Responses of Transformed Roots of *Atropa Belladonna* to Treatment with Salicylic Acid and other Phenolic Compounds

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Abstract

Treatment of transformed *Atropa belladonna* root cultures with high concentrations of salicylic acid (SA) resulted in the production of methylated salicylic acid derivatives but not of glucosylated derivatives. This response was induced by 0.1 mM SA whereas treatment with SA at concentrations higher than 1.2 mM caused growth retardation. Nontransformed roots of *A. belladonna* responded to the addition of SA in almost the same manner as transformed roots, which suggests that this is a natural response in *A. belladonna*. We could not detect methylated SA in cultures of *Nicotiana plumbaginifolia* and *Hyoscyamus niger*. Besides SA, several aromatic compounds were methylated by the transformed roots of *A. belladonna*, and other responses, such as the conversion of benzoic acid to benzyl alcohol, were also observed.

Salicylic acid (SA) is a phenolic compound produced and metabolized in the phenylpropanoid pathway in higher plants, and has several physiological functions including acting as the signal for systemic acquired resistance (Malamy *et al.*, 1990; Metraux *et al.*, 1990). However, excessive SA is harmful to plant growth and development (Manthe *et al.*, 1992). In plant cells, SA is detoxified by several processes, including glucosylation (Tanaka *et al.*, 1990; Edwards, 1994), methylation (Schulaev *et al.*, 1997), and binding with amino acids (Bourne *et al.*, 1991). Glucosylation of SA is reported in many plants, and is the most dominant response to SA feeding (Pierpoint, 1994).

We reported previously that the SA was monomethylated and dimethylated without any glucosylation when transformed roots of *Atropa belladonna* M8 were cultured in the presence of high concentrations (0.2 mM) of SA (Lee *et al.*, 2001). While the concentrations of SA decreased with time, methyl salicylate (MSA) and methyl-*O*-methoxybenzoate (MMB) were detected 12 and 14 h after the addition of SA, respectively. Therefore, we presumed that SA was converted to MSA, and

subsequently MSA was converted to MMB. In this paper we report the changes in the concentration of methylated SAs in the medium over time, and the specific substrates which *A. belladonna* can methylate.

Transformed root cultures were incubated in 200 ml of hormone-free half-strength Murashige and Skoog (1/2 MS) liquid medium containing 3% (w/v) sucrose in 300 ml conical flasks capped with aluminum foil. One gram (fresh weight) of roots was inoculated per flask and subcultured every 2 weeks. All cultures were maintained on a rotary shaker (100 strokes per minute) at 25 °C in the dark.

The concentration of SA in medium was varied to determine the amount of SA that is required to induce this response. Sterilized SA solution was added to 18-day-old root cultures to give a final concentration ranging from 0.01 to 2.0 mM. SA derivatives in the culture medium were quantified by HPLC analysis (Simadzu SCL-10Avp, SPD-M10Avp). Ten microliters of culturing medium was injected into a Tosoh ODS-120A column (4.6 mm i.d. x 250 mm) and carried by the mobile solution [acetonitrile : 10 mM SDS (adjusted to pH 3.4 with

1% H$_3$PO$_4$) = 2:3] at 1.2 ml min^{-1}. The concentrations of SA and its derivatives were recorded at 215 nm absorbance.

Fig. 1 shows the production of MSA and MMB over time following the addition of SA. SA at a concentration of at least 0.1 mM SA was required to induce a positive response, and at concentrations of 1.2–1.6 mM SA caused negative effects to the root, such as tissue browning and growth retardation.

