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Colleagues,

For your information please find attached a just published AHRI research paper in the journal Plant Cell and Environment (2013) Vol 36, pp 818-827 by Yu et al entitled "Enhanced rates of herbicide metabolism in low herbicide dose selected resistant *Lolium rigidum*."

For many years we have been studying non-target site based herbicide resistance (NTSR), especially in *Lolium*. In 2005 Paul Neve showed that recurrent selection of a normal herbicide susceptible *Lolium* population for just three generations at low diclofop-methyl dose resulted in resistance evolution (Neve & Powles, Theoretical & Applied Genetics 2005). Since this study further AHRI work conducted with initially herbicide susceptible *Lolium* populations selected at low, sub-lethal rates of metabolisable herbicides has resulted in low-dose selected rapid resistance evolution. It is now clear in a genetically diverse, cross-pollinated species such as *Lolium* that recurrent low herbicide dose can rapidly lead to herbicide resistance evolution. This is especially so for herbicides like diclofop-methyl which can be metabolised by P450's. In this latest paper (attached) there is clear evidence, as expected, that the basis of the non-target site based (NTSR) resistance is enhanced rates of herbicide metabolism. This enhanced metabolism is likely due to P450's, although direct evidence is yet to be obtained (see below).

The great threat of using herbicides at low rates on genetically diverse cross-pollinated species is the evolution of metabolism based herbicide resistance in which, as in this case, there can be non-target site cross resistance to very different herbicides, if they can be metabolised. Herbicides should always be used at full label rates.

This study and several others that we have conducted on NTSR due to enhanced rates of herbicide metabolism has now resulted in an international collaboration in which Bayer CropScience Frankfurt have employed Todd Gaines to conduct a next-generation sequencing project to identify the genes responsible for NTSR in *Lolium*. The objective is to identify the specific genes involved.

Thank you,

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Enhanced rates of herbicide metabolism in low herbicide-dose selected resistant *Lolium rigidum*

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ABSTRACT

Lolium rigidum is an obligately cross-pollinated, genetically diverse species and an economically important herbicide resistance-prone weed. Our previous work has demonstrated that recurrent selection of initially susceptible *L. rigidum* populations with low herbicide rates results in rapid herbicide resistance evolution. Here we report on the mechanisms endowing low-dose-selected diclofop-methyl resistance in *L. rigidum*. Results showed that resistance was not due to target-site ACCase mutations or overproduction, or differential herbicide leaf uptake and translocation. The *in vivo* de-esterification of diclofop-methyl into phytotoxic diclofop acid was rapid and similar in resistant versus susceptible populations. However, further metabolism of diclofop acid into non-toxic metabolites was always faster in resistant plants than susceptible plants, resulting in up to 2.6-fold lower level of diclofop acid in resistant plants. This corresponded well with up to twofold higher level of diclofop acid metabolites in resistant plants. The major polar metabolites of diclofop acid chromatographically resembled those of wheat, a naturally tolerant species. Clearly, recurrent selection at reduced herbicide rates selected for non-target-site-based enhanced rates of herbicide metabolism, likely involving cytochrome P450 monooxygenases.

Key-words: cytochrome P450; diclofop-methyl; low herbicide rate; resistance evolution.

INTRODUCTION

Lolium rigidum, an economically important global weed, is an obligate cross-pollinated, genetically diverse species able to rapidly evolve herbicide resistance under herbicide selection pressure. For example, resistance to acetyl CoA carboxylase (ACCase)-inhibiting herbicides (hereafter referred to as ACCase herbicides) was evident in *L. rigidum* within only 3 years of their use (Heap & Knight 1982; Tardif, Holtum & Powles 1993). What is striking was that there was cross-resistance to the completely dissimilar acetolactate synthase (ALS)-inhibiting herbicides (thereafter referred to as ALS herbicides) without any previous use of these herbicides (Heap & Knight 1986; Christopher *et al.* 1991). Resistance to ACCase herbicides can be endowed by target-site-based ACCase gene mutations and/or non-target-site-based enhanced herbicide metabolism (Délye 2005; Powles & Yu

2010). While target-site-based resistance-endowing ACCase mutations can be precisely determined and therefore have been widely reported, non-target-site resistance mechanisms have been under-investigated, underestimated and/or masked by target-site resistance mutations. Nevertheless, although direct evidence is often lacking, non-target-site-based metabolic resistance mediated by cytochrome P450 monooxygenases (P450s) and/or glutathione S-transferases (GSTs) is now increasingly implicated as a herbicide resistance mechanism (reviewed by Preston 2004; Powles & Yu 2010; Délye *et al.* 2011; Beckie & Tardif 2012). Cytochrome P450s and GSTs belong to large and biochemically diverse enzyme families. Some P450s or GSTs can metabolize certain herbicides to products with reduced or modified phytotoxicity, conferring herbicide tolerance in major crops, or resistance in weeds (reviewed by Werck-Reichhart, Hehn & Didierjean *et al.* 2000; Siminszky 2006; Yuan, Tranel & Stewart 2007; Cummins *et al.* 2011). It is likely that several P450 isoforms are involved in conferring P450-mediated metabolic resistance to ACCase herbicides and associated cross-resistance to the dissimilar ALS herbicides in *L. rigidum* (Christopher *et al.* 1991). Compared with high level understanding of the important roles of P450s and GSTs in insecticide resistance (Li, Schuler & Berenbaum 2007), metabolic herbicide resistance and evolution in plants are poorly understood.

Herbicide resistance evolution is a dynamic process influenced by the biology of a particular weed species, genetics and herbicide-related factors (Maxwell & Mortimer 1994; Jasieniuk, BruleBabel & Morrison 1996; Powles & Yu 2010). Among these, herbicide use rate (i.e. the intensity of selection pressure) is an important but often under-appreciated factor. Our recent studies with herbicide susceptible *L. rigidum* populations have demonstrated that recurrent selection with reduced rates (lower than registered field rates) of the P450-metabolizable ACCase herbicide diclofop-methyl results in rapid herbicide resistance evolution to this herbicide over only three generations (Neve & Powles 2005a,b; Manalil *et al.* 2011). The diclofop-resistant *L. rigidum* populations are concomitantly cross-resistant to other ACCase herbicides as well as, importantly, to certain metabolizable members of the chemically unrelated, dissimilar ALS herbicides (Neve & Powles 2005a; Manalil *et al.* 2011). This low-dose selected resistance is polygenic (Busi, Neve & Powles 2012) and postulated to be non-target-site-based metabolic resistance (Neve & Powles 2005a). Here, we establish that recurrent selection at low herbicide rates selects for non-target-site-based metabolic resistance, likely involving cytochrome P450s.

