed for agromining with *O. chalcidica* are as follows: plant density of 4 plants m$^{-2}$; plowed ultramafic soil with nonlimiting conditions of Ni availability; and adequate fertilization with N, P, K, S, and Ca. Results demonstrated an improvement in yield from 1.7 to 105 kg Ni ha$^{-1}$ after optimization, but little decrease in soil Ni concentration was recorded, although it is expected that long-term agromining would cause depletion of soil Ni ([177], Table 25.2). Nickel yield and overall efficiency of the process have increased due to development and subsequent optimization of an economically feasible hydrometallurgical process for producing ammonium nickel sulfate hexahydrate salt, which can be extracted from *O. chalcidica* ash at a purity of 88.8% [159], or even 99.1% [160].

Another pioneering study of a serpentine quarry in northwest Spain explored the viability of four herbaceous Brassicaceae annuals—*Odontarrhena serpillifolia*, *O. chalcidica*, *Bornmuellera emarginata*, and *N. caerulescens*—as candidates for use in agromining on mine tailings [158]. Quarries and mine tailings are particularly unfavorable environments for plants due to shallow, underdeveloped soil with low water-holding capacity, limited organic matter content, and nutrient deficiency [165], all of which are common symptoms of “serpentine syndrome,” a set of characteristics that makes ultramafic soils physiologically demanding habitats for plant growth [188]; these stressors are much more extreme in the case of disturbed ultramafic settings such as quarries and mines. In this study, plots were amended with composted sewage sludge or inorganic NPK fertilizers, and while biomass yields increased for both treatments, compost was much more effective. This was possibly due to a three- to six-fold increase in bacterial density, which is known to positively affect shoot biomass in Ni hyperaccumulators [189]. All species except *O. chalcidica* exhibited a decrease in aboveground Ni concentration following the addition of compost; however, the significant increase in biomass resulted in the greatest total yield of Ni. *O. chalcidica* exhibited the greatest overall yields of Ni, making it a favorable candidate for agromining in the Mediterranean region [158].

In subtropical regions, shrubs and trees, rather than herbaceous annuals, are utilized for prospective agromining practices. The first tropical “metal farm” in Sabah, Malaysia is using *Phyllanthus rufuschaneyi* (Phyllanthaceae: Fig. 25.2A), which yields high-Ni concentrations, often $>6500$ mg Ni kg$^{-1}$ [161]. High plant biomass and accompanying shoot Ni concentration provide much promise for the development of commercial agromining in subtropical regions using this

### TABLE 25.2 Economically important Ni hyperaccumulators.

<table>
<thead>
<tr>
<th>Species</th>
<th>Shoot Ni conc. (g kg$^{-1}$)</th>
<th>Ni yield (kg ha$^{-1}$)</th>
<th>Native region</th>
<th>Citations</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Odontarrhena chalcidica</em></td>
<td>11.5</td>
<td>105</td>
<td>Temperate—Mediterranean</td>
<td>[177]</td>
</tr>
<tr>
<td><em>Ophrys bertolonii</em></td>
<td>7</td>
<td>72</td>
<td>Temperate—Mediterranean</td>
<td>[186]</td>
</tr>
<tr>
<td><em>Phyllanthus rufuschaneyi</em></td>
<td>25</td>
<td>250*</td>
<td>Tropical—Sabah</td>
<td>[161]</td>
</tr>
<tr>
<td><em>Streptanthus polygaloides</em></td>
<td>10</td>
<td>100</td>
<td>Mediterranean—California</td>
<td>[21, 187]</td>
</tr>
<tr>
<td><em>Berkheya coddii</em></td>
<td>10</td>
<td>100</td>
<td>Temperate—South Africa</td>
<td>[58]</td>
</tr>
</tbody>
</table>

Shoot Ni concentration, biomass, and total Ni yield are based on greatest values from experimental data. Only taxa tested in agromining field experiments are included.

*Suggested value based on field experiments conducted to date.*
taxon, which could achieve a yield of 250 kg Ni ha\textsuperscript{-1}(\cite{156} Table 25.2). If replicated on a wide scale, this would be the greatest yield of Ni via agromining yet to be recorded anywhere in the world. The species has other favorable qualities in addition to high-Ni concentration and biomass, such as a multiple stems and rapid regeneration after harvest. However, some uncertainties exist concerning the wide-scale propagation of \textit{P. rafuschaneyi}—the species is susceptible to fungal infections under shade, and its dependence on a specialized pollinator (\textit{Epicephala} moths) poses a barrier for its broader geographic use \cite{190}.

