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ARTICLE

Application of Separated Leaf Cell Suspension to Xenobiotic Metabolism in Plant

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Metabolic profiles of ^{14}C-labeled primary metabolites from several pesticides, 4-cyanophenol (**1**), 3-phenoxybenzoic acid (**2**), 3-phenoxybenzyl alcohol (**3**), 3,5-dichloroaniline (**4**), and (1*RS*)-*trans*-2,2-dimethyl-3-(2-methylprop-1-enyl)cyclopropanecarboxylic acid (**5**), were examined by using enzymatically separated leaf cell suspension from seedlings of cabbage (*Brassica oleracea*) and tomato (*Lycopersicon esculentum*). After 1 day of incubation, the metabolites were extensively transformed in cabbage, whereas they were scarcely metabolized in tomato. The major metabolic pathways were the phase II reactions leading to a number of conjugates such as glucoside/malonylglucoside of **1**−**5**, malate of **2**, and glutamate of **4**. The oxidation of **1** and **2** was observed as a minor reaction to produce 4-hydroxybezoic acid and 3-(4-hydroxyphenoxy)benzoic acid. The chemical identities of the secondary metabolites were determined by various spectrometric analyses (LC-MS, LC-MS/MS, and NMR) and/or HPLC cochromatography with the synthetic reference standards. As a result, this separated leaf cell suspension system was found to well reproduce the in vivo plant metabolism.

KEYWORDS: Metabolism in cabbage and tomato; separated leaf cell suspension; phase II metabolism

INTRODUCTION

Numerous investigations on the metabolism of xenobiotics using plant tissue cultures such as suspension culture cells and callus have been conducted during the past 40 years, and considerable knowledge in this field has been accumulated (*1*−*6*). These research efforts have revealed that the transformation of xenobiotics in plant tissue cultures is qualitatively similar to but quantitatively different from in vivo metabolism. Typical reactions observed in these methods are oxidation at both aliphatic and aromatic carbons, hydrolysis by enzymes such as amidases and esterases, dechlorination, hydrogenation, and conjugation with carbohydrates, amino acids, and other organic acids. Although the validity of the plant tissue cultures is well-demonstrated, it is recognized that no single in vitro system is sufficient to fully reflect in vivo metabolism (*1*). First, an argument remains that the metabolic reactions in heterotrophic cells, that is, meristem, and those in phototrophic cells are not identical (*1*), because the operating enzymatic systems are not likely to be the same between two different types of cells in nature. Second, development and maintenance of plant tissue cultures require time and labor, which makes it difficult to keep several kinds of crops simultaneously. This disadvantage will lead to some difficulties in conducting comparative research on metabolic features among different plant species. To cope with these issues, the separated leaf cell suspension is considered to be a promising methods. In this method the mesophyll and palisade cells from leaves are separated to give a homogeneous mixture that maintains the metabolic functions of leaf and can be manipulated as a unicellular form. This is unlike the technique involving leaf disks or sections in which cells along the edges are exposed preferentially to a chemical but the individual cells in suspension alternatively permit an equal exposure to a chemical and, therefore, improvement on uptake and transformation rates is expected. Furthermore, this method can be theoretically applied to all kinds of plant species in a relatively simple manner because it requires only seedlings of the plant as a starting material. This simplicity in construction of the test system facilitates the comparison of metabolic pathways among the different crops. Techniques for the isolation of separated mesophyll cells from higher plants have been investigated for various purposes (*7*−*10*). Isolation of metabolically active cells constitutes a useful experimental system for biochemical, physiological, and cytochemical studies of cellular processes in plants. For example, it has been shown that these cells are capable of performing processes such as photosynthesis, respiration, and syntheses of RNA, protein, and lipid (*10*−*14*). However, sufficient investigation has not been conducted to examine the metabolic features of xenobiotics using this method (*15*). From this standpoint, we have conducted metabolism studies using the separated leaf cell suspension of cabbage (*Brassica oleracea*) and tomato (*Lycopersicon esculentum*) with phenolic, alcoholic, anilinic, and carboxylic compounds, which are the primary metabolites from various kinds of agrochemicals.

MATERIALS AND METHODS

Chemicals. The ^{14}C labels (**Figure 1**) of 4-cyanophenol (**1**), 3-phenoxybenzoic acid (**2**), 3-phenoxybenzyl alcohol (**3**), 3,5-dichloroaniline (**4**), and (1*RS*)-*trans*-2,2-dimethyl-3-(2-methylprop-1-enyl)cyclopropanecarboxylic acid (**5**) were prepared in our laboratory according to the reported

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methods (*16*−*19*). Compounds **1**−**4** were uniformly labeled at the phenyl ring (*phenyl*-^{14}C-**1** and -**4** and *phenoxyphenyl*-^{14}C-**2** and -**3**), and **5** was labeled at the 1-position of the cyclopropane ring (*cyclopropyl*-1-^{14}C-**5**). The specific activities were 0.82 (**1**), 2.45 (**2**), 2.25 (**3**), 2.22 (**4**), and 0.98 (**5**) GBq/mmol, and their radiochemical purity was $>$97% as determined by high-performance liquid chromatography (HPLC). The non-radiolabeled authentic standards, **1** and **4**, were purchased from Sigma-Aldrich Co. (Milwaukee, WI) and **2**, **3**, and **5** were synthesized in a similar manner to the corresponding radiolabels. The reference standards of 4-hydroxybenzoic acid (**7**) and 3-(4-hydroxyphenoxy)benzoic acid (**10**) were synthesized following the described methods (*20*). The glucose conjugates of **1** (**6**) and **4** (**13**) were synthesized by modifying the procedures reported by Sinnott et al. and Mitts et al. (*21*, *22*), and the malate conjugate of **2** (**9**) was synthesized according to the method reported in our recent paper (*23*). The chemical structures of **6**, **7**, **9**, **10**, and **13** (see **Figure 6**) were confirmed by ^{1}H NMR (δ_H vs TMSP, ppm) and LC-ESI-MS (*m*/*z*) spectrometries, as listed in **Table 1**.

