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Transformation of *Brassica napus* canola cultivars with *Arabidopsis thaliana* acetohydroxyacid synthase genes and analysis of herbicide resistance

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Summary. A survey of selected crop species and weeds was conducted to evaluate the inhibition of the enzyme acetohydroxyacid synthase (AHAS) and seedling growth in vitro by the sulfonylurea herbicides chlorsulfuron, DPX A7881, DPX L5300, DPX M6316 and the imidazolinone herbicides AC243,997, AC263,499, AC252,214. Particular attention was given to the *Brassica* species including canola cultivars and cruciferous weeds such as *B. kaber* (wild mustard) and *Thlaspi arvense* (stinkweed). Transgenic lines of *B. napus* cultivars Westar and Profit, which express the *Arabidopsis thaliana* wild-type AHAS gene or the mutant gene *csr1-1* at levels similar to the resident AHAS genes, were generated and compared. The mutant gene was essential for resistance to the sulfonylurea chlorsulfuron but not to DPX A7881, which appeared to be tolerated by certain *Brassica* species. Cross-resistance to the imidazolinones did not occur. The level of resistance to chlorsulfuron in transgenic canola greatly exceeded the levels that were toxic to the *Brassica* species or cruciferous weeds. Direct selection of transgenic lines with chlorsulfuron sprayed at field levels under greenhouse conditions was achieved.

Key words: *Brassica napus* – Transgenic – Acetohydroxyacid synthase – Sulfonylurea – Imidazolinone

Introduction

Weeds, especially the broad leaf cruciferous species, are a major problem for canola production in Canada. Yield losses of 12%–14% have been reported in the provinces of Alberta, Saskatchewan, and Manitoba after optimal cultural practices were implemented (Chandler et al. 1984). The development of herbicide-resistant *Brassica napus* and *B. campestris* canola is therefore a high priority for varietal development.

Herbicides that inhibit the enzyme acetohydroxyacid synthase (AHAS or ALS, EC 4.1.3.18), including the sulfonylureas (LaRossa und Schloss 1984) and imidazolinones (Shaner et al. 1984), are potential candidates for use with genetically altered canola. All of the cultivars registered in Canada are presently sensitive to these herbicides except for a new experimental sulfonylurea, DPX A7881 (Hutchison et al. 1987; Swanton and Chandler 1989). The older sulfonylureas, including chlorsulfuron (Glean, DuPont) and sulfometuron methyl (Oust, DuPont), have very restricted applications in agriculture, e.g., on land used only for cereals or in rotation with summer fallow. The persistence of residual activity in soils for long periods of time severely limits crop rotation (Beyer et al. 1987). Recently, short-residual sulfonylureas have been developed for cereals. Examples are DPX M6316 (Harmony, DuPont) and DPX L5300 (Express, DuPont), which are degraded 20–50 times faster by soil microbes and chemical hydrolysis, respectively (Beyer et al. 1987).

Several plant AHAS mutants have been described, including *B. napus* lines which are resistant to chlorsulfuron or the imidazolinones (Swanson et al. 1988, 1989). Resistance may be selective for a herbicide class, such as the sulfonylureas, as described for the *Arabidopsis thaliana* mutant GH50 (Haughn and Somerville 1988; Gabard et al. 1989). Cross-resistance may occur within a single class (Chaleff and Mauvais 1984) or between herbicide classes that inhibit AHAS (Saxena and King 1988). Recently, the transfer of selective sulfonylurea re-

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sistance into *Nicotiana tabacum* with the mutant *A. thaliana* AHAS gene, *csr1-1* (Haughn et al. 1988; Gabard et al. 1989; Charest et al. 1989), indicated the potential for deliberate and precise manipulation of herbicide resistance properties in transgenic plants using well-characterized mutant genes.

This report describes the generation and assessment of transgenic canola that expresses the mutant gene *csr1-1* at levels comparable with the resident AHAS genes. The *B. napus* cultivar employed, Profit, has just been registered in Canada. It is a high-yielding canola variety with tolerance to black leg disease. An extensive survey of herbicide activity with the major *Brassica* species and weeds that affect canola production was conducted to assess the general utility of these plants and to identify potential obstacles that may be encountered during field evaluations.

Materials and methods

Plant material

Sixteen different species were studied: *Brassica campestris* cv Tobin and cv Candle (canola), *B. juncea* cv Zaria (black mustard), *B. napus* cv Westar and cv Profit (canola), *B. hirta* cv Ochre (yellow mustard), *B. oleracea*, rapid cycling, *B. nigra*, rapid cycling (radish), *Crambe maritima*, *Thlaspi arvense* (stinkweed), *Medicago sativa* cv Rangelander (alfalfa), *Glycine max* cv Maple Presto (soybean), *Setaria viridis* (green foxtail), *Avena fatua* (wild oats), *Hordeum vulgare* cv Argyle (barley), *Triticum aestivum* cv Katepwa (wheat). *Brassica* species and cultivars were obtained from R. K. Downey (Agriculture Canada, Saskatoon). Rapid cycling *Brassicas* were obtained from P. Williams (University of Wisconsin, USA). *C. maritima* and *M. sativa* were gifts respectively from G. Baillargeon (Agriculture Canada, Ottawa) and D. Brown (Agriculture Canada, Ottawa). *T. arvense*, *S. viridis*, and *A. fatua* were received from D. Derksen (Agriculture Canada, Saskatoon). *G. max*, *H. vulgare*, and *T. aestivum* were obtained from the Seed Biology Laboratory (Agriculture Canada, Ottawa).

Batches from 100 to several thousand seeds were sown in flats containing a mixture of soil:peat:sand:perlite (1:2:1:1), with traces of lime and superphosphate. They were watered twice daily and supplemented weekly with a 20:20:20 NPK solution. Seedlings were grown in a greenhouse under sunlight during the summer with a temperature range of 10° to 35 °C, and under artifical lights (16 h daylight, 60 $\mu E \times m^{-2} \times sec^{-1}$ intensity) during fall and winter, with the temperature maintained at approximately 22 °C.

