

Uptake of salicylic acid 2-*O*- β -D-glucose into soybean tonoplast vesicles by an ATP-binding cassette transporter-type mechanism

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In soybean (*Glycine max* L.), salicylic acid (SA) is converted primarily to SA 2-*O*- β -D-glucose (SAG) in the cytoplasm and then accumulates exclusively in the vacuole. However, the mechanism involved in the vacuolar transport of SAG has not been investigated. The vacuolar transport of SAG was characterized by measuring the uptake of [¹⁴C]SAG into tonoplast vesicles isolated from etiolated soybean hypocotyls. The uptake of SAG was stimulated about six-fold when MgATP was included in the assay media. In contrast, the uptake of SA was only stimulated 1.25-fold by the addition of MgATP and was 2.2-fold less than the uptake of SAG providing an indication that the vacuolar uptake of SA is promoted by glucosylation. The ATP-dependent uptake of SAG was inhibited by increasing concentrations of vanadate (64% inhibition in the

presence of 500 μ M) but was not very sensitive to inhibition by bafilomycin A₁ (a specific inhibitor of vacuolar H⁺-ATPase; EC 3.6.1.3), and dissipation of the transtonoplast H⁺-electrochemical gradient. The SAG uptake exhibited Michaelis–Menten-type saturation kinetics with a K_m value of 90 μ M for SAG. SAG uptake was inhibited 60% by β -estradiol 17-(β -D-glucuronide), but glutathione conjugates and uncharged glucose conjugates were only slightly inhibitory. Based on the characteristics of SAG uptake into soybean tonoplast vesicles it is likely that this uptake occurs through an ATP-binding cassette transporter-type mechanism. However, this vacuolar uptake mechanism is not universal since the uptake of SAG by red beet (*Beta vulgaris* L.) tonoplast vesicles appears to involve an H⁺-antiport mechanism.

Introduction

Salicylic acid (SA) is a naturally occurring phenolic that is widely distributed throughout the plant kingdom (Raskin et al. 1990). SA is known to be an important signal molecule involved in a variety of plant responses including the regulation of thermogenesis in the inflorescences of *Arum* lilies (Raskin et al. 1987) and both local and systemic resistance of plants to pathogens (Malamy et al. 1990, Métraux et al. 1990, Delaney et al. 1994). In addition to these effects, SA is also thought to be an allelopathic chemical and is known to have toxic effects on cells due to its general phenolic structure and its ability to disrupt membrane integrity and interfere with ion absorption (Raskin 1992). Metabolism and conjugation of SA are an important part of the detoxification

mechanism used by plants to protect themselves from exogenously supplied or endogenously produced SA. Although a variety of SA metabolites and conjugates have been described in plant species, in most cases glucosylation appears to be the major route of SA metabolism. Glucosylation of SA can result in the formation of either SA 2-*O*- β -D-glucose (SAG) or the SA glucose ester (SGE). In the limited number of species examined, SAG appears to be the major glucose conjugate of SA that is formed (Tanaka et al. 1990, Schulz et al. 1993, Edwards 1994, Silverman et al. 1995, Lee and Raskin 1998). In soybean cell cultures, it was originally reported that exogenously administered SA was metabolized exclusively to SGE (Barz et al. 1978). However, it has recently

Abbreviations – ABC transporter, ATP-binding cassette transport protein; AMP-PNP, adenosine 5'-(β , γ -imino)triphosphate; Δ pH, transmembrane pH difference; $\Delta\psi$, transmembrane electrical potential difference; DNP-GS, *S*-(2,4-dinitrophenyl)glutathione; E₂17G, β -estradiol 17-(β -D-glucuronide); H⁺-ATPase, H⁺-translocating adenosine triphosphatase; H⁺-PPase, H⁺-translocating pyrophosphatase; HPS, hydroxyprimisulphuron; MRP, multidrug resistance-associated protein; *o*-NPG, *o*-nitrophenyl β -D-glucoside; SA, salicylic acid; SAG, salicylic acid 2-*O*- β -D-glucose; SAGT, salicylic acid glucosyltransferase; SGE, salicylic acid glucose ester.

been demonstrated that at least some soybean cell cultures (cv. Williams 82) metabolize SA primarily to SAG, lower levels of methyl salicylate 2-*O*- β -D-glucose and glucosylated 2,5-dihydroxybenzoic acid without any detectable SGE formation (Dean et al. 2003). In soybean, SA is converted to SAG by an inducible SA glucosyltransferase (SAGT), presumably in the cytoplasm, yet the SAG is stored exclusively in the vacuole (Dean et al. 2003). Therefore, it appears as if there is a transport mechanism in soybean cells that moves SAG from the cytoplasm across the tonoplast and into the vacuole.

Although it is generally assumed that glucose conjugates of natural products undergo carrier-mediated uptake into the vacuole, very little is known about these transport mechanisms (Martinoia et al. 2000). The three main mechanisms proposed for vacuolar transport of glucosides includes H^+ -antiport, conformational trapping and uptake by ATP binding cassette (ABC) transporters.

The tonoplast membrane possesses both a V-type H^+ -translocating adenosine triphosphatase (H^+ -ATPase) and an H^+ -translocating pyrophosphatase (H^+ -PPase; Rea and Sanders 1987) and depending on the energy source available these pumps transport H^+ from the cytoplasmic side of the membrane into the lumen of the vacuole forming both a pH gradient (ΔpH) and a membrane potential ($\Delta\psi$). This H^+ -electrochemical potential difference can then be used to drive transport across the membrane through an H^+ -antiporter or may be used for the pH-dependent conformational trapping of acylated compounds (Martinoia et al. 2000). It has been suggested that this latter mechanism is involved in the vacuolar accumulation of *Daucus carota* anthocyanin and apigenin 7-*O*-(6-*O*-malonylglucoside) from parsley (Matern et al. 1986, Hopp and Seitz 1987). Plant vacuolar membranes also contain ABC transporters that are able to move large organic anions into the vacuole (Rea et al. 1998, Rea 1999, Martinoia et al. 2000). ABC transporters are directly energized by ATP hydrolysis and therefore do not depend on the transmembrane H^+ -electrochemical gradient. These ABC transporters were originally described as glutathione conjugate pumps due to their ability to transport xenobiotic glutathione conjugates (Martinoia et al. 1993, Li et al. 1995). However, it was later revealed that ABC transporters may be involved in the transport of a variety of xenobiotics and natural product metabolites including glucose conjugates (Rea et al. 1998, Rea 1999, Martinoia et al. 2000).

