Theor Appl Genet (1992) 84:480–486

THEORETICAL AND APPLIED GENETICS

Characterization of transgenic sulfonylurea-resistant flax (*Linum usitatissimum*)

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Received August 12, 1991; Accepted November 29, 1991
Communicated by J. W. Snape

Summary. Fourteen transgenic flax (*Linum usitatissimum*) lines, carrying a mutant *Arabidopsis* acetolactate synthase (ALS) gene selected for resistance to chlorsulfuron, were characterized for resistance to two sulfonylurea herbicides. Progeny of 10 of the 14 lines segregated in a ratio of 3 resistant to 1 susceptible, indicating a single insertion. Progeny of 1 line segregated in a 15:1 ratio, indicating two insertions of the ALS gene at independent loci. Progeny from 3 lines did not segregate in a Mendelian fashion and were likely the products of chimeric shoots. Resistance to chlorsulfuron was stably inherited in all lines. At the enzyme level, the transgenic lines were 2.5 to more than 60 times more resistant to chlorsulfuron than the parental lines. The transgenic lines were 25–260 times more resistant to chlorsulfuron than the parental lines in root growth experiments and demonstrated resistance when grown in soil treated with 20 g ha^{-1} chlorsulfuron. The lines demonstrated less resistance to metsulfuron methyl; in root growth experiments, the transgenic lines were only 1.6–4.8 times more resistant to metsulfuron methyl than the parental lines. Resistance was demonstrated in the field at half (2.25 g ha^{-1}) and full (4.5 g ha^{-1}) rates of metsulfuron methyl.

Key words: Flax – Herbicide resistance – Sulfonylurea herbicides – Chlorsulfuron – Acetolactate synthase

Introduction

Flax (*Linum usitatissimum*) is an oilseed crop grown on the prairies of western Canada and is important for diversity in crop rotations. Since flax is a poor competitor, the use of herbicides for weed control is important for maximum yield production. Most herbicides registered for use in flax were initially developed for other crops, and damage to flax with some of these herbicides is not uncommon (Kneeshaw 1981).

Sulfonylurea herbicides are recommended for broadleaf weed control in wheat and barley. However, some sulfonylurea herbicides are very persistent in soil, and this can severely limit crop rotation flexibility for farmers. Flax and many other dicotyledonous species are sensitive to sulfonylurea residues in the soil, and an interval of up to 4 years may be required before these crops can be sown in sulfonylurea-treated soil (Anonymous 1990). Recently, high-level resistance to sulfonylurea herbicides has been generated by altering acetolactate synthase (ALS), the target site of sulfonylurea herbicides (Chaleff and Ray 1984; Haughn and Somerville 1986). Flax lines were transformed by *Agrobacterium tumefaciens* with a gene encoding a resistant ALS enzyme from *Arabidopsis*, generating 14 different, independently transformed lines (McHughen 1989). The gene for ALS was cloned from *Arabidopsis* and inserted into the plasmid pMON200 to produce the plasmid pGH6, which was then recombined into the disarmed Ti plasmid, pGV3850 (Haughn and Somerville 1986; Haughn et al. 1988). Also present on the plasmid was a gene conferring resistance to kanamycin, neomycin phosphotransferase II (*nptII*), and a nopaline synthase gene.

The objectives of the work reported here were to characterize the resistance of these lines to chlorsulfuron and their cross-resistance to metsulfuron methyl, another popular sulfonylurea herbicide. This was done at the enzyme level and at the whole plant level in root growth experiments, greenhouse tests, and a field test. Resistance in the field was examined to determine if flax was protected adequately from typically encountered residual levels of sulfonylurea herbicides in the soil.

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Materials and methods

Transgenic flax lines

Fourteen different transformed flax lines were evaluated. Eleven of the lines were obtained by transforming the cv 'NorLin', and three by transforming the cv 'McGregor'. In general putative clones of an initial shoot were generated by rooting adventitious shoots. One line within each group of clones was chosen for study, although in several instances clones of a line were also used.

