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## Glucosylation of Bisphenol A by Various Plant Species

By

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**K e y w o r d s :** Bisphenol A, glycosyltransferase, phytoremediation, glucoside.

### S u m m a r y

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The concentration of bisphenol A (BPA) in the culture medium of tobacco BY-2 cells decreased rapidly 2.5 h after the application. Four metabolites of BPA were observed in a methanol extract of the cells. They were determined to be BPA mono- $\beta$ -D-glucopyranoside and 3 highly glucosylated BPA. These results indicate that tobacco cells uptake BPA, then metabolized to  $\beta$ -glucosides. We also determined BGT activity in cell-free extracts of leaves of 43 plant species. The level of BGT activity correlated with the rate of BPA uptake in *Fabaceae* and *Brassicaceae*. The results indicate that BGT activity may be related to uptake of BPA in both species.

### I n t r o d u c t i o n

Bisphenol A (BPA: 4,4'-isopropylidenediphenol) has a weak estrogenic activity and used in the many types of plastics (ASH & ASH 1995). Annual world production of BPA has been estimated as more than 500,000 tons (STAPLES & al. 1998). BPA has been detected in terrestrial and aquatic environments (ALEXANDER & al. 1988) and it has been shown to affect embryonic and postnatal development of mammals (TAKAI & al. 2001), therefore the environmental impact of this compound is of concern (STAPLES & al. 1998). Plants have also been considered as suitable agents for the biodegradation of lipophilic compounds and would probably be more easily controlled in the environment (CUNNINGHAM & OW 1996). However, the ability of plant cells to take up and metabolize BPA had not been investigated earlier. In the present study, we

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(472)

have identified 4 types of glucosylated metabolites of BPA in tobacco suspension cultured cells. We determined BPA-glucosyltransferase (BGT) activity, which responsible for production of the metabolites, in the leaves of 43 plants species.

## Material and Methods

### Cell cultures and BPA treatment

Bisphenol A was supplemented with  $10 \text{ mg} \cdot \text{L}^{-1}$  at initial concentration in two weeks-old tobacco BY-2 suspension cultured cells. For a control BPA was added to freshly prepared culture medium in the absence of cells. They were incubated at  $25^\circ\text{C}$  with gentle shaking. Aliquots ( $10 \mu\text{L}$ ) of the medium were taken and analyzed by HPLC [C-18 column  $150 \times 3.9 \text{ mm}$  (Symmetry, Nihon Waters, Tokyo, Japan),  $40^\circ\text{C}$ , 40 % aqueous methanol as eluent at a flow rate of  $1 \text{ mL} \cdot \text{min}^{-1}$ . Absorption at  $217 \text{ nm}$  was monitored (NAKAJIMA & al. 2002).

### Analysis of the metabolites of BPA in BY-2 cells

A portion ( $50 \text{ mL}$ ) of a two-week-old BY-2 cultured cells was supplemented with  $10 \text{ mg} \cdot \text{L}^{-1}$  of BPA and incubated at  $25^\circ\text{C}$  for 24 h with shaking. At each sampling time,  $3 \text{ mL}$  of the culture was put into  $10 \text{ mL}$  of methanol and the mixture was allowed to stand for 24 h. The clear supernatant ( $50 \mu\text{L}$ ) was then subjected to HPLC using the same conditions as above.

### Isolation and identification of metabolites

Two liter of BY-2 cells supplemented with BPA at  $10 \text{ mg} \cdot \text{mL}^{-1}$ , was incubated at  $25^\circ\text{C}$  with gentle shaking. The cells were collected and extracted with 1 liter of methanol. The extract was evaporated to dryness, dissolved in  $200 \text{ mL}$  of 67 % aqueous methanol and the pH adjusted to 10 by the addition of a sodium hydroxide solution. Lipophilic material was removed by three times of extraction with  $200 \text{ mL}$  of chloroform and the aqueous phase was evaporated to dryness after the pH was adjusted to 4 with acetic acid. The residue was extracted with  $200 \text{ mL}$  of chloroform at  $50^\circ\text{C}$ . The chloroform-soluble material was evaporated to dryness and dissolved in  $2 \text{ mL}$  of water. The aqueous solution was then subjected to HPLC (as above) with 40 % aqueous methanol as eluent. Three fractions containing metabolites were combined, concentrated. Fraction "A" was re-separated with 23 % aqueous methanol as an eluent. Again the fractions containing metabolites were combined and were then evaporated to dryness. NMR spectrum was obtained from a JNM-ALPHA500 ( $500 \text{ MHz}$  for  $^1\text{H}$ , JEOL, Tokyo, Japan).

