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Herbicide Resistance Endowed by Enhanced Rates of Herbicide Metabolism in Wild Oat (*Avena* spp.)

M. S. Ahmad-Hamdani, Qin Yu, Heping Han, Gregory R. Cawthray, Shao F. Wang, and Stephen B. Powles*

The biochemical basis of resistance to the acetyl-coenzyme A carboxylase (ACCase)-inhibiting herbicide diclofop-methyl was investigated in a resistant wild oat population (R1), which does not exhibit a resistant ACCase. Rates of foliar uptake and translocation of [^{14}C]-diclofop were the same in the R1 vs. susceptible (S) populations. However, the level of phytotoxic diclofop acid was always found to be lower in the R1 vs. S plants, with a concomitant higher level (up to 1.7-fold) of nontoxic polar diclofop metabolites in R1 relative to the S plants. These results indicate that a non–target-site-based mechanism of enhanced rate of diclofop acid metabolism confers resistance in population R1. Moreover, the high-performance liquid chromotography elution profile of the major diclofop metabolites in R1 is similar to that of wheat, suggesting resistance in individuals of population R1 involves a wheat-like detoxification system mediated by cytochrome P450 monooxygenases. In addition, lower level of tissue diclofop acid was also observed using nonradioactive ultra-performance liquid chromatography–mass spectrometry analysis in resistant individuals of three other resistant wild oat populations (R2, R3, and R4) known to posses ACCase gene resistance mutations. These results establish that either one or at least two independent resistance mechanisms (target-site ACCase resistance mutations and non–target-site enhanced rates of herbicide metabolism) can be present in individual wild oat plants.

Nomenclature: Diclofop-methyl; wild oat, *Avena fatua* L. AVEFA.
Key words: Herbicide uptake and translocation, multiple resistance mechanisms, non-target-site based resistance.

Since 1978, ACCase-inhibiting herbicides (hereafter referred to as ACCase herbicides) have been globally used in world agriculture for POST grass weed control in a range of crops and situations. These herbicides are potent inhibitors of ACCase (EC 6.4.1.2). By inhibiting ACCase activity, fatty acid synthesis is inhibited, resulting in growth cessation in meristematic tissue and then plant death. Crop selectivity and efficacy of ACCase herbicides on many grass weed species, including wild oats has led to global use of ACCase herbicides in a variety of crops. Inevitably, persistent use of ACCase herbicides has led to the evolution of grass weed populations that are resistant to one or more ACCase herbicides (reviewed by Délye 2005; Powles and Yu 2010).

The biochemical basis of ACCase herbicide resistance has been studied in wild oat populations and a resistant form of ACCase has often been found (Cruz-Hipolito et al. 2011; Maneechote et al., 1994, 1997; Seefeldt et al. 1996; Shukla et al. 1997). Importantly, however, enhanced rates of ACCase herbicide metabolism have also been identified in ACCase herbicide-resistant wild oat populations (Cocker et al. 2000; Maneechote et al. 1997). Enhanced metabolism of ACCase herbicides has been more frequently reported in resistant populations of *Lolium* spp. than in other resistant weed species (Cocker et al. 2001; De Prado et al. 2005; Holtum et al. 1991; Preston and Powles 1998; Preston et al. 1996). Although direct evidence is often lacking, non–target-site resistance (enhanced herbicide metabolism) is being increasingly identified or implicated as a mechanism conferring resistance to ACCase herbicides in grass weed species such as blackgrass (*Alopecurus myosuroides* Huds.) (Cocker et al. 1999; Délye et al. 2007, 2011), wild oat (Beckie and Tardif 2012; Beckie et al. 2012; Cocker et al. 2000; Owen and Powles 2009), and ryegrass (*Lolium* spp.) (Cocker et al. 2001; Owen et al. 2007). The natural tolerance of wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) to ACCase herbicides is known to be due to rapid herbicide metabolism (reviewed by Devine and Shimabukuro 1994), which involves detoxification of the parent herbicide through a cytochrome P450 monooxygenase-mediated pathway (via oxidative hydroxylation) with subsequent sugar conjugation.

Cytochrome P450 monooxygenases (known as P450s) are membrane-bound and heme-iron–containing enzymes that use NADPH + H^+ as a cosubstrate by forming a complex with a cytochrome P450 reductase. P450s are among the largest superfamilies of enzymes and can be found in most organisms, with plants (especially monocots) possessing the highest numbers of P450 genes (reviewed by Nelson and Werck-Reichhart 2011; Siminszky 2006). In plants, P450s participate in secondary metabolism, including the initial metabolism of herbicides (reviewed by Preston 2004; Siminszky 2006; Powles and Yu 2010). Depending on the isoform (or isoforms) of P450 genes involved, P450-mediated herbicide resistance can display cross-resistance to herbicides of similar and dissimilar modes of action, including ACCase herbicides as reported in blackgrass, sterile oat (*Avena sterilis* L.), large crabgrass [*Digitaria sanguinalis* (L.) Scop.], rice (*Oryza sativa* L.) barnyardgrass [*Echinochloa crus-galli* (L.) Beauv.], and ryegrass (reviewed by Délye 2005; Powles and Yu 2010).

