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Acetolactate Synthase (ALS) Inhibitor-Resistant Wild Buckwheat (*Polygonum convolvulus*) in Alberta

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Wild buckwheat is the most abundant broadleaf weed across the Prairie region of western Canada. Acetolactate synthase (ALS)-inhibiting herbicides are commonly used to control this species and other broadleaf weeds in cereal crops. A field survey in Alberta in 2007 identified a single population that was putatively resistant to ALS-inhibiting herbicides. In herbicide resistance screening in the greenhouse, all F_1 progeny tested were resistant to the ALS-inhibiting herbicides thifensulfuron/tribenuron, a sulfonylurea herbicide, or florasulam, a triazolopyrimidine herbicide; dose response of shoot biomass indicated the population was 10- and 20-fold less sensitive to thifensulfuron/tribenuron and florasulam, respectively, than a susceptible control population. *ALS* gene sequencing of 24 F_1 progeny indicated that the $Trp_{574}Leu$ target-site mutation was responsible for conferring ALS-inhibitor resistance in this biotype, the first global report of ALS-inhibitor resistance for this species. Because this mutation typically endows high-level resistance across all five ALS-inhibitor classes, this wild buckwheat biotype may only be controlled by a different site-of-action herbicide.
Nomenclature: Florasulam; thifensulfuron; tribenuron; wild buckwheat, *Polygonum convolvulus* L. POLCO.
Key words: *ALS* gene sequencing, ALS-inhibitor resistance, herbicide resistance, target-site mutation.

Polygonum convolvulus es la maleza de hoja ancha más abundante a lo largo de la región Pradera del occidente de Canadá. Los herbicidas inhibidores de la Acetolactate synthase (ALS) son comúnmente usados para controlar esta especie y otras malezas de hoja ancha en el cultivo de cereales. Una investigación de campo realizada en Alberta en 2007, identificó una sola población que era putativamente resistente a los herbicidas inhibidores ALS. En la evaluación de resistencia a los herbicidas en el invernadero, toda la progenie F_1 evaluada fue resistente a los herbicidas inhibidores ALS thifensulfuron/tribenuron, un herbicida sulfonylurea, o florasulam, un herbicida triazolopyrimidine. La respuesta a dosis de la biomasa de la parte aérea de las plantas, indicó que la población fue 10 y 20 veces menos sensible a thifensulfuron/tribenuron y florasulam, respectivamente, que una población susceptible testigo. La secuencia genética ALS de 24 descendientes F_1 indicó que la mutación del sitio de acción -$Trp_{574}Leu$ fue responsable de conferir la resistencia al inhibidor ALS para este biotipo, siendo este el primer reporte global de resistencia al inhibidor ALS para esta especie. Ya que esta mutación, típicamente confiere un nivel alto de resistencia entre todas las cinco clases de inhibidores ALS, este biotipo de *P. convolvulus* podría ser controlado únicamente por un herbicida con un sitio de acción diferente.

Wild buckwheat, a member of the Polygonaceae, is a common nonnative, self-compatible annual weed occurring throughout much of Canada (Hume et al. 1983). In the Canadian Prairies of western Canada (provinces of Alberta, Saskatchewan, and Manitoba), wild buckwheat is the most abundant broadleaf weed, and the third most abundant species overall (Leeson et al. 2005). However, in Alberta, wild buckwheat is the most abundant weed (Leeson and Neeser 2011). Since the 1970s, there has been little change in its relative abundance ranking amongst weed species based on field surveys conducted after POST herbicide application (Leeson et al. 2005). The consistently high abundance of wild buckwheat during the past 40 yr may have been aided by its relatively long soil seed bank persistence, ranging from 6 to 10 yr (Van Acker 2009).

Wild buckwheat can cause significant crop yield loss (Friesen and Shebeski 1960; Hume et al. 1983). Moreover, the vines entangle crop plants; consequently, harvesting operations can be hampered by vines becoming entangled in the cutter bar, threshing drum, and cleaning and conveying mechanisms (Forsberg and Best 1964; Neururer 1961). The acetolactate synthase (ALS)-inhibiting herbicides thifensulfuron/tribenuron or florasulam are commonly applied in cereal crops to control wild buckwheat and other broadleaf weeds (Leeson et al. 2006).

