Research Article

Received: 29 June 2011 Revised: 23 August 2011 Accepted: 23 August 2011 Published online in Wiley Online Library: 3 February 2012

(wileyonlinelibrary.com) DOI 10.1002/ps.2289

Development of near-isogenic lines and identification of markers linked to auxinic herbicide resistance in wild mustard (*Sinapis arvensis* L.)

Jugulam Mithila,[a] Michael D. McLean,[a] Shu Chen,[b] and John Christopher Hall[a]*

Abstract

BACKGROUND: Auxinic herbicides are widely used for selective control of many broadleaf weeds, e.g. wild mustard. An auxinic-herbicide-resistant wild mustard biotype may offer an excellent model system to elucidate the mechanism of action of these herbicides. Classical genetic analyses demonstrate that the wild mustard auxinic herbicide resistance is determined by a single dominant gene. Availability of near-isogenic lines (NILs) of wild mustard with auxinic herbicide resistance (R) and herbicide susceptibility (S) will help to study the fitness penalty as well as the precise characterization of this gene.

RESULTS: Eight generations of backcrosses were performed, and homozygous auxinic-herbicide-resistant and auxinic-herbicide-susceptible NILs were identified from BC_8F_3 families. S plants produced significantly more biomass and seed compared with R plants, suggesting that wild mustard auxinic herbicide resistance may result in fitness reduction. It was also found that the serrated margin of the first true leaf was closely linked to auxinic herbicide resistance. Using the introgressed progeny, molecular markers linked to auxinic herbicide resistance were identified, and a genetic map was constructed.

CONCLUSION: The fitness penalty associated with the auxinic herbicide resistance gene may explain the relatively slow occurrence and spread of auxinic-herbicide-resistant weeds. The detection of the closely linked markers should hasten the identification and characterization of this gene.

Keywords: auxinic herbicides; backcross; dominance; fitness; markers; near-isogenic lines

1 INTRODUCTION

Wild mustard (*Brassica kaber* syn. *Sinapis arvensis*) is an annual dicotyledonous weed in the family Brassicaceae that commonly infests wheat and barley crops. Unlike oilseed rape (an important field crop of Brassicaceae), which is predominantly a self-pollinated crop,[1] wild mustard is a complete outcrosser.[2] Auxinic herbicides such as 2,4-D, MCPA and dicamba are widely used to control broadleaf weeds, including wild mustard, in cereal crops, while picloram is used for control of broadleaf weeds in non-agricultural areas (e.g. power-line corridors). These herbicides have been in use for more than 60 years, but their mode of action is not completely understood. Prolonged use of a combination of auxinic herbicides in wheat and barley fields in western Canada resulted in the evolution of resistance in wild mustard biotypes to these herbicides.[3] The wild mustard auxinic-herbicide-resistant biotypes were found to be highly resistant to picloram and dicamba (104-fold) and moderately resistant to 2,4-D (tenfold) and MCPA (18-fold).[3] In spite of extensive characterization of this species at morphological, physiological, biochemical and molecular levels,[4–16] the precise mechanism of auxinic herbicide resistance in wild mustard remains unknown.

Physiological and biochemical studies of auxinic-herbicide-resistant (R) and auxinic-herbicide-susceptible (S) wild mustard indicate that the resistance is not due to differences in uptake, translocation or metabolism of auxinic herbicides.[5] Classical genetic analyses demonstrate that the auxinic herbicide resistance in *S. arvensis* is determined by a single dominant gene.[14,17] Single gene inheritance, as opposed to multiple gene resistance, should facilitate the identification and precise characterization of the gene involved in auxinic herbicide resistance in wild mustard. However, for the precise characterization of the auxinic herbicide resistance gene (especially when the resistance is determined by a single gene), it is extremely useful to have near-isogenic lines (NILs) developed for the trait. NILs are two identical lines that differ only in the

* *Correspondence to: John Christopher Hall, School of Environmental Sciences, University of Guelph, Guelph, Ontario N1G 2 W1, Canada. E-mail: jchall@uoguelph.ca*

a *School of Environmental Sciences, University of Guelph, Guelph, Ontario, Canada*

b *Laboratory Services Division, University of Guelph, Guelph, Ontario, Canada*

genetic region that has been introduced from a donor parent line, and therefore are distinguished only by the presence or absence of the target gene and a small region of DNA flanking this gene.[18]

NILs have been developed previously by introgression using the traditional backcross method of breeding for transferring agronomically desirable traits from wild relatives to cultivated species in many crops, such as tomato,[19,20] rapeseed,[21] rice,[22] wheat[23] and lettuce.[24] Earlier, NILs were developed primarily to determine pleiotropy (i.e. a single gene determining more than one trait) or close linkage of traits.[25–27] However, these lines were also extensively used for gene mapping studies, especially for identifying markers tightly linked to agronomically important traits such as *Pseudomonas* resistance in tomato[28] and downy mildew resistance in lettuce.[24]

The development of NILs with weed species segregating for auxinic-herbicide-resistant and auxinic-herbicide-susceptible biotypes has not yet been reported. In fact, these lines are extremely useful in weed science for genetic analyses, as well as for understanding the basis of evolution of resistance to herbicides. Consequently, data obtained using NILs are highly reliable and hence useful in developing valid and dependable models that will predict the occurrence and evolution of herbicide resistance. Because of the lack of availability of these lines for herbicide resistance in weed species, the majority of research elucidating the genetic basis of herbicide resistance has always been conducted using genetically diverse weed populations (i.e. biotypes obtained from field populations), with a few exceptions, namely triazine-resistant groundsel (the herbicide triazine inhibits photosynthesis).[29] It has been emphasized that, in order to understand the effect of a resistant mutation on fitness of plant species, comparison of genetically uniform lines is recommended.[30,31]

In this research, NILs with auxinic herbicide resistance and susceptibility in wild mustard were developed using repeated backcrosses. These lines were used to perform growth room studies to examine parameters (e.g. plant biomass and seed yield) that contribute to the fitness of plants. In addition, using these lines, the authors also identified amplified fragment length polymorphism (AFLP)-based markers linked to auxinic herbicide resistance. Molecular markers such as restriction fragment length polymorphism (RFLP),[32] random amplified polymorphic DNA (RAPD)[33] and sequence tagged sites (STS)[34] have been identified in several crop species and used extensively for positional cloning as well as marker-assisted selection (MAS) in crop improvement. AFLP analysis was used in the present research because this technique is reproducible and robust and allows rapid evaluation of several thousand polymorphic loci. In addition, a morphological marker (leaf shape) closely linked to this gene was also identified. The availability of molecular and morphological markers linked to the gene of interest is a major step towards its subsequent identification.

