BRASSICA NAPUS MUTANTS WITH INCREASED CHLORSULFURON RESISTANCE

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SUMMARY

Mutants of *Brassica napus* with increased chlorsulfuron resistance were isolated following seed mutagenesis with ethyl methanesulfonate and the *in vitro* screening of second generation mutant seedlings in the presence of the herbicide. Inheritance studies in two mutant plants attributed the increased chlorsulfuron resistance to single dominant nuclear mutations. Lines homozygous for chlorsulfuron resistance showed similar growth to the wild-type in the absence of chlorsulfuron. The mutant lines retained active growth at chlorsulfuron applications up to 30 g/ha, whereas the wild-type line showed severe damage following chlorsulfuron applications as low as 5 g/ha.

Keywords: *Brassica napus*, herbicide resistance, sulfonylurea resistance, seed mutagenesis, mutation breeding

INTRODUCTION

The development of herbicide-resistant crop plants has been recently recognised as offering a number of new applications in agriculture, in addition to simple weed control (Field *et al.* 1993). These new opportunities involve enhancing agronomic practices associated with crop rotations and crop thinning, and the use of herbicide resistance as a genetic marker during seed production. Herbicide resistance marker genes will allow seed of specific cultivars to be conveniently maintained free from contaminating crop and weed seeds in seed production fields. It will also overcome problems associated with pollen contamination during hybrid seed production as the undesirable plants can be easily removed by the herbicide.

Plants with improved herbicide resistance have been developed using traditional plant breeding and selection, seed mutagenesis, and biotechnology approaches including cell selection, protoplast fusion and genetic engineering (Conner and Meredith 1989; Field *et al.* 1993). The development of plants with resistance to sulfonylurea herbicides has been successful in a number of plant species. This has involved the use of traditional breeding and evaluation (eg. Landi *et al.* 1989), pollen selection (Sari-Gorla *et al.* 1989), seed mutagenesis (Haughn and Somerville 1986; Sebastian and Chaleff 1987; Sebastian *et al.* 1989), *in vitro* selection in microspore, protoplast and cell cultures (Chaleff and Ray 1984; Jordan and McHughen 1987; Creason and Chaleff 1988; Swanson *et al.* 1988; Harms *et al.* 1991; Pofelis *et al.* 1992), and genetic engineering (eg. Haughn *et al.* 1988; Moses *et al.* 1993).

In this paper we report the use of seed mutagenesis for the development of *Brassica napus* (rape) mutants with improved resistance to chlorsulfuron (Glean), a sulfonylurea herbicide.

MATERIALS AND METHODS

Seed mutagenesis

A mutagenised population of a rapid cycling rape line (CrGC#5; ACaacc), originally obtained from the Crucifer Genetics Cooperative, University of Wisconsin,

was used in this study. Seeds were soaked overnight (16 h) in 0.3% ethyl methanesulfonate (EMS), washed in running water for 2-3 h, then dried on absorbent paper for 2 days. Seeds were sown in a greenhouse and transplanted out as 33 first generation mutant (M1) populations of 100 plants each, which were allowed to self pollinate and set seed. The seed from the 100 plants in each M1 population was harvested together to give 33 second generation mutant (M2) populations of 180-2580 seeds, resulting in a total M2 population size of 30,609 seeds (mean = 928 for a M2 population).

***In vitro* seedling screen for chlorsulfuron resistance**

Each M2 population was individually screened for chlorsulfuron-resistant seedlings using an *in vitro* assay. Seeds were surface sterilised by immersion in 1% sodium hypochlorite (plus a drop of Tween 20) for 10 minutes, followed by 2-3 rinses with sterile water. Seeds were then sown onto the surface of chlorsulfuron supplemented nutrient medium consisting of MS salts (Murashige and Skoog 1962) at pH 5.8, solidified with 0.8% (w/v) Gibco bacteriological agar. This medium was autoclaved for 15 min at 103 kPa, to which filter-sterilised chlorsulfuron was added to a final concentration of 10 µg/litre, just prior to dispensing 50 ml of the medium into pre-sterilised plastic pottles (85 mm diameter x 35 mm high). Up to approximately 80 seeds were sown into each pottle, then germinated at 24-26°C under light from cool white fluorescent lamps (80-100 µmol/m²/sec; 16h light:8h darkness daily). Seedlings were scored for chlorsulfuron resistance after 10-14 days.

Using this *in vitro* seedling screen, chlorsulfuron-resistant rape seedlings could be easily identified by the elongation of their root system into the chlorsulfuron supplemented medium. Such seedlings generally developed as though there was no chlorsulfuron in the medium. A total of eight seedlings were identified in the M2 populations with apparent resistance to chlorsulfuron: one each from populations 19, 26, and 28; two from population 33; and three from population 30. These seedlings were transferred to soil and allowed to self pollinate in a greenhouse. Four of the plants set seed, and their seedling progeny were screened *in vitro* for resistance to chlorsulfuron. Two of the initially selected plants failed to transmit chlorsulfuron resistance to their progeny and therefore appear to have been escapes through the selection procedure. The remaining two plants (19 and 30) had a high frequency of chlorsulfuron-resistant progeny (see results). Seven randomly selected chlorsulfuron-resistant progeny from each of the segregating populations derived from mutant rape lines 19 and 30, were transferred to soil and allowed to self pollinate in a greenhouse. The resulting progeny (M4 populations) were again screened *in vitro* for resistance to chlorsulfuron.