### Determination of BPA uptake and BGT activity in various plant species

The name of species for determination of BPA uptake and BGT activity were recored in Tables 1. Four weeks old seedlings were immersed in  $1.5 \text{ mL}$  of  $10 \text{ mg} \cdot \text{L}^{-1}$  BPA solution, then incubated at  $25^\circ\text{C}$  under white light ( $1000 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$  of photosynthetically active photon flux density). At each sampling time amount of BPA in the medium was determined by HPLC. For determination of BGT activity,  $1 \text{ g}$  of leaves were homogenized with  $5 \text{ mL}$  of extraction buffer ( $50 \text{ mM}$  Tris-HCl, pH7.5,  $1 \text{ mM}$  2-mercaptoethanol). After centrifugation, low molecular weight substances in the supernatant were removed by gel filtration. The enzyme reaction mixture contained  $50 \text{ mM}$  Tris-HCl pH7.5,  $1 \text{ mM}$  UDP-Glucose and  $0.5 \text{ mM}$  BPA. The reaction was performed at  $30^\circ\text{C}$  for 20 min, then stopped with a addition of 60 % of perchloric acid. Precipitate was removed by centrifugation, BPA- $\alpha$ - $\beta$ -D-glucopyranoside in the supernatant was determined by HPLC.

## Results and Discussion

Tobacco BY-2 cells uptake BPA and metabolize to glucosides

To determine whether plant cells can absorb BPA, tobacco BY-2 cells

were exposed to  $10 \text{ mg} \cdot \text{L}^{-1}$  of BPA in the medium. The BPA concentration rapidly decreased to one third of the initial value after 0.5 h, and to total disappearance by 2.5 h. In the absence of cells there was no reduction. These results indicate that BY-2 cells have the ability to take up BPA. We separated methanol extracts of the cells after BPA was added to the medium. Four metabolites were apparent in the extracts. They were coded A-1, A-2, B and L. Their chemical structure are determined to be BPA di- $\beta$ -D-glucopyranoside (A-1), BPA  $\beta$ -D-glucopyransyl-(1 $\rightarrow$ 4)-[ $\beta$ -D-glucopyransyl-(1 $\rightarrow$ 6)]  $\beta$ -D-glucopyranoside (A-2), BPA mono- $\beta$ -D-gentiobioside (B) and BPA-mono-D-glucopyranoside (L). Our results indicated that tobacco cells uptake BPA, then metabolized to  $\beta$ -glucosides.

BGT may be responsible for BPA uptake in *Fabaceae* and *Brassicaceae*

We determined whether the ability of seedlings to take up BPA correlated with the level of BPA specific glucosyltransferase (BGT) activity in the leaves. In 43 species we analyzed, the ability to absorb BPA varied (Table 1). The highest absorber was *Capsicum annuum* L. We could not detect any absorption in three species (*Cucurbita maxima*, *Canavalia gladiata* and *Lemna minor*). Among the 43 species tested, *Brassicaceae* plants had higher BGT activity (Table 1). We could not detect any activity in three species (*Citrullus vulgaris*, *Eichhornia crassipes*, and *Lycopersicon esculentum*) and could not determine that of *Brassica campestris* L. (pak-choi) by interfering substances. The level of BGT activity did not correlate with the rate of BPA absorption in 3 of 5 families tested. However, significant correlation was observed in *Fabaceae* and *Brassicaceae* plants (Fig. 1A, B). *Arabidopsis* had the highest absorption rate in *Brassicaceae* plants tested but it had the lowest BGT activity. While *Arabidopsis* showed extraordinary nature in the relation to BPA uptake and BGT activity, our results indicate that the level of BGT activity may be responsible for the rate of BPA absorption in *Fabaceae* and *Brassicaceae*.

In the previous study (NAKAJIMA & al. 2002), we showed that tobacco cells metabolize BPA to BPA mono- $\beta$ -D-glucopyranoside (BPAG); here we showed that all the plant leaves used in this study, except for those of 3 species, had at least a low level of BGT activity. These findings suggest that glucosylation might be a common metabolic process of BPA in plants. We demonstrated that BPAG accumulates in the leaves (NAKAJIMA & al. 2002). Therefore, plants may metabolize BPA by a BGT-catalyzed reaction, accumulate BPAG and its highly glucosylated forms in the leaves, and then eliminate it by detaching the leaves. Therefore, identification of the gene encoding BGT would open the way toward improvement of BPA uptake by ectopic expression of BGT in plant cells.

Table 1. Comparison of BPA uptake and BGT activity in leaves of several plant families. Three to five independent experiments were performed according to described in materials and methods. Average of the value and standard deviations (SD) are indicated (n=3-5). N.D.: Not determined, U.D.: Under detectable.