A random survey of 300 fields was conducted in Alberta in 2007 (Beckie et al. 2009). Of wild buckwheat populations sampled in 42 fields from across the province, preliminary herbicide resistance screening results indicated that a single population was putatively resistant to thifensulfuron/tribenuron and florasulam. Resistance to ALS inhibitors in this species had not been previously reported in Canada or elsewhere. Herein, we describe the ALS-inhibiting herbicide resistance screening and dose–response results, as well as the molecular basis for resistance in this wild buckwheat population.

Materials and Methods

Herbicide-Resistant Wild Buckwheat Population. The wild buckwheat population was sampled during a survey of 300 randomly selected fields throughout Alberta in 2007 (Beckie et al. 2009). The population (9091) was uniformly abundant throughout a 64-ha spring wheat (*Triticum aestivum* L.) field located in central Alberta (Figure 1). In the fall of 2007, over

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1,000 seeds were collected as a bulk sample from over 100 randomly selected plants throughout the field. In herbicide screening and dose–response experiments, population 2168 (Figure 1) served as the herbicide-susceptible (HS) control.

Herbicide Screening. In the spring of 2009 at Saskatoon, Saskatchewan, 100 seedlings (F_1 progeny) of the putative herbicide-resistant (HR) population were screened in the greenhouse for resistance to the sulfonylurea herbicide thifensulfuron/tribenuron and the triazolopyrimidine herbicide florasulam. Seeds were planted in 52 by 26 by 5 cm flats containing a potting mixture of soil, peat, vermiculite, and sand (3 : 2 : 2 : 2 by volume) plus a controlled-release fertilizer (15–9–12, 150 g 75 L^{-1}; Scotts Osmocote PLUS, Scotts-Sierra Horticultural Products Co., Mississauga, ON, Canada). Experiments were conducted in a greenhouse at 20/17 C day/night temperatures with a 16-h photoperiod, supplemented with 230 μmol m^{-2} s^{-1} illumination. Seedlings were sprayed at the two- to four-leaf stage with the ALS-inhibiting herbicides florasulam (Frontline, Dow AgroSciences, Calgary, AB, Canada) at 5 g ai ha^{-1}, or thifensulfuron/tribenuron (Refine Extra, E. I. duPont, Mississauga, ON, Canada) at 15 g ai ha^{-1} (thifensulfuron at 10 g ai ha^{-1} and tribenuron at 5 g ai ha^{-1}) plus a nonionic surfactant (Agral 90®, Norac Concepts Inc., Ottawa, ON, Canada) at 0.2% v/v. The herbicides were applied using a moving-nozzle cabinet sprayer equipped with a flat-fan nozzle tip (TeeJet 8002VS, Spraying Systems Co., Wheaton, IL) calibrated to deliver 200 L ha^{-1} of spray solution at 275 kPa in a single pass over the foliage. Plant response to herbicide application was visually scored after 2 wk as HS (0 = dead; 1 = nearly dead) or HR (2 = some injury but new growth; 3 = no injury). Although the scoring system is likely related to dose, rating levels were distinct when visually evaluating plants in the greenhouse.