Though barley is not believed to form the coumarin esculetin, this compound supplied exogenously is glucosylated in the cytoplasm of barley leaf mesophyll cells resulting in the formation of esculin, which accumulates exclusively in the vacuole. An H^+ -antiporter is believed to be involved in the transport of esculin across the tonoplast of the barley leaf mesophyll cells (Werner and Matile 1985). Very recently, it has been shown that uptake of *p*-hydroxycinnamic acid-glucoside and *p*-hydroxybenzoic acid glucoside (two naturally occurring plant phenolic glucosides) into red beet tonoplast vesicles depends on a transtonoplast H^+ gradient and is likely due to an H^+ -antiport mechanism

(Bartholomew et al. 2002). This same H^+ -antiport mechanism was also responsible for the uptake of 5-hydroxychlor-sulphuron-glucoside, an herbicide glucose conjugate (Bartholomew et al. 2002). Glucosylation appeared to be an essential prerequisite for recognition by the H^+ -antiporter since the unconjugated compounds were taken up at a dramatically lower rate. Uptake of the glutathione conjugates of the herbicide chlorimuron-ethyl (which possesses structural similarities to 5-hydroxychlor-sulphuron) and the glutathione conjugate of *trans*-cinnamic acid (which has a structural resemblance to *p*-hydroxycinnamic acid) into red beet tonoplast vesicles was directly energized by ATP and had properties consistent with the involvement of an ABC transporter (Walczak and Dean 2000, Bartholomew et al. 2002). Therefore, it appears as if in red beet, glucose conjugates cross the tonoplast via an H^+ -antiport mechanism and glutathione conjugates cross via an ABC transporter. In barley vacuoles, uptake of hydroxyprimisulphuron-glucoside (HPS-glucoside; a sulphonylurea herbicide glucoside similar in structure to the two used by Bartholomew et al. 2002) is taken up by an ABC transporter-type mechanism (Klein et al. 1996), whereas, isovitexin (apigenin 6-C-glucoside; a native barley flavonoid C-glucoside) and saponarin (apigenin 6-C-glucosyl-7-*O*-glucoside; the major native barley flavonoid) are transported across the tonoplast of barley by an H^+ -antiport mechanism (Klein et al. 1996, Frangne et al. 2002). However, saponarin uptake into the vacuoles of *Arabidopsis* (a species that does not produce saponarin) occurs through an ABC transporter-type mechanism (Frangne et al. 2002).

Based on these previous investigations, it seems likely that SAG may cross the tonoplast of soybean cells through either an H^+ -antiport or ABC transporter-like mechanism. Conformational trapping of SAG seems unlikely since soybean vacuoles accumulate SAG in an unaltered form (Dean et al. 2003).

The goal of this study was to characterize the transport mechanism involved in the vacuolar uptake of SAG. Soybean appeared to be a good model organism for this study since, as mentioned previously, SAG accumulates exclusively in the vacuole of soybean cells. In addition, the high rate of in vivo [^{14}C]SAG formation from exogenously supplied [^{14}C]SA provided a convenient method for the synthesis of high levels of [^{14}C]SAG for the uptake assays.

From this work, we have been able to demonstrate that SAG uptake into tonoplast vesicles isolated from etiolated soybean hypocotyls occurs through an ABC transporter-type mechanism and that glucosylation appears to be required for recognition by the transporter. However, this vacuolar uptake mechanism is not found in all species since SAG uptake into red beet tonoplast vesicles appears to involve an H^+ -antiporter.

Materials and methods

Chemicals

The [$7-^{14}C$]SA (55.5 mCi mmol $^{-1}$) was purchased from PerkinElmer Life Sciences (Boston, MA, USA). All

other chemicals were purchased from Sigma (St. Louis, MO, USA).

Plant material

Red beet (*Beta vulgaris* L.) storage roots were purchased from local markets. Soybean (*Glycine max* [L.] Merr. Pioneer variety 93B15) seed was planted in coarse sand and grown in the dark at 25°C for 7 days. The seeds were watered once a day with tap water. Soybean (*Glycine max* [L.] Merr. cv Williams 82) cell suspension cultures were grown in Murashige and Skoog (1962) media with 3% (w/v) sucrose and 0.4 mg ml⁻¹ 2,4-D. The cells were incubated on a rotary shaker (140 r.p.m) at room temperature under constant room light. The cells were subcultured weekly by adding the 7-day-old cultures to an equal volume of fresh media.

Preparation of DNP-GS and [¹⁴C]SAG

DNP-GS was prepared as described by Walczak and Dean (2000). In order to prepare [¹⁴C]SAG, 20 μCi of [7-¹⁴C]SA (10 mCi mmol⁻¹) was added to a 100-ml soybean cell suspension culture 3 days after subculture. After 24 h the cells were collected through vacuum filtration and ground in 80 ml of 90% ethanol with a mortar and pestle. The extract was centrifuged at 400g for 20 min. The supernatant was concentrated to about 0.5 ml in vacuo at 50°C. The concentrate was then diluted to 1 ml with acetic acid (1%):methanol (95:5) and centrifuged for 5 min at 11 000 g before HPLC purification. The extract (250 μl) was injected onto a 250 × 7 mm Allsphere 5 μm ODS-1 column (Alltech, Deerfield, IL, USA). Compounds were eluted from the column with a linear gradient from 95% acetic acid (1%) and 5% methanol to 50% acetic acid (1%) and 50% methanol in 20 min at a flow rate of 2 ml min⁻¹ followed by a 5-min linear gradient to 20% acetic acid (1%) and 80% methanol at a flow rate of 1.5 ml min⁻¹. Radioactive peaks eluting from the column were detected with a Triathler HPLC detector (Bioscan, Washington, DC, USA) fitted with a 25-μl flow cell. The major radioactive compound had a retention time of 12 min and was collected as a 2-ml fraction with a fraction collector. The fraction was loaded (1 ml per cartridge) onto a Sep-Pak C18 cartridge (Waters, Milford, MA, USA). The cartridge was washed with 2 ml of water to remove residual acetic acid and the [¹⁴C]SAG was eluted with methanol. The eluant was concentrated to dryness in vacuo and stored at -20°C. The [¹⁴C]SAG was dissolved in water before use in the uptake assays. Typical yields of purified [¹⁴C]SAG from [¹⁴C]SA were 20%. In order to determine the specific activity, the [¹⁴C]SAG was converted to [¹⁴C]SA by β-glucosidase treatment as described by Edwards (1994). The amount of SA was determined by monitoring the A₃₀₀ during an analytic HPLC separation (see next section) and comparing this value to a standard curve constructed from HPLC separations of authentic SA. The radioactivity associated

