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Non-target site resistance to flucarbazone, imazamethabenz and pinoxaden is controlled by three linked genes in *Avena fatua*

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Summary

Extensive herbicide usage has led to the evolution of resistant weed populations that cause substantial crop yield losses and increase production costs. The multiple herbicide-resistant (MHR) *Avena fatua* populations utilised in this study are resistant to members of all selective herbicide families, across five modes of action, available for *A. fatua* control in US small grain production, and thus pose significant agronomic and economic threats. Resistance to acetolactate synthase and acetyl-CoA carboxylase inhibitors is not conferred by known target site mutations, indicating that non-target site resistance (NTSR) mechanisms are involved. Understanding the inheritance of NTS MHR is of upmost importance for continued agricultural productivity in the face of the rapid increase in resistant weed populations worldwide. As few studies have examined the inheritance of NTSR in autogamous weeds, we investigated the inheritance and genetic control of NTSR in the highly autogamous, allohexaploid species *A. fatua*. We found that NTSR in MHR *A. fatua* is controlled by three separate, closely-linked nuclear genes for flucarbazone-sodium, imazamethabenz-methyl and pinoxaden. The single-gene NTSR inheritance patterns reported here contrast with other examples in allogamous species and illustrate the diversity of evolutionary responses to strong selection.

Keywords: NTSR, herbicide resistance, genetics, inheritance, wild-oat, xenobiotic.

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Introduction

Agricultural weeds are the primary cause of crop yield loss, and herbicides have been the most prominent and effective weed control practice for over 50 years (Liebman *et al.*, 2001). However, their extensive usage worldwide imposes strong selection for resistant weed populations, threatening their continued efficacy. Populations of approximately 250 species have evolved resistance to 23 of the 26 known herbicide mechanisms of action, representing over 160 different herbicides (Heap, 2017). Furthermore, 34% of these populations are resistant to two or more mechanisms of action, a phenomenon known as multiple herbicide resistance (MHR).

Resistance can be conferred by target site overexpression or mutations that alter herbicide binding, or non-target site resistance (NTSR) alterations, such as enhanced rates of herbicide metabolism, reduced absorption and/or translocation, sequestration or more generalised stress defence networks (Délye, 2013). The selection of NTSR is particularly threatening, as it is

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associated with unpredictable patterns of resistance to unrelated mechanisms of action (Délye *et al*., 2013) and to herbicides not yet commercially available (Petit *et al*., 2010a).

It has long been recognised that the underlying genetics and inheritance of herbicide resistance have profound implications for the occurrence, evolution, spread and management of resistant populations (Jasieniuk *et al*., 1996). It is particularly important to understand the genetic determinants of NTSR, due to the increasing number of reports that NTSR is the prominent type of resistance to the three most widely used herbicide mechanisms of action worldwide: acetyl-CoA carboxylase (ACCase) inhibitors, acetolactate synthase (ALS) inhibitors (for grasses) and 5-enolpyruvylshikimate-3-phosphate synthase inhibitors (Délye, 2013).

Non-target site resistance often results from a gradual selective process over multiple generations, and previous research in populations of *Alopecurus myosuroides* Huds., *Lolium rigidum* Gaudin and *Amaranthus tuberculatus* (Moq.) J.D. Sauer demonstrates that NTSR is conferred by multiple mechanisms and alleles (Busi *et al*., 2010; Petit *et al*., 2010b; Huffman *et al*., 2015). In an extreme case, resistance to ACCase and ALS inhibitors in an NTSR population of the allogamous species *A. myosuroides* is controlled by three loci for each herbicide (Petit *et al*., 2010b). In contrast to the numerous studies of NTSR in allogamous species (Preston, 2003; Busi *et al*., 2010, 2012; Petit *et al*., 2010b; Han *et al*., 2014; Huffman *et al*., 2015; Rosenhauer *et al*., 2015), only a few studies have investigated NTSR inheritance in autogamous species, with both showing that it was controlled by a single gene in *Echinochloa phyllopogon* (Stapf) Koso-Pol. (Iwakami *et al*., 2014) and *Conyza canadensis* (L.) Cronquist (Zelaya *et al*., 2004).

