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ORIGINAL ARTICLE

Genetic control of a cytochrome P450 metabolism-based herbicide resistance mechanism in *Lolium rigidum*

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The dynamics of herbicide resistance evolution in plants are influenced by many factors, especially the biochemical and genetic basis of resistance. Herbicide resistance can be endowed by enhanced rates of herbicide metabolism because of the activity of cytochrome P450 enzymes, although in weedy plants the genetic control of cytochrome P450-endowed herbicide resistance is poorly understood. In this study we have examined the genetic control of P450 metabolism-based herbicide resistance in a well-characterized *Lolium rigidum* biotype. The phenotypic resistance segregation in herbicide resistant and susceptible parents, F1, F2 and backcross (BC) families was analyzed as plant survival following treatment with the chemically unrelated herbicides diclofop-methyl or chlorsulfuron. Dominance and nuclear gene inheritance was observed in F1 families when treated at the recommended field doses of both herbicides. The segregation values of P450 herbicide resistance phenotypic traits observed in F2 and BC families was consistent with resistance endowed by two additive genes in most cases. In obligate out-crossing species such as *L. rigidum*, herbicide selection can easily result in accumulation of resistance genes within individuals.
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Introduction

For the past several decades herbicides have dominated weed control in crop production systems in many parts of the world. Because of strong and persistent herbicide selection pressure, herbicide-resistant populations have globally evolved in many weed species (Heap, 2010; Powles and Yu, 2010). Several determinants influence the speed of resistance evolution, including agro-ecosystem and herbicide factors, as well as plant genetic factors such as the number and initial frequency of resistance genes, level of resistance gene dominance and mode of inheritance (Darmency, 1994; Diggle and Neve, 2001). Thus, inheritance studies provide important insights for understanding herbicide resistance evolution, genetic structure of weed populations, adaptation dynamics and resistance management (Neve *et al*., 2009).

Plant cytochrome P450 enzymes have a paramount role in detoxification pathways and can be responsible for herbicide detoxification by metabolism (Schuler, 1996; Mizutani and Ohta, 2010). Herbicide metabolism/detoxification catalysed by cytochrome P450 enzymes is a prominent herbicide resistance mechanism evolved in some herbicide resistant weed populations (Powles and Yu, 2010). The current understanding of P450-mediated herbicide metabolism in plants suggests that likely there are multiple P450 isoforms with varying herbicide (substrate) specificity, thus variable capacity for herbicide metabolism (Siminszky, 2006; Powles and Yu, 2010). However, the molecular identification of specific P450 genes endowing herbicide resistance by metabolism remains to be elucidated. It remains unknown whether there is changed P450 substrate specificity (gene mutation), gene regulation or gene duplication and gene mutation (Schuler and Werck-Reichhart, 2003). Studies with a P450-based resistant *L. rigidum* population (biotype VLR69) indicated single (P450) metabolism-based genetic traits conferred resistance to a specific herbicide mode of action (Preston, 2003). In P450-based herbicide resistant *Alopecurus myosuroides* (Huds.) populations with both single major and additive genes have been implicated in resistance (Letouze and Gasquez, 2001; Petit *et al*., 2010).

We have extensively characterized a multiple herbicide resistant *L. rigidum* population (biotype SLR31). This biotype evolved resistance when selected with the acetyl-CoA-carboxylase (ACCase) inhibiting herbicide diclofop-methyl and, importantly, displayed cross resistance to the chemically unrelated acetolactate synthase-inhibiting herbicide chlorsulfuron (Heap and Knight, 1990). Subsequent research established that resistance to these two very different herbicide modes of action in this biotype is endowed by enhanced rates of P450-mediated herbicide metabolism (Christopher *et al*., 1991, 1994; Preston and

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Powles, 1998). However, neither the specific cytochrome P450 enzymes responsible for herbicide metabolism nor the genetic basis of P450 resistance have been identified.

Here, we focus on the genetic control of non-target-site based P450-mediated herbicide resistance evident in this well-characterized resistant *L. rigidum* population (SLR31). We purified a SLR31 population subset containing individuals with only P450 herbicide metabolism as a resistance mechanism (Vila-Aiub *et al*., 2005). To understand the inheritance and genetic basis of P450-based herbicide resistance in this population we hybridized P450-based resistant plants with herbicide susceptible plants and evaluated segregation of parental, F1, F2 and backcross (BC) families.

