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The formation, vacuolar localization, and tonoplast transport of salicylic acid glucose conjugates in tobacco cell suspension cultures

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Abstract The metabolism of salicylic acid (SA) in tobacco (*Nicotiana tabacum* L. cv. KY 14) cell suspension cultures was examined by adding [7-¹⁴C]SA to the cell cultures for 24 h and identifying the metabolites through high performance liquid chromatography analysis. The three major metabolites of SA were SA 2-*O*- β -D-glucose (SAG), methylsalicylate 2-*O*- β -D-glucose (MeSAG) and methylsalicylate. Studies on the intracellular localization of the metabolites revealed that all of the SAG associated with tobacco protoplasts was localized in the vacuole. However, the majority of the MeSAG was located outside the vacuole. The tobacco cells contained an SA inducible SA glucosyltransferase (SAGT) enzyme that formed SAG. The SAGT enzyme was not associated with the vacuole and appeared to be a cytoplasmic enzyme. The vacuolar transport of SAG was characterized by measuring the uptake of [¹⁴C]SAG into tonoplast vesicles isolated from tobacco cell cultures. SAG uptake was stimulated eightfold by the addition of MgATP. The ATP-dependent uptake of SAG was inhibited by bafilomycin A₁ (a specific inhibitor of the vacuolar H⁺-ATPase) and dissipation of the transtonoplast H⁺-electrochemical gradient. Vanadate was not an inhibitor of SAG uptake. Several β -glucose conjugates were strong inhibitors of SAG uptake, whereas glutathione and glucuronide conjugates were only marginally inhibitory. The SAG uptake exhibited Michaelis–Menten type saturation kinetics with a K_m and V_{max} value of 11 μ M and 205 pmol min⁻¹ mg⁻¹, respectively, for SAG. Based on the transport characteristics it appears as if the vacuolar uptake of SAG in tobacco cells occurs through an H⁺-antiport-type mechanism.

Keywords Antiporter · Glucose conjugate · *Nicotiana* · Salicylic acid · Tonoplast transport · Vacuole

Abbreviations ABC transporter: ATP-binding cassette transport protein · 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 · MeSA: Methyl salicylate · MeSAG: Methyl salicylate 2-*O*- β -D-glucoside · *o*-NPG: *o*-Nitrophenyl β -D-glucoside · *p*-NP α G: *p*-Nitrophenyl α -D-glucoside · *p*-NPG: *p*-Nitrophenyl β -D-glucoside · SA: Salicylic acid · SAG: Salicylic acid 2-*O*- β -D-glucoside · SAGT: Salicylic acid glucosyltransferase · SGE: Salicylic acid glucose ester · TMV: Tobacco mosaic virus

Introduction

Salicylic acid (SA) is a phenolic compound that appears to be distributed ubiquitously throughout the plant kingdom (Raskin et al. 1990). Exogenous applications of SA are known to result in a variety of plant responses (Raskin 1995). However, the discovery that SA regulated thermogenesis in inflorescences of *Arum* lilies was the first demonstration that endogenously produced SA had an important regulatory role in plants (Raskin et al. 1987). It was later determined that SA also had an important endogenous role in both local and systemic resistance of plants to pathogens (Malamy et al. 1990; Métraux et al. 1990; Delaney et al. 1994). In addition, it has been suggested that SA is an allelopathic compound that inhibits the growth and germination of competing plants (Shettel and Balke 1983). In tobacco, SA supplied exogenously or produced endogenously during a defense response is primarily metabolized to SA 2-*O*- β -D-glucose (SAG; Fig. 1) and minor amounts of the glucose ester of SA (SGE; Edwards 1994; Lee and Raskin 1998). These glucose conjugates are formed by an SA glucosyltrans-

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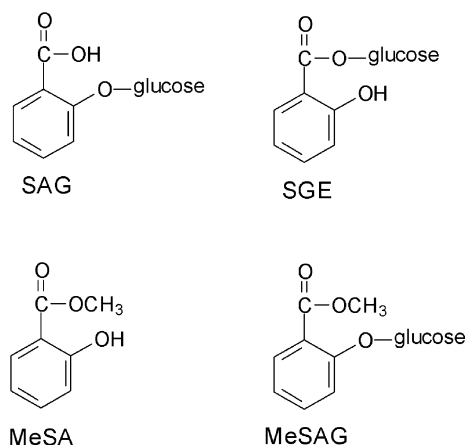


Fig. 1 Diagram of relevant salicylic acid (SA) metabolites and glucose conjugates. *MeSA* Methyl salicylate; *MeSAG* methyl salicylate 2-*O*- β -D-glucoside; *SAG* salicylic acid 2-*O*- β -D-glucoside; *SGE* salicylic acid glucose ester

ferase (SAGT) that is induced by SA, tobacco mosaic virus (TMV) and *Pseudomonas syringae* pv. *Phaseolicola* inoculation. Tobacco appeared to contain two SAGT enzymes, one that could form SAG and one that could form SGE. The first SAGT enzyme that was purified and cloned from tobacco was able to form both SAG and SGE (Lee and Raskin 1999).

In addition to SAG and SGE, it was also revealed that SA is metabolized to significant amounts of methyl salicylate (MeSA; Fig. 1) in TMV-inoculated tobacco leaves (Shulaev et al. 1997; Seskar et al. 1998). MeSA is thought to be an airborne signal capable of activating disease resistance. Though a variety of other SA metabolites have been described in a few other plant species (see citations in Lee and Raskin 1998), in most cases SAG is the major metabolite (Tanaka et al. 1990; Schulz et al. 1993; Silverman et al. 1995; Dean et al. 2003).