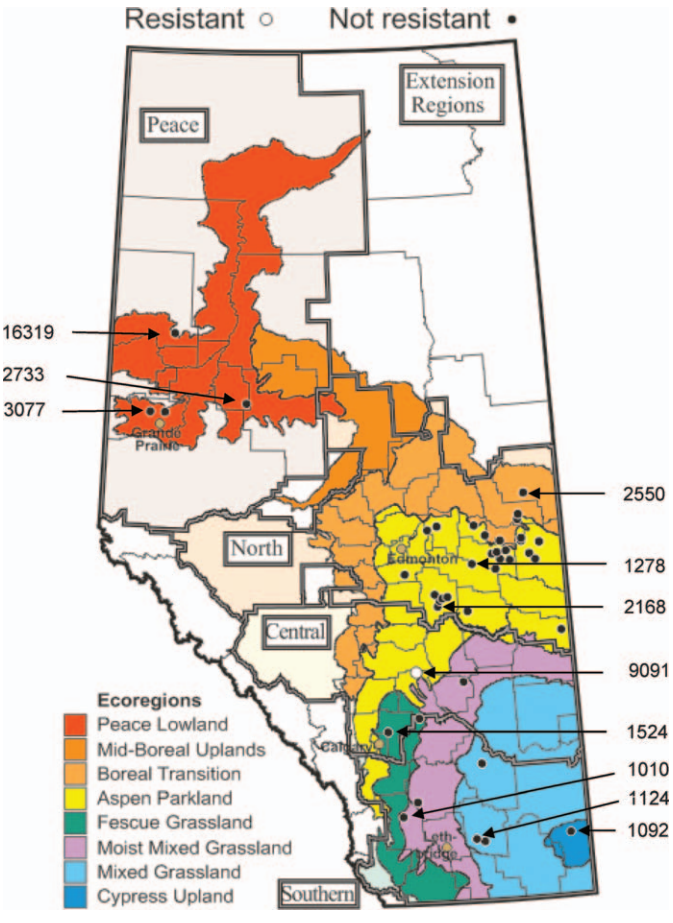

Figure 1. Location of an ALS inhibitor-resistant (9091) and 10 ALS inhibitor-susceptible wild buckwheat populations from a field survey in 2007 in Alberta, Canada (adapted from Beckie et al. 2009).

Following assessment of herbicide resistance, leaf tissue was sampled from 24 herbicide-treated F_1 progeny and samples were freeze-dried. In addition, leaf tissue was sampled from two individuals from each of 10 herbicide-untreated ALS inhibitor-susceptible wild buckwheat populations that originated from across Alberta (Figure 1). These populations had previously been confirmed as susceptible to ALS inhibitors in the 2007 field survey (Beckie et al. 2009).

Herbicide Dose–Response Experiments. The herbicide dose–response experiments involving the putative HR and HS control population were conducted in the greenhouse in the spring of 2011 at Saskatoon and repeated once. The experiments were arranged in a completely randomized design with four replications (one pot per replicate) per treatment. The dose response of the populations to each of the two herbicides (florasulam, thifensulfuron/tribenuron) were separate experiments.

Four seeds were planted 1 cm deep in 10-cm square pots containing a mixture of soil, peat, vermiculite, and sand (3 : 2 : 2 : 2 by volume) plus a controlled-release fertilizer (15–9–12; 150 g 75 L^{-1}). Greenhouse conditions were as described previously. Pots were watered daily to field capacity.

Seedlings (F_1 progeny) were treated at the two- to four-leaf stage with florasulam or thifensulfuron/tribenuron plus a nonionic surfactant at 0.2% v/v. Herbicide application methodology was described previously. Florasulam was applied at six doses (0.0625 to four times the recommended dose) and thifensulfuron/tribenuron at five doses (0.0625 to two times the recommended dose). A nontreated control was included. Two weeks after treatment, shoots were cut at soil level. Harvested biomass was dried at 60 C for 3 d, and weighed.

Dose-Response Data Analysis. Results of each experiment were combined across runs upon confirmation of homogeneity of variances (Steel and Torrie 1980). The two herbicide experiments were analyzed separately. Aboveground biomass (Y, percentage of nontreated control) was regressed against herbicide dose (x, g ha^{-1}) using the double exponential-decay model:

$$Y = ae^{-bx} + ce^{-dx} \quad [1]$$

where $a + c$ is the intercept (% of nontreated control) and b and d quantify the slope (Yoshimura et al. 2006). Data were fitted to the model using a derivative-free nonlinear regression procedure, provided with PROC NLIN (SAS 1999). Regression analyses were performed on treatment means averaged over replications as recommended by Gomez and Gomez (1984). The resistance factor or index was calculated

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Table 1. Primers for amplifying and sequencing 630 bp (210 amino acids) of the *ALS* gene.