Segregation tests for chlorsulfuron resistance

Seeds from the initial transgenic plants (T1) were tested for resistance to chlorsulfuron in a root growth assay. Approximately 30 seeds from each transgenic line and 10 seeds from the corresponding parental line were surface sterilized with a 2-min wash in 70% ethanol, two 10-min washes in 1.3% sodium hypochlorite, and two rinses in sterilized water. Seeds were germinated in Magenta boxes containing Murashige and Skoog basal medium (MS) (1962) containing 5 n*M* chlorsulfuron. The test period was 7 days: 2 days in a dark cabinet followed by 5 days in continuous light. Segregation ratios (number of resistant seedlings: number of sensitive seedlings) were established on the basis of root length.

Im some instances, cotyledon sections were removed from the seedlings after the test period and plated on MS with 1 mg L^{-1} benzyladenine and 0.02 mg L^{-1} naphthaleneacetic acid plus 400 n*M* chlorsulfuron or 100 mg L^{-1} kanamycin. Sections were scored, positive or negative, for callus formation.

Development of homozygous representatives

Homozygous representatives were obtained by allowing several T2 plants from each line to self-pollinate and then testing the progeny for segregation of chlorsulfuron resistance. In some cases homozygous representatives were not obtained until the T4 generation; in all cases resistance to chlorsulfuron was stably inherited from one generation to the next.

ALS assays

Characterization of the transgenic lines was performed with plants homozygous for the T-DNA insert. The ALS assay protocol used, described by Devine et al. (1991), was a modification of the method described by Chaleff and Mauvais (1984) and Singh et al. (1988). The enzyme was extracted from 2 to 3 g of flax leaf tissue, precipitated by ammonium sulfate fractionation, and desalted on a Pharmacia PD-10 Sephadex column. Fifty microliters of eluted protein was assayed in triplicate at final chlorsulfuron concentrations of 0, 10^{-8}, 10^{-7}, 10^{-6}, and 3×10^{-6} *M*. The ALS data were expressed as percentage of control (no herbicide) samples. I_{50} values (concentration of herbicide required to reduce enzyme activity by 50%) were determined by interpolating from the graph of ALS activity versus chlorsulfuron concentration.

Root growth experiments

The root growth assay described for the segregation tests was used to establish dose-response curves over a range of concentrations of chlorsulfuron and metsulfuron methyl. Twenty seeds from each transgenic line and 10 seeds from the corresponding parental line were surface-sterilized and germinated in Magenta boxes containing MS with 0, 50, 400, 1600, or 3200 n*M* chlorsulfuron or with 0, 12.5, 25, 50, or 100 n*M* metsulfuron methyl. The root length data were expressed as per cent of root length of controls (no herbicide) and plotted against herbicide concentration. GR_{50} values (concentration of herbicide required to reduce root growth to 50% of control plants) were determined by interpolating from graphs of root length versus herbicide concentration.

For these and the ALS experiments, ANOVAs were performed across parental and transgenic lines at each herbicide concentration. Least significant difference (LSD) tests were used to detect differences between parental and transgenic lines, and among transgenic lines.

Greenhouse experiments

Seedlings from lines 12103, 12113, 12115, and 12129 were transferred to untreated soil for 2 weeks, then transferred to chlorsulfuron-treated soil. Chlorsulfuron was sprayed onto soil in plastic trays at a rate equivalent to a field rate of 20 g ha^{-1}. The soil was then mixed and placed in 1-l milk cartons. Plants that survived were grown to maturity. Visual observations on germination, height, and vigour were recorded.

Field experiment

Six homozygous transgenic lines and their two parental lines, 'NorLin' and 'McGregor', were tested at the Kernen Crop Research Farm, Saskatoon, during the summer of 1989. Three rates of metsulfuron methyl were used; 0, 2.25, and 4.5 g ha^{-1}. Each rate was tested in duplicate. Each 2.44×4.88 m plot contained one row of each transgenic line and four rows each of 'NorLin' and 'McGregor'. The rows were spaced 30 cm apart and contained 25–30 seeds. The plots were separated by 1.22 m borders.