Materials and methods

Plant material

The multiple herbicide-resistant *L. rigidum* population SLR31 originated from Bordertown, South Australia (140°46′E, 36°18′S) and exhibits multiple resistance to herbicides with different modes of action (Burnet *et al*., 1994). Most individuals within this population display herbicide metabolism mediated by cytochrome P450, which endows resistance to several herbicides that inhibit ACCase-inhibiting herbicide and acetolactate synthase-inhibiting herbicide (Christopher *et al*., 1994). We removed a small proportion of the SLR31 population that additionally possess a target-site mutation (Ile-1781-Leu) in the ACCase gene (Zhang and Powles, 2006). The identification and purification of P450-based resistant plants (hereinafter referred to as R) within the SLR31 population was conducted according to methodology described in Vila-Aiub *et al*. (2005). The *L. rigidum* biotype (VLR1) known to be susceptible to all herbicides (hereinafter referred to as S) was used as the susceptible parent.

Generation of F1, F2 and BC populations

Plants of the P450-based R and S parental populations were grown in 18 cm diameter pots containing a potting mix (50% peatmoss: 50% river sand) and maintained outdoors during the normal winter growing season for this species. R parental plants that survived a high rate (3000 g ha^{-1}), eight times the field rate of diclofop-methyl, were kept for plant crossing. Single R and S plants growing individually in pots were moved to glasshouse conditions, paired according to floral synchronicity and enclosed within a plastic-coated cylinder (1.5 m height), which excluded foreign pollen and ensured cross-pollination only between R and S plants. At maturity, seeds were collected from 10 reciprocal crosses between R and S parents thus generating 20 F1 families comprising 10 F1 maternal R and paternal S (hereinafter referred to as F1 RS) and 10 F1 maternal S and paternal R (hereinafter referred to as F1 SR).

To generate BC families, RS F1 hybrids and parent S plants were grown in 18 cm diameter pots containing a standard potting mix in a controlled environment room with 12 h photoperiod at 25 °C (360 μmol m^{-2} s^{-1}) and 12 h dark period at 15 °C. After 40 days, the photoperiod was increased to 15 h to induce flowering and cross-pollination between the RS F1 and S plants coupled in pair crosses. A total of 10 BC families (hereinafter referred as to F1 BC) were obtained after crossing individual RS F1 plants back to S parental plants and collecting seed from each RS F1 mother plant.

To generate F2 families, 10 individuals each from different RS F1 families were grown individually in pots and randomly paired and enclosed at flowering time as described above. Seed from each plant was collected and kept separately to constitute a total of ten F2 families. F2 families originating from the specific pair crosses of F1 families (no. 1 × no. 14, no. 2 × no. 5, no. 4 × no. 11, no. 6 × no. 10 and no. 7 × no. 12) were subsequently tested for herbicide resistance segregation.

Herbicide screening of F1 families

The F1 hybrids obtained from both reciprocal crosses with R and S parental populations were assessed for herbicide resistance. Plants from parental lines (S and R) and six F1 families (three RS and three SR) were grown outdoors in plastic pots during the normal growing season for *L. rigidum*. At the two-leaf stage, seedlings were treated with 0, 94, 188, 375, 750 or 1500 g diclofop-methyl ha^{-1} (recommended rate 375 g ha^{-1}; Hoegrass EC 375 g a.i. l^{-1} plus 0.25% wetting agent BS1000). Alternatively, seedlings were treated with 0, 3, 6, 11, 23, 45 or 90 g chlorsulfuron ha^{-1} (recommended rate 20 g ha^{-1}; Glean DF 75 g a.i. kg^{-1} plus 0.25% wetting agent BS1000). Herbicide treatments were applied to plants using a dual nozzle cabinet sprayer with a delivery rate of 98 l ha^{-1} (200 kPa, 4 km h^{-1}). There were three replicates per treatment and 15 plants per replicate. Plant survival was evaluated 15 days after herbicide treatment. Two discriminative doses were identified for diclofop-methyl (375 and 1500 g ha^{-1}) and chlorsulfuron (22 and 90 g ha^{-1}) (Figure 1). The lower dose was chosen to observe resistance segregation at the same dose at which plants were selected in the field. The higher dose (4 ×) was chosen to observed resistance segregation with no interference of minor resistance genes. All the remaining F1 families (RS and SR) were tested similarly at these two specific herbicide rates. S and R parental lines were also included as control in bioassay studies.

