Properties of Enhanced Tonoplast Zinc Transport in Naturally Selected Zinc-Tolerant *Silene vulgaris*

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It was demonstrated recently that isolated tonoplast vesicles derived from plants of a Zn-tolerant ecotype of *Silene vulgaris* accumulate more Zn than vesicles derived from a Zn-sensitive ecotype. We have now characterized the tonoplast-transport system that causes this uptake difference and demonstrated its genetic correlation to Zn tolerance using plant crosses. We conclude that the tonoplast Zn uptake system of the tolerant ecotype differs greatly in its characteristics from that of the sensitive one, with the most prominent differences being its insensitivity to protonophores and ortho-vanadate and its stimulation by Mg-GTP. These differences in characteristics are most likely due to the fact that Zn can be taken up by two or more parallel pathways, which are not present in the same proportions in both ecotypes. In both ecotypes, Zn is actively transported across the tonoplast (temperature coefficient > 1.6), most likely as a free ion, since citrate does not accumulate in vesicles. Most importantly, the uptake difference found using the ecotypes was also found between homozygous Zn-tolerant and Zn-sensitive F_3 plants, proving the genetic correlation between increased tonoplast Zn transport and naturally selected Zn tolerance in *S. vulgaris*.

Silene vulgaris has many natural populations (ecotypes), some of which grow on soils that are enriched in various heavy metals (Ernst, 1974). These populations have evolved increased resistance to the metals present in the soil (Verkleij and Schat, 1990). Although the details of the genetic basis for the tolerance mechanisms are not yet clear (Schat and Vooijs, 1997), much is known about the physiological mechanisms of tolerance, especially in the case of Zn and Cd.

It has been demonstrated that enhanced Zn tolerance is not due to reduced uptake. Uptake studies have shown that roots of tolerant plants accumulate more Zn than those of sensitive plants (Mathys, 1975; Harmens et al., 1993b). Alternatively, detoxification of heavy metals could be achieved by intracellular binding to phytochelatins (Grill et al., 1987). However, this mechanism has not only been rejected as the mechanism underlying differential Cd tolerance in *S. vulgaris* (De Knecht et al., 1992), but its significance in the Zn-tolerance mechanism is also rebutted, since Zn is a poor inducer of phytochelatin sythase (Grill et al., 1989), and only low amounts of phytochelatins are produced upon Zn exposure in *S. vulgaris* (Harmens et al.,

1993a). Moreover, the accumulation of phytochelatins upon exposure to Zn is higher in the roots of sensitive plants than in those of tolerant plants (Harmens et al., 1993a). These findings support the hypothesis that naturally selected Zn tolerance might be based on enhanced compartmentation in the vacuole (Ernst, 1969; Mathys, 1975), as was suggested for Cd tolerance in *S. vulgaris* by De Knecht et al. (1995) and Chardonnens et al. (1998). This hypothesis is further supported by other reports showing elevated levels of heavy metals in vacuoles upon exposure of intact plants (Vögeli-Lange and Wagner, 1990; Brune et al., 1995).

As demonstrated by means of a split root experiment (Harmens et al., 1993b), the Zn-tolerance mechanism operates in root cells. Recently, we demonstrated that isolated tonoplast vesicles derived from roots of a Zn-tolerant ecotype of *S. vulgaris* take up 2 to 3 times more Zn than vesicles derived from Zn-sensitive plants when Zn is supplied as Zn citrate in the presence of Mg-ATP (Verkleij et al., 1998). In the present paper, this tonoplast transport of tolerant plants is further characterized using both direct filtration assays and fluorescence spectroscopy. The effect of incubation temperature, several known inhibitors of transport proteins, and Mg-GTP on the Zn uptake rate are studied in ecotypes with different Zn sensitivity. Additionally, a comparison is made between the uptake of Zn and the uptake of citrate to determine whether Zn is taken up as a cation or as a Zn citrate complex.

