

Formation and vacuolar localization of salicylic acid glucose conjugates in soybean cell suspension cultures

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The metabolism and intracellular localization of salicylic acid (SA) was investigated in soybean (*Glycine max* [L.] cv Williams 82) cell suspension cultures. [$7-^{14}\text{C}$]SA was added to the cell cultures, the metabolites were extracted from the cells at various time points and analysed by TLC and HPLC. The [$7-^{14}\text{C}$]SA was taken up rapidly from the culture media and converted primarily to SA 2-*O*- β -D-glucose (SAG). Lower levels of glucosylated 2,5-dihydroxybenzoic acid (gentisic acid) and methyl salicylate 2-*O*- β -D-glucose were also formed. Examination of the intracellular localization of the glucose conjugates revealed that all of the conjugates associated with the protoplasts were found in the vacuoles. An SA glucosyltransferase (SAGT) that could catalyse the formation of SAG

from SA and UDP-glucose could be extracted from soybean cells and assayed *in vitro*. Increasing concentrations of SA added to the culture media induced the SAGT activity. The highest levels of SAGT activity were observed in cells treated with 0.5 mM SA. The SAGT activity in these cells was 88-fold greater than the SAGT activity in the untreated cells. The intracellular localization of the SAGT activity was also examined and it was determined that the majority of the SAGT activity in the protoplasts was located outside the vacuole. Therefore, it appears as if SAG is formed from SA outside the vacuole, presumably in the cytoplasm, and then subsequently transported into the vacuole where it accumulates.

Introduction

Salicylic acid (SA; 2-hydroxybenzoic acid) is a naturally occurring phenolic compound found in most plant species examined (Raskin et al. 1990). SA is known to be involved in a variety of plant responses. It has been shown to regulate thermogenesis in inflorescences of *Arum* lilies (Raskin et al. 1987). It may serve as an allelopathic chemical that inhibits the growth of competing plants (Shettel and Balke 1983). SA is also known to be involved in both local and systemic resistance of plants to pathogens (Malamy et al. 1990, Métraux et al. 1990, Delaney et al. 1995). SA formed endogenously during plant defense responses or applied exogenously is typically converted to conjugated forms or other metabolites. Formation of 2,5-dihydroxybenzoic acid (2,5-DHBA, gentisic acid) and 2,3-dihydroxybenzoic acid from SA, cinnamic acid or benzoic acid occurs in various plant species (see citations in Lee and Raskin 1998). SA

is methylated in a number of plant species resulting in the formation of the volatile methyl salicylate ester (MeSA, see citations in Lee and Raskin 1998). The amino acid conjugate *N*-salicyloyl aspartic acid has been detected in wild grape and French bean (Steffan et al. 1988, Bourne et al. 1991). However, in many species glucosylation of SA appears to be the major route of metabolism. Glucosylation of SA can occur at either the hydroxyl or the carboxyl group of the molecule forming either the SA glucoside (SAG; SA 2-*O*- β -D-glucose) or the SA glucose ester (SGE; Fig. 1). Studies examining the metabolism of SA in rice, *Mallotus japonicus* and *Vicia faba* provided evidence that SA was primarily metabolized to SAG (Tanaka et al. 1990, Schulz et al. 1993, Silverman et al. 1995). The majority of the SA found in untreated and tobacco mosaic virus (TMV)-inoculated leaves of tobacco (*Nicotiana tabacum* L. 'Xanthi-nc' NN genotype)

Abbreviations – 2,5-DHBA, 2,5-dihydroxybenzoic acid (gentisic acid); MeSA, methyl salicylate; MeSAG, MeSA 2-*O*- β -D-glucose; SA, salicylic acid; SAG, SA 2-*O*- β -D-glucose; SAGT, SA glucosyltransferase; SGE, SA glucose ester; TMV, tobacco mosaic virus.

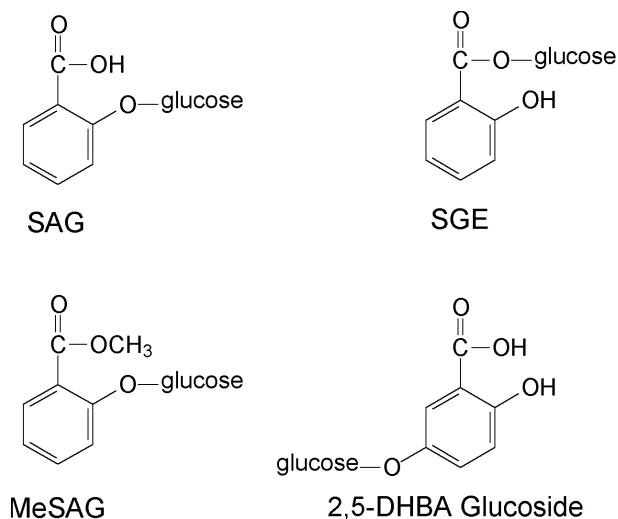


Fig. 1. Diagram of relevant SA metabolites and glucose conjugates.

was thought to exist primarily as SAG (Enyedi et al. 1992, Malamy et al. 1992); however, subsequent research revealed that small amounts of SGE could also be formed (Edwards 1994, Lee and Raskin 1998). In soybean cell suspension cultures, SA was reported to be exclusively metabolized to SGE (Barz et al. 1978) and in a variety of other plant species SA was metabolized primarily to the 5-*O*-glucoside of gentisic acid (Cooper-Driver et al. 1972, Schulz et al. 1993).

