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Glycosylation of Tetrabromobisphenol A in Pumpkin

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Abstract

Tetrabromobisphenol A (TBBPA) is the most widely used brominated flame retardant (BFR), and it bioaccumulates throughout the food chains. Its fate in the first trophic level, plants, is of special interest. In this study, a four-day hydroponic exposure of TBBPA at a concentration of 1 $\mu\text{mol L}^{-1}$ to pumpkin seedlings was conducted. A nontarget screening method for hydrophilic bromine-containing metabolites was modified, based on both typical isotope patterns of bromine and mass defect, and used to process mass spectra data. A total of 20 glycosylation and malonyl glycosylation metabolites were found for TBBPA in the pumpkin plants. Representative glycosyl TBBPA reference standards were synthesized to evaluate the contribution of this glycosylation process. Approximately 86% of parent TBBPA was metabolized to form those 20 glycosyl TBBPAs, showing that glycosylation was the most dominant metabolism pathway for TBBPA in pumpkin at the tested exposure concentration.

Graphical Abstract

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.9b02122.

Synthetic routes of five glycosyl TBBPA standards; NMR and MS² of five glycosyl TBBPA standards; algorithm of *findbr*; additional figures and tables (PDF)

The authors declare no competing financial interest.

1. INTRODUCTION

Tetrabromobisphenol A (TBBPA) is the most widely used brominated flame retardant all over the world.¹ Its annual application was estimated to be 6.43×10^6 tons just in China in 2017.² TBBPA was frequently detected in various environmental matrices.^{3–5} The concentrations of TBBPA were 4870 ng L^{-1} in Lake Chaohu, China, with multiple polluted rivers' inflow,⁶ and 2900 ng g^{-1} in the surface soil around an e-waste recycle workshop in northern Vietnam.⁷ Because of its high lipophilicity ($\log K_{ow} = 4.5\text{--}6.5$) and long half-life in soil (14.7–430 days) both in oxic and anoxic conditions,^{8,9} TBBPA bioaccumulates in the environment and can be transported through food chains from plants to animals and humans.^{5,8,10} TBBPA has been demonstrated to be an endocrine disrupter and can cause cytotoxicity and neural development toxicity.^{11–13} To understand its biogeochemical cycling, environmental risks, and fate, the transformation and metabolism of TBBPA in plants is very important.¹⁴

Pumpkin is a promising model plant to investigate the metabolism of halogenated organic compounds based on its ability to accumulate and transform DDT, PCBs, SCCPs, and PBDEs.^{15–19} Generally, exogenous organic pollutants undergo multiple metabolic pathways which are divided into three phases, Phase I, Phase II, and Phase III. Our previous study had shown that pumpkin plants could metabolize TBBPA to methylated and carbon chain cleaved metabolites, including TBBPA monomethyl ether (TBBPA MME), TBBPA dimethyl ether (TBBPA DME), and two single benzene ring metabolites through the phase I and II reactions. However, only 3.8% of parent TBBPA was transformed to those four metabolites.²⁰ A large amount of TBBPA was believed to be metabolized through other pathways. It was reported that TBBPA was sulfated and conjugated with glucuronide(s) in rats and conjugated with glucoside(s) in microalgae.^{21,22} Whereas a lack of research on both comprehensive metabolic pathways and quantitative evaluation of biotransformation makes the metabolism of TBBPA in organisms difficult to predict.²³ To better elucidate the unknown parallel metabolic reactions in plants, hydroponic exposure experiments using pumpkin with a relatively high TBBPA concentration of $1 \text{ } \mu\text{mol L}^{-1}$ were conducted in this research. Hydrophilic bromine-containing metabolites were the special focus of this research using liquid chromatography coupled with high resolution mass spectrometry (LC-HRMS). To minimize the effects of the intricate mass spectra of plant matrices on the determination

of trace metabolites, and to discover as many metabolites as possible, a sensitive and effective screening method was necessary.

