Antimony (Sb) is toxic to organisms including plants. Although it is not essential to organisms, plants take up Sb from the environment. In this study, we identified an antimonite [Sb(III)] transporter from Arabidopsis thaliana. We examined the Sb(III) tolerance of the disruption mutant plants of arsenite [As(III)] transporters, nodulin 26-like intrinsic proteins (NIPs), since Sb(III) is similar to As(III) in structure. One of the mutants, nip1;1, showed Sb(III) tolerance and accumulated less Sb. Furthermore, yeast expressing NIP1;1 accumulated twice as much Sb as control. These data indicate that NIP1;1 transports Sb(III) and determines the Sb(III) sensitivity of A. thaliana.

**Keywords:** Antimony • NIP • Transport.

**Abbreviations:** As, arsenic; ICP-MS, inductively coupled plasma mass spectrometry; MIP, major intrinsic protein; NIP, nodulin 26-like intrinsic protein; Sb, antimony.

Antimony (Sb) is toxic to organisms at elevated levels and, to our knowledge, its essentiality for organisms has not been established until now. The Sb content in the earth’s crust is very low and Sb-contaminated soil is mainly found in Sb-mining areas (Tschan et al. 2009). Until now, Sb contamination in the environment has not been a big problem in the world. However, Sb contamination is pointed out to be a potential problem (Maher 2009). Sb is used as a flame retardant in textiles, paper, plastics and adhesives, and is also contained in coal at high concentrations (Tschan et al. 2009). Combustion of those materials releases Sb into the environment, which can lead to the contamination of soil and water. Sb in the environment enters human bodies through the food chain, like eating and drinking contaminated crops and water, and may cause health problems (Pacyna and Pacyna 2001, Filella et al. 2009). Antimonite trioxide has been considered as potentially carcinogenic (Leonard and Gerber 1996).

In the environment, both inorganic and organic forms of Sb are present, and inorganic Sb is more abundant than the organic form. There are two major inorganic forms of Sb in the environment: antimonite [Sb(III)] and antimonate [Sb(V)]. Sb(III) is more toxic to organisms than Sb(V) (Filella et al. 2007). Both Sb(III) and Sb(V) are known to be taken up from the environment by organisms. An Sb(III) transporter has been identified in bacteria, yeast and mammals. No Sb(V) transporter has been identified so far (Filella et al. 2007). In those organisms, Sb(III) uptake is mediated by major intrinsic proteins (MIPs). The first Sb(III) transporter, GlpF, was identified in Escherichia coli by screening for Sb(III)-tolerant mutants (Sanders et al. 1997). GlpF belongs to an aquaglyceroporin subfamily of the MIP family. Subsequently, Fps1p, AQP9 and LmAQP1, which also belong to the aquaglyceroporin subfamily, have been known for their Sb(III) transport activity (Wysocki et al. 2001, Liu et al. 2002, Figarella et al. 2007). In addition to Sb(III), all of those transporters are also known to transport As(III). Since As(III) and Sb(III) belong to the same group (Group 15) in the periodic table, their chemical properties are similar. As(III) and Sb(III) exist in solution as As(OH)3 and Sb(OH)3, respectively, and bear similar structures. The pKₐ values of As(III) and Sb(III) are 9.2 and 11.8, and they exist as uncharged molecules in solution at neutral pH. These similar chemical properties are likely to be the basis for As(III) and Sb(III) being transported by the same transporter, aquaglyceroporins.

It has been known that Sb in soil enters plants, suggesting that Sb uptake is mediated by plant transporters (Tschan et al. 2009). However, transporters mediating Sb uptake have not been identified. In addition, little is known about Sb tolerance mechanisms. In this study, we identified the Sb(III) transporter in plants. Furthermore, we showed that the transporter is a key determinant of Sb(III) sensitivity in Arabidopsis thaliana.
To identify the Sb(III) transporter in plants, we focused on nodulin 26-like intrinsic proteins (NIPs), which are the equivalent of aquaglyceroporins in plants (Zardoya et al. 2002), for the following reasons. First, as mentioned above, As(III) transport in bacteria, yeast, and mammals is mediated by aquaglyceroporins. Second, in rice and A. thaliana, NIPs (Lsi1 and NIP1;1, respectively) are involved in As(III) uptake in roots (Ma et al. 2008, Kamiya et al. 2009). A. thaliana NIP1;1 is also the major determinant of As(III) tolerance in A. thaliana. Third, As(III) uptake in rice is inhibited by Sb(III) in a dose-dependent manner (Meharg and Jardine 2003). Fourth, a Sb(III)-tolerant yeast mutant expressing NIP7;1 is sensitive to Sb(III) (Bienert et al. 2008). Those data suggest that plant aquaglyceroporins have the ability to transport Sb(III).