The presence of SA glucosyltransferase (SAGT) enzymes that are capable of catalysing the formation of SA glucose conjugates from SA and UDP-glucose have been identified and in some cases characterized from oat roots (Yalpani et al. 1992a, b), tobacco (Enyedi and Raskin 1993, Edwards 1994, Lee and Raskin 1998, 1999), and *M. japonicus* (Tanaka et al. 1990). In oat and *M. japonicus* the SAGT activity was induced by SA (Tanaka et al. 1990, Yalpani et al. 1992a) and in tobacco, the SAGT activity was induced by SA, TMV and *Pseudomonas syringae* pv. *Phaseolicola* inoculation (Enyedi and Raskin 1993, Edwards 1994, Lee and Raskin 1998, 1999). In addition, tobacco appeared to contain two SAGT activities, one that resulted in the formation of the ester and one that resulted in the formation of the glucoside (Lee and Raskin 1998). Purification and cloning of a tobacco SAGT revealed that a single enzyme possesses both of these activities (Lee and Raskin 1999).

Glucosylation of SA is clearly an important route of SA metabolism in plants and although the role of these glucose conjugates is not yet clear, it has been suggested that glucosylation may significantly affect the signalling and defence responses mediated by SA or serve to protect the cell from the toxic effects of excess levels of SA (Malamy et al. 1992).

Although no direct evidence has ever been presented, it has been suggested that the glucose conjugates of SA once formed are sequestered in the vacuole (Ben-Tal and Cleland 1982, Tanaka et al. 1990). Vacuolar sequestra-

tion of SA conjugates certainly seems likely since it has been demonstrated that glucose conjugates of other plant hormones and growth regulators are found in the vacuole (Garcia-Martinez et al. 1981, Schmitt and Sandermann 1982, Bray and Zeevaart 1985, Lehmann and Glund 1986, Füsseder and Ziegler 1988).

In this report, the metabolism of SA to glucose conjugates and the vacuolar localization of these glucose conjugates are described. In contrast to a previous report that indicated that [¹⁴C]SA supplied to soybean cells was converted only to SGE (Barz et al. 1978), we have been able to demonstrate that SAG is the major metabolite of SA formed in soybean along with lower levels of glucosylated 2,5-DHBA and MeSA 2-*O*-β-D-glucose. In addition, we have provided the first direct evidence that the glucose conjugates of SA are localized in the vacuole and that the SAGT activity that forms SAG is located outside the vacuole.

Materials and methods

Cell suspension cultures

Soybean (*Glycine max* [L.] Merr. cv Williams 82) cell suspension cultures were grown in Murashige and Skoog (1962) media with 3% sucrose and 0.4 mg l⁻¹ 2,4-D. The cells were incubated on a rotary shaker (140 r.p.m.) 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) at a final concentration of 10 μM and a specific activity ranging from 1 to 5 μCi μmol⁻¹ in ethanol. The final ethanol volume was 0.1% of the culture media volume. Cells were collected through vacuum filtration on Whatman no. 1 filter paper (Whatman, Clifton, NJ, USA). The cells and the media were then frozen at -20°C. The frozen cells (approximately 6.5 g) were ground in 90% ethanol (50 ml) with a mortar and pestle. The extract was centrifuged at 400 g for 20 min. The resulting supernatant was concentrated in vacuo before TLC or HPLC analysis. Radioactivity was determined with a liquid scintillation counter.

Analysis of metabolites

TLC analysis was performed on silica gel LK6 plates (Whatman) developed in butanol:acetic acid:water (4:1:1). Radioactivity on the TLC plates was visualized with an imaging scanner (Bioscan, Washington DC, USA). 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. The eluant from the column was diverted to a fraction collector and the radioactivity in the 1-ml fractions was determined through liquid scintillation counting. Identification of conjugates through enzymatic hydrolysis with either β -glucosidase or esterase was determined as described by Edwards (1994). Identification of 2,5-DHBA, MeSA, and SA were confirmed through co-chromatography with authentic standards.

