Crop Protection 65 (2014) 57–63

Contents lists available at ScienceDirect

Crop Protection

journal homepage: www.elsevier.com/locate/cropro

Genetic inheritance of cytochrome P450-mediated metabolic resistance to chlorsulfuron in a multiple herbicide resistant *Lolium rigidum* population

H. Han [a], Q. Yu [a, *], M. Vila-Aiub [b], S.B. Powles [a]

[a] Australian Herbicide Resistance Initiative, School of Plant Biology, University of Western Australia, 35 Stirling Hwy, WA 6009, Australia
[b] IFEVA-CONICET, Facultad de Agronomía, Universidad de Buenos Aires (UBA), Argentina

ARTICLE INFO

Article history:
Received 20 March 2014
Received in revised form
18 June 2014
Accepted 28 June 2014
Available online

Keywords:
Cytochrome P450
Genetic inheritance
Herbicide resistance
Herbicide metabolism
Lolium rigidum

ABSTRACT

Field evolved resistance to acetolactate synthase (ALS)-inhibiting herbicides in a multiple resistant *Lolium rigidum* population (VLR69) is known to be mainly due to enhanced rates of herbicide metabolism, likely involving cytochrome P450 monooxygenases. The present study investigates genetic inheritance of P450-mediated metabolic resistance to the ALS-inhibiting herbicide chlorsulfuron. To this end, a P450-mediated, metabolism-based resistant sub-set of VLR69 was carefully selected using plant vegetative cloning, appropriate herbicide screen test and the known P450 inhibitor malathion. Both intermediate and near-dominant nuclear-encoded phenotypic resistance traits were observed in 14 reciprocal F_1 families. The segregation of phenotypic chlorsulfuron resistance in ψ-F_2 families was analysed using genetic inheritance models involving one or two loci. The results from four ψ-F_2 families revealed complex patterns of genetic inheritance of P450-mediated metabolic resistance in genetically diverse and cross-pollinated species *L. rigidum*: multiple loci are likely involved and interact with herbicide rates and environmental conditions in mediating the resistance phenotype.

1. Introduction

Non-target-site base herbicide resistance (NTSR) refers to any mechanisms preventing a lethal herbicide dose reaching the herbicide target site. NTSR has long been documented in major grass weeds and is now increasingly implicated as a common herbicide resistance mechanism (Powles and Yu, 2010; Délye et al., 2011; Délye, 2013). One common NTSR mechanism is enhanced rates of herbicide metabolism (hereinafter referred to as metabolic herbicide resistance) often involving cytochrome P450 monooxygenases (Preston, 2004; Yuan et al., 2007; Powles and Yu, 2010; Beckie and Tardif, 2012; Yu and Powles, 2014a,b). Plant cytochrome P450 monooxygenases (thereafter referred to as P450s) form a large family that catalyse a diverse array of biosynthetic reactions for lignin, pigments, hormones, fatty acids, UV protectants and defence compounds (Schuler, 1996). Most plant P450s catalyse hydroxylation and dealkylation reactions to form more reactive products. Due to their high number and biochemical diversity, some P450s can metabolise certain herbicides to products with reduced or modified phytotoxicity that can be further inactivated often by conjugation to glucose, and subsequent transport into the vacuole (Kreuz et al., 1996).

Some crops (e.g. wheat, maize) have inherent capacity for P450-mediated herbicide metabolism (reviewed by Werck-Reichhart et al., 2000; Siminszky, 2006). Herbicide selection on a large weed population exerts a strong selection pressure for individuals possessing similar P450s capable of metabolising herbicides. Many populations of *Lolium rigidum*, *Alopecurus myosuroides*, *Echinochloa phyllopogon* as well as other weed species have evolved metabolic resistance to ALS-, ACCase- and PSII-inhibiting herbicides (Powles and Yu, 2010; Yu and Powles, 2014a,b). Known P450 inhibitors suppress *in vivo* herbicide metabolism and can reverse resistance, indicating that P450s are involved in herbicide metabolic resistance (e.g. Christopher et al., 1991; Hall et al., 1995, 1997; Preston et al., 1996; Fisher et al., 2000; Yun et al., 2005; Yasuor et al., 2009). However, our understanding of genetic inheritance of metabolic herbicide resistance is very limited with only a few available studies on well-characterised resistant weed populations (Preston, 2003;

* Corresponding author. Tel.: +61 8 64887041; fax: +61 8 64887834.
E-mail address: qin.yu@uwa.edu.au (Q. Yu).

http://dx.doi.org/10.1016/j.cropro.2014.06.026
0261-2194/

Letouzé and Gasquez, 2001; Busi et al., 2011, 2013). Progress at the molecular level has thus far been limited (Duhoux and Délye, 2013; Iwakami et al., 2013).

A well-characterised *L. rigidum* population (VLR69) with multiple resistance to many herbicide sites-of-action including ALS-, ACCase- and PSII-inhibiting herbicide (Burnet et al., 1994b) displays P450s-mediated metabolic resistance (Preston et al., 1996). An earlier genetic inheritance study indicated that resistance to the ALS-inhibiting herbicide chlorsulfuron in this population is inherited as a monogenic trait (Preston, 2003). During the process of selecting P450-based, metabolically resistant phenotypes from VLR69, we further examined the genetic control of chlorsulfuron resistance and revealed a more complex genetic pattern than previously determined for the population (Preston, 2003). Factors (e.g. herbicide rate and temperature) interacting with metabolic resistance expression were also discussed.