Avena fatua L. is an annual, predominantly autogamous and allohexaploid monocotyledonous species. *Avena fatua* is a successful, persistent and economically important weed around the world, especially in the Northern Great Plains and Pacific Northwest of North America, where it infests over 11 million ha and causes crop losses of over $1 billion annually (Beckie *et al*., 2012). Due to extensive use of herbicides that inhibit ACCase, ALS and other targets, populations of resistant *A. fatua* have evolved in the USA (Lehnhoff *et al*., 2013; Keith *et al*., 2015) and worldwide (Heap, 2017). In Montana, producer complaints from a malt barley production area led us to investigate *A. fatua* infestations that were not controlled by pinoxaden. This complaint was unusual because it was the first year pinoxaden was commercially available and, due to its novel chemical characteristics, successful control was expected. From these, we derived two populations of resistant *A. fatua* termed MHR3 and MHR4 that are resistant to the ACCase inhibitors fenoxaprop-P-ethyl, tralkoxydim and pinoxaden, the ALS inhibitors imazamethabenz-methyl and flucarbazone-sodium, the growth inhibitor difenzoquat, the photosystem I inhibitor paraquat (MHR3 only) and the very long-chain fatty acid biosynthesis inhibitor triallate, with resistant/susceptible ratios ranging from 1.4 to 57 (Lehnhoff *et al*., 2013; Keith *et al*., 2015). These MHR populations are thus resistant to members of all selective herbicide families available for *A. fatua* control in small grain crops. The cytochrome P450 monooxygenase inhibitor malathion partially reversed the resistance phenotype for several herbicides (Keith *et al*., 2015), indicating that NTSR mechanisms are involved.

As relatively few studies have examined the inheritance of NTSR in autogamous weed species, we investigated the inheritance and genetic control of NTSR in MHR populations of the mostly autogamous species *A. fatua*.

Materials and methods

Parental populations

The MHR3 and MHR4 parental populations were derived from seeds collected in 2006 from two *A. fatua* populations not controlled by 60 g a.i. ha^{-1} pinoxaden in two production fields separated by approximately 8 km in Teton County, Montana, USA. Field-collected seeds (about 90% of which were resistant to 60 g a.i. ha^{-1} pinoxaden [Axial XL, 50.3 g a.i. L^{-1}, EC, Syngenta]), data not shown) were subjected to two generations of recurrent group selection (50 plants each generation) by spraying with the same dose of pinoxaden, after which 100% of plants were confirmed to be homozygous resistant to pinoxaden via dose–response experiments (Lehnhoff *et al*., 2013; Keith *et al*., 2015). From each generation of 50 plants, all seeds were harvested and a random selection of 50 seeds was used to initiate five additional generations without herbicide selection to homogenise the genetic background. Recurrent group selection was used as opposed to single seed descent, in order to maintain representative genetic backgrounds in the MHR3 and MHR4 populations. Further characterisation of these populations is reported in Lehnhoff *et al*. (2013) and Keith *et al*. (2015). The herbicide susceptible parental population HS2 is the highly inbred nondormant SH430 line used in seed dormancy research (Johnson *et al*., 1995). All plants used in this research were grown under a 16-h photoperiod of natural sunlight supplemented with mercury vapour lamps (165 μmol m^{-2} s^{-1}) at

25 ± 4°C in standard glasshouse soil mix [1:1:1 (by vol) Bozeman silt loam:Sunshine mix #12 (Sun Gro Horticulture, Inc., Bellevue, WA, USA):perlite] and fertilised weekly with 100 mg L^{-1} of Jack's Classic 20-20-20 All Purpose fertiliser (JR Peters Inc., Allentown, PA, USA).