Herbicide dose–responses in F1 and parental families

Plant survival data were transformed and expressed as percent as described by Knezevic *et al*. (2007). Data sets were fitted to a non-linear logistic model $Y = c + [(d-c)/[1 + (x/G)^b]$. Y denotes the plant survival expressed as percentage of the untreated control, d and c are upper and lower asymptotic values of Y, respectively, b the slope of the curve, G the herbicide dose at the point of inflection halfway between the upper and the lower asymptotes and x the herbicide dose (Streibig *et al*., 1993). Statistical differences in plant survival between the parental and F1 lines (that is, level of dominance of resistant alleles) after increasing diclofop-methyl and chlorsulfuron rates, were assessed by a lack-of-fit F-test applied to data sets fitted with the above non-linear logistic model. Regression assumptions were held under square root data transformation (that is, Box-Cox transformation lambda $\lambda = 0.5$).

Herbicide screening of F2 and BC populations

Similarly, 10 F2 and 10 BC families (F1 BC) were tested at the specific herbicide rates indicated above. S and R

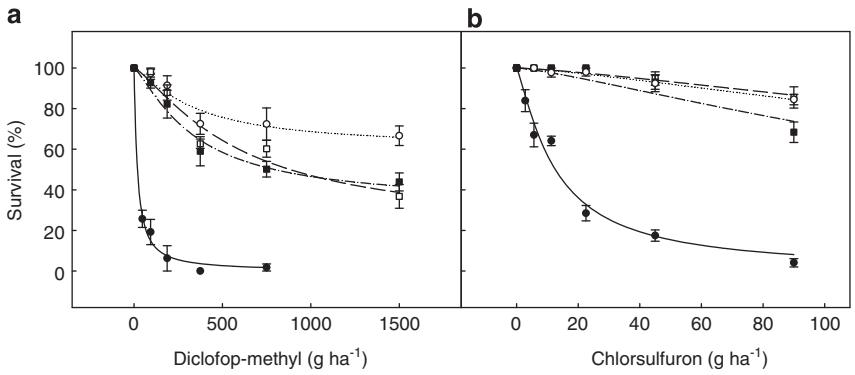

Figure 1 Survival response to a range of doses of diclofop-methyl (**a**) and chlorsulfuron (**b**) for R parental line (P450) (open circles and dotted line), parental S (VLR1) (solid circles and solid line), 3 SR F1 families SR F1 (♀ VLR1; ♂ P450) (solid squares and dash–dotted line) and 3 RS F1 families (♀ P450; ♂ VLR1) (open squares and dashed line). Symbols are mean ± s.e. ($n = 3$). Pooled data for F1 SR and F1 RS ($n = 9$).

parental and F1 plants were included as control. Two experiments repeated in time were conducted.

Genetic models and statistical analysis for resistance segregation

Resistance segregation in BC and F2 families: A goodness-of-fit χ^2-test was used to compare the observed plant survival segregating values for each F2 and BC family with predicted values according to both one- and two-gene resistance models (Table 1). The one-gene model is based on the assumption of one single major gene with incomplete dominance controlling P450-based diclofop-methyl or chlorsulfuron resistance (Supplementary Table S2-S3). In the two-gene model, the two genes are non-completely dominant and with additive effect to endow herbicide resistance (that is, plant survival) at the two doses tested for each herbicide (Tables 1–3).

Because of the relatively small size (n) with a few samples (for example, F2 no. 1) and subsequent low plant number (<5) in each class as survivor or dead plants, a comparison between Fischer exact test and χ^2-test was performed. No difference in probability (P) values was observed overall and therefore χ^2 was chosen as indicated by Yu *et al*. (2009) as the appropriate statistical test to assess statistical difference in the resistance segregation values at specific herbicide doses. P-values were obtained indicating the probability of type II error in rejecting the null hypothesis ($H_o = $ F2 segregation of two incompletely dominant genes in a 5R:6F1:5S ratio and BC segregation 1F1:3S ratio (Table 1). The significance level was $\alpha = 0.05$ and only P-values $\geqslant \alpha$ were accepted to statistically validate the gene segregation model for each F2 and BC family tested. A pooled χ^2 value was calculated considering all the lines tested as a single F2 or BC family. A heterogeneity χ^2-test was performed as detailed in Sokal and Rohlf (1969) to evaluate the direction of the deviations in the segregation values obtained.