2. Materials and methods

2.1. Original plant material

A well-characterised herbicide susceptible *L. rigidum* population (VLR1) and the multiple herbicide resistant population (VLR69) were used in this study. In VLR69, chlorsulfuron resistance is mainly non-target-site, metabolism based (Burnet et al., 1994a; Preston et al., 1996), but a small fraction of the population exhibits ALS target-site resistance (Burnet et al., 1994a) endowed by the ALS Pro-197-Glu substitution (Yu et al., 2008).

2.2. Herbicide treatment

Herbicides were applied using a laboratory spray cabinet equipped with two flat fan nozzles delivering 118 L ha^{-1} water at 200 kPa pressure. After treatment, plants were returned to the conditions as specified below and mortality (no active new growth) was determined 21 days later.

2.3. Selection of the metabolism-based resistant sub-population

Sulfometuron, unlike the wheat-selective sulfonylurea herbicide chlorsulfuron, is not rapidly metabolised by *L. rigidum* (Christopher et al., 1991, 1992). Therefore, resistant *L. rigidum* individuals solely due to enhanced metabolism of sulfonylurea herbicides remain susceptible to sulfometuron. Hence, *in vivo* response to sulfometuron can be used as an indicator of the resistance mechanism expressed in resistant *L. rigidum* plants (Christopher et al., 1992).

Individual plants from the original VLR69 population were vegetatively separated into three clones. These clones were trimmed, re-potted and allowed to regrow for two weeks. Then, two of the three clones of each plant were treated with a high rate of chlorsulfuron (400 g ha^{-1}) or sulfometuron (40 g ha^{-1}), whereas the third clone was an untreated control. Plants with clones surviving chlorsulfuron but not the sulfometuron treatment were classified as enhanced metabolism-based resistant and bulked to produce seeds.

2.4. Selection of the P450-mediated, metabolism-based resistant sub-set and generation of F_1 families

The insecticide malathion, a known P450 inhibitor, has been demonstrated as a specific synergist for chlorsulfuron in *L. rigidum*, where resistance is solely due to P450-mediated herbicide metabolism (Christopher et al., 1994) and, therefore, was used to determine P450 involvement in metabolic resistance in VLR69.

Each individual metabolism-based resistant plant was divided into four clones, and three of them were treated respectively with sulfometuron (40 g ha^{-1}), or chlorsulfuron (400 g ha^{-1}) in the absence and presence of malathion (1000 g ha^{-1}), and one clone used as an untreated control. Individuals with clones, that well survived chlorsulfuron but were killed by sulfometuron or chlorsulfuron plus malathion, were identified as P450-mediated metabolic resistant plants and bulked to produce a P450 sub-set and used as a resistant (R) parental population. Vegetative clones from the herbicide susceptible population VLR1 were treated with chlorsulfuron at 0, 60 and 120 g ha^{-1}, respectively, to confirm susceptibility. Chlorsulfuron resistant (R) plants exhibiting P450-based resistance and susceptible (S) plants derived from untreated clones were used for pair-crossing to produce F_1 families. Single R and S plants were grown individually in pots and at the flowering time they were randomly paired and grown in isolation. At maturity, seeds were harvested from 7 reciprocal crosses between R and S parents thus producing 14 F_1 families. The herbicide rates used to discriminate the P450 based R and S individuals as vegetative clones were determined in preliminary experiments.

2.5. Generation of pseudo F_2 (ψ-F_2) families

Four F_1 families showing phenotypic resistance level similar to the R parents were chosen to produce F_2 families. Seeds of F_1 families collected from S parent plants, along with S and P450 based R parent populations, were sown and grown outdoors in winter. Seedlings (50–100 per treatment) were treated with 200 g chlorsulfuron ha^{-1}. As *L. rigidum* is an obligate cross-pollinated diploid species, surviving plants from within each F_1 family were randomly paired, isolated and seeds produced from each pair were bulked to produce ψ-F_2 families. Four ψ-F_2 families with adequate amount of quality seeds were selected for subsequent herbicide screening.

2.6. Chlorsulfuron screening of F_1 and ψ-F_2 seedlings

Fourteen F_1 reciprocal families, together with the parental S (VLR1) and the P450 based R were assessed for chlorsulfuron resistance. Several preliminary experiments were conducted to determine the key chlorsulfuron rates (e.g. 100, 200, 400 g ha^{-1}) discriminating the parental R and S plants, at different experimental conditions and plant developmental stages prior to the full dose response experiments. The S plants were treated with chlorsulfuron at the 2–3 leaf stage at 0, 12.5, 25, 50, 100, 200, 400 g ha^{-1} and the P450 based R plants as well as F_1 families were treated at 0, 25, 50, 100, 200, 400, 800 g ha^{-1}. Two experiments were conducted. The first was conducted outdoors in the normal growing season of *L. rigidum* (May–June with average temperature of 10.5 °C). The second trial was conducted under glasshouse conditions in summer (October–January with average temperature of 22 °C, under natural sunlight). There were three replicates per treatment (chlorsulfuron rate) and 20 plants per replicate. Seedlings (120–140) from each of the four F_2 families, together with the parent S, R and the respective F_1s, were chlorsulfuron-treated at 200 and 400 g ha^{-1} in the winter growing season, and 100 and 200 g ha^{-1} under glasshouse conditions, as described above.

2.7. Assessing resistance segregation using vegetative cloning of a ψ-F_2 family

To confirm herbicide screening results in F_2 seedlings, 98 individuals from a ψ-F_2 family (F_2-11), together with 15 individuals respectively from the S and R parents, were each divided into 3 clones. Twelve days after cloning, the clones were each treated with

chlorsulfuron at 50, 100 or 200 g ha^{-1} and phenotypic assessment of R and S plants was carried out 21 days after treatment.