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MATERIALS AND METHODS

Plant material

Four *L. rigidum* populations were used in this study: two parent herbicide susceptible populations VLR1 (Neve & Powles 2005a) and WALR1 (Manalil *et al.* 2011) (hereafter referred to as S1 and S2, respectively), and two ACCase herbicide-resistant populations derived from low-dose diclofop-methyl selection of S1 (Neve & Powles 2005a, hereafter referred to as R1) and S2 (Manalil *et al.* 2011, hereafter referred to as R2). For ACCase *in vitro* activity and the ultra performance liquid chromatography-mass spectrometry (UPLC-MS) analysis, seeds were germinated in plastic trays (300 × 400 × 100 mm) containing potting mix (25% peat moss, 25% river sand and 50% mulched pine bark) and seedlings were grown in a glasshouse at day/night temperatures of 20–25/10–15 °C under natural light. For [¹⁴C]-diclofop-methyl uptake, translocation and metabolism experiments, seeds were germinated in small plastic cups (60 × 60 × 100 mm), and seedlings (one seedling per cup) were grown in a growth chamber at day/night temperatures of 18–20/13–15 °C, photon flux density of 500–600 μmol m⁻² s⁻¹, a photoperiod of 12 h and relative humidity of 70–75%.

Herbicide treatment

The ACCase herbicide diclofop-methyl was applied as a commercial formulation at rates as specified below in 118 L ha⁻¹ water, delivered in two passes at 200 kPa with a cabinet sprayer equipped with two flat fan nozzles. After spraying, plants were returned to the glasshouse or growth chamber at the particular temperatures indicated.

In vitro ACCase activity assay

Shoots of two- to three-leaf stage *L. rigidum* seedlings without herbicide treatment were harvested, snap-frozen in liquid N₂ and stored at –80 °C until use. Generally, one sample contained 30–40 individual plants (about 3–4 g fresh weight) and three samples were harvested for each population. ACCase extraction and partial purification and enzyme activity measurements were performed according to Yu *et al.* (2004). The specific ACCase activity and ACCase sensitivity to herbicide were determined in the absence and presence of fenoxaprop acid, respectively. Two subsamples from each extraction were assayed, and there were at least two to three extractions per population. Technical grade fenoxaprop acid (96% purity) was used in assays to determine the herbicide concentration causing 50% inhibition of ACCase activity (I₅₀). Fenoxaprop acid stock solution was prepared in 100 mM Tricine buffer (containing 10% acetone, with a final concentration of <1% acetone in the assay mix). Tissue soluble protein concentration was determined by the Bradford method (Bradford 1976).

[¹⁴C]-diclofop-methyl foliar uptake and translocation

[¹⁴C]-diclofop-methyl (dichlorophenyl-UL-¹⁴C) with specific activity of 6.6 MBq mg⁻¹ was obtained from Bayer Crop-Science® (Bayer Cropscience, Frankfurt, Germany). To mimic the field situation, resistant (R) and susceptible (S) seedlings at the two- to three-leaf stages were foliar-sprayed with (non-radiolabelled) diclofop 50 g ha⁻¹, which is one-eighth of the Australian recommended field rate of diclofop-methyl (375 g ha⁻¹), and gave 10% control of the S populations under the above experimental conditions. Three hours after the treatment, one droplet (1 μL) of the treatment solution containing [¹⁴C]-diclofop-methyl (approximately 0.76 kBq μL⁻¹) was applied to the midpoint of the adaxial surface of the second fully expanded leaf of each plant. [¹⁴C]-diclofop-methyl was dissolved in a small volume of acetonitrile and diluted in water containing 0.25% (v/v) non-ionic surfactant BS1000. The final diclofop concentration in the treatment solution was 0.45 mM (concentration equivalent to 17 g ha⁻¹). Five 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, OH, USA), and the radioactivity present in the rinse solution was quantified by liquid scintillation spectrometry (LSS) to determine unabsorbed radioactivity. Foliar uptake of [¹⁴C]-diclofop-methyl was calculated from the difference between the radioactivity applied and that in the rinse solution. 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, Tokyo, Japan) for 24 h before scanning for radioactivity. [¹⁴C]-diclofop-methyl translocation was visualized using a phosphor-imager (Bio-Rad PMI, Sydney, Australia), quantified and expressed as a percentage of signal intensity inside, above and below the application zone, over the total signal intensity in a defined resolvable ¹⁴C image by the volume analysis tool of the Quantity One software (version 4.6.7; Bio-Rad PMI). The total radioactivity applied to single plants (0.76 kBq) in this experiment helped to avoid local overexposure of the application site to the imaging plate (based on preliminary experiments).

Analysis of [¹⁴C]-diclofop-methyl metabolism using high-performance liquid chromatography (HPLC)

Two- to three-leaf stage R and S seedlings were first foliar-sprayed with 50 g ha⁻¹ diclofop-methyl as for the uptake and translocation experiments, and then treated with a solution containing [¹⁴C]-diclofop-methyl (approximately 5.2 kBq μL⁻¹), prepared as above but with a final diclofop concentration of about 3 mM (concentration equivalent to 115 g ha⁻¹). The treatment solution was applied as a 1 μL droplet and spread along the adaxial surface (close to the leaf base) of the second fully expanded leaf. Treated plants showing no symptoms of diclofop damage were harvested (above-ground) 48 and 96 h after treatment. The treated leaf

of each plant was rinsed as described above, blotted dry, snap-frozen in liquid N₂ and stored at -80 °C until extraction and analysis. Six plants of each population were bulked as a replicate sample for each time point and two to three replicate samples per time point per population were analysed.