2 MATERIALS AND METHODS

2.1 Wild mustard parental R and S lines

Picloram-resistant (R) and picloram-susceptible (S) wild mustard plants were raised from seeds collected from Gilbert Plains and Minto, Manitoba, Canada, respectively.[3] The resistant biotype was identified in a field repeatedly treated with a combination of MCPA, dicamba and 2,4-D for nearly a decade. The seeds were sown in 4″ plastic pots containing Promix (Plant Products, Bramalea, ON) and were placed in a growth chamber having a 16 h photoperiod and 22/15 °C day/night temperature. The light intensity and the relative humidity were maintained at 350 μmol s^{-1} m^{-2} and 65–75% respectively. Each pot contained one plant, and the plants were irrigated when required and were fertilized weekly with 20 : 20:20 (N : P : K). To confirm resistance to picloram (Tordon 22; Dow AgroSciences; for this research the authors chose to screen the plants using picloram because, as indicated in the introduction, wild mustard R biotypes were found to be highly resistant to this herbicide), the plants were treated with this herbicide at 100 g AI ha^{-1} at the 3–4-leaf stage of development (the procedure for screening for picloram resistance is described later); all these plants survived the initial herbicide treatment and were used for further experimentation.

2.2 Genetic crosses to generate F_1 wild mustard populations

The F_1 plants were generated from crosses between homozygous wild mustard auxinic-herbicide-resistant and auxinic-herbicide-susceptible plants. The plants were raised in a growth chamber (as described previously), and reciprocal crosses were performed. Emasculation (i.e. the removal of immature anthers before pollen dehiscence) and pollination were performed as reported previously.[14] At 4–5 weeks after pollination, mature siliques were harvested, and the F_1 seed from each cross was collected from both R and S plants. Upon screening with picloram, the heterozygous F_1 plants were used to generate backcross progeny.

2.3 Repeated backcrosses to generate near-isogenic lines of wild mustard

The first generation of backcross progeny (BC_1F_1) was produced by performing crosses between heterozygous auxinic-herbicide-resistant F_1 hybrid plants and homozygous recessive S (recurrent parent) plants. Ten plants in each of these F_1 sibling sets, as well as the S parental lines, were raised in a growth chamber (as described above). The S parent was used as a seed parent, whereas the F_1 was chosen as the pollen parent (Fig. 1). Cross-pollinations were performed (as described above) to obtain BC_1F_1 seed. Mature seed (BC_1F_1) was harvested from the S plant. Seeds of BC_1F_1 were planted in pots, and plants were grown in a growth chamber (as described previously) to generate BC_2F_1 seed. The BC_1F_1 progeny were evaluated for response to picloram (100 g AI ha^{-1}), and the S plants were discarded. The survivors of picloram treatment, i.e. heterozygous R plants, were used as the pollen parent and backcrossed to the recurrent parent to obtain BC_2F_1 progeny. Eight backcrosses were performed using this procedure, and in every generation the progeny were screened with picloram (100 g AI ha^{-1}) to select the heterozygous R plants. The survivors of progeny generated from BC_8F_1 seed (i.e. heterozygous R plants) were self-pollinated to generate BC_8F_2 seed. The seed from one such self-pollinated BC_8F_1 plant was collected, and 25 BC_8F_2 seedlings were raised in a growth chamber (as previously described). Upon flowering, each of these 25 plants was self-pollinated. Mature seed from each plant (~40–60 seeds) was harvested. From these BC_8F_2 (self-pollinated) seed, 20 individual BC_8F_3 families, each consisting of 20–30 plants, were raised in a growth chamber. When the BC_8F_3 seedlings were at the 3–4-leaf stage of development, each family was screened using picloram (100 g AI ha^{-1}) to identify homozygous auxinic-herbicide-resistant and auxinic-herbicide-susceptible lines (NILs) (Fig. 1). The remaining seed from the 20 families was stored.

2.4 Biomass and seed of NILs

NILs of wild mustard that were auxinic herbicide resistant and susceptible were raised in a growth chamber (as described

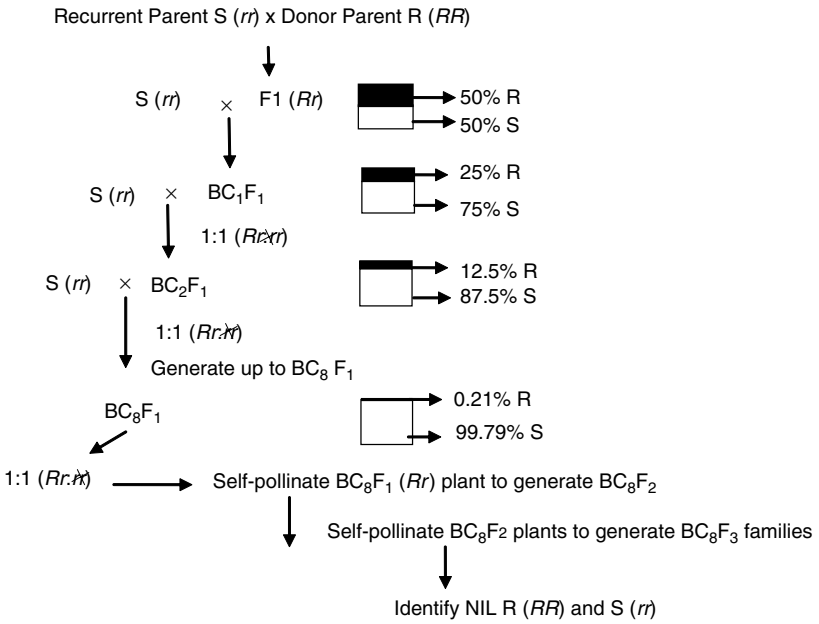

Figure 1. Backcross procedure illustrating the theoretical development of wild mustard NILs with auxinic herbicide resistance (R) and susceptibility (S) (eliminate *rr* plants upon treatment with 100 g AI ha^{-1} of picloram).

previously) to assess the parameters that contribute to the fitness of the genotypes. These plants were grown in 6″ pots. At least four plants of NIL R and S were treated with picloram (100 g AI ha^{-1}) at the four-leaf stage of development. The other plants were grown untreated. The following parameters were assessed in both treated and untreated plants of NILs:

- *Determination of plant dry weight*. The dry weight of each plant (roots and shoots) was determined 6 weeks after planting. The whole plant was lifted from the pot and the soil was rinsed with water. The plant parts (i.e. roots and shoots) were dried in an oven at 68 °C for 48 h, and dry weights were determined.
- *Determination of seed yield*. Four plants of each NIL R and S were allowed to pollinate naturally. The seed pods were harvested from each plant at maturity and the number of seeds per pod was recorded.

2.5 Assessment of leaf area and leaf shape

Previously, a morphological trait (a serrated leaf margin of the first true leaf) associated with auxinic herbicide resistance in wild mustard has been observed. To test whether this marker was linked to the resistance or the pleiotropic effect of the gene controlling auxinic herbicide resistance, parental R and S and NIL R and S (30 in each), as well as backcross (BC_4F_1) progeny, were raised in a growth chamber (as described previously). At 21 and 31 days after planting, data on leaf shape and leaf area, respectively, were recorded for the first true leaf of each seedling. Leaf area was measured using a leaf area meter. All the seedlings were scored as either R or S on the basis of the leaf shape (serrated or smooth) before being treated with picloram.