Chemicals

Seven different herbicides were studied. The imidazolinones AC 263,499 (imazethapyr, Pursuit), AC 252,214 (imazaquin, Scepter), and AC 243,997 (imazapyr, Arsenal) were gifts from E. R. Nestman, American Cyanamid (Willowdale, Canada). The others were sulfonylurea herbicides from DuPont, including chlorsulfuron (the active ingredient from Glean) and short residual sulfonylureas DPX A7881, DPX M6316 (Harmony) and DPX L5300 (Express). Chlorsulfuron was obtained from R. Chaleff (DuPont, Wilmington, DE, USA) and the others were received from F. Houston, (DuPont, Toronto, Canada). The imidazolinones and chlorsulfuron were technical-grade compounds (97.1%–98.75% purity), whereas the three short residual sulfonylureas were commercial grade (75% purity). All herbicides were routinely dissolved in double distilled water with dilute KOH (pH < 9.5).

AHAS activity

Plant material was harvested 2–5 weeks after germination depending on the growth rate of the different species in the greenhouse. One gram of fresh young growing leaves was frozen in liquid nitrogen and stored at −70 °C in aluminium foil. Frozen leaves were ground to a powder in a mortar containing liquid nitrogen. The powder was poured into 3 ml extraction buffer supplemented with 250 mg polyclar-ATg FW (BDH). AHAS activity was measured using the method described by Chaleff and Ray (1984) modified by Haughn and Somerville (1986). Herbicide levels used for inhibition were: 0, 0.05, 0.5, 1.5, 5, 50, 500 μM for AC 263,499 and AC 243,997. With AC 252,214 the maximum concentration used was 250 μM. Concentrations of 0, 1, 3, 10, 30, 100, 300 µg/l were used for DPX M6316, DPX A7881, and DPX L5300. For chlorsulfuron, the levels used were 3–1,000 µg/l. AHAS activity measurements were made in triplicate and the percent inhibition of AHAS activity at each concentration of herbicide was determined. Results are expressed as the concentration of herbicide (µg/l) needed to inhibit AHAS activity by 50% (I_{50}) and the percent inhibition of AHAS activity obtained at the highest level of herbicide used (P_{max}). Several experiments were repeated twice or more as required.

In vitro germination and growth

Seed samples were wrapped in filter paper bags (Whatman 3MM) and sterilized in a 2-l beaker with 70% EtOH, followed by 0.2% $HgCl_2$, and finally undiluted Javex (6% Na-hypochlorite; Bristol-Myers, Toronto, Ontario) for various times. A few droplets of Tween-20 were added to the last two solutions. Paper bags containing seeds were rinsed three times with sterile double distilled water and dried overnight under laminar flow. When contamination was persistent, the seed sterilization was repeated a few weeks later without $HgCl_2$ and seedling germination was repeated.

Ten to 50 seeds were sown under sterile conditions in GA7 Magenta boxes (Magenta Corporation, Chicago/IL, USA). Fifty or 20 ml of MS medium (Murashige and Skoog 1962) with B5 vitamins (Gamborg et al. 1968), 1% sucrose, and 0.6% agarose (Seakem ME; FMC) were used. Herbicide levels were 0, 1, 10, 30, 100, 300 µg/l for sulfonylureas and 0, 10, 30, 100, 300, 1000 nM for imidazolinones. Seedlings were germinated in incubators set at 20 °C, 16 h a day, 15 °C night except for soybeans and cereals, which were germinated at 28 °C days and 25 °C nights. Light intensity ranged from 44 to 70 $\mu E \times m^{-2} \times sec^{-1}$. Except for *A. fatua*, *H. vulgare*, *B. hirta*, and *C. maritima*, ten seedlings at each herbicide level were picked randomly and weighed after 2–4 weeks. The average fresh weight was calculated at each herbicide concentration and each measurement was duplicated. Several complete experiments were repeated twice. Results were expressed as the herbicide concentration (µg/l sulfonylurea; mg/l imidazolinone) needed to inhibit growth by 50% (G_{50}) and by the level of inhibition (%) reached at the highest level of herbicide used (P_{max}).

Genetic transformation

Stem segments (Fry et al. 1987) were prepared from *B. napus* cv Westar and cv Profit and inoculated with *Agrobacterium tumefaciens*, as described earlier (Charest et al. 1988). Selection was on kanamycin at 40 µg/ml or on chlorsulfuron at 16 µg/l (40 nM), as described earlier (Charest et al. 1988). Direct selection for

Table 1. Inhibition of AHAS activity and seedling growth by the sulfonylureas

Species	Chlorsulfron				DPX A7881				DPX M6316				DPX L5300			
	AHAS		Growth		AHAS		Growth		AHAS		Growth		AHAS		Growth	
	I_{50}[a] (μg/l)	P_{max}[b] (%)	G_{50}[c] (μg/l)	P_{max} (%)	I_{50} (μg/l)	P_{max} (%)	G_{50} (μg/l)	P_{max} (%)	I_{50} (μg/l)	P_{max} (%)	G_{50} (μg/l)	P_{max} (%)	I_{50} (μg/l)	P_{max} (%)	G_{50} (μg/l)	P_{max} (%)
B. campestris	19	100	10	88	19	94	NR[d]	25	3	98	6	95	44	89	NR	NT[e]
B. nigra	70	98	7	86	18	97	23	89	6	100	1	93	16	90	26	89
B. oleracea	16	100	1	80	20	90	95	59	10	98	8	66	100	85	NR	NI
B. juncea	7	100	7	81	4	100	190	75	3	100	4	88	6	100	NR	30
B. napus	26	95	20	84	6	97	NR	7	5	98	7	86	55	89	100	73
B. carinata	13	93	30	66	50	100	NR	NI	6	100	55	79	20	100	NR	NI
B. hirta	28	95	ND[f]	ND	85	85	I[g]	I	5	86	I	I	61	79	NR	NI
B. kaber	105	96	3	93	11	79	10	90	8	81	7	93	13	82	NR	NI
R. sativus	29	95	10	62	2	100	200	59	4	100	80	61	3	100	NR	NI
C. maritima	12	95	7	88	2	94	ND	ND	4	97	ND	ND	73	85	ND	ND
T. arvense	74	100	7	77	11	100	20	79	4	100	10	87	14	97	NR	NI
M. sativa	11	98	55	55	4	99	10	64	4	98	30	69	54	87	NR	NI
G. max	90	87	20	90	13	88	NR	10	14	94	5	80	150	72	NR	NI
S. viridis	150	89	NR	42	1	95	100	66	2	91	NR	43	75	83	NR	NI
A. fatua	18	100	NR	NI	16	85	NR	NI	16	93	NR	NI	50	83	NR	NI
H. vulgare	28	96	NR	NI	14	95	NR	NI	8	100	NR	NI	45	81	NR	NI
T. aestivum	26	80	NR	NI	15	95	NR	NI	6	95	NR	NI	135	83	NR	NI