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 N₂ with a pre-chilled mortar and pestle, and then homogenized with 5 mL of 80% (v/v) cold methanol. The crude homogenate was centrifuged at 8000 g for 15 min at 4 °C. The supernatant was decanted and the residue was re-extracted with 1.5 mL 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 85–95%). The pooled supernatant (8 mL) was spin-evaporated under vacuum to remove methanol and freeze-dried to dryness, resuspended in 300 µL 50% (v/v) methanol, and centrifuged at 14 000 g for 5 min. This step recovered 70–80% 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, USA). Separation was conducted on a Waters Spherisorb 5 µm ODS2 (250 mm long × 4.6 mm i.d.) column. Radioactivity was detected with an in-line Beta-RAM model 2B (IN/US Systems Inc., Pine Brook, NJ, USA) detector. The solvents used were 10% acetonitrile:89% water:1% acetic acid (v/v/v) (A), and 90% acetonitrile:9% water:1% acetic acid (v/v/v) (B). Chromatographic conditions were according to Holtum *et al.* (1991) and involved 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% B. The column was then flushed with 100% B for 10 min, before re-equilibration under the initial conditions for 10 min prior to the next injection. The flow rate of both the HPLC and the scintillant pump on the Beta-RAM detector was 1.5 mL min⁻¹. Samples were filtered through a 0.22 µm Teflon filter before injection. Injection volumes (80–120 µL) were adjusted to provide similar total radioactivity in paired R and S samples in each experiment. The proportion of the herbicide and metabolites was expressed as percentage peak area of total radioactivity in the sample injection. Wheat seedlings (variety EGA Bonnie Rock), known to efficiently metabolize diclofop-methyl, were included as a positive control. Growth conditions, herbicide treatment, sample harvesting and extraction for wheat were performed as described for R and S *L. rigidum* plants. To reduce variation within an experiment caused by handling a large number of samples, the experiments were conducted each time in pairs (S1 versus R1 or S2 versus R2).

Quantification of diclofop acid content in above-ground tissues using UPLC-MS

UPLC-MS was used to determine above-ground tissue diclofop acid content in an effort to develop a quick diagnostic

tool for enhanced herbicide metabolism via a non-radioactive approach. R and S seedlings at the two- to three-leaf stages were analysed following foliar spray of half the field rate (188 g ha⁻¹) of a commercial formulation of diclofop-methyl. This mimics spraying conditions in the field, thus giving a better and more accurate indication of herbicide metabolism status *in planta*. Above-ground plant parts were harvested at 24, 48 and 72 h after treatment. No visible symptoms of herbicide injury were observed for any of the plants at each harvest. Each harvest had two replicate samples of 10–15 plants and these were agitated in 300 mL water containing 20% methanol plus 0.2% (v/v) Triton® X-100, blotted dry, snap-frozen in liquid N₂, and stored at -80 °C until being freeze-dried. Plant tissue (about 0.1 g dry weight) was ground into powder in liquid N₂ with a mortar and pestle, and then extracted with 80% methanol (v/v) at room temperature. The sample was ultrasonicated for 10 min followed by 10 min centrifugation at 1000 g. The supernatant was filtered through a 0.25 µm membrane and used for UPLC-MS analysis. The UPLC-MS analysis was performed using an Agilent HPLC 1100 series equipped with a Luna 2.5 µm C18 (2) HPLC column (50 × 3.00 mm i.d., 2.5 µm particle size; Phenomenex, Torrance, CA, USA). The UPLC system was connected to an Agilent 6410 Triple Quadrupole LC-MS system (Agilent, Santa Clara, CA, USA) in multiple reaction monitoring (MRM) mode for ionization, and data were analysed by the Agilent MassHunter Workstation software (Agilent, Santa Clara, CA, USA). The mobile phase consisted of acetonitrile (A) and 10 mM ammonium formate (B) at pH 3 with a gradient of 10–100% A in 20 min and a flow rate of 0.5 mL min⁻¹. The column was washed with 100% A for 3 min, and then equilibrated with 10% A for 8 min. For MS analysis, MRM mode and negative ionization were applied. 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 (325–253) of the peak with those of the standard. Diclofop acid levels in the samples were quantified using a standard curve.

Statistical analysis

The I₅₀ was estimated by non-linear 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 close to the untreated controls, ED₅₀ is the dose giving 50% response, and *b* is the slope around ED₅₀. Estimates were obtained using Sigmaplot® version 12.0; Systat Software Inc., San Jose, CA, USA. Data were subjected to one-way analysis of variance (ANOVA) and significant differences between paired S and R populations in ACCase I₅₀ and herbicide uptake, translocation and metabolism were determined by the *t*-test (Graphpad Prism version 6.0; GraphPad Software, Inc., San Diego, CA, USA).

Population	ACCase specific activity (nmol HCO ⁻³ mg ⁻¹ protein min ⁻¹)	C	D	b	I ₅₀ (μM)
S1	8.14 (0.33) ^a	6.0 (5.3)	103 (6.1)	-0.69 (0.17)	1.07 (0.42) ^a
R1	8.20 (0.39) ^a	4.4 (4.4)	102 (5.2)	-0.67 (0.13)	0.86 (0.28) ^a
S2	9.3 (0.92) ^a	5.3 (3.1)	102 (3.7)	-0.76 (0.13)	1.02 (0.24) ^a
R2	8.28 (0.71) ^a	3.9 (2.8)	102 (3.3)	-0.7 (0.09)	1.03 (0.22) ^a

C is the lower limit, D the upper limit, and b the slope around I₅₀. Standard errors are in parentheses. Means with the same letter in a column for each paired S and R populations are not significantly different ($\alpha=0.05$) as determined by the *t*-test.

RESULTS

ACCase was herbicide susceptible in both S and R populations

In order to determine if target-site-based resistance mechanisms (ACCase gene resistance mutations or ACCase overproduction) were involved in herbicide resistance in these populations, ACCase activity was assayed in partially purified enzyme extracts of unselected S versus selected R plants. The extractable specific activity of ACCase from unselected S and R plants (Table 1) was found to be similar both between and across the S and R comparison pairs, and was equally sensitive to herbicide fenoxaprop acid inhibition (Fig. 1), with similar I₅₀ values (Table 1). These results clearly establish that, as expected, the resistance mechanism(s) in the two low-dose selected resistant populations (R1 and R2) is not ACCase based and therefore must be non-target-site based.

