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Further Characterization of Picloram-tolerant Mutants of *Nicotiana tabacum*

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Summary A genetic and preliminary biochemical analysis has been performed on four picloram-tolerant mutants of *Nicotiana tabacum* that were isolated from cell cultures. The four mutations define three distinct linkage groups. Mutant seedlings incorporate radioactively labeled picloram normally and do not modify or degrade the herbicide in a manner that alters its solubility characteristics.

Key words: Cell culture – Picloram-tolerance – Genetics – Uptake studies – *Nicotiana tabacum*

Introduction

Mutants of *Nicotiana tabacum* L. exhibiting an enhanced degree of tolerance for the herbicide picloram (4-amino-3,5,6-trichloropicolinic acid) previously have been selected by plating cell suspension cultures on medium containing a normally toxic concentration of the herbicide. Herbicide-tolerance is due to a single dominent nuclear mutation in three cases (*PmR1, PmR2,* and *PmR7*). The single nuclear allele effecting tolerance of a fourth mutant (*PmR6*) is semidominant (Chaleff and Parsons 1978). Additional *PmR* mutants have been isolated in subsequent experiments (Chaleff unpubl. results). The genetic relationship of several mutations conferring tolerance for picloram now has been established and the incorporation of radioactively labeled herbicide by homozygous mutant seedlings has been studied.

Materials and Methods

Crosses and Origin of Mutants

The isolation of the picloram-tolerant mutants *PmR1, PmR6,* and *PmR7* from cell suspension cultures derived from diploid plants of *Nicotiana tabacum* L. cv. 'Xanthi' has been described previously (Chaleff and Parsons 1978). Mutant *PmR85* was isolated by this same procedure in a subsequent experiment. Crosses were scored by plating surface-sterilized seeds on medium supplemented with 100 μM picloram (Chaleff and Parsons 1978). All plants employed in the biochemical studies were homozygous for the *PmR* allele in question. These plants were grown from seeds produced by homozygous mutant plants obtained by self-fertilization of the original heterozygous plant regenerated from cell culture. Two different homozygous isolates (4 and 11) of *PmR1* were employed. Plants heterozygous for two *PmR* alleles were constructed by crossing two homozygous mutant plants. Genetic linkage analyses were performed by testing for their response to picloram seeds produced by self-fertilization of doubly heterozygous plants and by crosses of such mutant plants with normal plants.

Incorporation of ^{14}C-Picloram

Seeds were surface-sterilized, rinsed thoroughly with sterile distilled water, and placed on medium in 25mm deep petri dishes. This medium contained the major salts and minor elements formulation of Murashige and Skoog (1962), but lacked sucrose and phytohormones and was solidified by the addition of 1.2% (w/v) potato destrose agar (Difco). The cultures were incubated for three weeks at 25±1° C under an equal mixture of Gro-lux and cool white fluorescent lamps (16h day). Each seedling then was transferred to a 25 mm deep petri dish (one seedling per dish) containing approximately 30ml of medium of the same composition, but supplemented with (2,6-^{14}C)-picloram to a final concentration of 1 μM. The radioactively labeled picloram (specific activity 10mCi/mmole) was furnished generously by The Dow Chemical Company; Midland, Michigan. After one week in the medium containing radioactively labeled picloram, seedlings were removed and rinsed three times with an aqueous solution of 1mM unlabeled picloram (pH 7.0). Seedlings were blotted dry, weighed, and extracted according to the following modification of the procedure of Hallmén and Eliasson (1972). Each extract was prepared by grinding together ten seedlings of a given genotype in a mortar and pestle with 5ml aliquots of 100mM phosphate buffer (pH 7.2). The supernatant solution was passed through a Whatman GF/A filter. By repeating this procedure five times a filtrate of 30ml was collected. After lowering its pH to 1.6 by the addition of 1N HCl, the filtrate was extracted three times with dichloromethane. The dichloromethane was evaporated and the residue dissolved in 5ml absolute ethanol. Aliquots of the aqueous and ethanol solutions were added to ACS (Amersham) and counted.

