Constitutively Elevated Salicylic Acid Signals Glutathione-Mediated Nickel Tolerance in Thlaspi Nickel Hyperaccumulators[1]

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Progress is being made in understanding the biochemical and molecular basis of nickel (Ni)/zinc (Zn) hyperaccumulation in Thlaspi; however, the molecular signaling pathways that control these mechanisms are not understood. We observed that elevated concentrations of salicylic acid (SA), a molecule known to be involved in signaling induced pathogen defense responses in plants, is a strong predictor of Ni hyperaccumulation in the six diverse Thlaspi species investigated, including the hyperaccumulators *Thlaspi goesingense*, *Thlaspi rosulare*, *Thlaspi oxyceras*, and *Thlaspi caerulescens* and the nonaccumulators *Thlaspi arvense* and *Thlaspi perfoliatum*. Furthermore, the SA metabolites phenylalanine, cinnamic acid, salicyloyl-glucose, and catechol are also elevated in the hyperaccumulator *T. goesingense* when compared to the nonaccumulators Arabidopsis (*Arabidopsis thaliana*) and *T. arvense*. Elevation of free SA levels in Arabidopsis, both genetically and by exogenous feeding, enhances the specific activity of serine acetyltransferase, leading to elevated glutathione and increased Ni resistance. Such SA-mediated Ni resistance in Arabidopsis phenocopies the glutathione-based Ni tolerance previously observed in Thlaspi, suggesting a biochemical linkage between SA and Ni tolerance in this genus. Intriguingly, the hyperaccumulator *T. goesingense* also shows enhanced sensitivity to the pathogen powdery mildew (*Erysiphe cruciferarum*) and fails to induce SA biosynthesis after infection. Nickel hyperaccumulation reverses this pathogen hypersensitivity, suggesting that the interaction between pathogen resistance and Ni tolerance and hyperaccumulation may have played a critical role in the evolution of metal hyperaccumulation in the Thlaspi genus.

Worldwide, more than 400 plant species are now known that hyperaccumulate various trace metals (Cd, Co, Cu, Mn, Ni, and Zn), nonmetals (Se; for review, see Reeves and Baker, 2000), and metalloids (As; Ma et al., 2001) in their shoots when growing in their native habitats. Of these, many are Brassicaceae family members, including numerous Thlaspi species that hyperaccumulate nickel (Ni) and zinc (Zn) up to 3% (30,000 μg g^{-1}) of their shoot dry weight. The extraordinary ability of these plants to hyperaccumulate Ni/Zn make them an ideal source of genetic material for the development of both mineral nutrient-fortified crops and plants suitable for phytoremediation of metal-polluted soils and waters (Guerinot and Salt, 2001). To develop a practical genetic model system for dissecting the mechanistic basis of metal hyperaccumulation, we have been studying numerous biannual Ni and Zn hyperaccumulators from the Brassicaceae, including many members of the Thlaspi genus collected from both serpentine and mine sites in Austria, France, Greece, Turkey, and the United States (Peer et al., 2003). To complement these broad-based studies, we have also focused on *Thlaspi goesingense* Hálácsy (Brassicaceae), a hyperaccumulator species found growing on naturally Ni-enriched serpentine soils in Redschlag, Austria, where it accumulates up to 1.2% of its shoot dry weight as Ni (Reeves and Brooks, 1983; Krämer et al., 1997; Wenzel and Jockwer, 1999).

Nickel hyperaccumulation in *T. goesingense* is primarily determined by its high degree of Ni tolerance (Krämer et al., 1997; Salt et al., 1999), achieved through an efficient system to pump and store Ni in the central vacuole of shoot cells (Krämer et al., 2000; Küpper et al., 2001). We observed that a member of the cation diffusion facilitator family, *TgMTP1*, is constitutively highly expressed in *T. goesingense* and may be playing a role in vacuolar sequestration of metal in the hyperaccumulator (Persans et al., 2001). Similar constitutively enhanced expression has also been observed for the *TgMTP1* homologs *ZTP1* and *AhMTP1* in the Zn hyperaccumulators *Thlaspi caerulescens* and *Arabidopsis halleri* (Assunção et al., 2001; Becher et al., 2004). Furthermore, in crosses between the Zn hyperaccumulator *A. halleri* and the nonaccumulating relative *Arabidopsis lyrata*, *AhMTP1* overexpression was found to cosegregate with Zn tolerance (Dräger et al., 2004). Intriguingly, recent data from our laboratory suggests that TgMTP1 may also be acting at the plasma membrane as a metal efflux pump (Kim et al., 2004). From our cellular Ni distribution studies (Krämer et al.,

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Table I. *Quantification of SA and metabolites in hyperaccumulator and nonaccumulator plants*

SA, salicyloyl-Glc, and catachol shown as μmol g^{-1} fresh weight; Phe and cinnamate as nmol g^{-1} fresh weight. Data represent average (n = 6 individual plants) ± SD.

	T. goesingense	*T. arvense*	Arabidopsis
Phe	54.4 ± 29.1	28.0 ± 7.2	18.1 ± 6.2
Cinnamic acid	52.1 ± 11.6	nd[a]	nd
SA	68.2 ± 11.5	0.13 ± 0.06	0.16 ± 0.08
Salicyloyl-Glc	502.8 ± 30.0	0.58 ± 0.06	0.10 ± 0.02
Catechol	316.2 ± 66.7	6.8 ± 2.2	nd

[a]Not detected.

2000), it is clear that a substantial amount of cellular Ni also accumulates outside of the vacuole, suggesting the need for a cytoplasmic-based tolerance mechanism. The recent identification of Ni^{2+} complexed to the high-affinity metal chelate nicotianamine in the Ni/Zn hyperaccumulator *T. caerulescens* (Vacchina et al., 2003) suggests that nicotianamine may play an important role in detoxification of extravacuolar Ni in hyperaccumulating plants. Constitutive overproduction of nicotianamine and the enzyme responsible for its biosynthesis, nicotianamine synthase, in *T. caerulescens* and the related hyperaccumulator *A. halleri* (Vacchina et al., 2003; Becher et al., 2004; Weber et al., 2004) strongly supports such a conclusion, and suggests that nicotianamine overproduction is a general mechanism underlying Ni/Zn hyperaccumulation in the Brassicaceae family. Recently, we observed that glutathione (GSH) concentrations in Thlaspi hyperaccumulators are also constitutively elevated, leading to enhanced tolerance to Ni-induced oxidative stress (Freeman et al., 2004). Further investigations determined that enhanced GSH in the hyperaccumulators is due to the constitutively enhanced activity of Ser acetyltransferase (SAT; Freeman et al., 2004). Such elevated GSH provides one mechanism whereby the hyperaccumulator is able to resist the oxidative damage caused by nonsequestered Ni^{2+}.