There are potentially many more Ni hyperaccumulators to be discovered from around the world and floristic surveys should be encouraged to document such species before they are lost from their native habitats (i.e., serpentine outcrops) that are undergoing drastic changes resulting from agriculture, deforestation, mining, exotic species invasions, and atmospheric deposition of previously limiting macronutrients such as nitrogen \cite{191}. For agromining to be successful at a worldwide scale, and without posing the environmental risks associated with moving hyperaccumulators from one region to another for agromining \cite{20}, region-specific plants will have to be utilized after carefully studying their biology and ecology and testing them in small-scale trials prior to additional testing on a larger scale \cite{155}. In this regard, the Global Hyperaccumulator Database (http://hyperaccumulators.smi.uq.edu.au/collection/) is an important resource for identifying region-specific Ni hyperaccumulators.

### 25.6 Conclusion

Plants can tolerate nearly every environment on Earth and have evolved adaptations to deal with extreme abiotic and biotic stressors. The unique nutritional and osmotic challenges associated with ultramafic soils are no different, and plants growing on these soils have developed traits to cope with limited essential nutrients, high heavy metal content, and low calcium to magnesium ratios \cite{33}. Heavy metal hyperaccumulation, defined as $\geq1000$ mg metal kg\textsuperscript{-1} dry weight for most metals (Ref. \cite{24} and also see Ref. \cite{192}), is a phenomenon born out of these harsh edaphic conditions and exhibited by more than 500 species worldwide \cite{193}. Nickel is by far the most commonly accumulated element and, as a result, much of the literature on hyperaccumulation concerns Ni \cite{194}.

Recent studies have begun to fully elucidate the physiological mechanisms plants use to take up, detoxify, transport, and store bioavailable Ni from the soil. The unique capacity of hyperaccumulators to take up Ni into the roots seems to be dependent on a number of factors, namely, soil pH, the presence of other heavy metals, the bioavailable Ni concentration, and the efficiency of pumps and transport proteins in root epidermal cell membranes \cite{53,144}. Nickel is chelated by various organic and amino acids upon entry to a root and translocated into aboveground tissue via xylem, where it is then primarily localized into leaf epidermal cell walls and vacuoles or to phloem tissue \cite{66,82,89,84,88,91}. The Ni hyperaccumulation process seems to draw heavily on existing transport and homeostasis mechanisms for other metals, namely, Zn and Fe, indicative of more recent evolution of the trait \cite{47,58,61}. There are four main hypotheses for the adaptive significance of Ni hyperaccumulation, the most supported being the \textit{elemental defense hypothesis} \cite{105}, which states that plants with elevated tissue Ni concentrations are better protected from unadapted pathogens, pests, and herbivores. Other hypotheses are that Ni hyperaccumulation evolved because certain species require high levels of Ni to carry out metabolic functions \cite{31,40}, Ni is involved in elemental allelopathy to inhibit the growth of nearby plants \cite{81}, and Ni is used as an osmoticum, aiding in drought tolerance \cite{84,88}.

The genes, expression patterns, and proteins that create this unique phenotype are slowly being discovered. So far a handful of genes involved in Zn and Fe transmembrane transport have been shown to play a role in Ni tolerance and accumulation by facilitating movement of Ni into aboveground tissues \cite{80,136,145,146}. Further, genes and gene products for the biosynthetic pathways controlling Ni chelator concentration and localization may also be involved in Ni transport and detoxification \cite{38,149,153}. Innovations in genetic sequencing and gene manipulation tools are making it ever easier to determine the genetic factors behind Ni hyperaccumulation and subsequently alter these factors to create transgenic strains of hyperaccumulators for biotechnological application (but see Refs. \cite{20,155} for environmental risks associated with genetic modification). Agromining is an expanding field and the efficiency of the practice is gradually increasing with greater interest in improving the economic viability of this new green technology \cite{154}. Optimized irrigation, fertilization, land management, and Ni extraction techniques (\cite{160,161,177}) have demonstrated a highly realistic prospect of implementing large-scale agromining operations in Mediterranean and tropical regions of the world. In addition, it is possible that long-term phytoextraction could result in rehabilitation of contaminated soils or mine wastes, due to depletion of toxic metals \cite{164,165}. Nickel hyperaccumulation is a fascinating adaptation to ultramafic soils worldwide and its study from physiological, ecological, genetic, and biotechnological perspectives has provided unique insights into the ways in which plants cope with harsh soil environments. Continued research will bring about more discoveries about the nature of Ni hyperaccumulation and its wide-ranging applications.
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PART | II Biotechnological aspects


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Further Reading