Foliar uptake and translocation of [¹⁴C]-diclofop were similar in S and R populations

Leaf [¹⁴C]-diclofop-methyl uptake was similar in both S and R plants, with more than 90% leaf uptake within 24 h (Table 2). At all sampling time points, there were no significant differences in rates of [¹⁴C]-diclofop-methyl uptake between the S and R plants.

In the translocation study, phosphor imaging revealed at all times a very similar [¹⁴C]-radioactivity distribution pattern between S and R plants (see Fig. 2a,b for representative images). The majority of the radioactivity remained in the treated leaves, and as quantified by the volume analysis, only a small amount of [¹⁴C] moved acropetally (upward) (average 10%, maximum 13%) or basipetally (downward) (average 15%, maximum 22%) from the application site, at 72 h after treatment (Table 2, Fig. 2). Overall, no significant differences in acropetal or basipetal translocation of [¹⁴C] between the S and R plants were found, except for a small but significant difference in acropetal translocation between S1 and R1 at 24 h. Therefore, diclofop resistance in these two resistant populations is not associated with differential herbicide foliar uptake or translocation.

Table 1. ACCase specific activity and parameters of the logistic analysis of ACCase herbicide dose required to cause 50% inhibition of ACCase activity (I₅₀) of the unselected susceptible (S) and selected resistant (R) *Lolium rigidum* populations

Resistant populations show faster [¹⁴C]-diclofop metabolism than S populations

The de-esterification of diclofop-methyl into the phytotoxic diclofop acid and further conversion into non-toxic polar metabolites in S and R plants were assessed 48 and 96 h after treatment. As shown in Fig. 3, diclofop-methyl, diclofop acid and its metabolites were clearly resolved at 32 min (peak 1), 29 min (peak 2), and between 2 and 15 min (peak 3 and unlabelled peaks), respectively, under our HPLC conditions. Diclofop-methyl is known to be rapidly de-esterified in

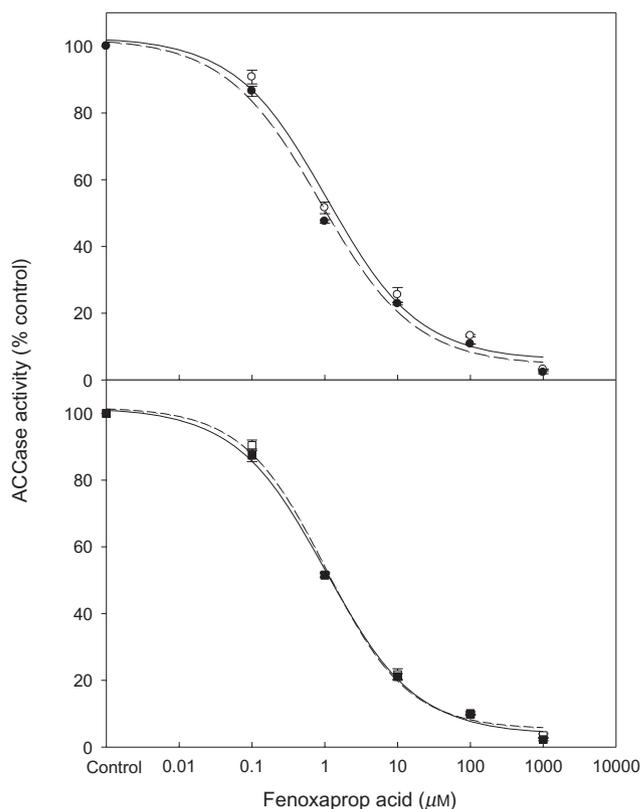


Figure 1. *In vitro* inhibition of ACCase activity by ACCase-inhibiting herbicide fenoxaprop acid of unselected susceptible (○ S1, □ S2) populations versus low-dose selected resistant (● R1, ■ R2) *Lolium rigidum* populations.

Population	Foliar uptake (% of [¹⁴ C]-diclofop applied)	Translocation (% of [¹⁴ C]-diclofop absorbed)	
		Acropetal	Basipetal
24 h			
S1	91 (0.64) ^a	10.3 (0.33) ^a	9.8 (0.21) ^a
R1	93 (0.68) ^a	8.2 (0.32) ^b	8.7 (0.74) ^a
S2	93 (0.61) ^a	8.8 (1.1) ^a	10.4 (1.14) ^a
R2	90 (1.63) ^a	10.0 (0.83) ^a	10.5 (0.74) ^a
48 h			
S1	94 (0.26) ^a	9.3 (1.23) ^a	14.3 (0.77) ^a
R1	95 (0.38) ^a	8.8 (0.52) ^a	15.0 (0.48) ^a
S2	95 (0.38) ^a	10.5 (1.37) ^a	16.6 (1.39) ^a
R2	95 (0.25) ^a	10.0 (0.61) ^a	17.1 (1.24) ^a
72 h			
S1	96 (0.17) ^a	10.9 (0.42) ^a	13.9 (0.16) ^a
R1	96 (0.30) ^a	11.6 (1.09) ^a	15.4 (0.43) ^b
S2	96 (0.17) ^a	13.4 (1.82) ^a	21.7 (2.64) ^a
R2	96 (0.25) ^a	13.2 (1.3) ^a	20.4 (0.1) ^a

Standard errors are in parentheses. Means with the same letter in a column for each paired S and R populations at each time point are not significantly different ($\alpha=0.05$) as determined by the *t*-test.

plants to phytotoxic diclofop acid (Shimabukuro, Walsh & Hoerauf 1979). As expected, all S and R plants rapidly de-esterified the absorbed diclofop-methyl to diclofop acid, with less than 5% of the applied [¹⁴C] being recovered as diclofop-methyl after 48 h (Table 3, Fig. 3). Importantly, however, the amount of radioactivity present as toxic diclofop acid was at all times significantly less (1.2- to 2.6-fold) in R1 than in S1. The level of diclofop acid did not differ between R2 and S2 plants until 96 h after treatment, with the level of diclofop acid being 1.5-fold lower in R2 than in S2 at this time point (Table 3).