In this study the segregation analysis in F2 and BC families was based on plant survival and it refers to the observed resistant (alive) or susceptible (dead) plants compared with the expected survival/mortality ratios calculated as suggested by both Preston (2003) and Tabashnik (2001) (Table 1). The expected survival ratio was corrected by weighting according to the observed performances of R and S parents and F1 lines. For example, to calculate the expected number of surviving resistant plants in F2 families, the number of treated plants was multiplied for the one-gene (0.25R:0.5F1:0.25S) or two-gene (0.3125R:0.375F1:0.3125S) expected segregation ratio multiplied by the mean plant survival ratio observed in the parental R line, F1 families and parental S line included in the segregation study as control of the phenotypic resistance

Table 1 Genotypic segregation pattern and expected phenotypic survival in F2 and BC families according to one-gene (A) and two-gene (B) resistance models

A

Alleles	*R1*	*r1*
R1	R1R1	R1r1
r1	r1R1	r1r1
F2 genotypic segregation 1R:2F1:1S (one-gene)		
R1		R1r1
r1		r1r1
BC genotypic segregation 1F1:1S (one-gene)		

B

Alleles	*R1R2*	*R1r2*	*r1R2*	*r1r2*
R1R2	R1R1 R2R2	R1R1 R2r2	R1r1 R2R2	R1r1 R2r2
R1r2	R1R1 R2r2	R1R1 r2r2	R1r1 R2r2	R1r2 r2r2
r1R2	R1r1 R2R2	R1r1 R2r2	r1r1 R2R2	r1r1 R2r2
r1r2	R1r1 R2r2	R1r1 r2r2	r1r1 R2r2	r1r1 r2r2
F2 genotypic segregation 5R:6F1:5S (two-genes)				
R1R2				R1r1 R2r2
R1r2				R1r2 r2r2
r1R2				r1r1 R2r2
r1r2				r1r1 r2r2
BC genotypic segregation 1F1:3S (two-genes)				

For the two-gene model, the presence of one dominant allele (R1 or R2) is not sufficient to endow resistance (genotypic combinations not highlighted). The presence of two dominant alleles (R1 and R2) endowed resistance equally as observed in F1 families (genotypic combinations highlighted in light grey). Three and four alleles (genotypes dark-grey highlighted) endowed resistance equally as observed in the parental resistant line.

Table 2 Observed resistance segregation values as plant survival in parental R (SLR31), S (VLR1), F1, F2 and BC lines following application of a low diclofop-methyl rate (375 g ha^{-1}) and a high rate (1500 g ha^{-1})

Family	*Diclofop-methyl 375 g ha^{-1}*					*Diclofop-methyl 1500 g ha^{-1}*				
	Treated	*Survival observed*	*Survival expected*	χ^2	P	*Treated*	*Survival observed*	*Survival expected*	χ^2	P
S	241	16				196	9			
R	237	205				232	178			
F1	571	396				572	252			
F2 no. 1	25	17	14	1.68	0.20	25	9	10	0.35	0.55
F2 no. 2	39	22	21	0.03	0.87	40	4	17	16.7	0.00
F2 no. 6	80	45	44	0.04	0.84	40	15	17	0.31	0.58
F2 no. 7	78	74	42	49.8	0.00	38	19	16	1.03	0.31
F2 no. 10	81	46	45	0.09	0.76	40	20	17	1.09	0.30
F2 no. 11	40	21	22	0.11	0.74	39	10	16	4.22	0.04
F2 no. 12	38	35	21	21.0	0.00	40	21	17	1.86	0.17
F2 no. 14	20	16	11	2.85	0.09	20	10	8	0.54	0.46
F2 no. 5						22	11	9	0.60	0.44
F2 no. 4						13	6	5	0.10	0.75
Pooled	401	276	221	30.5	<0.01	252	125	133	0.77	0.38
F1 BC no. 2	40	27	9	47.1	<0.01	40	22	6	53.4	0.00
F1 BC no. 4	41	29	9	55.4	<0.01	40	6	6	0.01	0.92
F1 BC no. 6	40	13	9	2.39	0.12	40	3	6	1.55	0.21
F1 BC no. 7	40	5	9	2.22	0.14	40	3	6	1.55	0.21
F1 BC no. 8	39	17	9	10.2	<0.01	40	4	6	0.63	0.43
F1 BC no. 9	39	9	9	0.01	0.91	40	1	6	3.91	0.05
F1 BC no. 10	40	4	9	3.50	0.06	40	2	6	2.87	0.09
F1 BC no. 11	40	8	9	0.12	0.73	40	3	6	1.55	0.21
F1 BC no. 13	40	18	9	11.9	<0.01	39	5	5	0.08	0.78
F1 BC no. 14	40	6	9	1.24	0.27	38	3	5	1.31	0.25
Pooled	399	136	89	31.9	<0.01	397	52	57	0.56	0.46

Expected survival values are calculated according to the two-gene model segregation suggested in Table 1.