In soybean, SA is converted to SAG in the cytoplasm by an inducible SAGT enzyme and the resulting SAG is stored exclusively in the vacuole (Dean et al. 2003). The glucose conjugates of other plant hormones and growth regulators have also been shown to be stored in the vacuole (Garcia-Martinez et al. 1981; Schmitt and Sandermann 1982; Bray and Zeevaart 1985; Lehmann and Glund 1986; Fussedder and Ziegler 1988). The vacuolar uptake of SAG from the cytoplasm of soybean cells occurs through a tonoplast localized ATP-binding cassette (ABC) transporter (Dean and Mills 2004). ABC transporters are directly energized by ATP and, therefore, do not depend on the formation of a proton gradient. Though originally described as glutathione conjugate pumps (Martinoia et al. 1993; Li et al. 1995), it was later revealed that ABC transporters could transport a variety of xenobiotic and natural product metabolites (Rea et al. 1998; Rea 1999; Martinoia et al. 2000) including glucose conjugates (Klein et al. 1996; Frangne et al. 2002). However, not all species use an

ABC transporter to move glucose conjugates across the tonoplast.

The tonoplast membrane of plant cells contains both a V-type H⁺-translocating adenosine triphosphatase (H⁺-ATPase) and an H⁺-translocating pyrophosphatase (H⁺-PPase; Rea and Sanders 1987). These tonoplast pumps, in the presence of the appropriate energy source, move protons from the cytosol into the lumen of the vacuole, resulting in the formation of both a pH gradient and a membrane potential. This H⁺-electrochemical potential difference can then be used to drive transport through H⁺-antiporters or may be used for the pH-dependent conformational trapping of acylated compounds (Matern et al. 1986; Hopp and Seitz 1987; Martinoia et al. 2000). An H⁺-antiporter is used by red beet cells to move SAG and other naturally occurring and xenobiotic glucose conjugates across the tonoplast (Bartholomew et al. 2002; Dean and Mills 2004).

In this study, we have re-examined the metabolism of SA in tobacco using cell suspension cultures and have found that in addition to SAG and MeSA, two previously identified metabolites of SA (Edwards 1994; Lee and Raskin 1998; Shulaev et al. 1997; Seskar et al. 1998), tobacco cells also metabolize SA to large amounts of MeSA 2-*O*- β -D-glucose (MeSAG; Fig. 1). We have also been able to demonstrate in this study that SAG is stored exclusively in the vacuole of tobacco cells even though it is synthesized outside the vacuole by an SA inducible SAGT enzyme. MeSAG, on the other hand, is not stored in the vacuole. In addition, we provide evidence that a tonoplast H⁺-antiporter is responsible for the movement of SAG from the cytoplasm into the vacuole of tobacco cells. This transport mechanism is distinct from the ABC transporter mechanism that is used for the recently characterized vacuolar uptake of SAG by soybean cells (Dean and Mills 2004).

Materials and methods

Cell suspension cultures

Tobacco (*Nicotiana tabacum* L. cv. KY 14) cell suspension cultures were maintained in Murashige and Skoog (1962) media with 3% (w/v) sucrose and 1 mg/l 2,4-D. The cells were incubated on a rotary shaker (140 rpm) at 25°C under constant room light. The cells were subcultured weekly by adding the 7-day-old cultures to an equal volume of fresh media.

In vivo [7-¹⁴C]-SA treatments

Cell cultures (50 ml) were treated 3 days after subculture with [7-¹⁴C]SA (PerkinElmer, Boston, MA, USA) in ethanol (0.1% v/v culture media volume) for 24 h at a final concentration of 10 μ M and a specific activity of either 4 or 8 μ Ci/ μ mol. Cells (12 g) were collected through vacuum filtration on grade 415 filter paper

(VWR, Bristol, CT, USA) and ground in 90% ethanol (20 ml) with a mortar and pestle. The extract was centrifuged at 400 g for 20 min. Acetic acid (1% v/v final concentration) was added to an aliquot of the supernatant, which was used directly for the high performance liquid chromatography (HPLC) analysis of MeSA. A separate aliquot of the supernatant was concentrated to dryness in vacuo and resuspended in HPLC start buffer.

Analysis of metabolites

HPLC separations were performed with a 150×4.6 mm Allsphere 5 μM ODS-1 column (Alltech, Deerfield, IL, USA) 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, which was held for 5 min. Eluant from the column was diverted to a fraction collector and the radioactivity in the 1-ml fractions was determined through liquid scintillation counting or radioactivity was determined in the eluant with a Triathler HPLC radioactivity detector (Bioscan, Washington, DC, USA) fitted with a 125 μl flow cell. Identification of conjugates through enzymatic hydrolysis with either β-glucosidase or esterase was determined as described by Edwards (1994). Identification of MeSA and SA was confirmed through cochromatography with authentic standards.

Preparation of protoplasts and vacuoles

Protoplast and vacuole preparations were performed as described by Dean et al. (2003) except that 3 mM EDTA was added to the protoplast lysis buffer and the overlay buffer.

Enzyme assays

Salicylic acid glucosyltransferase activity in the protoplasts versus vacuoles was determined using tobacco cells (3 days after subculture) that had been treated for 24 h with 0.1 mM SA. SAGT extraction buffer (Edwards 1994) was added to the protoplast and vacuole preparations and the membranes were disrupted with a sonifier. The samples were centrifuged (100,000 g for 20 min) and the supernatant was brought to 70% saturation with solid (NH₄)₂SO₄. The protein was collected by centrifugation at 16,000 g for 10 min. The pellet was suspended in SAGT extraction buffer and used directly for SAGT assays as described by Edwards (1994). The reaction product was separated from [¹⁴C]SA either through HPLC as described above or TLC as described by Dean et al. (2003). The assay for glucose-6-phosphate dehydrogenase (EC 1.1.1.49) activity (a cytoplasmic

marker enzyme) was performed as described by Simcox et al. (1977) and the assay for α-mannosidase activity (a vacuolar marker enzyme) was performed as described by Boller and Kende (1979).