Progress is being made in understanding the biochemical and molecular basis of Ni/Zn tolerance in Thlaspi and other hyperaccumulators. However, the molecular signaling pathways that control these mechanisms are not understood. Salicylic acid (SA) is a potent signaling molecule in plants and is well established to be involved in eliciting specific responses to biotic stresses (for review, see Dempsey et al., 1999; Shah, 2003). Furthermore, SA is also known to be involved in abiotic stress signaling, including plant responses to heavy metals. SA pretreatment alleviates Pb- and Hg-induced membrane damage in rice (*Oryza sativa*; Mishra and Choudhuri, 1999) and Cd toxicity in barley (*Hordeum vulgare*) and maize (*Zea mays*) seedlings (Pál et al., 2002; Metwally et al., 2003). Protection from oxidative damage caused by paraquat (Ananieva et al., 2004), heat (Larkindale and Knight, 2002), cold (Janda et al., 1999), NaCl (Tari et al., 2002), and water deficit (Bezrukova et al., 2001) has also been achieved by SA pretreatments. Interestingly, SA has been shown to accumulate in plants in response to various oxidizing stresses, including hydrogen peroxide (León et al., 1995), ozone (Sharma et al., 1996), and heat (Dat et al., 1998), and this correlates with accumulation of the antioxidant GSH and glutathione reductase (GR), the enzyme responsible for maintaining GSH in its reduced form (Dat et al., 1998; Srivastava and Dwivedi, 1998; Knörzer et al., 1999). It has been suggested that SA is directly involved in signaling these antioxidant responses (Larkindale and Knight, 2002), though the signaling mechanisms remain obscure.

However, a large number of the components of the SA-mediated pathogen response (PR) in plants have been identified (for review, see Dong, 2004; Pieterse and Van Loon, 2004). SA is known to activate PR via NPR1 (NONEXPRESSOR OF PR GENES1), a soluble protein localized to both the cytoplasm and the nucleus (Cao et al., 1997; Ryals et al., 1997; Despres et al., 2000). Nuclear localization of NPR1 is required for the induction of PR genes required for pathogen resistance (Kinkema et al., 2000). Relocalization of NPR1 to the nucleus is driven by the conversion of oligomers of

Table II. *SA, Ni, and Zn concentrations in various Thlaspi species*

Column 1, Metal concentrations from shoot tissue harvested from plants grown in a growth chamber in soil augmented with either 100 μg g^{-1} dry weight Ni or Zn (Peer et al., 2003). Column 2, Metal concentrations determined in shoot samples collected from plants growing in their native environment; data for *T. caerulescens* from Reeves et al. (2001).

Species	Habitat	SA[a]	Ni		Zn	
			1	2	1	2
		μmol g^{-1} fresh weight mean ± SD	*μg g^{-1} dry weight*			
T. arvense	Calcareous	0.17 ± 0.08	0.15	1	150	33
T. perfoliatum	Calcareous	0.24 ± 0.13	100	14	400	44
T. rosulare	Serpentine	1.64 ± 0.15	1,100	27,245	1,100	77
T. oxyceras	Serpentine	2.29 ± 0.63	1,000	16,930	200	77
T. caerulescens (Puy de Wolf)	Serpentine	5.41 ± 1.13	1,600	10,610	5,100	3,426
T. caerulescens (St Félix de Pallières)	Pb/Zn mine	9.29 ± 0.99	1,100	1	4,200	8,762

[a]Data represents the mean ± SD of quantifications from three to seven individual plants per species.

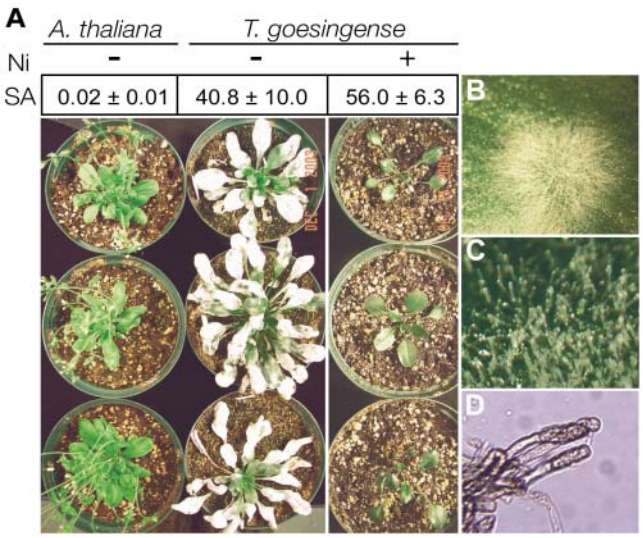

Figure 1. Response of hyperaccumulator and nonaccumulator to powdery mildew. A, Both Arabidopsis and *T. goesingense* were cocultivated for 35 d and allowed to become naturally inoculated with powdery mildew. During the growth period, plants were watered twice weekly with one-tenth Hoagland, except one group of *T. goesingense* plants received one-tenth Hoagland containing 200 μM $Ni(NO_3)_2$. Shoot tissue was harvested from all plants and analyzed for SA. Data represent mean μmol g^{-1} fresh weight SA ($n = 8$) ± SD. Infected *T. goesingense* leaves were examined for identification of powdery mildew. B, 1×; C, 6.3×; and D, 100× magnification.