A similar experiment was performed for nontransformed roots of *A. belladonna*, which was established and maintained at Tsukuba Medicinal Plant Research Station (Nakanishi *et al.*, 1998). The cultures were incubated in 100 ml of 1/2 MS liquid medium containing 3% (w/v) sucrose in 200 ml conical flasks, and maintained on a rotatry shaker (75 strokes per minute) at 25 °C in the dark. Roots (3 g fresh weight) were inoculated in each flask and
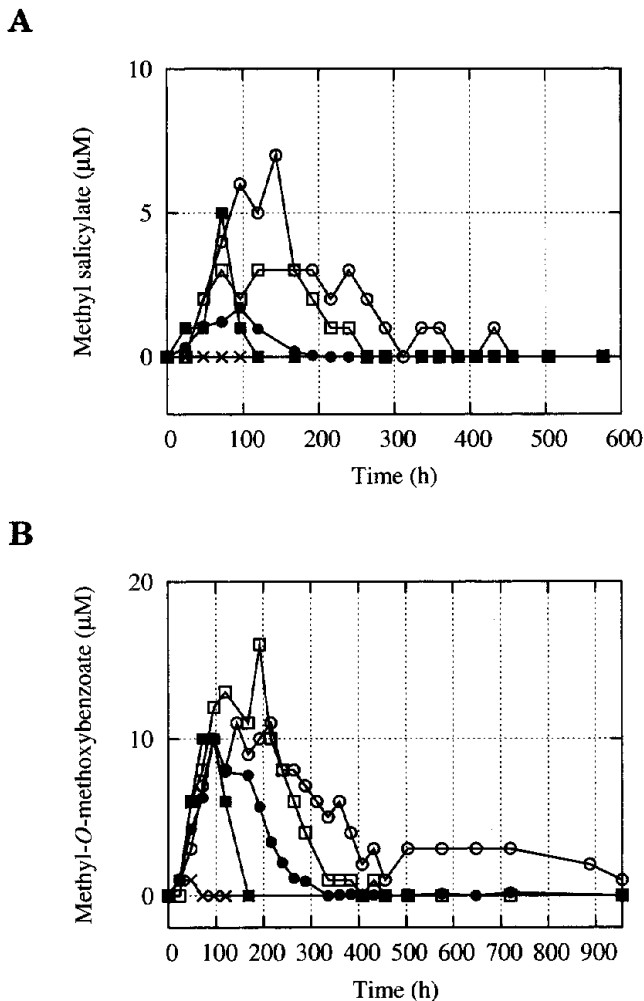
subcultured every month. Sterilized SA solution was added to 30-day-old cultures to give a final concentration of 0.8 mM. Two compounds were found in the medium 5 days after the addition of SA, and these were identified as MSA and MMB (**Fig. 2**) using the HPLC system. These results
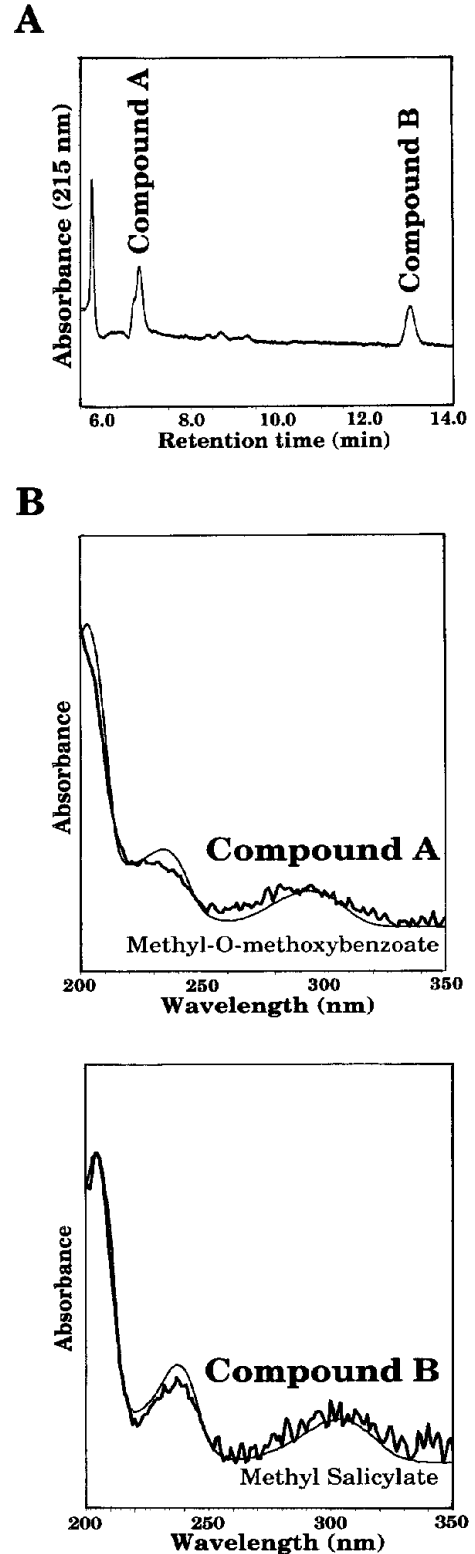
A