Preparation of protoplasts and vacuoles

Protoplasts were prepared as described by Taguchi et al. (2000). The protoplasts were collected through centrifugation (100 *g* for 6 min) and washed three times with buffer (1 *mM* KH_2PO_4 , pH 5.8, 0.6 *M* mannitol). Vacuoles were released from the protoplasts through osmotic shock. Before the protoplasts were collected from the third wash, they were placed on ice for 30 min. The protoplasts were collected through centrifugation and suspended in prewarmed (42°C) protoplast lysis buffer (10 *mM* HEPES-KOH, pH 7.5, 0.2 *M* sorbitol, 10% (w/v) Ficoll 400). The protoplasts were incubated in the lysis buffer at 42°C for 5 min. The mixture was then transferred to a Corex centrifuge tube and overlaid with 0.5 ml of buffer (10 *mM* HEPES-KOH, pH 7.5, 0.5 *M* mannitol). The tubes were centrifuged in a swinging bucket rotor at 5000 *g* at 10°C for 30 min. The vacuoles were collected from the interface between the lysis buffer and overlay buffer with a glass Pasteur pipette. Yields of both protoplasts and vacuoles were determined with a haemocytometer. Approximately 7 million protoplasts could be formed from 10 g cells and typical yields of vacuoles from protoplasts were about 20%. Microscopically, there was no visual evidence for the presence of protoplasts in the purified vacuole fraction, and the purified vacuoles appeared to be identical to those shown in previous studies (Mettler and Leonard 1979, Schmitt and Sandermann 1982).

Enzyme assays

For the studies examining the induction of SAGT activity by SA, the cells (3 days after subculture) were treated with various concentrations of SA in ethanol (0.1% v/v total tissue culture media) or ethanol alone (untreated cells) for 24 h. The SAGT activity was determined in crude extracts as described by Edwards (1994) except that the reaction products were separated from [7-¹⁴C]-SA through TLC as described above and the radiolabelled product was visualized with an imaging scanner. The protein concentration was determined by the method of Bradford (1976) using BSA as a protein standard.

In order to determine the localization of the SAGT activity in the protoplasts versus vacuoles, cells were treated as described above with 0.1 *mM* SA for 24 h. The protoplast and vacuole samples were isolated and

extraction buffer (100 *mM* K-PO_4 , pH 7, 10 *mM* Na-metabisulphite, 10 *mM* ascorbic acid, 28 *mM* 2-mercaptoethanol) was added to each sample (10 ml to the washed protoplast pellet and 10 ml to 1 ml of the purified vacuole suspension). Each suspension was sonicated and then centrifuged at 100 000 *g* for 20 min. The supernatant was brought to 70% saturation with solid ammonium sulphate and the protein pellet was collected by centrifugation at 16 000 *g* for 15 min. The pellet was suspended in extraction buffer and desalted on PD-10 columns (Amersham Biosciences, Piscataway, NJ, USA) into extraction buffer. The eluant from the protoplast preparation was used directly for the SAGT assays, but the eluant from vacuole preparation was concentrated with Centricon-10 concentrators (Amicon, Beverly, MA, USA).

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 the protoplast and vacuole samples for marker enzyme assays was conducted as described by Dombrowski et al. (1994).

Results

Time-dependent uptake and metabolism of [7-¹⁴C]SA

The [7-¹⁴C]SA that was added to the culture media was taken up rapidly by the soybean cells with nearly 80% of the radioactivity found in the cell extract supernatant after only a 2-h incubation (Fig. 2). The uptake of radioactivity by the cells was accompanied by a corresponding decrease in the radioactivity found in the culture media.

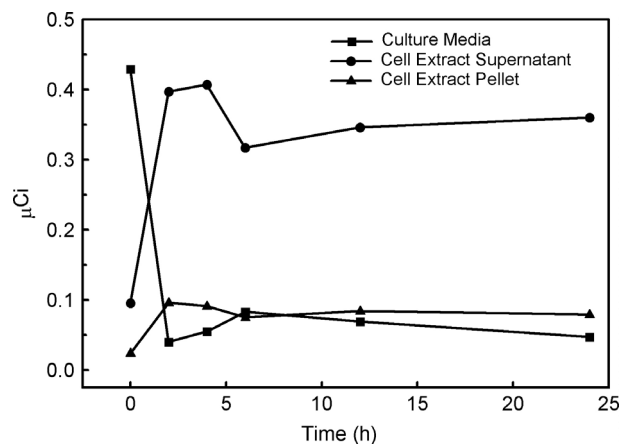


Fig. 2. Time-dependent uptake of [7-¹⁴C]SA by soybean cell suspension cultures. Cell cultures (50 ml) were treated with [7-¹⁴C]SA (10 μM final concentration with a specific activity of 1 $\mu\text{Ci } \mu\text{mol}^{-1}$) 3 days after subculture. At the time points indicated the cells and culture media were separated by filtration and frozen. The cells were ground in 90% ethanol and centrifuged. The cell extract supernatant and cell extract pellet refers to the supernatant and pellet, respectively, following centrifugation. Results shown are representative results of an experiment that was repeated twice.

The high percentage of radioactivity associated with the cell extract supernatant and the low percentage of radioactivity associated with the culture media remained relatively constant from 2 to 24 h after the addition of the [7-¹⁴C]SA. A small percentage of the added radioactivity was also found to be associated with the cell extract pellet. The amount of radioactivity associated with the pellet also remained relatively constant (between 1.5 and 1.9% of the total amount added) from 2 to 24 h after the addition of [7-¹⁴C]SA. At all time points examined, greater than 96% of all the radioactivity added could be accounted for in either the culture media, the cell extract supernatant or the cell extract pellet.