NPR1 into monomers, their interconversion being regulated by the reduction of disulfides within the NPR1 oligomer (Mou et al., 2003). In the nucleus, NPR1 interacts with certain Leu zipper transcription factors, including TGA1, to regulate SA-dependant gene expression. Interaction between NPR1 and TGA1 is also regulated by the reduction of a critical disulfide in TGA1 (Despres et al., 2003). At present, it is unclear how SA regulates the reduction of NPR1 and TGA1 (Dong, 2004). Plants containing a mutant *NPR1* allele are able to induce SA biosynthesis in response to a pathogen. However, *npr1* plants are hypersensitive to pathogens because SA cannot signal gene expression required for a normal PR. NPR1-independant SA signaling is also known to occur during plant responses to various viral pathogens (Kachroo et al., 2000; Takahashi et al., 2002), though the signaling pathway has still to be determined.

Here, we present evidence that the GSH-mediated Ni tolerance mechanism previously observed in Thlaspi hyperaccumulators (Freeman et al., 2004) is signaled by the constitutively elevated levels of SA observed in these Ni/Zn hyperaccumulators. In the four species of Thlaspi hyperaccumulators tested, SA concentrations are constitutively elevated compared to the nonaccumulators, and this is associated with the elevated SAT activity and GSH biosynthesis previously observed in these species (Freeman et al., 2004). We observe that both biochemical and genetic manipulations that increase SA in Arabidopsis (*Arabidopsis thaliana*) mimic the GSH-related phenotypes of the hyperaccumulating Thlaspi, and these biochemical changes in the nonaccumulator are associated with increased GSH-mediated Ni resistance. Such observations suggest that SA may be one of the regulators involved in coordinating certain key biochemical differences between Ni/Zn hyperaccumulators and nonaccumulator Thlaspi. Furthermore, our observation that SA mediates accumulation of GSH by activation of SAT, in an NPR1-independent manner, provides a novel mechanism for SA to control the NPR1 and TGA1 oxidation/reduction exchange that is critical for SA signaling of PR (Despres et al., 2003; Mou et al., 2003).

RESULTS

SA and Its Metabolites in Hyperaccumulator and Nonaccumulator Plants

SA, a molecule known to be involved in oxidative stress signaling, is accumulated constitutively in shoot tissue of the hyperaccumulator *T. goesingense* compared to the related nonaccumulators *Thlaspi arvense* and Arabidopsis (Table I), to levels comparable to those observed in the SA accumulators willow and wintergreen (Raskin et al., 1990). Similar elevated levels of SA were also observed in *T. goesingense* shoot tissue collected in the field at Redschlag, Austria (data not shown). Furthermore, SA is not observed in roots or xylem exudates, and concentrations are not significantly affected by Ni exposure (data not shown). Importantly, the SA metabolites Phe, cinnamic acid, salicyloyl-Glc, and catechol are also constitutively elevated in the shoot tissue of the hyperaccumulator *T. goesingense* compared to the nonaccumulators (Table I). A further comparison of shoot SA levels in the Ni

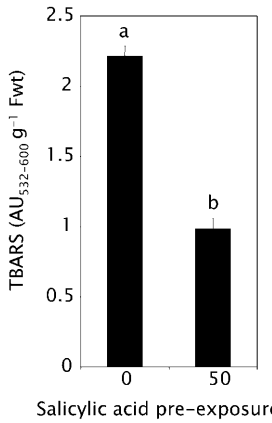

Figure 2. SA protection against Ni-induced lipid peroxidation in Arabidopsis. Arabidopsis (Col) were grown on solidified one-half Murashige and Skoog + B5 plates for 7 d then transferred to medium containing 0 or 50 μM SA for 14 d prior to transfer onto medium lacking SA but containing 100 μM $Ni(NO_3)_2$. After a further 8 d, shoot tissue was harvested and analyzed for TBARS. Data represents mean ($n = 9$) ± SD. Different lowercase letters represent a significant difference between means (Student's t test $P > 0.01$).

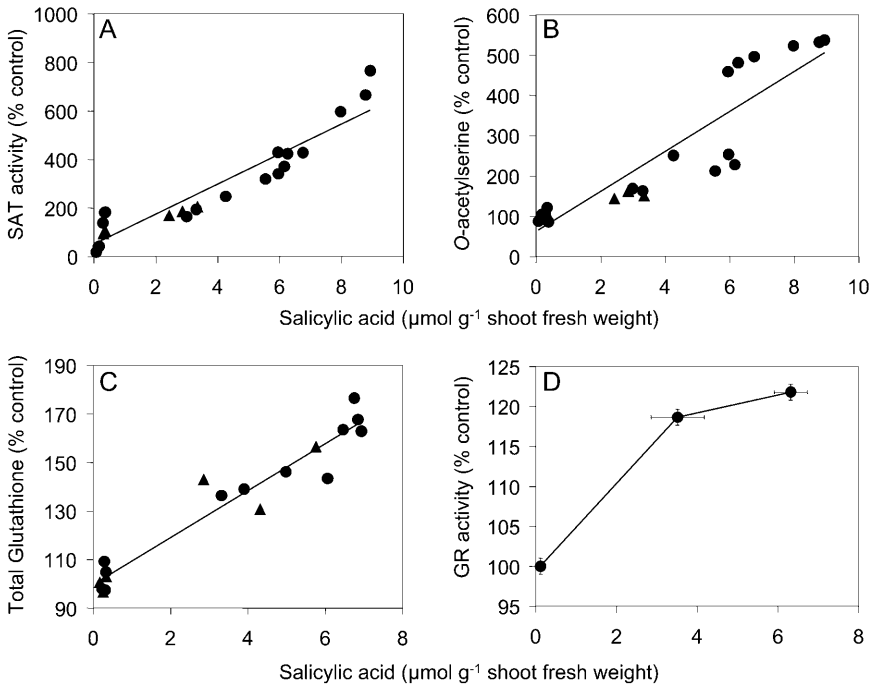

Figure 3. Effects of SA treatment on GSH metabolism. Arabidopsis was germinated and grown on solidified one-half Murashige and Skoog + B5 vitamin medium containing 0, 50, or 100 μM SA. After 14 d, shoot tissue was harvested and analyzed for SA and SAT activity (A), OAS (B), total GSH (C), and GR activity (D) in both wild-type Arabidopsis (Ws; circles) and *npr1-1* (triangles). Control levels of SAT activity, OAS, total GSH, and GR in Arabidopsis were 0.05 nmol OAS min^{-1} mg^{-1} total protein, 0.015 nmol g^{-1} fresh weight, 260.9 nmol g^{-1} fresh weight, and 11.7 nmol GSH mg^{-1} total protein min^{-1}, respectively, and 0.16 nmol OAS min^{-1} mg^{-1} total protein, 0.013 nmol g^{-1} fresh weight, and 271.4 nmol g^{-1} fresh weight, respectively, in *npr1-1*. Data for control values represent an average of three to six independent replicate analyses. GR activity and corresponding SA levels were measured on equivalent replicate samples, and data represent the average ($n = 3$) ± SD.

