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Genetics

Direct selection *in vitro* for herbicide-resistant mutants of *Nicotiana tabacum*

(cell culture/picloram tolerance/plant regeneration/Mendelian inheritance)

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ABSTRACT **Seven cell lines of *Nicotiana tabacum* resistant to the herbicide picloram were isolated from cell cultures. In crosses of plants regenerated from four cell lines, resistance was inherited as a single dominant Mendelian allele. Plants could not be regenerated from one cell line, and expression of resistance proved unstable in two others.**

In recent years plant cell culture techniques have opened a new sphere of genetic experimentation by making available for mutant selection large populations of cells from which plants can be regenerated. The promise that utilization of these methods offers for exploring basic questions of plant organization and function and for improving the performance and quality of crop species has been recognized widely. Nevertheless, although many variant cell lines have been isolated, in but a few cases have plants been regenerated and a genetic basis for the altered phenotype established (1–3).

We now report the selection among cultured cells of *Nicotiana tabacum* of clones resistant to the herbicide picloram (4-amino-3,5,6-trichloropicolinic acid) and the genetic characterization of mutant plants regenerated from such clones. Tobacco and most broad-leaved plants are very sensitive to picloram (4). It is hoped that picloram-tolerant mutants will prove useful both agronomically and in elucidating the mechanisms of action of this herbicide.

MATERIALS AND METHODS

Plant Material and Conditions of Culture. Seeds of *N. tabacum* Linnaeus cv. Xanthi (obtained from the Connecticut Agricultural Experiment Station, New Haven, CT) were surface-sterilized by immersion for 15 min in a solution of 10% Clorox and 0.1% sodium dodecyl sulfate. After being rinsed thoroughly with sterile distilled water, seeds were germinated on a Linsmaier and Skoog (5) medium containing 2% sucrose, 0.8% agar, and no hormones. Plants were grown aseptically on 50 ml of this same medium in 250-ml flasks. All media contained this same basic formulation, but differed in sucrose concentration and hormone composition. Callus cultures were initiated by macerating leaves from sterile plants on a medium supplemented with 3% sucrose, 2.0 mg of α-naphthaleneacetic acid, and 0.3 mg of kinetin per liter (C1 medium). C1 medium also was used for maintenance of callus and suspension (agar omitted) cultures. Shoot formation was induced by transferring callus to an agar medium containing 3% sucrose and 0.3 mg of indole-3-acetic acid and 3.0 mg of 6-(γ,γ-dimethylallylamino)purine per liter (shoot-induction medium). Shoot formation usually required several passages (three weeks per passage) on this medium. Root formation was accomplished on a medium containing 3% sucrose and supplemented with 0.1 mg of naphthaleneacetic acid per liter. Plantlets were grown to a height of 5–8 cm on a hormone-free medium containing 1% sucrose before transfer to soil.

Concentrated aqueous solutions of picloram, indoleacetic acid, and naphthaleneacetic acid were adjusted to pH 6.0 with KOH. Kinetin and dimethylallylaminopurine were dissolved in 0.1 M KOH. Picloram and hormone solutions were sterilized by filtration and added to autoclaved medium.

Chemicals. Naphthaleneacetic acid, indoleacetic acid, dimethylallylaminopurine, and kinetin were purchased from Sigma Chemical Company (St. Louis, MO). Picloram (analytical standard, 99% pure) was generously provided by the Dow Chemical Company (Midland, MI).

Cell Plating and Mutant Selection. Suspension cultures were filtered through cheesecloth and the filtrate was centrifuged for 10 min at 150 × *g*. The supernatant then was decanted and the cells were resuspended in an equal volume of fresh C1 liquid medium. Suspensions formed from several similar cultures were pooled, and 2 ml of this suspension was pipetted into each petri dish (9 cm) containing either C1 medium (control) or C1 medium supplemented with 500 μM picloram (selective medium). Plates were sealed with Parafilm and incubated at 25 ± 1°C under Gro-lux fluorescent lamps (16 hr/day). Resistant calluses appeared between 1 and 2 months later. Calluses that continued to grow during a second passage on selective medium were transferred to shoot-induction medium.

Scoring of Crosses. Seeds were surface-sterilized and plated on control (2% sucrose without hormones) and selective (supplemented with 100 μM picloram) media. In all crosses germination exceeded 99%. Normal seeds germinated on selective medium, but development was severely inhibited. After 14 days seedlings were stunted, swollen, and bleached. Although resistant seedlings did not grow as well on selective as on control medium, they could be distinguished readily from sensitive seedlings on selective medium by formation of roots and greening of the cotyledons.