A resistant wild oat population (R1) investigated in this study has high-level (> 20-fold) resistance to diclofop-methyl and cross-resistance to other ACCase herbicides (Ahmad-Hamdani et al. 2012). This resistance is largely not ACCase-based as sequencing of the ACCase carboxyl-transferase (CT) domain found that 89% of the parent individuals in this population did not contain any known ACCase gene resistance–endowing mutations (Yu et al. 2012). Thus, the objective of this study was to determine possible resistance mechanism (or mechanisms) in this R1 population and in three other resistant wild oat populations.

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* First, second, third, fourth, and sixth authors: Ph.D student, Associate Professor, Research Associate, Senior Research Officer, and Professor, respectively, Australian Herbicide Resistance Initiative (M086) and School of Plant Biology, the University of Western Australia (UWA), Crawley, WA 6009, Australia; fifth author: Senior Scientist, ChemCentre, Resources and Chemistry Precinct, Curtin University, Bentley, WA 6102, Australia, and Centre for Legumes in Mediterranean Agriculture UWA. Current address of first author: Department of Crop Science, Faculty of Agriculture, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia. Corresponding author's E-mail: qin.yu@uwa.edu.au

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Materials and Methods

Plant Material and Growth Condition. Population R1 (wild oat) exhibits high-level resistance to the ACCase herbicide diclofop-methyl (Ahmad-Hamdani et al. 2012), but does not possess known ACCase gene resistance mutations (Yu et al. 2012). Plants that survived diclofop-methyl and were established not to possess known ACCase mutations were allowed to self-pollinate to produce seeds. Progeny plants of these seeds were used for in vitro ACCase activity assay, [^{14}C]-diclofop-methyl uptake/translocation, and high-performance liquid chromatography (HPLC) metabolism studies. A known herbicide-susceptible population (S) was used as a control. For the nonradiolabeled ultra-performance liquid chromatography–mass spectrometry (UPLC-MS) diclofop metabolism study (see below), progeny of R1 plants that survived fenoxaprop-P-ethyl and did not have any known ACCase resistance mutations, and progeny of plants that possess a single ACCase resistance mutation (Ile-1781-Leu, Asp-2078-Gly, or Cys-2088-Arg) in wild oat (*Avena fatua* and *A. sterilis*), populations R2, R3, and R4 (Yu et al. 2012) were used. Seeds of two susceptible populations (the original S population and another susceptible wild oat population, hereafter referred to as S1) were used as controls. For germination and initial growth of all seedlings, seeds were lightly indented with two-tooth tweezers and placed on 0.6% agar-solidified water. The seeds were stored at 4 to 5 C for 10 d until they began to germinate, and then transferred to room temperature (20 to 25 C) for 3 d. For [^{14}C]-diclofop uptake/translocation and metabolism studies, individual seedlings were transplanted into small plastic cups (60 by 60 by 100 mm), one seedling per cup, and for in vitro ACCase activity assay, seedlings were transplanted into plastic trays (300 by 400 by 100 mm). For the UPLC-MS measurement, seedlings were transplanted into 180-mm-diam plastic pots, 10 plants in each pot. All cups, trays, and pots were filled with potting mix (50% composted pine bark, 25% peat, and 25% river sand). Plants were watered and grown in a controlled environment room (CER) with a day/night temperature of 20/15 C, a 12-h photoperiod with light intensity of approximately 500 μmol photon m^{-2} s^{-1}, and 65 to 75% relative humidity.

In vitro ACCase Activity Assay. Leaf material of R1 and S plants at the three- to four-leaf stage were harvested, snap-frozen in liquid N_2, and stored at −80 C until use. Generally, 20 to 25 individual plants were sampled to obtain approximately 3 g of leaf tissue. ACCase extraction and partial purification, and enzyme inhibition by ACCase herbicides were performed according to Maneechote et al. (1994) and Yu et al. (2004). Herbicide stock solutions were prepared in 100 mM Tricine buffer (containing 10% acetone for diclofop acid or fenoxaprop acid with a final concentration of $<$ 1% acetone in the assay mix). Technical-grade diclofop acid, fenoxaprop acid, clethodim (94 to 98% purity), and sethoxydim (52% purity) were used in the assays to determine the herbicide concentration causing 50% inhibition of ACCase activity (I_{50}). Tissue protein content was determined by the Bradford method (Bradford, 1976). Two subsamples from each extraction were assayed, and there were at least two independent extractions per population per herbicide-dose experiment.

[^{14}C]-Diclofop Foliar Uptake and Translocation. [^{14}C]-diclofop-methyl (dichlorophenyl-UL-^{14}C) was obtained from Bayer CropScience®(Frankfurd, Germany) (specific activity: 6.6 MBq mg^{-1}). At the two-leaf stage, R1 and S plants were treated with the solution containing [^{14}C]-diclofop-methyl (1.7 kBq μL^{-1}), prepared in a commercial formulation of diclofop-methyl (Hoegrass® 500 [Bayer CropScience, Pty. Ltd. Hawthorn, VIC, Australia]; plus 0.25% [v/v] nonionic surfactant BS1000)(Crop Care Australia, Murarie, QLD). The final diclofop concentration in the treatment solution was 1.88 mM (equivalent to 72 g ha^{-1}). The lower radioactivity applied to plants for this experiment as compared to the metabolism study (described below) helped to avoid overexposure of the imaging plate at the application zone due to limited translocation.