Establishing F_1–F_3 populations

Homozygous MHR3, MHR4 and HS2 plants were used as parents in reciprocal crosses to generate F_1 seed. Homozygosity of parental resistance was confirmed for MHR3 and MHR4 by zero injury from treatment with field use rates of flucarbazone-sodium (flucarbazone hereafter; 30 g a.i. ha^{-1} [Everest, 70% WDG, WDG, Arysta LifeScience North America LLC]), imazamethabenz-methyl (imazamethabenz hereafter; 94 g a.i. ha^{-1} [Assert, 299.6 g a.i. L^{-1}, EC, Nufarm Inc]) or pinoxaden (58 g a.i. ha^{-1}) as previously reported (Lehnhoff *et al.*, 2013; Keith *et al.*, 2015), susceptibility of HS2 plants by 100% injury from the same treatments (data not shown) and all three parents were screened with the PCR primer pairs below. Reciprocal crosses were performed using the methods of Brown (1980) for *Avena sativa* L. cross-pollination. The resulting 151 putative F_1 seeds were grown in the glasshouse, self-pollinated by wrapping individual panicles in clear polypropylene pollen-proof bags and F_2 seeds collected. Fifty-five successful F_1 crosses were confirmed by Touchdown PCR assays using primer pairs AJ010728 (Holland *et al.*, 2001) and AM83 (Pal *et al.*, 2002). Of these, four F_1 plants representing each pairwise reciprocal cross [(MHR3 × HS2), (HS2 × MHR3), (MHR4 × HS2), (HS2 × MHR4)] were bagged and self-pollinated to produce F_2 seeds. Ten F_2 families from each of the four F_1 plants were self-pollinated and advanced to the F_3 generation. In total, 100 F_3 plants from each pairwise cross (10 from each of the 10 F_2 families) were screened for herbicide response. Family sizes were calculated as described in Jasieniuk *et al.* (1996).

Herbicide screening

F_3 and parental HS2, MHR3 and MHR4 plants were screened for herbicide response using previously determined doses that were lethal to HS2 plants, while not severely injuring MHR plants (Lehnhoff *et al.*, 2013). One ACCase- and two ALS-inhibitor herbicides (127 g a.i. ha^{-1} imazamethabenz, 30 g a.i. ha^{-1} flucarbazone plus 0.125% (v/v) nonionic surfactant and 29 g a.i. ha^{-1} pinoxaden) were applied separately to three-leaf stage plants using a moving nozzle sprayer in 94 L water ha^{-1}. When the spray solution had dried, plants were returned to the glasshouse conditions described above for 3 week, after which plants were scored for visual injury (0 = no injury to 4 = plant death; Fig. 1). All herbicide applications were made in mid-morning to minimise potential environmental- and circadian-induced changes in plant response. In total, 100 F_3 plants from each pairwise cross and 20 parental plants were screened for each herbicide.

Data analysis

Data obtained from F_3 plants were used to derive the genotypes of each F_2 family. F_3 plants were classified as homozygous resistant or homozygous susceptible when injury ratings were within two standard errors of the mean injury rating for MHR or HS parents, respectively, or segregating for resistance if the above criteria were not met (Table 1). Because hexaploid *A. fatua* exhibits bivalent chromosome pairing with disomic inheritance, results were compared with Mendelian disomic inheritance segregation ratios. To confirm homozygosity, a second set of herbicide treatments was applied as described above to 20 additional F_3 plants from each F_2 family that were scored as homozygous resistant or susceptible.

To compare injury ratings among each F_2 family and determine whether each F_2 family from each F_1 cross could be combined for data analysis, a linear model was constructed for each herbicide screened, with fixed effects for F_1, F_2 and F_3. Models were fit using the lm function in R, and means were separated using Tukey's HSD *post hoc* tests (R, 2016). When appropriate ($P > 0.05$), data were pooled and chi-square tests were performed to determine the goodness of fit of F_2 family segregation ratios to known genetic models. To confirm chi-square test results, Fisher's exact tests were conducted in R using the xmulti function in the XNomial package (R, 2016). Tests for linkage among resistance genes to the three herbicides

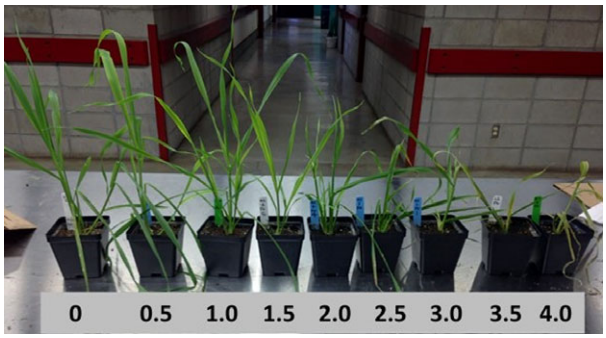

Fig. 1 Visual injury rating scale of *Avena fatua* F_3 plants used for genetic analysis of non-target site resistance. [Colour figure can be viewed at wileyonlinelibrary.com]

Table 1 Mean injury ratings (standard errors) used for F_3 family classifications of parental populations HS2, MHR3 and MHR4 *Avena fatua* treated with flucarbazone, imazamethabenz or pinoxaden