Primer	5′–3′ sequence	Region of homology[a]
ALSR5 (Diebold et al. 2003)	CAG (A/G)TA (A/T)GG TCC TGG (A/G)GT ATC C	1874–1896
RuTh-F-2 (Warwick et al. 2010)	GAA GAA TAA GCA ACC CCA TGT GTC	1194–1217

[a] Amino acids and nucleotide positions are numbered based on the amino acid sequence of *ALS* from *Arabidopsis* (Sathasivan et al. 1990).

as GR_{50} of the HR population divided by GR_{50} of the HS population, where GR_{50} is the dose resulting in a 50% reduction in aboveground biomass relative to the nontreated control.

***ALS* Gene Sequencing.** DNA was extracted from freeze-dried leaf tissue (10 to 20 mg) using Fast DNA SPIN kit (QBioGen, MP Biomedicals, Solon, OH) following the manufacturer's instructions. Two primers (Table 1) were utilized to amplify and sequence 630 bp (210 amino acids) of the *ALS* gene. These included a primer used to sequence the *ALS* gene of *Amaranthus* (Diebold et al. 2003) and that of Russian thistle (*Salsola tragus* L.) (Warwick et al. 2010); all other primers from Warwick et al. (2008, 2010) did not amplify the *ALS* gene of wild buckwheat. Polymerase chain reaction (PCR) amplifications were performed using Ready-To-Go (GE Healthcare UK Limited, Little Chalfont, Buckinghamshire, UK) PCR beads with approximately 50 ng of genomic DNA and 400 nM of each primer in a total of 25 µL. PCR was performed in a Mastercycler epGradient under the following conditions: 2 min incubation at 94 C; 40 cycles of 30 s at 94 C, 45 s at 60 C, and 1.5 min at 72 C; then 5 min at 72 C. PCR fragments were excised from a 1.2% agarose gel and purified using QIAquick Gel Extraction kit (QIAGEN Sciences, Germantown, MD) and sequenced directly. Sequencing reactions used 50 ng of purified PCR product, 160 nM of primer, and 0.5 µL of ABI BigDye™ Terminator reagent v. 3.1 (PE Corporation, PE Biosystems, Foster City, CA) in a final volume of 10 µL, and were run on a ABI 3130 DNA Sequencer (Applied Biosystems, Foster City, CA). Primers used for sequencing were the same as those used for PCR amplification. Sequences were determined and compared using Sequencher software v 4.7 (Gene Codes Corporation, Ann Arbor, MI). Polymorphism or nucleotide heterozygosity was based on the appearance of two peaks at a single nucleotide position on the chromatograms. Amino acids and nucleotide positions were numbered based on the amino acid sequence of *ALS* from mouseear cress [*Arabidopsis thaliana* (L.) Heynh.] (Sathasivan et al. 1990).

To study the genetic segregation of the "heterozygous" genotype, tissue samples were collected, freeze-dried, and genotyped from 35 herbicide-untreated F_2 progeny derived from 10 selfed F_1 plants (HR population 9091) that survived herbicide treatment. Greenhouse conditions for plant growth were similar to those described previously.

Results and Discussion

Herbicide Screening. The wild buckwheat population was homogeneous HR. All seedlings screened with florasulam or thifensulfuron/tribenuron were rated as HR (level 2). The latter herbicide had been applied POST in the wheat field in 2007, the year the survey was conducted. This herbicide application, as well as previous treatments of ALS inhibitors in the field, apparently resulted in the selection of a homogeneous HR population.