To identify an Sb(III) transporter in A. thaliana, we looked for Sb(III) tolerance in T-DNA insertion mutants of NIPs (nip1;1-1, nip1;1-2, nip1;2-1, nip1;2-2, nip5;1-1), since we expected that disruption mutants of an Sb(III) transporter(s) would be tolerant to Sb(III). NIP1;2 and NIP5;1 are highly expressed in roots in contrast to other NIPs according to the MPSS database (http://mpss.udel.edu/at/). NIP1;2 is most similar to NIP1;1 in amino acid sequence, and NIP5;1 is involved in boron uptake in roots.

The mutants seeds were sown onto half-strength Murashige–Skoog (MS) medium containing 0, 15, 50 or 100 µM Sb(III) and grown for 10 d. Growth inhibition was observed in wild-type plants at concentrations of 50 and 100 µM Sb(III). Among the lines tested, only nip1;1 mutant lines were tolerant to Sb(III) (Fig. 1). nip1;1-1 and nip1;1-2 are independently isolated T-DNA insertion lines (Kamiya et al., 2009). Tolerance was observed in both roots and shoot growth. Root lengths of nip1;1 mutant lines are more than twice as long as those of wild-type and the other mutants tested in the presence of 100 µM Sb(III). Shoot growth of nip1;1 mutant lines was also less affected by 100 µM Sb(III) than the wild-type plants. These observations established that NIP1;1 determines Sb(III) sensitivity. Disruption mutants of NIP1;2, which is the most similar to NIP1;1 in terms of amino acid sequence, and NIP5;1, a boron uptake transporter in roots (Takano et al. 2006), did not show Sb(III) tolerance.

The fact that disruption of NIP1;1 confers Sb(III) tolerance suggests that NIP1;1 is able to transport Sb(III). To observe the Sb(III) transport activity in planta, the Sb content of the mutants plants was determined. The seeds were grown for 10 d in the presence of 15 µM Sb(III), followed by Sb content determination by inductively coupled plasma mass spectrometry (ICP-MS). The Sb content of nip1;1 mutants is 50% lower than in wild-type plants (Fig. 2A), indicating that roughly the half of the Sb(III) uptake in A. thaliana is mediated by NIP1;1. This percentage is larger than is the case for As(III) (30% lower than in wild-type plants). Taken together with the Sb(III) tolerance experiment, this result strongly suggests that NIP1;1 is capable of transporting Sb(III) in planta.

To directly confirm the Sb(III) transport activity of NIP1;1, we used a yeast heterologous expression system. NIP1;1 was expressed in the yeast strain fps1Δ, which lacks a gene for glycerol, As(III) and Sb(III) uptake transport (fps1) (Wysocki et al. 2001). NIP1;1 was introduced into strain fps1Δ and the expression was driven by the glyceraldehyde 3-phosphate dehydrogenase promoter. The transformants were incubated with medium containing 100 µM Sb(III) for 1 h and the Sb content of the cells was determined by ICP-MS. The yeast carrying NIP1;1 accumulated twice as much Sb as control yeast (Fig. 2B), demonstrating that NIP1;1 is able to transport Sb(III).

In plants, very little is known about the mechanisms of Sb(III) transport and tolerance. In this study, we demonstrated that NIP1;1 is the determinant of Sb(III) tolerance and that Sb(III) transport is mediated by NIP1;1.
Antimonite transport by Arabidopsis NIP1;1

In Sb(III) determination in mutant plants (Fig. 2A), nip1;1 alone showed reduced Sb content, but nip1;2 and nip5;1 did not. In contrast, in As(III) uptake experiments, all of the mutants showed reduced As contents, that is, those transporters are involved in As(III) uptake (Kamiya et al. 2009). These results suggest that Sb(III) transport is mediated by NIP1;1 alone, and not by NIP1;2 or NIP5;1. NIP1;2 and NIP5;1 are the first examples of As(III) transporters that do not mediate Sb(III) uptake. This may arise from differences in substrate specificity among NIPs. Although As(III) and Sb(III) are similar in structure, their molecular sizes are different: the ionic radii of Sb(OH)\(_3\) and As(OH)\(_3\) are 0.76 Å and 0.58 Å, respectively (Rosen et al. 1995). NIP1;2 and NIP5;1 may not be permeable to large molecules.