The herbicide solution was applied as a 1-μl droplet to the midpoint of the adaxial surface of the second fully expanded leaf of each plant. Four plants (including roots) of each population were harvested at 24, 48, and 72 h after treatment. The treated leaf of each plant was rinsed with 20 ml 20% (v/v) ethanol plus 0.2% (v/v) Triton X-100 (Amresco, Solon, Ohio), and the radioactivity present in the rinse solution was quantified by liquid scintillation spectrometry (LSS) to determine unabsorbed radioactivity. Foliar uptake of [^{14}C]-diclofop was calculated from the difference between the radioactivity applied and that remained unabsorbed. The plants were blotted dry, pressed, and oven-dried at 70 C for 48 h, then exposed to a phosphor-imager plate (BAS-IP MS 2040; Fuji Photo Film Co. Ltd., Kamakura, Japan) for 72 h before scanning for radioactivity. [^{14}C]-diclofop translocation was visualized using a phosphor-imager (Bio-Rad PMI, Sydney, Australia) and quantified by the volume analysis tool of the Quantity One software (version 4.6.7, Bio-Rad PMI). A volume is the total signal intensity inside a defined boundary drawn on an image. [^{14}C]-diclofop translocation is then expressed as a percentage of signal intensity in the application zone, above and below the application zone, over the total signal intensity in a defined resolvable ^{14}C image. This experiment was conducted twice.

[^{14}C]-Diclofop Metabolism Study using HPLC. Two-leaf–stage seedlings of R1 and S plants were treated with a treatment solution containing [^{14}C]-diclofop-methyl (2.5 kBq μl^{-1}), prepared as in the uptake and translocation studies with a final diclofop concentration of 2.95 mM (equivalent to 113 g ha^{-1}). The radiolabeled herbicide solution was applied as a 1-μl droplet, spread along the adaxial surface (close to the leaf base to facilitate translocation to the new growth) of the second fully expanded leaf of the plants. Treated plants were harvested 48 and 72 h after treatment. The treated leaf of each plant was rinsed as described above. The plants were blotted dry, snap-frozen in liquid N_2, and stored at −80 C until use. Five plants of each population were bulked as a replicate for each time point and three replicates of each sample were analyzed.

Extraction, separation, and identification of the parent herbicide and its metabolites were modified from Holtum et al. (1991). Briefly, plant tissue was ground in liquid N_2 with a prechilled mortar and pestle, and then homogenized with 5 ml of 80% (v/v) cold methanol. The crude homogenate was centrifuged at 8,000 × g for 15 min at 4 C. The supernatant was decanted and the residue was re-extracted with 1.5 ml

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80% cold methanol, followed by a final extraction with 1.5 ml 50% (v/v) cold methanol. The supernatants were pooled and recovered radioactivity was determined by LSS (recovery was 85 to 95%). The pooled supernatant (about 7 ml) was evaporated to dryness under vacuum with a SpeedVac (Savant, Farmingdale, NY), resuspended in 300 µl 50% (v/v) methanol, and centrifuged at 14,000 × *g* for 5 min. This step recovered 70 to 80% radioactivity in the supernatant in both R and S samples.

Parent herbicide and its metabolites were separated by gradient reverse-phase HPLC equipped with a 600E dual-head pump with 717 plus autosampler (Waters; Milford, MA). Separation was done on a Waters Spherisorb 5-µm ODS2 (250 mm long by 4.6 mm inner diam [i.d.]) column. Radioactivity was detected with an in-line Beta-RAM model 2B (IN/US Systems Inc., Pine Brook, NJ) detector. The solvents used were 10% acetonitrile : 89% water : 1% acetic acid (v/v/v) (solvent A), and 90% acetonitrile : 9% water : 1% acetic acid (v/v/v) (solvent B). The chromatographic conditions were according to Holtum et al. (1991) involving a 10-min linear gradient from 30 to 35% solvent B, followed by a 12-min linear gradient from 35 to 50% solvent B, then a 3-min linear gradient from 50 to 100% solvent B. The column was then flushed with 100% solvent B for 10 min, before re-equlibration under the initial conditions for 10 min prior to the next injection. Samples were filtered through a 0.22-µm Teflon filter before injection. Injection volumes (between 80 and 130 µl) were adjusted to provide similar total radioactivity loading in both resistant and susceptible samples. The flow rate of both the HPLC and the scintillant pump on the Beta-RAM detector was 1.5 ml min^{-1}. The proportion of the herbicide and metabolites were expressed as a percentage peak area of total radioactivity in the sample injection. This experiment was conducted twice. Wheat seedlings (variety 'EGA Bonnie Rock') were included in the metabolism study as a positive control. Growth conditions, herbicide treatment, sample harvesting, and extraction for wheat were performed as described for populations R1 and S.

