JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

Cite This: J. Agric. Food Chem. 2018, 66, 13378−13385

pubs.acs.org/JAFC

Identity and Activity of 2,4-Dichlorophenoxyacetic Acid Metabolites in Wild Radish (*Raphanus raphanistrum*)

Danica E. Goggin,*[,†] Gareth L. Nealon,[‡] Gregory R. Cawthray,[§] Adrian Scaffidi,[∥] Mark J. Howard,[‡,∥,⊥] Stephen B. Powles,[†] and Gavin R. Flematti[∥]

[†]Australian Herbicide Resistance Initiative, School of Agriculture and Environment, [‡]Centre for Microscopy, Characterisation and Analysis, [§]School of Biological Sciences, and [∥]School of Molecular Sciences, The University of Western Australia, 35 Stirling Highway, Crawley, Western Australia 6009, Australia

[⊥]School of Chemistry, University of Leeds, Leeds LS2 9JT, United Kingdom

Supporting Information

ABSTRACT: Synthetic auxin herbicides, such as 2,4-dichlorophenoxyacetic acid (2,4-D), are widely used for selective control of broadleaf weeds in cereals and transgenic crops. Although the troublesome weed wild radish (*Raphanus raphanistrum*) has developed resistance to 2,4-D, no populations have yet displayed an enhanced capacity for metabolic detoxification of the herbicide, with both susceptible and resistant wild radish plants readily metabolizing 2,4-D. Using mass spectrometry and nuclear magnetic resonance, the major 2,4-D metabolite was identified as the glucose ester, and its structure was confirmed by synthesis. As expected, both the endogenous and synthetic compounds retained auxin activity in a bioassay. The lack of detectable 2,4-D hydroxylation in wild radish and the lability of the glucose ester suggest that metabolic 2,4-D resistance is unlikely to develop in this species.

KEYWORDS: *2,4-dichlorophenoxyacetic acid, auxin, herbicide resistance, metabolism, wild radish (Raphanus raphanistrum)*

1. INTRODUCTION

The synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D), which mimics the action of the key plant hormone indole-3-acetic acid, was discovered over 70 years ago and was quickly adopted as a herbicide, a promotor of plant growth in tissue culture, and an inhibitor of preharvest fruit drop.[1] Although the use of 2,4-D as a herbicide in the United States has declined from 18 million kg per year in the 1950s to its current level of around 1 million kg per year, it remains an essential component of selective dicot weed control in crops, turf areas, and roadsides worldwide.[2] The basis of selectivity of the synthetic auxins is mainly attributed to the greater capacity of monocotyledonous plants to metabolize these herbicides to inactive polar conjugates, but there is evidence that enhanced metabolism may not entirely account for the high levels of resistance seen in most grass species.[3,4]

The major pathways of 2,4-D metabolism in several commercially important cereal and legume species were identified in the 1970s as (1) direct conjugation of glucose or amino acids (predominantly aspartate and glutamate) to the carboxyl group of the 2,4-D molecule via ester or amide linkages and (2) hydroxylation of the phenol ring, followed by conjugation of a sugar (usually unidentified) through an ether linkage.[5−14] These conclusions were largely based on co-chromatography, where radiolabeled metabolites extracted from [^{14}C]-2,4-D-treated plants were chemically or enzymatically hydrolyzed and the retention times/R_f values of the hydrolysis products were compared to authentic standards. More recent studies have used liquid chromatography−mass spectrometry (LC−MS) to confirm the presence of 2,4-D amino acid conjugates[15] and some sugar conjugates of 2,4-dichlorophenol[16] in crude plant extracts.

The 2,4-D glucose ester (1-[(2,4-dichlorophenoxy)acetate]-β-D-glucopyranose) and most 2,4-D amino acid conjugates are readily cleaved back to the parent molecule by endogenous plant hydrolases and, thus, display auxin activity,[9,15,17] while ring-attached 2,4-D glycosides and their respective aglycones (predominantly 4-hydroxy-2,5-D and 4-hydroxy-2,3-D) are generally inactive.[8] Less commonly, 2,4-D has been found incorporated into larger inactive and/or insoluble molecules, such as 3-(2,4-dichlorophenoxy)propionic acid,[18] triacylglycerols,[19] and lignin and oligopeptides,[6] presumably as a mechanism to sequester 2,4-D in a low-toxicity form.

Wild radish (*Raphanus raphanistrum*), the major dicotyledonous weed of Western Australian cropping systems, is displaying increasing levels of resistance to 2,4-D.[20] In previous work, 13 populations were studied, which showed no evidence of differential metabolism between susceptible and resistant plants.[21,22] However, in this work, approximately 50% of applied 2,4-D was converted to compounds that did not match known metabolites and, thus, remain unidentified. As growers in Australia intensify their use of 2,4-D in response to resistance to other herbicides,[20] the increased selection pressure could potentially result in the appearance of populations with a greater capacity to detoxify 2,4-D, as has occurred in corn poppy[23] and nodding thistle.[24] Therefore, in

Received: September 28, 2018
Revised: November 29, 2018
Accepted: December 5, 2018
Published: December 5, 2018

DOI: 10.1021/acs.jafc.8b05300
J. Agric. Food Chem. 2018, 66, 13378−13385

this study, we investigated the identity and herbicidal activity of the major 2,4-D metabolites produced in wild radish.

2. MATERIALS AND METHODS

2.1. Chemicals and Instrumentation. Technical-grade 2,4-D acid was a gift from Nufarm (Laverton, Australia). Ring ^{14}C [U]-2,4-D (specific activity of 2.035 GBq mmol^{-1}) was obtained from American Radiolabeled Chemicals (St. Louis, MO, U.S.A.), and ring ^{13}C [U]-2,4-D was obtained from Cambridge Isotope Laboratories (Tewksbury, MA, U.S.A.). All other chemicals were from Sigma-Aldrich (Sydney, Australia), and solvents for extractions, synthesis, and purifications were of high-performance liquid chromatography (HPLC) grade, unless otherwise stated. HPLC was performed on an Agilent 1200 HPLC system equipped with a photodiode array detector and fraction collector. Plant extracts were separated on a reversed phase Grace Apollo C_{18} HPLC column (250 mm long, 10 mm inner diameter, and 5 μm particle size, Grace-Davison Discovery Sciences, Columbia, MD, U.S.A.) with a 33 × 7 mm guard column of the same material. An in-line β-RAM detector (IN/US Systems, Inc., Pine Brook, NJ, U.S.A.) was used to detect radioactivity in eluents from [^{14}C]-2,4-D-containing extracts. HPLC−mass spectrometry (MS) and high-resolution mass spectrometry (HRMS) were conducted using a Waters Alliance e2695 HPLC connected to a Waters LCT Premier XE time-of-flight (TOF) mass spectrometer with an electrospray ionization (ESI) source and direct injection valve. Ionization conditions were optimized using unlabeled 2,4-D in negative ionization mode. Cone and desolvation gas flows were set to 150 and 650 L h^{-1}, respectively. The capillary voltage was set at 3 kV, and the cone voltage was set at 20 V, with the source temperature at 80 °C and the desolvation temperature at 350 °C. HRMS calibration was achieved using leucine encephalin at 2 ng μL^{-1}. HPLC−MS separations were achieved using a reversed-phase Grace Alltima C_{18} HPLC column (250 mm long, 2.1 mm inner diameter, and 5 μm particle size, Grace-Davison Discovery Sciences, Columbia, MD, U.S.A.) with a 7.5 × 2.1 mm guard column of the same material. Nuclear magnetic resonance (NMR) spectra were acquired at 298 K on a Bruker Avance IIIHD 11.4 T spectrometer (^{1}H at 500.10 MHz and ^{13}C at 125.75 MHz) equipped with a 5 mm broad band fluorine observation (BBFO) probe or on a Bruker Avance IIIHD 14.1 T spectrometer (^{1}H at 600.13 MHz and ^{13}C at 150.90 MHz) using a 1.7 mm TXI microprobe (Bruker, Rheinstetten, Germany), with either deuteromethanol or deuteroacetone as the solvent. Chemical shifts are reported in parts per million relative to the residual solvent signals (δ 3.31 and 2.05 ppm for deuteromethanol and deuteroacetone, respectively).