[a] I_{50} was the level of herbicide (μg/l) needed to inhibit AHAS activity by 50%
[b] P_{max} was the percent inhibition achieved at the highest concentration of herbicide used
[c] G_{50} was the level of herbicide (μg/l) needed to inhibit seedling growth by 50%
[d] NR – I_{50} not reached
[e] NI – no inhibtion
[f] ND – not done
[g] I – inhibition observed but accurate measurements not obtained

chlorsulfuron resistance in the greenhouse was performed on unselected regenerants that had reached the two- to four-leaf stage. The plantlets were sprayed in groups of 50–100, individually planted in 5-in. diameter pots, with Glean at the equivalent of 10 g/ha in 0.1% Tween-20 (Sigma) as surfactant.

Southern blot analyses

Southern blots were performed essentially as described previously (Charest et al. 1990). In brief, 2 μg each of total DNA from various plants was digested with XbaI, electrophoresed on a 0.7% agarose gel, and transferred to a Nytran membrane (Schleicher and Schuell). The membrane was hybridized with a ^{32}P-labelled *A. thaliana* AHAS probe containing the 5′ half of the coding region, washed under stringent conditions (0.5% sodium lauroylsarcosine at 65 °C), and exposed to film at −80 °C.

RNase protection analyses

RNA probes were ^{32}P-labelled by in vitro transcription from the plasmid pGEM-4Z (Promega, Madison/WI) containing about the middle third of the coding region of the *A. thaliana* Landsberg erecta wild-type AHAS gene (Charest et al. 1990) or the *B. napus* cv Topas AHAS1 gene (R. Rutledge, T. Ouellet, J. Hattori, B. Miki, in preparation). The RNase protection assays were performed with minor modifications as described previously (Ouellet et al. 1990). Each RNA probe was hybridized (overnight, 45 °C) to 5 μg of total leaf RNA from control or transgenic plants, then treated with 200 μg/ml RNase A and 500 units/ml RNase T1 (45 min, 30 °C) to remove the nonhybridized RNAs. The protected fragments were electrophoresed on 5% polyacrylamide-7M area denaturing gels. The gels were dried and exposed to film. Autoradiograms were scanned on a Fisher FB910 densitometer to determine the relative amounts of specific AHAS mRNA in each sample.

Results

Activity of herbicides

The inhibition of seedling germination and growth in vitro by the sulfonylurea and imidazolinone herbicides were examined on culture medium. (Tables 1 and 2). A number of species was surveyed with particular attention to the *Brassica species* and cruciferous weeds that affect canola production in Canada. *Brassica napus* (canola), *B. campestris* (canola), *B. juncea* (mustard), and the weeds *B. kaber* (wild mustard) and *T. arvense* (stinkweed) were inhibited by each of the imidazolinones examined and by the sulfonylureas, chlorsulfuron, and DPX-M6316. Selective resistance to the sulfonylurea DPX-A7881 was observed for *B. napus, B. carinata, B. campestris,* and *G. max* to different extents. The sulfonylurea DPX-L5300 could not be examined due to the extremely high rate of

Table 2. Inhibition of AHAS activity and seedling growth by the imidazolinones

Species	AC 243, 997				AC 263, 499				AC 252, 214			
	AHAS		Growth		AHAS		Growth		AHAS		Growth	
	I_{50}[a] (mg/l)	P_{max}[b] (%)	G_{50}[c] (µg/l)	P_{max} (%)	I_{50} (mg/l)	P_{max} (%)	G_{50} (µg/l)	P_{max} (%)	I_{50} (mg/l)	P_{max} (%)	G_{50} (µg/l)	P_{max} (%)
B. campestris	0.5	100	53	83	0.3	100	69	77	0.2	100	9	90
B. nigra	0.8	100	24	77	0.5	100	61	79	0.2	100	23	79
B. oleracea	0.6	100	52	66	0.4	100	20	73	0.3	100	27	68
B. juncea	3.5	70	13	94	0.4	100	42	96	0.5	80	7	99
B. napus	1.2	86	131	67	0.4	100	26	84	0.4	95	16	87
B. carinata	0.5	72	14	83	0.5	90	13	75	1.1	73	9	88
B. hirta	0.9	90	NG[d]	NG	0.4	100	NG	NG	0.4	92	NG	NG
B. kaber	13.3	68	22	85	0.6	87	36	88	0.5	85	3	88
R. sativus	0.4	90	NR[e]	34	0.4	90	245	53	0.4	94	23	70
C. maritima	1.0	92	I[f]	I	0.3	95	I	I	0.5	97	I	I
T. arvense	2.5	87	NR	36	1.2	100	188	52	0.3	100	202	65
M. sativa	10.5	94	NR	NI[g]	8.7	85	NR	7	2.0	94	NR	20
G. max	9.1	85	NR	NI	3.2	92	NR	NI	2.5	88	NR	NI
S. viridis	2.6	66	222	59	0.4	94	43	77	0.5	82	53	76
A. fatua	0.6	100	NR	NI	0.4	100	NR	NI	0.3	96	NR	NI
H. vulgare	0.8	90	NR	NI	0.3	100	NR	NI	0.3	100	NR	NI
T. aestivum	2.6	74	NR	NI	0.6	89	NR	NI	0.5	92	NR	NI