A nontarget screening method which utilizes both the isotope pattern of bromine atoms and the mass defect (MD) was developed for recognition of all possible bromine-containing organic compounds.^{24,25} MD is the difference between the detected exact mass and the nominal mass of a fragment ion peak in the mass spectrum. The pattern of the MS isotope peak clusters was used to determine the number of bromine atoms of a bromine-containing compound based on the natural abundance ratio of the two bromine isotopes ⁷⁹Br and ⁸¹Br (close to 1:1). This method has been successfully applied to screen a total of 33 brominated compounds in plastic casings of electric/electronic devices even though some were at very low concentrations.²⁴

In this study, the method was modified with a script written in R language and applied to recognize potential metabolites. A total of 20 glycosylation and malonyl glycosylation metabolites of TBBPA were found for the first time in plants. Five reference standards of representative glycosyl TBBPAs were synthesized to further confirm the results. This is also the first quantitative estimation on the significance and importance of the glycosylation pathway for TBBPA metabolism in plants.

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents.

Tetrabromobisphenol A (TBBPA, solid, 0.25 g, 99%) was obtained from Dr. Ehrenstorfer GmbH (Augsburg, GER). The stock and working solutions of TBBPA were all dissolved in methanol. The working solution was diluted from stock solution (20 mmol L⁻¹) to the concentration of 2 mmol L⁻¹. Tetrachlorobisphenol A (TCBPA, 25 g, >98%), as the injection standard, was purchased from Tokyo Chemical Industry CO., Ltd. (Kitaku, Tokyo, Japan). Acetic acid and ammonia solutions were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). The formic acid (HPLC grade, >99%) was purchased from Dikma Technologies Inc. (CA, USA). Methanol (HPLC grade) was obtained from J.T. Baker (Philipsburg, USA). Ultrapure water (18.2 MΩ cm⁻¹) used in experiments was produced by a Milli-Q advantage A10 system (Billerica, USA). Cleanert polar enhanced polymer (PEP) cartridges (500 mg/6 mL) were purchased from Bonna-Agela Technologies (Tianjin, China).

2.2. Synthesis of Glycosyl TBBPAs.

According to the preliminary metabolites determination results, five representative glycosyl TBBPAs were synthesized as the reference standards to further confirm and quantify the glycosylation metabolites. The five standards have different typical conjugate structures, including (1) TBBPA mono-β-D-glucopyranoside (TBBPA MG, 99%), (2) TBBPA mono-β-D-(6-O-malonyl)-glucopyranoside (TBBPA MMG, 90%), (3) TBBPA di-β-D-glucopyranoside (TBBPA DG, 99%), (4) TBBPA mono-β-D-glucopyranosyl-(6-O-malonyl)-glucopyranoside (TBBPA MGMG, 90%), and (5) TBBPA di-β-D-(6-O-malonyl)-

glucopyranoside (TBBPA DMG, 80%). Their synthetic routes are shown in the Supporting Information, section S1. The NMR and MS information are shown in section S2.

2.3. Hydroponic Exposure.

Seeds of pumpkin (*Cucurbita maxima* × *C. moschata*, Taigu Yinong Seed Co., Ltd., Shanxi province, China) were immersed in pure water for 8 h to accelerate germination and then germinated at a temperature of approximately 28 °C. After the roots grew to about 2 cm, the seedlings were transferred to a sterile perlite bed which was placed in an illumination growth chamber at 25 °C for 16 h light and 22 °C for 8 h dark. Four days later, the robust seedlings of similar height (4–5 cm) were transferred into 50 mL glass reactors for further cultivation for 2 days. Then the hydroponic exposure was conducted.

Four groups were set for the exposure experiment, including a planted exposure group, planted blank control group, unplanted exposure control group, and root exudate and microorganism control group. Each group consisted of three parallel reactors. The reactor of the planted exposure group contained three pumpkin seedlings and 40 mL of autoclaved pure water which was added with TBBPA to the initial concentration of 1 $\mu\text{mol L}^{-1}$. With only pure water, pumpkin seedlings, and the same volume of methanol (20 μL , without TBBPA) were added into the blank reactors. These planted blank controls were used to control the cross contamination between reactors caused by volatilization and phytovolatilization during the exposure experiment. The unplanted exposure control group was used to control the possible transformation processes caused by photolysis, chemical degradation, etc. Only 40 mL of pure water containing TBBPA was added, and no pumpkin seedlings were put into the unplanted exposure controls. Root exudates and microorganism controls were used to evaluate the contributions of enzyme and compounds excreted from roots and the microorganisms associated with pumpkin plants to the transformation of TBBPA. Preparation of the root exudate and microorganism control group was similar to that in our previous work and another study.^{26,27} Three pumpkin seedlings were cultivated in a reactor with 40 mL of autoclaved pure water for 4 d to obtain the solution of root exudates which also contained the microorganism associated with pumpkin roots. After the pumpkin plants were removed, a TBBPA standard was added into the left solution and the solution was replenished with autoclaved pure water to 40 mL. These reactors then acted as the root exudate and microorganism controls. The exposure experiments were conducted in an exposure chamber which was set at the same environmental conditions as the growth chamber.