2.2. Plant Material. Five of the wild radish (*R. raphanistrum* L., Brassicaceae) populations (R1, R2, R3, R6, and R8) characterized in previous work,[22] which showed no differences in 2,4-D metabolism and retained most of the applied 2,4-D in the treated leaf, making for simpler processing of samples, were bulked together for this study. Seedlings were grown in potting mix (composted pine bark/river sand/peat moss, 2:1:1) and kept in a growth cabinet under a 12 h photoperiod of 200 μmol m^{-2} s^{-1} white light-emitting diode (LED) and incandescent light at day/night temperatures of 20/15 °C. Plants were watered daily and fertilized weekly with 1.5 g L^{-1} commercial soluble fertilizer (Diamond Red, N, 27%; P, 5.7%; K, 10.9%, plus trace elements, Campbells Fertilisers, Laverton North, Australia). When seedlings had reached the three-leaf stage, 2,4-D was applied as a 5 mM solution in 0.1% (v/v) Tween 20, with 10 droplets of 1 μL placed on the first two leaves of each seedling. Plants were then returned to the growth cabinet, and leaf tissue was harvested after 96 h. To identify HPLC fractions containing 2,4-D or its metabolites and to pinpoint MS fragments originating from 2,4-D, pilot studies were performed with (1) plants treated with 5 mM unlabeled 2,4-D plus 3 kBq per leaf of [^{14}C]-2,4-D for radio-HPLC analysis and (2) plants treated with a 1:1 (5 mM total) mixture of unlabeled 2,4-D and [^{13}C]-2,4-D for HPLC−MS analysis. To account for potential interfering signals from endogenous plant compounds, plants treated with 0.1% Tween 20 alone were extracted alongside the [^{13}C]-2,4-D-treated plants. No signals in the m/z regions of 2,4-D or its metabolites were detected in the control plants (data not shown).

2.3. Sample Processing. Treated leaves were excised from seedlings and ground to powder in liquid nitrogen in a mortar and pestle. The time between excision and freezing was <1 min for the pilot studies using labeled 2,4-D and <5 min for the preparative studies (leaves were kept on ice during this time). The frozen powder was ground further in cold 100% methanol[19] and filtered through prewetted Miracloth. The residue was extracted 3 more times in fresh methanol, and all filtered extracts were combined and clarified by centrifugation (2000g for 10 min at 4 °C). The extract was concentrated to near dryness in a rotary evaporator at 36 °C and reconstituted in a small volume of aqueous methanol (1:1, v/v). Recovery of ^{14}C from extracts of plants treated with [^{14}C]-2,4-D was >80%, as measured by liquid scintillation counting. Approximately 10 treated leaves per experiment were used for the pilot studies with labeled 2,4-D, and 80−100 treated leaves were used for each partial purification and subsequent structural analysis of metabolites from unlabeled 2,4-D.

2.4. Sample Fractionation and MS. An extract from [^{14}C]-2,4-D-treated plants was separated by HPLC using a linear gradient of 10−100% (v/v) acetonitrile in water containing 0.1% (v/v) formic acid over 40 min at 24 °C, at a flow rate of 4 mL min^{-1}, and the retention times of fractions containing a ^{14}C signal were noted. Parent 2,4-D eluted at 24.3 min, matching the retention time of an authentic [^{14}C]-2,4-D standard, along with a major metabolite (designated metabolite 1) at 18.1 min, a minor metabolite (designated metabolite 2) at 19.5 min, and a second minor metabolite (designated metabolite 3) at 22.3 min (Figure 1). Synthesized, [^{14}C]-labeled versions of potential metabolites were not used for radio-HPLC. The experiment was repeated with extracts from plants treated with a 1:1 mixture of unlabeled 2,4-D and [^{13}C]-2,4-D, and 4 mL fractions were collected from the column. Fractions corresponding to the retention times of parent 2,4-D and metabolites 1, 2, and 3 were evaporated to dryness under a stream of nitrogen, resuspended in a small volume of acetonitrile, and injected into the HPLC−mass spectrometer operating in both positive and negative ESI mode. Separation was achieved using a linear gradient of 10−100% (v/v) acetonitrile in water containing 0.1% (v/v) formic acid over 40 min at a flow rate of 0.3 mL min^{-1}. Mass ions and fragments with characteristic m/z differences of 6 (the difference between 2,4-D and ring-labeled [^{13}C]-2,4-D) plus the m/z 2 differences caused by natural chlorine isotopes were identified in the mass spectra, and high-resolution measurements were conducted. Tentative molecular ions [M − H]$^-$ and solvent adducts were identified containing the isotopic signature and were present at m/z 219 (parent 2,4-D), 427 (formate adduct of metabolite 1), 334 (metabolite 2), and 335 (metabolite 3). High-resolution mass spectra (ESI, −ve) of metabolite 1 as the formate adduct [M + CHOO]$^-$ found m/z 427.0189 ($C_{15}H_{17}O_{10}Cl_2$ requires 427.0199). Metabolite 2 [M − H]$^-$ gave m/z 333.9870 ($C_{12}H_{10}NO_6Cl_2$ requires 333.9885), while metabolite 3 [M − H]$^-$ gave m/z 334.9734 (closest logical match suggested $C_{12}H_9O_7Cl_2$, which requires 334.9725).