2.6 Screening of wild mustard plants for picloram resistance

Seedlings of F_1 backcrosses as well as NILs were treated with picloram (100 g AI ha^{-1}) at the 3–4-leaf stage of development using a motorized hood sprayer. When plants were screened for herbicide resistance, seedlings of wild mustard R and S plants (3–4-leaf stage of development) were included as positive and negative controls, respectively. The sprayer was equipped with a flat-fan nozzle (8002 E) and calibrated to deliver 200 L ha^{-1} at 276 kPa. One week after herbicide treatment, the seedlings were visually rated for injury and classified as R or S by comparing the injury response with those of R and S control plants. Susceptibility of the plants to picloram was assessed on the basis of epinasty (downward curling of the leaf petiole and stem). The frequencies of R and S phenotypes following treatment with picloram were tabulated.

2.7 AFLP analysis to identify markers linked to auxinic herbicide resistance

Wild mustard parental R and S, NIL R and S and progeny segregating for auxinic herbicide resistance and susceptibility from BC_1F_1, BC_7F_1 and BC_8F_1 were chosen for AFLP analysis. Genomic DNA was extracted from the leaf tissue of these plants using DNeasy® Plant Mini (QIAGEN, Mississauga, ON) following the manufacturer's protocol. The AFLP analysis[35] was based on the protocol of AFLP™ plant mapping kit (Applied Biosystems, Foster City, CA). Briefly, the genomic DNA (~250 ng) was digested with restriction enzymes EcoRI and MseI (New England Biolabs Ltd, Pickering, ON), and the resulting DNA fragments were ligated to EcoRI and MseI adapters with T4 DNA ligase (New England Biolabs Ltd). The resultant mixtures were diluted 18-fold in $TE_{0.1}$ (Tris and EDTA) buffer and used for preamplification with preselective primers and the AFLP Core Mix. PCR amplification was performed on GeneAmp® PCR System 9700. The preselective amplification products were diluted 11-fold in $TE_{0.1}$ buffer and used for selective amplification with AFLP selective primers as described in the selective amplification kits for regular and small plant genomes. The amplified DNA fragments were separated using an ABI 3730 genetic analyzer. The GS ROX 500 size standard was used as an internal size standard for each sample. Data were analyzed for size and intensity using GeneMapper® v.4.0 software.

For AFLP analysis, three *Eco*RI/*Mse*I primer combinations were initially tested using 20 plant samples from R and S plants.

Table 1. Segregation of resistant (R) and susceptible (S) wild mustard plants among backcross progeny following treatment with 100 g AI ha^{-1} of picloram[a]

	Segregation of plants			
Backcross[b]	R (*Rr*)	S (*rr*)	χ^{2c}	Probability[d]
BC_1F_1	26	23	0.18	0.67[e]
BC_2F_1	21	25	0.36	0.56[e]
BC_3F_1	18	15	0.27	0.60[e]
BC_4F_1	25	22	0.191	0.66[e]
BC_5F_1	17	20	0.24	0.62[e]
BC_6F_1	18	16	0.11	0.74[e]
BC_7F_1	23	20	0.21	0.65[e]
BC_8F_1	21	25	0.35	0.56[e]

[a] Resistance and susceptibility were assessed by comparing the response of progeny from backcrosses with the responses of seedlings from R and S parental populations following treatment with picloram.
[b] Backcross populations resulted from cross-pollination of S plants with heterozygous R plants.
[c] Chi-square values are the results of tests for goodness of fit to a 1 : 1 (R : S) segregation model.
[d] Probability of accepting or rejecting the null hypothesis.
[e] Accepting the null hypothesis that the backcross progeny segregated 1 : 1 (R : S).

Table 2. Segregation of resistant (R) and susceptible (S) plants among BC_8F_3 families (raised from self-pollinated BC_8F_2) following treatment with 100 g AI ha^{-1} of picloram[a]

	Segregation of plants			
BC_8F_3 family[b]	R	S	χ^{2c}	Probability[d]
Family 1	26 (*RR*)	0[f,g]		
Family 2	20 (*Rr*)	8 (*rr*)	0.191	0.66[e]
Family 3	18 (*Rr*)	7 (*rr*)	0.121	0.72[e]
Family 4	19 (*Rr*)	8 (*rr*)	0.308	0.58[e]
Family 5	0	30[f,g] (*rr*)		
Family 6	19 (*Rr*)	8 (*rr*)	0.308	0.58[e]
Family 7	21 (*Rr*)	8 (*rr*)	0.13	0.72[e]
Family 8	26 (*RR*)	0[f,g]		
Family 9	22 (*Rr*)	8 (*rr*)	0.04	0.84[e]
Family 10	19 (*Rr*)	7 (*rr*)	0.051	0.82[e]
Family 11	22 (*Rr*)	8 (*rr*)	0.04	0.84[e]
Family 12	21 (*Rr*)	8 (*rr*)	0.13	0.72[e]
Family 13	19 (*Rr*)	6 (*rr*)	0.013	0.91[e]
Family 14	21 (*Rr*)	6 (*rr*)	0.11	0.74[e]
Family 15	19 (*Rr*)	6 (*rr*)	0.013	0.91[e]
Family 16	0	21[f,g] (*rr*)		
Family 17	23 (*Rr*)	8 (*rr*)	0.013	0.89[e]
Family 18	0	25[f,g] (*rr*)		
Family 19	20 (*Rr*)	7 (*rr*)	0.012	0.91[e]
Family 20	19 (*Rr*)	8 (*rr*)	0.309	0.58[e]

[a] Resistance and susceptibility were assessed by comparing the response of progeny from backcrosses to the responses of seedlings from R and S parental populations following treatment with picloram.
[b] Progeny raised from self-pollination of BC_8F_2.
[c] Chi-square values are the results of tests for goodness of fit to a 3 : 1 (R : S) segregation model.
[d] Probability of accepting or rejecting the null hypothesis.
[e] Accepting the null hypothesis that the progeny segregate 3 : 1 (R : S).
[f] Rejecting the null hypothesis that the progeny segregate 3 : 1 (R : S).
[g] Not subjected to χ^2 because of zero expected value for one class.

After establishing that the AFLP procedures were functional, 128 additional *Eco*RI/*Mse*I primer combinations were tested using pooled samples consisting of three S or R plants from both BC_1F_1 and BC_8F_1. These combinations included *Eco*RI primers ending in nucleotides CAN and ARS with *Mse*I primers ending in AN and CN, and *Eco*RI primers ending in AN and TN with *Mse*I primers ending in CAN and CTN, where N is any of the four nucleotides, R = A or G and S = G or C. Seven AFLP primer combinations that resulted in potential S- or R-specific polymorphisms were selected to test 15 individual plants, and, of these, five primer combinations (*Eco*RI-TA/*Mse*I-CAC, *Eco*RI-TA/*Mse*I-CTG, *Eco*RI-ACT/*Mse*I-AA, *Eco*RI-ACG/*Mse*I-AC and *Eco*RI-ACG/*Mse*I-AG) were further tested on 50 plants from BC_1F_1, seven plants from BC_7F_1 and six plants from BC_8F_1 progeny.