Preparation of S-(2,4-dinitrophenyl)glutathione, [¹⁴C]SAG, and [¹⁴C]MeSAG

S-(2,4-dinitrophenyl)glutathione (DNP-GS) was prepared as described by Walczak and Dean (2000), and [¹⁴C]SAG was prepared using soybean (*Glycine max* [L.] Merr. cv Williams 82) cell suspension cultures as described by Dean and Mills (2004). The [¹⁴C]MeSAG was isolated from tobacco cell suspension cultures that had been treated with [¹⁴C]SA. With the exception of the cell culture species and the peak collected, the method used for the preparation of [¹⁴C]MeSAG was the same as the [¹⁴C]SAG preparation method described by Dean and Mills (2004).

Preparation of tonoplast membrane vesicles

Tonoplast vesicles from tobacco cell suspension cultures were prepared using the procedure developed for mung bean (*Vigna radiata*) as described by Rea et al. (1992). The starting material for a typical isolation was composed of 250 g of filtered tobacco cells frozen in liquid nitrogen and then homogenized in a blender together with 300 ml of the homogenization media described by Rea et al. (1992). The protein concentration was estimated by the method of Bradford (1976) using BSA as a protein standard.

Measurement of [¹⁴C]SAG, [¹⁴C]MeSAG and [¹⁴C]SA uptake into tonoplast vesicles

Measurement of [¹⁴C]SAG, [¹⁴C]MeSAG and [¹⁴C]SA uptake into tonoplast membrane vesicles was performed as described by Li et al. (1995). Unless otherwise stated the standard assay contained 50 μM of [¹⁴C]SAG, [¹⁴C]MeSAG or [¹⁴C]SA (ca. 10 mCi/mmol), 24 μl of tobacco tonoplast membrane vesicles and was allowed to proceed at 25°C for 15 min.

Results

Metabolism of [¹⁴C]SA

Nearly 90% of the radioactivity added as [¹⁴C]SA to tobacco cell suspension cultures was recovered following a 24-h incubation. Typically about 16% of the radioactivity was found in the culture media, 21% was present in the insoluble pellet (cell debris) following cell extraction and centrifugation, and 52% was found in the cell extract supernatant. HPLC analysis was used to identify and quantify the metabolites of [¹⁴C]SA in the

cell extract supernatant. Initial HPLC separations were conducted on an aliquot of the supernatant that was concentrated in vacuo and resuspended in HPLC start buffer. Three main peaks of radioactivity were consistently present in the elution profile from these separations (Fig. 2a). The metabolite collected in fractions 9 and 10 (Fig. 2a) and the metabolite(s) collected in fractions 13–15 came out in the same positions as SAG and MeSAG, respectively, that have recently been shown to be formed from SA in soybean cell suspension cultures (Dean and Mills 2004). The identity of these metabolites was confirmed following β -glucosidase and esterase treatment. The metabolite collected in fractions 9 and 10 contained one compound with a retention time of 9 min when the radioactivity in the HPLC eluant was detected with a flow through radioactivity detector (Fig. 3a). When this metabolite was treated with β -glucosidase, a

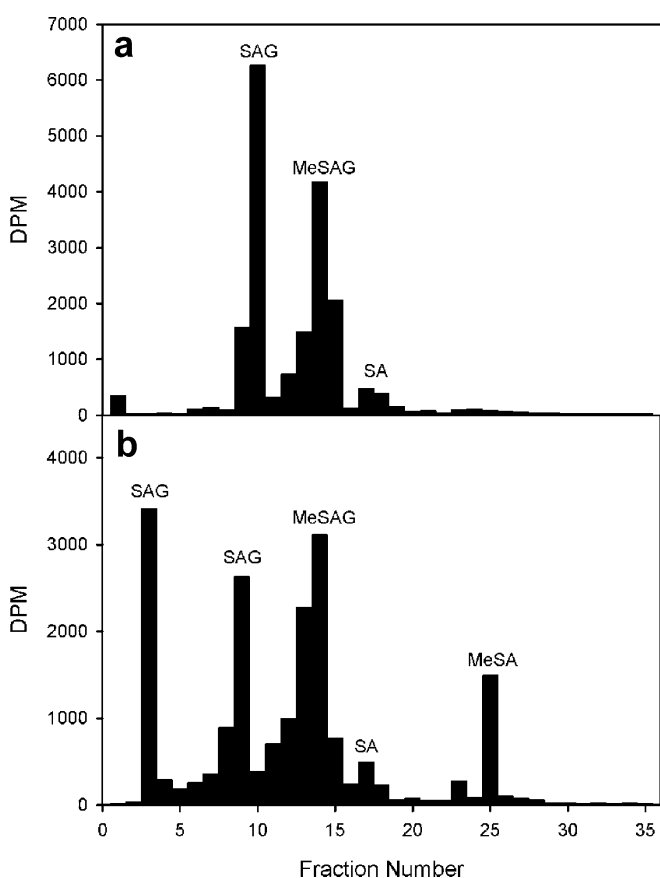
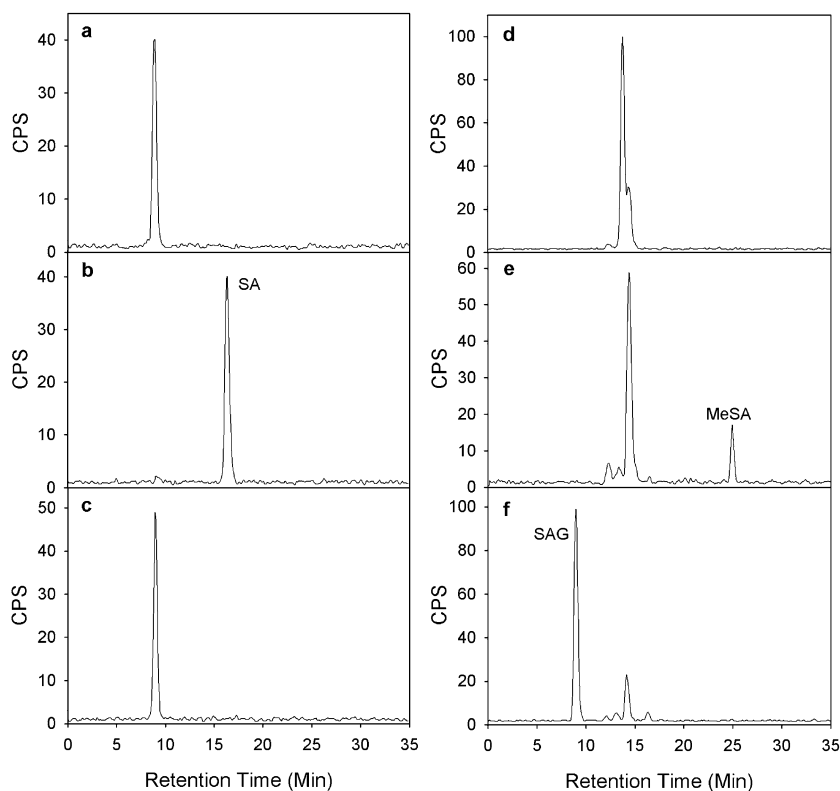