2.8. Data analysis

Chi-square goodness of fit test (χ^2) was used to compare the observed plant survival values with those expected for one or two loci segregation models (Table 1). p Values were obtained for comparison with the significant level ($\alpha = 0.05$) to indicate the probability of committing type I error in rejecting the null hypothesis (H_0 = resistance segregation in a given ψ-F_2 family is consistent with one or two loci model as specified in Table 1). Often plant survival does not always achieve 100% and zero, respectively, in the parental R and S population at the discriminating herbicide rates used, and herbicide response of the F_1 plants is prone to experimental/environmental conditions, especially for non-target-site resistance. Therefore, the expected survival ratio in ψ-F_2 families (except for the experiment using vegetative clones) for each segregation model (Table 1) was corrected by the frequency of survival observed in herbicide treated parental S and R and F_1 plants, as described by Tabashnik (1991), Preston (2003) and detailed in Busi et al. (2013). For example, for a particular chlorsulfuron dose and according to the two dominant loci model (Table 1B) with resistance segregation ratio of R:RS (F_1):S = 9:6:1, the expected number of survivors of each ψ-F_2 family was calculated as:

$$\text{Survivors (expected)} = N_t\left[0.5625 \times \text{Ob}_{r(R)} + 0.375 \times \text{Ob}_{r(RS)} + 0.0625 \times \text{Ob}_{r(S)}\right]$$

where N_t is the number of herbicide-treated plants, 0.5625, 0.375 and 0.0625 are, respectively, the theoretical resistance ratio for R, RS (F_1) and S plants, and the Ob_r is the observed % of plant survival at a specific herbicide rate for the resistant parent (R), F_1 (RS) and susceptible parent (S). The heterogeneity χ^2 test (Sokal and Rohlf, 1969) was conducted to examine variations among the ψ-F_2 families in resistance segregation.

Table 1
Genetic models used for analysis of resistance segregation in ψ-F_2 families of *Lolium rigidum*.

A: single locus model

Alleles	*R1*	*r1*
R1	*R1R1*	*R1r1*
r1	*r1R1*	*r1r1*

ψ-F_2 phenotypic segregation 1R:2F_1:1S
Assumption:
1. Plants with no dominant R alleles behave like the parental S.
2. Plants with one R allele behave like F_1 (light-grey highlighted).
3. Plants with two R alleles behave like the parental R (dark-grey highlighted).

B: two loci model, dominant

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*

ψ-F_2 phenotypic segregation 9R:6F_1:1S
Assumption:
1. Plants with no dominant R alleles behave like the parental S.
2. Plants with R allele(s) only at one locus (R1 or R2) behave like F_1.
3. Plants with R allele(s) at both loci (R1 and R2) behave like the parental R.

3. Results

3.1. Identification of P450-sub-set as resistant parents for F_1 crosses

The metabolism-based sub-population of VLR69 was generated in this experiment and individual plants from this population were further screened for P450-mediated resistance using appropriate herbicide treatment in combination with the cytochrome P450 inhibitor malathion. Of the total 45 plants analysed, 28 individuals (62%) were identified to be P450-based, highly resistant plants, and only one resistant individual (2%) with possible target-site resistance. Six individuals (13%) did not survive 400 g chlorsulfuron and therefore, not further characterised. It is interesting to note that 10 individuals (22%) were able to survive chlorsulfuron even in the presence of malathion but killed by sulfometuron, indicating NTSR but unlikely involving malathion-sensitive P450s. Clones of seven P450-based resistant individuals were selected to generate the resistant (R) parental sub-set. Seven paired-crosses between R and S individuals (from VLR1) were made, and seeds were harvested separately from each parent plant, generating 14 reciprocal F_1 families.

3.2. P450-based metabolic resistance to chlorsulfuron is nuclear-gene controlled with varying dominance

Dose response studies of 14 F_1 families, together with the parental S and R plants, were conducted outdoors in winter and in glasshouse in summer. Similar results were observed in both conditions. As expected, the parent R displayed high level chlorsulfuron resistance with 97–100% survival at 100 g ha^{-1}. The parental S was clearly susceptible to chlorsulfuron and can be controlled at 100 g ha^{-1}. There was no difference in chlorsulfuron dose response between the two reciprocal F_1 families of each pair-cross, indicating nuclear inheritance of P450-mediated chlorsulfuron resistance. However, there were variations in the mortality response of F_1 families to chlorsulfuron: three F_1 families showed a resistance level intermediate between the R and S parents (Fig. 1a), and four F_1 families showed a resistance level closer to the R parents (Fig. 1b), especially at the discriminating rates of 100–400 chlorsulfuron ha^{-1}.

3.3. P450-based metabolic resistance is controlled by one or two loci

Resistance segregation of four ψ-F_2 families was assessed along with their corresponding F_1 families, and the parental S and R populations. Survival data was analysed by chi-square analysis fitting single locus (1R:2F_1:1S) or two loci (9R:6F_1:1S) models (Table 1).