Quantification of Tissue Diclofop Acid Level using UPLC-MS. UPLC-MS (or alternatively LC-MS) is an analytical chemistry technique that combines the physical separation capabilities of liquid chromatography with the detection and identification capabilities of mass spectrometry. More importantly for this study, UPLC-MS analysis may be potentially used for the early indication of enhanced rates of herbicide metabolism in resistant plants because this method has higher sensitivity (capable of detecting low-level herbicides and their metabolites without much sample preparation) and reproducibility, compared to the time-consuming and less sensitive conventional HPLC analysis. In addition, LC-MS does not require the use of radiolabeled herbicide (expensive, often unavailable). Rather, whole plants were sprayed with a commercial formulation of diclofop-methyl prior to sample extraction and analysis. This mimics spraying conditions in the field (exposing the whole plant to herbicide application), thus giving a more realistic and more accurate indication of herbicide metabolism status in plants.

At the three- to four-leaf stage, R and S plants were treated with a commercial formulation of diclofop-methyl (Hoegrass® 500; Bayer CropScience) at a rate of 1,000 g ha^{-1} using a cabinet sprayer delivering 118 L ha^{-1} water at a pressure of 200 kPa. This rate is the minimum discriminating rate between the R and S plants under CER conditions. Plants (aboveground tissue) were harvested 48 and 72 h after treatment. No visible symptoms of herbicide injury were observed for any of the plants at either harvest. Each harvest had two replicate samples of 10 plants each. Harvested plants were agitated in 300 ml water, blotted dry, frozen in liquid N_2, and stored at −80 C until use. Plant tissue was ground to powder in liquid N_2 with a prechilled mortar and pestle, and extracted with 80% methanol (v/v) at room temperature. The sample was ultrasonicated (Unisonics, Brookvale, NSW, Australia) for 10 min followed by 10 min of centrifugation at 1,000 × *g*. The supernatant was filtered through a 0.25-µm membrane and used for UPLC-MS analysis.

The UPLC-MS/MS analysis was performed using an Agilent HPLC 1200 series connected to an Agilent 6410 Triple Quaddrupole LC-MS system (Agilent, Santa Clara, CA). The instrument control and data acquisition were performed using Agilent MassHunter Workstation software. The chromatographic analysis was performed using a Luna 2.5µ C18 (2) HPLC column (50 by 3.00 mm i.d., 2.5 µm particle size, Phenomenex, Torrance, CA). The mobile phase consisted of acetonitrile (solvent A) and 10 mM ammonium formate (solvent B) pH 3 with a gradient of 10 to 100% A in 20 min and the flow rate of 0.5 ml min^{-1}. The column was washed with 100% A for 3 min, then equilibrated with 10% A for 8 min.

For MS analysis, multiple reaction monitoring (MRM) mode and negative ionization were applied. The fragment voltage was 135 V and collision energy was 15 V, and the precursor and product ions of diclofop acid were monitored at 325 and 253, respectively. Diclofop acid was identified by comparing retention time and MRM transition (325 to 253) of the peak with those of the standard. Diclofop acid levels in the samples were quantified using a standard curve.

Statistical Analyses. The I_{50} was estimated by nonlinear regression using the logistic model:

$$y = C + \frac{D - C}{1 + (x/ED_{50})^b}$$

where *C* is the lower limit at indefinitely large doses, *D* is the upper limit which is close to the untreated controls, ED_{50} is the dose giving 50% response and b is the slope around ED_{50}. Estimates were obtained using Sigmaplot® (version 12.0, Systat Software Inc., San Jose, CA). Data was subjected to one-way ANOVA and significant differences between the R1 and S populations in uptake and translocation, metabolism, and I_{50} values were determined by unpaired *t* test (GraphPad Prism®: GraphPad Software, Inc., San Diego, CA), and significant differences in tissue diclofop acid content among S, R1, R2, R3, and R4 populations were analyzed using Duncan's test (SPSS software version 11.5 for Windows, Chicago , IL).

Results and Discussion

Resistance Is Not ACCase Based. ACCase activities were assayed on partially purified extracts of R1 vs. S leaf material. In the absence of herbicides, the specific activity of ACCase extracted from R1 and S plants (1.8 ± 0.1 and 1.6 ± 0.2 nmol fixed CO_2 mg^{-1} protein min^{-1}, respectively) were

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Table 1. Parameters of the nonlinear logistic analysis of the herbicide dose required to cause 50% inhibition of ACCase activity (I_{50}) in the susceptible (S) and progeny lines of resistant (R1) wild oat populations. Standard errors are in parentheses.

Population	Herbicide	D^a	C^a	b^a	I_{50} (μM)	r^2 (coefficient)
S	Diclofop	97.1 (1.9)	3.3 (6.6)	−0.91 (0.2)	7.9 (1.8) a[b]	0.99
R1		98.6 (1.2)	4.2 (5.8)	−0.9 (0.1)	10.8 (2.1) a	0.99
S	Fenoxaprop	107.9 (9.2)	−1.5 (6.5)	−0.6 (0.2)	1.9 (0.25) a	0.99
R1		111.5 (11.5)	−3.3 (7.5)	−0.5 (0.1)	1.8 (0.03) a	0.99
S	Sethoxydim	99.4 (0.9)	14.8 (1.3)	0.8 (0.04)	1.7 (0.13) a	0.99
R1		99.1 (1.2)	23.4 (1.9)	−0.8 (0.1)	1.8 (0.23) a	0.99
S	Clethodim	99.4 (0.5)	3.5 (1.5)	−0.8 (0.02)	0.5 (0.03) a	0.99
R1		98.6 (1.5)	−1.4 (6.9)	−0.8 (0.1)	0.9 (0.21) a	0.99

[a] Abbreviations: *C*, lower limit; *D*, upper limit; b, the slope around the dose giving 50% response.
[b] Means with same letters in the I_{50} column for each herbicide are not significantly different (*t* test, $\alpha = 0.05$).

similar. In the presence of ACCase-inhibiting herbicides, ACCase from R1 and S plants was equally sensitive to inhibition by fenoxaprop (Table 1), whereas the I_{50} values for diclofop, sethoxydim, and clethodim in population R1 were slightly higher than, but not significantly different from, the S population.