To demonstrate the significance of increased tonoplast Zn uptake in the mechanism of naturally selected Zn tolerance, we attempted to genetically link tonoplast Zn uptake with Zn tolerance. Previous experiments using crosses between Zn-sensitive and Zn-tolerant ecotypes have shown that tolerance in *S. vulgaris* is based on two major genes (Schat et al., 1996); therefore, it is possible to select homozygous tolerant and sensitive plants derived from crosses of a tolerant and sensitive ecotype. Replicating results obtained in uptake experiments using these selected lines would not only eliminate the possibility that any differences found were due to variations in the purity or protein content of the vesicle preparations of the different ecotypes, but, more importantly, it would very strongly link these results to Zn tolerance.

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Abbreviation: ABC, ATP-binding cassette.

MATERIALS AND METHODS

Plant Material

Seeds of *Silene vulgaris* (Moench) Garcke were collected from a Zn-sensitive population at the campus of the Vrije Universiteit (Amsterdam, The Netherlands) and from a Zn-tolerant population at a mine spoil near Plombières, Belgium. Plants were germinated and grown hydroponically as described by Verkleij et al. (1998). After 2 weeks, plants were used for tonoplast vesicle isolation.

Tonoplast Vesicle Isolation

Tonoplast vesicles were isolated from roots of nonexposed plants according to the method of Schumaker and Sze (1986), with slight modifications as described by Verkleij et al. (1998).

Assays of ATP Hydrolysis

Several assays of ATP hydrolysis were performed to determine the purity of the vesicle preparations and the percentage of right-side-out vesicles using a spectrophotometric measurement of phosphate, as described by Murphy and Riley (1962). The standard incubation was conducted as follows: 130 μL of a buffer containing 130 mM mannitol, 0.5 mM DTT, 1.3 mM Hepes/bis Tris propane, pH 7.4, and 7 mM KCl; 20 μL of a tonoplast protein sample (± 5 μg of vesicle protein) was added, followed by 150 μL of Mg-ATP to a final concentration of 2 mM. The vesicles were incubated for 30 min at 37°C, after which time the reaction was terminated by adding 4 mL of a reagent containing 2% (w/v) SDS, molybdate, and ascorbate (Murphy and Riley, 1962).

To determine the percentage of right-side-out vesicles, an incubation with 0.03% (w/v) Triton X-100 was compared with a standard incubation as described above.

To assess the purity of the vesicle preparation, the standard incubation was compared with incubation in a medium to which sodium molybdate and sodium vanadate were added to a final concentration of 0.26 mM, and NaN_3 was added to a final concentration of 2.63 mM.

The inhibition of V-type proton ATPases was assayed by adding 5 mM KNO_3 and 0.03% (w/v) Triton X-100 to the incubation medium and comparing this incubation with a standard incubation without KNO_3 but with Triton X-100.

Differences between the sensitive and the tolerant ecotype were tested using one-way analysis of variance.

Determination of ∆pH and ∆ψ

Prior to all direct filtration experiments, the effect of the concentration of chemicals used was tested with fluorescence spectroscopy. It was assessed that 5 mM NH_4Cl and 0.4 μg mL^{-1} gramicidin-D effectively dissipated the ∆pH of vesicles. Vesicles incubated with 5 mM KNO_3 were not able to generate a proton gradient upon the addition of Mg-ATP.

The effect of 0.5 mM Zn citrate, 50 μM ortho-vanadate, 5 mM NH_4Cl, or 0.4 μg mL^{-1} gramicidin-D on the maintenance of the proton gradient of isolated tonoplast vesicles was monitored using acridine orange quenching in fluorescence spectroscopy, as described by Verkleij et al. (1998). The effect of substances was expressed as the percentage of maximal proton gradient formation. Additionally, the effect of 0.5 mM Zn citrate on ∆ψ was monitored in similar experiments using 3 μM oxonol-V quenching (Scherman and Henry, 1980). Mg-ATP was added immediately after adding the vesicles to the incubation medium, and Zn citrate was added after 400 s. After another 100 s, gramicidin was added to dissipate the gradient. The effect of Zn was expressed as the percentage of change in fluorescence.