Macerozyme R-10 and Murashige−Skoog medium were purchased from Yakult Biochemicals Co., Ltd. (Nishinomiya, Japan) and Wako Pure Chemical Industries Ltd. (Osaka, Japan), respectively. Other reagents were of the purest grade commercially available.

Spectroscopy. ^{1}H NMR spectra were measured with a Varian Unity 400 FT-NMR spectrometer operating at 400.45 MHz with a 5 mm PFG ATB probe, using trimethylsilylpropionate-2,2,3,3-d_4 (TMSP) as an internal standard (δ = 0.0 ppm). Liquid chromatography−atmospheric pressure chemical ionization-mass spectrometry (LC-APCI-MS) and liquid chromatography−electrospray ionization-mass spectrometry (LC-ESI-MS) in positive and negative ion modes were simultaneously performed using a Waters Micromass ZQ spectrometer equipped with Waters Separation Module 2695 and Photodiode Array Detector 2996 as liquid chromatograph. Liquid chromatography−electrospray ionization−tandem mass spectrometry (LC-ESI-MS/MS) in both ion modes with a collision energy of 10−40 V was conducted using a Thermo Finnigan TSQuantum attached to an Agilent 1100 series liquid chromatograph. Samples dissolved in methanol were manually injected into an ionization source through Sumipax ODS A-212 column (150 mm × 6 mm i.d., 5 μm, SCAS Co., Ltd.) with a flow rate of 1.0 mL min^{-1} using the gradient system with acetonitrile (solvent A) and 0.1% formic acid in water (solvent B). The composition of the mobile phase was changed stepwise as follows: 0 min, %A−%B, 10−90; 40 min, %A−%B, 90−10 (method A).

Radioassay. Radioactivity in the plant cell extracts and culture medium was determined by liquid scintillation counting (LSC). An aliquot of the samples was mixed with 10 mL of Packard Scintillator Plus and counted on a Packard model 2900TR liquid scintillation counter equipped with an automatic external standard. The average background of the LSC instrument was 30 dpm, which was subtracted from the measured sample dpm. Radioactivity in the unextractable residues from the treated plants was measured by using a Packard model 307 sample oxidizer. The unextractable plant residues were air-dried at room temperature overnight, weighed with a Mettler model AE240, and subjected to combustion. The $^{14}CO_2$ produced was absorbed into 9 mL of Packard Carb-CO_2 absorber and mixed with 15 mL of Packard Permafluor scintillator, and the radioactivity was quantified by LSC. The efficiency of combustion was determined to be $>$90%.

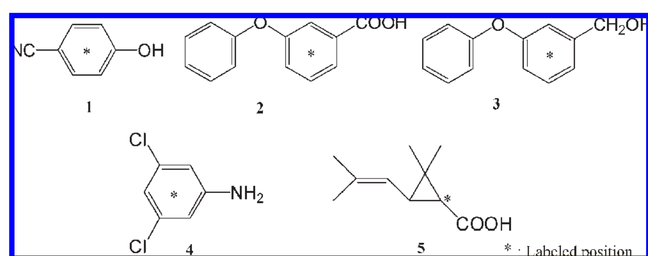

Figure 1. Chemical structures of ^{14}C-labeled **1**−**5**.

Chromatography. HPLC was carried out using a Shimadzu LC-20AT pump linked in series with a SPD-20A UV−vis detector and a Perkin-Elmer Radiomatic 610TR radiodetector equipped with a 500 μL liquid cell. Ultima-Flo AP (Packard Instrument Co.) was utilized as a scintillator. A Sumipax ODS A-212 column was employed for both analytical and preparative purposes at a flow rate of 1.0 mL min^{-1}. The following gradient systems were used for the typical analysis, separation, and purification of each metabolite: 0.01% trifluoroacetic acid (solvent A) and acetonitrile (solvent B), 0 min, %A−%B, 90−10; 40 min, %A−%B, 10−90; 40.1 min, %A−%B, 0−100; 50 min, %A−%B, 0−100 (method B); 1/33 M phosphate buffer (solvent A) and acetonitrile (solvent B), 0 min, %A−%B, 90−10; 40 min, %A−%B, 10−90 (method C). Retention times (min) of **1**−**15** in the method B were as follows: 19.8 (**1**), 30.1 (**2**), 28.5 (**3**), 30.8 (**4**), 28.9 (**5**), 16.4 (**6**), 12.3 (**7**), 25.0 (**8**), 27.0 (**9**), 22.0 (**10**), 19.1 (**11**), 22.8 (**12**), 18.8 (**13**), 23.3 (**14**), and 23.5 (**15**).