[a] I_{50} was the level of herbicide (mg/l) needed to inhibit AHAS activity by 50%
[b] P_{max} was the percent inhibition achieved at the highest concentration of herbicide used
[c] G_{50} was the level of herbicide (µg/l) needed to inhibit seedling growth by 50%
[d] NG – no germination
[e] NR – I_{50} not reached
[f] I – inhibition observed but not measured
[g] NI – no inhibition

degradation in culture medium at pH 5.8. Selective resistance to the imidazolinones was found only for the legumes *G. max* and *M. sativa*. Naturally occurring resistance to both the sulfonylureas and imidazolinones was evident only for the cereals *T. aestivum*, *H. vulgare*, and *A. fatua*.

A survey of AHAS activities in young leaves was performed (Tables 1 and 2). Naturally occurring AHAS forms that were not inhibited by the sulfonylureas and imidazolinones could not be found (Tables 1 and 2). This included AHAS from species that displayed resistant growth patterns. The concentrations needed to inhibit AHAS activity by 50% (I_{50}) and the maximum level of inhibition achieved (P_{max}) were compared. Generally, the sulfonylureas were more potent inhibitors of AHAS activity than the imidazolinones. In all cases, inhibition of AHAS was extensive; however, the I_{50} values varied greatly.

Transformation and selection of transgenic B. napus

The *A. tumefaciens* strain GV3101 harboring the co-integrate vectors pGV3850::p35SALSR or pGV3850::p35SALSW has been described in detail (Charest et al. 1990). The intermediate vector p35SALSR carried a chimaeric gene consisting of the coding and 3′ regions of the mutant *A. thaliana* AHAS gene, *csr1-1* (Haughn et al. 1988), fused to the 35S promoter and upstream sequences. The vector p35SALSW was identical except that the coding and 3′ regions of the AHAS gene were derived from a wild-type gene. Both vectors carried a chimaeric *35S-nptII-nos* gene (Saunders et al. 1987) as a selectable marker for plant transformation.

Transgenic *B. napus* lines were produced by *Agrobacterium*-mediated transformation of stem explants (Fry et al. 1987; Charest et al. 1988). Several lines potentially transformed with pGV3850::p35SALSR or pGV3850::p35SALSW were recovered by selection for resistance to 40 µg/ml kanamycin in culture (Charest et al. 1988). Biochemical analysis of eight separate lines revealed that none possessed high levels of AHAS activity resistant to chlorsulfuron. Direct selection for resistance to chlorsulfuron in culture with pGV3850::p35SALSR was attempted and discontinued after more than 6,000 inoculated explants of *B. napus* cv Profit failed to yield resistant shoots. *Brassica napus* cv Westar transgenic lines transformed with pGV3850::p35SALSW were identified by Southern and Northern blot analyses, following selection on kanamycin.

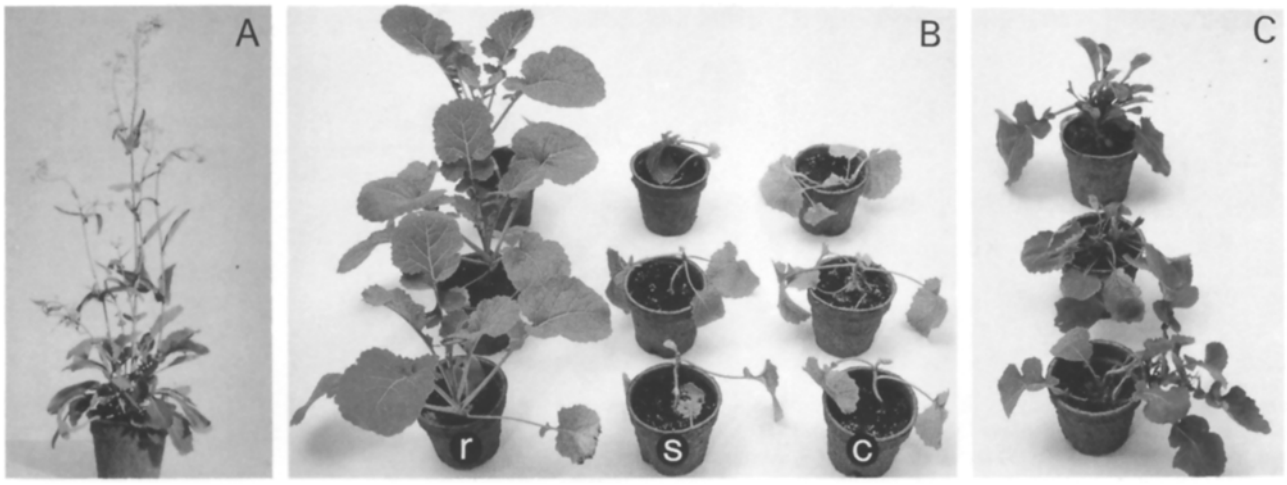


Fig. 1A–C. Chlorsulfuron resistance. **A** Regenerated *B. napus* cv Profit, line Can55, selected under greenhouse conditions by spraying with Glean at a concentration equivalent to 10 g/ha. **B** The progeny of Can55 after self-pollination segregating for chlorsulfuron resistance (r) and sensitivity (s), compared with untransformed controls (c), 2 weeks after spraying. **C** The survival of sensitive regenerated plants with abnormal morphology 4 weeks after spraying