Visual observations were made throughout the season. Number of seedlings emerged, days to flower, height at flowering, yield, and the presence of stress morphology were recorded. Orthogonal contrasts were used to compare parental and transgenic lines within each plot.

Results

Segregation tests for chlorsulfuron resistance

After the 7-day test period, root lengths of the transgenic and parental seedlings fell into two distinct groups. Root length in resistant transgenic seedlings ranged from approximately 5 to 7 cm; in sensitive transgenic seedlings, root lengths were similar to those of parental seedlings and ranged from approximately 0 to 2 cm. *t*-tests were used initially to dinstinguish these two groups; segregation ratios were examined using a goodness of fit chi-square test (Table 1). Of the 14 lines, 10 lines appeared to segregate in a 3:1 (resistant:sensitive) pattern, suggesting a single insertion. However, caution should be exercised in interpreting the results for lines 12116 and 12153, which had very low seed numbers; for these lines the segregation ratio can only suggest the pattern of inheritance. Line 12115 appeared to segregate in a 15:1 pattern, suggesting two independent inserts. Line 12129 did not fit any predicted ratio. There was a very low ratio of resistant to sensitive seedlings with lines 12087 and 12113, suggesting that they were chimeric plants containing both transformed and nontransformed cells.

Segregation ratios obtained from cotyledon callus tests on chlorsulfuron or kanamycin corroborated the

Table 1. Germination of segregants on 5 n*M* chlorsulfuron

Line	Segregation of progeny resistant : sensitive	Predicted ratio	Chi-square value
NorLin-derived:			
12087	3:40	–	–
12113	1:29	–	–
12115	24:1	15:1	ns
12116	3:1	3:1	ns
12129	16:16	3:1	**
12140	26:6	3:1	ns
12151	14:7	3:1	ns
12172	8:4	3:1	ns
12174	21:9	3:1	ns
12352	19:9	3:1	ns
12362	20:11	3:1	ns
McGregor-derived:			
12103	19:7	3:1	ns
12153	6:6	3:1	ns
12171	23:7	3:1	ns

–, No predicted ratio; ns, not significant at $P=0.01$; **, significant at $P=0.01$

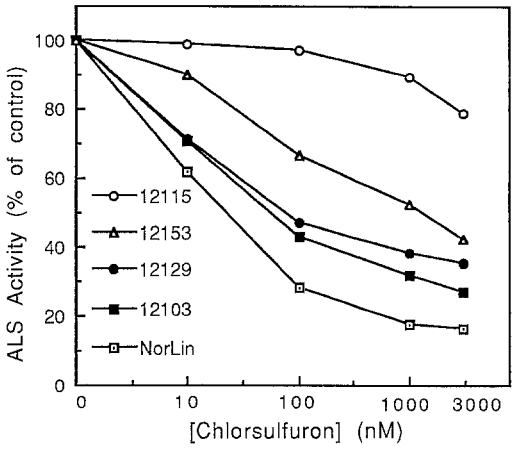


Fig. 1. Inhibition of ALS from 'NorLin' and 4 transgenic lines by chlorsulfuron. Based on LSD values, parental and transgenic lines were divided into five groups. A representative from each group is plotted in this figure

segregation patterns observed in the germination tests (data not shown).

ALS assays

On the basis of the ANOVA results, ALS from the flax lines could be distinguished into five groups, ranging from very sensitive to chlorsulfuron (e.g., 'NorLin', 'McGregor'), to very resistant (e.g., 12115, 12140). A representative of each group is presented in Fig. 1. Lines 12103 (Fig. 1) and 12352 (not shown) were very sensitive at the two highest chlorsulfuron concentrations and were in the second most sensitive group at the lower concentrations. The remaining lines can be sorted into an upper middle and lower middle group for which the boundary is less clear.