TLC and HPLC was used to determine the identity of the metabolites found in the cell extract supernatant and to follow their formation over time following the addition of [7-¹⁴C]SA. At all time points examined, there were never more than four peaks of radioactivity following TLC analysis (Fig. 3). The *R_f* values were 0.33, 0.45, 0.6, and 0.72, respectively, for metabolites 1 through 4.

Each metabolite was scraped from the TLC plate, treated with either β-glucosidase or esterase and analysed by HPLC. Metabolite 1 was eluted in fraction 7 following HPLC analysis (Fig. 4A). When this metabolite was treated with β-glucosidase, a compound with the same retention time as authentic 2,5-DHBA was released (Fig. 4B). Esterase treatment had no effect (Fig. 4C). Therefore, it was concluded that this metabolite was a glucoside of 2,5-DHBA. Metabolite 2 was eluted in fraction 10 and 11 (Fig. 5A). β-glucosidase treatment released a compound with the same retention time as authentic SA (Fig. 5B). Esterase treatment had no effect. Therefore, it was concluded that this metabolite was SAG. Metabolite 3 had a retention time between 15 and 17 min following HPLC analysis (Fig. 6A). Treat-

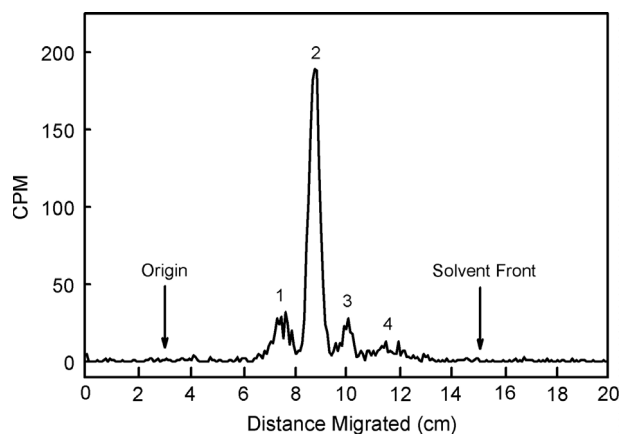


Fig. 3. TLC analysis of the [7-¹⁴C]SA metabolites found in the cell extract supernatant. The cells were treated for 24 h with [7-¹⁴C]SA as described in the legend of Fig. 2. The cell extract supernatant was concentrated in a rotary evaporator before spotting on silica gel TLC plates. The TLC plates were developed in butanol:acetic acid:water (4:1:1). Radioactivity on the plates was determined with a Bioscan imaging scanner. Radioactive metabolites are labelled 1 to 4.

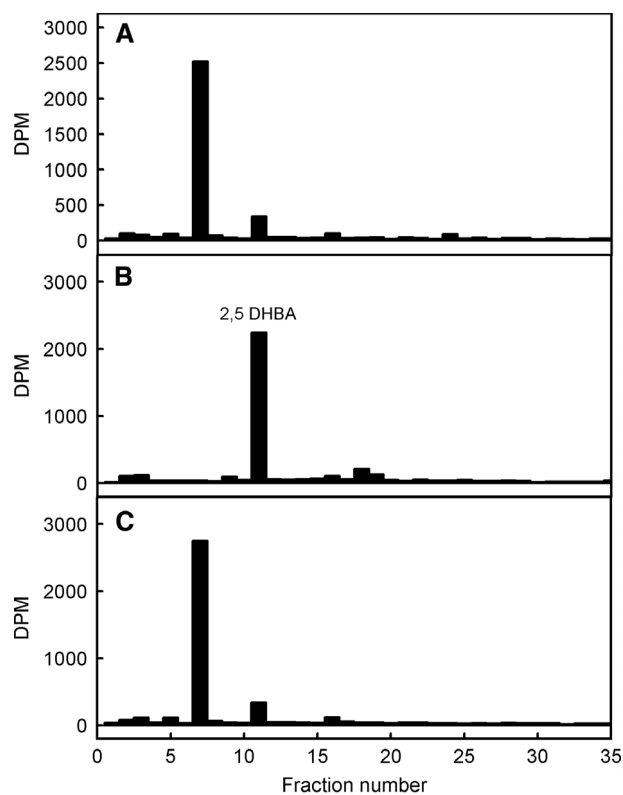


Fig. 4. HPLC analysis of metabolite 1. Metabolite 1 (Fig. 3) was scraped from the TLC plate, eluted with water, and analysed by HPLC as described in the Materials and Methods. (A) Untreated sample. (B) β-glucosidase-treated sample. (C) Esterase treated sample.