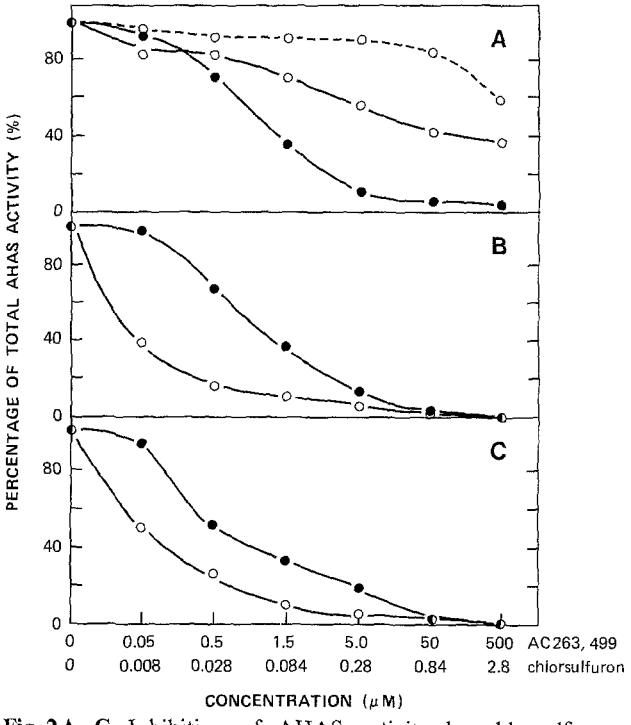


Fig. 2A–C. Inhibition of AHAS activity by chlorsulfuron (o–o) and AC 263, 499 (●–●). **A** AHAS activities from *B. napus* cv Profit, transgenic Line Can55 (*solid lines*) and *A. thaliana* mutant line GH50 (*dashed line*). **B** AHAS activity from *B. napus* cv Westar, transgenic line Can2. **C** AHAS activity from untransformed *B. napus* cv Profit

Screening for chlorsulfuron resistance under greenhouse conditions was pursued with plants regenerated from explants inoculated with *A. tumefaciens* harboring pGV3850::p35SALSR. Selection in culture was omitted. Plants were sprayed with Glean at a concentration equivalent to 10 g/ha with 0.1% Tween-20 as surfactant at the two- to four-leaf stage. The resistant plants were clearly distinguished from the sensitive ones within 2 weeks, and biochemical analyses were performed to confirm the presence of AHAS activity resistant to chlorsulfuron. Transgenic line Can55, which is described in detail below, was the only survivor in an experiment in which a total of 85 plantlets were sprayed. It flowered and set seed without obvious delays (Fig. 1A). Over 40% of the total AHAS activity was resistant to chlorsulfuron in young leaves that emerged within the 2-week period after spraying (Fig. 2A). In contrast, sensitive plants displayed atrophy of the apical meristems and extensive defoliation within 2–3 weeks. Growth of new leaves occurred on 5%–10% of the sensitive plants after 3–4 weeks; however, these plants had severe morphological abnormalities such as elongated leaves and lack of apical dominance (Fig. 1C). Chlorsulfuron-resistant AHAS activity was not detected in any of them.

Genetic analyses of transgenic lines

Two transgenic lines were chosen for detailed analyses. Line Can55 was derived from *B. napus* cv Profit transformed with vector pGV3850::p35SALSR. Line Can2 was derived from *B. napus* cv Westar transformed with vector pGV3850::p35SALSW. Analyses of AHAS activity in mature leaves of Can55 indicated that 40% of the total uninhibited AHAS activity remained resistant to chlorsulfuron at 2.8 μ*M* (Fig. 2A). At the same chlorsulfuron concentration, resistant AHAS activity from *A. thaliana* line GH50 comprised about 60% of the total uninhibited activity (Fig. 2A). Reconstruction experiments with both samples indicated that about 66% of the

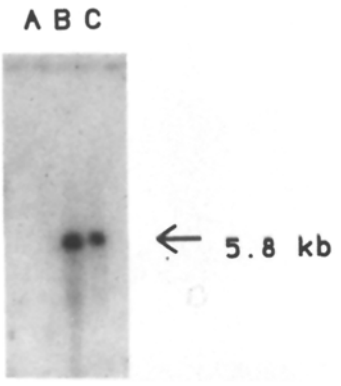
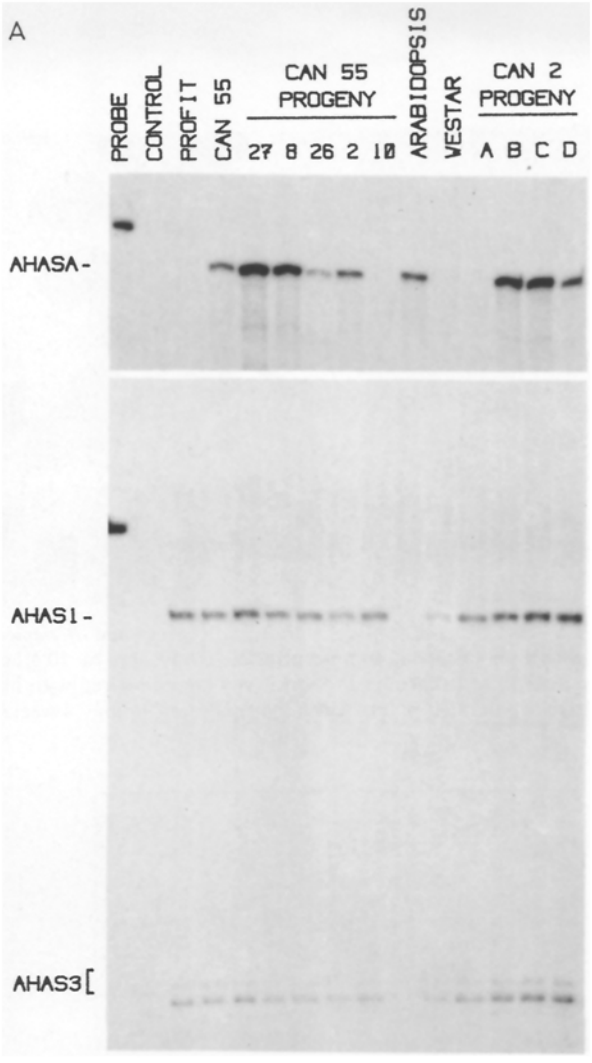


