Gene Flow and Multiple Herbicide Resistance in Escaped Canola Populations

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Gene flow among herbicide-resistant (HR) canola varieties can lead to the development of multiple HR canola plants, creating volunteer canola management challenges for producers. In western Canada, escaped populations of HR canola are ubiquitous outside of cultivated fields, yet the extent of gene flow resulting in herbicide resistance trait stacking in individuals within these populations remains unknown. The objectives of this study were to document the presence of single and multiple herbicide resistance traits and assess the extent of gene flow within escaped canola populations. Seed was collected from 16 escaped canola populations along the verges of fields and roadways in four agricultural regions in southern Manitoba from 2004 to 2006. Glyphosate resistance was found in 14 (88%) of these populations, glufosinate resistance in 13 (81%) populations, and imidazolinone resistance in five (31%) populations. Multiple herbicide resistance was observed at levels consistent with previously published canola outcrossing rates in 10 (62%) of the tested populations. In 2005 and 2006, maternal plants from two escaped populations were tested using trait indicator test strips for glyphosate and glufosinate resistance to confirm outcrossing events. In 2005, two of 13 tested maternal plants with single herbicide resistance traits produced progeny with both glyphosate and glufosinate resistance. In 2006, of 21 tested plants, 10 single HR maternal plants produced multiple HR progeny, and five nonresistant maternal plants produced resistant offspring. This is the first report indicating that intraspecific gene flow results in stacking of herbicide resistance traits in individuals within escaped canola populations, confirming that multiple HR canola volunteers are not confined to agricultural fields. Results of this study suggest that escaped populations of crop plants can contribute to the spread of genetically engineered novel traits, which has important implications for containment, especially for highly controversial pharmaceutical and industrial traits in crop plants.

Nomenclature: Glufosinate; glyphosate; canola, *Brassica napus* L. BRSNS.

Key words: Escaped volunteer, gene flow, genetically modified crops, herbicide resistance, outcrossing, roadside ditches.

Canola varieties genetically engineered for herbicide resistance were approved for large-scale commercial release in the United States and Canada in 1995 (for the purposes of this paper, canola refers exclusively to *Brassica napus* L.). Currently, three herbicide-resistant (HR) canola types are widely grown in western Canada, including transgenic varieties resistant to glyphosate or glufosinate, and mutagenic varieties resistant to certain imidazolinone herbicides. In 2005, glyphosate- and glufosinate-resistant canola varieties were grown on 42 and 47%, respectively, and imidazolinone resistant varieties on 9% of the 902,000 ha of canola grown in Manitoba, Canada (Manitoba Agricultural Services Corporation 2006a). Conventional non-HR canola represented only 2% of the area planted to canola. High adoption rates of HR canola varieties are largely associated with operational benefits, including improved weed control and ease of management (Beckie et al. 2006; Mauro and McLachlan 2008).

Despite these benefits, risks to producers associated with the cultivation of genetically engineered/modified (GM) crops are increasingly recognized. These risks include compromised weed control in zero-till systems (Friesen et al. 2003), increased herbicide costs (Van Acker et al. 2003), crop contamination with adventitious herbicide resistance traits, and even legal liability (Mauro and McLachlan 2008; Smyth et al. 2002). Yet post-release monitoring of HR crops has focused primarily on their impacts in natural ecosystems (Crawley et al. 1993; Crawley et al. 2001) and on gene flow to wild relatives (Scott and Wilkinson 1998; Warwick et al. 2003; Wilkinson et al. 2003). However, the importance of intraspecific gene flow within and between arable fields is increasingly recognized (Ellstrand 2001; Légère 2005; Smyth et al. 2002; Waines and Hegde 2003), raising concerns about the containment of herbicide resistance traits (Beckie et al. 2006) and implications for environmentally beneficial practices such as direct seeding in western Canada (Friesen et al. 2003). Among canola plants, where individual outcrossing rates range from 6 to 81% (Cuthbert and McVetty 2001), pollen-mediated gene flow may result in the relatively rapid movement of herbicide resistance genes. Outcrossing between adjacent canola fields with differing herbicide resistance traits has resulted in multiple HR canola volunteers in western Canada (Beckie et al. 2003; Hall et al. 2000). Multiple HR canola plants appear not to be subject to fitness costs (Simard et al. 2005), and herbicide resistance traits may potentially accumulate and spread in volunteers growing both in cultivated fields and adjacent habitats.

Canola is a weedy plant that frequently grows in disturbed habitats (Crawley and Brown 1995; Pessel et al. 2001), and escaped canola populations have become ubiquitous in rural landscapes throughout western Canada. Escaped populations most likely arise from seed dispersed by agricultural activities and transport (Crawley and Brown 1995), and commonly contain glyphosate and glufosinate resistance traits in areas where these HR varieties are widely grown (Yoshimura et al. 2006). Although localized escaped canola populations are believed to be transient (Crawley and Brown 2004), and thus of little management significance (Hall et al. 2005), such populations may contribute to the spread of herbicide resistance traits through intraspecific gene flow within a single generation. Where differing HR canola types are locally grown, gene flow within escaped canola populations has the potential to result in stacking of resistance traits in individuals in disturbed, noncrop habitats. Moreover, survival of canola plants over winter (Lawson et al. 2006; Simard et al. 2002) may contribute to increased persistence of herbicide resistance

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Table 1. Characteristics of escaped canola populations in the rural municipalities of Rhineland, MacDonald, Roland, and Dufferin, in southern Manitoba, Canada.

