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RESEARCH ARTICLE

Herbicide-Resistant Mutants from Tobacco Cell Cultures

R. S. Chaleff and T. B. Ray

Abstract. *Several mutants resistant to the herbicides chlorsulfuron and sulfometuron methyl were isolated form cultured cells of* Nicotiana tabacum. *Resistance was inherited as a single dominant or semidominant mutation in all cases. Linkage analysis of six mutants identified two unlinked genetic loci. Studies of plants homozygous for one mutation showed the mutant plants to be completely resistant to treatment with a concentration of chlorsulfuron 100 times higher than that which produces symptoms of phytotoxicity on normal plants.*

In recent years, many novel plant mutants have been isolated by the application of selective growth conditions to cultured cells (*1*). However, few of the variant phenotypes that have been reported so far are of potential agronomic value. This limited practical success of mutant selection in vitro results to a large degree from the genetic and developmental complexity of agronomically important characteristics. Many such traits are exclusively whole plant functions that are not expressed and, therefore, cannot be selected at the cellular level (*2, 3*).

One trait of agronomic interest that may be expressed by cultured cells is herbicide sensitivity. Herbicides that interfere with basic metabolic activities can be expected to inhibit growth of cultured cells as well as of the whole plant. In such cases, herbicide-tolerant mutants can be selected by culturing cells in the presence of a herbicide concentration that is toxic to normal cells. The feasibility of this experimental approach was demonstrated by the isolation of tobacco mutants exhibiting increased tolerance for picloram. Tolerance was expressed by regenerated plants and was transmitted to progeny as a dominant or semidominant nuclear allele (*1, 4, 5*).

We now report the isolation from tobacco cell cultures of mutants resistant to chlorsulfuron and sulfometuron methyl. Chlorsulfuron and sulfometuron methyl (Fig. 1) are, respectively, the active ingredients in the herbicides Glean and Oust (Du Pont). Important features of these compounds are very high herbicidal activities and low mammalian toxicities. The demonstration that chlorsulfuron rapidly inhibits cell division in root tips (*6, 7*) suggested that this herbicide interferes with a basic cellular function and, therefore, could be used to select resistant cell lines in vitro.

Isolation and Genetic Characterization of Mutants

Callus cultures initiated from young leaves of a haploid plant (H1) obtained by culturing anthers of *Nicotiana tabacum* cv. 'Xanthi' (*8*) were maintained on C1 agar medium (*4*). Resistant cell lines were selected by transferring callus to medium supplemented with either chlorsulfuron or sulfometuron methyl at 2 ppb (approximately $5.6 \times 10^{-9} M$). In several experiments, callus cultures were maintained on medium containing 1 m*M* ethylnitrosourea for 3 weeks prior to selection. Isolates C3 and C4 were obtained from cultures that had been mutagenically treated in this manner. Resistant cell lines were propagated on selective medium for two additional passages of 3 weeks each before plants were regenerated as described (*4*). The letter preceding the isolate number indicates the compound on which that isolate was recovered (S for sulfometuron methyl and C for chlorsulfuron).

Crosses of fertile plants regenerated from several isolates were made. The results of crosses with a diploid plant regenerated from isolate S4 (Table 1) demonstrate that the regenerated plant was homozygous for a single dominant nuclear mutation conferring resistance to sulfometuron methyl. Similar series of crosses with plants regenerated from other isolates (S1, S4, S5, S6, S7, C1, C2, C3, C4, and C5) established that, in these cases, resistance likewise resulted from single dominant or semidominant nuclear mutations. Plants regenerated from only one isolate (C3) were heterozygous. Apparently, the mutation conferring resistance on isolate C3 arose after diploidization of a haploid cell of the parental H1 cell line. In all other

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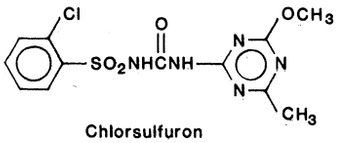
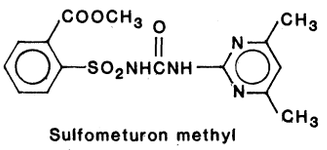
cases, mutation occurred either in a haploid cell that subsequently diploidized or in a diploid cell in which homozygosity was restored by somatic recombination.