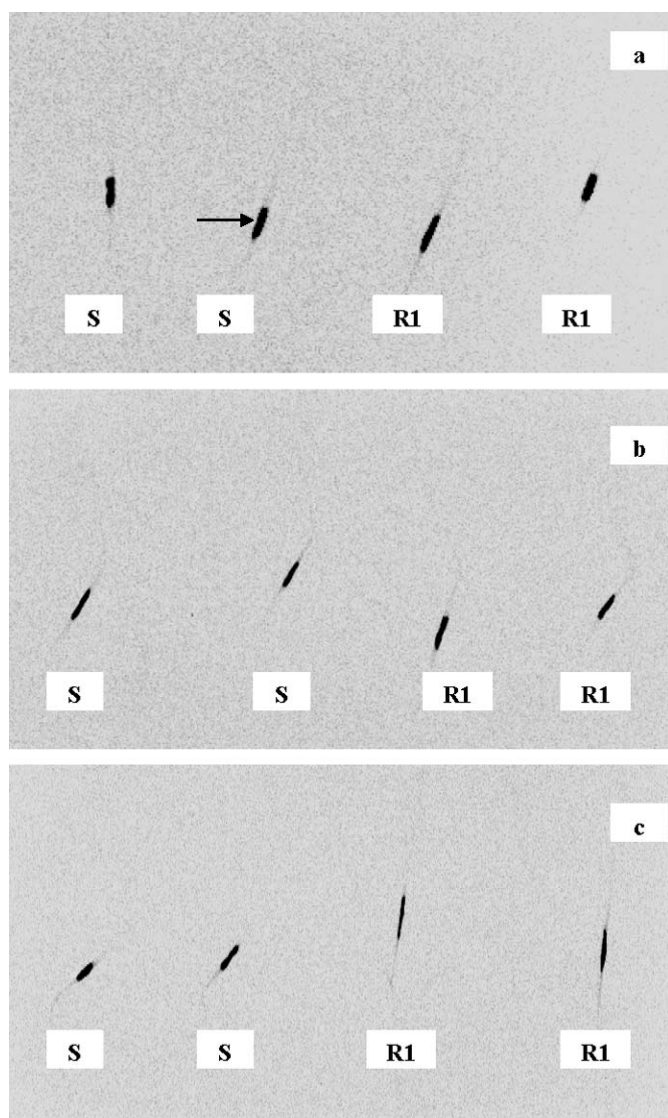

Figure 1. Phosphor-images comparing the translocation pattern of [^{14}C]-diclofop between susceptible (S) and resistant (R1) wild oat populations at 24 (a), 48 (b), and 72 (c) h after treatment. [^{14}C]-diclofop-methyl was applied as a 1-μl droplet to the midpoint (as shown by arrow) of the adaxial surface of the second leaf of each plant.

For the wild oat population R1 we have established through plastidic ACCase CT domain gene sequencing and polymerase chain reaction–based marker analysis that resistant individuals do not possess any known potential ACCase gene resistance mutations (Yu et al. 2012). The in vitro ACCase activity assay confirmed this as R1 plants have ACCase herbicide I_{50} values similar to S plants (Table 1). This result, and the similar specific ACCase activities observed in the R1 and S plants, establishes that the resistance mechanism (or mechanisms) in resistant individuals of population R1 is not ACCase target-site based.

Resistance Is Not Herbicide Foliar Uptake or Translocation Based. The [^{14}C]-diclofop-methyl rapidly entered leaves of both R1 and S plants, with more than 94% leaf uptake by 24 h after treatment (94.8 ± 2.3 and 94.2 ± 1.9 of total applied ^{14}C for R and S, respectively). At 48 h after treatment, 97% of the applied herbicide was within the leaf (97.5 ± 0.9 and 96.9 ± 1.1 for R1 and S, respectively), with no further leaf uptake evident in both populations at 72 h after treatment (97.7 ± 1.1 and 96.5 ± 1.5 for R1 and S, respectively). Leaf uptake could not explain resistance as ANOVA indicates that there were no significant differences in [^{14}C]-diclofop-methyl uptake between the R1 and S plants at any sampling time point. Likewise, resistance in R1 is not due to differential rates of herbicide translocation because the translocation study (phosphor-imaging) revealed very similar [^{14}C]-radioactivity distribution pattern between R1 and S plants at 24, 48, and 72 h after treatment (Figures 1 a–c). Almost all radioactivity remained in the treated leaves, as quantified by the volume analysis, with less than 5% of ^{14}C being translocated to other parts of the plants (Table 2). ANOVA showed no significant differences in acropetal (upward) or basipetal (downward) translocation of ^{14}C between the R1 and S plants at any sampling time point. Therefore, neither decreased herbicide absorption/uptake, nor reduced herbicide translocation contributes to diclofop-methyl resistance in R1plants.