with the SA was determined through liquid scintillation counting. The specific activity of the [¹⁴C]SAG used for the transport assays ranged from 9 to 11 mCi mmol⁻¹. The identification of this compound as [¹⁴C]SAG has been described by Dean et al. (2003). This identification was based on the observation that SA was released following treatment of the compound with β-glucosidase, but not by esterase treatment. In addition, [¹⁴C]SAG formed in vitro from [¹⁴C]SA and UDP-glucose in the presence of the soybean SAGT enzyme had the same TLC R_f value and HPLC retention time as the purified [¹⁴C]SAG synthesized in vivo. Recent mass spectrometer analysis of the compound provided additional evidence that the sample was SAG. The mass spectra were generated with a LCQ mass spectrometer (Thermo Finnigan, San Jose, CA, USA) using electrospray ionization in both the positive and negative ion mode. This analysis was performed at the University of Illinois at Chicago Research Resources Center.

Analytical HPLC

An analytical HPLC procedure was used to monitor alterations of the [¹⁴C]SAG following transport and to determine the specific activity and radiochemical purity of [¹⁴C]SAG. For this procedure, the sample was loaded onto a 150 × 4.6 mm Allsphere 5 μm ODS-1 HPLC column (Alltech) that was eluted at a flow rate of 1 ml min⁻¹ with a linear gradient from 95% acetic acid (1%) and 5% methanol to 50% acetic acid (1%) and 50% methanol in 20 min followed by an additional 5 min linear gradient to 20% acetic acid (1%) and 80% methanol that was held for 5 min. Radioactivity was detected with a Triathler HPLC detector fitted with a 250-μl flow cell.

Preparation of membrane vesicles

Tonoplast vesicles from peeled red beet storage roots or etiolated soybean hypocotyls were prepared using the procedure developed for mung bean (*Vigna radiata*) as described by Rea et al. (1992). If plasma membrane was collected, the step gradient consisted of 3.3 ml each of 10% (w/w), 23% (w/w) and 40% (w/w) sucrose. Tonoplast vesicles were collected from the 10/23% interface and plasma membrane was collected from the 23/40% interface. Washed tonoplast vesicle and plasma membrane pellets were suspended in 1 ml buffer (1.1 M glycerol, 1 mM Tris-EGTA, 2 mM DTT, 5 mM Tris-MES; pH 7.8) to protein concentrations of about 5 mg ml⁻¹ and 10 mg ml⁻¹, respectively. The protein concentration was estimated by the method of Bradford (1976) using BSA as a protein standard.

Measurement of [¹⁴C]SAG uptake, H-PPase and H-ATPase activity

Measurement of [¹⁴C]SAG uptake into membrane vesicles was measured as described by Li et al. (1995). Unless otherwise stated the standard assay contained 50 μM of [¹⁴C]SAG (approximately 10 mCi mmol⁻¹), 24 μl of

soybean membrane vesicles or 12 μl of red beet tonoplast vesicles and was allowed to proceed at 25°C for 15 min.

The H⁺-PPase or H⁺-ATPase activity was determined by measuring the decrease in the A_{490} of acridine orange as described by Giannini et al. (1991). For the measurement of the H⁺-PPase activity, the assay medium (1 ml) contained 0.4 M sorbitol, 50 mM KCl, 1.3 mM MgSO₄, 0.3 mM PP_i, 25 mM Tris-MES (pH 8.0) and 10 μM acridine orange. The assay was started by adding 20 μl of vesicles (approximately 200 μg protein). The H⁺-ATPase activity was determined in the same manner as the H⁺-PPase activity except that ATP (3 mM) replaced the PP_i, the MgSO₄ concentration was 3 mM, and the pH was 6.5. When added, the vanadate concentration in the H⁺-ATPase assay medium was 100 μM .

Results

Time-dependent uptake of SAG into soybean tonoplast vesicles in the presence or absence of MgATP

In the presence of MgATP, the in vitro uptake of SAG by tonoplast vesicles isolated from etiolated soybean hypocotyls increased for nearly 30 min (Fig. 1). However, from 30 to 120 min, no further increase in uptake was observed. SAG uptake increased over time in the absence of MgATP, however, these levels were considerably less than the levels observed in the presence of MgATP. At assay times of 8 and 15 min, the SAG uptake levels were 4.7- and 5.6-fold, respectively, greater in the presence of MgATP than in its absence. Unless