Site/year	Density[a]	Adjacent crop	Adjacent crop in previous year	Time since last canola crop in adjacent field
	plants m^{-2}			yr
Rhine1[b] 2004	2.8 (0.45)	Corn	Dry bean	10
Rhine2 2004	1.5 (0.23)	Corn	Dry bean	> 10
Rhine4 2004	5.9 (0.66)	GLU canola	n/a	0
MacD1 2004	1.0 (0.25)	GLY canola	Barley	0
MacD2 2004	n/a	GLU canola	Wheat	0
MacD4 2004	3.4 (0.74)	Alfalfa	Alfalfa	> 10
Rhine3 2005	n/a	Pinto bean	Corn	> 6
Rhine4 2005	1.9 (0.60)	Wheat	GLU canola	1
MacD1 2005	1.2 (0.29)	Barley	GLY canola	1
MacD2 2005	1.3 (0.34)	Wheat	GLU canola	1
MacD4 2005	0.3 (0.10)	Alfalfa	Alfalfa	> 10
MacD1 2006	0.5 (0.13)	IMI canola	Barley	0
Rol1 2006	10.4 (1.56)	Corn	Soybean	4
Rol2 2006	15.5 (1.29)	Corn	Barley	2
Duff1 2006	11.2 (0.77)	Corn	GLY canola	1
Duff2 2006	3.1 (0.41)	Wheat	GLY canola	1

[a] Density of escaped canola plants was counted in 30 1-by-1–m quadrats in the roadside ditches in August 2004, 2005, and 2006. Mean density is presented with standard errors in parentheses.

[b] Abbreviations: Rhine, Rhineland; MacD, MacDonald; Rol, Roland; Duff, Dufferin; GLY, glyphosate resistant; GLU, glufosinate resistant; IMI, imidazolinone resistant; n/a, not available.

traits. The movement of stacked traits from disturbed habitats into non-HR canola crops, or to canola crops possessing different herbicide resistance traits, has important implications for subsequent management of volunteer canola and choice of cropping system. Although escaped populations of HR canola appear to be widespread, the extent to which outcrossing within these populations contributes to the spread of herbicide resistance traits remains unknown.

The objectives of this study were to: (1) characterize the frequency of glyphosate, glufosinate, and imidazolinone resistance in roadside escaped canola populations in southern Manitoba over multiple years, and (2) determine whether intraspecific gene flow contributes to the spread and stacking of herbicide resistance traits within these escaped populations. Multiple HR progeny produced by outcrossing events were confirmed through phenotypic testing of maternal plants combined with herbicide screening of progeny.

Materials and Methods

Site Selection. To determine the herbicide resistance characteristics of escaped canola volunteers over several years, roadside canola populations were identified in the rural municipalities (RMs) of MacDonald (49°40′N, 97°30′W), Rhineland (49°9′N, 97°39′W), Roland (49°20′N, 97°54′W), and Dufferin (49°31′N, 98°4′W) in southern Manitoba. MacDonald and Dufferin, which cover 1,106 km^2 and 882 km^2, respectively, are among the most important canola-growing regions in the province, and canola is generally planted once every 3 or 4 yr in rotation. In Rhineland and Roland, which cover 923 km^2 and 466 km^2, respectively, canola is grown less frequently and in some fields has not been planted for over 30 yr (Table 1). However, one of the province's two canola crushing facilities is located in the Rhineland region, resulting in much transport of canola seed in the region.

Escaped canola populations were initially identified in the RMs of MacDonald and Rhineland in July 2004, and subsequently in the RMs of Roland and Dufferin in July 2006, at the time of canola flowering. Populations were located along paved highways or gravel grid roads, adjacent to cropped land. Current and past crop cultivation in adjacent fields were identified through interviews with landowners. All populations had an initial size of at least 30 flowering plants, and occupied 50 to 200 m (linear) of roadside habitat. Density of each escaped population was determined in August of each year in 30 randomly located permanent 1-by-1-m quadrats.

Seed Collection and Field Testing. In August and September of 2004, 2005, and 2006, seed was collected from mature escaped canola plants in the roadside populations. All plants were collected from outside the permanent quadrats to minimize impact on population dynamics within the quadrats. In 2004, six of eight monitored populations produced reproductive plants, and seed was collected from each of 45 escaped canola plants. In 2005, seed from 37 plants was collected from five of eight monitored populations. In 2006, seed from 47 plants was collected from 5 of 12 monitored populations. In all cases, all pods were collected from each plant. Three populations (Rhineland4, MacDonald2, and MacDonald4) produced viable seed in both 2004 and 2005. Only one population (MacDonald1) produced viable seed in all 3 yr of the study, attesting to the transient nature of these populations.

To assess gene flow in escaped canola populations, the maternal phenotypes of escaped canola plants growing in the MacDonald2 and Dufferin1 populations were tested in 2005 and 2006, respectively. In each of these two populations, a relatively large number of plants produced enough vegetative tissue to permit maternal phenotype testing, and sufficient seed for subsequent herbicide trials. In 2005, leaf tissue samples were collected from 21 marked plants from the MacDonald2 population in July, and tested for glyphosate and glufosinate resistance using trait indicator test strips.[1] Trait indicator test strips detect the presence in leaf tissue of proteins associated with glyphosate or glufosinate resistance. In September 2005 when seed had matured, seed of 13 of the tested maternal plants was collected. The remaining eight tested plants had died or had been mowed over the course of

the summer. In 2006, leaf tissue samples from 27 marked plants from the Dufferin1 population were tested for glyphosate and glufosinate resistance using test strips in July, and seed from 21 surviving plants was collected in September. In both years, collected seed from tested plants was included in herbicide screening trials (as described below) to determine the herbicide resistance characteristics of the progeny. Results of herbicide screening of plants from these two populations are shown in a separate figure, permitting examination of the herbicide resistance characteristics of progeny.