Observed limited diclofop translocation in this study is consistent with previous findings (Boldt and Putnam 1980; Cruz-Hipolito et al. 2011; Gronwald et al. 1992; Maneechote et al. 1997; Seefeldt et al. 1996), showing that more than 80% of the absorbed diclofop-methyl remained at the site of application. To date, reduced diclofop-methyl absorption/penetration in grass weed species has only rarely been reported in wild oat (Nandula and Messersmith 2003) and rigid ryegrass (*Lolium rigidum* Gaudin) (De Prado et al. 2005).

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Table 2. Translocation (acropetal and basipetal from the application point) of [^{14}C]-diclofop, expressed as a percentage of the total absorbed radioactivity by volume analysis in susceptible (S) and resistant (R1) wild oat plants at 24, 48, and 72 h after treatment.

		Radioactivity (% of [^{14}C]-diclofop absorbed)	
Population	Time (hours after treatment)	Acropetal translocation	Basipetal translocation
S	24	2.6 a[a]	2.6 a
R1	24	2.5 a	3.0 a
S	48	3.1 a	4.2 a
R1	48	2.9 a	3.8 a
S	72	4.1 a	4.1 a
R1	72	3.4 a	3.6 a

[a] Means with same letters in a column for each time point are not significantly different (t test, $\alpha = 0.05$).

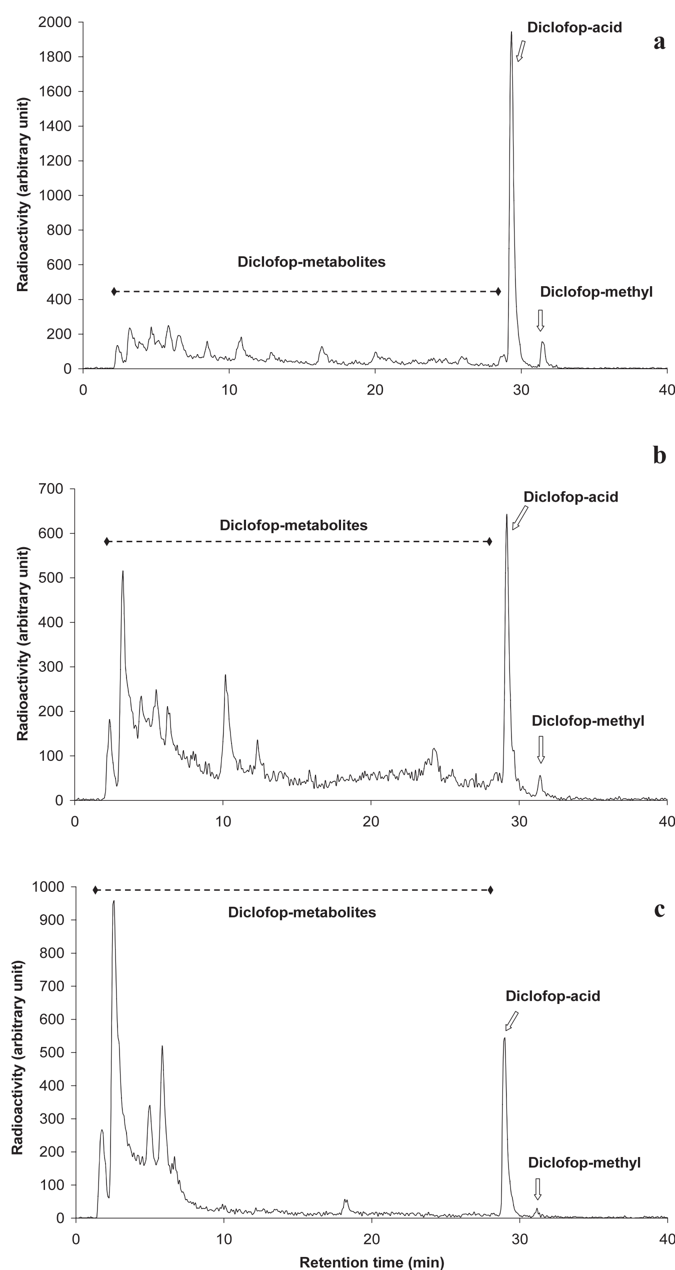

Figure 2. High-performance liquid chomotography chromatograms showing the elution profiles of diclofop-methyl and diclofop-acid and its metabolites in S (a) and R1 (b) wild oat populations, and in wheat (c), 48 h after treatment.

Table 3. Metabolism of [^{14}C]-diclofop by susceptible (S) and resistant (R1) wild oat plants 48 and 72 h after treatment. The proportion of the herbicide and its metabolites were expressed as a percentage peak area of total radioactivity in the sample injection by the high-performance liquid chromatography analysis.