hyperaccumulator *Thlaspi oxyceras* and the Ni/Zn hyperaccumulators *Thlaspi rosulare*, *T. caerulescens* (Puy de Wolf), and *T. caerulescens* (St Félix de Pallières) with the nonaccumulators *T. arvense* and *Thlaspi perfoliatum* reveals free SA levels to be constitutively between 7- and 50-fold higher in all the hyperaccumulators examined (Table II). Such evidence supports the conclusion that at least among the Thlaspi hyperaccumulators, elevated SA is associated with Ni hyperaccumulation. Intriguingly, constitutively elevated SA in *T. goesingense* is also associated with enhanced susceptibility to powdery mildew (*Erysiphe cruciferarum* Opiz ex L. Junell) when compared to the susceptible Arabidopsis Columbia (Col-0) ecotype (Adam et al., 1999; Fig. 1A). *T. goesingense* growing side by side with Arabidopsis consistently show severe infections with *E. cruciferarum* with extensive development of fungal conidia producing a powdery appearance on leaf surfaces (Fig. 1, B–D). This enhanced susceptibility to *E. cruciferarum* is suppressed by Ni (Fig. 1A), as has previously been observed for infection of the Ni hyperaccumulator *Streptanthus polygaloides* (Brassicaceae) with *Erysiphe polygonii* (Boyd et al., 1994). Furthermore, SA levels in *T. goesingense* are not significantly affected by pathogen infection (Fig. 1A). Investigations are currently under way in our laboratory to determine if *T. goesingense* lacks a biochemical and molecular response to *E. cruciferarum*.

The Role of SA in Ni Tolerance

To establish a role for SA in Ni/Zn hyperaccumulation, we performed experiments on the related nonaccumulator Arabidopsis. Pretreatment of Arabidopsis with SA for 14 d, prior to Ni exposure for 8 d, causes a 2.2-fold decrease in Ni-induced lipid peroxidation in shoot tissue, measured as the presence of thiobarbituric acid reactive species (TBARS; Fig. 2). SA treatment, which caused significant increases in SA accumulation in shoots (Fig. 3), was also observed to induce a linear increase in SAT activity in Arabidopsis

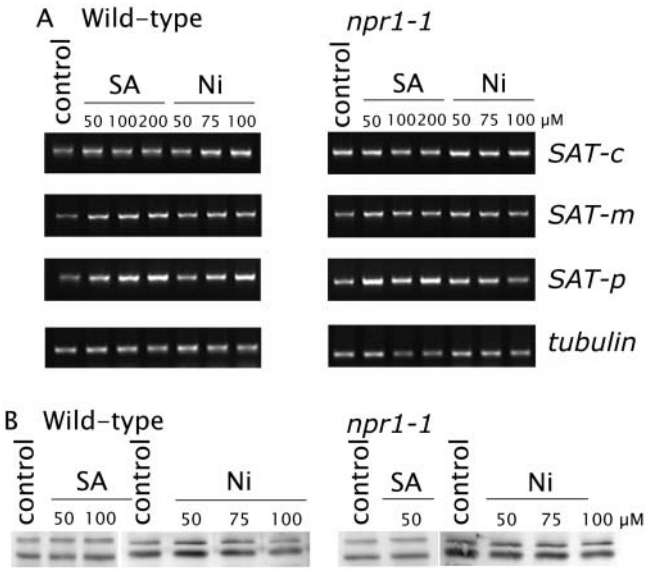

Figure 4. Transcriptional and translational regulation of SAT by SA and Ni^{2+} in Arabidopsis. Both wild-type Arabidopsis and *npr1-1* were grown on solidified one-half Murashige and Skoog + B5 vitamin medium for 14 d prior to transfer onto medium containing various concentrations of SA or Ni^{2+}. After a further 14 d, shoot tissue was harvested and analyzed by RT-PCR for steady-state levels of SAT mRNA (A) and by immunoblotting for SAT protein.

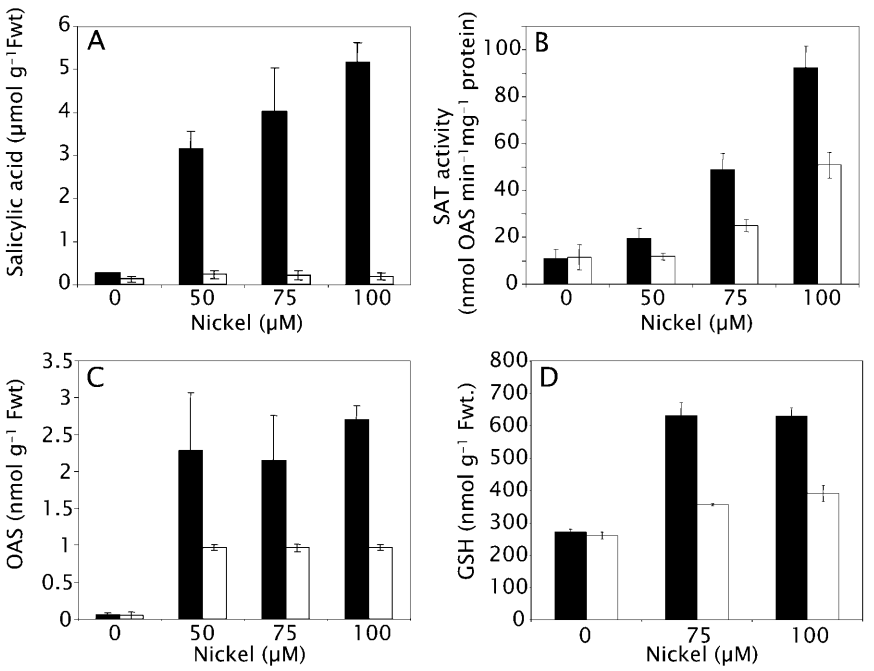

Figure 5. Regulation of SA and GSH metabolism by Ni^{2+} in wild-type Arabidopsis and the SA nonresponsive *npr1-1* mutant. Both wild-type Arabidopsis and *npr1-1* were grown on solidified one-half Murashige and Skoog + B5 vitamin medium for 7 d prior to transfer onto medium containing 0, 50, 75, or 100 μM $Ni(NO_3)_2$. After 14 d, shoot tissue was harvested and analyzed for SA (A), SAT activity (B), OAS (C), and total GSH (D). Bars represent wild type (white) and *npr1-1* (black). Data represent the mean ($n = 10$) ± SD.