ment with β-glucosidase resulted in a loss of about 36% of the initial radioactivity. This loss occurred presumably through the release of a semi-volatile compound. One of the main compounds formed following β-glucosidase treatment had the same retention time as authentic MeSA (Fig. 6B). However, this treatment did not result in a complete conversion to MeSA. Esterase treatment of metabolite 3 released a compound with the same retention time as SAG (Fig. 6C). However, as with the β-glucosidase treatment, the esterase treatment did not result in a complete conversion. Based on the evidence, it appears as if metabolite 3 represents more than one compound. However, one of these metabolites appears to be MeSA 2-*O*-β-D-glucose (MeSAG). Treatment with β-glucosidase released MeSA, some of which was volatilized and some of which was detected in the HPLC profile (Fig. 6B). Treatment of MeSA 2-*O*-β-D-glucoside with esterase removed the methyl group and resulted in the formation of SA 2-*O*-β-D-glucoside (Fig. 6C). Attempts to resolve metabolite 3 into more than one HPLC peak were unsuccessful. Therefore, this peak will be referred to as MeSAG/unknown. It does not appear as if this unknown is SGE since neither β-glucosidase nor esterase was able to completely convert this metabolite(s). Metabolite 4 (Fig. 3) was determined to be SA

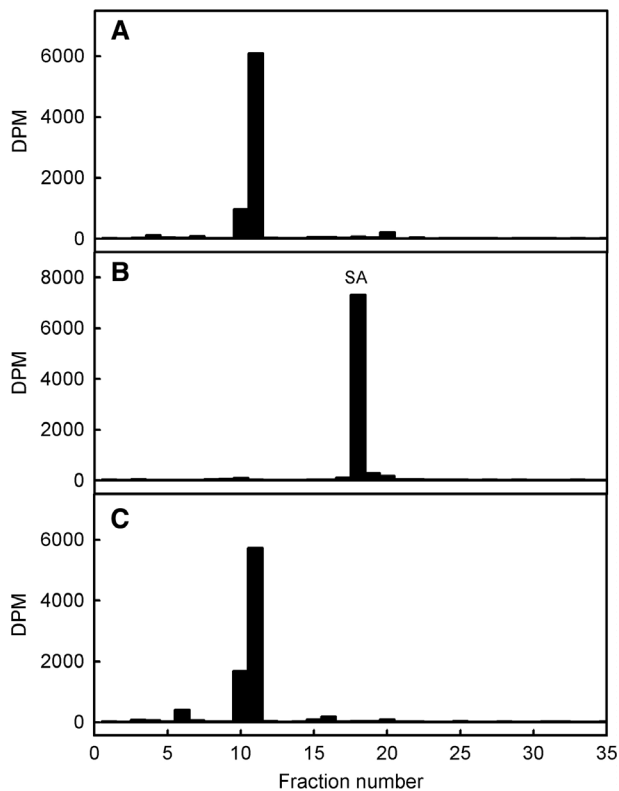


Fig. 5. HPLC analysis of metabolite 2. Metabolite 2 (Fig. 3) was scraped from the TLC plate, eluted with water, and analysed by HPLC as described in the Materials and Methods. (A) Untreated sample. (B) β -glucosidase-treated sample. (C) Esterase treated sample.

since it was not affected by either β -glucosidase or esterase and had the same R_f (TLC) and retention time (HPLC) as authentic SA (data not shown).

Two hours after the addition of $[7-^{14}\text{C}]$ SA the majority of the radioactivity in the cell extract supernatant was in the form of unaltered SA (Fig. 7). However, the amount of SA declined steadily from 4 to 6 h and by 12 and 24 h only trace amounts of SA were found in the cell extract supernatant. This decrease in the amount of SA was accompanied by a steady increase in the metabolites of $[7-^{14}\text{C}]$ SA. After 4 h, SAG was the major metabolite formed (Fig. 7). The amount of SAG formed by the cells reached its highest level 12 h after the addition of $[7-^{14}\text{C}]$ SA where it represented 73% of the total amount of radioactivity found in the cell extract supernatant. MeSAG/unknown was found to be present in the cell extract supernatant as early as 2 h after the addition of $[7-^{14}\text{C}]$ SA (Fig. 7). The levels of the MeSAG/unknown remained relatively constant from 2 to 24 h. By 24 h the levels represented 23% of the total radioactivity recovered in the cell extract supernatant. The glucoside of 2,5-DHBA was not detected at time points earlier than 6 h (Fig. 7). From 6 to 24 h, however, there was a steady but slight increase in this glucoside and by 24 h it represented only 18% of the total radioactivity recovered in the cell extract supernatant.