All experiments using fluorescence spectroscopy consisted of at least three replicates.

Uptake of Zn

All uptake experiments were performed according to the method of Verkleij et al. (1998), with one exception. Instead of loading the vesicles with K$^+$ to form an artificial proton gradient using nigericine, the vesicles were washed with resuspension buffer. A proton gradient was allowed to form in the presence of 3 mM Mg-ATP. Zn was supplied as Zn citrate. The uptake assay was started by the addition of Zn. In a pilot experiment, Zn uptake was shown to be optimal after 90 s, and was therefore measured after 90 s in all experiments. In direct filtration assays, the correction for aspecific binding of Zn to the outer side of the membrane was made by subtracting the Zn concentration measured in a simultaneous incubation of vesicles without ATP (Verkleij et al., 1998). In all experiments, Zn was measured using a flame atomic absorption spectrophotometer (model 1100B, Perkin-Elmer). All experiments were performed several times; each replicate is the average of a number of measurements made from a single vesicle isolation.

By adding 0.05% Triton X-100 to a vesicle incubation, it was tested whether Zn was actually transported into the lumen of the vesicles.

The proton gradient dependence of Zn uptake was measured by adding a protonophore to the incubation medium in direct filtration assays. NH_4Cl (5 mM) or gramicidin-D (0.4 μg mL^{-1}) was provided to the vesicles after 100 s, just prior to the addition of Mg-ATP. In other experiments, 50 μM ortho-vanadate was supplied to the vesicles. Zn was added 400 s after the addition of tonoplast vesicles, at which time the formation of the proton gradient was maximal. After 90 s of incubation, the vesicles were filtered and Zn uptake was measured using atomic absorption spectrophotometry. The effect of substances was expressed as the percentage of uptake in a reference situation, which was a simultaneous incubation with Zn in the presence of Mg-ATP.

The effect of substitution of ATP by GTP and the omission of Mg from the incubation medium were measured in similar experiments. The effect of each change in incubation circumstances was again expressed as a percentage of the reference situation.

In some experiments the incubation temperature was lowered from 24°C to 4°C, and the temperature coefficient (Q_{10}) for both ecotypes was calculated.

Uptake of Citrate

The uptake of citrate was measured using GC (model 5890 chromatograph, Hewlett-Packard) to determine the chemical species of Zn taken up by the tonoplast vesicles. Vesicles were incubated with either 0.1 or 0.5 mM Zn citrate, both in the presence and in the absence of 3 mM Mg-ATP.

After filtration over a 0.45-μm nitrocellulose filter (Schleicher & Schuell), vesicles were disrupted with 2 mL of 0.1% (v/v) trifluoroacetic acid. After measuring the Zn concentration of the samples using atomic absorption spectrophotometry, the volume was determined and 30 μL of 15 mM glutaric acid was added as an internal standard, followed by lyophilization of the samples.

The lyophilized samples were dissolved in 1.5 mL of water and mixed with 50 mg of Dowex anion-exchange resin (200–400 mesh, formate form; AG 1-X8, Bio-Rad). After sedimentation of the resin, the organic acids were released by adding 1.5 mL of 50% (v/v) HCOOH. Subsequently, 1.3 mL of the solution was supplied to 50 mg of Dowex cation-exchange resin (200–400 mesh, hydrogen form; AG 50W-X8, Bio-Rad). One milliliter of the supernatant was air-dried overnight in a vial (Chrompack, Raritan, NJ). The dried samples were resuspended in 1 mL of 96% ethanol, and this solution was evaporated with N_2 for 1.5 h. The dried samples were derivatized in two steps. First, they were oximated by adding 0.2 mL of $CHCl_3$ and 0.2 mL of Stox reagent (49805, Pierce), followed by a 30-min incubation at 75°C. Second, samples were sililated by adding 0.2 mL of bis-(trimethylsilyl)-trifluoroacetamide containing 1% trimethylchlorosilane (8251, Chrompack) and incubating for 5 min at 75°C. The concentration of organic acids in the samples was determined using the GC method described by Harmens et al. (1994).