B

A

B

Fig. 1 Time courses of methyl salicylate (A) and methyl-*O*-methoxybenzoate production (B) in *A. belladonna* transformed root culture medium treated with 0.01, 0.1, 0.4, 0.8, and 1.2 mM SA. The transformed root culture treated with 1.2 mM SA was damaged.

Symbols: ×, 0.01 mM SA; ■, 0.1 mM SA; ●, 0.4 mM SA; □, 0.8 mM SA; ○, 1.2 mM SA.

Fig. 2 HPLC profile of the culture medium of adventitious roots with photodiode array detection (A), and absorption spectra of compounds A and B (B).

suggest that T-DNA integration is not the cause of the methylation response, and therefore it appears that this is a natural response by *A. belladonna*.

We investigated whether a similar response to the addition of SA to culture media could be observed in other plant species. We tested *Nicotiana plumbaginifolia* and *Hyoscyamus niger* (Solanaceae), which taxonomically is close relative of *A. belladonna*. We employed transformed roots of *N. plumbaginifolia*, which were established by co-culture of leaf discs with *Agrobacterium rhizogenes* 1610, and nontransformed roots of *H. niger*, which were established and maintained at Tsukuba Medicinal Plant Research Station (Jung *et al.*, 2001).

Sterilized SA solution was added to 14-day-old transformed root cultures of *N. plumbaginifolia* and 30-day-old nontransformed root cultures of *H. niger*. They were incubated under the same conditions as the transformed root cultures of *A. belladonna*. In both cases the concentration of SA in the medium was decreased, but methylated SAs were not detected (data not shown).

We also investigated the responses of *A. belladonna* to the addition of compounds which are structurally similar to SA. For this purpose we examined the responses of transformed roots of *A. belladonna* to treatment with 13 aromatic compounds (Table 1). Seventy-two h after the addition of each compound, any compounds in the medium were extracted by *n*-hexane and identified by a GC-MS system (Shimadzu GCMS-QP5050A). A 5-μl extract was injected into a chromatographic

column (0.25 mm i.d. x 30 m, 0.25 μm; J&W Scientific DB-1) using helium gas as the carrier at a flow rate of 1.7 ml min^{-1}. The oven temperature was increased from 30 °C to 250 °C at a rate of 20 °C min^{-1}, and the ionization voltage was 70 eV.

As shown in **Table 1**, two compounds (MSA and salicyl alcohol) were methylated by *A. belladonna* transformed roots. The ability of methylation of MSA supports the hypothesis of the conversion sequence described above (i.e., SA → MSA → MMB). However, SA was detected in the culture medium to which MMB had been added (**Table 1**). This result suggests that MMB is converted to SA in *A. belladonna*. Interestingly, the result also suggests that *A. belladonna* was able to remove the acetyl group from the aromatic compounds, because the addition of acetyl SA induced the production of MSA and MMB (**Table 1**). Another manner of modification was also detected, when some other compounds (such as benzoic acid) were added (**Table 1**).