ALS I_{50} values were 1260, 78, 55, and 22 n*M* for lines 12153, 12129, 12103, and 'NorLin', respectively. Thus, ALS from the transgenic lines was 2.5 to over 60-fold more resistant to chlorsulfuron than that from the parental lines. An I_{50} value could not be determined for line 12115 because 50% inhibition was not reached over the concentration range of chlorsulfuron used (Fig. 1).

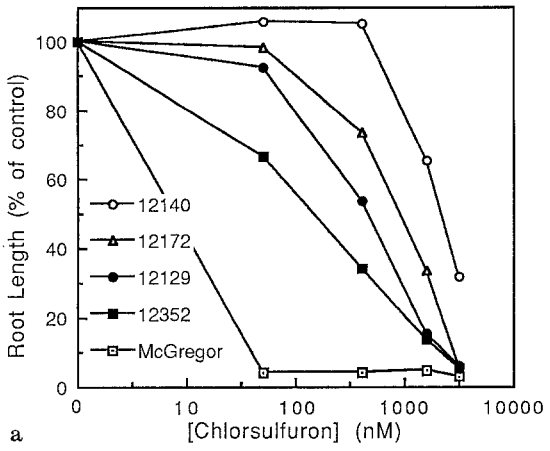
Root growth experiments

After 2 days in the dark, most seeds, especially those at low herbicide concentrations, had germinated and possessed a radicle 1–4 mm long. After 5 days of growth under the light bank, the roots of seedlings growing in agar with no herbicide ranged in length from approximately 5 to 7 cm. Often, seeds from transgenic lines failed to germinate at the highest concentrations of both chlorsulfuron and metsulfuron methyl.

The lines were ranked and divided into five different groups based on the ANOVA results. 'NorLin' and 'McGregor' were very sensitive, and lines 12140 and 12115 highly resistant, to chlorsulfuron and metsulfuron methyl. A representative of each group is presented in Fig. 2a (chlorsulfuron) or 2b (metsulfuron methyl). GR_{50} values from root growth experiments with chlorsulfuron were 2300, 1100, 480, 230, and 9 n*M* for lines 12140, 12172, 12129, 12352, and 'McGregor', respectively. For root growth experiments with metsulfuron methyl, GR_{50} values were 34, 22, 14, 11, and 7 for lines 12115, 12171, 12172, 12129, and 'NorLin', respectively. Thus, the transgenic lines were 25- to 260-fold more resistant to chlorsulfuron than the parental lines and 1.6 to 4.8-fold more resistant to metsulfuron methyl than the parental lines.

Greenhouse experiments

'NorLin' seedlings grown in the chlorsulfuron-treated soil were stunted compared to those grown in untreated soil; they also displayed what is referred to as "stress leaf morphology", where the leaves were clasped to the stem. 'NorLin' seedlings grown in untreated soil did not show this morphology. Seedlings of the transgenic lines that did not demonstrate resistance to chlorsulfuron in the cotyledon callus tests were stunted and displayed stress leaf morphology. Transgenic seedlings that demonstrated resistance in cotyledon callus tests were not stunted in chlorsulfuron-treated soil and displayed no stress morphology. Their growth appeared vigorous, similar to that shown by 'NorLin' seedlings in untreated soil.