Parental population	Herbicide	Mean injury*
HS2	Flucarbazone	3.10 (0.067)
MHR3	Flucarbazone	0.44 (0.056)
MHR4	Flucarbazone	0.20 (0.082)
HS2	Imazamethabenz	3.00 (0.000)
MHR3	Imazamethabenz	0.95 (0.189)
MHR4	Imazamethabenz	1.53 (0.230)
HS2	Pinoxaden	3.06 (0.056)
MHR3	Pinoxaden	0.50 (0.065)
MHR4	Pinoxaden	0.69 (0.091)

*Injury rating using a scale of 0 = no injury to 4 = plant death.

were conducted using both logarithm of odds (LOD) score analysis and recombination frequency analysis following the methods of Lathrop *et al.* (1984). LOD scores were considered significant when *P*-values were ≤ 0.05, and a detailed discussion on the interpretation of LOD scores is addressed in Nyholt (2000). Correlation analyses using the mean F_2 injury ratings were conducted in R using the rcorr function in the Hmisc package (R, 2016) to examine the pairwise correlation of injury ratings to the three herbicides tested in this study.

Results

Genetic analysis

Data were pooled for analysis based on non-significant injury rating differences among reciprocal crosses (data not shown) for plants screened with flucarbazone, imazamethabenz and pinoxaden. F_2 families for each herbicide segregated in a 1 homozygous resistant: 2 segregating: 1 homozygous susceptible ratio (Table 2), indicating that resistance is conferred by one gene for each herbicide. A lack of reciprocal cross differences indicated that resistance genes are nuclear and not cytoplasmic.

Linkage analysis

Data obtained from F_3 plants were used to derive the genotypes of each F_2 family. For linkage analysis, non-parental recombinants were classified as F_2 families that were resistant to only one of the herbicides screened. All other phenotypes, resistant to all or susceptible to all herbicides, were classified as parental types. The presence of non-parental recombinants indicates that the same gene is not responsible for resistance to all three herbicides screened in this study. Furthermore, if resistance genes segregate independently and are dominant, then the F_2 families should segregate in a 9:3:3:1 ratio. For example, for resistance to flucarbazone and pinoxaden, the F_2 families should segregate in the ratio of 9 resistant to both flucarbazone and pinoxaden: 3 resistant to flucarbazone and susceptible to pinoxaden: 3 susceptible to flucarbazone and resistant to pinoxaden: 1 susceptible to both flucarbazone and pinoxaden. For analysis, data from homozygous resistant and segregating families were grouped together to form the resistant category (Karlowsky *et al.*, 2006). Data for the resistance genes for flucarbazone/imazamethabenz and flucarbazone/pinoxaden did not fit this ratio, suggesting that the resistance genes for these herbicides are linked (Table 3). In contrast, data for imazamethabenz/pinoxaden fit a 9:3:3:1 ratio, suggesting independent assortment of resistance genes.

To further explore whether resistance genes are linked, recombination frequency analysis and the LOD score method were independently employed. Recombination frequencies were 32–33% for flucarbazone/imazamethabenz and 13% for flucarbazone/pinoxaden (Table 4). LOD scores for flucarbazone/imazamethabenz and flucarbazone/pinoxaden linkage were 1.14 ($P = 0.011$) and 5.01 ($P \leq 0.001$), respectively, indicating that the likelihood of linkage is 14 and 102 329 times greater than no linkage respectively.

Correlation analysis of the mean F_2 injury ratings between each pairwise comparison of herbicides resulted in correlation coefficients of 0.61 ($P \leq 0.001$) for flucarbazone/imazamethabenz injury ratings, 0.70 ($P \leq 0.001$) for flucarbazone/pinoxaden injury ratings and 0.72 ($P \leq 0.001$) for imazamethabenz/pinoxaden injury ratings (Fig. 2), where an absolute value of 1 indicates a perfect linear relationship and 0 indicates no linear relationship.