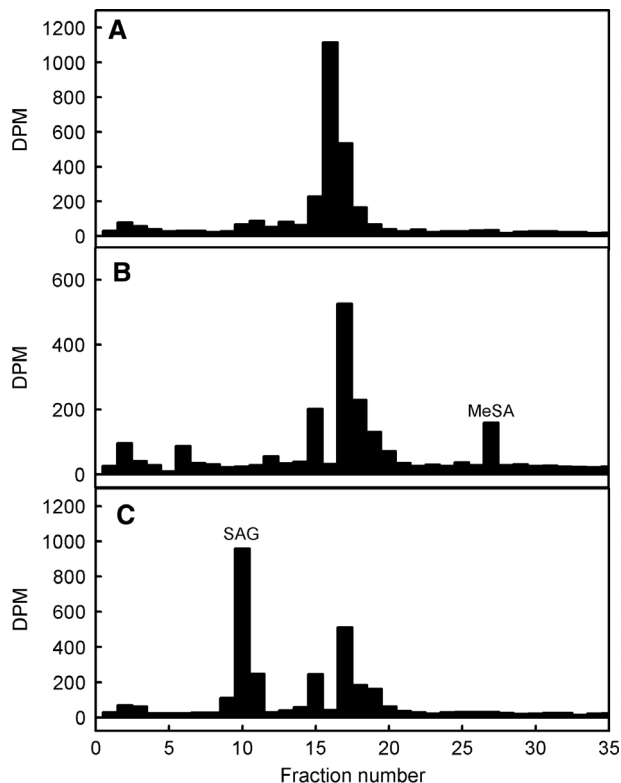


Fig. 6. HPLC analysis of metabolite 3. Metabolite 3 (Fig. 3) was scraped from the TLC plate, eluted with water, and analysed by HPLC as described in the Materials and Methods. (A) Untreated sample. (B) β -glucosidase treated sample. (C) Esterase treated sample.

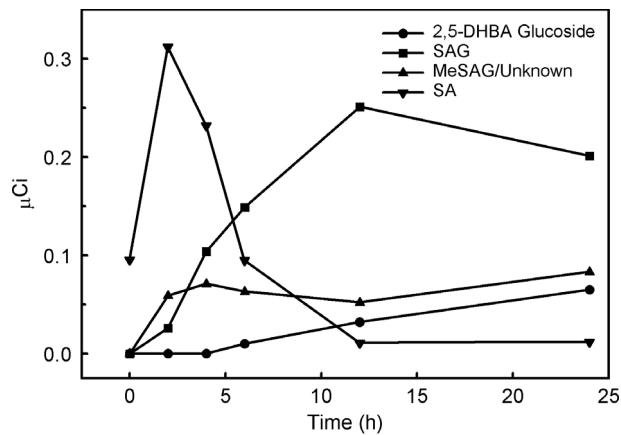


Fig. 7. Time-dependent formation of $[7-^{14}\text{C}]$ SA metabolites. The metabolites of $[7-^{14}\text{C}]$ SA in the cell extract supernatant described in the legend of Fig. 2 were separated by TLC. The relative amount of each metabolite at each time point indicated was determined with a Bioscan imaging scanner. Results shown are representative results of an experiment that was repeated twice.

SAGT activity

Very little in-vitro SAGT activity could be detected in crude enzyme extracts from untreated-soybean cell

suspension cultures (Fig. 8). However, a 24-h treatment with as little as 0.01 mM of SA resulted in about a nine-fold increase in the in-vitro SAGT activity (Fig. 8). The SAGT activity increased as the SA concentration added to the media increased up to 0.5 mM. The SAGT activity in the cells treated with 0.5 mM SA was about 88-fold greater than the SAGT activity observed in the untreated cells. Treatment of the cells with concentrations of SA higher than 0.5 mM did not result in any further increases in the SAGT activity, and in fact, the SAGT activity extracted from the cells treated with 1.0 mM SA was nearly nine-fold less than the SAGT activity extracted from the cells treated with 0.5 mM SA (Fig. 8).

In order to perform the in-vitro SAGT assay, it was necessary to separate the conjugated radiolabelled product from the [7-¹⁴C]SA added at the beginning of the assay. This was accomplished through TLC as previously described. In all of the TLC separations performed during the assays only one radiolabelled peak other than the [7-¹⁴C]SA was observed (Fig. 9). This peak was determined to be SAG based on its *R_f* value of 0.45, which was identical to the *R_f* value of the SAG formed in vivo. In addition, the in-vitro product was converted to SA following β-glucosidase treatment but treatment with esterase had no effect (data not shown) providing further evidence that this product was SAG.

Distribution of enzymes and SA metabolites in protoplasts and vacuoles

Vacuole preparations from the soybean cells were almost completely free of protoplasts as determined through a microscopic examination and were visibly similar to photographs of vacuoles shown in previous publications (Mettler and Leonard 1979, Schmitt and Sandermann 1982). In addition, the vacuole to protoplast ratios of the α-mannosidase activity (a vacuolar marker enzyme) and the glucose-6-phosphate dehydrogenase activity (a cytoplasmic marker enzyme) were consistent with what would be expected for a vacuolar preparation (Table 1).

A microscopic examination of soybean protoplasts stained with neutral red provided visual evidence that most of the protoplasts contained one large vacuole. If this is the case, then it appears as if all of the radioactivity in the protoplasts prepared from soybean cells treated with [7-¹⁴C]SA for 24 h was associated with the vacuole (Table 1). Of these radioactive metabolites the vacuole contained all of the 2,5-DHBA glucoside and all of the SAG. However, the protoplasts contained a higher concentration of the MeSAG/unknown than the vacuoles, but a lower concentration of SA.