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Results

Genetic Analysis

Self-fertilization of a plant regenerated from picloram-resistant cell line 85 produced 114 resistant, 252 intermediate, and 137 sensitive progeny. These results provided the first evidence that tolerance of *PmR85* for picloram is conferred by a single semidominant nuclear mutation. This suggestion was confirmed by further analysis. Callus cultures were initiated from leaves of six randomly chosen progeny of the regenerated plant and tested for growth on picloram. Two of the callus cultures were sensitive (indicating that the segregants from which they had been derived were normal) and four were resistant to the herbicide. The plants from which the resistant cell lines originated were self-fertilized and the resultant seeds plated on picloram-supplemented medium. In this manner, three heterozygous (*PmR85*/+) and one homozygous (*PmR85*/*PmR85*) mutant plant were identified.

Plants homozygous for different *PmR* alleles were crossed to produce doubly heterozygous individuals. The heterozygotes were selfed and crossed with normal plants to test for linkage of the *PmR* mutations. If two mutations are closely linked (or allelic), only resistant progeny will be obtained from both types of cross. However, if two mutations are unlinked, self-fertilization of the double heterozygote should produce 15 resistant: 1 sensitive progeny and one fourth of the progeny of a testcross should be sensitive. Crosses involving plants heterozygous for a semidominant mutation are expected to yield progeny displaying an intermediate degree of resistance. If the second mutant allele is dominant, 2 resistant: 1 intermediate: 1 sensitive progeny will be obtained from the testcross. However, if the second mutant allele also is semidominant and is not additive with the first (i.e. the double heterozygote is no more resistant than the single heterozygotes), the testcross should produce 3 intermediate: 1 sensitive progeny. Many different degrees of resistance were evident among progeny obtained from the self-fertilization of plants heterozygous for a semidominant mutation. But as the gradient of resistance exhibited by these progeny was not completely discontinuous, they could not be assigned to discrete classes with any certainty. Therefore, these progeny were scored only as intermediate or sensitive. It is evident from the results presented in Table 1 that the two dominant mutations *PmR1* and *PmR7* are linked. These data also establish that *PmR6* and *PmR85* are unlinked both to *PmR1* and *PmR7* and to each other.

Incorporation of ^{14}C-Picloram

Some plant species that are naturally tolerant of picloram appear to be so because of their ability to form water-soluble and presumably biologically inactive conjugates of the herbicide (Hallmén and Eliasson 1972; Hallmén 1974, 1975). In the present studies, therefore, it was considered important to determine not only the total amount of picloram incorporated by mutant seedlings, but the extent to which the herbicide is metabolized by the various mutants. By extracting seedlings according to a modification of the procedure of Hallmén and Eliasson (1972), the amounts of both unmodified (soluble in dichloromethane) and conjugated (soluble in water) picloram present in seedlings can be determined. In this manner it could be discovered whether the mutations being studied confer resistance to picloram either by reducing the ability of seedlings to incorporate the herbicide or by providing

Table 1. Segregation among progeny obtained from plants heterozygous for two *PmR* alleles

	Resistant		Intermediate		Sensitive	
	Obs	(Exp)	Obs	(Exp)	Obs	(Exp)
PmR1; *PmR7* × +	426				0	
PmR1; *PmR7* selfed	244				0	
+ × *PmR7*; *PmR6*	103	(116)	59	(58)	70	(58)
PmR7, *PmR6* selfed			228	(236)	24	(16)
+ × *PmR1*; *PmR6*	135	(131.5)	66	(65.75)	62	(65.75)
PmR1; *PmR6* selfed			240	(241)	17	(16)
+ × *PmR7*; *PmR85*	176	(169)	85	(84.5)	77	(84.5)
PmR7; *PmR85* × +	141	(149.5)	72	(74.75)	86	(74.75)
PmR7; *PmR85* selfed			538	(533)	31	(36)
+ × *PmR6*; *PmR85*	165	(167)	89	(83.5)	80	(83.5)
PmR6; *PmR85* × +	169	(164)	87	(82)	72	(82)
PmR6; *PmR85* selfed			230	(238)	24	(16)

Segregation ratios do not differ significantly from the expected values ($P > 0.05$)

them with the capacity to convert picloram into an innocuous water-soluble conjugate.