Thin-layer chromatography (TLC) was conducted using silica gel 60 F_{254} thin-layer chromatoplates (20 × 20 cm, 0.25 mm thickness, Merck KGaA). The cochromatography of **7** and **9** isolated from the separated suspension cells with the respective reference standard was carried out with the solvent system of toluene/ethyl formate/formic acid, 5:7:1 (v/v/v). An autoradiogram was prepared by exposing a TLC plate to a BAS-IIIs Fuji imaging plate for several hours. The radioactivity on the imaging plate was detected by using a Typhoon (Amersham Bioscience Co., Ltd.), and the non-radiolabeled reference standard was detected by exposing the chromatoplate to ultraviolet light. The typical R_f values of **7** and **9** were 0.60 and 0.35, respectively.

Plant Material and Maintenance. Seeds of cabbage (*B. oleracea* var. Kinkei 201) and tomato (*L. esculentum* var. Patio) were purchased from Japan Agricultural Co-operatives (Kakogawa, Japan). The seeds were sown in 50 mL plastic pots containing a moist compost (Kureha Chemical Co., Ltd.) and grown until third- to fourth-leaf stage in a temperature-controlled cabinet at 25 °C for day temperature and 22 °C for night temperature. Light with an intensity of 19500 lx at the level of primary leaves was supplied by a combination of fluorescent and incandescent lights with a 16 h light and 8 h dark sequence. Irrigation was appropriately conducted until sampling for approximately a month. Five grams of third- and fourth-leaves in fresh weight were used for the experiments.

Separation of the Parenchyma Cells and Treatment. Sampled leaves were immersed in 80% ethanol (v/v) for 30 s, sterilized by dipping into a 0.5% sodium hypochlorite solution for 10 min, and rinsed three times with sterilized deionized water. The leaves were then transferred into a homogenizer cup filled with 120 mL of Murashige−Skoog medium, cut into small pieces by scissors, and homogenized for 1 min in an AM-8 Ace homogenizer (Nihon Seiki Co., Ltd.) at 10000 rpm and 0 °C. Prior to the experiment, the osmotic pressure and pH of the MS medium were adjusted

Table 1. Analytical Data for the Reference Standards

compound	MS (*m*/*z*)	^{1}H NMR (ppm)
6	282 $[M + H]^+$	7.21−7.95 (d, 4H, aromatic-*H*), 3.43−5.25 (m, 7H, sugar-*H*)
7	137 $[M - H]^-$ 93, 69	6.80−7.86 (d, 4H, aromatic-*H*)
9	329 $[M - H]^-$ 213	2.97 (dd, *J* = 4.0 Hz, 2H, CHC*H*$_2$), 5.57 (t, *J* = 4.0 Hz, 1H, C*H*CH$_2$), 7.02−7.79 (m, 9H, aromatic-*H*),
10	275 $[M + HCOO]^-$ 229 $[M - H]^-$	6.89−7.88 (m, 8H, aromatic-*H*)
13	324 $[M + H]^+$	6.68−6.78 (d, 3H, aromatic-*H*), 6.21 (brd, *J* = 1.8 Hz, 1H, N*H*), 3.27−4.67 (m, 7H, sugar-*H*)

to 0.3−0.4 M and 6.5, respectively, and the prepared medium was sterilized by autoclaving for 20 min at 120 °C with an SS-325 autoclave (Tomy Seiki Co., Ltd.). Twenty-milliliter portions of the leaf homogenate were divided into 100 mL Erlenmeyer flasks, and 1% of Macerozyme R-10 was added to each. The methanol dose solutions of ^{14}C-**1**−**5** were individually prepared by isotopically diluting them with corresponding non-radiolabeled reference standards to give the specific activity of approximately 4.3 kBq μg^{-1}. Each of the dose solution was spiked into the leaf suspension medium at the volume of 100 μL, which produced the final exposure concentration of 20 $\mu g\ mL^{-1}$, vacuum-infiltrated for 1 min and incubated in a BR-180LF Bioshaker (Taitec Corp.) at 120 rpm and 25 °C in dark. Sampling was conducted at 1, 2, and 4 days of exposure.

Extraction and Isolation of Metabolites. The incubated samples were first divided into leaf cells and culture medium by filtration through a filter paper (pore size = 7 μm) and were stored in a freezer (< −20 °C) before analyses. The leaf cells were extracted using an AM-8 Ace homogenizer at 10000 rpm for 10 min with acetone/water (4:1, v/v). The homogenate was filtered through a filter paper (pore size = 1 μm), and the residue on the filter was extracted two additional times in the same way. The culture medium was partitioned three times with a 3-fold volume of ethyl acetate. Aliquots of the plant extracts as well as the organic and water layers from culture medium were radioassayed by LSC. The plant residues were air-dried in open vessels at room temperature for 1 week, and subsamples of the dried residues were subjected to combustion analysis to determine the remaining radioactivity.

For the purpose of isolating unknown metabolites for spectroscopic analyses, the plant extracts and the organic layer of the culture medium were individually subjected to the preparative purification by a solid-phase absorbent Waters Sep-Pak C18 cartridge. First, the concentrated extracts or organic layer were reconstituted in 5 mL of deionized water, and the mixture was loaded onto the Sep-Pak cartridge. The cartridge was successively washed with 30 mL of water followed by 30 mL of methanol, and the water/methanol wash was repeated three times. From LSC and HPLC analyses, most of the unknown metabolites were eluted in the methanol fraction. Further purification of the methanol fraction was conducted with HPLC using methods B and C.