2.8 Statistical analyses

Frequencies of R and S phenotypes were tabulated for F_1 and backcross populations. Chi-square tests were performed to determine the goodness of fit to specific genetic ratios. The analysis of other data parameters was performed using ANOVA. For the experiments that determined the fitness of wild mustard NILs, the data were collected from at least four replications, and all the experiments were conducted twice.

3 RESULTS

3.1 Selection of NILs

The progeny from all backcrosses (BC_1F_1 to BC_8F_1) showed similar responses following picloram application (100 g AI ha^{-1}) (Table 1). Seedlings exhibiting picloram injury 1 week after treatment showed severe epinasty and eventually died. Remaining backcross progeny showed no picloram injury, and chi-square tests confirmed 1 : 1 segregations (R [*Rr*] : S [*rr*]) among all backcross progeny, i.e. BC_1F_1 to BC_8F_1 progeny, as expected.[14] NILs were selected from BC_8F_3 families. Upon picloram application (100 g AI ha^{-1}), all plants in families 1 and 8 were unaffected by herbicide treatment, confirming that the genotype of these individuals was *RR* (Table 2). Conversely, in other families, i.e. families 5, 16 and 18, all plants exhibited epinasty and eventually died (Table 2), indicating that these families were of the *rr* genotype. Thus, these families constitute the chosen NILs with auxinic herbicide resistance (families 1 and 8) and susceptibility (families 5, 16 and 18).

3.2 Morphological marker and linkage to auxinic herbicide resistance

There were differences between R and S wild mustard biotypes with regard to the shape of first true leaves (Fig. 2). The R biotype plants exhibited a serrated leaf margin, whereas the S biotype plants had a smooth margin. The areas of first true leaves were also significantly greater for S than for R ($P = 0.05$) (Fig. 3). The areas of the first true leaves of BC_4F_1 (75 plants) were measured before treatment with picloram, and then scored for herbicide resistance (Table 3). The serrated leaf margin and its smaller leaf area were perfectly correlated (not shown). The segregation of leaf shape and auxinic herbicide resistance traits confirmed that the smaller leaf area/serrated leaf trait is linked and in phase with the R

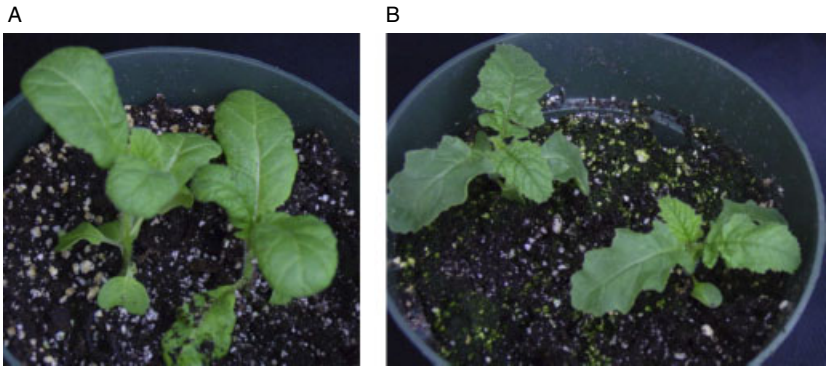

Figure 2. Wild mustard plants 21 days after planting (DAP). A and B denote S and R plants respectively.

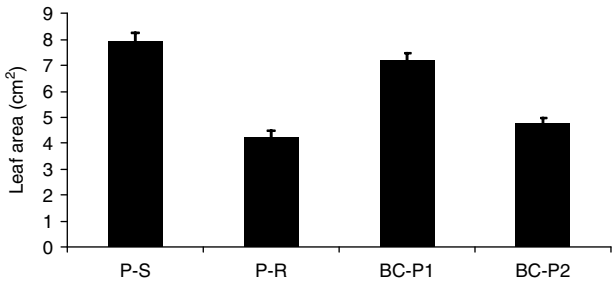

Figure 3. Area (cm^2) of the first true leaf of wild mustard (31 DAP). S: parental S; R: parental R; BC-P1and BC-P2 are BC_4F_1 progeny with larger leaf area and smaller leaf area respectively. Vertical bars represent the standard error of the means (SEM).

Table 3. Number of R and S plants of BC_4F_1 progeny with large and small leaf area upon treatment with 100 g AI ha^{-1} of picloram[a]

Phenotype	Small leaf area	Large leaf area
Resistant	30	5
Susceptible	6	34

[a] The χ^2 is set for the probability of leaf area unlinked to the resistance locus; the results ($\chi^2 = 37.9$, df = 3; $P < 0.001$) suggest that the gene loci that control leaf morphology and auxinic herbicide resistance are linked. Linkage percentage 14.6% based on the following calculation: Number of recombinants/total number of BC_4F_1 progeny $\times$ 100[11/75 = 0.146 $\times$ 100 = (14.6%)].

allele, and demonstrated that the loci controlling these two traits are separated by a distance of 14.6 cM (Table 3).

3.3 Biomass and seed of NILs

Analysis of growth and seed yield of NILs indicated that untreated S plants produced more whole-plant biomass and had a greater seed yield than R plants (Table 4). Also, the total plant biomass and seed yield of R plants that survived picloram treatment did not significantly differ from those of untreated R plants (Table 4).

3.4 AFLP analyses and construction of a genetic map

A total of 128 primer combinations were tested, and comparisons between auxinic-herbicide-resistant and auxinic-herbicide-susceptible plants were made. AFLP fragments ranging from 60 to 500 bps were used in the analyses. The majority of bands generated

Table 4. Growth and seed yield of auxinic-herbicide-resistant (R) and auxinic-herbicide-susceptible (S) NILs of wild mustard 6 weeks after planting[a]

	Untreated		Treated with 100 g AI ha^{-1} of picloram
Parameter	Susceptible	Resistant	Susceptible
Plant dry weight (g)	5.55 (0.07)	4.01* (0.04)	3.36 (0.01)
Shoot dry weight (g)	5.18 (0.07)	3.63* (0.12)	3.02 (0.10)
Root dry weight (g)	0.37 (0.01)	0.36 (0.01)	0.34 (0.01)
Siliques $plant^{-1}$	7.5	4.5*	3
Total seed yield $plant^{-1}$	25	10*	6

[a] The values in parentheses are the standard error of the means (SEM). An asterisk (*) indicates that the untreated R and S means are significantly different at $P = 0.05$. In all cases the untreated R and treated R plants are not significantly different ($P = 0.05$).

using R and S plants were the same (data not shown). However, some primer combinations produced DNA fragments that were present in R but not in S, and vice versa. The AFLP fragments that were present in R plants and absent in S plants were selected for linkage analysis (Fig. 4). The linkage of markers to the auxinic herbicide resistance locus was determined by analyzing cosegregation data from the backcross progeny. Consequently, six markers indicating polymorphisms between S and R were identified using primer combinations EcoRI-TA/MseI-CAC (M1), EcoRI-TA/MseI-CTG (M2), EcoRI-ACT/MseI-AA (M3), EcoRI-ACG/MseI-AC (M4,5) and EcoRI-ACG/MseI-AG (M6), and the nucleotide sequences of these markers were also obtained (see the Appendix for sequence information). Based on the recombination frequency calculated from the cosegregation data, a genetic map was constructed (Fig. 5). The genetic distance between the resistance locus and the closely linked markers ranged from ~1.5 to 26 cM in wild mustard. The marker M5 was found to be tightly linked to the resistance locus, at 1.5 cM away (Fig. 5).