Less than 10% of the total radioactivity present in any of the extracts remained adsorbed to the filter after the extracts were filtered and the filter rinsed with buffer. Therefore, little of the incorporated picloram is deposited in insoluble material and the amount of radioactivity present in the filtered extract is a near approximation of the total quantity of picloram incorporated by the seedlings.

All mutant seedlings incorporated more picloram than did the normal seedlings (Table 2). The relatively greater uptake of picloram by mutant seedlings probably is a consequence of their more vigorous growth on medium supplemented with 1 μM picloram. Incubation on this medium for one week apparently did not affect mutant seedlings, whereas normal seedlings were severely wilted and bleached after this period. Thus, it is to be expected that, during the period of incubation in the presence of radioactively labeled picloram, the metabolic activity of mutant seedlings is greater than that of normal seedlings and that mutant seedlings thereby are able to incorporate more of the herbicide. Unfortunately, because the amount of radioactivity incorporated would be below the limit of detection, the experiment could not be performed using a lower picloram concentration that does not affect growth of the normal seedlings. In any case, it is clear from the data presented that the *PmR1, PmR6, PmR7,* and *PmR85* mutations do not confer resistance to picloram by reducing or preventing uptake of the herbicide.

By partitioning the ^{14}C-picloram in the extracts between CH_2Cl_2 and H_2O fractions, the extent to which the herbicide has been modified can be determined from the relative distribution of radioactivity in the two fractions. If the mutant seedlings are able to convert (either by degradation or conjugation) picloram to a form that is more soluble in water, the ratio of radioactivity present in the H_2O to that present in the CH_2Cl_2 fraction would be higher for the mutant than for the normal seedlings.

The ratio of radioactivity present in H_2O to that present in CH_2Cl_2 is the same for *PmR1/PmR1* (Isolate 4), *PmR6/PmR6*, and *PmR85/PmR85* and is slightly lower than for normal seedlings. But this ratio is higher for the second *PmR1/PmR1* isolate (Isolate 11) than for both normal and *PmR7/PmR7* seedlings (Table 2). It is apparent, therefore, that the differences observed are either not meaningful or that they are due to variation at other genetic loci that segregate independently of the *PmR1* and *PmR7* loci (or locus).

Discussion

This paper reports the genetic and preliminary biochemical characterization of four picloram-tolerant mutants that were isolated from cultured cells of *N. tabacum*. Three of these mutants (*PmR1, PmR6,* and *PmR7*) were obtained from the same cell population (Chaleff and Parsons 1978). Therefore, since *PmR1* and *PmR7* are both dominant and linked, it is possible that they did not arise as independent mutations, but originated from daughter cells of a single progenitor. The genetic analysis has established that the four picloram-tolerant mutants studied define three linkage groups. It seems probable, therefore, that other loci capable of mutating to effect resistance to picloram would be revealed by the analysis of additional such mutants that have been isolated.

One possible mechanism by which tolerance for picloram could be achieved is exclusion of the herbicide from the cell. Such exclusion could result from a mutation that alters or inactivates the transport system by which picloram enters the cell. However, as all of the mutants analyzed incorporate more picloram than do normal seedlings, clearly their ability to transport the herbicide is unimpaired. This result is not unexpected, since mutations of genes encoding transport proteins are in general recessive and the *PmR* mutants are dominant or semidominant.

Tolerance for picloram also could be effected by mutations that introduce a capability to modify or degrade the herbicide. Following treatment with picloram of plants that are naturally sensitive to the herbicide, picloram is recovered primarily in the free (dichloromethane-soluble) form. In contrast, picloram is largely converted to water-soluble conjugates by plants that are naturally tolerant of the herbicide. On the basis of these results it has been proposed that natural tolerance for picloram results from the ability of these species to complex the herbicide into nontoxic forms (Hallmén and Eliasson 1972; Hallmén 1974, 1975). Apparently a similar detoxification mechanism does not operate in any of the *PmR* mutants, since the ratio of radioactivity present in the dichloromethane and aqueous fractions was nearly the same in extracts of mutant as in extracts of normal seedlings. Considerable variability is present in these data. But as the ratio obtained for one homozygous *PmR1/PmR1* isolate is higher and that for another is lower than the control value, the observed differences in these ratios cannot be the basis for the tolerance of these mutants for picloram.