RESULTS

^{14}C Distribution in the Test System. The recoveries of [^{14}C]-**1**−**5** from the test system after 1 day of incubation were 78.8% (**1**), 82.3% (**2**), 79.8% (**3**), 80.6% (**4**), and 78.6 (**5**)% of the applied ^{14}C. The unrecovered ^{14}C increased as the exposure period was extended, possibly due to azeotropic vaporization (data not shown). There were insignificant differences in the loss of ^{14}C from the test system among the test substances, although the Henry's law constants of **1**−**5** estimated by the EPI-Suite software (*24*) greatly varied by 3 orders (atm m^3 mol^{-1}): 5.42×10^{-9} (**1**), 2.37×10^{-9} (**2**), 4.75×10^{-9} (**3**), 1.05×10^{-6} (**4**), and 2.42×10^{-6} (**5**). The distribution of ^{14}C within the test system after 1 day of exposure was examined in duplicate, and the result is shown in **Table 2**. The radioactivity taken up by cabbage leaf cell was estimated to be 36.2% TRR(**1**), 46.3% TRR(**2**), 35.9% TRR(**3**), 50.8% TRR(**4**), and 47.2% TRR(**5**) by combining extractable and unextractable ^{14}C. For tomato leaf cell, an increase in rate of the ^{14}C taken up was observed compared to the one in cabbage, as 81.1% TRR (**1**), 79.7% TRR (**2**), 91.3% TRR (**3**), 84.3% TRR (**4**), and 86.6% TRR (**5**). The unextractable ^{14}C for **1**−**5** amounted to 3.0−5.4% TRR in cabbage and 2.4−6.9% TRR in tomato.

Distribution of Metabolites. The formation and decline of metabolites were examined in detail for [^{14}C]-**5** using the cabbage leaf cell suspension with exposure periods of 1, 2, and 4 days. The metabolic distribution reached a plateau within 1 day, and approximately 100% of the radioactivity detected in the cabbage leaf cell was the malonylglucose conjugate of **5** (**15**). On the basis of these data, the incubation period in the study examining metabolic profiles of [^{14}C]-**1**−**5** was determined to be 1 day after treatment.

Table 2. ^{14}C Distribution of **1**−**5** in the Cell Suspension System

	% TRR				
	1	**2**	**3**	**4**	**5**
			Cabbage		
leaf cell ^{14}C					
extractable	30.8	42.4	32.3	47.8	43.3
unextractable	5.4	3.9	3.6	3.0	3.9
medium ^{14}C	63.8	53.7	64.1	49.2	52.8
			Tomato		
leaf cell ^{14}C					
extractable	78.4	74.5	84.5	79.0	81.9
unextractable	2.4	5.2	6.9	5.3	4.7
medium ^{14}C	18.9	20.3	8.7	15.7	13.4

Table 3. Metabolic Distribution of **1**−**5** in the Cell Suspension System

		% TRR	
compound	metabolite	cabbage	tomato
1	leaf extract	30.8	78.4
	1	3.2	72.3
	6	27.6	6.1
	medium	63.8	18.9
	1	24.4	15.9
	6	9.4	3.0
	7	7.4	nd
2	leaf extract	42.4	74.5
	2	13.4	72.8
	8	6.6	1.7
	9	18.4	nd
	10	2.4	nd
	medium	53.7	20.3
	2	10.7	19.8
	8	34.3	0.5
	9	7.6	nd
	10	0.8	nd
3	leaf extract	32.3	84.5
	3	nd	84.5
	11	4.6	nd
	12	27.7	nd
	medium	64.1	8.7
	3	64.1	8.7
4	leaf extract	47.8	79.0
	4	nd	63.9
	13	23.7	15.1
	14	16.3	nd
	medium	49.2	15.7
	4	49.2	15.7
5	leaf extract	43.3	81.9
	5	nd	81.9
	15	43.3	nd
	medium	52.8	13.4
	5	52.8	13.4

The metabolic distribution in the extract of leaf cells is shown in **Table 3**. In general, the test compounds were extensively metabolized in the leaf cells of cabbage as compared with tomato cells. In cabbage, **6** was the only metabolite from **1** and amounted to 27.6% TRR with the remaining radioactivity (3.2% TRR) being unchanged **1**. Both **3** and **4** were fully transformed within 1 day of exposure as none of the corresponding test compounds remained in the extract of the leaf cells. The metabolites of **3** were glucoside