Fig. 3. Southern blot analyses of transgenic plants. Southern blot of untransformed *B. napus* DNA (*lane A*) and DNA from transgenic lines Can2 (*lane B*) and Can55 (*lane C*). About 2 μg of total DNA was digested with Xba I, run on a 0.7% agarose gel, and transferred to a Nytran membrane. The blot was hybridized with an *A. thaliana* AHAS probe. The *arrow* indicates the 5.8-kbp XbaI fragment

total AHAS activity of Can55 corresponded to enzyme encoded by *csr1-1* (data not shown). Resistance to the imidazolinone AC 263,499 was not detected (Fig. 2A). Similar measurements of AHAS activity in untransformed cv Profit (Fig. 2C), untransformed cv Westar (Tables 1 and 2), and transgenic line Can2 (Fig. 2B) revealed complete sensitivity to both chlorsulfuron and AC 263,499.

In two separate experiments, chlorsulfuron resistance segregated as a single dominant trait under greenhouse conditions. Among 58 selfed progeny of Can55, 44 were clearly resistant at 10 g/ha Glean after 2 weeks and 14 were sensitive, as were all ten untransformed control plants (Fig. 1B). At 50 g/ha Glean, 15 out of 20 plants were resistant. As shown in Fig. 3, Southern blot analyses revealed the presence in both Can55 and Can2 of the predicted 5.8-kbp XbaI fragment which hybridized with the *A. thaliana* AHAS gene probe. Northern blot analyses confirmed a single RNA band corresponding to *A. thaliana* AHAS in both lines (data not shown). Under the stringent conditions used, *B. napus* AHAS genes and transcripts were not visible with the *A. thaliana* AHAS probe, which consisted of the 5′ half of the coding region.

Among 12 resistant progeny of Can55, the proportion of uninhibited AHAS activity resistant to chlorsulfuron at 2.8 μ*M* ranged between 25% and 60% (Fig. 4B). A detailed analyses of transcript levels in these plants was conducted with an RNase protection assay (Ouellet et al. 1990) using RNA probes consisting of the middle third of the coding region of the *A. thaliana* AHAS (Fig. 4A, upper panel) and the *B. napus* AHAS1 (Fig. 4A, lower panel) genes. In the *B. napus* canola cultivars Westar, Profit, and Topas, the gene family consists of four distinct genes, AHAS1-4 (R. Rutledge, T. Ouellet, J. Hattori, B. Miki, in preparation). As found in another study



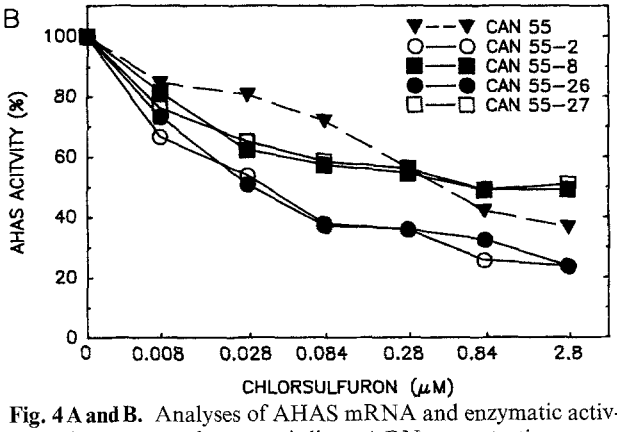


Fig. 4 A and B. Analyses of AHAS mRNA and enzymatic activity in the progeny of transgenic lines. **A** RNase protection assays on RNA from Can55 and its progeny and from Can2 progeny using an *A. thaliana* AHAS probe (*upper panel*) or a *B. napus* AHAS1 probe (lower panel). Probe, untreated RNA probe; control, control using only the probe and tRNA for the assay; Profit, *B. napus* cv Profit; Can55, *B. napus* cv Profit transgenic line Can55 and some selfed progeny that were resistant to chlorsulfuron (Can55-2 to 27) or sensitive to chlorsulfuron (Can55-10); *Arabidopsis*, *A. thaliana* cell suspension culture; Westar, *B. napus* cv Westar; Can2-A to D, progeny of *B. napus* cv Westar transgenic line Can2; AHASA, *A. thaliana* AHAS protected RNA fragment; AHAS1 and AHAS3, *B. napus* AHAS1 and AHAS3 specific RNA fragments. **B** Inhibition of AHAS activity by chlorsulfuron in Can55 and its progeny