Discussion

This study shows that NTSR in MHR3 and MHR4 *A. fatua* is controlled by a separate nuclear gene each for flucarbazone, imazamethabenz and pinoxaden. The most parsimonious explanation for these results would be a mutation in the genes encoding these herbicides' respective target enzymes. Target site resistance (TSR) for ALS and ACCase inhibitors is the most widely documented mechanism of resistance worldwide (Heap, 2017), and it has been documented for *A. fatua* populations resistant to diclofop-methyl and flucarbazone herbicides as reviewed in Beckie *et al.* (2012). However, we previously showed that MHR3 and MHR4 do not contain known target site mutations for ALS

Table 2 Segregation of resistance to flucarbazone, imazamethabenz and pinoxaden in the F_3 generation of pooled MHR3-4/HS2 *Avena fatua* crosses

Herbicide	Resistant	Segregating	Susceptible	Ratio	χ^2	Probability*
Number of families						
Flucarbazone	10	20	8	1:2:1	0.316	0.85
Imazamethabenz	10	18	10	1:2:1	0.105	0.95
Pinoxaden	8	24	6	1:2:1	2.842	0.24

*Probability values ≥0.05 indicate that data do not differ significantly from the test ratio.

Table 3 Chi-square and Fisher's exact tests to determine the presence of linkage between flucarbazone, imazamethabenz-methyl and pinoxaden resistance based on derived F_2 genotypes from F_3 family screening

Herbicide	Test ratio (observed)	χ^2	Probability*	Probability†
Flucarbazone/ Imazamethabenz	9:3:3:1 (20:10:2:6)‡	10.47	0.015	0.019
Flucarbazone/ Pinoxaden	9:3:3:1 (28:2:2:6)§	14.96	0.002	0.002
Imazamethabenz/ Pinoxaden	9:3:3:1 (21:7:5:5)¶	3.54	0.315	0.421

*Chi-square probability values ≥0.05 indicate that data do not differ significantly from the test ratio.
†Fisher's exact test probability values ≥0.05 indicate that data do not differ significantly from the test ratio.
‡Test ratio 9 resistant to both flucarbazone and imazamethabenz-methyl: 3 resistant to flucarbazone and susceptible to imazamethabenz-methyl: 3 susceptible to flucarbazone and resistant to imazamethabenz-methyl: 1 susceptible to both flucarbazone and imazamethabenz-methyl (428 plants).
§Test ratio 9 resistant to both flucarbazone and pinoxaden: 3 resistant to flucarbazone and susceptible to pinoxaden: 3 susceptible to flucarbazone and resistant to pinoxaden: 1 susceptible to both flucarbazone and pinoxaden (428 plants).
¶Test ratio 9 resistant to both imazamethabenz-methyl and pinoxaden: 3 resistant to imazamethabenz-methyl and susceptible to pinoxaden: 3 susceptible to imazamethabenz-methyl and resistant to pinoxaden: 1 susceptible to both imazamethabenz-methyl and pinoxaden (373 plants).

Table 4 Linkage analysis of resistance genes in the F_2 generation of pooled MHR3-4/HS2 *Avena fatua* crosses

Herbicide comparison	LOD recombination frequency	LOD score	*P*-value	Recombination frequency
Flucarbazone vs. imazamethabenz	0.33	1.14	0.011	0.32
Flucarbazone vs. pinoxaden	0.13	5.01	<0.001	0.13

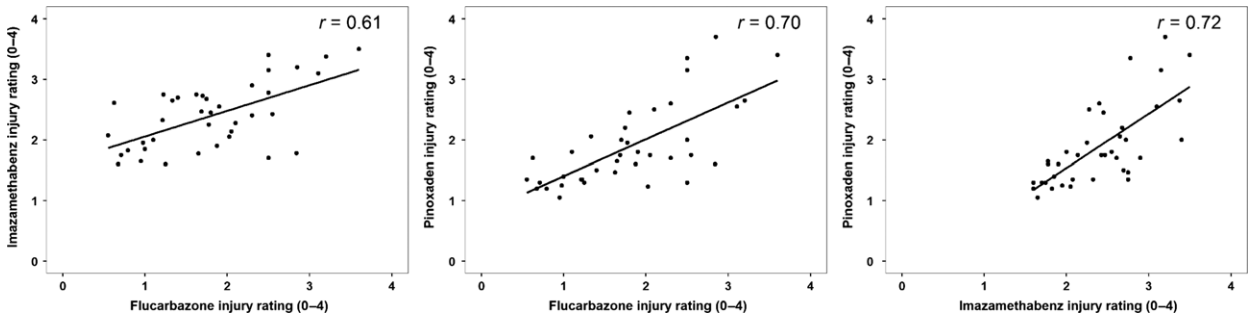

Fig. 2 Correlation plots of mean pairwise *Avena fatua* F_2 injury ratings 3 weeks after flucarbazone, imazamethabenz-methyl or pinoxaden herbicide application.

or ACCase inhibitors via transcriptome sequencing, cDNA and genomic DNA sequencing, and allele-specific PCR assays (Keith *et al*., 2015). While it is possible that MHR3 and MHR4 contain novel target site mutations, our recent RNA-Seq results strongly support the involvement of NTSR mechanisms (Keith *et al*., 2017).