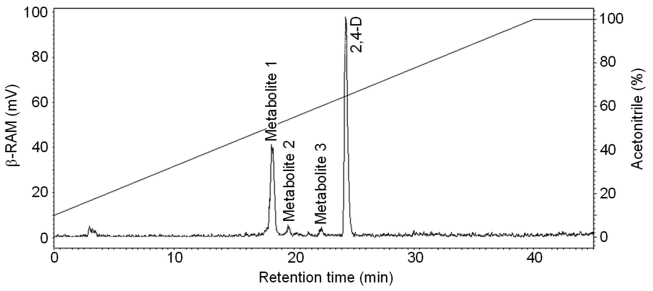

Figure 1. HPLC chromatogram of a leaf extract from wild radish plants treated with [^{14}C]-2,4-D. Only ^{14}C-labeled compounds are registered by the β-RAM detector. The diagonal line shows the concentration of acetonitrile in the mobile phase over time.

DOI: 10.1021/acs.jafc.8b05300
J. Agric. Food Chem. 2018, 66, 13378−13385

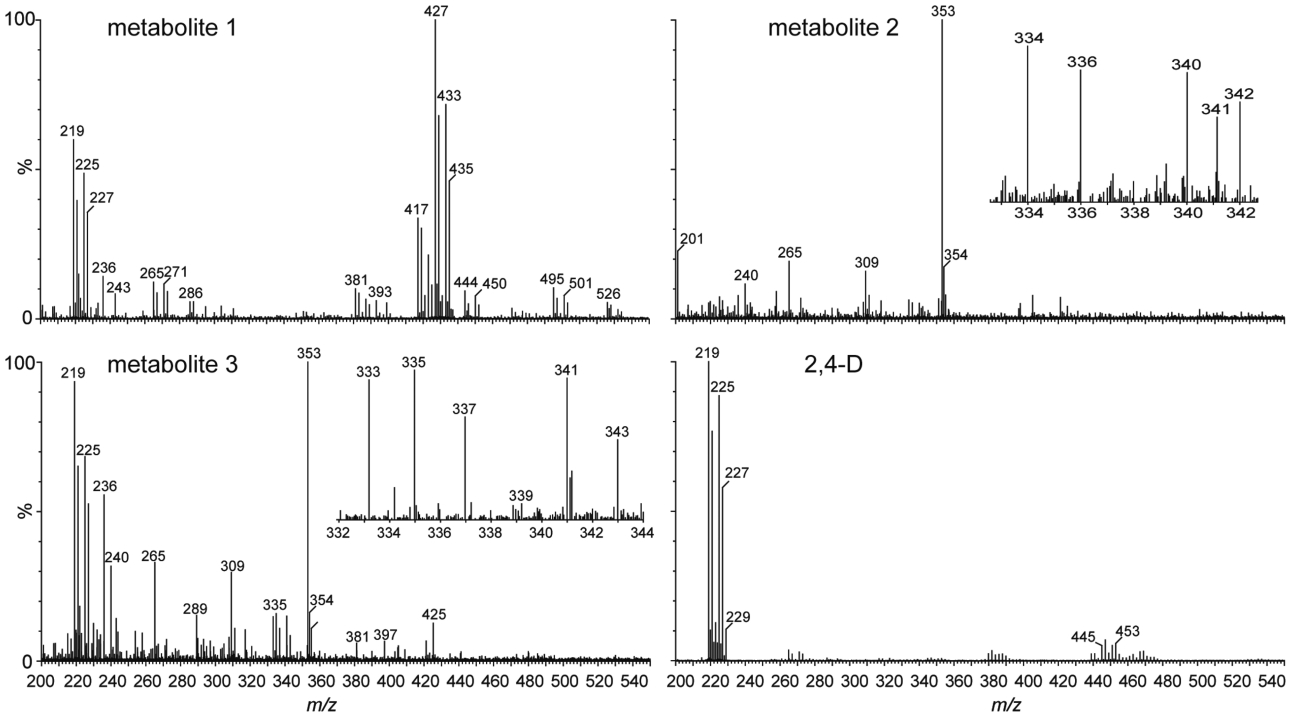

Figure 2. Mass spectra of the 2,4-D metabolites 1, 2, and 3 and parent 2,4-D isolated from 1:1 [^{12}C]/[^{13}C]-2,4-D-treated wild radish plants.

The major metabolite observed, metabolite 1, was purified from extracts of leaves treated with unlabeled 2,4-D by C_{18} silica (50 g, Grace-Vydac reversed-phase C_{18}, 40−63 μm, p/n 5134095, Hesperian, CA, U.S.A.) filtration eluted with 100% water (200 mL), followed by 3:2 methanol/water (200 mL) and methanol (200 mL). The 3:2 methanol/water fraction was evaporated under reduced pressure and separated using semi-preparative HPLC as above. The fraction containing metabolite 1 eluted at ca. 19 min, as confirmed by direct injection into the mass spectrometer, and this was purified further on the same column, eluting with 1:1 methanol/water (containing 0.1% formic acid) at 3.5 mL min^{-1}, to provide the purified compound eluting at 23.5 min (Figure S1 of the Supporting Information). This sample was subjected to ^{1}H and two-dimensional (2D) NMR analysis. ^{1}H NMR [600 MHz, $(CD_3)_2CO$]: δ 3.36 (dd, J = 9.0 and 8.2 Hz, 1H), 3.41−3.45 (m, 2H), 3.50 (dd, J = 9.0 and 9.0 Hz, 1H), 3.69 (dd, J = 11.9 and 4.3 Hz, 1H), 3.81 (dd, J = 11.9 and 2.1 Hz, 1H), 4.96 (s, 2H), 5.61 (d, J = 8.1 Hz, 1H), 7.13 (d, J = 8.9 Hz, 1H), 7.31 (dd, J = 8.9 and 2.6 Hz, 1H), 7.48 (d, J = 2.6 Hz, 1H) (Figure S2 of the Supporting Information).

2.5. Synthesis of 1-[(2,4-Dichlorophenoxy)acetate]-β-D-glucopyranose. Diethyl azodicarboxylate (220 μL, 1.4 mmol) was added to glucose (160 mg, 0.9 mmol), 2,4-D (200 mg, 0.9 mmol), and triphenylphosphine (240 mg, 0.9 mmol) in dimethylformamide (5 mL) at 0 °C. The reaction was slowly warmed to room temperature and allowed to stand overnight. The reaction was concentrated under reduced pressure and subjected to flash chromatography (1:19 methanol/ethyl acetate) to give a mixture of α and β anomers (1.3:1 ratio) as a colorless gum (258 mg, 76%). To isolate exclusively the β anomer, a small sample of the above mixture was separated using semi-preparative HPLC as above, except that separation was achieved using a flow rate of 4 mL min^{-1} and a mobile phase of a 1:3 mixture of acetonitrile/water. The β anomer was isolated as a colorless gum. ^{1}H NMR [500 MHz, $(CD_3)_2CO$]: δ 3.37 (dd, J = 9.0 and 8.2 Hz, 1H), 3.42−3.47 (m, 2H), 3.51 (dd, J = 9.0 and 9.0 Hz, 1H), 3.70 (dd, J = 11.9 and 4.7 Hz, 1H), 3.82 (dd, J = 11.9 and 2.1 Hz, 1H), 4.96 (s, 2H), 5.62 (d, J = 8.1 Hz, 1H), 7.11 (d, J = 8.9 Hz, 1H), 7.31 (dd, J = 8.9 and 2.6 Hz, 1H), 7.48 (d, J = 2.6 Hz, 1H) (Figure S3 of the Supporting Information). ^{13}C NMR [125.8 MHz, $(CD_3)_2CO$]: δ 62.4, 66.4, 71.0, 73.9, 77.7, 78.6, 96.0, 116.1, 124.2, 126.9, 128.7, 130.6, 153.6, 167.8 (Figure S4 of the Supporting Information). HRMS (ESI, −ve) [M + CHOO]$^-$ m/z: 427.0188 ($C_{15}H_{17}O_{10}Cl_2$ requires 427.0199).