A decrease in diclofop acid level over time was correlated with an increase in polar and less polar metabolites of diclofop acid in both S and R plants. However, there was always significantly more radioactivity associated with diclofop acid metabolites in the R plants than the S plants (Table 3). For instance, at 48 h after treatment, the percentage of total metabolites in R1 plants was 1.5-fold greater than S1 plants. By 96 h after treatment, the percentage of total metabolites was twofold greater in R1 than in S1, and 1.3-fold higher in R2 than in S2 (Table 3). Moreover, the magnitude of decrease in diclofop acid between the S and R populations was especially correlated with the increase in the major polar metabolite resolved at 2.8 min (peak 3 in Fig. 3, Table 3). These demonstrate that while both S and R plants are able to metabolize diclofop acid, the low-dose-selected R plants can metabolize diclofop acid faster than the S plants.

As wheat is known to rapidly metabolize diclofop to non-toxic metabolites (Shimabukuro *et al.* 1979), the diclofop metabolism pattern at 96 h after treatment between S and R *L. rigidum* populations was compared with that of wheat (Fig. 3). As expected, wheat rapidly metabolized diclofop acid with only 3% radioactivity remaining as diclofop acid (data not shown), as compared with about 20 and 60% in R and S plants, respectively. Although the specific metabolites

Table 2. Foliar uptake and translocation (acropetal and basipetal from the application site) of [¹⁴C]-diclofop-methyl in unselected susceptible (S) and selected resistant (R) *Lolium rigidum* plants 24, 48 and 72 h after treatment

were not identified, HPLC elution profiles showed similar but not identical metabolism patterns between *L. rigidum* and wheat. For instance, both had polar metabolite peaks with retention times between 2 and 5 min, but *L. rigidum* plants also had less-polar metabolite peaks at retention times between 10 and 15 min, and wheat at around 20 min (Fig. 3), respectively. The major polar metabolites in wheat have been identified as aryl-hydroxylated diclofop acid and aryl-hydroxylated sugar conjugates (Shimabukuro *et al.* 1979).

Resistant populations had a lower tissue diclofop acid content than S populations

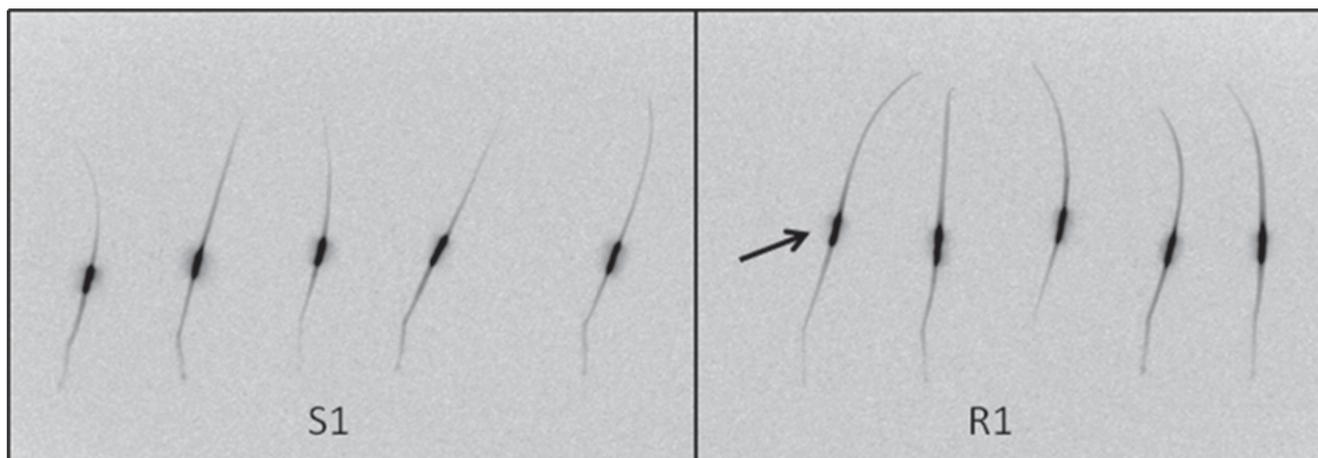
In addition to radioactive HPLC study, the diclofop acid content in above-ground seedling tissues of S and R plants was determined by non-radioactive UPLC-MS (Table 4). This method revealed that at all times, the diclofop acid content in diclofop-treated R1 was significantly lower (up to 2.2-fold) than in S1 (Table 3). Diclofop acid content between R2 and S2 plants did not significantly differ until 72 h after treatment, with the diclofop acid level being 1.3-fold lower in R2 than in S2. The magnitude of difference in tissue diclofop acid content between R and S populations measured by non-radioactive UPLS-MS was similar to the difference revealed by the [¹⁴C]-diclofop HPLC study (Table 3). These UPLS-MS results support the [¹⁴C]-diclofop metabolism studies showing a faster rate of diclofop acid metabolism in R versus S *L. rigidum*.

DISCUSSION

Low-dose-selected resistance is not target-site ACCase based

Our previous study (Neve & Powles 2005a) indicated that ACCase isolated from the low-dose-selected R1 population

(a)



(b)