(Fig. 3A), along with increases in its product *O*-acetyl-L-Ser (OAS) and the downstream metabolite GSH (Fig. 3, B and C). The enzyme responsible for maintaining a reduced pool of GSH, GR is also activated after SA exposure (Fig. 3D). Furthermore, these metabolic changes are also observed in the SA-signaling mutant *npr1-1* (Fig. 3), suggesting that SAT activity is regulated by SA in an NPR1-independent manner. Increases in GSH, driven by overexpression of SAT, have previously been shown by the authors to decease the level of both Ni induced reactive oxygen species (ROS) and lipid peroxidation in Arabidopsis (Freeman et al., 2004), establishing the link between elevated GSH and reduced Ni-induced ROS. Qualitative measurement of the different SAT mRNAs by reverse transcription (RT)-PCR reveals that SA exposure does not cause changes in steady-state SAT mRNA (Fig. 4A), and immunoblotting using antiserum that cross-reacts with all three SAT isoforms shows no major changes in the accumulation of SAT (Fig. 4B) in either wild-type or *npr1-1*. Based on this evidence, SA appears to regulate SAT activity posttranslationally via an NPR1-independent pathway. Such regulation is consistent with the observation that SA pretreatment induces enhanced accumulation of GSH and protection against Ni toxicity, since GSH is known to protect against Ni-induced lipid peroxidation in Arabidopsis and Thlaspi hyperaccumulators (Freeman et al., 2004).

To develop further evidence for a role of SA in regulating GSH-mediated Ni resistance via activation of SAT, we observed that the SA-signaling mutant *npr1-1* hyperaccumulates SA on exposure to Ni (Fig. 5A). This accumulation of SA in *npr1-1* is associated with enhanced increases in SAT activity (Fig. 5B), OAS, and GSH (Fig. 5, C and D), in a similar manner to that observed in wild-type Arabidopsis after exposure to exogenous SA (Fig. 3). The enhanced SAT activity observed in *npr1-1* after Ni exposure is not related to increased SAT mRNA (Fig. 4A) or SAT protein (Fig. 4B), suggesting a posttranslational regulatory mechanism, as observed for SA induced SAT activity (Figs. 3 and 4). These metabolic changes in *npr1-1* are also associated with increased Ni resistance (Figs. 6 and 7A) similar to that observed in SA-pretreated plants (Fig. 2). Mutation of *NPR1* leads to a 2-fold increase in Ni resistance, quantified as an increase in the I_{50} for inhibition of root growth from 75 μM to 150 μM Ni (Fig. 7B). Such Ni resistance in *npr1-1* is completely abolished when plants are grown in the presence of the

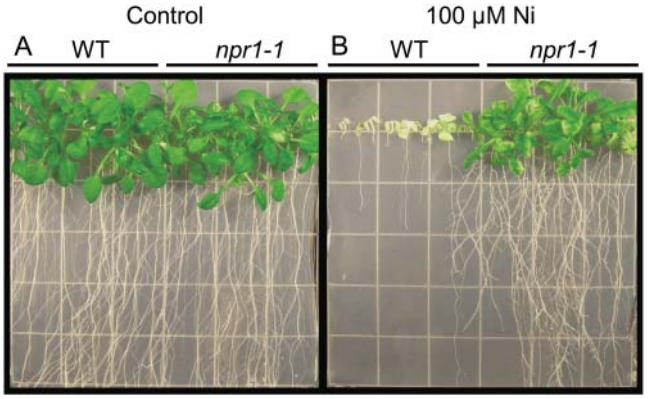

Figure 6. Ni resistance of wild-type Arabidopsis and *npr1-1* mutant. Both wild-type Arabidopsis and *npr1-1* were germinated and grown on solidified one-half Murashige and Skoog + B5 vitamin medium without (A) and with (B) 100 μM $Ni(NO_3)_2$ for 30 d.