Although our results show that NTSR in MHR3 and MHR4 *A. fatua* is controlled by a separate nuclear gene for each herbicide screened, it is possible that numbers of non-parental types and subsequent estimates of recombination could have been inflated by (1) insufficient F_3 plants in which heterozygous F_2 families might be mistaken for homozygous due to chance, (2) plants scored as susceptible may have been injured by another factor that caused a homozygous resistant family to appear to be segregating, or (3) plants scored as resistant were escapes from herbicide treatment. We believe that (2) can be eliminated because no untreated F_3 plants exhibited significant injury symptoms, and our extensive experience with herbicide applications rules out (3). Factor (1) is nonetheless possible but highly unlikely, as F_2 families like 40j were scored as homozygous resistant to flucarbazone and segregating for pinoxaden resistance, demonstrating the presence of recombination and that resistant phenotypes are not controlled by the same gene (Table 5).

Linkage analysis indicates that the resistance genes for these herbicides are linked. Similarly, positive correlations between pairwise comparisons of mean F_2 injury ratings in response to each herbicide (Fig. 2) further demonstrate that independent segregation of individual resistance genes is not occurring. Although data for imazamethabenz/pinoxaden linkage fit the 9:3:3:1 ratio (suggesting independence of resistance genes), these data may be have been influenced by the large range of injury scores from the parental MHR3 and MHR4 populations treated with imazamethabenz (Table 1), indicating that the herbicide screening dose was above that required for clearly distinguishing between resistant and susceptible phenotypes. While the 9:3:3:1 ratio was not rejected in this case, correlation data nonetheless support some degree of linkage or interaction between genes for imazamethabenz and pinoxaden resistance.

The linkage patterns documented here may help clarify why these populations were resistant to pinoxaden prior to its first field use. Adoption of the ALS inhibitors imazamethabenz and flucarbazone occurred in 1988 and 1998, respectively, and the ACCase inhibitor pinoxaden was introduced in 2005. Cross-resistance between these two families is well documented (Bagavathiannan *et al*., 2014), and it is possible that previous applications of these two ALS inhibitors simultaneously selected for pinoxaden resistance due to the linkage of resistance genes. This idea is supported by the high LOD score associated with flucarbazone and pinoxaden resistance, reflecting that the likelihood of linkage is 102 329 times greater than no linkage. An alternative hypothesis for these linkage patterns is that elements of NTSR pathways selected by flucarbazone may also have roles in pinoxaden resistance, allowing plant survival at the pinoxaden field use rate. Evolution of NTSR would then occur by a stepwise increase in the expression of NTSR pathways over subsequent generations.

Linkage of NTSR genes has also been reported in *L. rigidum*, where resistance genes for diclofop-methyl/chlorsulfuron (Busi *et al*., 2010) and simazine/chlorotoluron (Preston, 2003) are linked, as well as in *A. myosuroides* where resistance genes for fenoxaprop/clodinafop (Petit *et al*., 2010b), fenoxaprop/pinoxaden (Petit *et al*., 2010b; Rosenhauer *et al*., 2015) and fenoxaprop/flufenacet (Rosenhauer *et al*., 2015) are linked. These results support the hypothesis that accumulations of adaptive mutations are generally located in regions of strongly reduced recombination (Aeschbacher & Bürger, 2014).

Our findings are similar to those of Karlowsky *et al*. (2006) who investigated the inheritance of MHR in the UMWO12-01 and UMWO12-03 Canadian *A. fatua* populations. They reported that separate single dominant or semi-dominant nuclear genes conferred resistance to imazamethabenz, flamprop-M-methyl and fenoxaprop-P-ethyl in both populations. Furthermore, genes conferring resistance to imazamethabenz and flamprop-M-methyl were linked. However, the physiological mechanisms conferring MHR in UMWO12-01 and UMWO12-03 are unknown (Karlowsky, 2004), in contrast to the MHR3 and MHR4 *A. fatua* populations discussed here. However, as TSR is generally much more prevalent than NTSR (Heap, 2017), it seems likely that resistance in UMWO12-01 and UMWO12-03 is due to TSR.