The root length of wild-type plants grown under 50 µM Sb(III) is similar to that of wild-type plants under 15 µM As(III) (Fig. 1; Kamiya et al. 2009). In addition, when plants were treated with 15 µM Sb(III) and 5 µM As(III), the Sb and As content of wild-type plants is about 250 µg/g DW and 300 µg/g DW, respectively (Fig. 2A; Kamiya et al. 2009). These data indicate that As(III) is more toxic than Sb(III) and the Arabidopsis roots have a higher affinity for As(III) uptake than for Sb(III) uptake, that is, As(III) enters the plants easily compared with Sb(III). The degree of toxicity seems to be consistent with the degree of permeability of Sb(III) and Sb(III) into plants. Although Sb(III) is transported by NIP1;1 but not by NIP1;2 or NIP5;1, As(III) is transported by NIP1;2 and NIP5;1 as well as NIP1;1. Furthermore, the expression level of NIP5;1 in roots is higher than that of NIP1;1 according to the MPSS database. The abundance of Sb(III) transporters may result in a much higher influx of As(III) than Sb(III) and in the severe growth inhibition by As(III). Therefore, the difference in the number of transporters involved in uptake is likely to determine the degree of As(III) and Sb(III) toxicity for plants. In addition to the difference in permeability, the difference in reactivity between As(III) and Sb(III) to thiol residues of proteins would be another reason for the toxicity difference. Although no information about the reactivity difference is available, if As(III) has a higher affinity than Sb(III) for the thiol of target proteins, As(III) will cause toxicity at lower concentrations than Sb(III).

We observed Sb accumulation in nip1;1 mutant plants and the yeast strain fps1Δ when these are defective in the Sb(III) transporter. For example, in nip1;1 mutant plants, Sb(III) uptake was reduced by 50%. The other half is not explained by uptake mediated by NIP1;1. Until now, aquaglyceroporin is the only transporter to show Sb(III) transport activity. There would exist additional uptake mechanisms in plants as well as in yeast.

In summary, we demonstrated that NIP1;1 transports Sb(III) and disruption of NIP1;1 confers Sb(III) tolerance on A. thaliana. To our knowledge, this represents the first identification of an Sb(III) transporter in plants. Sb(III) is rare in the natural environment but is widely used in industries throughout the world. Understanding the Sb cycle in organisms as well as in the earth contributes to the correct risk assessment of Sb, the action for contamination and the correct understanding of the danger of Sb in the future.

Materials and Methods

The seeds, growth conditions and media used in this study were described previously (Kamiya et al. 2009). For antimonite [Sb(III)] treatment of plants and yeast, potassium antimonyl tartrate (Wako, Osaka, Japan) was added to the medium at the concentrations shown in individual experiments.

Seeds were sown on half-strength MS medium containing 15 µM Sb(III). After incubation for 2 d at 4°C, the plates were
placed vertically and the plants were grown at 22°C for 10 d under a 16-h light/8-h dark photoperiod. The plants were washed with distilled water three times, dried at 65°C for >2 d and subjected to Sb determination with ICP-MS as described below.

NIP1:1 cDNA was amplified by PCR using 5′- GAGAATTCATGGCGATATCTCGGG-3′ and 5′- GAGAGTCGACTCAAGTGCTACCGATTCTCA-3′ (EcoRI and SalI sites are underlined). The resulting fragment was digested with EcoRI and SalI, and inserted into the EcoRI–SalI site of pKT10 (Tanaka et al. 1990). The construct was transformed into yeast (S. cerevisiae) (Tanaka et al. 1990). The constract was transformed into yeast (US EPA 1992). 

The mass 123 was monitored as the Sb signal. 125 Te was digested with concentrated HNO₃ at 110°C. After complete digestion, the samples were dissolved in 0.08 N HNO₃ with ICP-MS as described below. 

The yeasts were collected by centrifugation and suspended in ice-cold SD medium three times. The time point (1 h) was determined by referring to previous work (Liu et al. 2002). It was demonstrated that the same yeast strain expressing the same yeast transformants were incubated with AHCW/Glc medium overnight and then precultured with SD medium (0.67 M yeast nitrogen base w/o amino acid, 2% glucose) supplemented with auxotrophic requirements until the OD₆₀₀ reached about 0.5. The yeasts were dried at 65°C and subjected to Sb determination with ICP-MS as described below. 

The samples, including dried plants and yeasts, were digested with concentrated HNO₃ at 110°C. After complete digestion, the samples were dissolved in 0.08 N HNO₃ containing 10 ppb Ge. 72Ge was used as an internal standard. The mass 123 was monitored as the Sb signal. 125Te was simultaneously monitored and the actual Sb signal was calculated with the equation given in the EPA 200.8 method (US EPA 1992).

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