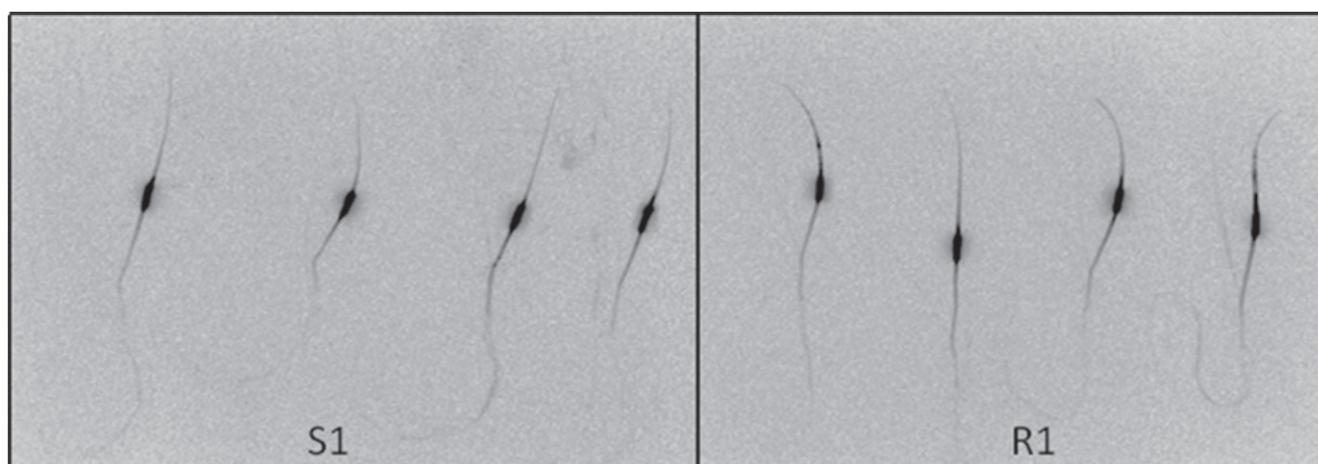


Figure 2. Phosphor imaging of [^{14}C]-diclofop-methyl translocation in unselected S1 versus low-dose selected R1 *Lolium rigidum* populations 24 h (a) and 48 h (b) after diclofop treatment. The arrow indicates the herbicide application site.

was equally as susceptible to the ACCase herbicide diclofop acid as the unselected S1 population. As almost all known ACCase resistance mutations identified so far confer resistance to the ACCase herbicide fenoxaprop-P-ethyl (Powles & Yu 2010), fenoxaprop acid was used in the current study for the *in vitro* ACCase susceptibility test. The results clearly showed that the two low-dose selected R populations had an ACCase as sensitive to fenoxaprop acid as their two parent unselected S populations (Table 1), providing clear evidence that resistance in these two R populations is not ACCase based. Likewise, ACCase overproduction is an unlikely resistance mechanism, as in the absence of the herbicide the two R populations displayed similar ACCase specific activity to the S populations (Table 1). Therefore, neither reduced ACCase sensitivity nor ACCase overproduction is associated with resistance in the two low-dose-selected R populations. This was expected as only a small number of susceptible *L. rigidum* plants (S1, S2) were subjected to low-dose diclofop selection (Neve & Powles 2005a; Manalil *et al.* 2011), and the chance of selecting for target ACCase gene mutations is

low, as in susceptible populations ACCase gene resistance mutations are initially very rare. Similarly, high frequencies (average 0.4%) of phenotypic resistance to the recommended field rate of diclofop in many previously untreated *L. rigidum* populations are orders of magnitude greater than expected rates of ACCase gene mutations conferring target-site ACCase resistance in another study (Neve & Powles 2005b). Therefore, resistance in these low-dose selected *L. rigidum* populations is due to non-target-site mechanisms.

Low-dose-selected resistance is not due to differential rates of herbicide uptake or translocation

In the present study, both S and R plants showed similar (and rapid) foliar uptake of [^{14}C]-diclofop-methyl (Table 2), establishing that reduced herbicide leaf penetration does not contribute to diclofop resistance in the R populations. However, long-distance translocation of ACCase herbicides such as diclofop-methyl can be limited. As shown in Fig. 2 and