2.4. Sampling and Sample Preparation.

After a four-day exposure, the roots, stems and leaves of the pumpkin seedlings as well as the corresponding solution in each reactor were collected. This sampling time was set according to our preliminary experiments in which most of the metabolites showed relatively high peaks at the fourth day. The hydroponic solution was immediately extracted and purified by PEP cartridges after sampling.

All the plant tissues were gently washed by pure water, freeze-dried, and then stored at –20 °C for further analysis. The root and stem (0.04–0.05 g dry weight (dw)) and leaf (0.17–

0.25 g dw) samples were put into 2 mL centrifuge tubes with small steel balls (4 mm in diameter). The centrifuge tube was shaken at 30 Hz for 2 min on a Tissuelyser II (QIAGEN, Hilden, Germany) with 1 mL methanol as the extraction solvent. Then the supernatant was transferred into a clean 10 mL glass tube after centrifugation at 10000 rpm for 5 min. The extraction process was conducted three times, the supernatant was combined and vaporized to dryness by a gentle flow of nitrogen, redissolved in 5 mL of water containing 5% methanol and loaded onto a PEP cartridge for purification.

The PEP SPE columns were activated by 5 mL of methanol and 5 mL of water in sequence before use. After the samples were loaded, the columns were rinsed by 5 mL of water with 5% methanol and 2% acetic acid, and 5 mL of water with 5% methanol and 2% ammonia in sequence, then eluted by 5 mL of methanol with 2% ammonia and 8% water, the eluates were vaporized to dryness and solvent exchanged with methanol, and added with 40 pmol TCBPA for instrumental analysis.

2.5. Instrumental Analysis.

The metabolites whose reference standards were synthesized were quantified by an Agilent 1290 Series LC system coupled with an Agilent 6460 Triple Quadrupole MS/MS system (LC–MS/MS) with an electron spray ionization source (ESI) using a ZORBAX ODS column (150 mm × 3 mm × 5 μ m, Agilent Technologies). Gradient elution mode was used with the water (Solvent A) and the methanol containing 1% formic acid (Solvent B) as the mobile phases. The initial ratio of A/B was set at 70:30 and held for 1 min, the ratio was changed to 25:75 within 4 min, then to 100% solvent B within 6 min, and finally back to the initial ratio within 1 min and held for 4 min. The flow rate was 0.5 mL min⁻¹. The injection volume was 5 μ L and column temperature was 35 °C. Multiple reaction monitoring (MRM) mode was used. The detailed parameters of the MS/MS system and MRM mode are shown in Tables S1 and S2.

Other glycosylation metabolites of TBBPA were identified and approximately quantified by UltiMate 3000 LC-Orbitrap Tribrid HRMS (Thermo Scientific, Waltham, MA) coupled with negative heated electrospray-ionization (H-ESI). The chromatographic conditions were the same as those of the Agilent 1290 Series LC system. The parameters of HRMS are shown in Table S3. The full scan mass ranged from 200 to 1200 m/z with a resolution of 120 000 (fwhm at 200 m/z). The automatic gain control (AGC) target was assigned a value of 2.0e⁵. For MS² scan, the collision energy was set at 10 eV in negative mode and the orbitrap resolution was set at 30000 fwhm, AGC target at 5.0e⁴.