Growth Tests. Suspension cultures were not used for growth tests because of the altered growth habit of callus derived from mutant plants and the effect of picloram on this growth habit. When grown on unsupplemented C1 medium, mutant callus is less friable than normal callus, but it becomes progressively more friable on media containing increasing concentrations of picloram. Because it was expected that the degree of dispersion of the cells would influence the growth rate of suspension cultures, growth tests were performed on callus cultures. These callus cultures were obtained from F_1 plants produced by the selfing of *PmR1*/+ (the plant regenerated from the originally selected mutant cell line). Cultures used as a source of inocula were initiated and maintained on unsupplemented C1 medium and transferred to fresh medium 3 days prior to initiation of the growth tests. A concentrated picloram solution was diluted serially, filter-sterilized, and added to C1 medium containing 0.6% agar. A sterile 5.5-cm diameter filter disc (Whatman no.

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1) was placed on the surface of the medium in each petri dish and approximately 50 mg of tissue was spread on the filter. After 2 weeks at 25 ± 1°C the filter was transferred to a Buchner funnel and rinsed thoroughly with distilled water. The callus then was scraped from the surface of the filter and weighed.

Nomenclature of Crosses. In this paper the term F_1 has been used to designate progeny resulting from the first cross of a heterozygous mutant plant regenerated from cell culture. Because this term usually refers to heterozygous progeny produced by crossing two homozygous parents, the authors recognize that to many this usage may seem inappropriate and even confusing. This difficulty makes apparent the need for defining a new terminology to describe a heterozygous plant regenerated from culture and its progeny. However, until general discussion and agreement on the introduction of new terminology, we have opted for the familiar nomenclature employed in this paper.

RESULTS

Mutation Frequency. Several features of the growth of plant cells in culture make it difficult to estimate the frequency of spontaneous mutation from picloram sensitivity to picloram resistance. Cultured tobacco cells grow as aggregates and even following filtration through cheesecloth the number of cells per aggregate is too large to permit an accurate count of the initial cell number. In addition, because growth of nonmutant cells is greatly but not completely impaired by 500 μM picloram (Table 1), mutants appear at a total frequency that is the sum of the mutation frequency (mutations present in the initial cell population) and rate (mutations occurring per generation). Furthermore, without a genetic analysis of regenerated plants, the number of resistant cell lines that are true mutants is not known. Also unknown is the number of loci at which mutations can produce the resistance phenotype.

In one experiment the approximate size of the initial cell population was determined by counting (as best we could) the number of cells in an aliquot of the suspension culture. The cell cultures employed in this particular experiment were derived from a haploid plant that had been produced by anther culture (6). From an initial population of 2.7×10^6 cells, 54 resistant cell lines were isolated. This number represents only those cell lines that continued to exhibit resistance during a second passage on medium supplemented with 500 μM picloram. If it is assumed that all of these resistant cell lines resulted from independent mutations and that the contribution of the mutation rate is negligible, a spontaneous mutation frequency of 2×10^{-5} can be calculated. Of course this frequency may represent the sum of the mutation frequencies for more than one genetic locus and is based on the assumption that resistance occurs only by genetic events and not by epigenetic events as well.

Genetic Characterization of Mutants. In the first plating of cell cultures derived from a diploid plant on medium containing 500 μM picloram, seven resistant cell lines (CL1–7) were isolated. The characterization of these cell lines exemplifies some of the difficulties encountered in plant cell genetics research. More than ten plants were regenerated from each of six resistant cell lines, but no plants could be regenerated from CL4. Callus cultures initiated from leaves of regenerated plants were tested for resistance to growth inhibition on medium containing 500 μM picloram. Callus derived from plants regenerated from five cell lines continued to exhibit herbicide resistance. Despite the fact that CL3 was stably resistant to picloram, even after several passages in its absence, resistance was not expressed by callus cultures obtained from ten plants regenerated from this cell line.

Because cell cultures derived from diploid plants were used in the initial mutant isolation experiment, regenerated plants would be expected to be heterozygous for a dominant allele. These alleles have been assigned the general symbol *PmR* (for picloram resistance) followed by the number of the originally isolated resistant cell line. This notation was adopted for the sake of clarity even though it is not yet known whether the mutations arose independently or whether more than a single gene is involved. Thus, putatively heterozygous diploid plants regenerated from CL1 are designated *PmR1/+*.