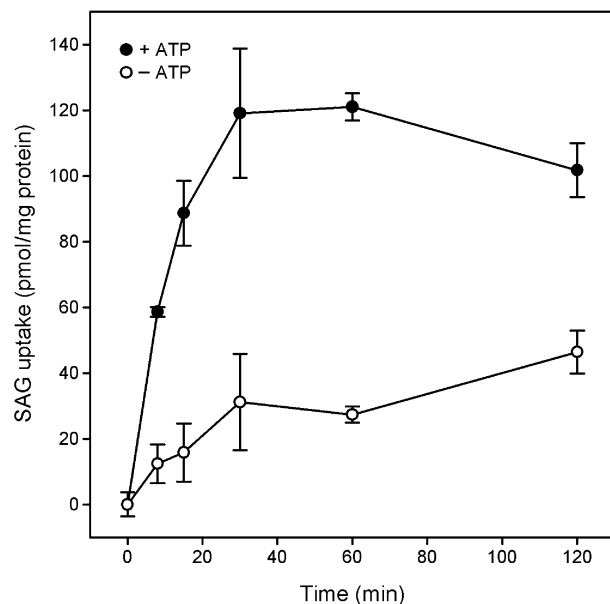


Fig. 1. Time course of [¹⁴C]SAG uptake into soybean tonoplast vesicles in the presence or absence of MgATP. Uptake was measured using the procedure described by Li et al. (1995) in the presence (+MgATP) or absence (-MgATP) of 3 mM MgATP. Values shown are means of three replicates \pm SD. All values were corrected for radioactivity that was detected on the filters at time 0.

otherwise stated, all subsequent uptake assays were allowed to proceed for 15 min since at this time point the greatest difference between uptake in the presence and absence of MgATP was observed.

Tonoplast vesicles prepared by the same method used in this study have an internal volume of 10 $\mu\text{l mg}^{-1}$ protein and constitute a 1:1 mixture of right-side-out and inside-out vesicles (Poole et al. 1985, Rea et al. 1987, Zhen et al. 1994). Using these assumptions and the [¹⁴C]SAG uptake values from a 30-min assay (Fig. 1), an internal [¹⁴C]SAG concentration of 24 μM can be calculated. Since the external [¹⁴C]SAG concentration used in the assay was 50 μM , accumulation of [¹⁴C]SAG against a concentration gradient in these uptake assays could not be demonstrated.

Effects of various assay conditions on the uptake of [¹⁴C]SAG into soybean tonoplast vesicles

Since MgATP clearly enhanced the uptake of SAG by soybean tonoplast vesicles, the next goal was to determine if this uptake was directly or indirectly energized by MgATP. Indirect enhancement of uptake in the presence of MgATP may involve an antiporter that couples the movement of SAG with that of protons. A V-type ATPase in the presence of MgATP would form the H⁺ gradient in tonoplast vesicles. Bafilomycin A₁ is a specific inhibitor of V-type ATPases (Dröse et al. 1993) and Gramicidin D is a cation-selective ionophore. The inclusion of either of these compounds into the assay medium would limit the extent of any uptake driven by an MgATP generated H⁺ gradient. However, neither of these compounds had much of an affect on the MgATP-dependent SAG uptake rates of soybean tonoplast vesicles. In the presence of bafilomycin A₁ and gramicidin D the SAG uptake rates were 91 and 83%, respectively, of the control rates (+ MgATP only; Table 1). On the other hand, increasing concentrations of vanadate were strongly inhibitory to the SAG uptake by soybean tonoplast vesicles (Fig. 2). The addition of 125 μM of vanadate into the SAG uptake assay medium reduced the MgATP-dependent uptake rates by 38% (Fig. 2). The addition of higher vanadate concentrations decreased the uptake rate by 82% (1 mM; Fig. 2). Vanadate is a phosphoryl transition-state analogue known to be a strong inhibitor of enzymes, including ABC transporters, which form a phosphoenzyme intermediate. The uptake of SAG also appears to be dependent on ATP hydrolysis since adenosine 5'-(β,γ -imino)triphosphate (AMP-PNP), a non-hydrolysable analogue of ATP, did not support uptake (Table 1). ABC transporters are also not energized by AMP-PNP (Martinoia et al. 1993, Li et al. 1995).

SA versus SAG uptake into soybean tonoplast vesicles

There was very little difference between the uptake of SA by soybean tonoplast vesicles in the presence and absence of MgATP (Table 1). The uptake of SA in the

Table 1. Uptake of SAG and SA into soybean and red beet tonoplast vesicles and the effects of various assay conditions. The uptake was measured in the standard 15 min assay containing either [¹⁴C]SAG or [¹⁴C]SA (10 mCi mmol⁻¹). Bafilomycin A₁ and gramicidin D were dissolved in ethanol and compared with control assays that also contained ethanol (1%). All other compounds were dissolved in water. All values shown were corrected for non-specific binding of either [¹⁴C]SAG or [¹⁴C]SA to the filters. The [¹⁴C]SAG uptake in the presence of 3 mM MgATP was used as the control value. Values shown are the means of three replicates ± SD. The soybean control values used for bafilomycin A₁ and gramicidin D calculations were 9.4 ± 0.3 and 9.8 ± 1.0 pmol min⁻¹ mg⁻¹, respectively. The soybean control value used for all other assay conditions was 5.6 ± 0.8 pmol min⁻¹ mg⁻¹. The red beet control value was 1239.45 ± 26.37 pmol min⁻¹ mg⁻¹. ND, not determined. F2, results shown in Fig. 2.

Substrate (50 μM)	Assay conditions	Percentage of control	
		Soybean	Red beet
SAG	-MgATP	16.8 ± 2.0	-0.3 ± 0.4
SAG	+3 mM MgATP (control)	100 ± 14.2	100 ± 2.1
SAG	-MgATP + 3 mM AMP-PNP	29.8 ± 8.4	ND
SAG	+3 mM MgATP + 0.25 mM vanadate	F2	98.5 ± 1.5
SAG	+3 mM MgATP + 0.4 μM bafilomycin A ₁	91.3 ± 3.0	1.4 ± 0.1
SAG	+3 mM MgATP + 5 μM gramicidin D	82.9 ± 8.8	21.0 ± 1.8
SA	-MgATP	36.5 ± 10.2	3.8 ± 0.5
SA	+MgATP	45.8 ± 11.3	5.2 ± 0.6

presence of MgATP was only 1.25-fold greater than the uptake in the absence of MgATP. In addition, the uptake of SA in the presence of MgATP was 2.2-fold less than the MgATP-dependent uptake of SAG (Table 1).