Genetic analysis of mutant S4 was pursued further by self-fertilizing a heterozygous plant produced by two successive backcrosses of the regenerated mutant plant to normal plants. Callus cultures established from the resulting progeny were tested for growth on medium supplemented with sulfometuron methyl. In addition, seeds produced by self-fertilization of these plants were germinated on herbicide-supplemented medium. All 40 progeny of the *S4*/+ heterozygote that gave rise to resistant callus cultures also produced resistant progeny. Only sensitive seeds were obtained from 16 progeny that gave rise to sensitive callus cultures. Cosegregation of resistances of the derivative callus cultures and of the seedlings indicates that resistances at both levels of differentiation result from the same mutation. Self-fertilization of the progeny of the *S4*/+ heterozygote also enabled homozygous and heterozygous individuals to be distinguished. Of the 56 progeny examined in this manner, 12 proved to be homozygous mutant, 28 were heterozygous, and 16 were homozygous normal plants. These results are consistent with the expected segregation ratio of 1:2:1.



Chlorsulfuron



Sulfometuron methyl

Fig. 1. Chemical structures of chlorsulfuron and sulfometuron methyl.

Linkage analysis of six mutations was performed by first crossing two homozygous mutant plants to construct individuals heterozygous for two mutant alleles. These doubly heterozygous plants were then crossed with normal plants. If two mutations are allelic (or closely linked), only resistant progeny will be obtained from the testcross. However, if two mutations are unlinked, one-fourth of the progeny of the testcross will be sensitive. The linkage data (Table 2) establish that the six mutations are located in two distinct genetic regions and therefore represent at least that number of genetic loci. Mutations S1, S5, S6, and C3 define one region and mutations S4 and C4 define a second region.

It is interesting that no recessive mutations were obtained in these experiments. Because the parental cell line H1 was initiated from a haploid plant and all but one of the mutants recovered were homozygous, presumably the isolation of recessive mutations was possible in this system. It appears, therefore, that either there is no mechanism by which resistance to these herbicides can be achieved by a recessive mutation or that the frequency of such mutations is much lower than that of dominant mutations.

All the resistant isolates that were analyzed genetically proved to be true mutants. That is, in all cases resistance was transmitted through sexual crosses in accord with conventional inheritance patterns. Moreover, in all cases the resistance phenotype was expressed at both the callus and seedling stages. These findings contrast with the results of previous experiments in which resistance to the herbicide picloram was selected (*4*). In these earlier studies, resistant callus cultures were produced by plants from only five of six resistant cell lines from which plants were regenerated. Plants regenerated from one picloram-resistant cell line gave rise only to sensitive callus and germination of seeds produced by plants regenerated from another cell line was sensitive to the herbicide.

Table 1. Segregation among progeny of crosses with a mutant S4 regenerated plant. A plant regenerated from the resistant cell line S4 is designated R. Normal plants (N) were grown from seeds of the parent, *N. tabacum* cv. 'Xanthi.' Seeds were surface-sterilized (*4*) and plated on both unsupplemented control medium and selective medium containing sulfometuron methyl at 200 ppb. Media consisted of one-half the concentration of Murashige and Skoog (*10*) salts and 100 mg of myoinositol, 10 g of sucrose, and 16 g of potato dextrose agar per liter.

Cross	Number of Individuals			
	Resistant		Sensitive	
	Observed	(Expected)	Observed	(Expected)
N selfed	0		459	
R selfed	176		0	
R × N	311		0	
N × R	52		0	
(R × N) × N	39	(38)	37	(38)
(R × N) selfed	100	(100.5)	34	(33.5)

Table 2. Linkage analysis.