4 DISCUSSION

Auxinic herbicides are widely used by cereal growers around the globe because of their selectivity and low cost. In spite of their use for more than 60 years, the development of resistance to auxinic herbicides has been slow, and to date only 28 weed biotypes have developed resistance to these herbicides.[36] Earlier research indicates that the resistance to auxinic herbicides in wild mustard

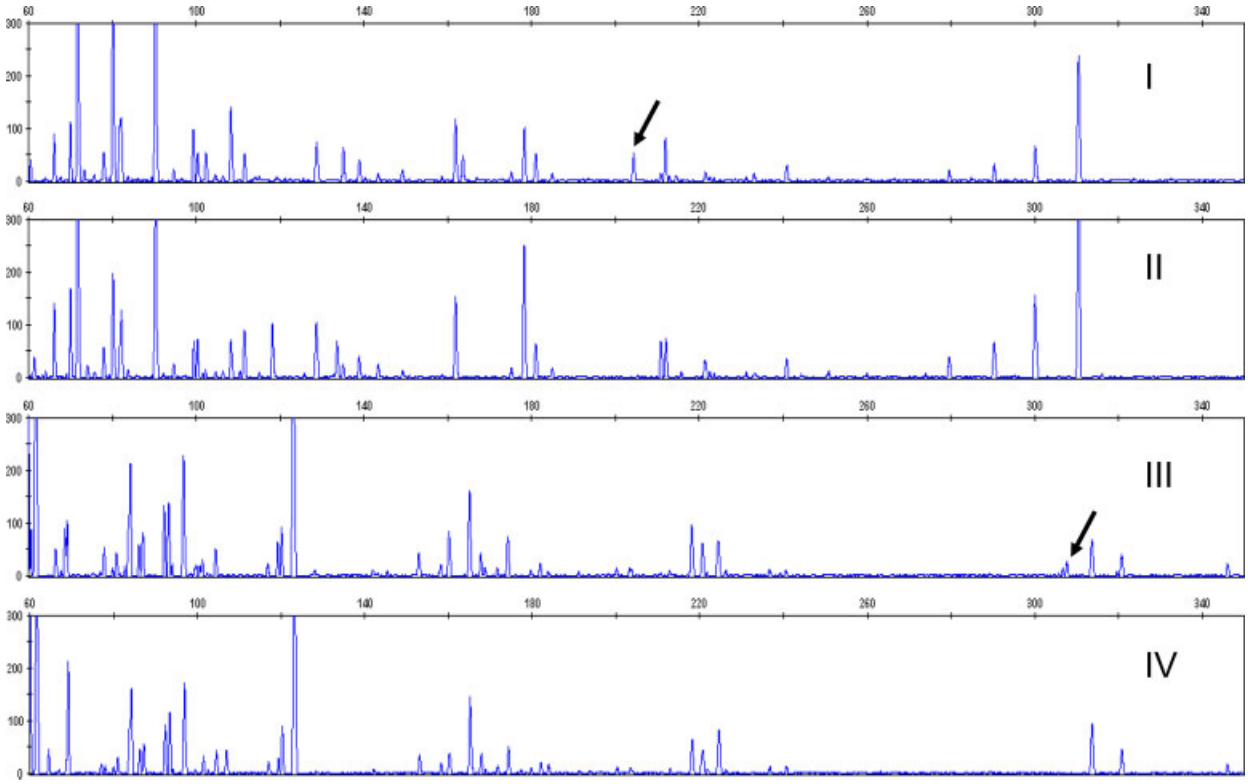

Figure 4. AFLP electropherograms illustrating polymorphism between bulked DNA samples of wild mustard NILs. Panels I and III show specific peaks representing markers 2 and 5 (arrow), respectively, present in resistant and absent (panels II and IV) in susceptible samples.

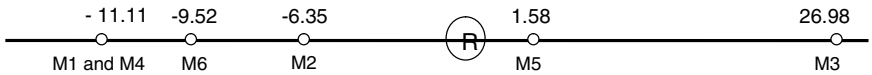

Figure 5. Linkage map of wild mustard. M1, M2, M3, M4, M5 and M6 are AFLP markers (1 to 6) and their measured distances (cM) from the auxinic herbicide resistance locus.

is not due to differences in herbicide uptake, translocation or metabolism.[5] Furthermore, it has also been demonstrated that the auxinic herbicide resistance in wild mustard is determined by a single dominant gene.[14,17] A single dominant gene determines herbicide resistance in the majority of herbicide-resistant weed biotypes.[37,38] However, in a few weed species, a single recessive gene determining herbicide resistance has also been reported.[39,40]

This report describes the development of NILs selected for auxinic herbicide resistance and susceptibility in a weed species, i.e. wild mustard. Although these lines are near-isogenic, it is not clearly known how much genomic DNA from the R biotype has been removed by recurrent backcrosses. The expected rate of recovery of the recurrent parent is calculated by $1-(1/2)^n$, where n is the number of backcross generations.[41] Therefore, after eight generations of backcrosses, in NILs 99.79% of initial differences between R and S plants will be identical; however, considerable deviation from theoretical values has also been reported.[42] Further evaluation of the present NILs would be required for more accurate estimates.

Mutations resulting in herbicide resistance could have a fitness cost, either owing to pleiotropic effects of the resistance gene or owing to linkage of the resistance gene with one or more other loci that impose the fitness cost. NILs provide a tremendous resource for determining whether a gene (or genes linked to it) carries a fitness penalty.[31] Thus far, fitness studies in weed species using NILs have been performed only for triazine-resistant and triazine-susceptible lines,[43–45,29] where it was shown that the mutation resulting in triazine resistance is associated with reduced fitness. Similarly, when wild mustard NILs were compared, the R lines produced significantly less biomass and seed (Table 4). Theoretically, if fitness costs were associated with the herbicide resistance mutation or due to closely linked genes, then, in the absence of selection, S plants would be expected to replace most of the R types.[31] The results of this research suggest that a fitness penalty is associated with wild mustard auxinic herbicide resistance (Table 4). Potentially, this fitness penalty may be one of the reasons for the slow occurrence and spread of auxinic-herbicide-resistant weeds. The present results support those reported by Debreuil *et al.*,[46] who used the same wild mustard R and S biotypes (but not NILs) under field conditions, and reported that, in the absence of auxinic herbicide, the S biotype produced more plant biomass.