Fig. 2 HPLC analysis of the [^{14}C]SA metabolites found in the cell extract supernatant of tobacco cell suspension cultures. Cell cultures (50 ml) were treated for 24 h with [^{14}C]SA and extracted as described in Materials and methods. An aliquot of the cell extract supernatant was concentrated in vacuo and resuspended in HPLC start buffer before analysis (a). Acetic acid (1% v/v final concentration) was added to a separate aliquot of the cell extract supernatant which was then directly injected onto the HPLC for analysis (b). The radioactivity in each 1-ml fraction was determined through liquid scintillation counting. The identity of the SAG and MeSAG labeled fractions was confirmed through HPLC analysis of the β -glucosidase and esterase treated samples (see Fig. 3). The identity of the SA and MeSA labeled fractions was confirmed by comparing retention times to authentic standards

single compound with the same retention time as SA was released (Fig. 3b). Esterase treatment of this metabolite had no effect (Fig. 3c). Therefore, it was concluded that this metabolite represented SAG. The metabolite collected in fractions 13–15 (Fig. 2a) appeared to contain at least two compounds that we were unable to completely separate (Fig. 3d). Treatment of these metabolites with β -glucosidase resulted in the loss of approximately 66% of the initial radioactivity. This loss of radioactivity was presumably due to the release of a volatile compound. This volatile compound appears to be MeSA, since we were able to detect the presence of a compound with the same retention time as MeSA in the HPLC profile (Fig. 3e). Treatment of this metabolite with esterase resulted in the formation of SAG (Fig. 3f). Based on the evidence presented, it appears as if the major compound in this group of metabolites is MeSAG. Treatment with β -glucosidase released MeSA, a portion of which was lost as a volatile compound. Treatment with esterase removed the methyl group from the MeSAG, resulting in the formation of SAG. The minor compounds in this group of metabolites did not appear to be glucose conjugates or esters of SA, since they were not affected by β -glucosidase or esterase treatment (Fig. 3d–f).

In order to obtain accurate retention times of the radioactive peaks that eluted early in the HPLC profile, it was necessary to remove the cell extraction buffer (90% ethanol) by concentrating the sample and re-suspending it in the starting mobile phase for the HPLC separations. However, concentration of the sample prevented the detection of any MeSA that might be formed from SA by the tobacco cells. Since it is well known that tobacco is able to form MeSA from SA (Shulaev et al. 1997; Seskar et al. 1998), we conducted a separate HPLC analysis of SA metabolites using cell extract supernatants that were not concentrated (Fig 2b). An additional metabolite with the same retention time as MeSA was observed in these HPLC profiles (Fig. 2b). During these separations, the SAG was eluted as two separate peaks (Fig. 2b). The first peak of SAG (fraction 3) did not effectively interact with the column due to the presence of ethanol in the injected sample. Therefore, the amounts of SAG and MeSAG were determined using HPLC analysis of concentrated supernatants and the amount of MeSA was determined in supernatants that were not concentrated. Using this type of analysis, it was determined that in the cell extract supernatant of tobacco cells treated for 24 h with [^{14}C]SA, 33.5, 28.5, and 6.5% of the exogenously supplied SA is typically metabolized into SAG, MeSAG, and MeSA, respectively. However, since MeSA is somewhat volatile, the amount detected may underestimate the actual amount formed. Approximately 0.3% of the SA remains unchanged or was released from a metabolite during sample preparation and analysis.

Fig. 3 Identification of [^{14}C]SAG and [^{14}C]MeSAG as in vivo metabolites of [^{14}C]SA in the cell extract supernatant of tobacco cell suspension cultures. Fractions 9 and 10 and fractions 13 through 15 shown in Fig. 2a were collected and the metabolites analyzed by HPLC. **a** Fractions 9 and 10 untreated. **b** Fractions 9 and 10 treated with β -glucosidase. **c** Fractions 9 and 10 treated with esterase. **d** Fractions 13 through 15 untreated. **e** Fractions 13 through 15 treated with β -glucosidase. **f** Fractions 13 through 15 treated with esterase. CPS Counts per second



Cellular localization of SA metabolites and SAGT activity

Following a microscopic examination of tobacco protoplasts, it was possible to observe one large central vacuole in each protoplast. Using this 1:1 ratio of vacuoles to protoplasts, it appears as if the majority of the SAG and substantial amounts of SA are localized in the vacuole of tobacco protoplasts (Table 1). In contrast, only 38% of the MeSAG contained in the protoplasts appeared to be localized in the vacuole (Table 1). The volatile MeSA was lost during the preparation of the protoplasts and vacuoles; therefore, we were unable to determine the subcellular localization of this metabolite.