Cross	Number of individuals				Cross	Number of individuals			
	Resistant		Sensitive			Resistant		Sensitive	
	Obs.	(Exp.)	Obs.	(Exp.)		Obs.	(Exp.)	Obs.	(Exp.)
+/+ × (*S1*/*S1* × *S6*/*S6*)	109		2*		(*C3*/*C3* × *S5*/*S5*) × +/+	220		7*	
(*S1*/*S1* × *S6*/*S6*) × +/+	54		2*		(*C4*/*C4* × *C3*/*C3*) × +/+	211	(215.25)	76	(71.25)
+/+ × (*S1*/*S1* × *S5*/*S5*)	109		6*		+/+ × (*C4*/*C4* × *S6*/*S6*)	48	(44.25)	11	(14.75)
(*S1*/*S1* × *S5*/*S5*) × +/+	58		1*		(*C4*/*C4* × *S6*/*S6*) × +/+	39	(40.5)	15	(13.5)
+/+ × (*S4*/*S4* × *S1*/*S1*)	42	(41.25)	13	(13.75)	+/+ × (*S4*/*S4* × *C4*/*C4*)	50		0	
(*S4*/*S4* × *S1*/*S1*) × +/+	93	(91.5)	29	(30.5)	(*S4*/*S4* × *C4*/*C4*) × +/+	120		0	
+/+ × (*S5*/*S5* × *S6*/*S6*)	38		0		(*S5*/*S5* × *C4*/*C4*) × +/+	58	(60)	22	(20)
(*S5*/*S5* × *S6*/*S6*) × +/+	61		0						
+/+ × (*C3*/*C3* × *S4*/*S4*)	78	(77.25)	25	(25.75)					
(*C3*/*C3* × *S4*/*S4*) × +/+	212	(219)	80	(73)					

*Similar numbers of poorly growing individuals were observed among control populations plated on nonselective medium. Therefore, these poorly growing individuals appearing on herbicide-supplemented medium probably do not represent herbicide-sensitive individuals arising by either independent assortment or recombination between two mutations conferring herbicide-resistance, but segregation of an independent lethal mutation that is unrelated to the seedling response to the herbicide.

Table 3. Growth responses to chlorsulfuron of cell lines derived from isolates produced by self-fertilization of an *S4/+* heterozygote. Approximately 50 mg of callus tissue was spread on a 7-cm diameter filter disk (Whatman No. 1) placed on the surface of the medium in each petri dish. Cultures were incubated for 14 days at 25° ± 1°C on a 16-hour light/8-hour dark cycle. Tissue was then scraped from each filter disk and weighed. Final fresh weights are presented in milligrams as the means and standard errors of the means for *N* cultures. Differences of means larger than two standard deviations are regarded as significant.

Chlorsulfuron (ppb)	Isolate 1 (+/+)			Isolate 7 (+/+)			Isolate 23 (*S4*/+)			Isolate 24 (*S4*/+)			Isolate 16 (*S4*/*S4*)			Isolate 30 (*S4*/*S4*)		
	Weight (mg)	Percent	*N*	Weight (mg)	Percent	*N*	Weight (mg)	Percent	*N*	Weight (mg)	Percent	*N*	Weight (mg)	Percent	*N*	Weight (mg)	Percent	*N*
0	2982 ± 173	100	10	2332 ± 111	100	10	2670 ± 115	100	25	2874 ± 164	100	22	3286 ± 198	100	18	2752 ± 205	100	10
0.1	2287 ± 198	76.7	10	2170 ± 94	93.1	10	2803 ± 153	105.0	10	2895 ± 187	100.7	16	3755 ± 259	114.3	10	3235 ± 159	117.6	10
0.3	1204 ± 107	40.4	9	1395 ± 116	59.8	8	2559 ± 174	95.8	20	2842 ± 124	98.9	19	3778 ± 219	115.0	12	3157 ± 76	114.7	8
1.0	106 ± 9	3.6	9	215 ± 33	9.2	10	2505 ± 134	93.8	18	2515 ± 155	87.5	10	3148 ± 196	95.8	13	2707 ± 250	98.4	10
3.0	55 ± 3	1.8	9	58 ± 4	2.5	10	2401 ± 218	89.9	10	2099 ± 129	73.0	18	2958 ± 161	90.0	11	3287 ± 136	119.4	10
10.0							1537 ± 90	57.6	15	2196 ± 235	76.4	12	3171 ± 260	96.5	18	2975 ± 139	108.1	10
30.0							749 ± 54	28.1	15	1585 ± 129	55.1	12	2625 ± 202	79.9	17	2486 ± 170	90.3	10