RESULTS

Properties of Vesicles

The results of the assays of ATP hydrolysis (Table I) show that the vesicle preparations of different ecotypes have identical purity and inhibition rates by NO_3^-, although the tolerant ecotype has a slightly lower percentage of right-side-out vesicles than the sensitive ecotype (36.2% and 44.5%, respectively). Differences between ecotypes in the percentages of right-side-out vesicles, inhibition by nitrate, and purity were tested using one-way analysis of variance and found not to be significant (P > 0.05) in all cases.

Effect of Zn on ΔpH and $\Delta\psi$

The formation and maintenance of the proton gradient by isolated tonoplast vesicles of both ecotypes were monitored using acridine orange quenching. Upon addition of

Table I. *Assays of ATP hydrolysis performed with tonoplast vesicles derived from a tolerant and a sensitive ecotype of S. vulgaris*

Values are means ± SE of four to five replicates.

Treatment	ATPase Activity	
	Sensitive	Tolerant
	μmol Pi mg^{-1} vesicle protein h^{-1}	
No Triton X-100	13.5 ± 2.03	16.8 ± 4.3
Plus 0.03% Triton X-100	30.7 ± 0.8	46.4 ± 0.9
No inhibitors	13.5 ± 2.03	16.8 ± 4.3
Plus molybdate, vanadate, and NaN$_3$	12.1 ± 0.6	19.4 ± 2.8
No NO$_3^-$	13.5 ± 2.03	16.8 ± 4.3
Plus NO$_3^-$	8.7 ± 0.7	11.15 ± 0.9

Mg-ATP the formation of a proton gradient was observed in both ecotypes (Fig. 1A). The addition of 0.5 mM Zn citrate led to a slight decrease in ΔpH, which was similar in both ecotypes. Upon addition of either NH_4Cl or gramicidin, the proton gradient of the vesicles diminished (Fig. 1A). The addition of ortho-vanadate did not affect the proton gradient in either ecotype.

In both ecotypes the formation of an electrical gradient was observed upon addition of Mg-ATP using oxonol-V fluorescence quenching (Fig. 1, B and C). When Zn citrate was added, fluorescence in the sensitive ecotype recovered by 19% (Fig. 1B), indicating that net positive charge was moving out of the vesicles. The fluorescence in the tolerant ecotype did not recover at all, but was increased by 8% (Fig. 1C), indicating that net positive charge was moving into the vesicles.

Differential Sensitivities of Zn Transport to Inhibitors and Ionophores

The results of the Zn-uptake experiments are shown in Table II. At a concentration of 0.5 mM Zn citrate in the incubation medium, and in the presence of Mg-ATP, isolated tonoplast vesicles from the sensitive ecotype took up 1.85 ± 0.12 μmol mg^{-1} vesicle protein. Uptake by vesicles from the tolerant ecotype was 3.68 ± 0.79 μmol mg^{-1} vesicle protein. In the presence of 0.05% Triton 0.70 ± 0.08 and 0.69 ± 0.21 μmol mg^{-1} vesicle protein was measured in sensitive and tolerant plants, respectively, which proves that Zn is indeed taken up by the vesicles.

The uptake of Zn by isolated tonoplast vesicles derived from tolerant plants was not influenced by the addition of a protonophore, whereas the uptake of vesicles from sensitive plants was strongly reduced in all experiments: by 52% with NH_4Cl and by 22% with gramicidin. The addition of ortho-vanadate did not affect the Zn uptake in the tolerant ecotype, but the uptake of the sensitive ecotype was reduced to 42%.

When ATP was replaced by GTP, a 59% increase in Zn uptake was found in the tolerant ecotype, whereas the sensitive ecotype was inhibited by 39%. The omission of Mg diminished the Zn uptake in both ecotypes by over 80%.