Subsamples of between 25 and 100 seeds from canola plants collected in 2004 and 2006 were tested for germinability on filter paper in petri dishes, while ensuring sufficient seeds remained for herbicide screening. Extremely wet weather in 2005 resulted in low seed production and all seeds were required for herbicide screening tests, precluding any germination tests that year. Average germination of seeds from escaped plants was 98% (standard error [SE] 0.6) in 2004, and 96% (SE 1.1) in 2006. Percentage germination per individual sample ranged from 71 to 100% in both years. One-thousand seed weights were determined for seed collected from escaped plants in each of the 3 yr.

Herbicide Resistance Trials. Herbicide trials were conducted in 2004, 2005, and 2006 to determine the proportion of escaped canola progeny exhibiting single and multiple herbicide resistance. A subset of 28 seeds from each maternal canola plant was tested in two separate herbicide trials. Seedlings were grown indoors in a growth chamber with a 16-h photoperiod and 21/17 C day/night temperatures to the three- to four-leaf stage ($\approx$ 14 d). Numbers of seedlings were counted before herbicide application, and only those plants with at least 20 progeny (seedlings) were included in herbicide trials. Herbicide screening of between 18 to 24 progeny results in a 0.995 to 0.999 probability of detecting segregation from selfed heterozygous maternal plants (Rédei 1982), where the trait of interest is controlled by a single dominant nuclear gene. In the case of glyphosate and glufosinate resistance, segregation will occur among the progeny of hemizygous maternal plants that carry one herbicide resistance allele, but lack a second allele on the homologous chromosome (Hall et al. 2000).

In the first herbicide trial, seedlings were sprayed with glyphosate at 700 g ae ha^{-1} and assessed for survival after 7 d. Surviving seedlings were subsequently sprayed with glufosinate at 600 g ai ha^{-1} and further survival assessed after an additional 4 d. In the second herbicide trial, the order of herbicide application was reversed, and seedlings were first sprayed with glufosinate at 400 g ha^{-1} and assessed for survival after 4 d. Surviving seedlings were then sprayed with glyphosate at 700 g ha^{-1} and further survival assessed after 7 d. Growth response of seedlings in both trials was compared with that of 12 seedlings each of known glyphosate-resistant (LG 3235), glufosinate-resistant (SW Flare), and susceptible (46A65) cultivars. As glyphosate and glufosinate resistance are conferred by dominant nuclear alleles from two different genes (Beckie et al. 2003; Hall et al. 2000), both homozygous dominant and hemizygous seedlings will exhibit resistance, whereas homozygous recessive plants will be susceptible. Seedlings sprayed with these herbicides were scored as either resistant or susceptible to glyphosate and glufosinate, and resistance to the first herbicide was assessed before application

of the second. The results of glyphosate and glufosinate herbicide trials were verified by testing at least 50% of surviving, putatively double HR seedlings with trait indicator test strips for both glyphosate and glufosinate resistance.

A third herbicide trial was conducted to detect imidazolinone resistance for those escaped canola plants that produced sufficient seed (n = 42 plants in 2004, n = 24 in 2005, and n = 46 in 2006). Twenty-eight seeds from each maternal plant were planted and seedlings were sprayed at the three- to four-leaf stage with thifensulfuron at 10 g ai ha^{-1} + nonionic surfactant at 0.2% v/v. The imidazolinone resistance trait also confers resistance to thifensulfuron, a sulfonylurea herbicide, which was registered and commercially available for use on mutagenic imidazolinone-resistant canola varieties until 2002 (Friesen et al. 2003) and which provides a more predictable plant response than imazethapyr or imazamox in controlled environments (personal observation). Resistance to acetolactate synthase inhibitors (imidazolinones and sulfonylureas) is determined by an additive two-gene system in canola (Tan et al. 2005), and, depending on their genetics, outcrossed second- and third-generation plants may exhibit intermediate resistance. Canola seedlings sprayed indoors with thifensulfuron are visually distinguishable as either resistant, intermediate, or susceptible. Growth response of seedlings was compared with that of known imidazolinone-resistant (45A77) and susceptible (46A65) cultivars, and seedlings were scored as resistant, intermediate, or susceptible. Generally the time required for satisfactory development of plant injury response after the application of thifensulfuron (10 d) precludes any sequential herbicide application, as surviving seedlings are then too advanced for normal herbicide activity. In 2006, because of the large number of thifensulfuron survivors, these plants were subsequently sprayed with glyphosate. Surviving seedlings in all instances were tested for glyphosate and glufosinate resistance using test strips.