Table 3 Observed resistance segregation values as plant survival in parental R (SLR31), S (VLR1), F1, F2 and BC lines following application of a low chlorsulfuron rate (22 g ha^{-1}) and high rate (90 g ha^{-1})

Family	*Chlorsulfuron 23 g ha^{-1}*					*Chlorsulfuron 90 g ha^{-1}*				
	Treated	*Survival observed*	*Survival expected*	χ^2	P	*Treated*	*Survival observed*	*Survival expected*	χ^2	P
S	243	148				247	78			
R	242	233				230	206			
F1	590	531				589	390			
F2 no. 1	25	20	21	0.15	0.70	26	12	16	3.04	0.08
F2 no. 2	40	35	33	0.60	0.44	40	22	25	1.01	0.32
F2 no. 6	80	63	66	1.0	0.33	81	30	51	13.1	<0.01
F2 no. 7	79	60	65	2.67	0.10	79	76	50	40.9	<0.01
F2 no. 10	82	48	68	34.2	<0.01	80	23	50	36.5	<0.01
F2 no. 11	39	37	32	3.96	0.05	39	30	24	3.38	0.07
F2 no. 12	39	39	32	8.06	<0.01	40	39	25	20.7	<0.01
F2 no. 14	20	15	17	0.87	0.35	20	4	13	15.6	<0.01
F2 no. 5						22	19	14	5.27	0.02
F2 no. 4						13	10	8	1.13	0.29
Pooled	404	317	335	5.52	0.02	440	265	276	1.14	0.29
F1 BC no. 2	39	24	27	0.79	0.37	38	4	15	13.9	<0.01
F1 BC no. 4	40	34	27	5.22	0.02	38	24	15	8.30	<0.01
F1 BC no. 6	40	30	27	0.86	0.35	40	14	16	0.46	0.50
F1 BC no. 7	38	26	26	0.00	0.97	40	13	16	1.00	0.32
F1 BC no. 8	40	19	27	7.88	<0.01	39	10	16	3.46	0.06
F1 BC no. 9	38	29	26	1.16	0.28	40	16	16	0.00	0.98
F1 BC no. 10	40	30	27	0.86	0.35	40	15	16	0.12	0.72
F1 BC no. 11	40	39	27	15.9	<0.01	40	19	16	0.88	0.35
F1 BC no. 13	40	38	27	13.3	<0.01	40	25	16	8.24	<0.01
F1 BC no. 14	39	22	27	2.49	0.12	40	9	16	5.23	0.02
Pooled	394	291	269	5.9	0.02	395	149	159	1.04	0.31

Expected survival values are calculated according to the two-gene model segregation suggested in Table 1.

level. Similarly, in the BC the observed number of survivors was multiplied by a different segregation ratio (0.5F1:0.5S) or (0.25F1:0.75S) according to the one- or two-gene model, respectively (Table 1). Among the surviving resistant plants it was not possible to visually identify different or intermediate resistance phenotypes following herbicide treatments.

Results

Diclofop-methyl (ACCase-inhibiting herbicide) dose–response of R, S and F1 families

As earlier reported (Christopher *et al*., 1991), the dose–response study revealed the R parental line to be clearly resistant to diclofop-methyl whereas the S parental line was susceptible (Figure 1a). The overall resistance level of six F1 families ($n=6$) in comparison with the R ($P=0.908$) and S ($P<0.001$) parents and the similarity in plant survival between the RS and SR F1 families at the diclofop-methyl rates in the dose–response studies ($P=0.59$), establishes nuclear inheritance of resistance and near complete dominance of the gene traits endowing diclofop-methyl resistance (Figure 1a). At high diclofop-methyl rate (1500 g ha^{-1}) incomplete dominance was observed between the F1 families and the R parental line ($\chi^2=5.90$; $P=0.02$) (Figure 1a). A similar response to diclofop-methyl (375 or 1500 g ha^{-1}) was observed in all the F1 families tested in this study (data not shown) to generate the F2 families ($P>0.13$). This indicated low impact of the S parent (VLR1) genetic variability on the expression of the resistance in F1 crosses used to assess resistance segregation patterns in the F2 and BC families.