Lowering the incubation temperature from 24°C to 4°C eliminated the Zn uptake in vesicles of both ecotypes. From the uptake rates found at both temperatures the Q_{10} value for the Zn uptake process was calculated. The Q_{10} of the
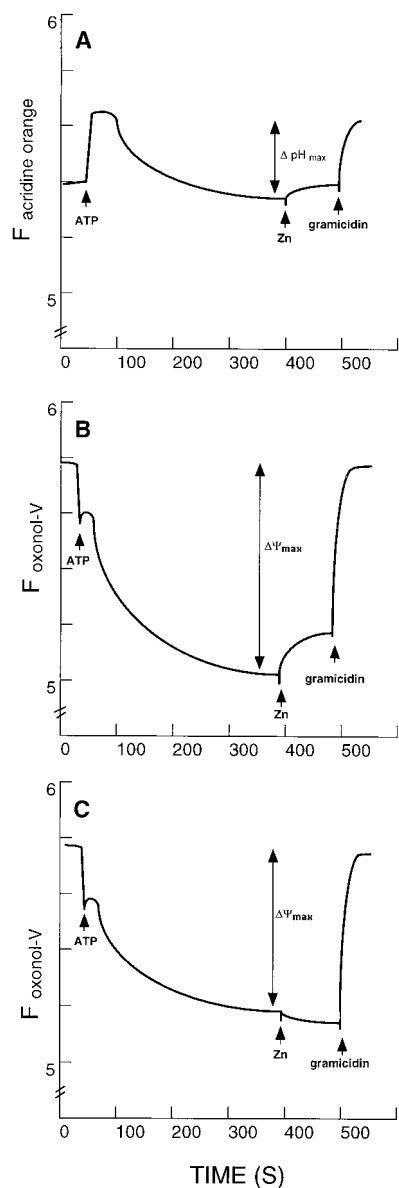
Figure 1. The effect of 0.5 mM Zn citrate on the ΔpH and $\Delta \psi$ of *S. vulgaris* tonoplast vesicles. Fluorescence (F) of acridine orange and oxonol-V were measured in arbitrary units. A, Effect of 0.5 mM Zn citrate on the ΔpH of tonoplast vesicles of a sensitive and tolerant ecotype of *S. vulgaris*, measured using acridine orange quenching (this graph is similar for both ecotypes). B and C, Effect of 0.5 mM Zn citrate on the $\Delta \psi$ of tonoplast vesicles of a Zn-sensitive (B) and a Zn-tolerant (C) ecotype was measured using oxonol-V fluorescence. In all cases, Mg-ATP was added immediately after adding the vesicles, and Zn citrate was added after 400 s. After another 100 s, NH_4Cl or gramicidin was added to disrupt the gradient.

tolerant ecotype was 2.4, and that of the sensitive ecotype was 1.6.

Uptake of Citrate

Tonoplast vesicles were incubated with either 0.1 or 0.5 mM Zn citrate in the presence or in the absence of Mg-ATP.

After measuring Zn with flame atomic absorption spectrophotometry, the citrate concentration of the samples was determined using GC. The Zn concentration in the vesicles increased with the external Zn concentration, and was higher in the presence of Mg-ATP for both ecotypes (Fig. 2). At 0.5 mM Zn citrate in the presence of Mg-ATP, vesicles derived from tolerant plants took up significantly more Zn than vesicles derived from sensitive plants (2.9 versus 0.9 μmol mg^{-1} vesicle protein). However, the uptake of citrate by the vesicles of either ecotype was independent of both the amount of citrate present in the incubation medium and the presence of Mg-ATP. The discrepancy between Zn uptake and citrate accumulation was demonstrated most clearly by vesicles of tolerant plants incubated with 0.5 mM Zn citrate in the presence of Mg-ATP (Fig. 2B). In these plants the amount of Zn in the vesicles was strongly increased (2.9 μmol mg^{-1} vesicle protein), whereas the amount of citrate remained very low (0.6. μmol mg^{-1} vesicle protein).