Resistance at the Cellular Level

The occurrence of spontaneous genetic variability in cultured plant cells has been extensively documented (*1*, *3*). Because of the frequency of this phenomenon, a variant phenotype cannot be attributed solely to a single genetic event. Rather, a given phenotype that is conditioned primarily by one mutation is probably modified by additional mutations accumulated during maintenance of the cell line in culture. For example, two isolates produced by self-fertilization of a plant heterozygous for a mutation conferring picloram resistance, although both homozygous for the same *PmR* mutation, displayed different degrees of resistance to this herbicide (*4*). Accordingly, in our studies two precautions were taken in evaluating the growth responses of mutant cell lines to chlorsulfuron. First, to eliminate some of the background variability that might other-

Table 4. Relative resistances of several homozygous mutant cell lines to sulfometuron methyl and chlorsulfuron. Data are means and standard errors of means of fresh weights of *N* cultures. With the exception of S4, mutant callus cultures were established from R1 plants (progeny produced by self-fertilization of a regenerated plant). The S4 mutant cell line was initiated from isolate 16 (see Table 3). All mutant cell lines are homozygous. H1 was derived from the same haploid plant from which the callus cultures used in all the mutant isolation experiments originated.

Cell line	Control		Sulfometuron methyl at 10 ppb			Chlorsulfuron at 10 ppb		
	Weight (mg)	*N*	Weight (mg)	Percent	*N*	Weight (mg)	Percent	*N*
H1	2573 ± 126	7	32 ± 2	1.2	9	33 ± 2	1.3	9
S1/S1	2676 ± 90	8	172 ± 14	6.4	10	309 ± 34	11.5	10
S4/S4	3286 ± 198	18	1105 ± 80	33.6	15	3171 ± 260	96.5	18
S5/S5	2639 ± 129	8	344 ± 55	13.0	10	725 ± 95	27.5	10
S6/S6	3348 ± 159	9	887 ± 92	26.5	10	1076 ± 108	32.1	10
C3/C3	2186 ± 165	8	1369 ± 63	62.6	10	1896 ± 119	86.7	10
C4/C4	4137 ± 254	9	3473 ± 323	83.9	10	3453 ± 231	83.5	10