When the experiment was conducted outdoors in the normal winter growing season, the parental S had with only 2% survival at 200 and 0.5% at 400 g chlorsulfuron ha^{-1}. As expected, parental R was resistant with 95% and 85% survival at the two respective chlorsulfuron rates (Table 2a, b). In F_1 families, the survival rate ranged from 76–93% (200 g ha^{-1}) to 49–78% (400 g ha^{-1}). This variation in survival response in the F_1 families indicates that defining a single rate (either at the low or high end) to simultaneously discriminate R, F_1 and S plants is challenging, although the single rates used are effective at discriminating the R and S parent plants. We have consistently observed that herbicide response of especially metabolism-based resistant F_1 plants is prone to changes in experimental/environmental conditions. As such the performance of related F_1 families has to be assessed together with the corresponding F_2 family and corrections on the expected survival ratio of the RS (F_1) need to be made. At 200 g chlorsulfuron ha^{-1}, the

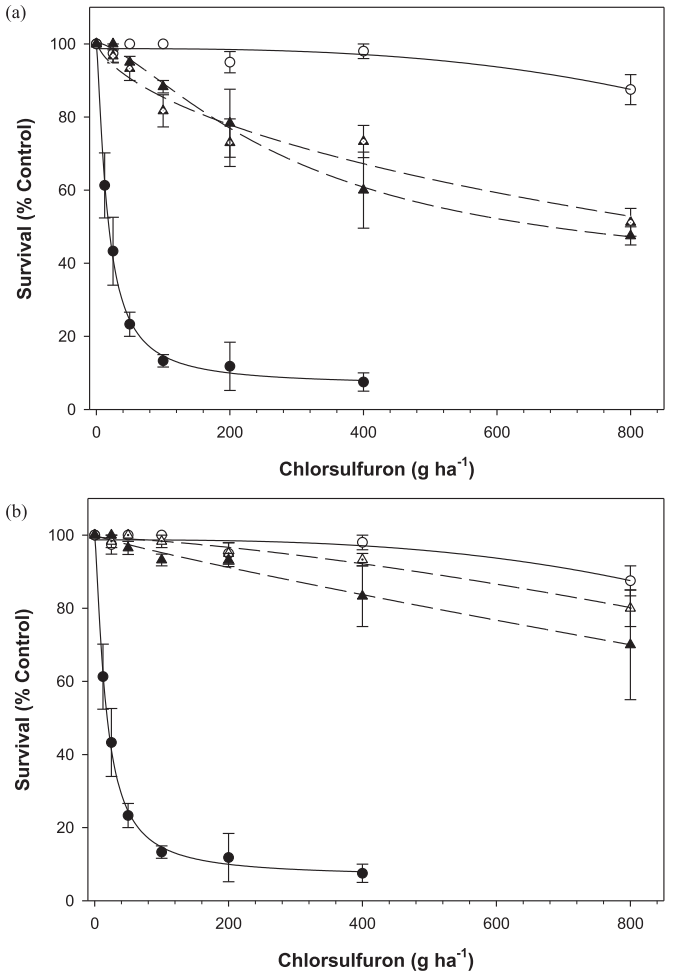

Fig. 1. Dose response to chlorsulfuron of representative reciprocal F_1 families (triangle), the parental P450-based resistant (open circle) and susceptible VLR1 (solid circle) populations, showing (a) intermediate and (b) near-dominant resistance pattern. Results were representative from the experiment conducted in glasshouse conditions.

chi-square test revealed one ψ-F_2 family (F_2-3) fitted the single locus model ($p = 0.84$) (Table 2a). The other three ψ-F_2 families clearly did not fit the single locus model ($p < 0.01$), due to higher than expected number of survivors. Instead, their resistance segregation indicated the involvement of at least two loci (Table 2a). The heterogeneity test was highly significant ($p \leq 0.01$) for each genetic model, indicating variations among the four ψ-F_2 families in resistance segregation at 200 g chlorsulfuron ha^{-1} (Table 2a). At 400 g chlorsulfuron ha^{-1}, none of the four ψ-F_2 families displayed resistance segregation patterns that fitted the monogenic model ($p < 0.05$) (Table 2b) again due to higher number of observed survivors. Rather, the polygenic genetic model involving at least two loci could generally explain the observed resistance segregation ratios in the four ψ-F_2 families. Heterogeneity test was not significant ($p > 0.1$) at 400 g chlorsulfuron ha^{-1} (Table 2b), indicating low variability among ψ-F_2 families in resistance segregation at the higher herbicide rate.

When the experiment was conducted in the glasshouse under warmer conditions, the parental S was similarly controlled by chlorsulfuron with only 5% and 3% survival respectively at 100 and 200 g ha^{-1}, while parental R showed 97% and 91% survival, respectively, at the two rates (Table 3a, b). Survival rates in F_1 families ranged between 55–91% (100 g ha^{-1}) and 49–85% (200 g ha^{-1}). Alike the winter experiment, at 100 g chlorsulfuron ha^{-1}, the observed resistance segregation pattern in the F_2-3 family

Table 2
Chi-square analysis and heterogeneity test for goodness of fit of the observed segregation for chlorsulfuron resistance in the four ψ-F_2 families of *Lolium rigidum* to the expected ratios of single or two loci model. The experiment was conducted outdoor in winter growing season and plants were treated with chlorsulfuron at (a) 200 and (b) 400 g ha^{-1}. The expected survival ratio for each model at each herbicide rate was corrected by the frequency of observed survival in the parental R, S and the respective F_1 families.