		Radioactivity (% of [^{14}C] recovered in the extracts)		
Population	Time (hours after treatment)	Diclofop metabolites	Diclofop-acid	Diclofop-methyl
S	48	42.53 a[a]	53.63 a	3.84 a
R1	48	71.50 b	26.44 b	2.05 b
S	72	59.69 a	37.00 a	3.32 a
R1	72	78.06 b	20.48 b	1.46 b

[a] Means with different letters in a column for each time point are significantly different (t test, $\alpha = 0.05$).

Similarly, translocation of [^{14}C]-diclofop was not found to differ between R and S biotypes of various grass weed species (De Prado et al. 2000; Holtum et al. 1991; Maneechote et al. 1994, 1997; Marles et al. 1993; Shimabukuro et al. 1979; Tardif and Powles 1994).

Resistance Is Due to Differential Rates of Diclofop Acid Metabolism. The initial de-esterification of diclofop-methyl into phytotoxic diclofop acid and then further conversion into nontoxic polar metabolites in R1 vs. S plants was assessed 48 and 72 h after treatment (Table 3). As shown in Figure 2, diclofop-methyl, diclofop acid, and its polar metabolites were clearly resolved at 32, 29, and 2 to 28 min, respectively, under our HPLC conditions. Both R1 and S plants rapidly de-esterified the absorbed diclofop-methyl to diclofop acid, with HPLC analysis revealing less than 5% of the applied ^{14}C being recovered as diclofop-methyl after 48 and 72 h. R1 plants showed significantly more rapid de-esterification of diclofop-methyl than the S plants (Table 3), establishing that differential rates of de-esterification could not explain resistance. Importantly, the amount of radioactivity present as phytotoxic diclofop acid was always significantly higher in S vs. R plants. By 48 h after treatment, the S plants had twofold higher levels of diclofop acid than the R1 plants. At 72 h, the level of diclofop acid decreased in both populations, but the diclofop acid pool in the S plants was still double (1.9-fold) that in R1 plants.

The decrease in diclofop acid correlated with an increase in nontoxic polar metabolites in both the R1 and S populations. Importantly, at all times, R1 plants had significantly more (1.3- to 1.7-fold) radioactivity in polar metabolites than the S plants (Table 3). At 72 h after treatment, the net conversion of diclofop acid to polar metabolites was 78% in R1 vs. 60% in S plants. These results show that although both R and S plants are able to metabolize the phytotoxic diclofop acid to nontoxic products, R1 plants metabolize diclofop acid faster than S plants. This faster herbicide metabolic rates minimize the level of phytotoxic diclofop acid reaching the target site ACCase, thus limiting injury in R1 plants.

Enhanced diclofop acid metabolism rates have been reported in resistant sterile oat (Maneechote et al. 1997), blackgrass (Hall et al. 1997), rigid ryegrass (Holtum et al. 1991), and Italian ryegrass (*Lolium multiflorum* Lam.)(Cocker et al. 2001). Although the increase in the rate of metabolism of diclofop acid in wild oat population R1 is relatively small, this non–target-site-based mechanism may be sufficient to account for the higher resistance to diclofop-methyl at the whole-plant level, similar to observations in resistant blackgrass (Hall et al. 1997),

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rigid ryegrass (Preston and Powles 1998), and Italian ryegrass (Cocker et al. 2001). Likewise, a modest increase (1.3- to 1.5-fold) in metabolism rates of the ACCase herbicide fluazifop acid has been found to confer considerable levels (7- to 20-fold) of resistance in large crabgrass (Hidayat and Preston, 1997) and wall barley (*Hordeum leporinum* Link.) (Matthews et al. 2000).

The diclofop metabolism pattern between R and S wild oat populations was compared with wheat (Figure 2). HPLC elution profiles showed similar, but not identical, metabolism patterns between wild oat and wheat: both had polar metabolite peaks with retention times between 2 and 6 min, but R1 plants also had metabolite peaks with lower polarity at retention time between 10 and 28 min (Figure 2).

Wheat is well known to tolerate ACCase herbicides due to enhanced rates of herbicide metabolism (Donald and Shimabukuro, 1980; Jacobson and Shimabukuro 1984; Romano et al. 1993). Following de-esterification of diclofop-methyl, diclofop acid is subject to the irreversible process of aryl hydroxylation, followed by conjugation to an acidic aryl glycoside (Shimabukuro et al. 1979; Tanaka et al. 1990). The major polar metabolites in wheat have been identified as mainly aryl-hydroxylated diclofop acid and aryl-hydroxylated sugar conjugates; less common metabolites, though not identified, are probably ester-sugar conjugates (Shimabukuro et al. 1979). Aryl hydroxylation of diclofop in wheat is catalyzed by cytochrome P450 monooxygenase (Zimmerlin and Durst 1990), with wheat lauric acid hydroxylase responsible for aryl hydroxylation of diclofop acid in wheat microsomes (Zimmerlin et al. 1992). Thus, phytotoxic diclofop acid is removed and the nontoxic aryl glycoside accumulates rapidly in wheat, as well as in resistant grass species (Devine and Shimabukuro 1994). In susceptible oat or wild oat, however, the diclofop acid is reversibly conjugated with glucose to form an ester, which can be rehydrolized to release phytotoxic diclofop acid, causing phytotoxicity to the plants (Shimabukuro et al. 1987). Although we did not further identify diclofop acid metabolites, the major polar metabolites in R1 plants were clearly resolved and are chromatographically similar to those in wheat. These results suggest that enhanced rates of diclofop acid metabolism in wild oat is likely to involve a wheat-like P450-mediated metabolic detoxification pathway, and that there is a greater capacity in resistant plants than in susceptible plants. The nature of the polar metabolites and enzymes/genes involved in diclofop metabolism in R1 remain to be determined.