Results and Discussion

Population Persistence and Reproductive Output of Escaped Canola Volunteers. In the roadside canola populations observed in this study, many canola plants were mowed or died over the course of each growing season (data not shown), and relatively few plants survived to produce seed. Additionally, on the basis of soil sampling at each of the roadside canola sites and subsequent cycles of emergence monitoring of these soil samples in the greenhouse (following the protocol described in De Corby et al. 2007), it appears that roadside canola seedbanks are small (less than five germinable seeds per square meter) and lack substantive dormancy (A. Knispel, unpublished data). Low numbers of reproductive plants and lack of seed dormancy likely contribute to the transience of the escaped canola populations observed in this study. Indeed, very few of the populations observed in this study produced reproductive plants in consecutive years at the same location; only 25% of populations produced seed over 2 yr and only one produced seed in all 3 yr. Escaped rapeseed (*Brassica napus* L.) populations observed over multiple years in England also were found to be transient (Crawley and Brown 1995; Crawley and Brown 2004). However, this apparent transience is likely offset at a landscape scale by yearly seed addition from agricultural sources.

Table 2. Number of escaped canola plants growing in roadside populations producing glyphosate- and glufosinate-resistant progeny in 2004, 2005, and 2006 in southern Manitoba, Canada. The percentage range of the proportion of resistant progeny per maternal plant is given in parentheses; values ranged from 0 to 100%.

Site/year	Maternal plants tested	Plants with GLY[a]-resistant progeny	Plants with GLU-resistant progeny	Plants with double HR progeny
	no.	— no. (percentage range) —		
Rhine1 2004	5	5 (18–75%)	5 (38–100%)	5 (4–65%)
Rhine2 2004	4	4 (63–96%)	0	0
Rhine4 2004	10	7 (4–79%)	8 (4–100%)	3 (4–21%)
MacD1 2004	10	8 (4–100%)	0	0
MacD2 2004	10	9 (4–100%)	9 (4–100%)	9 (4–25%)
MacD4 2004	6	6 (4–100%)	3 (4–10%)	2 (4–10%)
Rhine3 2005	7	7 (68–84%)	1 (4%)	1 (7%)
Rhine4 2005	2	0	2 (25–100%)	0
MacD1 2005	7	0	7 (4–100%)	0
MacD2 2005	13	5 (7–100%)	9 (7–100%)	2 (7%)
MacD4 2005	8	3 (4–100%)	6 (4–100%)	0
MacD1 2006	4	4 (86–100%)	1 (7%)	2 (4–7%)
Rol1 2006	10	7 (4–100%)	9 (4–100%)	5 (4–25%)
Rol2 2006	8	6 (13–100%)	7 (4–96%)	5 (4–11%)
Duff1 2006	21	20 (5–82%)	14 (4–52%)	10 (4–7%)
Duff2 2006	4	4 (59–86%)	0	0

[a] Abbreviations: Rhine, Rhineland; MacD, MacDonald; Rol, Roland; Duff, Dufferin; GLY, glyphosate; GLU, glufosinate. HR, herbicide resistant.

Interestingly, although populations are highly transient, seed yields from individual escaped canola plants were comparable with those of cropped canola plants in southern Manitoba. In our study in 2004, escaped canola plants produced an average of 1,667 seeds per plant (SE 297), with an average 1,000-seed weight of 3.6 g (SE 0.1). In 2005, because of very wet and cold conditions, escaped canola plants produced substantially less seed, averaging 355 seeds per plant (SE 111), with an average 1,000-seed weight of 3.0 g (SE 0.1). In 2006, escaped canola plants produced an average of 883 seeds per plant (SE 121), with an average 1,000-seed weight of 3.1 g (SE 0.1). On the basis of the 10-yr provincial average canola yield of 1,677 kg ha^{-1} (Manitoba Agricultural Services Corporation 2007) and given typical crop stand densities, in-field canola plants produce approximately 700 seeds per plant with an average 1,000-seed weight of 3.0 g (Gulden et al. 2003). The comparable reproductive output of escaped canola volunteers and cropped canola plants in western Canada, as well as the high viability of seed from escaped plants in this study (98% in 2004 and 96% in 2006), suggests that seed inputs from mature plants should contribute to population persistence in roadside habitats. Additionally, in the spring of 2006, overwintered canola rosettes were observed in many arable fields in Manitoba (Lawson et al. 2006) and HR canola plants also survived the winter in roadside habitats (personal observation). These plants may have further contributed to population persistence. However, to date, little work has focused on the persistence of escaped canola populations at either the population or landscape scale in western Canada.

Glyphosate and Glufosinate Resistance. In all 3 yr of this study, all escaped canola populations exhibited the presence of glyphosate or glufosinate (or both) herbicide resistance traits, and in all cases test strip results confirmed the results of the herbicide screening trials of progeny. In 2004, glyphosate resistance was identified in all six populations and in 39 (87%) of the 45 tested maternal plants, and glufosinate resistance was found in four of six populations and in 56% of tested maternal plants (Table 2). In 2005 samples, glyphosate resistance was found in only three of the five populations and

15 (41%) of the 37 tested maternal plants. However, glufosinate resistance was found in all populations and in 68% of maternal plants. In 2006, glyphosate resistance was again found in all populations and in 41 of the 47 maternal plants tested (87%). In that same year, glufosinate resistance was found in four of the five populations and in 66% of the tested maternal plants. These results indicate that transgenic herbicide resistance is widespread in roadside canola populations, reflecting the popularity of glyphosate- and glufosinate-resistant canola varieties with farmers in southern Manitoba. Similar correspondence between frequency of transgenic herbicide resistance traits in escaped canola plants and cultivated acreage was also observed in the neighboring province of Saskatchewan in 2005 (Yoshimura et al. 2006). These results suggest that HR canola plants will remain common in noncrop, ruderal environments as long as HR canola varieties continue to be widely cultivated in western Canada, especially as HR canola plants seem not to be subject to large fitness costs (Simard et al. 2005).