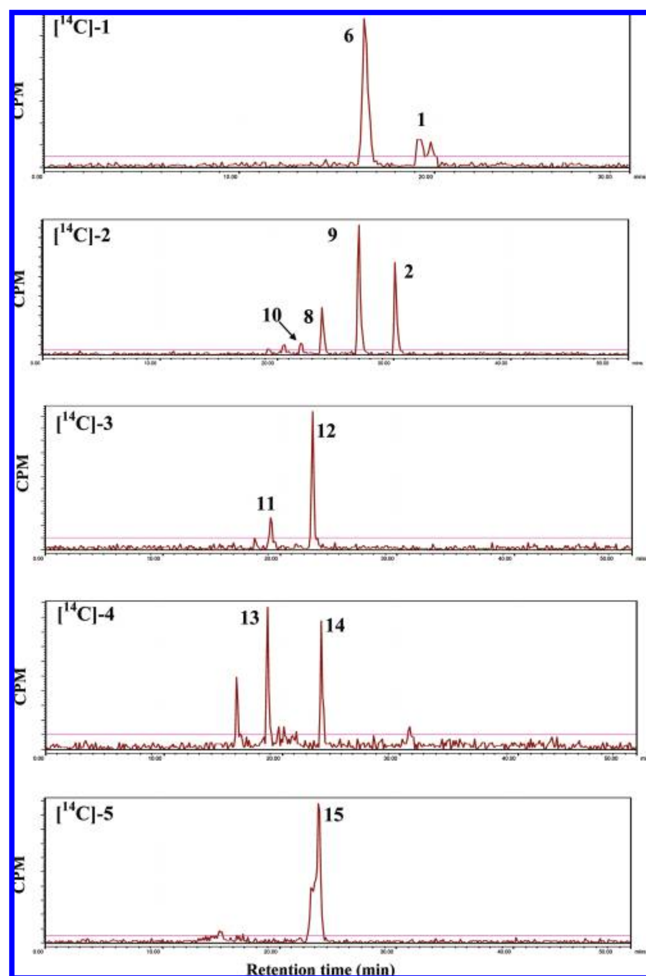

Figure 2. Representative HPLC chromatograms of cabbage leaf extracts.

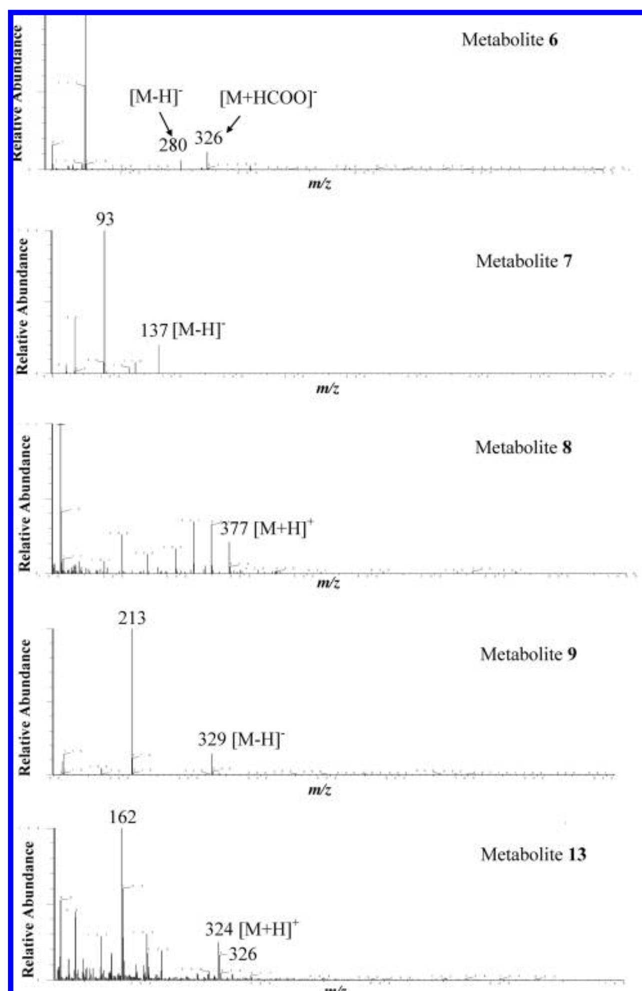

Figure 3. Representative LC-MS spectra of metabolites.

(**11**) and malonylglucoside (**12**) conjugates, each at 4.6 and 27.7% TRR, and **4** was metabolized to glucoside (**13**) and glutamate (**14**) conjugates at 23.7 and 16.3% TRR, respectively. In the case of **2** and **5**, differences were observed in the metabolic profiles, such as **2** being transformed to three major metabolites, glucoside (**8**) and malate (**9**) conjugates, and a hydroxylated free form (**10**) at 6.6, 18.4, and 2.4% TRR, respectively, whereas **5** was solely metabolized to its malonylglucoside conjugate (**15**) at 43.3% TRR. The representative HPLC chromatograms of the leaf cell extracts are shown in **Figure 2**. Some of the metabolites derived from **1** and **2** were detected in the culture medium. The metabolic distribution of **1** in the medium of cabbage was 9.4% TRR for **6** and 7.4% TRR for **7**. In the case of **2**, metabolites **8**, **9**, and **10** were detected in the cabbage culture medium at 34.3, 7.6, and 0.8% TRR, respectively (**Table 3**). With respect to tomato plant, **1**, **2**, and **4** were transformed to their corresponding glucose conjugates at the levels of 9.1% TRR (**6**), 2.2% TRR (**8**), and 15.1% TRR (**13**), respectively, within the total test system. No transformation products were observed for **3** or **5**.

Identification of Metabolites. For the phenolic compound **1**, the chemical structure of metabolite **6** was tentatively confirmed by spectrometric analyses. LC-APCI-MS and LC-ESI-MS in negative ion mode clarified the molecular weight of **6** as 280 ($[M-H]^-$), which showed a 162 amu increase from that of **1** (**Figure 3**). ^{1}H NMR analysis of isolated **6** had shown a number of protons detected at 3.38−5.18 ppm specifically corresponding to the signals derived from carbohydrate (**Figure 5**). The chemical structure of **6** was finally confirmed by conducting HPLC cochromatography with the reference standard. LC-MS analyses of **7** showed the pseudoion at 137 ($[M-H]^-$) (**Figure 3**). Comparison of ^{1}H NMR spectra of **1** and **7** suggested no substitutional change at the phenyl ring (**Figure 5**). The HPLC and TLC cochromatographies revealed the definitive chemical structure of **7** as 4-hydroxybenzoic acid.