Plant Crosses and Selection for Zn Tolerance

To unambiguously link Zn tolerance to differences observed in tonoplast vesicle Zn uptake, plants of the tolerant and the sensitive ecotype were crossed. To obtain homozygous Zn-sensitive and Zn-tolerant genotypes of Amsterdam $\times$ Plombières plants, sets of F_1 plants were numbered and crossed in pairs, resulting in F_2 families designated as 1×2, 3×4, and 7×8. Of these, 200 plants per family were tested for Zn tolerance according to the method of Schat et al. (1996).

Table II. *Properties of Zn uptake. The uptake of Zn in the presence of test compounds is expressed as a percentage $\pm$ SE of up to 12 replicates of the Zn uptake in the reference situation at 90 s, incubation of vesicles at room temperature (24°C), with 0.5 mM Zn citrate, in the presence of MgATP*

The average net uptake for vesicles in the reference situation was 1.85 ± 0.12 and 3.68 ± 0.73 μmol mg^{-1} vesicle protein for the sensitive and the tolerant ecotype, respectively. All values were corrected for the amount of Zn measured without the addition of Mg-ATP (0.61 ± 0.03 μmol mg^{-1} vesicle protein for both ecotypes) prior to the calculation of percentages.

Treatment	Zn Uptake	
	Sensitive	Tolerant
	%	
Mg-ATP (24°C)	100	100
Mg-ATP (4°C)	45.5 ± 12.2	18.7 ± 3.0
Mg-GTP	61.4 ± 8.3	159.0 ± 13.3
ATP	18.3 ± 5.5	11.7 ± 1.6
Mg-ATP + ortho-vanadate	42.4 ± 4.6	101.0 ± 6.9
Mg-ATP + NH_4Cl	48.0 ± 3.5	87.1 ± 12.5
Mg-ATP + gramicidin	77.9 ± 5.0	98.9 ± 36.0
Mg-ATP + NH_4Cl + bafilomycin	58	132.7 ± 10.2
Mg-ATP + Triton X-100	9.8 ± 7.0	22.9 ± 14.8

F_2 plants showing complete inhibition of root growth below 700 μM $ZnSO_4$ in the test solution (<6% of the amount of plants tested) were qualified as homozygous sensitive; plants that continued growing at concentrations over 4000 μM $ZnSO_4$ in the test solution (also <6% of the amount of plants tested) were qualified as homozygous tolerant. The homozygous plants were transferred to fresh nutrient solution (without additional Zn) to form new roots, put on soil, and intercrossed to obtain enough seeds to preculture plants for tonoplast vesicle isolation and Zn uptake experiments, as described above.

The results of the uptake experiments using selected lines are shown in Table III. Increased uptake of Zn by tonoplast vesicles cosegregated with Zn tolerance. Vesicles derived from tolerant F_3 plants took up more Zn than those derived from sensitive plants at all concentrations of Zn citrate. These results were found using different sensitive and tolerant F_3 lines. This cosegregation of Zn uptake with tolerance proves that enhanced uptake of Zn across the tonoplast is genetically correlated with naturally selected Zn tolerance.
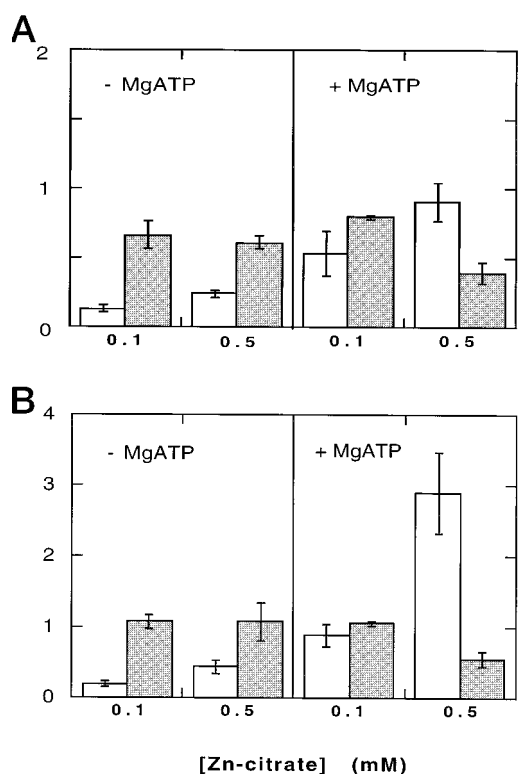