Chlorsulfuron (acetolactate synthase-inhibiting herbicide) dose–response of R, S and F1 families

As expected (Christopher *et al*., 1991), the parental R line displayed high level chlorsulfuron resistance whereas the S parent was susceptible (Figure 1b). The overall response to a range of chlorsulfuron doses observed for both the RS and SR F1 families was similar to the R line when analyzed by a lack-of-fit F test ($P=0.636$) (Figure 1b). Therefore, the overall R, S and F1 plant responses indicated that gene traits endowing chlorsulfuron resistance are nuclear inherited and expressed with complete dominance at the chlorsulfuron rates tested in F1 plants ($\chi^2=0.76$; $P=0.54$) (Figure 1b).

Diclofop-methyl resistance segregation of F2 families

Plants from the S, R and F1 families were included as control in this study. As expected, the S plants exhibited susceptibility with only 7% survival at 375 g diclofop-methyl ha^{-1} and 5% at 1500 g ha^{-1}. In contrast, in the R and F1 lines resistance was evident with 86 and 69% survival at 375 g diclofop-methyl ha^{-1} and 77 and 44% survival at 1500 g ha^{-1}, respectively (Table 2). According to a two-gene segregation resistance model (Table 1), the expected plant survival values (and reciprocal mortality) were estimated by assuming 31.25% (5:16) of the F2 plants responding to diclofop-methyl as the R line, 37.5% (6:16) as F1 families and 31.25% (5:16) as the S parental line. At 375 g diclofop-methyl ha^{-1} the segregation observed in six F2 families agreed with the two-gene model (Table 3). In two families (nos. 12 and 7), originating from the same F1 pair cross, survival was higher than expected (Table 2). Heterogeneity test was not significant when those two F2 families were excluded ($P=0.64$). The parallel hypothesis of one gene control for the resistance segregation observed in F2 families (thai is, 0.25% R: 50% F1: 25% S) could explain five of eight families tested at this rate with no significant deviation from the proposed model (Supplementary Table S2).

When diclofop-methyl was applied at a high rate of 1500 g ha^{-1}, the F2 segregation pattern observed fitted both the models for most of the families. Only two families (nos. 2 and 11) exhibited lower than expected survival values (Table 2). Similarly, the hypothesis of one gene could explain the segregation of eight families (Supplementary Table S2). Although χ^2-test of pooled data was not significantly different from the one- or two-gene model ($P>0.31$), the heterogeneity test was highly significant ($P<0.01$). Homogeneity of resistance segregation was observed only after removing the families not explained by the proposed genetic models ($P>0.82$).

Diclofop-methyl resistance segregation of BC families

In F1 BC populations treated at 375 g diclofop-methyl ha^{-1} the observed resistance segregation of six families fitted well the expected two-gene segregation (Table 2). Four families exhibited survival values higher than expected and this resulted in significant heterogeneity in F1 BC segregating families ($P<0.01$). Conversely, at the same diclofop-methyl rate, only four F1 BC families showed a resistance segregation pattern consistent with a one-gene model (Supplementary Table S2). Pooled χ^2-test analysis was not significant for a one-gene segregation model in F1 BC families whereas, it was significantly different for a two-gene model (Table 2). However, there was significant heterogeneity in segregation values with both one- and two-gene models ($P<0.01$).

At a high rate of 1500 g diclofop-methyl ha^{-1}, the observed segregation values for nine F1 BC families were best explained by a two-gene model (Table 2). A one-gene model fitted the segregation values of only two F1 BC families (Supplementary Table S2). The χ^2-test for pooled values was significantly different for one-gene model whereas, there was good fit assuming two gene segregation (Table 2). There was significant heterogeneity in segregation for the BC families studied at 1500 g diclofop-methyl ha^{-1} ($P<0.01$).

Chlorsulfuron resistance segregation of F2 families

For the S parental population there was 61% survival at 22 g chlorsulfuron ha^{-1} and 32% at 90 g ha^{-1} (in pot studies susceptible *L. rigidum* plants can survive chlorsulfuron, see Christopher *et al*., 1992). In contrast, high level chlorsulfuron resistance was observed in the R line, which displayed 90% survival at the highest tested rate 90 g ha^{-1} (Figure 1). In F1 families, plant survival ranged between 90 and 66% at 22 and 90 g chlorsulfuron ha^{-1}, respectively (Table 3). For six F2 families treated at the lower rate of chlorsulfuron (22 g ha^{-1}) segregation occurred according to a two-gene model prediction. Two F2 families (nos. 10 and 12) showed higher than expected survival values (Table 3). Only four F2 families were well

explained by a one single gene segregation model (Supplementary Table S3). Pooled values resulted in significant deviation from one-gene and two-gene model, although to a lesser extent with the two-gene hypothesis. Heterogeneity in segregation for different F2 families was high ($P<0.01$).