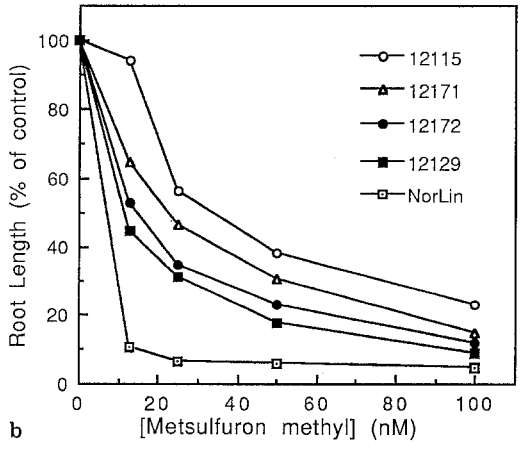


Fig. 2. **a** Dose-response curve of root length versus chlorsulfuron concentration for 'McGregor' and 4 transgenic lines. Seeds were germinated in MS containing a range of concentrations of chlorsulfuron. Based on LSD values, parental and transgenic lines were divided into five groups. A representative from each group is plotted in this figure. **b** Dose-response curve of root length versus metsulfuron methyl concentration for 'NorLin' and 4 transgenic lines. Seeds were germinated in MS containing a range of concentrations of metsulfuron methyl. Based on LSD values, parental and transgenic lines were divided into five groups. A representative from each group is plotted in this figure

Field experiment

Both parental and transgenic lines grew vigorously in the untreated soil. At the full rate of metsulfuron methyl (4.5 g ha^{-1}), and to a lesser extent at the half rate, the parental lines showed severe stunting and stress leaf morphology. Many parental seedlings wilted and died before harvest, whereas most transgenic lines appeared vigorous at all rates of herbicide application. Some 'NorLin' and 'McGregor' plants grew vigorously in the herbicide-treated plots. Seeds from plants in which this occurred were tested in a 5 n*M* chlorsulfuron root growth test, but no resistance was observed, indicating that their vigorous growth was not the result of chlorsulfuron resistance.

Table 2. Comparison of various field characteristics between the parental lines, 'NorLin' and 'McGregor', and transgenic lines 12087, 12103, 12115, 12116, 12140, and 12174. Treatment effects were examined using orthogonal contrasts

Rate (g/ha)	NorLin			McGregor		
	Transgenic lines ($n=5$)	Parental lines ($n=4$)	T/P[a] (%)	Transgenic lines ($n=1$)	Parental lines ($n=4$)	T/P (%)
	Emergence (%)					
0	64.4	43.7	147**	52.0	37.1	140**
2.25	58.0	54.6	106**	54.0	49.5	109**
4.5	56.0	47.9	117**	60.0	30.4	197**
	Time to flower (days)					
0	54.7	55.3	ns	60.0	59.7	ns
2.25	53.7	53.6	ns	60.0	59.7	ns
4.5	54.9	56.4	97**	60.0	59.1	ns
	Seed yield (g/plant)					
0	2.96	3.32	ns	3.84	4.30	ns
2.25	3.16	2.82	ns	3.68	3.05	ns
4.5	3.71	1.61	230**	3.74	1.06	353**
	Height (cm)					
0	52.0	51.4	ns	58.5	57.4	102*
2.25	49.5	33.8	146**	55.2	39.6	139**
4.5	47.6	22.6	211**	48.1	24.9	197**

ns, Not significant; * significant at $P=0.05$; ** significant at $P=0.01$

[a] T/P is the ratio of each characteristic for the transgenic lines to the parental line, expressed as percent

Emergence varied significantly between the replicates of the field trial, and also among lines, with transgenic lines showing greater emergence than their parental counterparts (P = 0.01). However, this variability was unrelated to herbicide treatment (Table 2). The transgenic lines flowered in the same number of days as their parental lines in the untreated plots (Table 2). 'NorLin' and 'NorLin' transgenic lines flowered earlier than 'McGregor' and 'McGregor' transgenic lines, as expected.

Seed yield of the transgenic lines was similar to that of their parental lines in untreated plots and plots treated with 2.25 g ha^{-1} metsulfuron methyl (Table 2). At the full rate of herbicide, the transgenic lines yielded more seed than the parental lines (P = 0.01).

'NorLin' transgenic lines were equal in height to 'NorLin' in untreated plots. The 'McGregor' transgenic line (12103) was taller than its parental line in the untreated plots by an average of 1.1 cm (P = 0.05, Table 2). In the treated plots, the transgenic lines were taller than their parental lines (P = 0.01).