Figure 2. The concentration of Zn and citrate in isolated tonoplast vesicles derived from sensitive (A) and tolerant (B) plants. Tonoplast vesicles were incubated with either 0.1 or 0.5 mM Zn citrate in the presence or absence of Mg-ATP. After measuring Zn with flame atomic absorption spectrophotometry, the citrate concentration of the samples was determined using GC (see "Materials and Methods"). Bars represent means of three to six samples. Error bars represent SE values. White bars, Zinc (μmol/mg vesicle protein); shaded bars, citrate (μmol/mg vesicle protein).

Table III. *Zn uptake by homozygous sensitive and tolerant F_3 plants of Amsterdam × Plombières*

Values are means ± SE of three to five replicates for F_3 family 3 × 4, and the results of a single isolation for the other lines. Results were corrected for nonspecific binding of Zn to the vesicles by subtracting the Zn concentration (0.3, 0.5, or 0.8 mM) measured in a reference incubation without Mg-ATP.

F_3 Family	Zn Uptake		
	0.3 mM	0.5 mM	0.8 mM
	μmol mg^{-1} vesicle protein		
3 × 4 (sensitive)	0.39 ± 0.1	1.10 ± 0.1	1.94 ± 0.5
3 × 4 (tolerant)	1.00 ± 0.2	2.11 ± 0.9	3.26 ± 1.0
1 × 2 (sensitive)		0.82	1.28
7 × 8 (tolerant)		1.35	3.67

DISCUSSION

Our results provide the first direct evidence, to our knowledge, for an important role of tonoplast transport in naturally selected heavy metal tolerance. Although other authors have reported transport of heavy metals into isolated tonoplast vesicles (Salt and Wagner, 1993; Salt and Rauser, 1995; Gries and Wagner, 1998), we were to our knowledge the first to report differences in heavy metal uptake rate between tonoplast vesicles derived from ecotypes of one species showing differential heavy metal tolerance (Verkleij et al., 1998). This finding strongly supported the hypothesis that the tonoplast plays an essential role in naturally selected Zn tolerance, as was recently also strongly suggested for Zn hyperaccumulation in *Thlaspi caerulescens* (Lasat et al., 1998). However, this is the first time (again, to our knowledge) that enhanced tonoplast transport has been found to be genetically correlated with tolerance. Therefore, it is of great interest to characterize the transport system that causes this uptake difference in *S. vulgaris*.

The uptake of Cd by tonoplast vesicles derived from oat roots demonstrated by Salt and Wagner (1993) and Gries and Wagner (1998) is due to H^+-coupled antiport activity. In our experiments a very small decrease in the ΔpH of vesicles of both ecotypes was observed upon addition of Zn (Fig. 1A), suggesting that one pathway for Zn uptake in both sensitive and tolerant plants is via a H^+-coupled Zn antiport. Although this is a major pathway in vesicles from sensitive plants (Table II), most of the Zn uptake in tolerant plants was not sensitive to the ΔpH. These results suggest that in tolerant plants an additional uptake system must be present. This is further supported by measurements of the $\Delta\psi$ across the tonoplast (Fig. 1, B and C). In contrast to the sensitive ecotype, the tolerant ecotype did not show efflux of positive ions upon addition of 0.5 mM Zn (Fig. 1B) and was not reconcilable with high H^+-coupled Zn antiport activity in the tolerant ecotype. We conclude that H^+-coupled Zn antiport activity is most certainly not the major mechanism underlying increased Zn tolerance in *S. vulgaris*.