Uptake of SAG and SA into red beet tonoplast vesicles

The uptake mechanism of SAG by red beet tonoplast vesicles was strikingly different than the uptake mechanism observed using soybean. Uptake of SAG by red beet tonoplast vesicles in the absence of MgATP was essentially undetectable (Table 1). However, in the presence of

MgATP, SAG uptake was easily measured and was 100- to 200-fold greater than the SAG uptake rate measured with soybean (Table 1). The inhibitor and ionophore sensitivity was also dramatically different. The addition of bafilomycin A₁ and gramicidin D resulted in 98.6% and 79% losses, respectively, of the SAG uptake by red beet tonoplast vesicles (Table 1). However, in the presence of 250 μM vanadate, 98.5% of the MgATP-dependent SAG uptake was retained (Table 1). As with soybean tonoplast vesicles, there was very little difference observed between the uptake of SA by red beet tonoplast vesicles in the presence and absence of MgATP. However, the uptake of SA by red beet tonoplast vesicles was 25-fold greater than the uptake of SA by soybean tonoplast vesicles (Table 1).

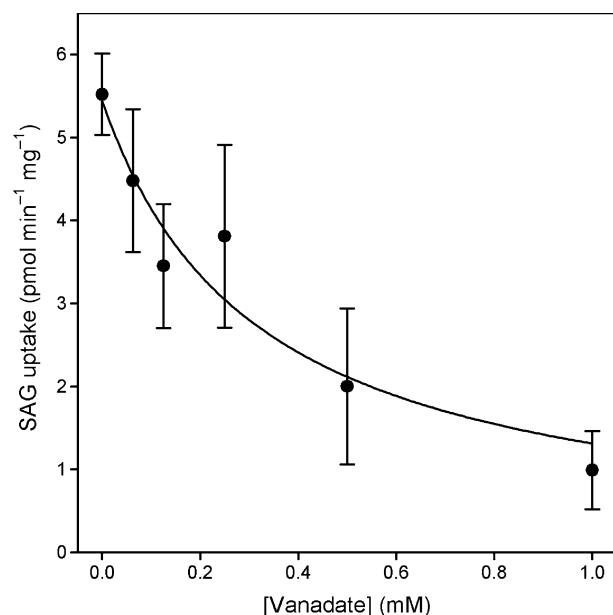


Fig. 2. Effects of increasing vanadate concentrations on MgATP-dependent uptake of [¹⁴C]SAG into soybean tonoplast vesicles. Uptake of [¹⁴C]SAG was measured in the standard assay as described by Li et al. (1995). All values were corrected for radioactivity detected on the filters following assays conducted in the absence of MgATP. Each data point represents the mean of three replicates ± SD.

HPLC analysis of [¹⁴C]SAG after uptake

A single radioactive peak with a retention time of 9.5 min was detected in the HPLC profile of the purified [¹⁴C]SAG added at the start of the uptake assays (Fig. 3A). At the end of both the soybean (Fig. 3B) and red beet (Fig. 3C) tonoplast uptake assays, the radioactivity on the washed filters was re-examined. In both cases, there was only one radioactive peak that had the same HPLC retention time (9.5 min) as the [¹⁴C]SAG added at the start of the assay (Fig. 3A-C). Therefore, the [¹⁴C]SAG used for the uptake assays had a high degree of radiochemical purity and remained unaltered following uptake into either the soybean or red beet tonoplast vesicles.

Competitive inhibitors of SAG uptake into soybean tonoplast vesicles

MgATP-dependent SAG uptake assays in the presence of a variety of potential competitive inhibitors were performed in order to elucidate the substrate specificity of the soybean tonoplast transporter. Salicin, a glucose conjugate similar in structure to SAG, resulted in only a 12% inhibition of SAG uptake (Fig. 4). Other structurally

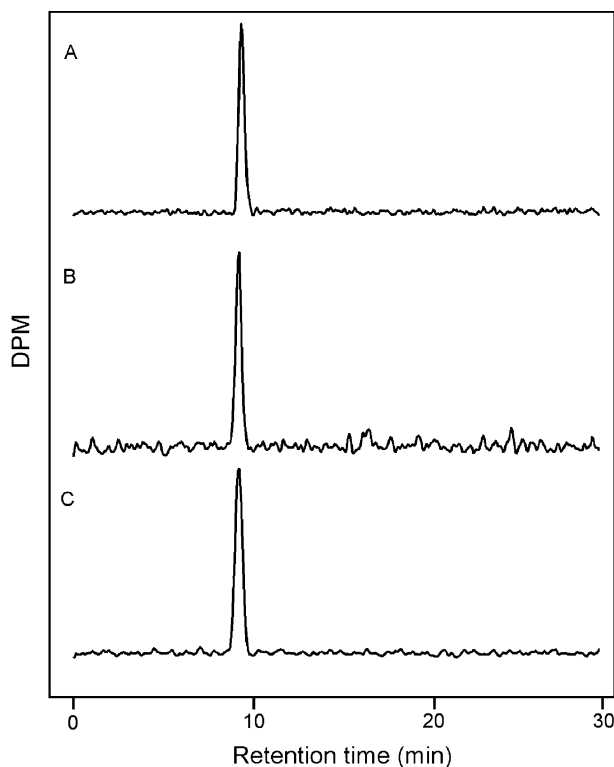


Fig. 3. HPLC profile of [^{14}C]SAG before (A) and after uptake into soybean (B) or red beet (C) tonoplast vesicles. [^{14}C]SAG uptake into red beet or soybean tonoplast vesicles was performed as described by Li et al. (1995) except radioactivity on the filters was eluted with 90% ethanol. The ethanol extracts were concentrated to dryness and suspended in acetic acid (1%):methanol (95:5) for HPLC analysis. HPLC separation was performed according to the analytical method described in the Materials and methods. Radioactivity in the profile was detected with a Triathler HPLC detector using a 250- μl flow cell. Approximately 1.4–2.3 nCi of each sample were analysed.

unrelated glucose conjugates such as esculin (a coumarin glucoside), arbutin (a hydroquinone glucoside), and *o*-nitrophenyl β -D-glucoside (*o*-NPG) were only marginally inhibitory (20–30% inhibition; Fig. 4). The glutathione conjugate of 1-chloro-2,4-dinitrobenzene (DNP-GS), a known substrate of vacuolar ABC transporters, was also only marginally inhibitory (35% inhibition; Fig. 4). SA and glucose, the individual structural components of SAG, had very little effect on SAG uptake (20 and 14% inhibition, respectively; Fig. 4). The strongest inhibition (60% inhibition; Fig. 4) of SAG uptake occurred in the presence of β -estradiol 17-(β -D-glucuronide) (E_2 17G). E_2 17G presumably does not occur in plants, but is a known substrate of ABC transporters (Liu et al. 2001).