The α -mannosidase activity was used as a marker enzyme for the vacuoles and the glucose-6-phosphate dehydrogenase activity was used as a marker enzyme for the cytoplasm. The vacuole to protoplast ratios of these two enzymes were consistent with what would be expected for a vacuolar preparation (Table 1).

Since the tobacco SAGT enzyme is inducible (Enyedi and Raskin 1993; Edwards 1994; Lee and Raskin 1998), it was necessary to determine the subcellular distribution of the SAGT activity in tobacco cells that had been treated for 24 h with SA. However, since SA results in changes in the cell wall (Siegrist et al. 1994) that interfere with the activity of the protoplasting enzymes, it was necessary to use an SA concentration that would result

Table 1 Distribution of salicylic acid (SA) metabolites and enzymes in protoplasts and vacuoles from tobacco cell suspension cultures

	Contents of SA metabolites and marker enzymes		
	In 10^6 vacuoles	In 10^6 protoplasts	Vacuoles/ protoplasts (%)
SAG (nmol)	2.13	1.49	143
MeSAG/unknown (nmol)	0.68	1.8	38
SA (nmol)	0.58	0.72	81
SAGT (pmol min^{-1})	0.50 ± 0.12	6.58 ± 0.95	7.6
α -Mannosidase (nmol min^{-1})	2.34 ± 0.09	2.39 ± 0.04	97.9
Glucose-6-phosphate dehydrogenase (nmol min^{-1})	2.71 ± 3.65	9.6 ± 1.46	28.2

Tobacco cells were treated 3 days after subculture with [^{14}C]SA (10 μM final concentration with a specific activity of 4 $\mu\text{Ci}/\mu\text{mol}$) for 24 h before the isolation of the protoplasts and vacuoles. Radioactive metabolites were separated into a fraction collector by HPLC and radioactivity determined by liquid scintillation count-

ing. The SAGT activity was determined in cells that had been treated with 0.1 mM SA for 24 h. Results shown for metabolites are representative results from experiments that were repeated at least twice. Enzyme rate values are the means of three replicates \pm SD

in detectable SAGT activity with minimal interference in protoplast generation. The best results were obtained with tobacco cells treated for 24 h with 0.1 mM SA. The SAGT activity in the cells treated with 0.1 mM SA was 1.4-fold higher than the SAGT activity in the untreated cells (data not shown). The SAGT activity of these tobacco cells resulted in the formation of only one major product. This product had the same HPLC retention time as SAG. In addition, SA was released from the product following β -glucosidase treatment, while treatment with esterase had no effect (data not shown). These results provided further evidence that the product of the SAGT activity in this tobacco cultivar was primarily SAG. Examination of the subcellular distribution of the SAGT activity revealed that the majority of the activity was located outside the vacuole (Table 1).

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

In the absence of MgATP, only a slight increase in the uptake of SAG by tobacco tonoplast vesicles was observed over time (Fig. 4). In contrast, the uptake of SAG increased dramatically over time when MgATP was included in the assays (Fig. 4). This increase in uptake was linear for 30 min. There was no further increase in SAG uptake from 30 min to 2 h. The standard reaction time for all subsequent uptake assays was 15 min.

The HPLC retention time of [14 C]SAG remained unchanged after uptake into the tobacco tonoplast ves-

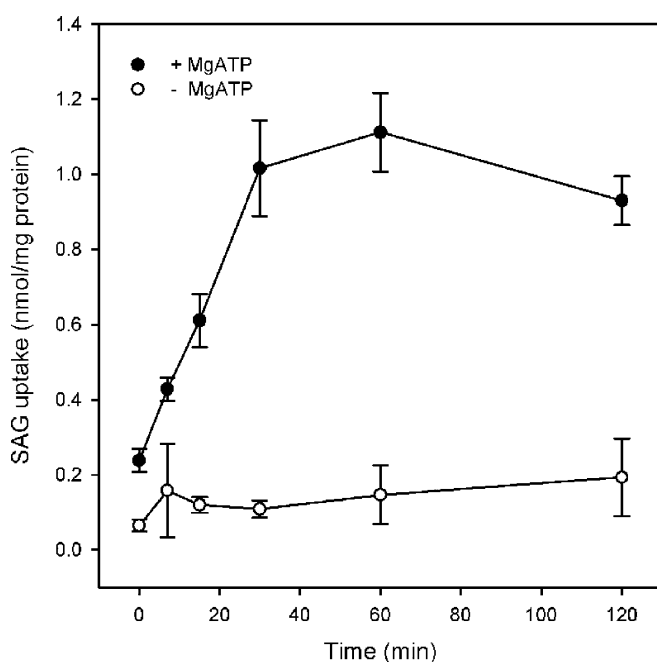


Fig. 4 Time course of [14 C]SAG uptake into tobacco tonoplast vesicles in the presence or absence of MgATP. Uptake was measured using the procedure described by Li et al. (1995) in the presence (+MgATP) or absence (-MgATP) of 3 mM MgATP. Values shown are the means of three replicates \pm SD

icles, indicating that the compound was not altered during transport (data not shown).

Tonoplast vesicles prepared in the same manner as described in this study have been reported to have an internal volume of $10 \mu\text{l mg}^{-1}$ membrane protein and consist of 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 uptake value at 30 min (Fig. 4), an internal SAG concentration of $200 \mu\text{M}$ can be calculated. Since the external concentration of SAG was $50 \mu\text{M}$, tobacco tonoplast vesicles were able to accumulate SAG against a concentration gradient.