Assessing whole plant resistance to chlorsulfuron

Seeds selected from M4 populations (19c and 30a) were germinated on moist filter paper and uniform seedlings transplanted one each into 10 cm diameter pots filled with 4 parts shredded bark: 1 part sand supplemented with a base dressing of fertiliser. When plants reached the four leaf stage, they were sprayed with chlorsulfuron at rates equivalent to 0-30 g/ha using a CO_2 pressurized knapsack sprayer capable of delivering 250 litres/ha at 120 kPa. The experiment involved five replicate plants at each chlorsulfuron dose using a randomized complete block design. Plants were grown for 4 weeks in a greenhouse, after which a visual assessment of their appearance was made (1= dead, 10= healthy). Shoot fresh and dry weights were measured.

Analysis of variance was performed on the data using the SAS statistical programme (SAS Institute Inc., Cary, NC, USA).

RESULTS AND DISCUSSION

Selection for chlorsulfuron-resistant mutants

The growth of wild-type rape seedlings on the *in vitro* medium supplemented with 10 µg/litre chlorsulfuron was severely suppressed after 7-10 days, and never developed beyond the cotyledon stage. The cotyledons developed curled margins and increased anthocyanin pigmentation on their abaxial surface. The seedlings had shortened hypocotyls, usually 2-3 cm, and never greater than 5 cm long. Their roots were also severely stunted (never exceeding 2 cm in length) and had curled apical tips and no branches. In contrast, seedlings grown in the absence of chlorsulfuron

maintained green cotyledons and readily initiated apical shoot elongation and true leaf development within 7-10 days. Their hypocotyls were 5-7 cm long, and their root systems 5-8 cm long with considerable root branching.

Inheritance of chlorsulfuron resistance

The M3 progeny of rape plants 19 and 30 showed clear segregation for seedlings that were chlorsulfuron-resistant and chlorsulfuron-susceptible (Table 1). Chi-square tests for goodness of fit confirmed that the observed frequencies of resistant versus susceptible individuals occurred in 3:1 segregation ratios expected if the originally selected rape plants were heterozygous for a single dominant mutation for chlorsulfuron resistance. As expected, chlorsulfuron-resistant plants (M4 populations) segregated for individuals that were either true breeding for chlorsulfuron resistance (homozygotes), or segregated for response to chlorsulfuron in 3:1 ratios of resistant and susceptible individuals (heterozygotes) (Table 1). The observed number of homozygous and heterozygous plants was consistent with the 1:2 ratio expected for a single dominant mutation conferring chlorsulfuron resistance (Chi-square = 0.04; P=0.95).

Whole plant resistance to chlorsulfuron

The growth of the mutant rape lines 19c and 30a (both homozygous for chlorsulfuron resistance) were compared to the original wild-type under greenhouse conditions. Similar results were obtained from the visual scores, and the fresh and dry weight determinations (Table 2). All three lines showed similar growth when untreated with the herbicide. The wild-type plants showed severe damage at all chlorsulfuron rates, even as low as 5 g/ha. In contrast, lines 19c and 30a both showed increased herbicide resistance, with all plants maintaining active growth following chlorsulfuron applications as high as 30 g/ha. However, some growth depreciation was observed in both mutant lines following chlorsulfuron application, which was more evident in line 19c. This was apparent as a slight chlorosis of younger leaves, with a suppression of apical growth and initiation of lateral bud development.

TABLE 1: Segregation of chlorsulfuron resistance among the progeny of the rape mutants (ns = non significance at the 5% probability level).

Rape line	Observed number of seedlings		Chi-square
	Resistant	Sensitive	for 3:1 ratio
M3 generation			
Wild-type	0	151	-
	0	143	-
19	53	23	1.12 ns
30	56	28	3.11 ns
M4 generation			
Wild-type	0	166	-
	0	139	-
	0	142	-
19a	37	16	0.76 ns
19b	26	10	0.15 ns
19c	149	0	-
19d	80	35	1.81 ns
19e	111	0	-
19f	105	0	-
19g	89	26	0.35 ns
30a	150	0	-
30b	93	0	-
30c	55	14	0.82 ns
30d	54	16	0.17 ns
30e	49	17	0.02 ns
30f	29	13	0.79 ns
30g	24	4	1.71 ns

TABLE 2: Response of rape lines 4 weeks after spraying with different rates of chlorsulfuron. For visual assessment 1=dead, 10=healthy. All other data are percent reductions in weight.

Growth parameter	Rape line	Chlorsulfuron rate (g ai/ha) 5	10	15	20	25	30
Visual assessment	Wild-type	2.8	2.0	1.4	1.6	1.4	1.8
SEM = 0.5	19c	6.4	7.2	5.0	5.8	5.2	5.0
	30a	9.8	8.8	6.8	6.2	6.8	5.6
Fresh weight	Wild-type	88.7	87.4	90.1	91.1	89.7	89.5
SEM = 6.1	19c	30.4	43.1	48.5	18.4	35.5	44.5
	30a	22.5	12.2	16.4	24.9	35.0	38.6
Dry weight	Wild-type	62.4	68.0	76.3	77.0	73.5	75.9
SEM = 6.5	19c	28.3	42.2	58.7	12.2	33.3	41.5
	30a	28.2	19.5	25.0	27.6	46.2	40.9

CONCLUSION

Using rapid cycling rape as an experimental system, this study has demonstrated the utility of seed mutagenesis as an approach for developing mutants of crop plants with increased resistance to sulfonylurea herbicides. Although the mutants described in this paper would have insufficient resistance to permit the application of sufficient herbicide to kill weeds without affecting the growth of rape plants, the level of resistance would allow the use of herbicide resistance as a genetic marker during seed production and permit some novel approaches to crop rotations and crop thinning (Field *et al.* 1993).

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