We examined the ability of the crude cell extract of transformed roots of *A. belladonna* to methylate the aromatic compounds *in vitro*. The extraction procedure basically followed the protocol of Akashi *et al.* (2000). Sterilized SA was added to the transformed root cultures to give a final concentration of 0.4 mM, and roots (about 50 g) were homogenized in a mortar and pestle with twofold volume (w/v) of extraction buffer and 0.5-fold weight of sea sand and polyclar VT. The extraction buffer consisted of 0.2 M sodium phosphate buffer (pH 7.5) containing 10% (w/v) sucrose, 14 mM 2-mercaptoethanol, 2

Table 1 The compounds detected in the culture media of transformed roots of *A. belladonna* after the addition of aromatic compounds, and the products of conversion with the crude cell-free extracts. (n.d.; not detected, n.t.; not tested)

Compound	Culture medium	Crude cell extract
MSA	SA, MMB	MMB
MMB	SA	n.d.
acetyl salicylic acid	MSA, MMB	MSA, MMB
benzoic acid	benzyl alcohol	n.d.
catechol	n.d.	n.t.
cyclohexane carboxylic acid	hydroxymethyl cyclohexane	n.t.
ferulic acid	n.d.	n.t.
gallic acid	n.d.	n.t.
m-hydroxy benzoic acid	n.d.	n.d.
p-hydroxy benzoic acid	n.d.	n.d.
1-hydroxy-2-naphthoic acid	2-hydroxy-1-naphthalenecarboxaldehyde	n.t.
phloroglucinol	n.d.	n.t.
pyrogallol-4-carboxylic acid	n.d.	n.t.
salicyl alcohol	2-methoxy benzylalcohol	2-methoxy benzylalcoho

mM EDTA, 0.5% (w/v) sodium ascorbate, 10 μg ml^{-1} pepstatin A, and 0.5 mM (p-Amidinophenyl)-methanesulfonyl fluoride hydrochloride (p-APMSF). Pepstatin A, p-APMSF, and polyclar VT were obtained from Wako Pure Chemical Industries. The homogenate was filtered through eight layers of cheesecloth, and the filtrate was centrifuged at 15,000 g for 15 min. The supernatant was stirred for 30 min with half the amount of Dowex 1-x2 (equilibrated with 0.2 M K-Pi buffer, pH 7.5). The solution obtained by filtration through a glass-sintered filter was fractionated with $(NH_4)_2SO_4$. The precipitate between 55 to 75% saturation was dissolved in extraction buffer, passed through a Sephadex G-25 column, and the resultant solution was used for the assay. For the reaction, a mixture containing 1 ml of cell-free extract, 50 μl of 2-methoxyethanol, 65.2 μM substrate (SA, MSA, salicyl alcohol, acetyl SA, and benzoic acid were tested), and 932.4 μM S-adenosyl-L-methionine in a total volume of 1110 μl was incubated for 3 h at 28 °C The products were extracted (and the reaction was stopped) by n-hexane, and identified by the GC-MS system. As shown in **Table 1**, SA, MSA, and salicyl alcohol were methylated, and acethyl SA were converted to MSA and MMB. On the other hand, when benzoic acid, m-hydroxy benzoic acid, or p-hydroxy benzoic acid were used as a substrate, we could detect neither methylated benzoic acids nor any other modified benzoic acids.

Further investigation will be needed to elucidate the function of these methylation responses in A. belladonna. SA methyl transferase of A. belladonna (AbSAMT1), a homolog of SA methyl transferase of Clarkia breweri, was isolated recently (Fukami et al., 2002). AbSAMT1 might be involved in these responses, because it was induced by the addition of abundant SA into the culture medium. However, other enzymes may also contribute to this phenomenon, because AbSAMT1 could not catalyze the conversion from MSA to MMB, which is clearly observed in this response. Purification and characterization of the methyl transferases that contribute to these responses in transformed roots of A. belladonna, especially the methyl transferase of MSA, are in progress in our laboratory.

Acknowledgements

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