2.6. Preparative Thin-Layer Chromatography (TLC). Approximately 30 leaves treated with unlabeled 2,4-D or control leaves treated with 0.1% (v/v) Tween 20 alone were homogenized in methanol as above and partitioned against diethyl ether and then 1-butanol[21] to separate the polar metabolites 1 and 2 from the less polar metabolite 3 and parent 2,4-D. Concentrated fractions were resuspended in 100% methanol and applied to 10 × 10 cm aluminum-backed silica gel TLC plates (Sigma-Aldrich, Sydney, Australia; two plates per fraction), which were developed in toluene/2-butanone/acetic acid (45:55:3).[19] Regions corresponding to the R_f values of parent 2,4-D (0.5), metabolite 3 (0.32), and combined metabolites 1 + 2 (0.0−0.1, overlapping), as identified in previous experiments using [^{14}C]-2,4-D-treated plants,[21] were scraped off the plates and extracted 3 times in methanol. The supernatants were placed into pre-weighed tubes, evaporated to dryness, and stored at −80 °C until used in bioassays. Two independent experiments were performed.

2.7. Bioassay for Auxin Activity. The average proportion of recovered 2,4-D equivalents in each TLC band was determined in previous experiments with [^{14}C]-2,4-D to be parent 2,4-D, 47%; metabolites 1 + 2, 34%; and metabolite 3, 19%.[22] Therefore, on the basis of these proportions and an assumed post-TLC recovery of 50% of applied 2,4-D (also calculated from previous [^{14}C]-2,4-D experiments), the preparative TLC bands extracted above were resuspended in 100% ethanol in volumes adjusted so that each fraction contained the same theoretical concentration (3.2 mM) of 2,4-D equivalents. The untreated control fractions were resuspended to the same mass of solids per microliter as the corresponding treated fractions, to account for possible growth inhibition by endogenous plant compounds present in the fraction.

A dose−response experiment was conducted using authentic 2,4-D, synthesized 2,4-D glucose ester, or TLC fractions from untreated and 2,4-D-treated plants, all diluted in 0.6% (w/v) agar in 90 mm diameter Petri dishes.[21] Authentic 2,4-D was used at concentrations of 0, 10^{-12}, 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 5× 10^{-7}, and 10^{-6} M, and 2,4-D glucose ester and the TLC fractions were used at concentrations of 0, 10^{-8}, 10^{-7}, 5× 10^{-7}, and 10^{-6} M 2,4-D equivalents (concentrations were estimated in the case of the TLC fractions). The radicle length

DOI: 10.1021/acs.jafc.8b05300
J. Agric. Food Chem. 2018, 66, 13378−13385

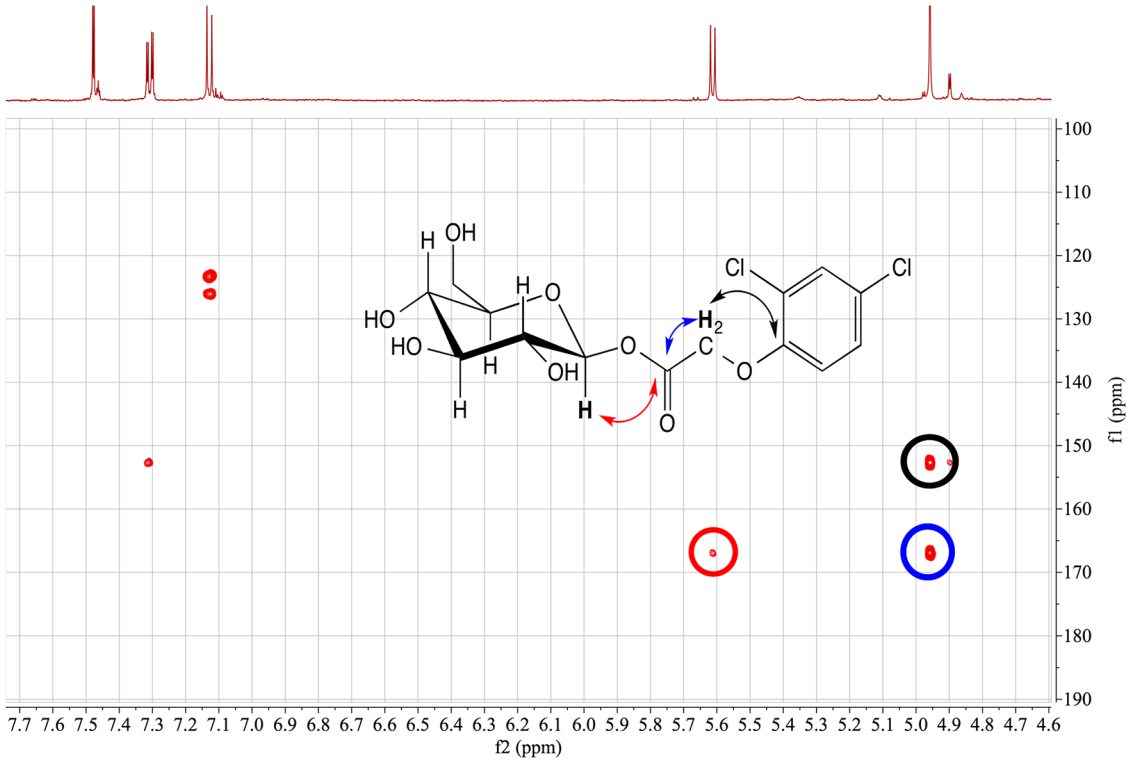

Figure 3. Selected region of the $^{1}H-^{13}C$ HMBC of metabolite 1 showing the existence of correlations between the anomeric proton and the carbonyl group of the ester and the methylene bridge protons with the same ester and the chlorinated aromatic ring.

of 3-day-old seedlings from a well-characterized 2,4-D-susceptible population[21] was measured before placing seedlings on treatment agar and again after 7 days. There were three replicates of each treatment, with three seedlings per replicate. The experiment was performed twice, using the two sets of TLC extracts, and the results were combined.