Varying frequencies of herbicide resistance traits in escaped canola populations suggests that noncrop habitats receive substantial seed input from agricultural sources. Indeed, canola plants from one escaped population (MacDonald1) exhibited only glyphosate resistance in 2004, only glufosinate resistance in 2005, and both glyphosate and glufosinate resistance in 2006 (Table 2), indicating that ongoing addition of new seed represents an important source of herbicide resistance traits in roadside populations. In this study, escaped canola plants producing 100% resistant progeny (Table 2), inferred to be homozygous for the dominant glyphosate or glufosinate alleles conferring resistance, likely arose from whole seed contamination of roadside habitats. Canola seed may be lost through seeding or harvest (or both) operations, and in this study 9 (56%) of the 16 escaped canola populations were adjacent to a canola field or adjacent to a field planted to canola in the previous year (Table 1). However, seed losses during agricultural transport are the most likely cause of escaped HR canola plants. Indeed, producers identified agricultural transport intensity as being high on the roads adjacent to 14 (88%) of the 16 escaped populations, and the majority (75%) of the escaped

Table 3. Number of escaped canola plants growing in roadside populations producing intermediate and fully imidazolinone-resistant progeny in 2004, 2005, and 2006 in southern Manitoba, Canada. Percentage of resistant progeny per maternal plant varied from 0 to 100%, and ranges are included in parentheses.

Site/year[a]	Maternal plants tested	Plants with IMI[b] intermediate progeny	Plants with IMI resistant progeny
	no.	————————— no. (percentage range) —————————	
Rhine1 2004	5	3 (4–8%)	0
Rhine2 2004	4	0	0
Rhine4 2004	10	0	0
MacD1 2004	9	2 (4–11%)	2 (100%)
MacD2 2004	10	1 (68%)	0
MacD4 2004	4	3 (8–100%)	1 (100%)
Rhine3 2005	6	0	0
MacD1 2005	5	0	0
MacD2 2005	11	0	0
MacD4 2005	2	0	0
MacD1 2006	3	0	0
Rol1 2006	10	0	0
Rol2 2006	8	0	0
Duff1 2006	21	2 (48–100%)	2 (52–100%)
Duff2 2006	4	0	0

[a] Note: There was insufficient seed available from the Rhine4 2005 population for imidazolinone resistance testing.
[b] Abbreviations: Rhine, Rhineland; MacD, MacDonald; Rol, Roland; Duff, Dufferin; IMI, imidazolinone.

populations examined over 3 yr were located along provincial roads and highways. Transport has also been identified as the likely source of escaped canola plants both in western Canada (Yoshimura et al. 2006) and in countries such as Japan where whole HR canola seed is imported for processing, but where HR varieties are not currently cultivated (Aono et al. 2006).

Within escaped populations, intraspecific gene flow may frequently occur within a single generation, thereby facilitating the spread of herbicide resistance traits within a relatively short time frame (Beckie et al. 2003; Hall et al. 2000) and potentially accelerating the development and spread of multiple HR canola volunteers. Escaped maternal canola plants tested in this study also produced progeny that segregated for the resistance trait, with resistance ranging between 4 and 96%, for both glyphosate and glufosinate resistance traits (Table 2). These proportions may be used to infer maternal genotypes, according to segregation ratios of offspring (Rédei 1982). Plants that produced high proportions (i.e., 50 to 99%) of resistant offspring were assumed to have been hemizygous for either the glyphosate or the glufosinate resistance allele, as wholly self-fertilized hemizygous plants are expected to produce offspring in a 3 : 1 resistant : susceptible ratio. Seed giving rise to plants hemizygous for herbicide resistance traits is present in commercial HR pedigreed seedlots (Beckie et al. 2003), and may be introduced into roadside habitats by nearby agricultural activities. Alternatively, these hemizygous maternal plants may have been the second generation of escaped canola resulting from the movement of resistance traits via pollen-mediated gene flow (PMGF).

Low proportions (i.e., 4 to 30%) of glyphosate resistance were observed in the progeny of 14 (31%) of 45 tested maternal plants in 2004, 3 (8%) of 37 tested maternal plants in 2005, and 6 (13%) of 47 tested maternal plants in 2006 (Table 2). Similarly low levels of glufosinate resistance were observed in the progeny of 9 (20%) of 45 tested maternal plants in 2004, 6 (16%) of 37 tested maternal plants in 2005, and 23 (49%) of 47 tested maternal plants in 2006. These low levels of resistant progeny are consistent with reported rates of plant-to-plant outcrossing in canola, which average approximately 20% for closely spaced plants (Cuthbert and McVetty 2001; Rakow and Woods 1987). These levels of herbicide

resistance are thus likely the result of intraspecific PMGF, occurring either between escaped plants and adjacent crops, or among escaped canola plants. Escaped canola populations containing herbicide resistance traits such as those examined here are ubiquitous in canola-growing regions of western Canada (Yoshimura et al. 2006), and may thereby serve as pollen sources or pollen sinks, contributing to the spread of herbicide resistance traits across the landscape and complicating volunteer control.