Herbicide Dose Response. For the florasulam experiment, the GR_{50} values for the putative HR and HS population were 19.9 and 1.0 g ai ha^{-1}, respectively (Figure 2A). Therefore, the HR population was nearly 20 times less sensitive to florasulam than the HS population. For the thifensulfuron/tribenuron experiment, the GR_{50} values for the HR and HS population were 13.3 and 1.3 g ai ha^{-1}, respectively; the

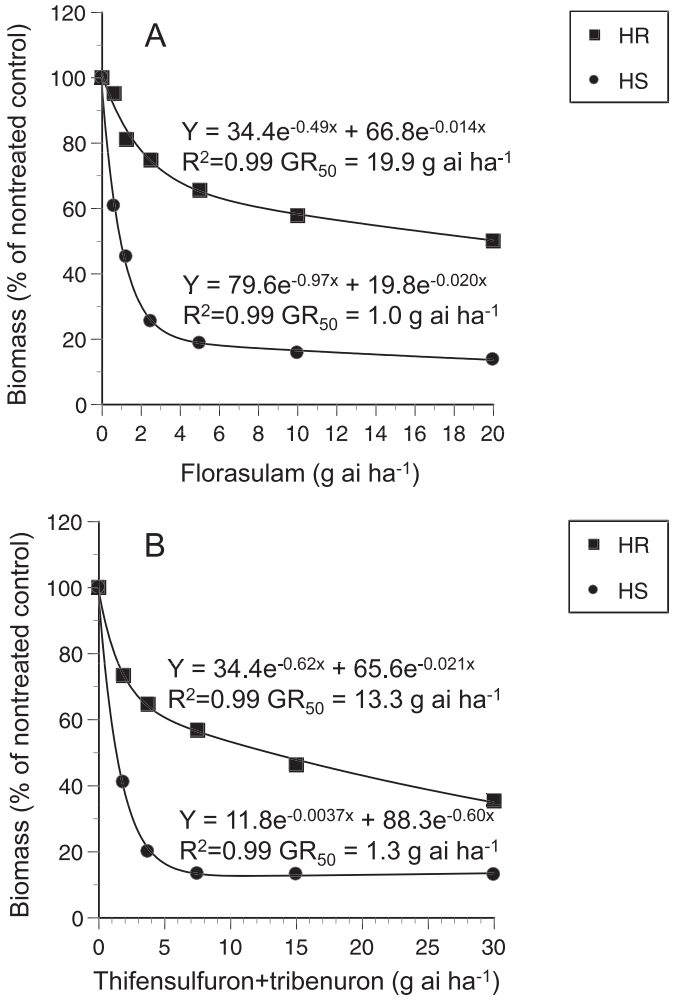

Figure 2. Shoot biomass response of an ALS inhibitor-resistant (HR) wild buckwheat population (9091) to increasing dose of florasulam (A) or thifensulfuron/tribenuron (B) (HS = herbicide-susceptible control). The dose–response equation is described in the text.

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Table 2. Target-site mutations in the *ALS* gene resulting in amino acid changes with Trp/Leu574 mutation conferring herbicide resistance (HR) in a wild buckwheat biotype from Alberta, Canada (HS = herbicide susceptible).[a]

		Arabidopsis amino acid number				
Population-Individual	Resistance phenotype	439	Trp 574	592	595	611
1010S-1, 2	HS	Pro/Ala	Trp	Leu/Ser	Ser/Ala	Pro
1092S-1, 2	HS	Pro/Ala	Trp	Leu/Ser	Ser/Ala	Pro
1124S-1, 2	HS	Pro/Ala	Trp	Leu/Ser	Ser/Ala	Pro
1278N-1, 2	HS	Pro/Ala	Trp	Leu/Ser	Ser/Ala	Pro
1524C-1, 2	HS	Pro/Ala	Trp	Leu/Ser	Ser/Ala	Pro
2168N-1, 2	HS	Pro/Ala	Trp	Leu/Ser	Ser/Ala	Pro
2550N-1, 2	HS	Pro/Ala	Trp	Leu/Ser	Ser/Ala	Pro
2733P-1, 2	HS	Pro/Ala	Trp	Leu/Ser	Ser/Ala	Pro
3077P-1, 2	HS	Pro/Ala	Trp	Leu/Ser	Ser/Ala	Pro
16319P-1, 2	HS	Pro/Ala	Trp	Leu/Ser	Ser/Ala	Pro
9091C-1 to -4, 7 to 17, 19-24	HR	Pro/Ala	**Trp/Leu**	Leu/Ser	Ser/Ala	Pro
9091C-5, -6, -18	HR	Pro/Ala	**Trp/Leu**	Leu/Ser	Ser/Ala	Pro/Leu
Heterozygote bases causing amino acid changes		(C/G)CT	T(G/T)G	T(T/C)A	(T/G)CT	C(C/T)T
		Pro/Ala	Trp/Leu	Leu/Ser	Ser/Ala	Pro/Leu