2.8. Statistical Analysis. Root elongation at each concentration of "treatment" TLC fractions (i.e., those extracted from 2,4-D-treated plants) was expressed as a percentage of root elongation at the corresponding concentration of "control" TLC fractions (i.e., those extracted from untreated plants) to account for potential inhibitory effects of the endogenous plant compounds co-migrating with the 2,4-D metabolites on TLC. Dose−response curves were created using the drc package in R,[25] in which the data were fitted to a three-parameter log-logistic model with the following equation:

$$y = \frac{d}{1 + \exp(b(\log x - \log e))}$$

where d is the upper limit of the root elongation data, b is the slope of the curve, x is the herbicide dose, and e is the dose at which root elongation was inhibited by 50% (otherwise known as ED_{50}).[26] The 2,4-D dose−response equation was used to calculate the concentration of 2,4-D equivalents in the TLC fractions (x) based on their inhibition of root elongation (y), and the ED_{50} values for the TLC fractions were subsequently obtained using the drc package in R.

3. RESULTS

3.1. Identification of 2,4-D Metabolites. Treatment of plants with $[^{14}C]$-2,4-D and subsequent extraction and separation by HPLC showed one major (metabolite 1) and two minor (metabolites 2 and 3) radiolabeled metabolites of 2,4-D (Figure 1). To determine the mass of these metabolites, the experiment was repeated with an extract from plants treated with a stable isotope-labeled mixture (1:1 $[^{12}C]/[^{13}C]$-2,4-D), using the same method and instrument. Fractions with elution times corresponding to those of the original radioactive fractions were then collected and analyzed by HPLC−MS, looking for a specific isotope pattern separated by 6 mass units. MS showed an isotopic series of ions in negative ESI corresponding to metabolite 1 at m/z 417, 427, 495, and 526 (Figure 2). For the minor metabolites 2 and 3, only minor isotopic signals were observed at m/z 334 and 335, respectively (Figure 2).

The major metabolite 1 at first did not match any known metabolites based on HRMS measurements, and therefore, isolation was attempted. Minor metabolite 2 had an accurate mass in good agreement with the common aspartic acid conjugate of 2,4-D,[15] while metabolite 3 had a mass that also did not match any known 2,4-D metabolites. The identities of metabolites 2 and 3 were not investigated further as a result of their abundance being too low for isolation.

3.2. Isolation of Metabolite 1. For isolation of metabolite 1, unlabeled 2,4-D was applied to plants in a scaled-up experiment. Plant metabolites were extracted with methanol, and the crude extract was pre-filtered through C_{18} silica with solvent mixtures consisting of increasing concentrations of methanol and water. The 3:2 (v/v) methanol/water fraction was separated using the same C_{18} semi-preparative HPLC method as above to provide a fraction containing metabolite 1, as determined by direct injection into the mass spectrometer. This fraction was purified further with semi-preparative HPLC using 1:1 (v/v) methanol/water (containing 0.1% formic acid) to provide a sufficiently pure sample for NMR analysis. The NMR spectra indicated that all of the features of 2,4-D were present, along with a sugar moiety that was inferred to be attached to the carboxylate group. This was supported by a heteronuclear multiple bond correlation (HMBC) of the anomeric proton to the carbonyl group of the carboxylate

DOI: 10.1021/acs.jafc.8b05300
J. Agric. Food Chem. 2018, 66, 13378−13385

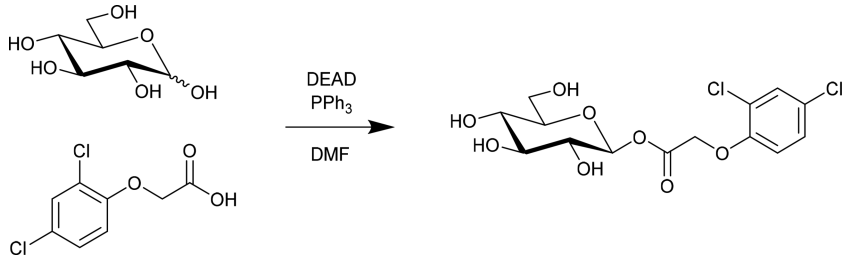

Figure 4. Synthesis of 2,4-D glucose ester from 2,4-D and β-D-glucose.

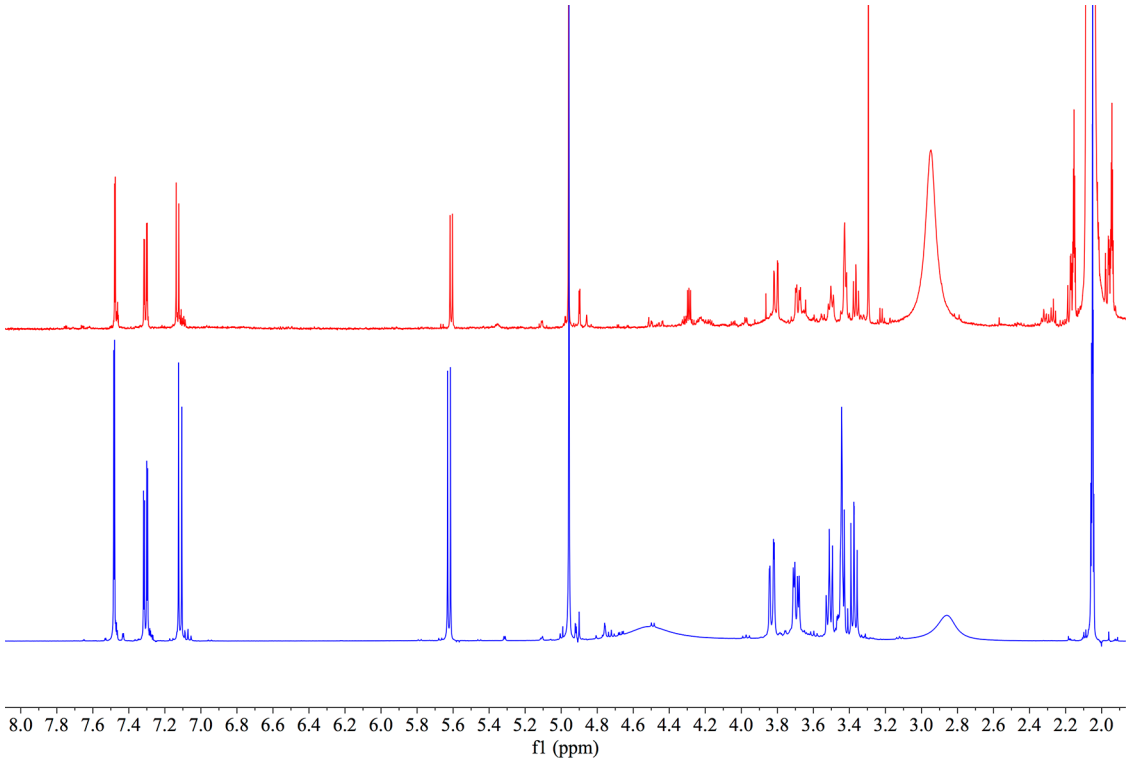

Figure 5. Overlaid ^{1}H NMR spectra of (top) isolated metabolite 1 and (bottom) synthetic 2,4-D glucose ester.