2.6. Nontarget Screening Method.

According to the basic rules and working flow reported by Ballesteros-Gómez et al.,²⁴ a modified nontarget screening method was developed. Because of a lack of information on the script in that article, a new R script was established to distinguish the potential bromine-containing metabolites within a large number of chromatographic peaks obtained from UltiMate 3000 LC-Orbitrap Tribrid HRMS in this study. The processes were as follow: (1) The exported data file (.raw) was transformed into mzXML format by the MSConvert.²⁸ (2) The R script was run on RStudio (version 3.4.3). The function *getdata2* defined by

enviGCMS and xcms packages was used to translate the data (.mzXML) into XCMSnExp objects. The modified function *findbr*, as shown in section S3, was used to obtain the information on the exact mass values and retention times (RTs) of the fragment ions from the XCMSnExp objects. The MD values were also automatically calculated by the script using the following equations:^{24,25,29}

$$\text{scaled mass} = \text{exact mass} \times \text{scale factor}$$

$$\text{scaled MD} = \text{nominal scaled mass (rounded up)} - \text{scaled mass}$$

The scale factor is $-H/+Br$ (78/77.91051) which is commonly used for identification of Br containing compounds.^{24,30,31} Then the scaled MD plot was plotted using scaled MDs versus the scaled exact masses. (3) The potential TBBPA metabolites could be quickly and intuitively distinguished through the MD plot. According to the isotope pattern of bromine, the abundance ratio between the highest peak $I_{m/z}$ and the peak $I_{m/z+2}$ is lower than 100/49 for a brominated compound. Thus, taking the fluctuations of the instrument response into account, a threshold of 100/40 was set as one of the criteria to distinguish the metabolites. As shown in Figure 1, the data points fitting both of the following requirements would be the potential metabolites: (i) the isotope peak cluster at the same RT (with an error of 1 s) had a pair of peaks with m/z difference of 2.000 ± 0.003 ; (ii) this pair of peaks had similar MD values and their abundance ratio was lower than the threshold. (4) The molecular weights and compositions of the recognized brominated metabolites were then calculated based on the exact mass of a quasi-molecular ion using Xcalibur software (Thermo Scientific). The deviation between the m/z values of detection and calculation should be less than 5 ppm. The number of Br atoms in the calculated composition should be consistent with the isotope patterns. Their chemical structures were identified by typical MS and MS² ions and the synthesized standards.

2.7. Quantitative Analysis of Glycosyl TBBPAs.

Quantification of glycosyl TBBPAs which have synthesized reference standards was carried out by comparison with external standard calibration curves. Glycosylation metabolites without reference standards were also approximately quantified using those synthesized standards with similar chemical structures. The response factors of compounds with similar chemical structures were generally considered equal or similar.^{27,32} The approximate quantification for other glycosyl TBBPAs was as follows. (1) Compounds with one glycosyl group were quasi-quantified by the standard of TBBPA MG. (2) Compounds with two glycosyl groups were quasi-quantified by the standard of TBBPA DG. (3) Compounds with one glycosyl group and one malonyl-glycosyl group were quasi-quantified by the standard of TBBPA MMG.

2.8. Quality Assurance and Quality Control (QA/QC).

The glassware was heated in a muffle furnace at 400 °C for 4 h before use. No TBBPA and concerned metabolites were found in the procedural blanks. Two solvent blanks were injected following every three samples to avoid instrumental residue between injections. The

spiked recoveries and method detection limits (MDLs) of synthesized glycosyl TBBPAs of water, root, stem, and leaf samples are shown in Tables S4 and S5. All of the reported data were corrected by corresponding spiked recoveries.

3. RESULTS AND DISCUSSION

3.1. Metabolites of TBBPA.

The TBBPA metabolites in various pumpkin tissues were determined. A MD plot with all LC–HRMS data points obtained from a root sample is shown in Figure S1. There are thousands of raw data points. However, after the data are processed by the R script, as shown in Figure 1, only the screened potential metabolites remain. A cluster of data points represents a cluster of isotope peaks belonging to one or more compound(s). The isotope pattern of the peak cluster gives the information on bromine numbers. The exact mass of the quasi-molecular ion $[M-H]^-$ in negative ESI mode can give the possible molecular weight and composition. According to the bromine numbers, the molecular weight, and the typical ions, the parent TBBPA and the reported metabolite TBBPA MME were determined,²⁰ and a total of 20 unknown metabolites (M1–M16) were preliminarily inferred to be glycosyl TBBPAs. To confirm this result, five glycosyl TBBPA standards were synthesized and determined. Their ionization behavior (section S2) resulted in the formation of $[M-H]^-$ ions as well as other quasi-molecular ions such as $[M+Cl]^-$, $[M+NO_3]^-$, and $[M+HCOOH-H]^-$ of glycosyl TBBPAs under negative ESI mode. Similar MS behaviors of the unknown metabolites to the standards verified that these unknown metabolites were glycosylation products. The detailed MS information on glycosylation metabolites is listed in Table S6. Among the glycosylation metabolites marked with black circles in Figure 1, M1, M6, M8, and M10 all contained two isomers a and b.