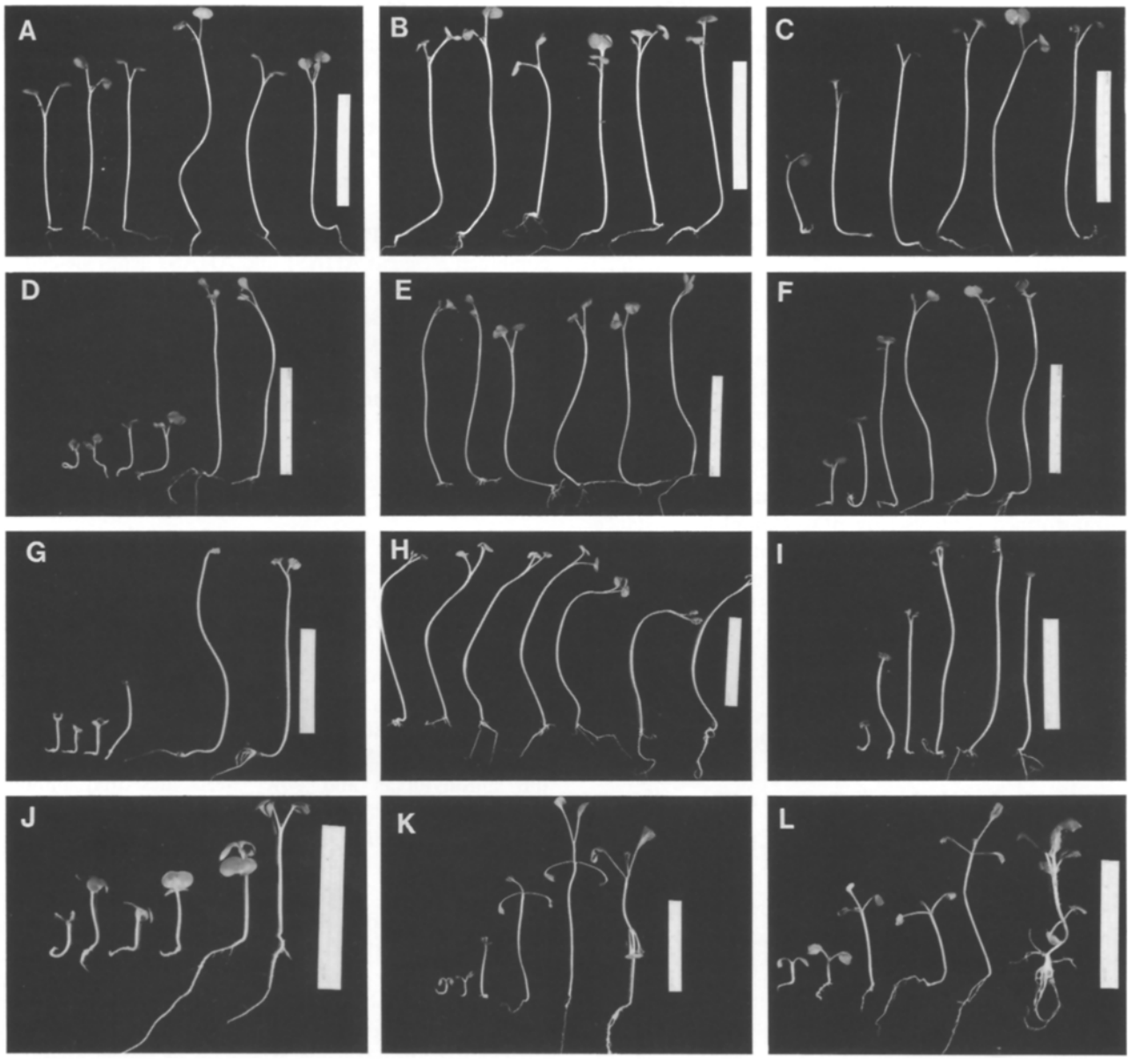


Fig. 5A–L. Inhibition of seedling growth in culture by chlorsulfuron (A, D, G, J; 300, 100, 30, 10, 1, 0 µg/1), DPX A7881 (B, E, H, K; 300, 100, 30, 10, 1, 0 µg/1), and AC 263, 499 (C, F, İ, L; 1000, 300, 100, 30, 10, 0 n*M*). Seedlings of *B. napus* cv Profit transgenic line Can55 resistant to chlorsulfuron (**A**) and DPX A7881 (**B**) or sensitive to AC 263, 499 (**C**). Seedlings of *B. napus* cv Westar transgenic line Can2 sensitive to chlorsulfuron (**D**), resistant to DPX A7881 (**E**), and sensitive to AC 263, 499 (**F**). Seedlings of untransformed *B. napus* cv Westar sensitive to chlorsulfuron (**G**), resistant to DPX A7881 (**H**), and sensitive to AC 263, 499 (**I**). Seedlings of *B. kaber* sensitive chlorsulfuron (**J**), DPX A7881 (**K**), and AC 263, 499 (**L**)

with cultivars Westar and Topas (T. Ouellet, R. Rutledge, B. Miki, in preparation), only AHAS1 and AHAS3 were expressed in leaf tissue of Can55 (cv Profit) and Can2 (cv Westar; Fig. 4A, lower panel). *Arabidopsis thaliana* AHAS mRNA accounted for about 60% of the total AHAS mRNA in Can55 (Fig. 4A). The level of *B. napus* AHAS mRNA was not altered in the transgenic lines compared with untransformed plants (Fig. 4A). The level of *A. thaliana* AHAS mRNA varied among the progeny of Can55 (range: 30%–80%, Fig. 4A) in proportion to the amount of chlorsulfuron-resistant AHAS activity (Fig. 4B). Can55-10 was a sensitive plant and no *A. thaliana* AHAS mRNA could be detected (Fig. 4A). Can55-8 and Can55-27 possessed levels of chlorsulfuron-resistant AHAS activity and *A. thaliana* AHAS mRNA that were greater than found in the Can55 parent line. Can55-2 and Can55-26 had similar or lower levels of both. The level of *A. thaliana* wild-type AHAS mRNA in

progeny of Can2 was comparable (range: 20%–30%, Fig. 4A) to that found in progeny of Can55.

Herbicide resistance

The inhibition of seedling germination and growth was examined over a range of herbicide concentrations in culture medium, pH5.8, as discussed for Tables 1 and 2. The progeny of Can55 self-pollination segregated for resistance (Fig. 5A) and sensitivity (data not shown) to chlorsulfuron. Complete resistance to chlorsulfuron was apparent at 300 µg/l. This represented at least a 30-fold increase in resistance above untransformed cultivar Profit or Westar (Fig. 5G), the sensitive progeny of Can55 (data not shown), and the weeds *B. kaber* and *T. arvense* (Table 1 and Fig. 5). A marginal stimulation in growth was noted at low chlorsulfuron levels (10 µg/l, Fig. 5A). Similar experiments with progeny of Can2 revealed sensitivity to chlorsulfuron at the same levels as untransformed controls (Fig. 5D). Complete resistance to the sulfonylurea DPX-A7882 was noted over the same concentration ranges as with chlorsulfuron for progeny of both Can55 (Fig. 5B), Can2 (Fig. 5E), and untransformed controls (Fig. 5H). Inhibition of Can55 (Fig. 5C) and Can2 (Fig. 5F) seedling growth by AC 263,499 was at the same level as for untransformed controls (Fig. 5I). In contrast to the pattern of selective resistance displayed by the transgenic and untransformed *B. napus* lines, the cruciferous weed species *B. kaber* (Fig. 5J–L) and *T. arvense* (Tables 1 and 2) were sensitive to chlorsulfuron, DPX A7881, and AC 263,499.