The benzoic acid derivative **2** was found to undergo three metabolic transformations. LC-APCI-MS and LC-ESI-MS analyses of **8** in positive and negative ion modes showed the molecular weight of 376, suggesting the glucose conjugate of **2** (**Figure 3**). The existence of monosaccharide was also confirmed by ^{1}H NMR spectrum of **8**, having glucose proton signals at 3.50−5.29 ppm (**Figure 5**). The major pathway was formation of the metabolite **9**, which was subjected to LC-APCI-MS and LC-ESI-MS analyses in both ion modes. The molecular weight of **9** isolated from the culture medium was estimated to be 330, by the negative-ion LC-ESI-MS peak. The pseudoion was observed at 329 $[M-H]^-$ together with the daughter one corresponding to $[M - \text{malate} - H]^-$ at m/z 213 (**Figure 3**). The ^{1}H NMR spectrum showed the presence of nine aromatic protons at 7.05−7.86 ppm, very similar to those of **2**, indicating that no structural change occurred in the phenoxyphenyl moiety (**Figure 5**). The chemical identity of **9** was finally confirmed by both HPLC and TLC cochromatographies with the reference standard of malate conjugate of **2**. The chemical structure of **10** was determined as 3-(4-hydroxyphenoxy)benzoic acid both from the molecular weight of 230 in LC-MS and by HPLC cochromatography with its reference standard.

In the case of **3**, the major pathway was malonylglucosyl conjugation, which has been reported for metabolites of various

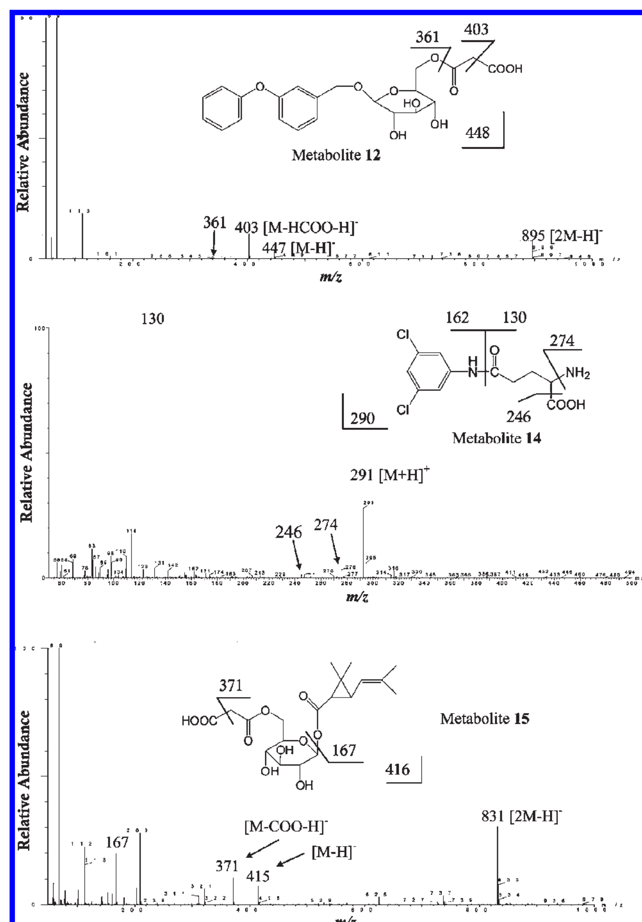

Figure 4. MS fragment patterns of metabolites **12**, **14**, and **15**.

pesticides. The LC-APCI-MS and LC-ESI-MS analyses of the isolated **12** in negative ion mode showed the m/z value of a parent ion at 447. The profiles of the daughter ions corresponding to $[M - COOH]^-$ at m/z 403 and $[M - \text{malonate} - H]^-$ at m/z 361 (**Figure 4**) were compared in detail with those of the phenol metabolite from tolclofos-methyl previously reported (*25*). The chemical structure of the minor component **11** was determined as the *O*-glucoside conjugate of **3** by the molecular weight of 363 $[M + \text{glucoside} + H]^+$ in LC-MS.

For the anilinic derivative **4**, the *N*-glucoside **13** was formed as a major metabolite. LC-ESI-MS in positive ion mode clarified the molecular weight to be 323 with a daughter ion at 162 corresponding to monosaccharide (**Figure 3**). The chemical structure of **13** was also confirmed by HPLC cochromatography with the synthetic standard. To examine the chemical identity of **14**, LC-APCI-MS and LC-ESI-MS analyses were first conducted in both ion modes. The molecular weight of **14** was estimated to be 291 ($[M + H]^+$) in positive ESI ion mode. From an increase of the m/z value by 129 amu as compared to **4**, glutamic acid was considered to attach to the amino group of **4**. In the thorough examination by LC-MS/MS analysis, daughter ions were detected at m/z 274, 245, and 130, assigned as successive dissociation of an amino group, a carboxyl group, and a glutamate structure (**Figure 4**), which are totally consistent with the observation by Mutlib et al. (*26*).

In the case of **5**, the only metabolite detected was **15**. The typical fragment profile of a malonylglucose conjugate was observed in LC-MS analysis; that is, the parent ion at 415 $[M-H]^-$ with the daughter ions corresponding to $[M-COOH]^-$ at m/z 371 and $[M - \text{malonylglucose} - H]^-$ at m/z 167 (**Figure 4**).

On the basis of these identifications, the metabolic pathways of **1**−**5** are summarized in **Figure 6**.