Effects of various assay conditions on the uptake of SAG

Since the presence of MgATP clearly resulted in an increase in SAG uptake, 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. In the presence of MgATP, the proton gradient would be formed by the tonoplast localized V-type ATPase. Direct enhancement of uptake by ATP may involve an ABC transporter type mechanism. Bafilomycin A₁ is a specific inhibitor of V-type ATPases (Dröse et al. 1993) and gramicidin D is a cation selective ionophore. Both of these compounds would prevent the formation of a proton gradient needed to support an antiport mechanism. Both bafilomycin A₁ and gramicidin D were strongly inhibitory to SAG uptake (Table 2). Bafilomycin A₁ resulted in nearly an 80% inhibition in uptake and gramicidin D resulted in nearly a 60% inhibition (Table 2). Vanadate is a phosphoryl transition-state analog known to be a strong inhibitor of enzymes, including ABC transporters, which form a phosphoenzyme intermediate. The inclusion of vanadate

Table 2 Uptake of [14 C]SAG by tobacco tonoplast vesicles and the effects of various assay conditions

SAG uptake	
Assay conditions	Percentage of control
-MgATP	11.4 \pm 3.7
+ 3 mM MgATP (control)	100 \pm 3.0
+ 3 mM MgATP + 0.25 mM vanadate	95.5 \pm 0.8
+ 3 mM MgATP + 0.4 μM bafilomycin A ₁	19.6 \pm 4.9
+ 3 mM MgATP + 5 μM gramicidin D	38.7 \pm 10.2
-MgATP + 3 mM MgPPi	86.7 \pm 4.7

The uptake was measured in the standard 15-min assay except that all assays contained ethanol (1% v/v) so that uptake rates could be compared to assays conducted in the presence of bafilomycin A₁ and gramicidin D which were dissolved in ethanol. The [14 C]SAG uptake in the presence of 3 mM MgATP was $93.4 \pm 2.8 \text{ pmol min}^{-1} \text{ mg}^{-1}$ and was used as the control value. Values shown are the means of three replicates \pm SD

in the assay medium had very little effect on the ATP-stimulated uptake of SAG by tobacco tonoplast vesicles. The uptake of SAG into tobacco tonoplast vesicles was also stimulated by the addition of MgPPi to the assay medium (Table 2), indicating that the H⁺ gradient generated by the tonoplast H⁺-PPase could also drive the uptake of SAG. Based on these results, it appears likely that SAG uptake into tobacco tonoplast vesicles occurs through an H⁺-antiporter-type mechanism.

Comparison of SA, SAG and MeSAG uptake

There was very little difference between the uptake of SA and MeSAG by tobacco tonoplast vesicles in the presence and absence of MgATP (Table 3). The uptake of SA in the presence of MgATP was only 1.6-fold greater than the uptake in the absence of MgATP, and the uptake of MeSAG in the presence of MgATP was only 1.4-fold greater than the uptake in the absence of MgATP. In contrast, SAG uptake was increased 8.7-fold in the presence of MgATP and was 2.5-fold and 2.7-fold, respectively, greater than the MgATP-stimulated uptake of SA and MeSAG (Table 3).

Kinetics of SAG uptake

MgATP-dependent SAG uptake into tobacco 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 used to calculate apparent K_m and V_{max} values of 11 μM and 205 $\text{pmol min}^{-1} \text{mg}^{-1}$, respectively.

Competitive inhibitors of SAG uptake

In order to gain some insight into the substrate specificity of the transporter, the effects of a number of potential competitive inhibitors on the MgATP-dependent uptake of SAG by tobacco tonoplast vesicles was examined. Salicin (2-[hydroxymethyl]phenyl- β -D-glucoside), esculin (6,7-dihydroxycoumarin-6- β -D-glucoside), arbutin (hydroquinone- β -D-glucoside), and *o*-nitrophenyl- β -D-glucoside (*o*-NPG) were strongly inhibitory (61–73% inhibition) to SAG uptake (Fig. 6). However, *p*-nitrophenyl- β -D-glucoside (*p*-NPG) was only marginally

Table 3 Comparison of [¹⁴C]SA, [¹⁴C]SAG and [¹⁴C]MeSAG uptake by tobacco tonoplast vesicles

Substrate	Uptake ($\text{pmol min}^{-1} \text{mg}^{-1}$)	
	–MgATP	+MgATP
SA	11.7 ± 1.1	18.7 ± 1.2
SAG	5.4 ± 0.8	46.9 ± 1.0
MeSAG	12.2 ± 1.7	17.2 ± 0.7

The uptake was measured in the standard 15-min assay with or without 3 mM MgATP. Values shown are the means of three replicates ± SD

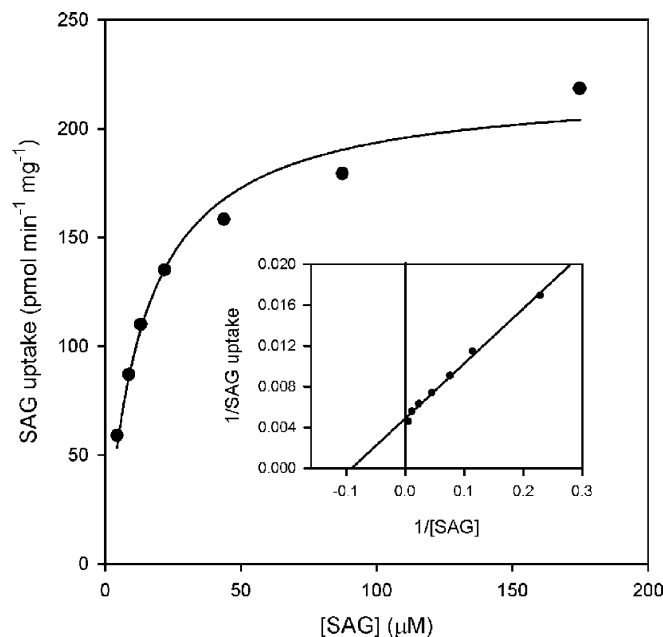


Fig. 5 Effects of [¹⁴C]SAG concentration on the MgATP-dependent uptake into tobacco tonoplast vesicles. Uptake was measured in a 15-min assay as described by Li et al. (1995). Uptake rates in the presence of 3 mM MgATP at each [¹⁴C]SAG concentration were corrected for radioactivity detected on filters during assays performed in the absence of MgATP. Each point represents the mean of duplicate uptake measurements. Results shown are representative of an experiment that was repeated twice. The inset shows a double-reciprocal plot of the data points. Apparent K_m and V_{max} values were calculated to be 11 μM and 205 $\text{pmol min}^{-1} \text{mg}^{-1}$, respectively