Monogenic control of TSR is widespread, as a single point mutation in the gene encoding a herbicide's target enzyme is frequently sufficient to confer resistance (Jasieniuk *et al*., 1996). In contrast, NTSR is generally thought to evolve by 'gene stacking' in which herbicide application initially selects for slightly less sensitive genotypes, which then cross-pollinate over multiple generations to create recombinant genotypes with combinations of parental alleles conferring enhanced herbicide resistance (Délye *et al*., 2011). Significantly, this hypothesis pertains only to allogamous weed species, which are represented by the vast majority of NTSR and MHR populations studied to date [(Heap, 2017); see examples below]. However, our

Table 5 *Avena fatua* F_2 mean (standard errors) injury ratings 3 weeks after herbicide treatment

Cross*	F_2	Mean F_2 injury†		
		Flucarbazone	Imazamethabenz	Pinoxaden
MHR3 × HS2	316a	1.78 (0.27)	2.25 (0.28)	1.95 (0.27)
	316b	2.05 (0.20)	2.14 (0.13)	1.75 (0.29)
	316c	0.55 (0.05)	2.08 (0.27)	1.35 (0.15)
	316d	1.88 (0.32)	1.90 (0.15)	1.60 (0.24)
	316e	1.90 (0.40)	2.55 (0.23)	1.80 (0.28)
	316f	1.40 (0.25)	2.69 (0.13)	1.50 (0.22)
	316g	2.84 (0.09)	1.78 (0.24)	1.60 (0.31)
	316h	0.95 (0.16)	1.65 (0.11)	1.05 (0.09)
	316i	2.30 (0.25)	2.90 (0.18)	1.70 (0.17)
	316j	2.03 (0.34)	2.05 (0.25)	1.23 (0.12)
HS2 × MHR3	38a	1.63 (0.31)	2.75 (0.25)	1.47 (0.11)
	38b	1.70 (0.25)	2.73 (0.23)	2.00 (0.29)
	38c	1.65 (0.33)	1.78 (0.31)	1.65 (0.15)
	38d	0.79 (0.09)	1.83 (0.21)	1.20 (0.27)
	38e	3.20 (0.13)	3.38 (0.15)	2.65 (0.15)
	38f	1.69 (0.12)	2.47 (0.26)	1.75 (0.25)
	38g	2.55 (0.20)	2.43 (0.23)	1.75 (0.37)
	38h	2.10 (0.26)	2.28 (0.27)	2.50 (0.31)
	38i	1.21 (0.14)	2.33 (0.20)	1.35 (0.11)
	38j	1.75 (0.26)	2.68 (0.29)	2.20 (0.37)
MHR4 × HS2	40a	2.50 (0.14)	3.15 (0.18)	3.15 (0.28)
	40b	2.50 (0.31)	3.40 (0.19)	2.00 (0.27)
	40c	3.11 (0.09)	3.10 (0.15)	2.55 (0.31)
	40d	0.98 (0.24)	1.95 (0.11)	1.25 (0.17)
	40e	1.80 (0.29)	2.45 (0.17)	2.45 (0.26)
	40f	2.50 (0.08)	2.78 (0.17)	3.35 (0.27)
	40g	0.68 (0.07)	1.60 (0.16)	1.20 (0.23)
	40h	1.33 (0.21)	2.65 (0.22)	2.06 (0.24)
	40i	1.23 (0.33)	2.75 (0.19)	1.35 (0.21)
	40j	0.63 (0.05)	2.61 (0.39)	1.70 (0.40)
HS2 × MHR4	45a	1.25 (0.11)	1.60 (0.10)	1.30 (0.12)
	45b	2.50 (0.22)	1.70 (0.12)	1.30 (0.34)
	45c	3.60 (0.19)	3.50 (0.27)	3.40 (0.24)
	45d	2.85 (0.10)	3.20 (0.20)	3.70 (0.20)
	45e	0.71 (0.06)	1.75 (0.22)	1.30 (0.44)
	45f	1.00 (0.00)	1.85 (0.15)	1.40 (0.19)
	45i	2.30 (0.37)	2.40 (0.40)	2.60 (0.51)
	45j	1.10 (0.10)	2.00 (0.00)	1.80 (0.20)