Discussion

Segregation ratios of seed from T1 plants can give an indication of how the inserted T-DNA is inherited and how many inserts are present. In this study, 11 of the 14 transgenic flax lines demonstrated Mendelian inheritance. Genes inserted using *Agrobacterium* are most commonly inherited in a Mendelian fashion. For example, in one study of 124 lines of transgenic *Arabidopsis*, 90% demonstrated Mendelian inheritance of the inserted gene (Feldmann and Marks 1987). Similarly, of 44 lines of transgenic tobacco plants, 40 demonstrated Mendelian inheritance (Budar et al. 1986).

Explanations for the higher than expected number of sensitive seedlings observed for lines 12087, 12113, and 12129 include the following: loss of the T-DNA insert before or during meiosis (Nelson et al. 1988); gene inactivation, for example by methylation (Heberle-Bors et al. 1988); or the formation of chimeric shoots containing both transformed and nontransformed cells (McHughen and Jordan 1989; Olszewski et al. 1988). If shoot organogenesis involves several initials, then chimeric shoots may arise from both transformed and nontransformed cells of the callus mass. If only one initial is involved, there may be inactivation or loss of T-DNA in some cells early in shoot differentiation (McHughen and Jordan 1989). The germ line obtained from these plants may have been derived from any combination of transformed and nontransformed cells, producing non-Mendelian segregation ratios. Given the high number of sensitive seedlings obtained, it is likely that chimeric shoots are the most probable explanation for lines 12087 and 12113.

Line 12129 was probably also chimeric, with approximately half of the germ line derived from nontransformed cells, resulting in half of the seeds testing positive for resistance to chlorsulfuron in the segregation test. An embryo-lethal mutation, in which the T-DNA may have inserted into a gene required for some aspect of embryo or seed development, was eliminated as the cause for the non-Mendelian inheritance pattern observed in line 12129. This condition would be lethal in the homozygous state, resulting in a 2:1 ratio of resistant:sensitive seedlings, with resistant seedlings being heterozygous for the inserted gene (Herberle-Bors et al. 1988). The chi-square test for 2:1 segregation for line 12129 was non-significant ($P = 0.01$); however, progeny of line 12129 were obtained that were homozygous for resistance to chlorsulfuron, thereby eliminating this explanation.

Cotyledon callus tests were useful when the results of segregation tests were inconclusive. Kanamycin resistance in the cotyledon callus tests correlated with resistance to chlorsulfuron. In another study, complete correlation was observed in 26 transgenic *Brassica* plants for kanamycin resistance and phosphinothricin acetyltransferase expression (De Block et al. 1989). In some instances, however, the loss of expression of one gene or another can occur (e.g., McCormick et al. 1986; Spielmann and Simpson 1986). Explanations for these null phenotypes include gene inactivation, gene instability, or transfer of an incomplete copy of the T-DNA. Thus, it cannot be assumed that all genes on a T-DNA insert will be integrated, maintained, and expressed. Southern analysis of 12 of the transgenic lines used in this study confirmed the presence of the T-DNA insert (data not shown), although copy number was not determined. Untransformed plants were used as controls; sensitive segregates were not included in this analysis.

Expression of chlorsulfuron resistance was stably maintained through succeeding generations. The stable inheritance of inserted genes is an important requirement for comercialization of crops. When backcrossed, the meiotic instability of transgenic tobacco plants was 0.06% (Muller et al. 1987). The authors concluded that this level was acceptable for commercialization, since it was not greater than the spontaneous mutation rate. Since flax is a self-pollinating species, the nature of the cross would not contribute to meiotic instability.

'NorLin' and the 'NorLin'-dervied transgenic lines flowered much earlier than 'McGregor' and 'McGregor' transgenic lines (Table 2), reflecting a previously documented difference between the two cultivars (Gubbels et al. 1984). Seed from 'NorLin' and 'McGregor' plants that grew vigorously in the herbicide-treated plots, flowered, and set seed, did not demonstrate chlorsulfuron resistance in laboratory germination tests. Their vigorous growth in herbicide-treated plots in the field was likely the result of uneven herbicide incorporation in the soil.