inhibitory (36% inhibition), while *p*-nitrophenyl- α -D-glucoside (*p*-NP α G) did not result in any inhibition of SAG uptake (Fig. 6). DNP-GS and β -estradiol 17-(β -D-glucuronide) (E₂17G) were also only marginally inhibitory (29% and 37% inhibition, respectively) to SAG uptake. Glucose and SA (the individual components of SAG) did not result in any inhibition of SAG uptake (Fig. 6).

Discussion

We have been able to demonstrate that SAG, MeSAG and MeSA are the main metabolites of SA formed in tobacco cell cultures. Though SAG and MeSA formation from SA in tobacco has been reported in previous investigations (Edwards 1994; Lee and Raskin 1998; Shulaev et al. 1997; Seskar et al. 1998), to the best of our knowledge, this is the first demonstration that tobacco can convert SA into MeSAG. MeSAG has also been detected in soybean cell cultures (Dean et al. 2003) and in the fruits of several *Passiflora* species (Chassagne et al. 1997). MeSA is believed to function as an airborne signal that induces defense mechanisms in non-infected areas of the infected plant or in neighboring plants (Shulaev et al. 1997; Seskar et al. 1998). MeSAG may serve as a convenient non-volatile storage form that re-

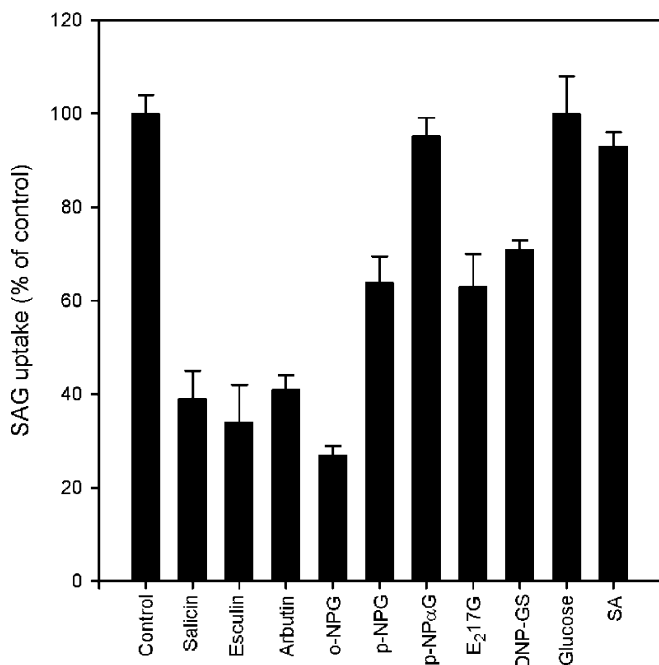


Fig. 6 Effects of potential competitive inhibitors on the MgATP-dependent uptake of [¹⁴C]SAG into tobacco tonoplast vesicles. Uptake of [¹⁴C]SAG (50 μ M) was measured as described by Li et al. (1995). SA was dissolved in ethanol and compared to control assays that also contained ethanol (1%). E₂17G was 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

leases MeSA during a defense response, or it is possible that the formation of MeSAG may protect the plant cell from the high intracellular levels of MeSA formed during a defense response. Further investigations will be required to determine the significance of MeSAG formation from SA in tobacco.

Though in most studies SAG appears to be the main metabolite of SA formed in tobacco, there are differences in the identities of the other metabolites. MeSAG was likely not detected in previous studies either due to differences in the tobacco cultivar or the cell type used during the investigation. Previous studies examined SA metabolism in leaf tissue, whereas we examined SA metabolism in cell cultures. These differences may also account for why, in contrast to previous studies (Edwards 1994; Lee and Raskin 1998), we were unable to detect SGE as a metabolite of SA in tobacco.

We have previously been able to demonstrate that all of the SAG formed from SA is primarily stored in the vacuole of soybean cells (Dean et al. 2003). However, only about 56% of the MeSAG found in the protoplasts of soybean cells was associated with the vacuole (Dean et al. 2003). Similar results were observed when the subcellular distribution of the SA conjugates was examined in tobacco cells. We were able to determine that all of the SAG was associated with the vacuole, while the MeSAG was primarily located outside the

vacuole (Table 1). High levels of SA were also found in the vacuole, but the vacuolar levels of SA were substantially less than the levels of SAG.

It is well known that tobacco contains an SAGT enzyme(s) that can conjugate SA to glucose. This enzyme is induced by SA treatment, and incompatible pathogens (Enyedi and Raskin 1993; Edwards 1994; Lee and Raskin 1998). It has been reported that the SAGT enzyme isolated from tobacco leaves forms both SAG and SGE (Edwards 1994; Lee and Raskin 1998; 1999). However, the SAGT that we have extracted from tobacco cell cultures appears to form only SAG. The inability of the cell culture enzyme to form SGE is consistent with our inability to detect SGE formation *in vivo*. We have been able to demonstrate that the tobacco SAGT activity is not associated with the vacuole (Table 1). It is possible that SAGT is a cytoplasmic enzyme, since it has been found that most other glucosyltransferases have a cytosolic location (see citations in Werner and Matile 1985; Martinoia et al. 2000; Jones and Vogt 2001) even though the glucose conjugate products are stored in the vacuole (Oba et al. 1981; Rataboul et al. 1985; Werner and Matile 1985; Yazaki et al. 1995; Taguchi et al. 2000).