Family	Number of plants	Observed survival	Single locus segregation 1R:2F_1:1S			Two loci segregation 9R:6F_1:1S		
			Expected survival	χ^2	p	Expected survival	χ^2	p
(a) At 200 g chlorsulfuron ha^{-1}								
ψ-F_2								
F_2-3	99	71	70	0.04	0.84	88	27.7	0.00
F_2-4	92	79	60	17.1	0.00	78	0.14	0.71
F_2-8	127	109	79	29.9	0.00	104	1.15	0.28
F_2-11	91	79	65	10.5	0.00	81	0.40	0.53
Total	409	338	274	44.8	0.00	351	3.16	0.07
Heterogeneity test				12.8	0.01		26.2	0.00
Parent								
S	190	4						
R	188	179						
[a]F_1 family for								
F_2-3	148	138						
F_2-4	136	111						
F_2-8	138	105						
F_2-11	143	133						
(b) At 400 g chlorsulfuron ha^{-1}								
ψ-F_2								
F_2-3	96	68	58	4.36	0.04	74	2.2	0.14
F_2-4	90	69	51	15.3	0.00	65	1.02	0.31
F_2-8	98	61	47	8.23	0.00	65	0.73	0.39
F_2-11	99	74	59	9.24	0.00	75	0.01	0.91
Total	383	272	215	35.0	0.00	278	0.52	0.47
Heterogeneity test				2.18	0.54		3.44	0.33
Parent								
S	189	1						
R	188	160						
F_1 family for								
F_2-3	146	114						
F_2-4	97	62						
F_2-8	100	49						
F_2-11	100	73						

[a] Only those F_1 families derived from seeds collected from the susceptible parents were used.

fitted the single locus model ($p = 0.5$), and resistance segregation in F_2-4 and F_2-8 families can be again explained by the presence of at least two loci (Table 3a). However, resistance segregation pattern in the F_2-11 family fitted the monogenic locus model ($p = 0.66$) which is different from what was observed in the winter experiment showing two loci segregation. At the higher chlorsulfuron rate (200 g ha^{-1}), resistance segregation in two ψ-F_2 families (F_2-4, F_2-8) followed the two loci model and in the F_2-11 family it seemed to follow a single locus model ($p = 0.15$) (Table 3b). In contrast, at this higher chlorsulfuron rate, resistance segregation in the F_2-3 family did not fit either one or two loci pattern ($p \leq 0.03$). Heterogeneity for different ψ-F_2 families was high ($p \leq 0.01$) at both low and higher chlorsulfuron rates (Table 3a, b).

As the resistance segregation pattern in the F_2-11 family was variable between the two experiments conducted in different environmental conditions, an additional experiment was

Table 3
Chi-square analysis and heterogeneity test for goodness of fit of the observed segregation for chlorsulfuron resistance in the four ψ-F_2 families of *Lolium rigidum* to the expected ratios of single or two loci model. The experiment was conducted in glasshouse warmer conditions and plants were treated with chlorsulfuron at (a) 100 and (b) 200 g ha^{-1}. The expected survival ratio for each model at each herbicide rate was corrected by the frequency of observed survival in the parental R, S and the respective F_1 families.

Family	Number of plants	Observed survival	Single locus segregation 1R:2F_1:1S			Two loci segregation 9R:6F_1:1S		
			Expected survival	χ^2	p	Expected survival	χ^2	p
(a) At 100 g chlorsulfuron ha^{-1}								
ψ-F_2								
F_2-3	132	89	93	0.45	0.50	116	54.6	0.00
F_2-4	119	91	63	26.9	0.00	90	0.10	0.75
F_2-8	142	121	93	23.9	0.00	121	0.01	0.93
F_2-11	142	103	101	0.20	0.66	126	37.3	0.00
Total	535	404	349	24.8	0.00	453	33.7	0.00
Heterogeneity test				26.7	0.00		58.2	0.0
Parent								
S	196	10						
R	183	177						
F_1 family for								
F_2-3	140	125						
F_2-4	143	78						
F_2-8	144	116						
F_2-11	141	128						
(b) At 200 g chlorsulfuron ha^{-1}								
ψ-F_2								
F_2-3	129	77	65	4.69	0.03	92	8.53	0.00
F_2-4	116	85	56	30.1	0.00	81	0.82	0.37
F_2-8	136	106	84	15.1	0.00	109	0.39	0.53
F_2-11	147	105	97	2.08	0.15	122	13.9	0.00
Total	528	373	301	40.2	0.00	403	9.71	0.00
Heterogeneity test				11.7	0.01		14.0	0.00
Parent								
S	190	6						
R	187	170						
F_1 family for								
F_2-3	137	73						
F_2-4	146	71						
F_2-8	145	111						
F_2-11	142	120						

conducted in the glasshouse with the F_2-11 family using vegetative clones. In this experiment, the phenotype (R or non-R) segregation of each 98 individual F_2-11 plants was determined using three chlorsulfuron rates, relative to the performance of parental R and S plants. Data in Table 4 confirmed that monogenic locus model is the only fit ($p = 0.73$) in the warmer glasshouse conditions.

Generally, P450-based phenotypic chlorsulfuron resistance in the four ψ-F_2 families falls into three groups: group I (F_2-3 family) involving between one and two loci, affected by herbicide rates; group II (F_2-11 family) involving between one and two loci, influenced by environmental conditions (e.g. temperatures); group II (F_2-4 and F_2-8 families) involving at least two loci regardless of herbicide rates and environmental conditions tested.

4. Discussion

In the present study, we identified and selected P450-metabolism-based chlorsulfuron-resistant individuals from the multiple herbicide resistant VLR69 population to investigate the genetic control of P450-based metabolic resistance with a final objective of identification and cloning of P450 and other genes involved. Contrary to a previous study showing that metabolic chlorsulfuron resistance in the multiple herbicide resistant VLR69 population is controlled by a single nuclear major gene with partial dominance (Preston, 2003), here we reveal a more complex genetic control of P450-based metabolic resistance: individuals differ in resistance dominance levels, in the number of resistance loci involved and likely in resistance gene expression levels affected by environmental conditions.