It is highly unlikely that Zn is associated with the membrane, as is the case for Ni in oat (Gries and Wagner, 1998), since almost no Zn is found in vesicles incubated without

Mg-ATP (Verkleij et al., 1998). The dependence of Zn transport on the presence of Mg-ATP could be due to binding of ATP to the transporter or to ATP hydrolysis. Since we found that ∆pH is not responsible for Zn transport, ATP hydrolysis by the transporting protein is likely to be the driving force of Zn transport. Indeed, Zn uptake was strongly reduced, both when the cofactor Mg was omitted, and when the incubation temperature was reduced from 24°C to 4°C. The Q_{10} values of the Zn uptake process calculated for both the sensitive and the tolerant ecotype (1.6 and 2.4, respectively) suggest biological activity rather than an nonspecific physical process as the cause of Zn accumulation.

Citrate concentrations in vesicles incubated with Zn citrate did not increase with the Zn concentration (Fig. 2), which strongly indicates that Zn is most likely taken up as Zn^{2+}. Further support for this hypothesis was obtained in experiments in which Zn and citrate were supplied to vesicles in a ratio of 1:2. In these experiments, Zn uptake was strongly reduced, whereas the replacement of citrate by malate in another experiment increased the uptake rate (data not shown).

It is plausible that the tonoplast of both ecotypes contains many cation transporters that are able to transport Zn when it is the most abundant substrate, as was the case in our assays. These transporters probably have a variety of characteristics that might account for some of the variation found in transport, especially in the sensitive ecotype. In the tolerant ecotype this variation was smaller, possibly because transport may have been dominated by one specific Zn transporter that might effectuate Zn tolerance. It is possible that the Zn-tolerant ecotype shows increased Zn uptake because it contains more units of a Zn-transport system constitutively present in all ecotypes. However, the transport system responsible for the increased uptake in vesicles of tolerant plants may also be due to an additional or a modified system not present on the tonoplast of sensitive plants.

The transport protein responsible for enhanced Zn transport across the vacuolar membrane of Zn-tolerant *S. vulgaris* might belong to the ABC superfamily of membrane transporters, which are directly energized by Mg-ATP and are able to transport a large number of substances, such as sugars, peptides, and inorganic ions (Rea et al., 1998). For instance, the YCF1 (yeast C̱d f̱actor) protein from *Saccharomyces cerevisiae*, which confers Cd resistance in this species, is a Mg-ATP-dependent, uncoupler-insensitive ABC protein (Li et al., 1996). However, this protein is inhibited by ortho-vanadate, as is the transport of chlorophyll catabolites in oilseed rape (Hinder et al., 1996), in which ATP could be partially replaced by GTP. ABC-protein-mediated transport with specificity for GTP hydrolysis is known from *Escherichia coli* (Zhong and Tai, 1998). Hwang et al. (1997) described two types of Ca^{2+}-pumping ATPases in carrot, one of which can hydrolyze GTP nearly as well as ATP, and is present on vacuolar membranes. In our experiments, however, Zn uptake by vesicles from the tolerant ecotype was strongly stimulated (59%) by the replacement of ATP by GTP. The latter finding, together with the insensitivity of Zn uptake to vanadate, suggests that transport in this case is not mediated by an ABC protein. Alternatively, the Zn-transport system in Zn-tolerant *S. vulgaris* might be related to one of several GTP-binding proteins present on the tonoplast of spinach, which have a molecular mass of 20 to 55 kD (Perroud et al., 1997).

We conclude that tonoplast vesicles derived from Zn-tolerant *S. vulgaris* possess a transport system that probably actively transports ionic Zn into the vesicles. The fact that increased Zn uptake by isolated tonoplast vesicles cosegregates with Zn tolerance in crosses very clearly demonstrates the importance of root tonoplast Zn transport in naturally selected Zn tolerance. The transport process in the tolerant ecotype is dependent on incubation temperature and the presence of Mg-ATP or Mg-GTP, and is not due to Zn^{2+}/H^+ antiport activity. The nature of the protein responsible for increased Zn transport, however, remains to be investigated. In vivo, this transport system most likely detoxifies Zn by transporting cytosolic Zn into the vacuole, a process that takes place far more efficiently in the Zn-tolerant than in the Zn-sensitive ecotype.

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