If SAG formation occurs in the cytoplasm and is then moved into the vacuole, then the tobacco cell tonoplast must have a transport mechanism responsible for this vacuolar accumulation. We attempted to characterize this tonoplast transporter and were able to demonstrate that the uptake of SAG by tobacco tonoplast vesicles was strongly stimulated by MgATP and MgPPi. The ATP-stimulated uptake was inhibited by compounds that either dissipate or prevent the formation of a proton gradient. Since the MgATP-dependent uptake required the presence of a proton gradient, it appears as if an H⁺-antiporter may be the primary type of transporter responsible for the vacuolar transport of SAG in tobacco cells. This is further supported by the stimulation of SAG uptake in the presence of MgPPi. In the presence of MgPPi, the tonoplast H⁺-PPase would also be able to generate a proton gradient that could be used to drive the vacuolar transport of SAG. The involvement of an ABC-transporter-type mechanism seems unlikely, since the presence of vanadate (a strong inhibitor of ABC transporters) had little influence on the uptake of SAG. Also, pH-dependent conformational trapping seems unlikely, since the SAG was not altered following vacuolar transport (data not shown). Unlike SAG uptake, the uptake of MeSAG and SA was only slightly stimulated by ATP. The low level of MeSAG uptake *in vitro* by tobacco tonoplast vesicles (Table 3) is consistent with the finding that very little MeSAG is found in the vacuole *in vivo* (Table 1). The somewhat higher level of SA found in the vacuoles (Table 1) is a bit surprising, since the *in vitro* levels of SA uptake by tobacco tonoplast vesicles (Table 3) were also low and similar to the uptake levels for MeSAG.

All of the neutral β -glucose conjugates examined except for *p*-NPG resulted in strong inhibition of the to-

bacco tonoplast H^+ -antiporter. These same conjugates had very little effect on the ABC-transporter-mediated uptake of SAG by soybean tonoplast vesicles. The only strong inhibitor of the soybean ABC transporter was E₂17G. E₂17G is a known substrate of the multidrug resistance-associated protein subclass of plant ABC transporters (Liu et al. 2001) and it is thought that SAG and E₂17G share the same transport pathway through the protein (Dean and Mills 2004). However, E₂17G was not a strong inhibitor of the tobacco H^+ -antiporter. Therefore, the tobacco tonoplast H^+ -transporter appears to be more specific for glucose conjugates than the soybean ABC transporter. The uptake of SAG by the tobacco H^+ -antiporter was not inhibited by *p*-NP α G. However, *p*-NPG, the β -D-glucose equivalent, resulted in a 30% inhibition. Since the β -glucose conjugate resulted in a stronger inhibition than the α -glucose conjugate, it is possible to speculate that the tobacco tonoplast H^+ -antiporter may be specific for β -glucose conjugates.

Uptake of a variety of glucose conjugates (including SAG) into red beet tonoplast vesicles has been shown to occur through an H^+ -antiport mechanism (Bartholomew et al. 2002; Dean and Mills 2004). The structural requirements for transportability of the substrate by the red beet tonoplast H^+ -antiporter include the presence of a negative charge and a β -glucose attachment (Bartholomew et al. 2002). The tobacco tonoplast H^+ -antiporter appears to have characteristics that are similar to the red beet H^+ -antiporter. SAG is easily transported, since it carries the negative charge and the β -glucose attachment, whereas SA and MeSAG are poorly transported because of the lack of a glucose attachment and a negative charge, respectively. These same structural features also appear to be required for the vacuolar uptake of glucose conjugates by an ABC-transporter-type mechanism (Dean and Mills 2004).

The tobacco H^+ -antiporter had a K_m value for SAG of 11 μ M and a V_{max} value of 205 pmol min⁻¹ mg⁻¹. This K_m value for SAG is eightfold less and the V_{max} value is nearly 12-fold greater than the respective values obtained for the soybean tonoplast ABC transporter that is responsible for the vacuolar uptake of SAG (Dean and Mills 2004). Therefore, the tobacco SAG tonoplast H^+ -antiporter appears to have a higher affinity for SAG than the soybean ABC transporter. In addition, the higher uptake rate into tobacco tonoplast vesicles during the in vitro assays would explain why it was possible to measure the accumulation of SAG against a concentration gradient using tonoplast vesicles from this species, while we were unable to observe SAG uptake against a concentration gradient using soybean tonoplast vesicles (Dean and Mills 2004).

In conclusion, we have been able to demonstrate that tobacco cell suspension cultures metabolize SA to SAG, MeSAG and MeSA. SAG is stored primarily in the vacuole, while MeSAG is located outside the vacuole; the significance of this difference is not clear. The SAG is likely formed in the cytoplasm by an SA-inducible SAGT enzyme. The resulting SAG is then transported across the

tonoplast by an H^+ -antiporter. This transport mechanism is distinct from the ABC transporter that is used by soybean cells. However, both species accumulate SAG exclusively in the vacuole, suggesting that both transport mechanisms may be equally effective in vivo. Though the significance of SA glucosylation and vacuolar storage is still not clear, it is possible that it is part of a detoxification pathway used to protect plant cells from the elevated levels of SA produced endogenously during a defense response (Enyedi et al. 1992; Malamy et al. 1992) or encountered exogenously during allelopathic interactions with other plants (Raskin 1992). Though glucosylation of SA alone may result in detoxification, vacuolar sequestration of the SAG product might ensure that there is no end-product inhibition of the cytosolic SAGT enzyme. On the other hand, rather than being just an end-product of SA detoxification, it is also possible that glucosylation and vacuolar storage of SA may be reversible, which might have a significant role in controlling the amount of "free" SA available to serve as a signal molecule during defense responses.

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