The kinetics of SAG uptake into soybean tonoplast vesicles

MgATP-dependent SAG uptake into soybean tonoplast vesicles exhibited Michaelis–Menten-type saturation kinetics (Fig. 5) in regard to SAG concentration. A double reciprocal plot of the data (inset Fig. 5) was

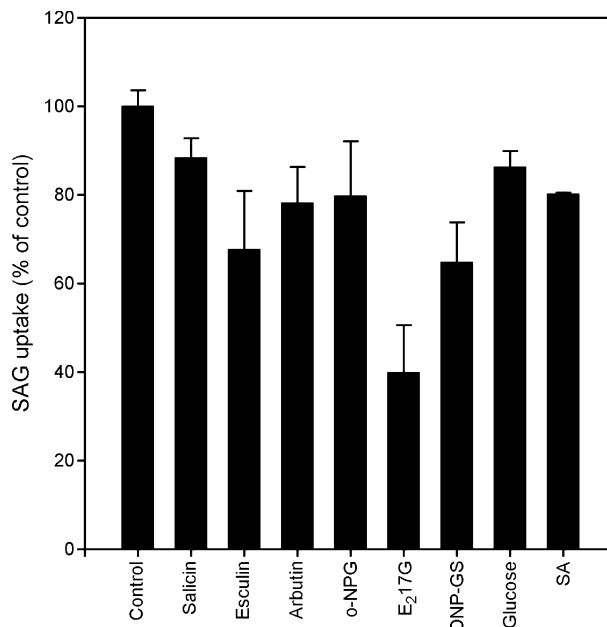


Fig. 4. Effects of potential competitive inhibitors on the MgATP-dependent uptake of [^{14}C]SAG into soybean tonoplast vesicles. Uptake of [^{14}C]SAG (50 μM) was measured as described by Li et al. (1995). SA was dissolved in ethanol and compared to control assays that also contained ethanol (1%). E_2 17G was dissolved in 0.1 M Tris (pH 8.0). All other compounds were dissolved in water. Each compound was added to a final concentration of 0.5 mM. All values were corrected for radioactivity that was detected on the filters following assays in the absence of MgATP. Each bar represents the mean of three replicates \pm SD. The results shown are representative of an experiment that was repeated twice. The control value of MgATP-dependent uptake was $4.28 \pm 0.15 \text{ pmol min}^{-1} \text{ mg}^{-1}$.

used to calculate K_m and V_{max} values of 91 μM and 17 $\text{pmol min}^{-1} \text{ mg}^{-1}$, respectively.

Association of SAG uptake with soybean tonoplast vesicles

Throughout this study, the soybean membrane vesicles collected from the 10/23% sucrose interface (see Preparation of membrane vesicles in the Materials and methods) have been referred to as tonoplast membrane vesicles. However, in order to support the hypothesis that the SAG uptake was associated with the tonoplast fraction and to determine whether the plasma membrane also possessed an MgATP-dependent mechanism for the transport of SAG, it seemed necessary to compare the SAG uptake rates of these two membrane fractions. The H^+ -PPase activity was used as a marker activity for tonoplast vesicles and the vanadate sensitive H^+ -ATPase activity was used as a marker activity for the plasma membrane vesicles. The H^+ -PPase, vanadate insensitive H^+ -ATPase, and SAG uptake activity were all primarily associated with the membrane vesicles collected from the 10/23% interface (Table 2). The vanadate sensitive H^+ -ATPase activity was associated with the membrane vesicles collected from the 23/40% step gradient interface. There was no measurable H^+ -PPase activity associated with this fraction and the SAG uptake activity was

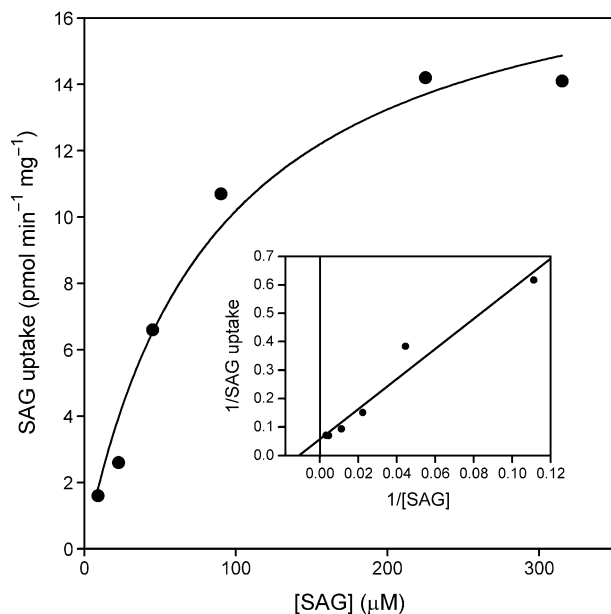


Fig. 5. Effects of [¹⁴C]SAG concentration on the MgATP-dependent uptake into soybean tonoplast vesicles. Uptake was measured in a 15-min assay as described by Li et al. (1995). Uptake rates in the presence of 3 mM MgATP at each [¹⁴C]SAG concentration were corrected for radioactivity detected on filters during assays performed in the absence of MgATP. Each point represents the mean of duplicate measurements, each of which was within 5.5% of the other. Results shown are representative of an experiment that was repeated twice. The inset shows a double-reciprocal plot of the data points. Values for K_m and V_{max} were calculated to be 91 μM and 17 $\text{pmol min}^{-1} \text{mg}^{-1}$, respectively.