Comparison of parental and transgenic lines in untreated plots with respect to time to flower, height, and yield suggests that the addition of the new gene had no deleterious effects on the transgenic lines (Table 2). In laboratory, greenhouse, and field testing, no adverse effects on agronomic characteristics have been observed for transgenic tobacco (Cuozzo et al. 1988; Stalker et al. 1988) or transgenic tomato (Nelson et al. 1988). Larger field trials will confirm whether the performance of these transgenic flax lines is equivalent to that of the parental cultivars.

Differences in resistance between the transgenic and parental lines were not as dramatic in the enzyme assays as they were in the root growth experiments. At the whole plant level, the resistant enzyme may be produced in sufficient quantities to override the effect of the herbicide on the sensitive enzyme. That is, sufficient branched chain amino acids for the plants' needs may be produced by the resistant enzyme so that growth is not inhibited. At the enzyme level, however, inhibition of the combined resistant and sensitive enzymes is assessed (Haughn et al. 1988); thus in most resistant plants, some inhibition of ALS is detected even at low herbicide concentrations.

The degree of resistance demonstrated by the transgenic lines was consistent across the root growth experiments, the ALS assays, and the field experiment. Lines 12115 and 12140 were consistently highly resistant, and line 12352 was the only consistently highly sensitive line across all concentrations of both herbicides in the root growth experiments. Similarly, lines 12115 and 12140 were highly resistant and line 12352 was highly sensitive in the ALS assays. Other lines varied in the level of resistance demonstrated in the different experiments, but were intermediate between the two extremes.

Consistency in the level of resistance demonstrated by transgenic lines across different tests is a common phenomenon. For example, protein levels from immunoblot analysis correlated with resistance levels observed, both in a leaf section assay and in greenhouse tests in transgenic tobacco plants carrying a bromoxynil detoxification gene (Stalker et al. 1988). Results from ALS assays, callus growth tests, germination tests, and greenhouse tests of transgenic tobacco genes carrying a resistant ALS gene were also consistent (Falco et al. 1987).

The degree of cross-resistance of the transgenic lines to metsulfuron methyl in root growth experiments was low but significant. That is, the transgenic lines were clearly more resistant to metsulfuron methyl than the parental lines, but the degree of resistance did not approach that demonstrated for chlorsulfuron. Not only was the degree of resistance lower, but the range of resistance demonstrated by the transgenic lines was less with metsulfuron methyl than with chlorsulfuron. It appears that lines highly resistant to chlorsulfuron (for example, line 12140) do not show the same proportionate degree of resistance to metsulfuron methyl.

One might expect a high degree of cross-resistance within the same group of herbicides, especially given that there are examples of cross-resistance both within and between different groups of herbicides (e.g., Devine et al. 1991). However, with the same mutant gene as used in these experiments, no cross-resistance to triasulfuron was observed with transgenic *Brassica* plants, while cross-resistance to three short-residual sulfonylurea herbicides could be demonstrated (Gabard et al. 1989). This suggests that the mutation in this particular ALS gene affects the binding of each sulfonylurea herbicide differently, and it is not possible to predict degrees of cross-resistance to other herbicides acting on the same target enzyme.

Despite the low degree of cross-resistance to metsulfuron methyl demonstrated by the transgenic lines in the laboratory, protection in the field appeared to be adequate. This suggests that the gene may offer protection from other sulfonylurea herbicides at the field level, even if the level of cross-resistance demonstrated in the laboratory is low. Since resistance was demonstrated at the full rate of metsulfuron methyl in the field, these transgenic lines will be protected from typical residual levels of metsulfuron methyl. Further work should carry the two highly resistant lines through larger field trials to confirm acceptable performance with respect to agronomic characteristics and to test for cross-resistance to other herbicides that inhibit ALS.

Acknowledgements. S.M. was the recipient of a Canadian Pacific Fellowship in Biotechnology. The assistance of F.A. Holm with the field experiment is gratefully acknowledged.

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