It was also possible to identify a simple morphological marker linked to auxinic herbicide resistance. Morphological markers linked to agronomically important traits have been identified in several crop species. For example, the gene (*Ltn*) responsible for leaf tip necrosis in wheat was found to be linked to leaf and stripe rusts caused by *Puccinia* species.[25,47] Also, it was suggested that the chromosomal region possessing the genes for leaf tip necrosis in wheat also carries genes associated with moderate resistance

to spot blotch.[27] It is shown that the gene controlling leaf shape was 14.6 cM from the auxinic herbicide resistance locus in wild mustard (Table 3).

Using NILs, tightly linked molecular markers have been identified and extensively used for the characterization and the identification of traits such as a *Pseudomonas* resistance gene in tomato,[28] a downy mildew resistance gene in lettuce,[24] a bacterial blight resistance gene in rice[48] and a powdery mildew resistance gene in wheat.[49,23] When NILs are used for the identification of closely linked markers to a target gene, the possibility of obtaining a linked DNA marker depends on: (a) the number of DNA bands screened, and hence the number of primers screened through a PCR-based procedure; (b) the extent of DNA sequence differences in the area surrounding the target region;[28] (c) the sizes of introgressed DNAs in the NIL population presenting the selected or desired phenotype, which in turn depends on the number of BC generations performed to produce the NIL population. Also, it was suggested that pooling of DNA from different lines for the detection of markers linked to the target gene may reduce the identification of false positive markers associated with the target gene.[50] Thus, in this research, the DNA from R and S plants have been pooled for initial AFLP analyses in order to reduce the chances of identifying false positives. Furthermore, the DNA from advanced generation of backcross progeny (BC_8F_1) was used to increase the stringency of selecting the closely linked markers to the auxinic herbicide resistance gene in wild mustard. The AFLP markers that were identified using NILs in this research will be extremely useful for fine-scale mapping and provide useful DNA sequence tags[51] that may be used to determine the genetic locus involved in auxinic herbicide resistance in wild mustard.

As auxinic herbicides mimic several biochemical and physiological responses caused by natural auxin IAA, it is important to relate the possible mechanism of action of these herbicides to recently discovered auxin receptors and the mode of action of auxin. TIR1 (transport inhibitor response 1) and AFB (auxin F box) families of proteins were identified as auxin receptors.[52,53] IAA, as well as synthetic auxins such as NAA and 2,4-D, binds directly to the TIR1 pocket and acts as a molecular glue. This interaction results in recruitment and degradation of Aux/IAA repressor proteins. Auxin perception by TIR1 is required for Aux/IAA degradation and subsequent molecular and biochemical processes associated with auxin.[54,55] Furthermore, it has been reported that AFB4, an auxin F-box protein, is the major target of picloram.[56] The closest AFLP marker (M5) identified in this research, with about 371 nucleotide bps, may not provide sufficient information regarding this marker's sequence homology to TIR1 or its homologues. Nevertheless, there is potential for further analysis of this fragment to obtain longer sequence information using the bacterial artificial chromosome (BAC) library cloning procedures, which may assist in the identification of the gene involved in the auxinic herbicide resistance in wild mustard. Additionally, as suggested by Cai *et al.*,[57] a marker linked to the target gene at a distance less than 5 cM can also be used directly in marker-assisted selection. The marker M5 (1.58 cM) thus also has potential to be used in a breeding programme for identifying auxinic-herbicide-resistant plants. More importantly, the results of this research will assist in designing future experiments directed towards uncovering the complete mechanism of action of auxinic herbicides, which has eluded researchers for more than six decades. Furthermore, the present finding that wild mustard auxinic herbicide resistance (naturally occurring) is associated with a fitness penalty may be one of the reasons for the low occurrence and spread of auxinic herbicide resistance, in spite of the extensive and prolonged use of these herbicides.

ACKNOWLEDGEMENTS

This research was supported by grants to JCH from the Natural Science and Engineering Research Council of Canada (NSERC) and the Ontario Ministry of Agriculture and Food (OMAF). The post-doctoral fellowship for MJ from NSERC is also gratefully acknowledged.

5 APPENDIX

5.1 Nucleotide sequences of the AFLP markers

5.1.1 Marker 1 (282 bp)

GACTGCGTACCAATTCTACAAATACAAGATTGCCACTCAAGAAGTG
AATGAATATATCAAAACCTTTGTCAGGAAGTTGAAAGCTGGACTTC
CCTGCTGAGTACCCGCCTGAAGTTTGATACGCAGAGAAAGAGAGG
TTGCTACTAGCAAACACCTGCTCCTCAGCGCCATTACCTCTCTGGAA
GCTCAGAGTAAAGCCGCTTTCCATCTGCATAGGCTTTGTATCATGA
GTGGTACCCCATGCAAGCTTTTCTTCAGACCTCGTGTTACTCAGGA
CTCATC

5.1.2 Marker 2 (205 bp)

GACTGCGTACCAATTCTACCCTACATTGTATACGACAAATTTGGAC
AGGTAGCAGCTGAAGAATCTATAATCTACGATTATTTTGTGTGTAG
TGCAGACCTTTTGTTTGGACCTTTTGTTTTGTCTTTTGTGTTGTGT
TATAGTTGGCTTGCGTTTGAGTTTCTATACTAACGTTTTGGTCAGG
CTCAGTTACTCAGGACTCATC

5.1.3 Marker 3 (273 bp)

GACTGCGTACCAATTCACTGCAGGAGTAAAAATCTTTGAATTCTGC
CTTAGAATATAAGGTGAGAGTGGATCCCCCTGACGTAAATCTCTCT
GTGGAGTTAGAAGCCCTCTAAGTGTCTTTCAATAACACTTTATATT
GTAATGATGATATACATTCCATCATAAGCTAAATCCACTGGGGATC
AAAACCCATTTCGTGGAACAATGCCTGAATAAAAGCTAATTATATC
CTATCATATGCTTTGCTCATATTCGTTTTACTCAGGACTCATC

5.1.4 Marker 4 (483 bp)

TTAGCGGCCGCGAATTCGCCCTTGACTGCGTACCAATTCACGGCCC
AAAACCTTTTCAACTTTACCAACCAACTTCAGGTTACTACGCTTGT
TAGTTCTAGCCAAAAAGCGTTCCTCTGTACCACGTTCGATCACTCT
TTTTGGCAAAGCAATCTTATCATCAGAGTTACTCATCGTTTCACCT
GAAACAATTCAAGATGGTAATGTGAAAGATATGTTTTATAATGACT
TATAAATCCTAATTCATTATTACCTACTGGTATCTCTTGAGGGTCA
ATGATTGGGGAGGCAGCATCATCATTCATAGTATCATCTGATTCAT
GAGGTAATGCTAACTCGAATGCCTACACACCATTATGTCAACACAA
ATGAAGTGATAGTTTACTCAGGACTCATCAAGGGCGAATTCGTTTA
AACCTGCAGGACTAGTCCCTTTAGTGAGGGTTAATTCTGAGCTTGG
CGTAATCATGGTCATAGCTGTTT