14-fold less than the uptake activity of the membrane vesicles collected from the 10/23% sucrose interface. Therefore, it appears as if the tonoplast membrane vesicles were in fact collected from the 10/23% step gradient interface and that the majority of the MgATP-dependent SAG uptake activity was associated with this fraction.

Discussion

It has recently been determined that SA supplied exogenously to soybean cell suspension cultures is converted primarily to SAG (Dean et al. 2003). Though conversion of SA to SAG by a substrate inducible SAGT presumably occurs in the cytoplasm, SAG accumulates exclusively in the vacuole (Dean et al. 2003). Therefore, a

vacuolar transport mechanism for SAG must exist in soybean cells. We have attempted to characterize the transport of SAG in vitro using [¹⁴C]SAG synthesized by soybean cell suspension cultures and tonoplast membrane vesicles isolated from etiolated soybean hypocotyls. Using this system, we have been able to demonstrate that uptake of SAG into soybean tonoplast vesicles is stimulated about six-fold by the inclusion of MgATP into the assay medium. However, since the uptake of SAG in the absence of MgATP increased slowly but steadily over time, we could not eliminate the possibility that some uptake occurs through an ATP-independent mechanism. The ATP-dependent uptake of SAG into soybean tonoplast vesicles was not inhibited by agents that dissipate or prevent the formation of the H^+ -electrochemical gradient that would be formed by the vacuolar ATPase in the presence of ATP and therefore does not involve an H^+ -antiport mechanism. SAG uptake into soybean tonoplast vesicles was strongly inhibited by vanadate, a phosphoryl transition state analogue. Based on this information, it appears likely that SAG uptake into soybean tonoplast vesicles occurs through an ABC transporter-type mechanism.

A complete inventory of the ABC protein superfamily in *Arabidopsis* has recently been completed (Sánchez-Fernández et al. 2001, Martinoia et al. 2002). However, despite this wealth of knowledge on the organization and sequences of the ABC genes, very little is known about the function of ABC proteins in plants. Most ABC proteins are membrane-bound transporters that have the ability to transport a range of substances (Theodoulou 2000, Sánchez-Fernández et al. 2001, Martinoia et al. 2002). In plants, one subclass of ABC transporters known as the multidrug resistance-associated proteins (MRPs) have been shown to be involved in the vacuolar transport of a variety of large amphipathic organic anions including glutathione conjugates, glucuronide conjugates, and chlorophyll catabolites (Liu et al. 2001). Although glutathione conjugation and vacuolar sequestration appear to be important routes of herbicide metabolism, it has been hard to demonstrate that plants use glutathione conjugation to detoxify endogenously produced compounds (Walbot et al. 2000). In addition, glucuronide conjugates are only formed in a few plant species (Harborne 1988). However, in plants, glucosylation of a variety of natural products is widespread and many of these glucose conjugates are thought to

Table 2. ATP-dependent SAG uptake, H^+ -PPase, and H^+ -ATPase activity of soybean membrane fractions purified through a sucrose step gradient. SAG uptake was measured using [¹⁴C]SAG (10 mCi mmol⁻¹) in the standard 15 min assay. The uptake rate in the presence of MgATP was corrected for uptake rates in the absence of MgATP. H^+ translocation in the presence of PP_i (H^+ -PPase) or ATP (H^+ -ATPase) was determined by measuring the decreasing ΔA_{490} of acridine orange. Values shown are means of three replicates \pm SD. ND, not detected.

Membrane fraction	SAG uptake activity ($\text{pmol min}^{-1} \text{mg}^{-1}$)	H^+ -PPase activity ($\Delta A_{490} \text{ min}^{-1} \text{mg}^{-1} \times 10^{-2}$)	H^+ -ATPase activity (- vanadate) ($\Delta A_{490} \text{ min}^{-1} \text{mg}^{-1} \times 10^{-2}$)	H^+ -ATPase activity (+ vanadate) ($\Delta A_{490} \text{ min}^{-1} \text{mg}^{-1} \times 10^{-2}$)	H^+ -ATPase (+ vanadate)/ H^+ -ATPase (- vanadate) (%)
10/23% interface	4.43 \pm 0.54	11.92 \pm 1.94	11.81 \pm 3.68	9.23 \pm 3.63	78.2
23/40% interface	0.32 \pm 0.06	ND	2.53 \pm 0.56	0.67 \pm 0.23	26.5

accumulate in the vacuole (Wink 1997). Most glucose conjugates are thought to enter the vacuole through an H^+ -antiport system (Werner and Matile 1985, Bartholomew et al. 2002). However, the uptake of HPS-glucoside into barley vacuoles and the uptake of saponarin into isolated *Arabidopsis* vacuoles are thought to occur through an ABC transporter-type mechanism (Klein et al. 1996, Frangne et al. 2002). Both of these compounds could be considered xenobiotics since HPS-glucoside is an herbicide conjugate and *Arabidopsis* does not endogenously form saponarin. In contrast, SAG is a very common glucose conjugate formed in a variety of plants from both exogenously supplied and endogenously produced SA (Klick and Herrmann 1988, Tanaka et al. 1990, Enyedi et al. 1992, Schulz et al. 1993, Edwards 1994, Silverman et al. 1995, Lee and Raskin 1998, Dean et al. 2003).

Although the uptake of SAG by soybean tonoplast vesicles appears to involve an ABC transporter-like mechanism, this mechanism is not used by all plant species. We have shown that the uptake of SAG into red beet tonoplast vesicles is enhanced by MgATP, is not inhibited by vanadate, and depends on the formation of the H^+ -electrochemical gradient. Based on these results it appears likely that in red beet cells the vacuolar uptake of SAG occurs through an H^+ -antiport mechanism. An H^+ -antiport mechanism has also been suggested for the uptake of a number of other herbicide and phenolic glucosides by red beet tonoplast vesicles (Bartholomew et al. 2002). It should be noted that the rate of SAG uptake by red beet tonoplast vesicles is two orders of magnitude greater than the uptake of SAG by soybean tonoplast vesicles. Although the significance of this is not clear, this may be somewhat surprising since it is generally assumed that uptake by direct energization mechanisms (e.g. ABC transporters) is more efficient than secondary active transport (e.g. H^+ -antiport; Kreuz et al. 1996).