Since very little SAGT activity could be detected in untreated soybean cells (Fig. 8), the subcellular distribution of the SAGT activity was examined in protoplasts and vacuoles isolated from SA-treated soybean cells. Although the 0.5 mM SA treatment resulted in the maximum induction of SAGT (Fig. 8), this level of SA interfered with the generation of protoplasts. This may have

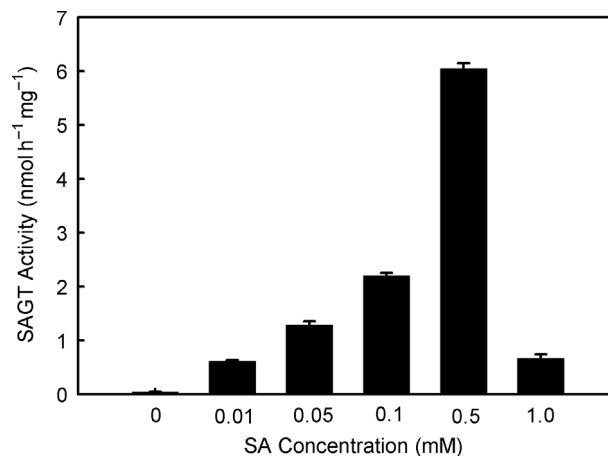


Fig. 8. Effects of SA on SA glucosyltransferase (SAGT) activity. The cells were treated with the various concentrations of SA for 24 h. The SAGT activity was determined as described in the Materials and Methods. Values shown are means ± SD of three replicates.

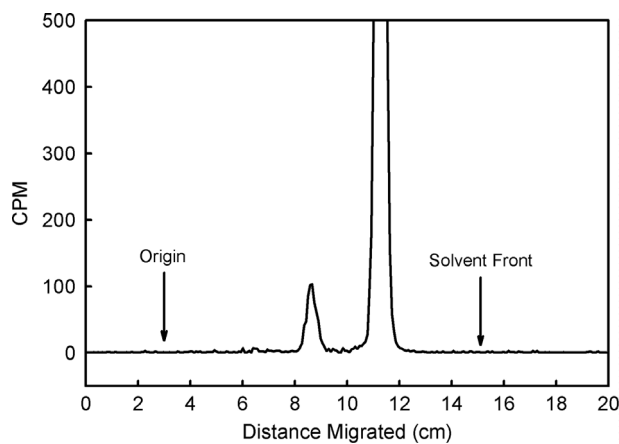


Fig. 9. TLC analysis of the SAGT product formed in vitro. The SAGT assay was performed with crude extracts prepared from soybean cells that were treated for 24 h with 0.1 mM SA. Following the assay, the reaction media was spotted on a silica gel TLC plate and the plate was developed in butanol : acetic acid : water (4 : 1 : 1). Radioactivity on the plate was visualized with a Bioscan imaging scanner.

been the result of SA-enhanced changes in the cell wall that interfered with the activity of the protoplasting enzymes (Siegrist et al. 1994). Therefore, the subcellular localization of the SAGT activity was determined in cells treated for 24 h with 0.1 mM SA. Examination of the SAGT activity in the vacuoles versus protoplasts revealed that the majority of the SAGT activity was localized outside the vacuole (Table 1).

Discussion

The most detailed investigations on the in-vivo metabolism of SA have been conducted using tobacco (Edwards 1994, Lee and Raskin 1998). From these studies, it has

Table 1. Distribution of enzymes and SA metabolites in protoplasts and vacuoles from soybean cell suspension cultures. Soybean cells were treated 3 days after subculture with [7-¹⁴C]SA (10 μM final concentration with a specific activity of 2 μCi μmol⁻¹) for 24 h before the isolation of the protoplasts and vacuoles. Radioactive metabolites were separated by HPLC and amounts determined by liquid scintillation counting. The SAGT activity was determined in cells that had been treated with 0.1 mM SA for 24 h. Results shown are representative results from experiments that were repeated at least twice.

	In 10 ⁶ vacuoles	In 10 ⁶ protoplasts	Vacuoles/protoplasts (%)
Total metabolites (nmol)	9	8	112.5
2,5-DHBA glucoside (nmol)	2.18	2.0	109
SAG (nmol)	4.5	3.9	115
MeSAG/unknown (nmol)	0.51	0.91	56
SA (nmol)	1.7	1.2	142
SAGT (pmol min ⁻¹)	0.25	9.69	2.6
α-mannosidase (nmol min ⁻¹)	2.96	4.09	72
Glucose-6-phosphate dehydrogenase (nmol min ⁻¹)	3.4	23.4	14.5

been determined that tobacco primarily converts SA to SAG, however, SGE, other uncharacterized 2-*O*-glycosides and CO₂ are also produced (Edwards 1994, Lee and Raskin 1998). The metabolism of [7-¹⁴C]SA in soybean cell suspension cultures appears to very different. Although SAG was the major metabolite formed, soybean cells were also able to convert SA into a 2,5-DHBA glucoside and MeSAG. This is in contrast to an earlier study in which it was reported that soybean exclusively converted SA to SGE (Barz et al. 1978). We were unable to find any evidence that soybean cells formed SGE from [7-¹⁴C]SA. This discrepancy between the two studies may be due to differences in the soybean cultivars used, the culture conditions, or the extraction and separation techniques. Whatever the reason, it seems clear that the metabolism of SA in soybean is much more complex than has originally been reported.