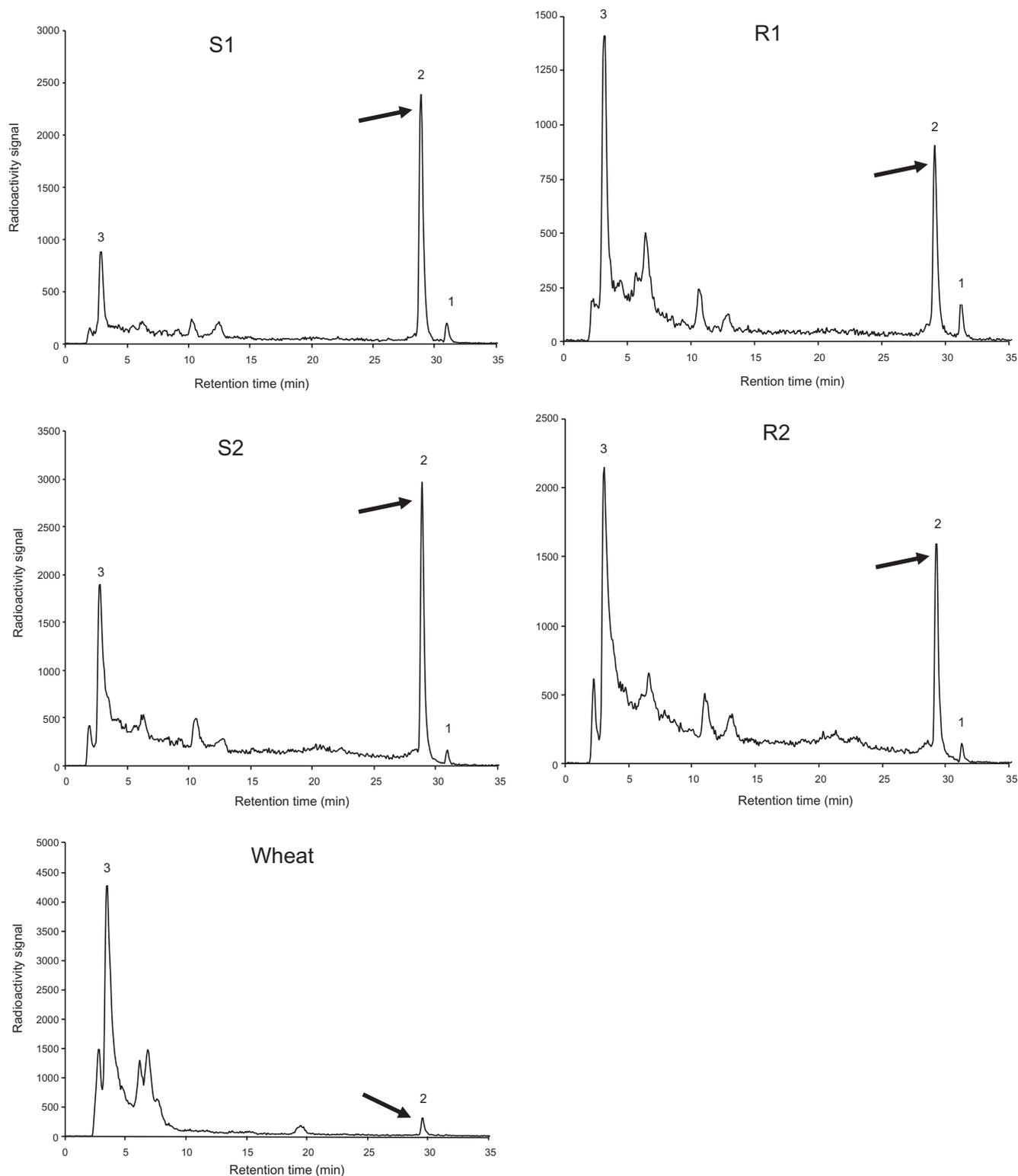


Figure 3. High-performance liquid chromatography (HPLC) chromatograms comparing the elution profiles of diclofop-methyl (peak 1), phototoxic diclofop acid (arrowed peak 2) and its metabolites (the major polar metabolite peak 3 plus unlabelled peaks) in unselected (S) and low-dose-selected (R) *Lolium rigidum* populations, and in wheat (peak 3 possibly diclofop aryl glycoside), 96 h after treatment.

Table 3. Metabolism of [¹⁴C]-diclofop-methyl by unselected susceptible (S) and selected resistant (R) *Lolium rigidum* plants 48 and 96 h after treatment, as quantified by radioactive HPLC analysis

Treatment duration	Population	Radiolabel (% of radioactivity recovered)			
		Major metabolite (peak 3 in Fig. 3)	Total metabolites	Diclofop acid	Diclofop-methyl
48 h	S1	8 (1.34) ^a	25 (0.2) ^a	72 (0.06) ^a	3.4 (0.3) ^a
	R1	12 (0.58) ^a	38 (3.1) ^b	58 (3.1) ^b	4.4 (0.05) ^a
	S2	14 (0.4) ^a	46 (0.7) ^a	52 (0.8) ^a	2.6 (0.06) ^a
	R2	19 (1.5) ^a	44 (3.2) ^a	54 (2.8) ^a	2.4 (0.4) ^a
96 h	S1	19 (0.1) ^a	36 (3.1) ^a	61 (3.6) ^a	3.4 (0.5) ^a
	R1	44 (0.9) ^b	74 (1.3) ^b	23 (0.5) ^b	4.0 (0.8) ^a
	S2	36 (0.6) ^a	58 (0.7) ^a	40 (0.6) ^a	1.5 (0.06) ^a
	R2	47 (0.1) ^b	73 (2.7) ^b	26 (2.6) ^b	1.5 (0.08) ^a

The proportion of the parent herbicide and metabolites was expressed as a percentage peak area of total radioactivity in the sample injection. Means with different letters in a column for each paired S and R populations at each time point are significantly different ($\alpha=0.05$) as determined by the *t*-test.

HPLC, high-performance liquid chromatography.

Table 2, a low amount (<20%) of [¹⁴C] in both S and R plants (Table 3) was translocated from the application site within 48 h; thus, differential translocation is unlikely to be a resistance mechanism in these populations. This observation is consistent with previous findings in *Lolium* spp. and other grass species (Gronwald *et al.* 1992; Seefeldt *et al.* 1996; Manechote, Preston & Powles 1997; Cruz-Hipolito *et al.* 2011), showing that 80–90% of absorbed diclofop-methyl remained at the site of application. Although increased rates of herbicide metabolism (as discussed below) often lead to decreased translocation due to the more polar, larger molecular weight, and decreased phloem mobility of the herbicide metabolite relative to the parent herbicide, this was not statistically (Table 2) nor visually (Fig. 2) evident (if any) under our experimental conditions. Therefore, neither reduced herbicide uptake nor translocation is likely to occur in the two low-dose selected R *L. rigidum* populations studied.

Table 4. Tissue diclofop acid content in unselected susceptible (S) and selected resistant (R) *Lolium rigidum* populations, as measured by non-radioactive LC-MS analysis

Population	Diclofop acid ($\mu\text{g g}^{-1}$ dry weight)		
	24 h	48 h	72 h
S1	51.6 (2.76) ^a	29.8 (0.89) ^a	16.7 (0.2) ^a
R1	23.5 (0.93) ^b	16.0 (0.33) ^b	9.2 (0.18) ^b
S2	41.6 (1.69) ^a	25.2 (0.95) ^a	17.2 (1.09) ^a
R2	40.8 (2.48) ^a	23.6 (1.17) ^a	13.2 (0.25) ^b

Seedlings at the two- to three-leaf stages were treated with diclofop-methyl 188 g ha⁻¹. Standard errors are in parentheses. Means with different letters in a column for each paired S and R populations at each time point are significantly different ($\alpha=0.05$) as determined by the *t*-test.

LC-MS, liquid chromatography-mass spectrometry.