*MHR3 and MHR4 are multiple herbicide-resistant populations, and HS2 is the herbicide susceptible population.
†Injury rating using a scale of 0 = no injury to 4 = plant death.

results from the highly autogamous species *A. fatua* and those of Iwakami *et al.* (2014) and Zelaya *et al.* (2004) support an alternative hypothesis more relevant for autogamous species: NTSR evolves through a 'step-by-step' process of consecutive selection events in the same autogamous genetic lines over time, via spontaneous mutations and standing genetic variation (Neve *et al.*, 2009).

Our results also contrast with the majority of NTSR inheritance studies reporting that NTSR is multigenic in populations of the allogamous species *L. rigidum* (Preston, 2003; Busi *et al.*, 2010, 2012; Han *et al.*, 2014), *A. myosuroides* (Petit *et al.*, 2010b; Rosenhauer *et al.*, 2015), *A. tuberculatus* (Huffman *et al.*, 2015) and *Papaver rhoeas* L. (Scarabel *et al.*, 2015). In contrast, monogenic NTSR has only been reported in the autogamous species *E. phyllopogon* (Iwakami *et al.*, 2014), with one report in the allogamous species *L. rigidum* (Yu *et al.*, 2009).

In addition to contrasting mating systems, differences in genome structure and ploidy level may influence the evolution of NTSR. The majority of research investigating NTSR inheritance has been conducted with allogamous diploid weedy species as discussed above, which maintain high levels of heterozygosity and allow resistance alleles to spread quickly through

cross-pollination. In contrast, autogamy promotes homozygosity and resistance alleles are not spread via pollen flow. In addition to being a highly autogamous species, *A. fatua* is also an allohexaploid ($2n = 6 \times = 42$), with six chromosome sets from three progenitor species (Yang *et al.*, 1999). Polyploidy results in genomewide genetic redundancy, providing novel opportunities for adaptive evolution. For example, gene duplication due to polyploidisation leads to the retention of functional gene copies, or homoeologs, at many loci. The result is that *A. fatua* and other polyploid weedy species can accumulate and fix normally deleterious herbicide resistance gene mutations, facilitating the evolution of NTSR.

To date, the only study investigating the impacts of polyploidy on herbicide resistance evolution was conducted on *A. fatua* populations with known target site mutations in the three homoeologous ACCase genes *Acc1;1*, *Acc1;2* and *Acc1;3* (Yu *et al.*, 2013). Yu *et al.* (2013) reported that the low levels of resistance observed in these populations may be due to dilution effects of resistance mutations by the maintenance of sensitive homoeologs. Thus, polyploidy may play contrasting roles in herbicide resistance evolution by (1) minimising the deleterious effects of herbicide resistance gene mutations, while at the same time (2) buffering the effect of herbicide resistance genes with homoeologous sensitive alleles. This gene dosage effect has not yet been investigated in weedy species with NTSR, so the implications drawn from Yu *et al.* (2013) may not be directly applicable to the current findings. Furthermore, the details of homoeologous gene expression, gene silencing and possible epigenetic regulation of homoeologs are essentially unknown in weedy species including *A. fatua*, although they provide valuable opportunities for developing a deeper understanding of NTS herbicide resistance evolution.

Understanding the inheritance of NTS MHR is of upmost importance for continued agricultural productivity in the face of rapid increases in resistant weed populations worldwide. The single-gene NTSR inheritance patterns reported here add to our understanding and illustrate the diversity of evolutionary responses to strong selection. Overall, these results from *A. fatua* demonstrate the importance of investigating the genetic basis of NTSR on a species by species, population by population level, to develop a complete understanding of this phenomenon. Current herbicide resistance management strategies include using different herbicide mechanisms of action in annual rotations, tank mixtures and/or sequential applications (Norsworthy *et al.*, 2012). However, these strategies will be ineffective at controlling or slowing the evolution of NTSR as described here, as it is associated with unpredictable patterns of resistance and to herbicides not yet commercially available (Petit *et al.*, 2010a). It is clear that, if herbicides are indeed non-renewable resources (Duke, 2012), alternative ecologically based approaches for sustainable weed management will be required in order to extend the utility of herbicides and lead to successful management of current and future NTS MHR weed populations.

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