Regenerated plants that gave rise to resistant callus were selfed and backcrossed. The segregation of resistance and sensitivity among the progeny was determined by plating surface-sterilized seeds on medium supplemented with 100 μM picloram. No resistance was detected among seeds produced by the self-fertilization of *PmR5/+*. However, germination of seeds produced by crosses of *PmR1/+*, *PmR2/+*, *PmR6/+*, and *PmR7/+* showed some degree of resistance to picloram. The numbers of resistant and sensitive progeny resulting from these crosses are presented in Table 2. These data are consistent with the ratios 3 resistant to 1 sensitive and 1 resistant to 1 sensitive that are expected from self- and backcrosses, respectively, of a heterozygous individual in the case in which resistance is conferred by a dominant allele of a single nuclear gene.

In platings on a higher picloram concentration (500 μM) of seeds produced from crosses of *PmR6/+*, two distinct degrees of resistance to picloram were observed. In seedlings of one class (resistant), root and cotyledon development was affected less by picloram than in those of the other (intermediate) class. In one plating of 242 seeds, 64 proved resistant, 117 intermediate, and 61 sensitive. From these results it is evident that *PmR6* is not completely dominant to the normal allele.

Table 1. Growth response to picloram of callus derived from F_1 isolates produced by self-fertilization of *PmR1/+*

Picloram, μM	Normal (+/+)			Isolate 18 (+/+)			Isolate 3 (*PmR1/+*)			Isolate 4 (*PmR1/PmR1*)			Isolate 11 (*PmR1/PmR1*)		
	Wt, mg	%	*n*	Wt, mg	%	*n*	Wt, mg	%	*n*	Wt, mg	%	*n*	Wt, mg	%	*n*
0	1471 ± 68	100	5	1295 ± 64	100	7	2301 ± 388	100	13	1381 ± 63	100	8	1160 ± 85	100	6
10	457 ± 32	31.1	5	271 ± 18	20.9	3	1894 ± 309	82.3	5	1289 ± 104	93.3	5	—	—	—
50	378 ± 27	25.7	4	246 ± 7	19.0	3	1944 ± 187	84.5	8	1224 ± 111	88.6	10	1094 ± 80	94.3	3
100	335 ± 20	22.8	5	232 ± 15	17.9	3	1494 ± 166	64.9	8	1276 ± 89	92.4	10	—	—	—
200	266 ± 17	18.1	3	207 ± 16	16.0	5	1702 ± 166	74.0	11	1554 ± 71	112.5	6	881 ± 121	76.0	6
500	219 ± 10	14.9	5	161 ± 14	12.4	5	1677 ± 113	72.9	6	1134 ± 121	82.1	5	—	—	—
1000	48 ± 2	3.2	5	48 ± 4	3.7	4	495 ± 114	21.5	7	464 ± 44	33.6	9	341 ± 69	29.4	4

Petri dishes were inoculated with approximately 50 mg of callus. After 14 days at 25 ± 1°C, cultures were washed with distilled water and weighed. Final fresh weights are given as means and standard errors of means for *n* cultures. The relative response to picloram of each cell line is calculated as percent of growth (mean final fresh weight) in the absence of picloram.

Table 2. Segregation among progeny derived from crosses of plants regenerated from several picloram-resistant cell lines

Cross	Number of individuals: Resistant Observed	Resistant Expected	Sensitive Observed	Sensitive Expected	P
+/+ (from seed) selfed	0		503		
+/+ (from callus) selfed	0		154		
PmR1/+ selfed	380	380	127	127	1.00
PmR1/+ × +/+	27	30	33	30	0.44
+/+ × *PmR1*/+	79	82	85	82	0.64
PmR2/+ selfed	308	311	106	104	0.79
PmR6/+ selfed	132	130	41	43	0.73
PmR6/+ × +/+	67	70	72	70	0.66
PmR7/+ selfed	488	490	165	163	0.86

Surface-sterilized seeds were plated on medium supplemented with 100 μM picloram. Plates were scored after 14 days at 25 ± 1°C. The probability *P* that observed = expected was determined from a χ^2 calculation.

No resistant progeny were produced by a plant regenerated from a normal callus culture that had not been selected for resistance to picloram (Table 2). Therefore, it is evident that resistance among seeds of *PmR*/+ plants does not result merely from passage through culture, but is a consequence of a rare event for which selection is necessary.