5.1.5 Marker 5 (371 bp)

TTAGCGGCCGCGAATTCGCCCTTGACTGCGTACCAATTCACGGCCG
CGAGACATTGGTGACGAGGTTACAGCACTGCTTGACGGTTTTTGAC
GACACAATCGGTATAAGCGGGAGGAGATGAGGAACTCGCGACGG
AATAATGTTTGTTTAGAATAAGAAAAATACATAGGTGTTGTAAAAA
AAAAAAAGGGGAGAAGAGAAAGTTATTTCTGGAAATATAAAAATG
GTAAATACATACTCTAATTGTAAAGGGTCACGAGAGAGAGGATAA
GAAATGTAAGCTAGGACTGACTAGGGGATAGGGGTGTTTTCGGTT
TACTCAGGACTCATCAAGGGCGAATTCGTTTAAACCTGCAGGACTA
GTCCCTTT

5.1.6 Marker 6 (106 bp)

GACTGCGTACCAATTCACGAGGTGGATAATGGGCAGGTCTGATTGT
TCCATAGAAATAGCTTACCTTGTGATCTGAGGAATGATCTAGCTTT
ACTCAGGACTCATC

REFERENCES

1 Becker HC, Damgaard C and Karlsson B, Environmental variation for out-crossing rate in rapeseed (*Brassica napus*). *Theor Appl Genet* **84**:303–306 (1992).
2 Stevens JP and Kay QON, The number, dominance relationships and frequencies of self-incompatibility alleles in a natural population of *Sinapis arvensis* L. in South Wales. *Heredity* **62**:199–205 (1989).
3 Heap IM and Morrison IN, Resistance to auxin-type herbicides in wild mustard (*Sinapis arvensis* L.) populations in western Canada. *Annual Meeting of Weed Science Society of America*, Abstract 32, p. 164 (1992).
4 Hall JC, Alam SMM and Murr DP, Ethylene biosynthesis following foliar application of picloram to biotypes of wild mustard (*Sinapis arvensis* L.) susceptible or resistant to auxinic herbicides. *Pestic Biochem Physiol* **47**:36–43 (1993).
5 Penuik MG, Romano ML and Hall JC, Physiological investigations into the resistance of wild mustard (*Sinapis arvensis* L.) biotype to auxinic herbicide. *Weed Res* **33**:431–440 (1993).
6 Hall JC and Romano ML, Morphological and physiological differences between the auxinic herbicide susceptible (S) and resistant (R) wild mustard (*Sinapis arvensis*) biotypes. *Pestic Biochem Physiol* **52**:149–155 (1995).
7 Webb SR and Hall JC, Auxinic herbicide-resistant and susceptible wild mustard (*Sinapis arvensis* L.) biotypes: effect of auxinic herbicides on seedling growth and auxin-binding activity. *Pestic Biochem Physiol* **52**:137–148 (1995).
8 Deshpande S and Hall JC, Comparison of flash-induced light scattering transients and proton efflux from auxinic-herbicide resistant and susceptible wild mustard protoplasts: a possible role for calcium in mediating auxinic herbicide resistance. *Biochim Biophys Acta* **1244**:69–78 (1995).
9 Deshpande S and Hall JC, ATP-dependent auxin- and auxinic herbicide-induced volume changes in isolated protoplast suspensions from *Sinapis arvensis* L. *Pestic Biochem Physiol* **56**:26–43 (1996).
10 Deshpande S and Hall JC, Auxinic herbicide resistance may be modulated at an auxin binding site in wild mustard (*Sinapis arvensis* L.): a light scattering study. *Pestic Biochem Physiol* **66**:41–48 (2000).
11 Hall JC, Webb SR and Deshpande S, An overview of auxinic herbicide resistance: wild mustard as a case study, in *Molecular Genetics and Evolution of Pesticide Resistance*, ed. by Brown TM. American Chemical Society, Washington, DC, pp. 28–43 (1996).
12 Wang YS, Deshpande S and Hall JC, Calcium may mediate auxinic herbicide resistance in wild mustard. *Weed Sci* **49**:2–7 (2001).
13 Zheng HG and Hall JC, Understanding auxinic herbicide resistance in *Sinapis arvensis* L.: physiological, biochemical and molecular genetic approaches. *Weed Sci* **49**:276–281 (2001).
14 Jugulam M, McLean MD and Hall JC, Inheritance of picloram and 2,4-D resistance in wild mustard (*Brassica kaber*). *Weed Sci* **53**:417–423 (2005).
15 Mithila J and Hall JC, Comparison of ABP1 over-expressing *Arabidopsis* and under-expressing tobacco with an auxinic herbicide-resistant wild mustard (*Brassica kaber*) biotype. *Plant Sci* **169**:21–28 (2005).
16 Mithila J and Hall JC, Production of an auxinic herbicide-resistant micropore-derived haploid wild mustard (*Sinapis arvensis* L.) plant. *Crop Prot* **27**:357–362 (2007).
17 Jasieniuk M, Morrison IN and Brule-Babel AL, Inheritance of dicamba resistance in wild mustard (*Brassica kaber*). *Weed Sci* **43**:192–195 (1995).
18 Muehlbauer GJ, Specht JE, Thomas-Compton MA, Staswick PE and Bernard RL, Near-isogenic lines: a potential resource in the integration of conventional and molecular marker linkage maps. *Crop Sci* **28**:729–735 (1988).
19 Young ND, Zamir D, Ganal MW and Tanksley SD, Use of near-isogenic lines and simultaneous probing to identify DNA markers tightly linked to the Tm-2a gene in tomato. *Genetics* **120**:579–585 (1988).
20 Monforte AJ and Tanksley SD, Development of a set of near isogenic and backcross recombinant inbred lines containing most of the *Lycopersicon hirsutum* genome in *L. esculentum* genetic backcground: a tool for gene mapping and gene discovery. *Genome* **43**:803–813 (2000).
21 Janeja HS, Banga SK, Bhaskar PB and Banga SS, Alloplasmic male sterile *Brassica napus* with *Enarthrocarpus lyratus* cytoplasm: introgression and molecular mapping of an *E. lyratus* chromosome segment carrying a fertility restoring gene. *Genome* **46**:792–797 (2003).
22 Ram T, Majumber ND and Mishra B, Introgression of broad-spectrum blast resistance gene(s) into cultivated rice (*Oryza sativa* ssp *indica*) from wild rice *O. rufipogon*. *Curr Sci* **92**:225–230 (2007).
23 Zhou R, Zhu Z, Kong X, Huo N, Tian Q, Li P, *et al*, Development of wheat near-isogenic lines for powdery mildew resistance. *Theor Appl Genet* **110**:640–648 (2005).
24 Paran I, Kesseli R and Michelmore R, Identification of restriction fragment length polymorphism and random amplified polymorphic DNA markers linked to downy mildew resistance genes in lettuce, using near-isogenic lines *Genome* **34**:1021–1027 (1991).
25 Dyck PL, Genetics of adult-plant leaf rust resistance in 'Chinese Spring' and 'Sturdy' wheats. *Crop Sci* **31**:309–311 (1991).
26 Singh RP, Association between gene *Lr34* for leaf rust resistance and leaf tip necrosis in wheat. *Crop Sci* **32**:874–878 (1992).
27 Joshi AK, Chand R, Kumar S and Singh RP, Leaf tip necrosis: a phenotypic marker associated with resistance to spot blotch disease in wheat. *Crop Sci* **44**:792–796 (2004).
28 Martin GB, Williams JGK and Tanksley SD, Rapid identification of markers linked to a *Pseudomonas* resistance gene in tomato by using random primers and near-isogenic lines. *Proc Natl Acad Sci USA* **88**:2336–2340 (1991).
29 McCloskey WB and Holt JS, Effect of growth temperature on biomass production of nearly isonuclear triazine-resistant and susceptible common groundsel (*Senecio vulgaris* L.). *Plant Cell Environ* **14**:699–705 (1991).
30 Vila-Aiub MM, Neve P and Powles SB, Fitness costs associated with evolved herbicide resistance alleles in plants. *New Phytol* **184**:751–767 (2009).
31 Jasieniuk M, Brule-Babel AL and Morrison IN, Evolution and genetics of herbicide-resistance in weeds. *Weed Sci* **44**:176–193 (1996).
32 Tanksley SD, Young ND, Paterson AH and Bonierbale MW, RFLP mapping in plant breeding: new tools for an old science. *Bio/Tech* **7**:257–263 (1989).
33 Haley SD, Miklas PN, Stavely JR, Byrum J and Kelly JD, Identification of RAPD markers linked to a major rust resistance gene block in common bean. *Theor Appl Genet* **86**:505–512 (1993).
34 Chelkowski J, Gokla L and Stepien L, Application of STS markers for leaf rust resistance genes in near-isogenic lines of spring wheat cv. Thatcher. *J Appl Genet* **44**:323–338 (2003).
35 Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, Hornes M, *et al*, AFLP: a new technique for DNA finger printing. *Nucl Acid Res* **23**:4407–4414 (1995).
36 *International Survey of Herbicide Resistant Weeds*. [Online]. WSSA. Available: http://www.weedscience.org/summary/MOASummary.asp/ [15 June 2011].
37 Shaaltiel Y, Chua NH, Gepstein S and Gressel J, Dominant pleitropy controls enzymes co-segregating with paraquat resistance in *Conyza bonariensis*. *Theor Appl Genet* **75**:850–856 (1988).
38 Yamasue Y, Kamiyama K, Hanoika Y and Kusanagi T, Paraquat resistance and its inheritance in seed germination of foliar resistant biotypes of *Erigeron canadensis* and *E. sumatrensis*. *Pestic Biochem Physiol* **100**:630–636 (1992).
39 Sabba RP, Ray IM, Lownds N and Sterling TM, Inheritance of resistance to clopyralid and picloram in yellow starthistle (*Centaurea solstitialis*) is controlled by a single nuclear recessive gene. *J Hered* **94**:523–527 (2003).
40 Van Eerd LL, McLean MD, Stephenson SR and Hall JC, Resistance to quinlorac and ALS-inhibitor herbicides in *Galium spurium* is conferred by two distinct genes. *Weed Res* **44**:355–365 (2004).
41 Briggs FN and Allard RW, The current status of backcross method of plant breeding. *Agron J* **45**:131–138 (1953).
42 Young ND and Tanksley SD, RFLP analysis of the size of chromosomal segments retained around the *Tm-2* locus of tomato during backcross breeding. *Theor Appl Genet* **77**:353–359 (1989).
43 Beversdorf WD, Hume DJ and Donnelly-Vanderloo MJ, Agronomic performance of triazine-resistant and susceptible reciprocal spring canola hybrids. *Crop Sci* **28**:932–934 (1998).
44 Gressel J and Ben-Sinai G, Low intraspecific competitive fitness in a triazine resistant, nearly isogenic line of *Brassica napus*, in *Molecular*