Family	Species	BPA Uptake ( $\mu\text{g}\cdot\text{h}^{-1}\cdot\text{gfw leaf}^{-1}$ )		BGT activity ( $\text{nmol}\cdot\text{h}^{-1}\cdot\text{mg protein}^{-1}$ )	
		average	S.D.	average	S.D.
Brassicaceae	<i>Raphanus sativus</i> L.	1.86	0.11	9.84	0.32
	<i>hortensis</i> Backer				
	<i>Brassica rapa</i> L. var.	2.26	0.33	13.65	0.55
	<i>capitata</i> L.				
	<i>Brassica oleracea</i>	2.66	0.37	19.14	4.50
	L.var. <i>capitata</i> L				
	<i>Brassica napus</i> L..	2.09	0.19	20.10	2.42
	<i>Raphanus sativus</i> L.	1.75	0.38	5.54	1.25
	var. <i>sativus</i> L.				
	<i>Brassica rapa</i> L. var.	1.56	0.36	6.30	0.37
	<i>amplexicaulis</i>				
	<i>Arabidopsis</i>	6.49	2.26	2.60	0.04
	<i>thaliana</i> L.				
Cucubitateae	<i>Brassica campestris</i> L	3.04	0.77	N.D.	N.D.
	(Pak-choi)				
	<i>Brassica</i>	1.02	0.68	4.75	0.11
	<i>campestris</i> L.				
	(Tsai-shin)				
	<i>Citrullus vulgaris</i> L	6.83	1.34	U.D.	U.D.
	<i>Cucurbita maxima</i> L.	U.D.	U.D.	1.42	0.18
	Duchesne				
	<i>Cucumis sativus</i> L.	11.72	2.29	1.06	0.04
	<i>Cucumis melo</i> L. var.	12.38	4.59	0.39	0.11
	<i>makuwa</i> Makino				
	<i>Litchi chinensis</i>	43.10	1.20	0.17	0.04
	<i>Lagenaria leucantha</i>	11.73	2.40	0.69	0.12
Fabaceae	<i>Luffa cylindrica</i> Roem	11.73	2.40	0.87	0.14
	<i>Vigna angularis</i> Ohwi	29.28	4.05	3.09	0.07
	et.				
	<i>Vigna sinensis</i>	14.34	2.10	1.29	0.23
	<i>Glycine max</i>	11.06	3.46	0.19	0.04
	<i>Phaseolus vulgaris</i> L.	5.09	3.44	0.06	0.03
	<i>Pisum sativum</i> L.	43.08	12.92	3.48	0.33
	<i>Vicia faba</i> L	5.92	0.83	1.49	0.07
	<i>Canavalia gladiata</i>	U.D.	U.D.	1.31	0.003
	DC.				
	<i>Triticum aestivum</i> L.	4.81	0.48	1.54	1.14
	<i>Sorghum nervosum</i>	8.49	1.18	0.56	0.01
	Bess				
Poaceae	<i>Zea mays</i> L.	5.71	1.86	0.21	0.04
	<i>Oryza Sativa</i> L	18.31	3.89	0.46	0.02
	<i>Lagurus ovatus</i> L.	27.90	3.45	17.67	1.98
	<i>Cymbopogon citratus</i>	30.86	15.60	0.23	0.01
	<i>Capsicum annuum</i>	7.92	1.12	3.68	0.20
	L.Var <i>angulosum</i>				



	<i>Nicotiana tabacum</i> L.	3.28	0.30	0.58	0.10
<i>Solanaceae</i>	<i>Lycopersicon</i> <i>esculentum</i> Mill.	16.71	1.93	U.D.	U.D.
	<i>Solanum melongena</i>	38.60	9.28	0.27	0.02
	<i>Capsicum annuum</i> L.	49.31	5.44	1.76	0.57
	<i>Physalis alkekengi</i> <i>L.val.francheti</i> <i>Makino.</i>	23.66	1.99	9.82	1.34
	<i>Lemna minor</i> L.	U.D.	U.D.	0.23	0.01
	<i>Sesamum indicum</i> L.	22.36	3.78	1.14	0.12
<i>Others</i>	<i>Ricinus communis</i> L.	4.30	0.65	6.88	0.96
	<i>Gossypium</i> <i>indicum</i> L.	5.11	0.46	7.80	0.74
	<i>Daucus carota</i> L.	16.73	4.71	0.08	0.27
	<i>Helianthus annuus</i> L.	6.30	1.78	0.12	0.04
	<i>Echinodorus</i> <i>amazonicus</i>	13.15	3.32	0.02	0.01
	<i>Eichhornia crassipes</i> <i>Solems-Laub.</i>	9.11	1.93	U.D.	U.D.

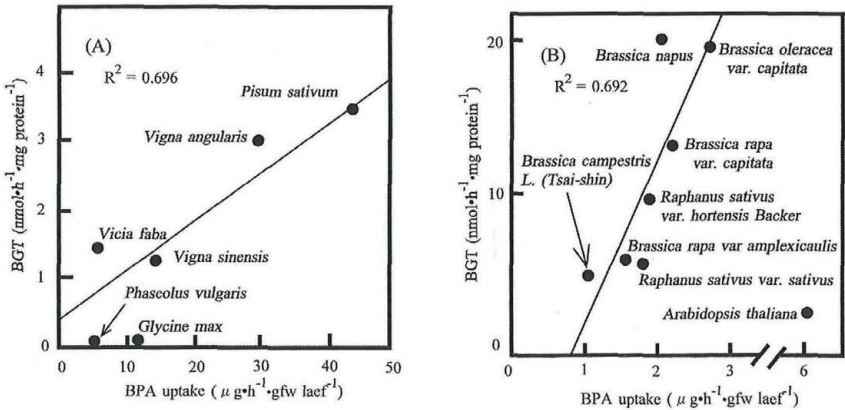


Fig. 1. Correlation between BPA uptake and BGT activity in leaves of *Fabaceae* (A) and *Brassicaceae* (B).

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