At every time point examined throughout the in-vivo [7-¹⁴C]SA uptake studies, greater than 96% of the radioactivity could be recovered. Therefore, significant formation of CO₂ from a 24-h exposure to [7-¹⁴C]SA did not occur in soybean. The lack of CO₂ production during SA metabolism in soybean was also reported by Barz et al. (1978). This differs from observations of tobacco leaves in which significant amounts of [¹⁴C]CO₂ were formed following the addition of [7-¹⁴C]SA (Edwards 1994). Therefore, unlike tobacco, the decarboxylation of SA does not appear to be a significant pathway of SA metabolism in soybean. Also, unlike tobacco, soybean cells formed a 2,5-DHBA glucoside and MeSAG from [7-¹⁴C]SA. The 2,5 DHBA glucoside is probably a 5-*O*-glucoside since this metabolite of SA has been found in several other plant species (Cooper-Driver et al. 1972, Schulz et al. 1993). Although it is known that SA formed endogenously in TMV-inoculated tobacco can be converted to MeSA (Shulaev et al. 1997, Seskar et al. 1998) and that MeSAG is found in the fruit of *Passiflora* species (Chassagne et al. 1997), to the best of our knowledge, this is the first report of the formation of MeSAG from exogenously supplied SA. MeSA is thought to function as an airborne signal that induces defence mechanisms in surrounding plants or non-infected areas of the infected plant. Conjugation of MeSA to glucose

may serve as a convenient non-volatile storage form of this signal molecule.

Although the vacuolar localization of glucose conjugates of plant hormones and growth regulators has been well documented, it is surprising that this has never been confirmed for the glucose conjugates of SA. Our results clearly demonstrate for the first time that all glucose conjugates of SA are stored in the vacuole (Table 1). However, it is not clear why we consistently observed higher levels of the MeSAG/unknown in the protoplasts than in the vacuoles (Table 1) even though all of the radioactivity in the protoplasts was associated with the vacuoles. It is possible that the unknown metabolite may be more unstable than the glucosides and releases SA during the preparation of the vacuoles. This is consistent with our results since when compared to the protoplasts, the lower level of the MeSAG/unknown in the vacuoles was associated with higher levels of SA (Table 1).

In addition to demonstrating that all of the SA glucose conjugates are located in the vacuole, we have also been able to demonstrate for the first time that most of the SAGT activity is found outside the vacuole (Table 1). It appears likely that the SAGT activity is located in the cytoplasm since it is typically a soluble enzyme and most other glucosyltransferase enzymes have a cytosolic localization (see citations in Werner and Matile 1985, Martinoia et al. 2000, Jones and Vogt 2001). It has previously been demonstrated that the glucosylation of other secondary compounds by their corresponding glucosyltransferases also occurs outside the vacuole even though the conjugates 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).

The in-vitro soybean SAGT activity appears to be different than the tobacco SAGT activity. SGE was the main product formed during the in-vitro tobacco SAGT assay even though SAG was the primary in-vivo product observed (Edwards 1994, Lee and Raskin 1998). Purification and cloning of the pathogen-inducible tobacco SAGT revealed that one enzyme could form both SAG and SGE (Lee and Raskin 1999). In soybean, SAG was the only reaction product formed during the in-vitro SAGT assay (Fig. 9).

Since the SA glucose conjugates are formed outside the vacuole and stored predominantly in the vacuole, it appears as if there is a transport mechanism responsible for the movement of the conjugates across the tonoplast membrane. Although it is generally assumed that glucose conjugates of secondary compounds undergo carrier-mediated uptake into the vacuole, very little is known about how the transport occurs (Martinoia et al. 2000). Based on the limited studies that have been conducted, it is possible that the glucose conjugates of SA may move across the tonoplast via an H⁺-antiport system (Werner and Matile 1985, Klein et al. 1996, Bartholomew et al. 2002). Alternatively, ATP-binding cassette transporters which are directly energized by MgATP and do not depend on a transmembrane H⁺-electrochemical potential difference may also move glucose conjugates (Klein et al. 1996, Frangne et al. 2002). We are currently in the process of determining how the glucosides of SA are transported across the tonoplast membrane of plant cells. Transport of SA glucose conjugates from the cytoplasm across the tonoplast for vacuolar sequestration may be as important as the glucosylation of SA in regard to either protecting the cell from toxic levels of SA or influencing the availability of SA produced endogenously during a defence response.

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