Low-dose-selected resistance is due to enhanced rates of herbicide metabolism

The present study demonstrates that R plants exhibited significantly faster rates of diclofop acid metabolism than did the S plants (Fig. 3, Table 3). More rapid metabolism reduces the level of phytotoxic diclofop acid in the tissue (Table 4), thus limiting herbicide damage in resistant plants. Enhanced rates of diclofop acid metabolism have been documented as a resistance mechanism in field evolved resistant populations of *Lolium* spp. (Holtum *et al.* 1991; Preston & Powles 1998; Cocker *et al.* 2001), *Alopecurus myosuroides* (Hall, Moss & Powles 1997) and *Avena sterilis* (Manechote *et al.* 1997). In addition, enhanced rates of metabolism of other ACCase herbicides have been reported in resistant populations of *Digitaria sanguinalis* (Hidayat & Preston 1997) and *Hordeum leporinum* (Matthews, Powles & Preston 2000). It is noted that, in the current study, as in all the above-mentioned studies, the observed *in vivo* increase in rates of herbicide metabolism in R versus S biotypes was generally modest (up to two- to threefold). We consider this measurable small increase in herbicide metabolism is sufficient to account for the relatively higher level of resistance observed at the whole plant level for R1 (25-fold, Neve & Powles 2005a) and R2 (18-fold, Manalil *et al.* 2011). The reasons are (1) the resistance level measured *in planta* (as whole plant LD₅₀ or GR₅₀) is not always consistent with that measured *in vivo* by herbicide metabolism assay or *in vitro* by target-enzyme inhibition assays (I₅₀), as different biological/physiological/biochemical processes were measured in different cases; (2) in the present studies, the whole above-ground materials were used for HPLC assay. The majority of the C¹⁴ remained in the fully expanded application leaf during the experimental period (Fig. 2, Table 2). Therefore, diclofop metabolism was largely measured in the fully expanded leaves. This may underestimate the difference between the R and S plants in rates of

herbicide metabolism *in planta*, as the most active metabolic activities occur in meristem tissue (new growth). Further evidence that supports involvement of enhanced herbicide metabolism in low-rate-induced rapid resistance evolution in *Lolium* is that resistance evolution is greater (both the magnitude and the rate) under recurrent selection of metabolizable herbicides (e.g. diclofop-methyl, pyroxasulfone) than less or non-metabolizable herbicides (e.g. glyphosate) (Neve & Powles 2005a; Busi & Powles 2009; Manalil *et al.* 2011; Busi *et al.* 2012b). While we believe enhanced herbicide metabolism is a major contributing factor for rapid resistance evolution in *Lolium*, other unknown/undetectable non-target-site resistance mechanisms of relatively minor effects (e.g. slightly enhanced antioxidant capacity and cellular herbicide sequestration and slightly reduced de-esterification of diclofop-methyl) are also possible.

Wheat is well known to tolerate diclofop-methyl due to rapid metabolism of diclofop acid via ring hydroxylation and subsequent sugar conjugation to form non-phytotoxic aryl glycoside (Shimabukuro *et al.* 1979; Shimabukuro, Walsh & Jacobson 1987; Tanaka *et al.* 1990). Ring hydroxylation of diclofop acid is catalysed by cytochrome-P450 monooxygenases (Zimmerlin & Durst 1990). Although we did not specifically identify diclofop acid metabolites, the major polar metabolites in resistant *L. rigidum* plants were clearly resolved and are chromatographically similar to those (aryl-hydroxylated diclofop acid and aryl glycoside of diclofop) in wheat (especially peak 3 in Fig. 3). This, together with evidence from early studies with resistant *L. rigidum* populations showing the presence of ring-hydroxylated diclofop acid in tissue extracts (Shimabukuro & Hoffer 1991) and *in vivo* inhibition of diclofop acid metabolism by the P450 inhibitor 1-aminobenzotriazole (ABT) (Preston *et al.* 1996), suggests that enhanced rates of diclofop acid metabolism in the two low-dose-selected resistant *L. rigidum* populations (R1 and R2) are likely to involve a wheat-like P450-mediated metabolic detoxification pathway, and that there is a greater herbicide metabolism capacity in R plants than in S plants. As our genetic studies revealed polygenic (with additive effect) control of diclofop resistance in these low diclofop-methyl rate selected populations (Busi *et al.* 2012a), diclofop acid metabolism in *L. rigidum* likely involves more than one P450 gene/isoenzymes or more complex metabolic pathways involving both P450s and GSTs. This may explain the cross-resistance to certain dissimilar ALS herbicides observed in these resistant populations (Neve & Powles 2005a; Manalil *et al.* 2011), as these ALS herbicides are known to be metabolized by P450s (e.g. Christopher *et al.* 1991; Preston *et al.* 1996). However, other non-target-site resistance mechanisms of probably minor effects (such as those mentioned above) cannot be ruled out. The specific genes/enzymes involved in non-target-site resistance, especially in diclofop acid metabolism, remain to be determined.

As the existence of phenotypic variation for diclofop-methyl susceptibility at low herbicide application rates has been demonstrated in cross-pollinated *Lolium* (Neve & Powles 2005b), it is likely that recurrent selection with diclofop-methyl at sub-lethal doses selected for individuals

with higher levels of herbicide-metabolizing enzyme (e.g. cytochrome P450s and GSTs) gene expression and/or with different iso-alleles, and that these mechanisms have been enriched by cross-pollination in the progeny both at the population and individual levels. These selected resistant populations are, therefore, very suitable for RNA transcriptome gene expression profiling to identify the specific genes involved. Next generation RNA sequencing (RNA-Seq) on the R and S populations is underway.

To conclude, this study was conducted with two initially herbicide susceptible *L. rigidum* populations that evolved resistance from three generations of recurrent selection with low rates of the herbicide diclofop-methyl (Neve & Powles 2005a; Manalil *et al.* 2011). This polygenic resistance is due to enhanced capacity for diclofop metabolism, likely endowed by P450 genes as evidenced by (1) the higher rates of diclofop acid metabolism in R plants; (2) the similarity to known P450-mediated diclofop metabolism in wheat; and (3) the evidence of cross-resistance to ALS herbicides with a completely different mode of action that are known to be metabolized by P450s. However, direct evidence of P450-based herbicide metabolism remains to be established, and involvement of other detoxification enzymes, such as GSTs, is also possible. Clearly (and especially for cross-pollinated plant species), low-dose herbicide application, within the range of genetic variation for herbicide susceptibility, is a dangerous practice as it can rapidly select those individuals with a greater capacity for herbicide metabolism, and this enables rapid resistance evolution. Metabolic resistance can greatly reduce herbicide options because it has the potential to endow resistance to herbicides of different modes of action including those still in development or yet to be conceived. Metabolic resistance can also compromise the efficacy of herbicide sequences, mixtures or rotations aiming to delay resistance evolution. Therefore, herbicides should be always used at the registered recommended rates.

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