Under greenhouse conditions chlorsulfuron resistance was observed after spraying with Glean at approximately 10, 50, and 100 g/ha; however, inhibition of growth and delays in flowering occurred at 50 and 100 g/ha (data not shown).

Discussion

The recovery of transgenic *B. napus* cv Profit, line Can55, was achieved by direct selection for resistance to chlorsulfuron at field levels under greenhouse conditions. The success of this strategy indicated the feasibility of employing extremely simple T-DNA, such as a mutant AHAS gene flanked only by T-DNA borders for future experiments. For maximum efficiency of this approach, optimal conditions for transformation of *B. napus* (Charest et al. 1988, 1989; De Block et al. 1989; Moloney et al. 1989) will be required. Although the unusually high frequency of *Brassica* transformants that poorly express marker genes has been observed (DeBlock et al. 1989), the inefficiency of selection for kanamycin resistance with *B. napus* cv Profit appeared to be more severe than in other studies (Charest et al. 1988), necessitating the approaches used.

Arabidopsis thaliana AHAS appeared to function extremely well in transgenic *B. napus*. This was anticipated from other studies (R. Rutledge, T. Ouellet, J. Hattori, B. Miki, in preparation) that showed extensive homology among the predicted amino acid sequences of *A. thaliana* AHAS and *B. napus* AHAS1 and AHAS3, which are the two resident genes expressed in leaf tissues (T. Ouellet, R. Rutledge, B. Miki, in preparation). Expression of *A. thaliana* AHAS mRNA to levels slightly above that of the combined *B. napus* AHAS1 and AHAS3 genes yielded a proportionate amount of the total AHAS activity as the chlorsulfuron-resistant form. The mutation in the *csr1-1* gene was clearly essential for resistance, since expression of the wild-type *A. thaliana* AHAS gene at comparable levels did not confer resistance to chlorsulfuron or the imidazolinone AC 263,499. The level of expression of the *A. thaliana* genes in both Can55 and Can2 was much lower than expected, considering the use of the 35S promoter. Much higher proportions of chlorsulfuron-resistant AHAS activity have been achieved in transgenic tobacco with the same vectors (Charest et al. 1990).

AHAS that is naturally resistant to the sulfonylureas or imidazolinones was not found in any of the species examined. As shown by others (Ray 1986), the concentration of herbicide needed to inhibit AHAS by 50% (I_{50}) varied greatly among species; however, the extent of inhibition was generally very high. This finding supports the use of mutant AHAS genes for achieving resistance to the sulfonylureas and other herbicides that inhibit AHAS. Previous studies have shown that the mutation in the *A. thaliana csr1-1* gene (Haughn et al. 1988) can confer resistance to chlorsulfuron, DPX A7881, DPX M6316, DPX L5300, but not to the sulfonylurea CGA131,036 or to the imidazolinones AC 293,997, AC 263,499, AC 252,214 (Gabard et al. 1989). Other mutants of *Datura innoxia* (Saxena and King 1988) and *B. napus* cv Topas (Swanson et al. 1989) have been described with selective resistance to the imidazolinones.

The agricultural value of mutant or transgenic crop varieties that express such genes depends on the patterns of naturally occurring resistance among them and the weeds that affect their production. The biochemical basis for selective resistance to the sulfonylureas and the imidazolinones involves metabolic mechanisms for herbicide detoxification. An example is the rapid hydroxylation of chlorsulfuron followed by glycosylation in species such as the grasses, night shade, and flax (Sweetser et al. 1982; Hutchinson et al. 1984). Under field conditions, selective resistance is dependent on a number of factors such as soil type, microbial activity, moisture, climate, and movement in soil (Blair and Martin 1988). The analysis of seedling growth inhibition in vitro provided a rapid and uniform basis for comparison of herbicide activity with several species and varieties. The data generally confirmed field observations; for instance, the

cereals were resistant to both the sulfonylureas and the imidazolinones and the legumes were resistant to only the imidazolinones. A major limitation was the rapid hydrolysis of DPX L5300 in culture medium which prohibited analysis of this short-residual sulfonylurea (Ferguson et al. 1985).

All of the cruciferous species surveyed in vitro were inhibited by each of the imidazolinones, the sulfonylurea chlorsulfuron, and the short-residual sulfonylurea DPX M6316 (Sionis et al. 1985). In progeny of transgenic line Can55, resistance to chlorsulfuron in vitro was elevated to levels more than 30-fold greater than the toxic levels for untransformed *B. napus* and other cruciferous species such as *B. kaber, T. arvense, R. sativa, B. nigra, B. juncea,* or *B. campestris*. Under greenhouse conditions, resistance to field levels of Glean was observed. Both the *B. napus* and *B. campestris* canola cultivars and *B. carinata* displayed selective resistance in vitro to the sulfonylurea, DPX A7881 (Hutchison et al. 1987). Field studies have also shown that DPX A7881 can selectively remove *B. kaber* from *B. napus* canola (Swanton and Chandler 1989). The in vitro studies indicate that other oilseed *Brassica* species such as *B. juncea* and other cruciferous weeds such as *T. arvense* are sensitive to DPX A7881. Under the conditions used, chlorsulfuron and DPX M6316 were more potent herbicides than DPX A7881. These results indicated that selective resistance to sulfonylureas in *Brassica* crops resulting from the introduction of mutant genes into transgenic plants or from inherent detoxification mechanisms both have great potential for the control of broad leaf cruciferous weeds. Transformation with well-characterized mutant genes provides much greater versatility and precision in manipulating the spectrum of herbicides and the crop species that can be considered. The use of genes equivalent to *csr1-1* in other crops could also extend the range of applications for herbicides such as DPX A7881.

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