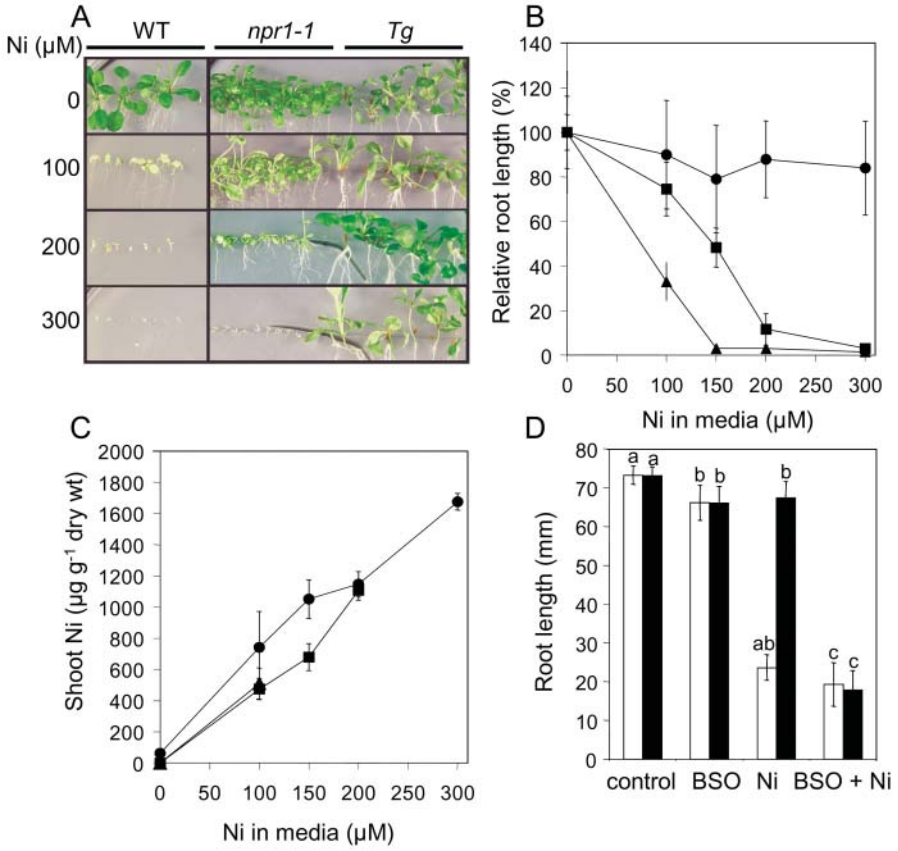

Figure 7. Ni resistance and accumulation of wild-type Arabidopsis, *npr1-1*, and *T. goesingense* (*Tg*). A, Plants were germinated and grown for 30 d on solidified one-half Murashige and Skoog + B5 vitamin medium containing various concentrations of $Ni(NO3)_2$. B, Root lengths plotted as a percentage of the root length after growth in the absence of Ni for *T. goesingense* (circles), Arabidopsis *npr1-1* (squares), and Arabidopsis wild type (triangles). Data represent means ($n = 20$) ± SD. C, Nickel concentrations in shoot tissue of *T. goesingense* (circles), Arabidopsis *npr1-1* (squares), and Arabidopsis wild type (triangles). Data represent means ($n = 9$) ± SD. D, Wild-type Arabidopsis (white bars) and *npr1-1* (black bars) were germinated and grown on solidified one-half Murashige and Skoog + B5 vitamin medium containing various combinations of 100 μM $Ni(NO_3)_2$ and 100 μM buthionine sulfoximine. After 30 d, root lengths were measured and data reported as mean ($n = 30$) ± SD. Lowercase letters (a, b, and c) represent significantly different means using the mixed procedure function in SAS ($P < 0.01$).

GSH biosynthetic inhibitor buthionine sulfoximine (Meister, 1988; Fig. 7D), strongly supporting a role for GSH in the enhanced Ni resistance of *npr1-1*. Though significant, this increase in Ni resistance in *npr1-1* is still lower than that observed in the Ni hyperaccumulator *T. goesingense* (Fig. 7, A and B). Interestingly, Arabidopsis wild-type, *npr1-1*, and *T. goesingense* accumulate equal amounts of Ni over the range of Ni concentrations in the medium at which the plants are viable (Fig. 7C), confirming that Ni tolerance rather than Ni uptake rates are what distinguish the hyperaccumulator *T. goesingense* from the nonaccumulator (Krämer et al., 1997), at least in in vitro culture.

DISCUSSION

Elevated concentrations of free SA are found to be a strong predictor of Ni hyperaccumulation across six different species of Thlaspi hyperaccumulator and nonaccumulators (Tables I and II). Furthermore, the SA upstream metabolites Phe and cinnamic acid, from the Phe ammonia lyase SA biosynthetic pathway, are also elevated in the hyperaccumulator, along with the downstream metabolites salicyloyl-Glc and catechol (Table I). Such perturbations suggest SA metabolism in the Thlaspi hyperaccumulators is permanently activated. SA is known to be involved in plant PRs, including signaling both the hypersensitive response (HR) and systemic acquired resistance (for review, see Shah, 2003). Furthermore, SA has been implicated in plant responses to heavy metals, including Pb and Hg in rice (Mishra and Choudhuri, 1999), and Cd in barley and maize seedlings (Pál et al., 2002; Metwally et al., 2003). However, the mechanism of SA-mediated metal resistance was not elucidated. Cadmium exposure has also been shown to induce accumulation of SA (Metwally et al., 2003), and exposure to Al, Cu, and Cd elicits expression of a pathogenesis related (PR2) protein in wheat (*Triticum aestivum*) roots (Cruz-Ortega and Ownby, 1993). Such observations suggest that plant responses to both metal toxicity and pathogens share certain mechanistic commonalities, possibly signaled by the formation of ROS (Overmyer et al., 2003). Our observation that preexposure of Arabidopsis to SA reduces Ni toxicity and increases SAT activity and accumulation of GSH supports a connection between SA and GSH-mediated Ni resistance. Furthermore, SA appears to act by regulating SAT activity posttranslationally, since direct assays of SAT, measured as production of OAS after addition of the substrates Ser and acetyl-CoA to a desalted extract, show increased activity without increases in either SAT mRNA or protein. Such increased SAT-specific activity observed in vitro could be due to either altered phosphorylation status or alteration of the stability of

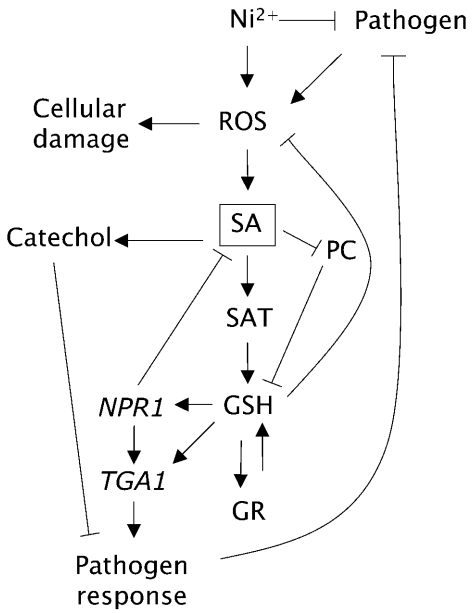

Figure 8. Model of the role of SA in response to biotic and abiotic Ni stress in Thlaspi hyperaccumulators. PC, Phytochelatins; NPR1, nonexpressor of PR genes; TGA1, basic domain/Leu zipper transcription factor.

the regulatory complex between SAT and OAS (thiol) lyase, both known to alter SAT activity in plants (Bogdanova and Hell, 1997; Yoo and Harmon, 1997). Reduction in the Cys sensitivity of one or more of the SAT isoforms (Inoue et al., 1999), possibly mediated by phosphorylation, could also contribute to the increased in vivo SAT activity suggested by the elevation of OAS after SA exposure. Answers to these intriguing questions await further study.