In these experiments most of the radioactivity extracted from tobacco seedlings, which are very sensitive to picloram, was recovered in the aqueous fraction. In contrast, Hallmén (1975) found the majority of picloram present in the dichloromethane fraction of extracts of the susceptible species sunflower (*Helianthus annuus* L.) and spruce (*Picea abies* (L.) Karst.). It is possible that tobacco is an exception to the proposal (Hallmén and Eliasson 1972; Hallmén 1974, 1975) that picloram remains predominantly in the free form in susceptible species. But this apparent discrepancy also could be due to differences

Table 2. Incorporation of ^{14}C-picloram by homozygous mutant tobacco seedlings (cpm per mg fresh weight)

	PmR1/PmR1 (Isolate 4)				*PmR1/PmR1* (Isolate 11)				*PmR6/PmR6*			
Expt. No.	Total	H_2O	CH_2Cl_2	$\frac{H_2O}{CH_2Cl_2}$	Total	H_2O	CH_2Cl_2	$\frac{H_2O}{CH_2Cl_2}$	Total	H_2O	CH_2Cl_2	$\frac{H_2O}{CH_2Cl_2}$
1	48.5	38.9	9.6	4.0	46.7	38.3	8.4	4.5				
2	36.3	30.1	6.2	4.9	37.0	32.9	4.1	8.0				
3	45.1	35.4	9.7	3.6	42.1	37.7	4.4	8.6				
4	34.6	26.0	8.6	3.0					26.6	19.6	7.0	2.8
5	41.9	33.6	8.3	4.0					32.0	26.5	5.5	4.8
6	30.4	22.6	7.8	2.9					32.9	26.8	6.1	4.4
7									35.1	27.1	8.0	3.4
8									42.6	33.2	9.4	3.5
9												
Mean ± S.E.M.	39.5 ± 2.8			3.7 ± 0.3	41.9 ± 2.8			7.0 ± 1.3	33.8 ± 2.6			3.8 ± 0.4
	PmR7/PmR7				*PmR85/PmR85*				+/+			
Expt. No.	Total	H_2O	CH_2Cl_2	$\frac{H_2O}{CH_2Cl_2}$	Total	H_2O	CH_2Cl_2	$\frac{H_2O}{CH_2Cl_2}$	Total	H_2O	CH_2Cl_2	$\frac{H_2O}{CH_2Cl_2}$
1	51.5	45.2	6.3	7.2					35.1	29.6	5.5	5.4
2	51.5	46.6	4.9	9.4					23.1	20.3	2.8	7.3
3	52.9	45.9	7.0	6.5					34.7	30.1	4.6	6.5
4	29.1	23.5	5.6	4.2					20.1	16.1	4.0	4.1
5	36.6	31.3	5.3	6.0					23.1	19.4	3.7	5.3
6					38.8	30.0	8.8	3.4	18.3	14.3	4.0	3.5
7					48.0	39.0	9.0	4.3	26.8	22.2	4.6	4.8
8					57.5	46.7	10.8	4.3	28.2	23.0	5.2	4.4
9					40.0	30.8	9.2	3.4	25.0	19.7	5.3	3.7
					42.8	32.9	9.9	3.3				
Mean ± S.E.M.	44.3 ± 4.8			6.7 ± 0.8	45.4 ± 3.4			3.7 ± 0.2	26.0 ± 2.0			5.0 ± 0.4

in the extraction procedures used. For example, whereas Hallmén and Eliasson (1972) and Hallmén (1974, 1975) added trichloroacetic acid to extracts to precipitate soluble proteins, this step was omitted in the present studies.

As the *PmR* mutants do not form more water-soluble conjugates of picloram than do normal seedlings, the mechanism of their tolerance for the herbicide remains to be explained. One possibility is that mobility of the herbicide is somehow restricted in the mutant seedlings. But it is unlikely that this mechanism would confer resistance to cultured cells, which is the property by which the mutants were selected. Another possibility is that picloram is sequestered within the mutant cells or either degraded or modified in a manner that does not alter its solubility characteristics. Tolerance for picloram also would result from a mutational alteration of an intracellular protein receptor that reduced its affinity for the herbicide. Further experimentation is required to discern which, if any, of these possible mechanisms is operating in the *PmR* mutants.

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