When chlorsulfuron was applied at 90 g ha^{-1}, four F2 families segregated according to a two-gene model, whereas three families displayed higher or lower than expected plant survival (Table 3). At the same rate 90 g chlorsufuron ha^{-1}, the resistance segregation of only three F2 families was explained by one single-gene model (Supplementary Table S3). Pooled segregating values did not significantly deviate from one- or two-gene model but, as expected, heterogeneity in F2 segregating values was highly significant ($P<0.01$).

Chlorsulfuron resistance segregation of BC families

At the lower dose of 22 g chlorsulfuron ha^{-1} six F1 BC families segregated according to a two-gene model. Four F1 BC families showed higher than expected survival (Table 3). A one-gene model could explain the resistance segregation pattern of five F1 BC families (Supplementary Table S3). Significant heterogeneity in segregation was observed with BC families at this chlorsulfuron rate. However, pooled values for BC segregation were not statistically different from expected segregation of a one-gene model (Supplementary Table S3).

At 90 g chlorsulfuron ha^{-1}, resistance segregation of six F1 BC families fitted the proposed two-gene model (Table 3). Two F1 BC families exhibited survival values higher than expected and other two F1 BC families displayed lower than expected survival. Similarly, a one-gene model explained the segregation of six F1 BC families (Supplementary Table S3). Pooled segregation values indicated two genes as genetic control with no significant deviation from observed and expected survival values (Table 3).

Discussion

Dominance of P450 metabolism-based herbicide resistance

In the resistant *L. rigidum* biotype SLR31 the herbicide dose–response studies revealed high level P450 metabolism-based resistance to the chemically unrelated herbicides diclofop-methyl and chlorsulfuron in the parental R line and in F1 families (Figure 1). In the F1 reciprocal crosses, nuclear inheritance and high level of dominance over susceptibility was evident by comparison with the parental R and S families. Nuclear inheritance allows resistance alleles to be moved both through pollen and seed. In the obligate cross-pollinated species *L. rigidum* pollen-mediated flow of nuclear inherited resistance genes occurs at considerable distance (Busi *et al*., 2008) and accumulation within individuals of different resistance genes is frequent (that is, multiple resistance) (Christopher *et al*., 1992; Zhang and Powles, 2006). At the recommended field rate of either herbicide, resistance genes were dominant meaning that heterozygous plants survive normal herbicide field use rates and resistant phenotypes can rapidly increase in populations under herbicide field selection (Jasieniuk *et al*., 1996). This is in contrast to several examples of successful management of insecticide resistant insect pests in which heterozygous resistant individuals are killed by the recommended insecticide field rates (Roush and McKenzie, 1987). Thus, heterozygous insecticide resistance alleles are often functionally recessive in the field under insecticide selection and this phenomenon has provided the basis for successful application of a high-dose refuge strategy to significantly delay and minimize insecticide resistance evolution in transgenic Bt crops (Tabashnik, 2008). In our study at high diclofop-methyl rates incomplete dominance was evident (Figure 1) but, herbicides cannot be used at very high doses because of regulatory restrictions, crop selectivity or cost. Therefore, for this metabolism-based resistant *L. rigidum* population heterozygous resistant weed genotypes (F1) cannot be controlled at normal herbicide recommended doses.