F_1 progeny produced by selfing *PmR1*/+ were self-fertilized, and the seeds were plated on selective medium to determine the F_2 segregation patterns. The results in Table 3 demonstrate that of 22 F_1 plants, 5 were homozygous mutant (*PmR1*/*PmR1*), 6 were homozygous normal (+/+), and 11 were heterozygous (*PmR1*/+). The fit of the composition of the F_1 to the theoretical pattern 1:2:1 confirms that the original regenerated plant was heterozygous for a dominant resistance allele. Furthermore, the recovery of the expected number of homozygous mutant individuals shows that, at least under laboratory conditions, the *PmR1* mutation does not reduce viability.

Additional evidence for Mendelian inheritance is provided by the results of plating on selective medium seeds obtained from selfing several plants produced by backcrosses. These experiments demonstrated that both heterozygous and homozygous normal individuals were produced in the crosses +/+ × *PmR1*/+ (4 +/+ and 1 *PmR1*/+) and *PmR6*/+ × +/+ (4 +/+ and 2 *PmR6*/+).

Table 3. F_2 progeny of random F_1 isolates obtained from self-fertilization of *PmR1*/+

F_1 isolate	Number of F_2 individuals: Resistant	Sensitive	Presumed genotype
1	405	126	*PmR1*/+
2	186	71	*PmR1*/+
3	358	134	*PmR1*/+
4	330	0	*PmR1*/*PmR1*
5	0	118	+/+
6	213	71	*PmR1*/+
7	0	247	+/+
8	0	312	+/+
9	0	324	+/+
10	146	0	*PmR1*/*PmR1*
11	408	0	*PmR1*/*PmR1*
12	461	0	*PmR1*/*PmR1*
13	418	155	*PmR1*/+
14	0	316	+/+
15	347	118	*PmR1*/+
16	198	79	*PmR1*/+
17	303	83	*PmR1*/+
18	0	333	+/+
19	343	151	*PmR1*/+
20	222	0	*PmR1*/*PmR1*
21	372	107	*PmR1*/+
22	245	92	*PmR1*/+

Because plant cells accumulate chromosomal abnormalities in culture (7, 8), it is desirable to determine the chromosome number of plants regenerated from selected cell lines. However, inasmuch as plants regenerated from cell lines that have not been cloned may be chimeral, chromosomes of F_1 rather than of regenerated plants were counted. A normal diploid complement ($2n = 48$) was observed in metaphase plates in corolla tissue prepared from two heterozygous F_1 progeny (isolates 17 and 22) of *PmR1*/+.

Growth Tests. The degree of resistance conferred by the *PmR1* allele was examined by means of growth tests of callus cultures derived from F_1 plants. The genotypes of these plants were determined from segregation patterns of progeny seeds plated on picloram-supplemented medium (Table 3). A callus culture produced from a normal plant grown from seeds (not regenerated from culture or involved in crosses with mutants) also was included in these experiments. It is clear from the final fresh weight of cultures grown in the absence or picloram that the *PmR1* mutation has no deleterious effect on the growth rate (Table 1). It also is evident that resistance to growth inhibition expressed by derivative callus cultures corresponds to resistance to inhibition of seed germination. That is, resistance to picloram was exhibited by callus cultures initiated from those plants that produced resistant seeds (isolates 3, 4, and 11) and not by those that produced sensitive seeds (normal and isolate 18).

The growth response to increasing picloram concentrations of callus obtained from a normal F_1 segregant (isolate 18) is nearly identical to that of callus obtained from a normal seed-derived plant. Growth of both callus cultures is inhibited to 70% or more by only 10 μM picloram and completely by 1 mM. In contrast, severe growth inhibition of cultures obtained from mutant plants is not observed at picloram concentrations below 1 mM. Growth of callus derived from one homozygous mutant plant (isolate 4) appears more resistant to picloram than does that of callus derived from a heterozygous plant (isolate 3). However, the response of callus cultures obtained from another homozygous mutant segregant (isolate 11) is more similar to the response of cultures derived from isolate 3 than to that of cultures derived from isolate 4. These results suggest that the growth response of these callus cultures to picloram may not be conditioned solely by the *PmR1* allele and that the parent

plant from which these segregants were obtained could have been heterozygous for alleles at other loci that also influenced the degree of picloram resistance expressed in culture. From these data it appears that *PmR1* is completely dominant over the normal allele. However, an additional possibility to be considered is that alleles of other loci (either present initially in that plant from which the original cell suspensions were derived or accumulated during maintenance of callus in culture) can modify the effect of *PmR1* and that these also were segregating in the cross.