The structures of these glycosylation metabolites of TBBPA were further characterized using their MS² spectra obtained from the quasi-molecular ion peak with the highest abundance. The results show that most glycosylation metabolites contained the product ion of m/z 542.74444 which represents the structure of TBBPA, indicating that these metabolites were conjugation products of TBBPA. Because of the consistency of chromatographic and mass spectrographic behaviors with the synthesized standards, four of the metabolites, M3, M4, M5, and M13, were confirmed as TBBPA MG, TBBPA MMG, TBBPA DG, and TBBPA DMG, respectively. Their confidence levels were level 1.³³ The MS² behaviors of the synthesized standards were also referenced to identify the structures of other glycosylation metabolites.

TBBPA MG has a glucopyranose substitution ($C_6H_{10}O_5$, 162.05273) on one of the benzene rings of TBBPA and shows a typical loss of a glycosyl group on MS² (Figure SS2–6). The dissociation behaviors of the product ions for M1a, M1b, and M2 (Figure S2) are similar to that of the TBBPA MG standard, showing that only one glycosyl group was lost. And the quasi-molecular ions ($[M-H]^-$) of the M1 (a and b) and M2 lost a pentose group ($C_5H_8O_4$, 132.04199) and a deoxyhexose group ($C_6H_{10}O_4$, 146.05762), respectively. Though the pentose and the deoxyhexose moieties could not be characterized using current data, several most likely candidates are supplied according to previous studies on glycosylation products for other phenolic compounds.^{34–41} The pentose moieties in M1a and M1b were inferred to

be apiose, arabinose, xylose, and so on; the deoxyhexose moiety in M2 were inferred to be rhamnose.

TBBPA DG has two glucose groups that bond to both benzene rings of TBBPA (both sides). Its MS² spectrum (Figure SS2–7) shows a neutral loss of a formic acid (HCOOH, 46.00537) from the most abundant quasi-molecular ion [M+HCOOH-H][−] and then sequential neutral losses of the two glucose groups (C₆H₁₀O₅, 162.05255). The neutral loss patterns of the quasi-molecular ion [M+HCOOH-H][−] of M6a, M6b (Figure S3a), and M7 (Figure S3b) are similar to that of TBBPA DG, with a neutral loss of HCOOH, and further losses of the two sugar groups. The two sugar groups of M6 isomers (a and b) are a hexose group and a pentose group. The two sugar groups of M7 are a hexose group and a deoxyhexose group, respectively.

As shown in Figure SS2–8 and Figure SS2–9, TBBPA MMG has one β-D-(6-*O*-malonyl)-glucopyranoside group (C₉H₁₂O₈, 248.05273) and TBBPA MGMG has one malonyl disaccharide group (C₁₅H₂₂O₁₃, 410.10790). Neutral losses of CO₂ (43.98901) and C₃H₂O₃ (86.00353) from [M-H][−] could be considered as the typical fragment ions for compounds containing a malonyl group. In Figure SS2–9, cleavage of the C–O bond between the two hexose groups of the disaccharide was not observed, consistent with the MS behaviors of the disaccharide-substituted metabolites of other organic compounds.^{38,41,42} M12 has the characteristic malonyl, glucopyranosyl, and malonyl-glucopyranosyl ions (Figure S4a), but it displayed different fragmentations from the disaccharide-substituted TBBPA MGMG. Depending on the collision differences between M12 and TBBPA MGMG shown in Figure SS2–9 and Figure S4a, M12 was believed to have two monosaccharide substitutions bonded to both of the benzene rings of TBBPA. It is TBBPA mono-β-D-glucopyranosidemono-β-D-(6-*O*-malonyl)-glucopyranoside (TBBPA MGGMG). The MS² spectra of M11 and M10 (Figure S4b,c) are similar to that of M12. However, M11 and M10 have neutral losses of deoxyhexose and pentose groups, respectively.