Table 4
Chi-square analysis for goodness of fit of observed segregation for chlorsulfuron resistance in the F_2-11 family of *Lolium rigidum*, using vegetative clones. Three clones were obtained from each individual plants and were each treated with chlorsulfuron at 50, 100, 200 g ha^{-1} respectively. Phenotypic assessment for resistance (R) and susceptibility (S) was made relative to the response of susceptible and resistant parental plants.

Total plants treated	Resistant individuals observed	Single locus segregation (3R:1S)		
		Resistance individuals expected	χ^2	p
98	75	74	0.10	0.73

4.1. Genetic dominance of P450-based metabolic resistance to chlorsulfuron

Among seven F_1 families analysed, three families displayed intermediate and four families displayed near dominant pattern of chlorsulfuron resistance (Fig. 1). This result basically concurs with the early study by Preston (2003) showing an intermediate type of response in the same population and by Busi et al. (2011) showing a dominance pattern in a different P450 metabolism-based resistant *L. rigidum* population (SLR31). Differences are likely due to (1) genetic variability expected for parental R plants within and between *L. rigidum* populations used to generate F_1 families, (2) differences in the range of chlorsulfuron rates used, and/or (3) difference in experimental/environmental conditions which have more influence on the F_1 than the parental plants. For example, in Busi et al. (2011) chlorsulfuron rates from 0 to 90 g ha^{-1} were used in comparison to 0–800 g ha^{-1} used in our study (Fig. 1). In addition, it is also possible that intermediate type resistance is sometimes caused by parental heterozygosity (e.g. using RS × SS instead of RR × SS structure for pair crossing) (Yu et al., 2009). This is highly likely for NTSR in obligate outcrosser where molecular basis is unknown and therefore, resistance genotypes are difficult to determine.

4.2. Genetic control of P450-based metabolic resistance to chlorsulfuron

This study shows that P450-mediated chlorsulfuron resistance in VLR69 is controlled by from a single locus to at least two loci (Tables 2 and 3), reflecting diversity in the genetic control of this metabolic resistance. This result is consistent with previous genetic studies demonstrating both mono- and poly-genic control of diclofop-methyl or chlorsulfuron resistance (Busi et al., 2011, 2013) in metabolism-based resistant *L. rigidum* populations (Neve and Powles, 2005; Yu et al., 2013). This level of genetic complexity has also been demonstrated in NTSR to ACCase- and ALS-inhibiting herbicides in *A. myosuroides* (Petit et al., 2010). However, a monogenic pattern for chlorsulfuron resistance in the population VLR69 was previously determined by Preston (2003). The inconsistency is likely due to different strategies in selection of individual plants for pair-crossing, environmental conditions (e.g. temperatures) during

the experiments, and genetic inheritance models used. For example, in the Preston (2003) study, plants possessing metabolism-based resistance were selected while in our current study, plants possessing metabolism-based, malathion-sensitive resistance were used. In fact in this study, 20% of individuals from within VLR69 showed malathion-insensitive resistance and were consequently eliminated in our study. In addition, the resistance segregation pattern was only tested for fitting a single gene model in Preston (2003) study. Nevertheless, fitting one genetic model does not exclude possibility of fitting other models.

In this work we were also able to show that segregation of metabolic herbicide resistance was affected by herbicide rates. For example, at relatively low discriminating chlorsulfuron rates (200 versus 400, 100 versus 200 in two experiments respectively) resistance segregation in F_2-3 fit one locus model (Tables 2a and 3a), but at higher chlorsulfuron rates it doesn't fit with more than expected survivors (Tables 2b and 3b). Interestingly, the results have also showed that resistance genetic control in one ψ-F_2-family (F_2-11) was affected by environmental conditions (e.g. temperature) (Tables 2 and 3). P450-based metabolic resistance to chlorsulfuron seems to vary between winter outdoor (Table 2) and summer glasshouse conditions (Table 3). This is expected for polygenic and quantitative resistance traits whose expression is prone to G × E effects, and thus complicated by inheritable and non-inheritable genetic factors (e.g. constitutively versus induced expression of genes). In fact, impact of environmental conditions (e.g. temperatures) on phenotypic herbicide resistance has been observed for non-target-site resistance to paraquat (Purba et al., 1995; Yu et al., 2004) and glyphosate resistance (Ge et al., 2011; Vila-Aiub et al., 2013).

In the current study, Mendelian inheritance models were used as herbicide effects on mortality of R and S individuals are distinct. However, these only reflect simple cases (dominant) of one or two loci models. Other additive models have been used for analysis of resistance segregation pattern in other resistant *L. rigidum* populations (Busi et al., 2011, 2013). As polygenic loci interaction is theoretically infinite and genotype versus phenotype is not always one to one relation for resistance traits, we understand that it is difficult to resolve exactly how many loci are involved in metabolic herbicide resistance using the basic Mendelian genetic models. Nevertheless, in essence, our data demonstrates that genetic control of P450-based metabolic resistance to ALS-inhibiting herbicide chlorsulfuron in an *L. rigidum* population (VLR69) is more complex than the previous single locus model as proposed by Preston et al. (2003), and is likely of polygenic and quantitative nature. Individuals likely differ in the number of loci involved in phenotypic chlorsulfuron resistance which is further modified by herbicide rates and environmental variations. As the P450 inhibitor malathion can reverse resistance, between at least one to several malathion-sensitive P450s may be involved in chlorsulfuron resistance in individuals of the resistant population. In addition, other enzymes interacting with or downstream of P450 (such as GST and glucosyl transferase (GT)) may also play a role in metabolic herbicide resistance. However, we cannot entirely exclude the possibility of environmental and/or random effects on resistance penetrance/expressivity and thus skewing results away from the single-gene, full penetrance model. As a long-term goal, it would be interesting to isolate highly homozygous isogenic lines (e.g. R1R1r2r2r3r3, r1r1R2R2r3r3, r1r1r2r2R3R3), and evaluate the environmental influence on resistance of these isolated lines. This would also help demonstrate polygenic resistance in the original parents. Recently, a few P450 genes have been identified in resistant populations of *E. phyllopogon* conferring resistance to ALS-inhibiting herbicides (Iwakami et al., 2013, 2014). P450, GT and other genes were also found to be involved in resistance to ACCase- and ALS-inhibiting herbicides in a number of resistant *L. rigidum* populations (Gaines et al., 2014). Given the complex genetic nature of metabolic herbicide resistance in cross-pollinated weed species, identifying all P450 and other genes/alleles involved remains challenging whereas comprehensive genomic approaches such as next generation transcriptome sequencing (RNA-seq) opens up new research opportunities. The resistant and susceptible parental as well as segregating ψ-F_2 families highly characterised in this study are valuable resources for resistance gene discovery.