DISCUSSION

Despite their potential utility for selecting mutants of higher plants, cell culture techniques so far have provided few agronomically beneficial variants. Moreover, in but few of the reported instances has a genetic analysis been performed to characterize the genetic nature of the altered phenotype (1, 3, 9). Selection for increased herbicide tolerance provides an experimental system that explores the applicability of cell culture at once both to problems of immediate practical importance and to more fundamental genetic and physiological studies. The selection of cell lines of white clover (*Trifolium repens*) for greater tolerance of several phenoxy herbicides has been reported (10). Cell lines of *N. tabacum* resistant to amitrole (3-amino-1,2,4-triazole) also have been isolated. Although several plantlets were regenerated from the amitrole-resistant cell lines, no study of heritability or of expression of resistance in the whole plant had been made at the time of the report (11).

In the present study, cell lines of *N. tabacum* were selected for resistance to the herbicide picloram and regenerated plants were characterized genetically. These experiments illustrate some of the promise and problems that cell culture offers as a method for selecting mutants in higher plants. Of seven resistant cell lines initially isolated, only four gave rise to plants in which resistance proved stable and heritable. Plants could not be regenerated from one cell line. A second cell line, although stably resistant in culture even after many passages in the absence of picloram, gave rise to plants from which only sensitive callus cultures could be recovered. This result demonstrates that stability of a variant phenotype in culture in the absence of selection is not an adequate criterion for defining the genetic basis for the altered phenotype and indeed may not distinguish between genetic and epigenetic events.

Resistant callus cultures were recovered from plants regenerated from a third cell line (CL5). However, germination of seeds from these plants was not resistant to the herbicide. The failure to obtain resistant seeds could mean that even expression of an altered phenotype in secondary cultures derived from regenerated plants is not indicative of a genetic change. Alternatively, it is possible that resistance in this cell line is due to a mutational alteration that confers resistance upon cultured cells, but not the seed. It cannot be assumed that a trait selected at the level of the undifferentiated cell in culture will be expressed in any of the highly differentiated cells that constitute the intact plant. These then are some of the sundry sources of frustration that are to be anticipated in such experiments. Yet another difficulty was encountered in subsequent mutant selection experiments in which resistant cell lines were isolated from which only sterile plants could be regenerated.

In the four cases in which picloram resistance was transmitted across sexual generations, three (*PmR1*, *PmR2*, and *PmR7*) behaved as dominant alleles and one (*PmR6*) as a semidominant allele of single nuclear genes. In one case (*PmR1*) the complete dominance of the resistance phenotype was confirmed by growth tests of callus cultures derived from plants of the three different genotypes segregating in the F_1 (+/+, *PmR1*/+, *PmR1*/*PmR1*). The recovery of only dominant and semidominant mutations was to be expected because the isolation of recessive mutations was essentially precluded by the use of diploid cells in the initial selection experiments. Because the four resistant isolates were recovered from the same experiment it is possible that they did not originate independently. However, the semidominant phenotype of *PmR6* shows that this mutation, at least, is probably distinct from the others even though it may be only a different allele of the same locus. Genetic crosses can test the allelism of the four mutants.

The results of the growth tests suggest that heterozygosity of plants regenerated from resistant cell lines may not be restricted to the *PmR* locus in question. Allelic forms of other genes that modify the *PmR1*-mediated resistance to growth inhibition by picloram also may have been segregating in these crosses. The presence of such modifying alleles would not be surprising, because others have observed that genetic instability results from passage through culture (7, 8). However, these results serve as a reminder that genetic and physiological studies conducted on mutants selected in culture should be considered as only preliminary until new alleles have been outcrossed to a uniform genetic background.

The value of cell culture as a means for selecting and introducing agronomically desirable new alleles has remained unproved because in too few cases has the expression of a characteristic selected at the cellular level been demonstrated in the mature plant. Germination of seeds of the four mutants is resistant to inhibition by 100 μM picloram. However, if seedlings are left in the presence of picloram they will not develop further and soon begin to deteriorate. In an initial attempt to detect expression of resistance in the plant, normal (+/+) and *PmR1*/*PmR1* aseptically grown plantlets (approximately 5 cm high) were transferred to medium containing 50 μM picloram. The normal plants rapidly bleached and died whereas growth of the mutant plants was inhibited only slightly. After one month callus tissue formed from the stems of the mutant plants and then these plants began to degenerate. These observations justify experiments to evaluate tolerance of the mutants to picloram in the field.

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