group of 2,4-D (Figure 3). This structural assignment agreed with a quasi-molecular ion $[M + CHOO^{-}]^{-}$ observed at m/z 427, but previous TLC experiments[21] and the observation of higher m/z values for this metabolite initially raised some doubt. It was possible that glucose was sulfated[16] or had another conjugate that was NMR silent. However, to our knowledge, no NMR spectra have been reported for the 2,4-D glucose conjugate that could be used to verify the structure. Previous identification in the literature has been based on β-glucosidase digestion of water-soluble [^{14}C]-labeled metabolites, followed by chromatographic analysis of the aglycones.[7,8,12] Hence, to unambiguously confirm the identity of metabolite 1 as the glucose ester of 2,4-D, a synthesis was undertaken.

3.3. Synthesis of 2,4-D Glucose Ester. The synthesis of 2,4-D glucose ester was achieved by a Mitsunobu reaction between 2,4-D and glucose (Figure 4). The reaction yielded the β anomer as the major isomer, which was isolated by semi-preparative HPLC. The synthesized ester provided NMR spectroscopic data (Figure 5) along with HPLC retention time and mass spectrometric data that correlated perfectly with that of metabolite 1 obtained from 2,4-D-treated plants, thus confirming the identity of this metabolite as 2,4-D glucose ester.

3.4. Auxin Activity of 2,4-D Metabolites. In terms of inhibition of seedling root elongation, the ED_{50} value for authentic 2,4-D was 1.02 nM, which was significantly ($p < 0.05$) lower than that for the synthesized 2,4-D glucose ester (14.7 nM) (Figure 6a). The parent 2,4-D and metabolite 1 + 2 bands extracted from TLC plates both caused increasing inhibition of root elongation with an increasing concentration (panels b and c of Figure 6). The ED_{50} values for extracted parent 2,4-D and metabolite 1 + 2 were calculated at 1.27 and 1.45 nM, respectively, which were significantly lower than that of synthesized 2,4-D glucose ester but not different from that of authentic 2,4-D. The TLC fraction corresponding to metabolite 3 caused a relatively consistent inhibition of root elongation at all concentrations used, and thus, the data could not be fitted to a dose−response curve (Figure 6d).

4. DISCUSSION

This study has identified the glucose ester as the major metabolite of 2,4-D in wild radish, a prominent weed species. This is, to our knowledge, the first reported NMR analysis of 2,4-D glucose ester isolated from a biological extract and

DOI: 10.1021/acs.jafc.8b05300
J. Agric. Food Chem. 2018, 66, 13378−13385

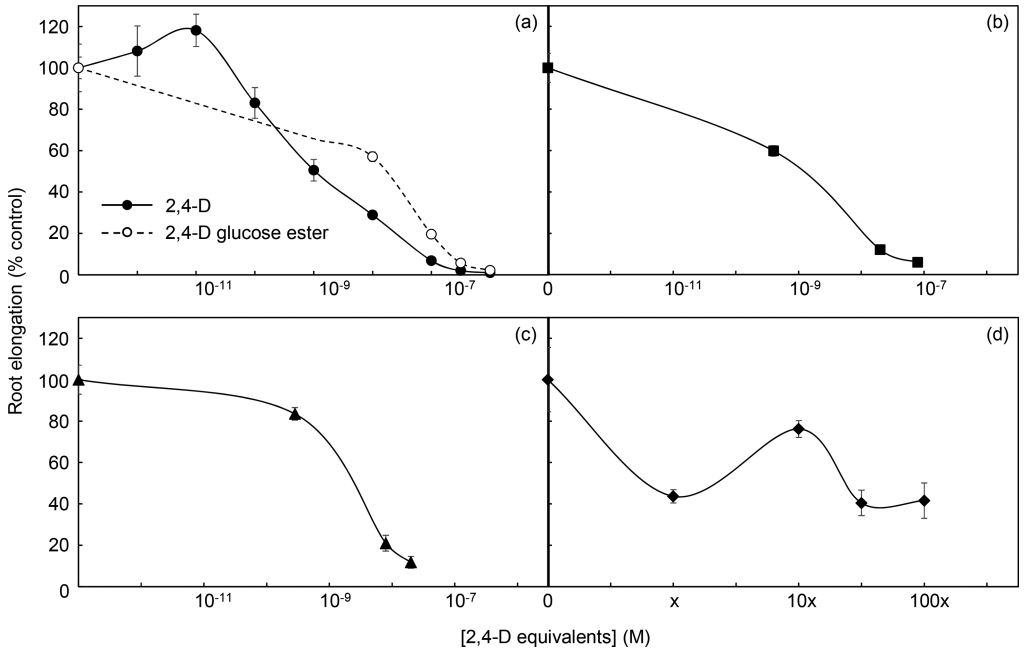

Figure 6. Response of seedling roots to 2,4-D and its metabolites. Preparative TLC was used to partially purify 2,4-D and its metabolites from extracts of 2,4-D-treated leaves, and their effect on seedling root elongation was measured. Dose−response curves are shown for (a) authentic 2,4-D and synthesized 2,4-D glucose ester, (b) TLC parent 2,4-D band, (c) TLC metabolite 1 + 2 band, and (d) TLC metabolite 3 band. Concentrations of 2,4-D equivalents in the TLC bands were calculated from the dose−response curve equation of authentic 2,4-D. Values are means ± standard error (SE), with data pooled from two independent experiments (n = 3 for each).

confirmed by organic synthesis. Very little work has been performed on 2,4-D metabolism in the Brassicaceae family, and no study has detected the glucose ester. In an investigation targeting hydroxylated 2,4-D metabolites, trace levels of 4-OH-2,5-D were found in 2,4-D-treated wild mustard (*Brassica kaber*).[27] In a study focused on the potential for amino acid conjugation of 2,4-D in *Arabidopsis thaliana*, small amounts of the aspartyl and glutamyl 2,4-D amides were isolated.[15] Wild radish may also produce low levels of the aspartyl amide of 2,4-D based on the mass spectral characteristics of a minor metabolite examined in the current study. The more comprehensive surveys of 2,4-D metabolism in non-cruciferous species have detected trace or low amounts of the glucose ester in dicots[10,11] and high levels in some grasses.[9,28]

As expected,[9] both laboratory-synthesized 2,4-D glucose ester and the partially purified wild radish TLC fraction containing this molecule displayed auxin activity. The fact that ED_{50} of the TLC fraction was 10-fold lower than that of the synthesized glucose ester (and almost identical to that of parent 2,4-D) suggests that the ester in the TLC fraction was hydrolyzed back to 2,4-D during extraction from the acidic silica of the TLC plate, in line with the high acid and base lability demonstrated in previous studies.[21,28] The TLC fraction containing the unidentified minor metabolite 3 caused a relatively consistent, concentration-independent level of root growth inhibition in the bioassay, suggesting that the plant may hydrolyze this metabolite to an auxin-active form at very slow rates.