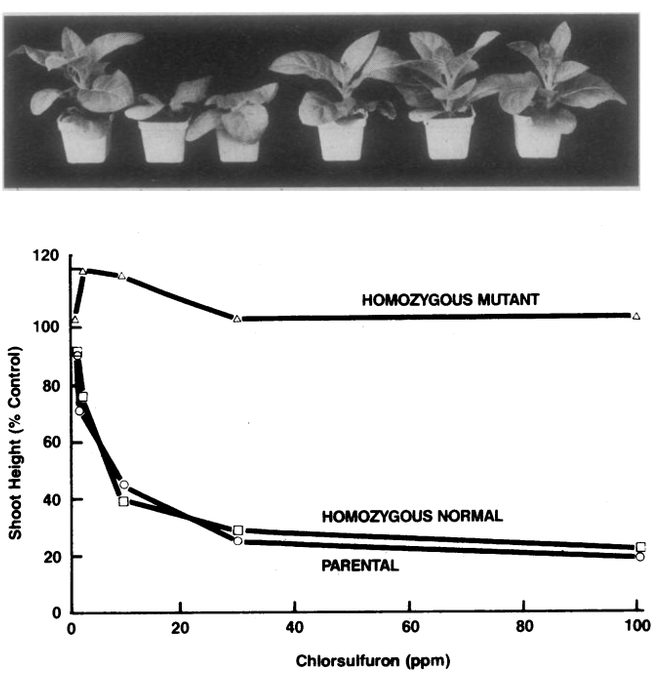


Fig. 2 (top). Effects of foliar application of chlorsulfuron to normal (+/+) and homozygous mutant (*S4/S4*) tobacco plants. Left to right; normal control; normal, treatment with chlorsulfuron at 30 ppm; normal, treatment at 100 ppm; mutant control; mutant, treatment at 30 ppm; mutant, treatment at 100 ppm. At the four- to five-leaf stage, each plant was sprayed with 5 ml of an aqueous solution containing the indicated concentration of chlorsulfuron, 0.2 percent Tween 20, and 10 percent (by volume) acetone. Controls received 5 ml of a solution lacking chlorsulfuron. Sensitive plants are progeny of a normal segregant (isolate 1) and resistant plants are progeny of a homozygous mutant segregant (isolate 16) obtained by self-fertilization of a heterozygous mutant individual (*S4/+*). The photograph was taken 2 weeks after treatment. Fig. 3 (bottom). Growth responses of normal (+/+) and homozygous mutant (*S4/S4*) tobacco plants to a foliar application of chlorsulfuron. Normal and mutant plants are progeny of isolates 1 and 16, respectively, as explained in the text and the legend to Fig. 2. Plants designated "parental" are from seeds of the *N. tabacum* cv. 'Xanthi' line from which cell cultures were established. Plants were sprayed as described in the legend to Fig. 2. Shoot height was measured 2 weeks after treatment. Each point represents the average height of four plants. Average heights of untreated control plants were: parental, 11.1 ± 0.8 cm; normal, 10.1 ± 0.7 cm; mutant, 9.2 ± 0.8 cm. At no point did the standard error of the mean exceed 10 percent of the mean.

wise confuse analysis of the effect of the primary mutation, the cell lines assayed were established from plants obtained by self-fertilization of a *S4/+* heterozygote produced by two successive backcrosses of a regenerated mutant plant to normal plants. Second, growth responses were determined for cell cultures initiated from two individuals of the same genotype with respect to the S4 mutation. The genotypes of these cell lines were confirmed by progeny analyses of the plants from which they were derived. The results of these growth tests are presented in Table 3.

Although the S4 mutation was selected on the basis of resistance to sulfometuron methyl, it was soon recognized that this mutation confers an even higher degree of resistance to chlorsulfuron. Therefore, chlorsulfuron was used in the growth tests. Because of the structural similarity of these two sulfonylurea herbicides (Fig. 1), this cross-resistance is not surprising. Cosegregation of resistances to chlorsulfuron and sulfometuron methyl demonstrated that both resistances result from the same (or closely linked) mutation (or mutations). Callus cultures were established from 34 progeny of a testcross of a *S4/+* heterozygote. Of these cultures, 8 were resistant and 16 were sensitive to both herbicides. None of the cultures was only resistant to either one or the other compound.

Some differences were observed between the growth responses of cell lines derived from individuals of the same genotype with respect to the S4 mutation (Table 3). For example, growth of one normal cell line (isolate 1) was significantly inhibited by chlorsulfuron at 0.1 ppb, whereas growth of the other normal cell line (isolate 7) was not. In general, isolate 1 appeared to be more sensitive to the herbicide than isolate 7; in contrast, the two homozygous mutant cell lines responded similarly to chlorsulfuron. Growth of neither homozygous mutant cell line was significantly inhibited by concentrations of chlorsulfuron below 30 ppb. Because growth of the homozygous mutant lines is less severely affected by chlorsulfuron at 30 ppb than is growth of the cell line derived from isolate 7 (the more tolerant of the two normal lines) by 0.3 ppb, it appears that the S4 mutation in a homozygous state increases the tolerance of cultured cells for chlorsulfuron at least 100 times. Both heterozygous lines are more sensitive to the herbicide than are the homozygous mutant lines (especially evident at higher concentrations). This result indicates that the S4 mutation is semidominant.