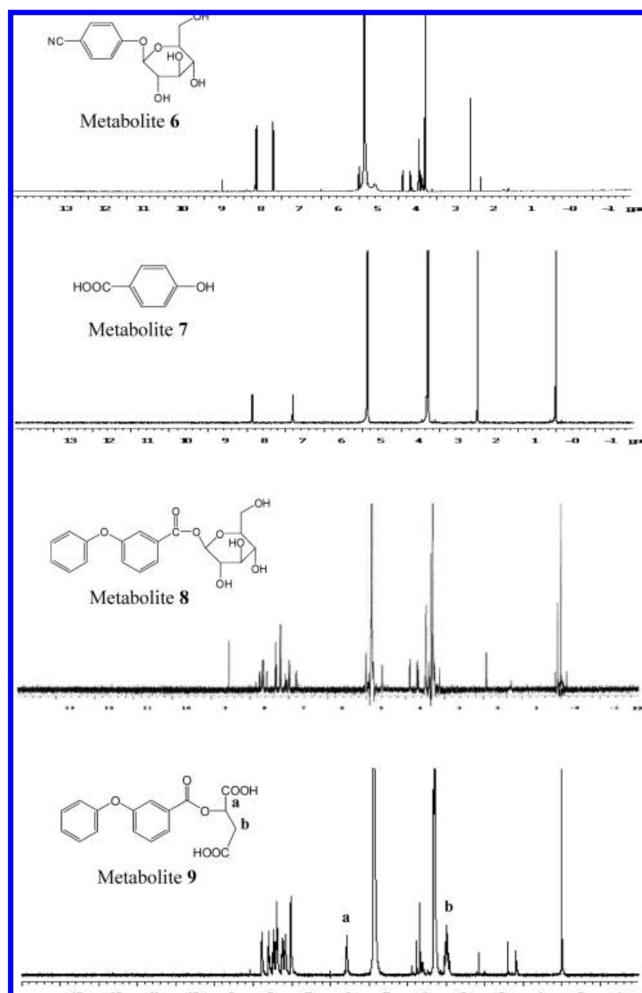

Figure 5. Representative NMR spectra of metabolites.

DISCUSSION

An insignificant difference between the identical crop species was observed on the amount of radioactivity incorporated into the separated leaf cells for **1**−**5**, which was 35.9−50.8% TRR for cabbage and 79.7−91.3% TRR for tomato. Because the pH of the culture medium was adjusted to 6.5, **1**, **3**, and **4** existed mostly as a nonionized form, whereas weak acids **2** and **5** would be mostly dissociated considering their dissociation constant (pK_a), as listed in **Table 4**. Generally, lipophilicity is one of the important factors that determines the uptake of chemicals into plants from both root and leaf surface, and it is assessed by the value of the octan-1-ol/water partition coefficient (log P). For undissociated compounds (*27*−*29*), the translocation from root to shoot versus log P plot showed a Gaussian curve with its maximum peak at 2−3, which indicates the optimum uptake to plant. In the case of dissociated ions, Kleirer (*30*) has developed a model to predict the phloem mobility in which the regressions between log P and the permeability for electrolytes will be still effective by assuming that the dissociated molecules have a 3.5 log unit reduction from log P of its undissociated form. If these theories were simply applied to the leaf cell suspension, the uptake of **2** and **5** should be extremely lower than that of **1**, **3**, and **4**, but this was not the case observed in our study. First, the difference in permeability between bilayer and multilayer structures of cell membrane is supposed. The application of the optimum log P concept for the chemical uptake is considered to be more limited in root and phloem cells, which require compounds to penetrate through the multilayer of

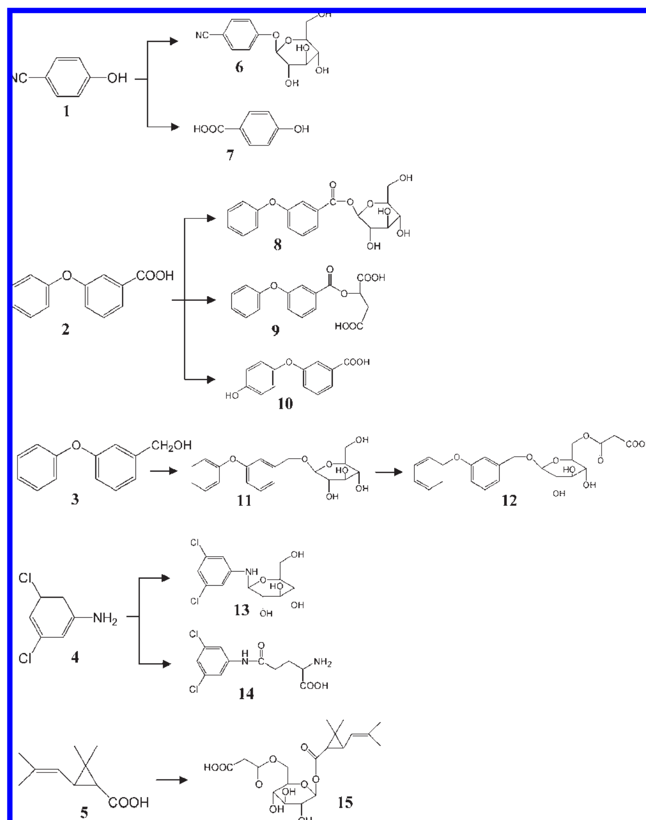

Figure 6. Proposed metabolic pathways of **1**−**5**.