Form and Function of Plant Genome, ed. by van Vloten-Doting L, Groot GSP and Hall TC. Plenum Press, New York, NY, pp. 489–504 (1989).
45 Jacob BF, Duesing JH, Antonovices J and Patterson DT, Growth performance of triazine-resistant and -susceptible biotypes of *Solanum nigrum* over a range of temperatures. *Can J Bot* **66**:847–850 (1988).
46 Debreuil DJ, Friesen LF and Morrison IN, Growth and seed return of auxin-type herbicide resistant wild mustard (*Brassica kaber*) in wheat. *Weed Sci* **44**:872–878 (1996).
47 McIntosh RA, Close genetic linkage of genes conferring adult plant resistance to leaf rust and stripe rust in wheat. *Plant Pathol* **41**:523–527 (1992).
48 Zhang HB, Choi S, Woo SS, Li Z and Wing RA, Construction and characterization of two rice bacterial artificial chromosome libraries from the parents of a permanent recombinant inbred mapping population. *Mol Breed* **2**:11–24 (1995).
49 Tao W, Liu D, Liu J, Feng Y and Chen P, Genetic mapping of the powdery mildew resistance gene Pm6 in wheat by RFLP analysis. *Theor Appl Genet* **100**:564–568 (2000).
50 Michelmore RW, Paran I and Kesseli RV, Identification of markers linked to disease-resistance genes by bulked segregation analysis: a rapid method to detect markers in specific genomic regions by suing segregating populations. *Proc Natl Acad Sci USA* **88**:9828–9832 (1991).
51 de Temmerman P, Visvikis S, Boerwinkle E and Siest G, Study of the sequence tagged site (STS) in the beginning of human apo A4 gene region. *Nucleic Acids Res* **18**:5576 (1990).
52 Dharmasiri N, Dharmasiri S and Estelle M, The F-box protein TIR1 is an auxin receptor. *Nature* **435**:441–445 (2005).
53 Kepinski S and Leyser O, The *Arabidopsis* F-box protein TIR1 is an auxin receptor. *Nature* **435**:446–451 (2005).
54 Guilfoyle T, Sticking with auxin. *Nature* **446**:621–622 (2007).
55 Tan X, Calderon-Villalobos LIA, Sharon M, Zheng C, Robinson CV, Estelle M, *et al*, Mechanism of auxin perception by the TIR1 ubiquitin ligase. *Nature* **446**:640–645 (2007).
56 Greenham K, Santner A, Castillejo C, Mooney S, Sairanen I, Ljung K, *et al*, The AFB4 axing receptor is a negative regulator of auxin signaling in seedling. *Curr Biol* **21**:520–525 (2011).
57 Cai HW, Gao Z-s, Yuyama N and Ogawa N, Identification of AFLP markers closely linked to the rhm gene for resistance to Southern Corn Leaf Blight in maize by using bulked segregant analysis. *Mol Gen Genomics* **269**:299–303 (2003).