Notable phenotypic differences between callus cultures derived from several mutant isolates are revealed by their relative resistances to chlorsulfuron and sulfometuron methyl (Table 4). As was already mentioned, mutant S4 is more resistant to chlorsulfuron than to sulfometuron methyl, the compound on which it was isolated. Mutant C4, which is genetically linked to S4 but is of independent origin and was selected on the basis of resistance to chlorsulfuron, displays the same high degree of resistance to both compounds. Although mutants S1, S4, S5, and S6 were isolated in the same experiment, the different degrees of resistance shown by these mutants suggest that they arose independently. These data indicate that there is no correlation between the resistance phenotypes of the mutants and their genetic linkage relationships.

Resistance at the Whole Plant Level

The seedling growth assay used in scoring crosses gave the first indication that mutations selected at the cellular level for resistance to chlorsulfuron and sulfometuron methyl were expressed by the whole plant. Sensitive seeds germinated on selective medium but were bleached and did not develop roots or leaves. In contrast, resistant seedlings were green and developed normally on this medium, albeit at a slower rate than in the absence of the herbicide.

Further studies on the effect of the S4 mutation on the whole plant response to chlorsulfuron were conducted with plants of the parental variety and progeny of homozygous mutant and homozygous normal isolates (No. 16 and No. 1, respectively) of the previously described backcross program. Accordingly, plants of the latter two types were derived, but separated by four generations, from the initial S4 mutant plant regenerated from callus culture.

Plants at the four- to five-leaf stage were treated with foliar sprays of several chlorsulfuron concentrations between 1 and 100 ppm. Two weeks after treatment the plants were evaluated for phytotoxicity, and the shoot heights were measured. Both parental and homozygous normal plants developed symptoms of phytotoxicity in response to the treatment with chlorsulfuron at 1 ppm. At this lowest concentration, the apical buds and youngest leaves of these sensitive plants were chlorotic. At 3 ppm, chlorosis was more severe and growth retardation was readily apparent. These symptoms were more pronounced with increasing chlorsulfuron concentrations. Sensitive plants treated with chlorsulfuron at 100 ppm showed extreme growth inhibition, chlorosis, and necrosis (Fig. 2). In contrast, homozygous mutant plants were unaffected by treatment with chlorsulfuron, even at the highest concentration of 100 ppm (Fig. 2). Measurements of shoot height (Fig. 3) show that growth of homozygous normal and parental plants was significantly inhibited by chlorsulfuron at 3 ppm, whereas treatment at 100 ppm had no effect on growth of homozygous mutant plants. The appearance of phytotoxicity symptoms on sensitive plants treated with chlorsulfuron at 1 ppm but not on homozygous mutant plants treated with 100 ppm indicates that the S4 mutation in a homozygous state confers at least a 100-fold increase in tolerance for the herbicide upon the whole plant. Thus, the degree of tolerance conferred by the S4 mutation on the whole plant corresponds well with the effect of this mutation on cultured cells (Table 3). The magnitude of this effect is sufficiently large to be properly referred to as resistance (*9*).

Although detailed studies of only one mutation (S4) are reported, additional mutations conferring resistance to chlorsulfuron and sulfometuron methyl were isolated in these experiments. Seedling growth responses, which were used as a basis for scoring genetic crosses, indicate that all these mutations can be expressed in the whole plant. Resistance of all isolates studied to date results from single genetically stable dominant or semidominant nuclear mutations.

Our experimental approach affords an alternative means for accomplishing the desired differential effect of a herbicide on the weed and crop species. In contrast to the traditional method of screening chemical compounds for specificity of herbicidal activity, we used cell culture to screen genomes to identify tolerant mutant forms of the crop species. This approach would both facilitate the introduction of new herbicides and expand the spectrum of applicability of existing ones.

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