Imidazolinone Resistance. Imidazolinone resistance was less frequent among escaped canola populations in this study than glyphosate and glufosinate resistance. In 2004, imidazolinone resistance was observed in four of the six tested populations and in progeny from 12 (29%) of 42 tested maternal plants (Table 3). In 2005, imidazolinone resistance was not observed in any of the tested populations; in 2006, imidazolinone resistance was observed in only one of the five tested populations, and in progeny from 4 (9%) of 46 tested maternal plants. Progeny exhibiting full resistance (as visually assessed) were rare, occurring only from three maternal plants (7%) in 2004 and two maternal plants (4%) in 2006. Most imidazolinone-resistant plants produced progeny exhibiting only partial resistance to the herbicide, and therefore probably were not homozygous for the two genes conferring the commercial resistance trait (Tan et al. 2005). Plants with partial imidazolinone resistance are likely the result of outcrossing events giving rise to heterozygous plants, containing some combination of the four resistance alleles. Also, there would be further segregation of these alleles with each subsequent generation of escaped canola plants. The low observed frequency of imidazolinone resistance in escaped canola populations reflects the lower hectarage planted to imidazolinone-resistant canola varieties in Manitoba. In 2004, only 14% of canola grown in Manitoba was imidazolinone resistant and in 2005 this proportion had further dropped to 9% (Manitoba Agricultural Services Corporation 2006a). These patterns contrast with those of the more prevalent glyphosate and glufosinate traits, and again suggest that the prevalence of traits in escaped canola

populations reflects the presence of the same types in surrounding fields.

Multiple Herbicide Resistance. Pollen-mediated gene flow has resulted in the stacking of herbicide resistance traits in canola volunteers in commercial fields (Beckie et al. 2003; Hall et al. 2000), creating management problems for producers. Such trait stacking may also occur in noncrop habitats where plants with differing herbicide resistance traits are present. In this study, one-third of the total tested maternal plants (43 of 129), from 10 populations over 3 yr, produced progeny resistant to both glyphosate and glufosinate at levels consistent with outcrossing rates in canola (i.e., < 30%) (Table 2). Although imidazolinone resistance was relatively rare in the escaped canola populations, low levels of stacked imidazolinone–glyphosate resistance were observed in the progeny of seven maternal plants in 2004 and of two plants in 2006; imidazolinone–glufosinate resistance was observed in the progeny of four plants in 2004 (data not shown). A single plant from the Rhineland1 population in 2004 produced a seedling with resistance to all three herbicides. This triple HR seedling would had to have been produced through two distinct outcrossing events (Ellstrand 2001), with one of those events involving at least one double HR parent plant. Such outcrossing events may occur either within commercial agricultural fields or during the production of pedigreed seed (Beckie et al. 2003; Hall et al. 2000), or within escaped canola populations containing multiple resistance traits. In this study, conditions necessary for transgene stacking existed within escaped canola populations, as the majority of populations tested contained multiple herbicide resistance traits, and three populations in 2004 and one in 2006 contained all three herbicide resistance traits (Tables 2 and 3). Indeed, all plants producing double or triple HR offspring were detected within populations containing multiple resistance traits. Correspondingly, none of the plants from populations containing only one herbicide resistance trait produced multiple HR progeny (Table 2). Rates of outcrossing are reported to increase with increasing proximity of canola plants (Cuthbert and McVetty 2001), indicating that adjacent plants within escaped populations would serve as the most likely source of gene flow to create multiple HR progeny.

Whereas low levels of double HR progeny likely arose through outcrossing events in the immediate preceding (maternal) generation, the higher proportions of multiple resistance (i.e., 60 to 99%) observed are well above those expected for single-season outcrossing events and are unlikely to be produced through PMGF within one generation in canola. In 2004, two escaped plants produced high levels of double HR offspring, one producing 65% glyphosate–glufosinate-resistant progeny (Table 2) and the other producing 68% glyphosate–imidazolinone-resistant offspring (data not shown). These levels of double HR progeny are close to those expected for multiple HR maternal plants hemizygous/heterozygous for two herbicide resistance traits, which would produce approximately 56% (9 : 7) double HR progeny according to Mendelian segregation ratios for self-fertilized maternal plants heterozygous for two independent nuclear dominant genes (Rédei 1982). Pedigreed HR canola seedlots containing adventitious herbicide resistance traits (Friesen et al. 2003) may serve as a source of seed producing multiple HR plants, which can be introduced into noncrop habitats by agricultural activities. Alternatively, given the apparent frequency of outcrossing events occurring among canola plants in escaped populations in this study, these putative double HR maternal plants might be the progeny of escaped plants from the previous year.

Outcrossing within Escaped Canola Populations. To confirm the production of multiple HR progeny through outcrossing events within escaped canola populations, both the leaf tissue and the progeny of certain escaped plants in two populations were tested for glyphosate and glufosinate resistance. A comparison of the herbicide resistance traits of maternal plants with those of their progeny allows identification of outcrossing events within escaped canola populations or with adjacent canola crops. Without such testing, it is impossible to conclusively determine if the multiple HR progeny of escaped canola plants are the result of outcrossing events or introduction of multiple HR seed into roadside habitats (Aono et al. 2006). However, this method may underestimate actual outcrossing rates, as gene flow between canola plants with the same herbicide resistance traits will not be detected. The objective of this study was to detect the occurrence of outcrossing events in escaped canola populations, rather than to precisely quantify the rate at which this occurs. Additionally, given that levels of outcrossing are expected to be low, and that seed and space limitations precluded the testing of larger numbers of progeny from each maternal plant, outcrossing events were not always similarly detected among the progeny of the same maternal plants in the two herbicide trials. However, the use of two parallel glyphosate/glufosinate herbicide trials doubled the sample size used to detect double HR progeny.