Competition experiments were conducted in order to gain some insight into the substrate specificity of the SAG transport by soybean tonoplast vesicles. The greatest inhibition of SAG uptake into soybean tonoplast vesicles was observed in the presence of E_217G . E_217G is a steroid glucuronide conjugate that is presumably not formed in plants. However, some plants such as rye do form glucuronide conjugates of certain flavones in the mesophyll layer of primary leaves and these accumulate in the vacuole (Schulz and Weissenböck 1986, Klein et al. 1998). In rye, the vacuolar uptake of flavone glucuronides and E_217G has been shown to involve an ABC transporter-type mechanism (Klein et al. 1998, 2000, 2001). However, plants that do not synthesize glucuronide conjugates also exhibit vacuolar uptake of these conjugates (Klein et al. 1998, 2001). MRPs can transport both E_217G and DNP-GS, however, the transport pathways through the protein are distinct (Liu et al. 2001). In some cases, DNP-GS will stimulate transport of the glucuronide conjugate, however, in most cases glucuronide conjugate transport is insensitive to or only slightly

inhibited by DNP-GS (Liu et al. 2001). In our competition experiments, we observed strong inhibition of SAG uptake by E_217G , but only slight inhibition with DNP-GS. This inhibition pattern is consistent with the involvement of an MRP in SAG uptake and provides evidence that SAG and E_217G share the same transport pathway through the protein. Unlike SAG, which carries a negative charge on the SA component of the conjugate and E_217G , which carries the negative charge on the glucuronic acid component, the other glucose conjugates examined as potential inhibitors were all uncharged substances. These compounds were examined because it has been shown that the uptake of saponarin, also an uncharged glucoside, is also mediated by an ABC-transporter-type mechanism (Frangne et al. 2002). However, all of the uncharged glucosides examined including salicin, which is very similar in structure to SAG, had very little effect on SAG uptake. These findings together with the demonstration that SA uptake into soybean tonoplast was much slower than SAG uptake indicates that both the negative charge and glucose conjugation are required for transport of SAG through this soybean ABC transporter. We have demonstrated that uptake of SA into red beet tonoplast vesicles by an H^+ -antiport mechanism also requires glucosylation. Bartholomew et al. (2002) have also recently demonstrated that the uptake of other phenolic glucosides into red beet tonoplast vesicles by an H^+ -antiport mechanism depends on glucosylation and the presence of a negative charge. Therefore, regardless of the vacuolar transport mechanism used by a plant species, glucosylation and a negative charge are likely required for the vacuolar uptake of SA.

Although the K_m value for SAG uptake by the putative soybean ABC transporter is comparable with the K_m values reported with other substrates for ABC transporters from other species (Li et al. 1995), the uptake is slower and is not sustained long enough in vitro to demonstrate accumulation of SAG against a concentration gradient. The low level of SAG transport may be due to a low number of constitutive transporters in the vacuolar membrane. Since the tonoplast vesicles used in the in vitro uptake assays were isolated from untreated soybean hypocotyls and it has been demonstrated that SAGT activity is virtually undetectable in untreated soybean cells but is rapidly induced in the presence of SA (Dean et al. 2003), it is possible that in soybean there is a coordinated SA induction of the SAGT activity and the transporter responsible for the vacuolar sequestration of the resulting glucose conjugate. This possibility is further supported by the demonstration that SA treatment of barley resulted in a 30% stimulation of the vacuolar uptake of HPS-glucoside (a compound that is taken up into barley vacuoles by an ABC transporter-type mechanism), and the specific induction of the expression of three *Arabidopsis* MRP genes by SA (Gaillard et al. 1994, Kolukisaoglu et al. 2002). Alternatively, the low level of SAG uptake may be due to the loss of a cytoplasmic factor during the vesicle isolation that would normally facilitate SAG uptake. In maize, a cytoplasmic

glutathione *S*-transferase known as BZ2 is required for the vacuolar sequestration of cyanidin-3-glucoside (Marrs et al. 1995). Cyanidin-3-glucoside is believed to be transported into the vacuole by an ABC transporter-type mechanism (Marrs et al. 1995). BZ2 is thought to serve as an anthocyanin carrier protein that delivers and facilitates the transport of anthocyanin through the ABC transporter (Mueller et al. 2000, Walbot et al. 2000). Further investigations will be required to determine whether a similar cytoplasmic factor facilitates the uptake of SAG and to determine whether the transporter responsible for SAG uptake is induced by SA.

In conclusion, we have been able to demonstrate that uptake of SAG by soybean tonoplast vesicles occurs through an ABC transporter-type mechanism whereas SAG uptake into red beet tonoplast vesicles occurs through an H⁺-antiport mechanism. Therefore, depending on the species two distinct vacuolar transport mechanisms for SAG uptake exist in plants. The examination of additional species will be necessary in order to determine which mechanism predominates throughout the plant kingdom. Regardless of the mechanism, the glucosylation and vacuolar sequestration of SA likely has an important biochemical role within the plant cell. For example, since SA is thought to be an allelopathic chemical, glucosylation and vacuolar storage may serve to protect plant cells from toxic levels of SA that are excreted into the rhizosphere by competing plants. In addition, it has been shown in tobacco that endogenous levels of both SA and SAG increase in leaves that have been infected with tobacco mosaic virus (Enyedi et al. 1992, Malamy et al. 1992). In tobacco and in other plants, the elevated levels of SA are thought to be involved in both local and systemic disease resistance (Malamy et al. 1990, Métraux et al. 1990, Delaney et al. 1994). It is possible that the formation and vacuolar storage of SAG protects the plant cells from the elevated levels of SA produced during the defence response and controls the amount of unconjugated SA available to serve as a signal molecule. Though glucosylation of SA to SAG is thought to be a detoxification step, it is not known whether this step alone is sufficient to reduce the toxicity of SA. Vacuolar sequestration of SAG would provide further protection and would also ensure that there would be no end-product inhibition of the SA glucosyltransferase enzyme (s) located in the cytoplasm.

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