M14 and M15 both contain the same fragment ion as TBBPA MG ([M-H][−], 704.79498). M16 contains a fragment ion of 772.78241, which was probably a dehydrated ion of TBBPA MMG [M-H₂O-H][−]. Thus, M14, M15, and M16 were all glycosylation metabolites of TBBPA. However, their glycosyl substitutions were difficult to identify because of the limited MS and MS² information.

Besides the glycosyl TBBPAs, three glycosylation metabolites containing three bromine atoms were also found. The MS² spectrum of M9 (Figure S5) shows that the quasi-molecular ion [M+HCOOH-H][−] has a neutral loss of a formic acid (HCOOH) and sequential neutral losses of two hexose groups (C₆H₁₀O₅), similar to the MS² behaviors of TBBPA DG, indicating that M9 has two hexose groups like TBBPA DG. In addition, M9 shows the typical anion of tribromobisphenol A (TriBBPA) at *m/z* 462.83632,²² suggesting that M9 is the conjugation metabolites of TriBBPA. Therefore, M9 was finally inferred to be TriBBPA DG with confidence level 2b.³³ The MS² spectra of M8a and M8b show that [M-C₆H₁₀O₅-H][−] (640.88379) and [M-C₆H₁₀O₅-C₆H₁₀O₅-H][−] (478.83148) were generated with one or two hexose group(s) lost. Of the two ions, [M-C₆H₁₀O₅-C₆H₁₀O₅-H][−] is the anion of TriBBPA bonded with an oxygen atom. According to the RTs of M8a, M8b, and M9, the

polarity of M8a and M8b is lower than that of M9. Thus, the oxygen atom bonded with TriBBPA does not belong to a hydroxyl group. However, the detailed chemical structures of M8a and M8b could not be inferred based on the present data.

3.2. Possible Glycosylation Pathways.

Glycosylation metabolites of TBBPA were only found in the planted exposure systems, and they were not detected in parent TBBPA standards as impurities; neither were they detected in any of the control groups. No glycosyl TBBPAs were found in the exudate and microorganism controls illustrating that the microorganism and the root exudates could not metabolize TBBPA to glycosylation metabolites. Glycosyl TBBPAs were all transformed under the action of the pumpkin seedlings.

According to these qualification results, the glycosyl substitutions of the metabolites were bonded with an oxygen atom of the phenolic hydroxyl groups of TBBPA and TriBBPA through the O-1-glycosidic bond. The glycosylation metabolites of TBBPA were classified into seven categories. (1) glycosyl TBBPAs with a monosaccharide conjugated with one of the phenolic rings; (2) with a malonylated monosaccharide substitution; (3) with two monosaccharides conjugated with two phenolic rings, respectively; (4) glycosyl TBBPAs with less brominated glycosylation metabolites; (5) with a monosaccharide and a malonylated monosaccharide substitution on two phenolic groups, respectively; (6) the glycosylation metabolites with confidence level < 3; and (7) with two malonylated monosaccharides on two phenolic groups, respectively. Thus, the possible glycosylation pathways of TBBPA were proposed in Figure 2.

3.3. Distribution of the Glycosylation Metabolites.

TBBPA MG, TBBPA MMG, TBBPA DG, and TBBPA DMG were further quantitatively analyzed for the planted exposure systems (Table 1) using the synthesized standards. The four glycosylation metabolites were mainly found in the roots, only small amounts were detected in solutions and stems, and none of them were found in the leaves, indicating that the glycosylation metabolism mainly took place in roots. Because the root exudate did not metabolize the TBBPA to glycosyl TBBPAs, the glycosylation metabolites found in solutions were released from roots. As shown in Table 1, for metabolites of a glycosyl substitution conjugated with one benzene ring, TBBPA MG and TBBPA MMG were released into the hydroponic solution, and they accounted for 23.3% and 37.4% of their total amounts in the whole planted exposure group, much higher than that of TBBPA DG (0.8%) and TBBPA DMG (4.7%) of which the glycosyl substitutions conjugated with two benzene rings. Thus, single benzene ring-conjugated glycosylation metabolites were more easily released into solution.