In 2005, among the 21 tested maternal canola plants from the escaped MacDonald2 population, six plants were identified as glyphosate resistant, 13 plants as glufosinate resistant, and only two plants as neither glyphosate nor glufosinate resistant. In 2004, fields adjacent to the MacDonald2 population were seeded to glufosinate-resistant canola, and seed losses during seeding or harvest may have contributed to the escaped population in 2005. Additionally, producers that were interviewed identified the adjacent road as having high agricultural traffic intensity, which may also have served as a source of seed for the escaped population. In September 2005, 13 tested maternal plants were harvested from the MacDonald2 population for herbicide screening of the progeny. Maternal phenotypes inferred from herbicide trial results were consistent with test strip results, though the production of a small proportion of susceptible offspring by certain HR maternal plants (Y_4 and $U_{1, 2, 4, 6}$; Figures 1A and 1C) indicates that these plants were hemizygous for the glyphosate and glufosinate resistance traits. Low levels of double resistance were identified among the progeny of two maternal plants (Y_3 and U_7) that expressed only single resistance traits in their leaf tissue (Figures 1A and 1C), confirming that double HR progeny in the escaped population resulted from outcrossing events. As fields adjacent to the MacDonald2 population were seeded to wheat (*Triticum aestivum* L.) and oats (*Avena sativa* L.) in 2005, the most likely source of pollen contributing to outcrossing events was canola plants within the escaped population itself, which contained both glyphosate- and glufosinate-resistant plants.
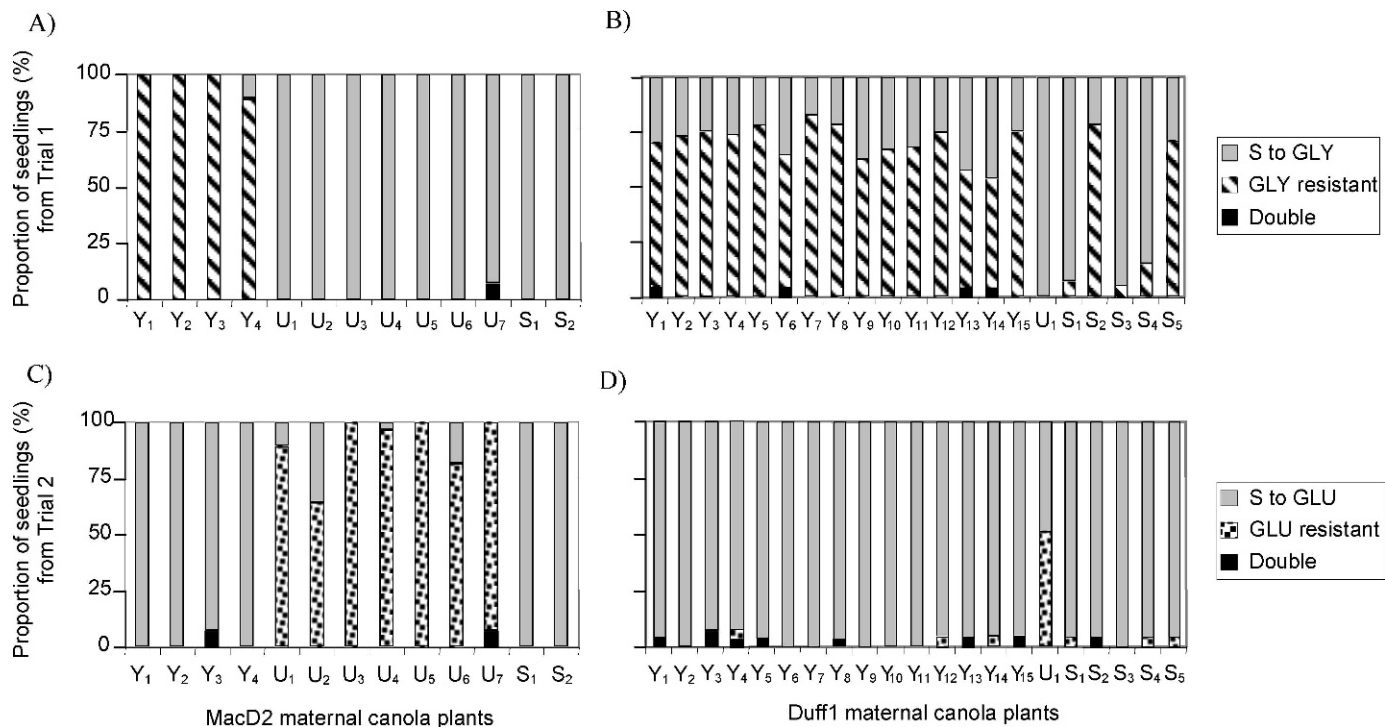
Figure 1. Proportions of herbicide-resistant progeny from 13 escaped canola plants collected from the MacDonald2 population in 2005 (A, C) and from 21 escaped canola plants collected from the Dufferin1 population in 2006 (B, D). Seedlings (progeny) were sprayed with glyphosate and then glufosinate in trial 1 (A, B), and sprayed with glufosinate and then glyphosate in trial 2 (C, D). Proportions of tested seedlings (20 to 28 seedlings per maternal plant in each trial) exhibiting single and double resistance to glyphosate and glufosinate are depicted by the different shades in each stacked bar. Phenotypes of maternal canola plants were determined using test strips. *Abbreviations: S, susceptible; GLY, glyphosate; GLU, glufosinate; Y, glyphosate–resistant; U, glufosinate–resistant.