In terms of 2,4-D resistance evolution in weeds, it appears that even the most resistant wild radish populations thus far characterized have little to no capacity for metabolic detoxification of 2,4-D.[22] In particular, there is no chemical, structural (current study), or pharmacological[21] evidence for the presence of low-toxicity, ring-hydroxylated 2,4-D metabolites. Therefore, it can be surmised that repeated selection of wild radish with 2,4-D in the field has thus far not resulted in increased expression/activity of potential 2,4-D-hydroxylating enzymes, such as cytochrome P450 monooxygenase[29] or iron(II)/α-ketoglutarate-dependent hydroxylase[30] (see also Table S1 of the Supporting Information). The enzyme responsible for the observed reversible conjugation of glucose with 2,4-D is likely to be a UDP-glycosyltransferase (UGT). A few members of the large UGT superfamily have been identified as having activity against 2,4-D but at much lower levels (1−15%) than against the natural auxins indole-3-acetic acid and indole-3-butyric acid.[31−33] A cursory search of the gene expression data generated in a transcriptomic study on the response of wild radish to 2,4-D at 2, 8, and 24 h after application[22] revealed consistent upregulation of five UGT genes in both resistant and susceptible plants (Table S1 of the Supporting Information). The most 2,4-D-responsive of these, UGT74E2, is known to have low but measurable activity against 2,4-D.[32] Thus, UGT74E2 could potentially be responsible for glucose conjugation of 2,4-D in wild radish, but this needs to be confirmed experimentally. It is more difficult to pinpoint a potential candidate for the 2,4-D glucose hydrolase from the existing transcriptomic data, because its expression may not have been affected by application of free 2,4-D within the 24 h time frame of the transcriptomic experiment.

Metabolic detoxification of 2,4-D is likely to be a rare (at best) resistance mechanism in wild radish based on the findings that (1) there is no differential metabolism of this herbicide across the 13 independently evolved populations studied thus far[22] and (2) the major metabolite, 2,4-D glucose ester, retains auxin activity. Therefore, there is little opportunity to manage 2,4-D resistance in wild radish with the use of chemical inhibitors of 2,4-D-metabolizing enzymes.

DOI: 10.1021/acs.jafc.8b05300
J. Agric. Food Chem. 2018, 66, 13378−13385

It is likely that, in most of the populations studied to date, as yet unidentified alterations in auxin perception and/or signaling are responsible for resistance,[22] which will probably require biological or genetic solutions rather than chemical methods of resistance amelioration.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.8b05300.

HPLC chromatograms showing the semi-preparative HPLC separation of metabolite 1 from wild radish extracts (Figure S1), full ^{1}H NMR spectrum of isolated metabolite 1 (Figure S2), ^{1}H NMR spectrum of synthetic 2,4-D glucose ester (Figure S3), and ^{13}C NMR spectrum of synthetic 2,4-D glucose ester (Figure S4) (PDF)

Expression of potential 2,4-D-metabolizing enzymes in 2,4-D-treated wild radish plants (Table S1) (XLSX)

■ AUTHOR INFORMATION

Corresponding Author

*Telephone: +61-8-6488-1512. E-mail: danica.goggin@uwa.edu.au.

ORCID

Danica E. Goggin: 0000-0003-3856-9801

Funding

This work was funded by the Australian Research Council (Grant LP150100161).

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors acknowledge the facilities and scientific and technical assistance of the Australian Microscopy & Microanalysis Research Facility at the Centre for Microscopy, Characterisation & Analysis, The University of Western Australia, a facility funded by the university and the state and commonwealth governments. The authors thank Roberto Busi (The University of Western Australia) for statistical advice, Chad Sayer (Nufarm, Australia) for helpful discussions, and two anonymous reviewers for their suggestions on improving the manuscript.

■ REFERENCES

(1) Tukey, H. B. 2,4-D, a potent growth regulator of plants. *Sci. Mon.* **1947**, *64* (2), 93−97.

(2) Peterson, M. A.; McMaster, S. A.; Riechers, D. E.; Skelton, J.; Stahlman, P. W. 2,4-D past, present and future: A review. *Weed Technol.* **2016**, *30* (30), 303−345.

(3) Song, Y. Insight into the mode of action of 2,4-dichlorophenoxyacetic acid (2,4-D) as an herbicide. *J. Integr. Plant Biol.* **2014**, *56*, 106−113.

(4) Grossmann, K. Auxin herbicides: Current status of mechanism and mode of action. *Pest Manage. Sci.* **2010**, *66* (2), 113−120.

(5) Montague, M. J.; Enns, R. K.; Siegel, N. R.; Jaworski, E. G. (1981) A comparison of 2,4-dichlorophenoxyacetic acid metabolism in cultured soybean cells and in embryogenic carrot cells. *Plant Physiol.* **1981**, *67* (67), 603−607.

(6) Scheel, D.; Sandermann, H., Jr. Metabolism of 2,4-dichlorophenoxyacetic acid in cell suspension cultures of soybean (*Glycine max* L.) and wheat (*Triticum aestivum* L.) I: General results. *Planta* **1981**, *152* (3), 248−252.

(7) Davidonis, G. H.; Hamilton, R. H.; Mumma, R. O. Metabolism of 2,4-dichlorophenoxyacetic acid in soybean root callus and differentiated soybean root cultures as a function of concentration and tissue age. *Plant Physiol.* **1978**, *62*, 80−83.

(8) Bristol, D. W.; Ghanuni, A. M.; Oleson, A. E. Metabolism of 2,4-dichlorophenoxyacetic acid by wheat cell suspension cultures. *J. Agric. Food Chem.* **1977**, *25*, 1308−1314.

(9) Chkanikov, D. I.; Makeyev, A. M.; Pavlova, N. N.; Grygoryeva, L. V.; Dubovoi, V. P.; Klimov, O. V. Variety of 2,4-D metabolic pathways in plants; its significance in developing analytical methods for herbicides residues. *Arch. Environ. Contam. Toxicol.* **1977**, *5* (1), 97−103.

(10) Feung, C.-S.; Hamilton, R. H.; Mumma, R. O. Metabolism of 2,4-dichlorophenoxyacetic acid. VII. Comparison of metabolites from five species of plant callus tissue cultures. *J. Agric. Food Chem.* **1975**, *23*, 373−376.