Multiple ACCase Herbicide Resistance Mechanisms in Wild Oats. Diclofop acid levels in leaf material of two susceptible (S and S1) and four resistant (R1, R2, R3, and R4) wild oat populations were determined by the UPLC-MS method (Table 4). The average diclofop acid content of the S and S1 populations was used as a control for each harvesting time. At each sampling time point, the diclofop acid level was significantly lower in all four R populations compared to the two S populations. Resistant plants with no known ACCase resistance mutations (population R1) had the lowest level of diclofop acid (13%), suggesting they had the highest rate of diclofop acid metabolism. Importantly, significantly lower diclofop acid levels were also observed in resistant individuals possessing ACCase resistance mutations (populations R2, R3, and R4), indicating the possible co-occurrence of non–target-site enhanced diclofop metabolism and a resistant ACCase

Table 4. Liquid chromatography–mass spectrometry analysis of tissue diclofop levels (μg g^{-1} Fresh weight) in the susceptible (S, S1) and resistant (R2, R3, and R4) wild oat populations at 48 and 72 h after treatment.

Population (mutation)	Time (hours after treatment): 48 h		72 h	
	Diclofop acid	% S control	Diclofop acid	% S control
S populations (S + S1)	4.79 a[a]	100	2.94 a	100
R1, no ACCase mutation	0.62 d	13	0.62 e	21
R2 (1781)	2.30 bc	48	1.17 cd	40
R2 (2078)	2.90 b	61	0.86 de	29
R2 (2088)	1.88 c	39	1.45 bc	49
R3 (2078)	2.80 b	58	1.57 bc	53
R3 (2088)	1.68 c	35	1.31 bcd	44
R4 (2078)	1.72 c	36	1.68 b	57

[a] Means with different letters in a column for each time point are significantly different (*t* test, $\alpha = 0.05$).

(Table 4). As differential foliar uptake of diclofop methyl or de-esterification is unlikely in R and S populations, the lower levels of diclofop acid in R populations is likely due to faster rates of diclofop metabolism than in the S populations.

Previously, it was shown that resistant plants in populations R2, R3, and R4 all possess target-site ACCase gene resistance mutations (Yu et al. 2012). Often, studies to establish herbicide resistance mechanisms examine only for target-site gene mutations and therefore ignore the distinct likelihood that non–target-site resistance mechanisms may coexist. Based on the observations that high-level diclofop-methyl resistance (> 20-fold) observed at the whole plant level (Ahmad-Hamdani et al., 2011) was not consistent with the low diclofop ACCase I_{50} R/S ratios (1.7- to 2-fold; Yu et al. 2012), we hypothesized that in addition to ACCase gene mutations these resistant populations R2, R3, and R4 may also possess a non–target-site resistance mechanism (or mechanisms). The UPLC-MS analysis, showing lower level of diclofop acid content in these target-site-resistance populations, indicates that enhanced rates of diclofop acid metabolism are likely present in these plants (Table 4). A genetic segregation study will help to resolve these multiple resistance mechanisms in these wild oat populations.

Multiple resistance mechanisms to ACCase herbicides in grass weed species have been reported in diploid *ryegrass* (Tardif and Powles 1994; Preston et al. 1996; Preston and Powles 1998; Scarabel et al. 2011), diploid blackgrass (Hall et al. 1997; Letouzé and Gasquez 2001), and tetraploid barley grass (Matthews et al. 2000). Nevertheless, unlike the obligate cross-pollinated species such as rigid ryegrass and blackgrass, with high frequency of gene/allele exchange, wild oats are predominantly self-pollinated, which means there is very limited gene exchange between individuals. This is probably why multiple resistance mechanisms to ACCase herbicides in wild oat had previously only been established in one study (Maneechote et al. 1997). Here, this study provides molecular and biochemical evidence that at least two distinct mechanisms, target-site ACCase gene mutations and non–target-site enhanced rates of herbicide metabolism coexist in the same individuals and confer high-level resistance to ACCase herbicides.

To conclude, ACCase herbicide resistance in the population R1, which has no ACCase gene resistance mutations, is due to enhanced rates of ACCase herbicide metabolism. The wheat-like herbicide metabolism pattern is likely to involve cytochrome P450 monooxygenase, which can also confer

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cross-resistance to other ACCase herbicides. Three other ACCase herbicide-resistant wild oat populations that exhibit ACCase gene mutations also exhibit a lower level of tissue diclofop acid (likely due to enhanced rates of herbicide metabolism). Thus it is evident that highly self-pollinated hexaploid wild oat can accumulate multiple (target-site and non–target-site) resistance mechanisms to herbicides of the same mode of action, conferring high-level resistance. Importantly, all four resistant wild oat populations examined in this research displayed enhanced herbicide metabolism, indicating that it is probably a common resistance mechanism in ACCase herbicide-resistant wild oat populations, although more resistant populations need to be examined.

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