Outcrossing events were much more frequent in the Dufferin1 escaped population in 2006, introducing adventitious resistance traits into the progeny of single HR and susceptible maternal plants. Among the 27 tested maternal plants, 17 were identified as glyphosate resistant, two as glufosinate resistant, and eight maternal plants as neither glyphosate nor glufosinate resistant. In 2005, the field adjacent to this escaped population was seeded to glyphosate-resistant canola, and seeding or harvest activities may have contributed seed to the escaped population. As before, producers also indicated that agricultural traffic intensity was high on the adjacent road, and this may also have served as a seed source. In September 2006, 21 tested maternal plants were harvested from the Dufferin1 population for herbicide screening of the progeny. Again, maternal phenotypes inferred from herbicide trials were consistent with test strip results. However, all HR maternal plants from the Dufferin1 population produced some susceptible offspring (Figures 1B and 1D), indicating that these plants were hemizygous for the herbicide resistance traits. Progeny from 15 of the tested plants expressed herbicide resistance traits acquired through outcrossing events. These included 10 of the 15 glyphosate-resistant maternal plants ($Y_{1, 3-6, 8, 12-15}$) that produced double HR or glufosinate-resistant (or both) progeny, and five susceptible plants (S_{1-5}) that produced glyphosate-resistant progeny (Figures 1B and 1D). Surprisingly, two susceptible maternal plants ($S_{2, 5}$) produced high levels (i.e., 71 to 79%) of glyphosate-resistant progeny, well above the average rate of outcrossing in canola (Figure 1B). In 2006, fields adjacent to the Dufferin1 population were seeded to corn (*Zea mays* L.) and glufosinate-resistant canola, and would not have provided a source of canola pollen containing the glyphosate resistance

trait. The large number of glyphosate-resistant plants present within the escaped population itself likely provided a considerable pollen source, and the proximity of escaped canola plants may have contributed to high levels of outcrossing. Similarly, very high individual plant outcrossing rates (i.e., 82%) have been reported for closely spaced (i.e., 10 cm) canola plants in field plots (Cuthbert and McVetty 2001). That outcrossing can also occur at such high levels in escaped canola populations suggests that these populations may contribute considerably to the spread and stacking of herbicide resistance traits in agricultural landscapes. Because of the widespread planting of hybrid canola varieties (Manitoba Agricultural Services Corporation 2006b), there may also be male sterility traits present in escaped canola populations; however, this was not assessed in this study.

The progeny of four susceptible maternal canola plants ($S_{1, 2, 4, 5}$) acquired glufosinate resistance traits also through outcrossing events, and one maternal plant (S_2) produced a double HR seedling (Figure 1 D), which necessarily would have arisen through PMGF from a double HR paternal plant. Movement of multiple herbicide resistance traits among escaped canola plants or between escaped populations and neighboring canola crops has important implications for farmers. Adventitious herbicide resistance traits may potentially contaminate canola crops of other varieties, complicating volunteer control (Friesen et al. 2003), compromising purity standards (Smyth et al. 2002), and putting producers at legal risk (Mauro and McLachlan 2008).

Implications of this Study. Escaped canola populations are ubiquitous in agricultural landscapes in western Canada. Although transient in nature, these populations appear to

receive substantial seed- and pollen-mediated gene flow from neighboring fields and other agricultural sources, which contributes to their persistence. Localized escaped populations may also facilitate gene flow between separate agricultural fields, and, indeed, may comprise larger metapopulations that persist at higher scales of organization (A. Knispel, unpublished data). The results of our study confirm that herbicide resistance traits are common in escaped canola populations in agricultural regions where these resistant varieties are widely grown. Additionally, intraspecific gene flow occurs frequently and at high levels in escaped canola populations, resulting in the development of multiple HR progeny within these noncrop habitats. The rapid spread and stacking of herbicide resistance genes in escaped populations may increase chances of contamination of adjacent fields and may compromise agricultural production systems, especially non-GM, zero-till, and organic systems. The presence and movement of herbicide resistance traits in noncrop environments confirms the difficulty of retracting novel plant traits once approved for unconfined release (Marvier and Van Acker 2005), and has important implications for the contamination of non-GM and organic crops (Friesen et al. 2003; Mauro and McLachlan 2008; Smyth et al. 2002). Given the widespread cultivation of GM-HR canola in western Canada, and the common presence of escaped HR plants in roadside habitats, satisfactory isolation distances for non-HR crops are increasingly difficult to maintain (Beckie et al. 2006). This study also provides insight into the potentially detrimental spread and persistence of future novel plant traits once approved for unconfined release, and has important implications for the introduction of even more controversial pharmaceutical and industrial traits into crop plants (Ellstrand 2001). Such traits represent substantial potential liabilities for producers (Smyth et al. 2002), which would be compounded by dispersal, persistence, and outcrossing in noncrop habitats and with neighboring fields. Given the current concern over GM trait confinement (Demeke et al. 2006), the observed prevalence of escaped canola populations with herbicide resistance traits and the frequency of outcrossing events within these escaped populations warrants increased caution when regulating and releasing other GM plant traits in the future.

Sources of Materials

[1] TraitChek™ test strips, Strategic Diagnostics Inc., 111 Pencader Drive, Newark, Delaware 19702.

Acknowledgments

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