Acknowledgements

This research is funded by the Grains Research and Development Corporation of Australia (GRDC) (Grant number: UWA0012). The authors thank Drs Michael Christoffers and Roberto Busi for their valuable comments on early manuscript drafts, and Mr Michael Ashworth for proofreading the manuscript.

References

Beckie, H.J., Tardif, F.J., 2012. Herbicide cross resistance in weeds. Crop Prot. 35, 15–28.

Burnet, M.W.M., Christopher, J.T., Holtum, J.A.M., Powles, S.B., 1994a. Identification of two mechanisms of resistance to sulfonylurea herbicides within one population of rigid ryegrass (*Lolium rigidum*) using a selective germination medium. Weed Sci. 42, 468–473.

Burnet, M.W.M., Holtum, J.A.M., Powles, S.B., 1994b. Resistance to nine herbicide classes in a population of rigid ryegrass (*Lolium rigidum*). Weed Sci. 42, 369–377.

Busi, R., Vila-Aiub, M.M., Powles, S.B., 2011. Genetic control of a cytochrome P450 metabolism-based herbicide resistance mechanism in *Lolium rigidum*. Heredity 106, 817–824.

Busi, R., Neve, P., Powles, S.B., 2013. Evolved polygenic herbicide resistance in *Lolium rigidum* by low-dose herbicide selection within standing genetic variation. Evol. Appl. 6, 231–242.

Christopher, J., Preston, C., Powles, S.B., 1994. Malathion antagonizes metabolism-based chlorsulfuron resistance in *Lolium rigidum*. Pestic. Biochem. Phys. 49, 172–182.

Christopher, J.T., Powles, S.B., Holtum, J.A.M., 1992. Resistance to acetolactate synthase-inhibiting herbicides in annual ryegrass (*Lolium rigidum*) involves at least two mechanisms. Plant Physiol. 100, 1909–1913.

Christopher, J.T., Powles, S.B., Liljegren, D.R., Holtum, J.A.M., 1991. Cross resistance to herbicides in annual ryegrass (*Lolium rigidum*). II. Chlorsulfuron resistance involves a wheat-like detoxification system. Plant Physiol. 95, 1036–1043.

Délye, C., 2013. Unravelling the genetic bases of non-target-site-based resistance (NTSR) to herbicides: a major challenge for weed science in the forthcoming decade. Pest Manag. Sci. 69, 176–187.

Délye, C., Gardin, J.A.C., Boucansaud, K., Chauvel, B., Petit, C., 2011. Non-target-site-based resistance should be at the centre of herbicide resistance research attention: blackgrass (*Alopecurus myosuroides*) as an illustration. Weed Res. 51, 433–437.

Duhoux, A., Délye, C., 2013. Reference genes to study herbicide stress response in *Lolium* sp: up-regulation of P450 genes in plants resistant to acetolactate-synthase inhibitors. PLOS One 8, e63576.

Fisher, A.J., Bayer, D.E., Carriere, M.D., Ateh, C.M., Yim, K.O., 2000. Mechanisms of resistance to bispyribac-sodium in an *Echinochloa phyllopogon* accession. Pestic. Biochem. Phys. 28, 156–165.

Gaines, T.A., Lorentz, L., Figge, A., Herrmann, J., Maiwald, F., Ott, M.C., Han, H., Busi, R., Yu, Q., Powles, S.B., Beffa, R., 2014. RNA-Seq transcriptome analysis to identify genes involved in metabolism-based diclofop resistance in *Lolium rigidum*. Plant J. 78, 865–876.

Ge, X., d'Avignon, D.A., Ackerman, J.J., Duncan, B., Spaur, M.B., Sammons, R.D., 2011. Glyphosate-resistant horseweed made sensitive to glyphosate: low-temperature suppression of glyphosate vacuolar sequestration revealed by ^{31}P NMR. Pest Manag. Sci. 67, 1215–1221.

Hall, L.M., Moss, S.R., Powles, S.B., 1995. Mechanism of resistance to chlortoluron in two biotypes of the grass weed *Alopecurus myosuroides*. Pestic. Biochem. Phys. 53, 180–192.

Hall, L.M., Moss, S.R., Powles, S.B., 1997. Mechanisms of resistance to aryloxyphenoxypropionate herbicides in two biotypes of *Alopecurus myosuroides* (black grass): herbicide metabolism as a cross resistance mechanism. Pest Manag. Sci. 57, 87–98.