Table 4. Physicochemical Properties of **1**−**5**

compound	log P^a	pK_a^{a}	Henry's law constant^b
1	1.60	8.00	5.42×10^{-9}
2	3.91	3.95	2.37×10^{-9}
3	3.13	14.19	4.75×10^{-9}
4	2.69	2.37	1.05×10^{-6}
5	3.48	5.31	2.42×10^{-6}

[a] Experimental values for **1**, **2**, and **4** from refs *48*−*50*. Calculated values using EPI-suite and ACD/pK_a DB (version 4.56) for **3** and **5** from refs *51* and *52*. [b] Calculated values using EPI-Suite, in atm m^3 mol^{-1} (*24*).

membranes, whereas separated leaf cells possess only a single set of cell membrane and cell wall. Second, the complicated balance of lignin in terms of distribution and quantity in cell wall is considered to be an important factor. Zeier et al. studied the distribution of lignin in the root zone using an immune electron microscopic method and found that liginin epitopes are specifically located at the Casparian strip and suberin lamella but less in the primary cell wall of the root cell (*31*). With regard to the cell wall of leaf, Abedon et al. have clarified that 30% of the total organic compound was liginin; however, the localization of liginin is much less specific compared to the one in the root (*32*). Concomitantly, ^{14}C taken up by the separated leaf cell of tomato was much greater than that taken up by cabbage. This may be explained by the similar viewpoint as described in this section because the constituents of grass cell wall vary extensively depending upon the grass species and the stage of development (*33*, *34*).

Interestingly, metabolites of **1** and **2** in the leaf cell suspension were detected in the culture medium, which facilitated their isolation and purification for spectroscopic analyses. A number of researchers have reported that significant amounts of polar metabolites are excreted to the extracellular media from the suspension culture cells (*35*−*37*) and separated leaf cells (*15*). Recently, the membrane transport of the plant secondary metabolites has been extensively studied and the transporters and/or channels of plants are shown as deeply involved in the membrane transport of alkaloids, terpenoids, phenol, and wax in plant cell. In general, glucosidation plays an important role in the detoxification of endogenous secondary metabolites or xenobiotics in plants, and these glucosides are specifically transferred and accumulated to vacuole. The ATP-binding cassette (ABC) transporters are reported to be involved in the vacuole transportation/storage of such glycosides, glucronides, and glutathione conjugates (*38*). According to the studies, it is suggested that the different types of transporter exist on specific transportation of either for endogenous and exogenous substances. For instance, a flavonoid glucoside, isovitexin in barely, was transported into the isolated vacuoles of barley via a proton motive force, whereas a glucoside of hydroxypromosulfron, a sulfonylurea herbicide, was incorporated by directly energized mechanism (*39*). Also, several members of mammalian ABC transporters are definitely identified as possessing the efflux carriers of plant secondary metabolites within its cell membrane (*40*), and we think this fact strongly supports the possibility of similar types of transporters/channels existing in plants that effectively convey metabolites from inner cells to outer atmosphere.

The major secondary metabolites observed in our leaf cell suspension system exposed to **1**−**5** were the glucose/malonylglucose conjugates. Lamoureux and Rusness have reported that glucoside conjugation via linkages with amino, hydroxy, or carboxy groups is the most common xenobiotic conjugating reaction in plant (*41*). Concomitantly, the metabolic fates of the pyrethroids in intact plants have been extensively studied in the past (*42*). The major metabolic process for pyrethroids in intact plants is hydrolytic cleavage of the ester bond to form their corresponding alcohol and acid moieties, such as **3** and **5**, the former of which is further oxidized to **2**. These primary metabolites were successively transformed to their mono- and disaccharide derivatives including β-glucopyranose conjugate as shown in the studies with permethrin and deltamethrin (*43*, *44*). Xenobiotic glucose conjugates frequently undergo additional metabolism by conjugation of the glucose with other endogeneous molecules, and the malonic acid is confirmed to be the most common candidate (*41*). Taguchi et al. have clarified that malonyltransferase is involved in the malonylation of the glucose conjugates for flavonoid and naphthol in tobacco (*45*), and the reaction is considered to be substrate dependent, which is in good agreement with our observation as **1**, **2**, and **4** were detected as glucosyl conjugates, whereas **3** and **5** were further metabolized to their malonyl glucose forms. Conjugation with small peptides and organic acids is also widely known for xenobiotic metabolism in plants. Chirikishvili et al. have reported that benzoic acids exposed to the seedlings of maize and pea existed mostly as conjugates with numerous kinds of peptides and organic acids, which are described to facilitate transportation/storage into vacuole (*46*). According to this paper, the glutamic acid is the most abundant amino acid conjugate observed among the various compositions of the peptide in nature. In the case of cabbage, the malate conjugate is evaluated as the typical conjugate produced for phenylpropanoid (*47*).

In conclusion, the results presented in our studies substantiate the view that xenobiotic metabolism conducted using the separated leaf cell suspension is a useful adjunct to the conventional whole plant approach. Our experiments have shown that the oxidative reactions and/or conjugation reactions observed in vivo were also found in the separated leaf cell suspension for compounds **1**−**5**. Furthermore, the species difference detected in

plants was considered to be reflected in this test system, with respect to both the nature of phase I and phase II metabolites formed and the amount of parent compounds remained. Especially, the cabbage leaf cell suspension clearly demonstrated the high efficiency of xenobiotic catabolism showing quantitative transformation of the substrates within a day. From this fact, we believe that this system will be an effective tool for the research on metabolic features in whole plant.

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