[a] Bolding denotes target-site mutation. GenBank accession numbers for the following nucleotide sequences: HR-9091, JF826439; HR-9091-5, JF826440; HS-1010, JF826441.

resultant resistance factor or index was 10.2 (Figure 2B). Therefore, the HR population was highly resistant (i.e., resistance factor $\geq$ 10) to both herbicides.

***ALS* Gene Sequencing.** A total of 630 bp (210 amino acids) of the *ALS* gene were sequenced (corresponding to *Arabidopsis* amino acids 407 to 616). This included the region containing the Trp_{574} amino acid site that has previously been associated with *ALS* resistance in weeds, but did not cover target-mutation sites Ala_{122}, Pro_{197}, Ala_{205}, Asp_{376}, Ser_{653}, or Gly_{654} that are also reported to confer *ALS* resistance (Beckie and Tardif 2011; Tranel et al. 2011). Eleven nucleotide positions exhibited heterozygous mutations; these were found to be common to all samples and neutral (i.e., did not create any amino acid change). Five heterozygous nucleotide positions resulted in amino acid substitutions and the possibility of two different amino acids being coded at positions 439 (Pro/Ala), 574 (Trp/Leu), 592 (Leu/Ser), 595 (Ser/Ala), and 611 (Pro/Leu) (Table 2). However, only one of these, Trp/Leu_{574}, has previously been associated with ALS-inhibitor resistance. All 24 HR wild buckwheat plants sequenced were identified as T(G/T)G-heterozygous for Trp_{574}, coding for either Trp or Leu. All 20 wild buckwheat HS controls were homozygous (TGG), coding for Trp at position 574. Heterozygous nucleotides at amino acid position 611 caused a Pro/Leu amino acid mutation in only three of the HR wild buckwheat samples, whereas all other samples coded for Pro at this position.

Heterozygosity of Trp/Leu at position 574 appears to be fixed since all 35 progeny derived from 10 selfed 9091 HR plants were found to have a heterozygous genotype. Segregation of molecular types, i.e., Trp_{574}, Trp/Leu_{574}, and Leu_{574}, as would be expected at a single locus was not observed. Such lack of segregation for herbicide resistance in progeny is consistent with the tetraploid genome structure of wild buckwheat (Hume et al. 1983) and the likely occurrence of two copies of the *ALS* gene. The presence of more than one *ALS* gene confounds the ability of the molecular techniques to accurately identify "true" heterozygotes in this study.

In summary, the Trp/Leu_{574} mutation was found in all 24 individuals (F_1 progeny derived from field-collected seed) of the parental wild buckwheat population, confirming the first global case of ALS-inhibitor resistance in this species. The presence of this mutation is consistent with the high-level resistance of the population to florasulam, a triazolopyrimidine class ALS inhibitor, and thifensulfuron/tribenuron, a sulfonylurea ALS inhibitor. This mutation is apparently widespread among HR populations of a number of broadleaf weeds, including kochia [*Kochia scoparia* (L.) Shrad.] (Warwick et al. 2008) and Russian thistle (Warwick et al. 2010). Because this mutation typically confers broad cross-resistance across the five ALS-inhibitor herbicide classes (Beckie and Tardif 2011), a different site-of-action herbicide is likely required to control this HR wild buckwheat population.

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