(11) Feung, C.-S.; Hamilton, R. H.; Mumma, R. O. Metabolism of 2,4-dichlorophenoxyacetic acid. V. Identification of metabolites in soybean callus tissue cultures. *J. Agric. Food Chem.* **1973**, *21*, 637−640.

(12) Feung, C.-S.; Hamilton, R. H.; Witham, F. H. Metabolism of 2,4-dichlorophenoxyacetic acid by soybean cotyledon callus tissue cultures. *J. Agric. Food Chem.* **1971**, *19* (3), 475−479.

(13) Crosby, D. G. Metabolites of 2,4-dichlorophenoxyacetic acid (2,4-D) in bean plants. *J. Agric. Food Chem.* **1964**, *12*, 3−6.

(14) Klämbt, H.-D. Wachstumsinduktion und Wuchsstoffmetabolismus im Weizenkoleoptilzylinder III. Stoffwechselprodukte der Naphthyl-1-essigsäure und 2,4-Dichlorphenoxyessigsäure und der vergleich mit jenen der Indol-3-essigsäure und Benzoesäure. *Planta* **1961**, *57*, 339−353.

(15) Eyer, L.; Vain, T.; Pařízková, B.; Oklestkova, J.; Barbez, E.; Kozubíková, H.; Pospíšil, T.; Wierzbicka, R.; Kleine-Vehn, J.; Fránek, M.; Strnad, M.; Robert, S.; Novak, O. 2,4-D and IAA amino acid conjugates show distinct metabolism in *Arabidopsis*. *PLoS One* **2016**, *11*, No. e0159269.

(16) Laurent, F.; Debrauwer, L.; Rathahao, E.; Scalla, R. 2,4-dichlorophenoxyacetic acid metabolism in transgenic tolerant cotton (*Gossypium hirsutum*). *J. Agric. Food Chem.* **2000**, *48*, 5307−5311.

(17) Feung, C.-S.; Hamilton, R. H.; Mumma, R. O. Metabolism of 2,4-dichlorophenoxyacetic acid. 11. Herbicidal properties of amino acid conjugates. *J. Agric. Food Chem.* **1977**, *25*, 898−900.

(18) Hagin, R. D.; Linscott, D. L.; Dawson, J. E. 2,4-D metabolism in resistant grasses. *J. Agric. Food Chem.* **1970**, *18*, 848−850.

(19) López-Villalobos, A.; Hornung, R.; Dodds, P. F. Hydrophobic metabolites of 2,4-dichlorophenoxyacetic acid (2,4-D) in cultured coconut tissue. *Phytochemistry* **2004**, *65*, 2763−2774.

(20) Owen, M. J.; Martinez, N. J.; Powles, S. B. (2015) Multiple herbicide-resistant wild radish (*Raphanus raphanistrum*) populations dominate Western Australian cropping fields. *Crop Pasture Sci.* **2015**, *66*, 1079−1085.

(21) Goggin, D. E.; Cawthray, G. R.; Powles, S. B. 2,4-D resistance in wild radish: Reduced herbicide translocation via inhibition of cellular transport. *J. Exp. Bot.* **2016**, *67*, 3223−3235.

(22) Goggin, D. E.; Kaur, P.; Owen, M. J.; Powles, S. B. 2,4-D and dicamba resistance mechanisms in wild radish: Subtle, complex and population-specific? *Ann. Bot.* **2018**, *122*, 627.

(23) Rey-Caballero, J.; Menéndez, J.; Giné-Bordonaba, J.; Salas, M.; Alcántara, R.; Torra, J. Unravelling the resistance mechanisms to 2,4-D (2,4-dichlorophenoxyacetic acid) in corn poppy (*Papaver rhoeas*). *Pestic. Biochem. Physiol.* **2016**, *133*, 67−72.

(24) Harrington, K. C.; Woolley, D. J. (2006) Investigations of how phenoxy-resistant *Carduus nutans* biotypes survive herbicide spraying. *N. Z. J. Agric. Res.* **2006**, *49* (49), 465−474.

(25) R Core Team. *R: A Language and Environment for Statistical Computing*; R Foundation for Statistical Computing: Vienna, Austria, 2015; https://www.R-project.org/.

(26) Ritz, C.; Streibig, J. C. Bioassay analysis using R. *J. Stat. Softw.* **2005**, *12* (5), 1−22.

(27) Fleeker, J.; Steen, R. Hydroxylation of 2,4-D in several weed species. *Weed Sci.* **1971**, *19* (5), 507−510.

(28) Hamburg, A.; Puvanesarajah, V.; Burnett, T. J.; Barnekow, D. E.; Premkumar, N. D.; Smith, G. A. Comparative degradation of [^{14}C]-2,4-dichlorophenoxyacetic acid in wheat and potato after foliar application and in wheat, radish, lettuce, and apple after soil application. *J. Agric. Food Chem.* **2001**, *49*, 146−155.
(29) Yuan, J. S.; Tranel, P. J.; Stewart, C. N., Jr. (2007) Non-target-site herbicide resistance: A family business. *Trends Plant Sci.* **2007**, *12* (12), 6−13.
(30) Keith, B. K.; Kalinina, E. B.; Dyer, W. E. Differentially expressed genes in dicamba-resistant and dicamba-susceptible biotypes. *Weed Biol. Manage.* **2011**, *11*, 224−234.
(31) Jackson, R. G.; Lim, E.-K.; Li, Y.; Kowalczyk, M.; Sandberg, G.; Hoggett, J.; Ashford, D. A.; Bowles, D. J. Identification and biochemical characterization of an *Arabidopsis* indole-3-acetic acid glucosyltransferase. *J. Biol. Chem.* **2001**, *276*, 4350−4356.
(32) Tognetti, V. B.; Van Aken, O.; Morreel, K.; Vandenbroucke, K.; van de Cotte, B.; De Clercq, I.; Chiwocha, S.; Fenske, R.; Prinsen, E.; Boerjan, W.; Genty, B.; Stubbs, K. A.; Inzé, D.; Van Breusegem, F. Perturbation of indole-3-butyric acid homeostasis by the UDP-glucosyltransferase *UGT74E2* modulates *Arabidopsis* architecture and water stress tolerance. *Plant Cell* **2010**, *22*, 2660−2679.
(33) Jin, S.-H.; Ma, X.-M.; Han, P.; Wang, B.; Sun, Y.-G.; Zhang, G.-Z.; Li, Y.-J.; Hou, B.-K. UGT74D1 is a novel auxin glycosyltransferase from *Arabidopsis thaliana*. *PLoS One* **2013**, *8* (4), No. e61705.

DOI: 10.1021/acs.jafc.8b05300
J. Agric. Food Chem. 2018, 66, 13378−13385