Iwakami, S., Uchino, A., Kataoka, Y., Shibaike, H., Watanabe, H., Inamura, T., 2014. Cytochrome P450 genes induced by bispyribac-sodium treatment in a multiple-herbicide resistant biotypes of *Echinochloa phyllopogon*. Pest Manag. Sci. 70, 549–558.

Iwakami, S., Endo, M., Saika, H., Okuno, J., Nakamura, N., Watanable, H., Yokoyama, M., Toki, S., Uchino, A., Inamura, T., 2013. Cytochrome P450 genes involved in the resistance to different classes of ALS inhibitors in *Echinochloa*

phyllopogon. In: Global Herbicide Resistance Challenge Conference. Perth, Australia.
Kreuz, K., Tommasini, R., Martinola, E., 1996. Old enzymes for a new job. Plant Physiol. 111, 349–353.
Letouzé, A., Gasquez, J., 2001. Inheritance of fenoxaprop-*P*-ethyl resistance in a blackgrass (*Alopecurus myosuroides* Huds.) population. Theor. Appl. Genet. 103, 288–296.
Neve, P., Powles, S.B., 2005. Recurrent selection with reduced herbicide rates results in the rapid evolution of herbicide resistance in *Lolium rigidum*. Theor. Appl. Genet. 110, 1154–1166.
Petit, C., Duhieu, B., Boucansaud, K., Délye, C., 2010. Complex genetic control of non-target-site-based resistance to herbicides inhibiting acetyl-coenzyme A carboxylase and acetolactate synthase in *Alopecurus myosuroides* Huds. Fr. Pest Manag. Sci. 66, 168–177.
Powles, S.B., Yu, Q., 2010. Evolution in action: plants resistant to herbicides. Annu. Rev. Plant Biol. 61, 317–347.
Preston, C., 2003. Inheritance and linkage of metabolism-based herbicide cross-resistance in rigid ryegrass (*Lolium rigidum*). Weed Sci. 51, 4–12.
Preston, C., 2004. Herbicide resistance in weeds endowed by enhanced detoxification: complications for management. Pest Manag. Sci. 52, 448–453.
Preston, C., Tardif, F.J., Christopher, J.T., Powles, S.B., 1996. Multiple resistance to dissimilar herbicide chemistries in a biotype of *Lolium rigidum* due to enhanced activity of several herbicide degrading enzymes. Pestic. Biochem. Phys. 54, 123–134.
Purba, E., Preston, C., Powles, S.B., 1995. The mechanism of resistance to paraquat is strongly temperature dependent in resistant *Hordeum leporinum* link and *H. glaucum* Steud. Planta 196, 464–468.
Schuler, M.A., 1996. Plant cytochrome P450 monooxygenases. Crit. Rev. Plant Sci. 15, 235–284.
Siminszky, B., 2006. Plant cytochrome P450-mediated herbicide metabolism. Phytochem. Rev. 5, 445–458.
Sokal, R.R., Rohlf, F.J., 1969. Biometry. W.H. Freeman and Company, San Francisco, p. 776.
Tabashnik, B.E., 1991. Determining the model of inheritance of pesticide resistance with backcross experiments. J. Econ. Entomol. 84, 703–712.
Vila-Aiub, M.M., Gundel, P., Yu, Q., Powles, S.B., 2013. Glyphosate resistance in *Sorghum halepense* and *Lolium rigidum* is reduced at suboptimal growing temperatures. Pest Manag. Sci. 69, 228–232.
Werck-Reichhart, D., Hehn, A., Didierjean, L., 2000. Cytochromes P450 for engineering herbicide tolerance. Trends Plant Sci. 5, 116–123.
Yasuor, H., Osuna, M.D., Ortiz, A., Saldain, N.E., Eckert, J.W., Fisher, A., 2009. Mechanism of resistance to penoxsulam in late watergrass (*Echinochloa phyllopogon* (Stapf) koss). J. Agric. Food Chem. 57, 3653–3660.
Yu, Q., Han, H.P., Cawthray, G., Wang, S.F., Powles, S.B., 2013. Enhanced rates of herbicide metabolism in low herbicide-dose selected resistant *Lolium rigidum*. Plant Cell Environ. 36, 818–827.
Yu, Q., Cairns, A., Powles, S.B., 2004. Paraquat resistance in a population of *Lolium rigidum*. Funct. Plant Biol. 31, 247–254.
Yu, Q., Han, H., Powles, S.B., 2008. Mutations of the ALS gene endowing resistance to ALS-inhibiting herbicides in *Lolium rigidum* populations. Pest Manag. Sci. 64, 1229–1236.
Yu, Q., Han, H., Nguyen, L., Forster, J.W., Powles, S.B., 2009. Paraquat resistance in a *Lolium rigidum* population is governed by one major nuclear gene. Theor. Appl. Genet. 118, 1601–1608.
Yu, Q., Powles, S.B., 2014a. Resistance to AHAS inhibitor herbicides: current understanding. Pest Manag. Sci. 70, 1340–1350.
Yu, Q., Powles, S.B., 2014b. Metabolism-based herbicide resistance and cross-resistance in crop weeds: a threat to herbicide sustainability and global crop production. Invited review for Plant Physiol. (Accepted).
Yuan, J.S., Tranel, P.J., Stewart, C.N., 2007. Non-target-site herbicide resistance: a family business. Trends Plant Sci. 12, 6–13.
Yun, M.S., Yogo, Y., Miura, R., Yamasue, Y., Fischer, A., 2005. Cytochrome P-450 monooxygenase activity in herbicide-resistant and susceptible late watergrass (*Echinochloa phyllopogon*). Pestic. Biochem. Physiol. 83, 107–114.