Increases in SAT activity and GSH were also observed in the SA-signaling mutant *npr1-1*, demonstrating that this regulation of SAT and GSH is independent of the NPR1 signaling pathway. The signaling mutant *npr1-1* lacks the ability to negatively regulate SA levels and SA hyperaccumulates in this mutant after challenge with a pathogen (Delaney et al., 1995) or after Ni exposure (Fig. 5A), demonstrating that both pathogens and Ni activate SA accumulation, possibly signed via an oxidative burst. SA hyperaccumulation in *npr1-1* was also found to be associated with increased SAT activity, GSH accumulation, and increased Ni resistance in this mutant. Based on such evidence, we propose that constitutively elevated SA in the hyperaccumulators acts to posttranslationally up-regulate SAT activity, causing constitutively elevated GSH and Ni tolerance, as previously observed in these hyperaccumulator species (Freeman et al., 2004).

Based on such observations, we propose the following model for the role of SA in Thlaspi Ni hyperaccumulators (Fig. 8). SA activates SAT posttranslationally causing accumulation of GSH and activation of GR (Knörzer et al., 1996) to maintain an enhanced pool of reduced GSH (Freeman et al., 2004). Increased GSH pools allow Ni-hyperaccumulating Thlaspi to resist Ni-induced oxidative stress (Freeman et al., 2004). Furthermore, SA potentially blocks phytochelatin synthase activity (Pál et al., 2002), inhibiting phytochelatin biosynthesis in response to Ni and conserving GSH to act as an antioxidant. Such inhibition is consistent with the lack of accumulation of phytochelatin-Ni complexes in *T. goesingense* (Freeman et al., 2004). The recent paradoxical observation that overexpression of phytochelatin synthase activity confers Cd sensitivity in Arabidopsis supports this view that depletion of GSH pools for phytochelatin synthesis can be detrimental to metal tolerance (Lee et al., 2003). Considering that SA normally signals HR and systemic acquired resistance in the nonaccumulator Arabidopsis (for review, see Dempsey et al., 1999), it is curious how Thlaspi hyperaccumulators are able to maintain constitutively high SA levels without initiating a PR. We propose that in the hyperaccumulator, the elevated levels of catecol, a potential breakdown product of SA, inhibits the initiation of a PR, as proposed previously in Arabidopsis (Van Wees and Glazebrook, 2003). However, it is also possible that mutations in other components of the SA-signaling cascade may exist in Thlaspi hyperaccumulators. This remains to be determined. The observation that in the absence of Ni in the growth medium *T. goesingense* is more susceptible to powdery mildew and does not increase SA synthesis or show any visible signs of a PR supports the conclusion that SA-mediated PRs in *T. goesingense* are suppressed. A similar increase in sensitivity to powdery mildew (*Erysiphe orontii*) occurs in the Arabidopsis mutant *npr1-1* (Reuber et al., 1998), and *npr1-1* also suppresses the increased resistance to powdery mildew (*Erysiphe cichoracearum*) of *edr1* (Frye et al., 2001). Normally, NPR1 acts to negatively regulate SA and signal SA-dependant gene expression via interaction with Leu zipper transcription factors, including TGA1. SA-dependent activation of NPR1, and its interaction with TGA1, has recently been shown to require reduction of critical disulfides in both proteins (Despres et al., 2003; Mou et al., 2003). Our observation of the SA-dependant activation of SAT, and the concomitant accumulation of GSH, provides one possible mechanism for such reduction via increased GSH levels. In *npr1-1*, SA-dependent signaling of GSH accumulation remains functional, allowing enhanced resistance to Ni. Furthermore, the inability of *npr1-1* to sense SA potentially suppresses its ability to initiate HR in response to the oxidative burst associated with Ni exposure, possibly further enhancing the Ni resistance of *npr1-1*. The fact that accumulation of Ni in *T. goesingense* enhances resistance to *E. cruciferarum* (Fig. 1) suggests that Ni hyperaccumulation may be compensating for the loss of normal PRs in these plants. Such a model has significant implications for both the mechanism of Ni tolerance in Thlaspi hyperaccumulators and the selective pressures driving their evolution.

MATERIALS AND METHODS

Plant Material

Seeds of the Ni hyperaccumulator *Thlaspi goesingense* (Hálácsy) were collected from an ultramafic site in Redschlag, Austria (Krämer et al., 1997). Shoot tissue samples from the Redschlag population of *T. goesingense* were collected, rapidly frozen on site, shipped to the United States in dry ice, and frozen at −80°C before extraction and HPLC analysis of SA levels. Seeds of the nonaccumulator *Thlaspi arvense* were collected from a calcareous soil at Col de Gleize, France (44°37′164″N, 6°03′959″E). All other accessions were as described by Peer et al. (2003) except seeds of Arabidopsis (*Arabidopsis thaliana*; Ws and Col-0), which were purchased from Lehle seeds (Round Rock, TX). Arabidopsis and various Thlaspi species were grown in a growth room (24°C/20°C, 10 h/14 h light/dark, 120 μmol m^{-2} s^{-1} photosynthetic photon flux) in artificial soil mix (Metro mix; Scotts, Marysville, OH). Arabidopsis was grown for 5 weeks and *T. goesingense* for 6 weeks, and plants compared were of equal size and leaf number. Shoot tissue was harvested at the rosette stage 5 h after the onset of the light period and immediately frozen in liquid nitrogen. Plants propagated on plates were grown on one-half strength Murashige and Skoog medium + B5 vitamins containing 0.8% agar on light racks in an environmentally controlled room (24°C/20°C, 14 h/10 h light/dark, 120 μmol m^{-2} s^{-1} photosynthetic photon flux).