Polygenetic nature of P450 metabolism-based resistance in *L. rigidum*

For many cases of herbicide-resistant weed populations (Darmency, 1994) or plants resistant to high concentration of heavy metals (Macnair, 1993), single-gene inheritance is known. For example, a single-nucleotide mutation in a specific region of a herbicide target-site gene can cause an amino-acid substitution that endows high level herbicide resistance without loss of enzymatic functionality (Yu *et al*., 2007). Conversely, the mechanistic basis for non-target-site resistance in the *L. rigidum* population SLR31 is mediated by cytochrome P450 herbicide metabolism minimizing toxic herbicide concentration reaching the susceptible herbicide target site of action (Christopher *et al*., 1991, 1994; Preston and Powles, 1998). Cytochrome P450s are a large family of plant enzymes, some of which are capable of herbicide metabolism, at least in grass species (Powles and Yu, 2010). In plants there are many P450 genes evident (300 genes have been identified in the *Arabidopsis* genome) and chemical defense seems to be a major reason for plant P450 diversification (Yuan *et al*., 2007; Mizutani and Ohta, 2010). In this inheritance study the P450-based herbicide metabolism in one *L. rigidum* population is likely conferred by two genes. Segregation analysis revealed that a two-gene model best fitted the segregation pattern observed in the F2 and BC families. In particular, the two-gene model segregation was evident in BC families treated with diclofop-methyl, where approximately 25% of the treated plants survived herbicide treatment according to a 1R:3S segregation ratio (Table 2). Moreover, the two-gene model always explained the observed segregation values for resistance better than one-gene model in F2 or BC families, treated as distinct sub-populations with low or high doses of two different herbicides (Tables 2 and 3). It is emphasized that the genetic model used in this study is valid at the population level for phenotypes originally selected with a high diclofop-methyl dose and subsequently used to generate F1, F2 and BC lines. However, populations of cross-pollinated species are rarely homogeneous and therefore a higher degree of genetic complexity within a population can exist (Petit *et al*., 2010).

The history of herbicide selection of the *L. rigidum* population SLR31 (Heap and Knight, 1990) indicates that

resistance to acetolactate synthase-inhibiting herbicides was conferred by the resistance mechanism that evolved from selection with the ACCase-inhibiting herbicide diclofop-methyl (Christopher *et al*., 1991). Despite diclofop-methyl selection only, high level resistance to diclofop-methyl and the chemically unrelated chlorsulfuron was evident (Christopher *et al*., 1992) (Figure 1). Similarly, in a preliminary assay R parental, F1 and F2 cloned plants sequentially treated with diclofop-methyl and chlorsulfuron or vice versa, displayed resistance to both herbicides (Supplementary Table S4). Therefore, we hypothesize that the same P450 genes (that is, herbicide-metabolizing cytochrome P450 isozymes) likely endow resistance to these two unrelated herbicide modes of action (Christopher *et al*., 1991; Werck-Reichhart *et al*., 2000). Definite evidence requires molecular identification of the specific P450 genes involved.

The genetic basis of P450-mediated metabolism-based chlortoluron resistance or non-target-site resistance to the ACCase herbicide pinoxaden in *A. myosuroides* has been shown to be controlled by multiple additive genes (Chauvel, 1991; Petit *et al*., 2010). Mackenzie *et al*. (1995) claimed polygenetic, quantitatively inherited chlorsulfuron resistance in *Lolium perenne*. Conversely, in another resistant *L. rigidum* population with a complex herbicide selection history, distinct single P450 major genes may each be responsible for resistance to a herbicide group (Preston, 2003). In addition, as reported by Neve and Powles (2005), we speculate that other resistance gene(s) of minor effect may have been enriched under herbicide selection and have a complementary effect in survival of resistant phenotypes. In our study this may have led to distortion from the proposed two-gene segregation model with additional (higher than expected) plant survival at lower herbicide rates (Tables 2 and 3), as similarly reported by Wang *et al*. (1996) in herbicide-resistant *Setaria italica* (L.). Complete control of the S parental line (100% plant mortality) was never achieved at any diclofop-methyl and chlorsulfuron herbicide dose (Tables 2 and 3). Therefore, minor yet efficient genes endowing low-level diclofop-methyl or chlorsulfuron resistance may have been introduced in the F1 families by pair-crossing R with S parental plants. This may have contributed to heterogeneity of resistance segregation values often observed in F2 and BC families following herbicide treatments.

Implications of P450-based herbicide resistance genetics

In this study a high percentage of susceptibility in BC families was evident especially in response to diclofop-methyl. This phenomenon suggests that if herbicide use is discontinued and if there is back-crossing of R with S individuals, high level cytochrome P450-based herbicide resistance may be significantly regressed over two generations. Therefore, management strategies that involve discontinuity in use of P450-metabolized herbicides may help reduce the frequency of metabolism-based herbicide resistance across different chemical groups endowed by additive gene accumulation. In this *L. rigidum* population the likely success of this resistance management practice is also aided by the known expression of a fitness cost associated with P450 herbicide metabolism resistance (Vila-Aiub *et al*., 2005).

Conflict of interest

The authors declare no conflict of interest.

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