TBBPA DMG was the most produced metabolite among those four glycosyl TBBPAs (Table 1). The malonyl glycosylation products of exogenous substances generally became more stable, water-soluble, and were more easily translocated into vacuoles than the parent compound,⁴² and were then stored as the malonyl glucopyranose after malonyl modification.^{43,44} However, its occurrence in solution suggested that special excretion pathways might

exist in pumpkin, consistent with the report on excretion of malonyl glycosylation products of naphthol from *Arabidopsis* and tobacco.⁴⁵

3.4. Contribution of the Glycosylation Metabolism.

At the end of exposure, the recoveries of parent TBBPA in the planted exposure group were only $17.3 \pm 3.7\%$. According to the results of planted blank controls (no TBBPA was detected in the end) and unplanted exposure controls (with good recovery of $98.4 \pm 2.1\%$), it was obvious that there was no cross-contamination and loss through photolysis, mineralization, or volatilization in the exposure systems. Therefore, a great deal of TBBPA was metabolized by the pumpkin plants. According to the equimolar transformation reaction from TBBPA to glycosyl TBBPA, transformed TBBPA was in the same molarity as that of produced glycosylation metabolites. The amounts of TBBPA that formed TBBPA MG, TBBPA MMG, TBBPA DG, and TBBPA DMG were 269 ± 74 , 3529 ± 868 , 2681 ± 766 , and 23952 ± 2922 pmol, respectively, totally accounting for $76.1 \pm 9.6\%$ of the initial exposed TBBPA (40000 pmol).

Other glycosylation metabolites were also approximately quantified. M1a, M1b, and M2 were quasi-quantified by the standard of TBBPA MG. M6a, M6b, M7, M8a, M8b, and M9 were quasi-quantified by the standard of TBBPA DG. M10a, M10b, M11, M12, M14, M15, and M16 were quantified by the standard of TBBPA MMG. The amounts of other glycosylation metabolites in the planted exposure system are shown in Table S7. The total amount of other glycosylation metabolites was 4105 ± 732 pmol.

Taking the quantified and approximately quantified results into account, the total transformed TBBPA accounted for $86.3 \pm 11.3\%$ of the exposed parent TBBPA. Besides the methylation and carbon chain cleavage metabolism pathways reported in our previous study,²⁰ glycosylation is also an important metabolism pathway for TBBPA in pumpkin. Though various glycosylation metabolites have been found for chlorophenols, triclosan and 2,4-dibromophenol in algae and carrot,^{40,46,47} this study was the first report on the quantitative assessment of the entire glycosylation process in plants.

Though a relatively high concentration than the environmental level was used in the hydroponic exposure, the results can reflect the environmental behavior of TBBPA to some extent. Generally, glycosylation is considered as a detoxic pathway for organic pollutants in organisms. The large amounts of glycosylation products of TBBPA emphasizes the important role of the glycosylation metabolic pathways of TBBPA in plants. Besides, the glycosylation of environmental pollutants such as TBBPA is an extra energy-consuming process for plants. Every formation of one *O*-1-glycosidic bond consumes at least the energy equivalent to the hydrolysis of adenosine triphosphate (ATP) to form adenosine diphosphate (ADP).⁴⁰ It was calculated that the pumpkin plants consumed an additional ~ 2.4 mol ATP to convert 1 mol TBBPA to the end product of TBBPA DMG found in this experiment. Thus, the energy consumption for the global pollutant TBBPA by glycosylation metabolism in plants is truly surprising and commands greater research attention. The possible further metabolism processes of those glycosylation metabolites require deeper studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Mass defect plot of a typical root sample after running with the R script. The quasi-molecular ion composition of TBBPA metabolites is marked. The data points circled by red and green ovals indicate that the compound contains four and three Br atoms, respectively.

Figure 2.

Proposed glycosylation pathways of TBBPA in pumpkin. Gly means glycosyl groups, including hexose, deoxyhexose, and pentose. Mal means a malonyl group.

Table 1.

Quantitative Analysis of TBBPA MG, TBBPA MMG, TBBPA DG, and TBBPA DMG in Different Compartments (Solution, Root, Stem and Leaf) of the Planted Exposure Group

compounds	solution (pmol)	root (pmol)	stem (pmol)	leaf (pmol)
TBBPA MG	64.0 ± 19.0	200.5 ± 63.6	5.13 ± 1.54	<MDL
TBBPA MMG	1319 ± 608	2156 ± 278	53.8 ± 7.0	<MDL
TBBPA DG	20.8 ± 2.8	2609 ± 773	51.2 ± 26.7	<MDL
TBBPA DMG	1125 ± 358	22202 ± 2800	624 ± 431	<MDL