Metabolite Quantification

Liquid N_2 frozen tissue stored at −80°C was extracted in ice-cold methanol at 4°C for 24 h, then phase extracted in ice-cold water/chloroform at 4°C for 12 h (Rhodes et al., 1986), with the addition of a norvaline internal standard. Extracts were derivatized and analyzed for all amino acids including OAS and Phe using AccQ Tag amino acid analysis following manufacturer's instructions (Waters, Milford, MA). SA, salicyloyl-Glc, catechol, and cinnamic acid were quantified by HPLC at 25°C using a Nova-Pak C-18 column with a flow rate of 1 mL min^{-1} over 22 min using a methanol gradient (solvent A, water and 1% formate; and solvent B, 100% methanol and 1% formate) of 10% to 40% B (10 min), 40% to 50% B (5 min), 50% to 100% B (2.5 min), 100% to 40% B (2.5 min), 40% to 10% B (1 min), and 10% B (1 min). Phenolic compounds were detected using both fluorescence (Ex_{254} and Em_{395}) and A_{280}. Analyses were performed using Waters Alliance HPLC system equipped with Millenium software, 2695 Separations Module, 2475 fluorescence detector, and 2996 Photodiode array detector. Standard curves were established using amino acid standard H (Pierce Chemical, Rockford, IL; catalog no. NCI0180), OAS (Sigma-Aldrich, St. Louis; catalog no. A–6262), SA (Sigma-Aldrich; catalog no. S–6271), cinnamic acid (Sigma-Aldrich; catalog no. 96340), and catechol (Sigma-Aldrich; catalog no. C9510). Identity of SA, salicyloyl-Glc, catechol, and cinnamic acid from plant samples were confirmed by liquid chromatography mass spectrometry/mass spectrometry (LC-MS/MS). Extracts from *T. goesingense* and other Thlaspi hyperaccumulators were fractionated as described above for phenolics and 30 s fractions collected. Fractions corresponding to the correct retention time and with the correct UV absorption spectrum were taken to dryness, resuspended in 50% methanol, and identified using accurate mass LC-MS/MS by identification of the correct parent ion with a Waters system equipped with MassLynx 4.0 software, AllianceHT Separations Module, 2996 Photodiode array detector, and Micromass Q-Tof micro mass spectrometer. Identity of SA and salicyloyl-Glc was further confirmed after fragmentation of the appropriate parent ions. Identification of OAS was confirmed using gas chromatography-mass spectrometry after methanol/chloroform/water extraction, standard amino acid fractionation (Rhodes et al., 1986), and derivatization with *N*-methyl-*N*-(*t*-butyldimethylsilyl)-trifluoroacetamide in pyridine. Thiols were derivatized with monobromobimane and quantified using HPLC as described (Tsakraklides et al., 2002).

Enzyme Assays

Freshly frozen Arabidopsis leaf tissue was immediately assayed for SAT activity in Sephadex G-25 fine desalted plant extracts after homogenization in 100 mM KH_2PO_4/K_2HPO_4, pH 7.6, by quantification of OAS produced after addition of Ser and acetyl-CoA, with a final reaction mixture of 100 μL containing 1 mM L-Ser, 0.1 mM acetyl-CoA, 1.25 mM Na_2EDTA, and 0.1 mM norvaline (internal standard) in 100 mM KH_2PO_4/K_2HPO_4, pH 7.6, at 25°C (Błaszcyk et al., 2002). Enzyme assays were stopped during the linear phase of the reaction at 20 min by the addition of ice-cold methanol/chloroform (2:1, v/v) and stored overnight at −80°C. OAS in the aqueous phase was derivatized and quantified using AccQ Tag following the manufacturer's instructions (Waters). GR was assayed in desalted total plant extracts in 100 mM Na_2HPO_4, 1 mM EDTA, pH 7.5, with HCl, using dithionitrobenzoate (Smith et al., 1988).

Immunoblot Analysis

SDS-PAGE was performed as described previously (Laemmli, 1970). Crude protein extracts from Arabidopsis were obtained by grinding shoot tissue samples in liquid nitrogen and mixing the frozen powdered plant material in a 2:1 ratio (w/v) with SDS sample buffer. The mixture was boiled for 10 min and centrifuged at 16,000*g*, and the supernatant was assayed for total protein using bicinchoninic acid (Pierce Chemical), and equal amounts of protein (30 μg) loaded onto an SDS-PAGE gel. For immunoblotting, proteins were transferred from the SDS-PAGE gel onto an Immuno-Blot polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA) using electrophoretic semidry blotting. Ser acetyltransferase was visualized on the membrane using polyclonal primary antibodies raised against Arabidopsis SAT-m in rabbits and a secondary anti-IgG antibody raised in goat and conjugated to alkaline phosphatase. Blots were developed by the addition of nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate.

RT-PCR-Based Assessment of SAT Transcript Levels

For RT-PCR analysis, 5 μg of total RNA was isolated from Arabidopsis (Col-0) and *npr1-1* shoot tissue after SA or Ni treatments and used as templates for RT using Superscript II (Invitrogen, Carlsbad, CA). Arabidopsis *SAT-m*, *SAT-p*, *SAT-c*, and *β-tubulin* were amplified from the RT reaction by PCR (94°C 5 min [94°C 30 s, 60°C 1 min, 72°C 2 min] × 30 cycles and 72°C 5 min) using Taq DNA polymerase (Promega, Madison, WI) and the appropriate pair of gene-specific primers (*SAT-m*, forward, 5′-GTCACAAGTCGCCGCCACTTCACA-3′ and reverse, 5′-AATTACATAATCCGACCACTCGG-3′; *SAT-p*, forward, 5′-TGCATCCACACATGCCGAACCGGT-3′ and reverse, 5′-AATTACATAATCCGACCACTCGG-3′; *SAT-c*, forward, 5′-GCCGGAGAACTCCGACATCAATCT-3′ and reverse, 5′-TATGATGTAGTCTGACCATTCCGA-3′; and *β-tubulin* forward, 5′-CGTGGATCACAGCAATCAAGAGCC-3′ and reverse, 5′-CCTCCTGCACTTCCACTTGGTCTTC-3′).

Quantification of Shoot Nickel Concentrations

After appropriate growth periods, shoot tissue was carefully harvested for inductively coupled plasma MS analysis of metal levels. Metal analysis was performed on dried shoot tissue as described previously (Lahner et al., 2003).

Quantification of Total Lipid Peroxidation

Peroxidized lipids were assayed as the presence of TBARS. Shoot tissue was harvested and assayed for TBARS as described previously (Murphy et al., 1999). During the period of metal exposure, all plants remained viable and continued growing.

Statistical Methods

All statistical analyses were performed using SAS software version 8e (SAS Institute, Cary, NC).

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