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Changes in frequencies of variegated leaves in NMU treated tobacco

Evidence for a differential response to NMU

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Summary. Seeds of *Nicotiana tabacum* were allowed to imbibe water for 1 h and were then treated with 5–20 mM N-nitroso-N-methylurea (NMU) for 1 h. Seedlings were planted out separately and leaves no. 1–6 were scored individually after expansion was complete: frequencies of leaves with mutant sectors and the percentage of leaf area that was mutant were determined for the numbered leaves. Treatment with 5 mM NMU gave few mutant plants but after 10–20 mM NMU 50–98% of plants were mutant. The frequency of mutant leaves increased from leaf no. 1 to leaves no. 3–4; as much as a 5.6-fold increase, from leaf no. 1 to no. 3 was found. There appears to be differential sensitivity to NMU: it is lower in the initial cells for leaf no. 1 than in the initials for leaves no. 3 or 4. Leaves no. 1–4 arise from four different groups of initial cells: mutations appearing in two or more of leaves no. 1–4 must, therefore, arise independently of one another. From mutants found in leaves no. 1–4 it is estimated that the mean number of mutations per seedling was 2.68 after treatment with 20 mM NMU. Mean percentage of leaf surface area occupied by mutant sectors increased from 14% to 29.4% as NMU concentration increased from 10 to 20 mM. It also increased significantly from leaf no. 1 to leaves no. 3–6 after treatment with 15 or 20 mM NMU but not after 10 mM NMU: this suggests that mutagen treatment may affect the formation of mutant homoplasmon cells and their contribution to leaf primordia.

Key words: Nitrosomethyl urea – Plastid mutation – Tobacco – Multiple mutations

Introduction

Nitrosomethylurea (NMU) induces mutations in plastid DNA (ctDNA). The ctDNA mutations reported include changes that reduce plastid pigmentation and produce variegated plants (Hagemann 1982; Hosticka and Hanson 1984) or make plastids antibiotic resistant (Fluhr et al. 1985).

In those three reports the NMU was applied to seeds. By varying the duration of the treatment or the NMU concentration at least 60% of seedlings survived long enough to produce several leaves. Of the survivors, more than 90% had sectors of mutant cells. In many cases variegation continued to appear in later leaves and extended into influorescences: these variegated plants yielded mutant progeny (Hagemann 1982; Hosticka and Hanson 1984; Fluhr et al. 1985).

Seeds of tobacco are small, approximately 0.7 mm long (Avery 1933; Davidson and Whitaker 1986). For studies of induced mutation tobacco seeds are interesting for several reasons: 1. Leaf primordia are not present in the seed embryos but are initiated some time after cotyledons are fully expanded (Hannam 1968; Davidson and Whitaker 1986); 2. The first two primordia are initiated at opposite ends of the shoot meristem (Hannam 1968; Williams 1975); they arise from different cells and differ in the frequencies of leaves with mutant sectors (Davidson and Whitaker 1986); 3. Leaf no. 3 is initiated between leaves no. 1 and 2 and by a different group of meristematic cells; it has a higher percentage of variegated leaves than no. 1 and 2. It appears that there is differential sensitivity to NMU in different cells of the shoot meristem of tobacco seeds, even though it consists of only 60–100 cells (Davidson and Whitaker 1986). We also suggest that ctDNA mutations in the first leaves of tobacco seedlings arise independently for each leaf. This suggestion has two implications: first, that the number of ctDNA mutations induced is higher than the estimates obtained by scoring percent variegation in mature plants; secondly, that a greater range of ctDNA mutants may be present in the first leaves to grow from the mutagenised seeds shoot apex than are induced in the cells that give rise to mature leaves and influorescences.

In order to confirm the differential sensitivity, to NMU, of the initial cells destined to give rise to the first

leaves of tobacco seedlings we have treated seeds with 5–20 mM NMU. A 1 h treatment with 5 mM NMU produces few variegated leaves but 10–20 mM NMU induced mutants in high frequencies. With the three higher concentrations tested, i.e. 10, 15 and 20 mM NMU there was a progressive increase in the percentage of mutant plants. With each concentration, moreover, we observed an increase, from leaf no. 1 to leaf no. 3, in the percentage of leaves with a mutant sector. In seeds treated with 10 mM NMU, for example, leaf no. 3 showed a 5.7-fold increase over leaf no. 1 in the percentage of leaves with mutant sectors. With 10, 15 and 20 mM NMU the initial cells for leaf no. 3 show a greater sensitivity to the mutagen than the initial cells for leaves no. 1 and 2. Leaves no. 4–5 are formed as the meristem undergoes a transition: leaf primordia for leaf no. 5 and all later leaves arise in a spiral pattern (Williams 1975). Paralleling this change in the growth pattern of the shoot meristem we find a decrease in the frequency of leaves with mutant sectors. With 10–20 mM NMU the percentage of mutant leaves decreases from leaf no. 4 to leaf no. 6. This is further evidence for differential sensitivity of initial cells that are destined to give rise to different leaves of the tobacco seedling shoot.

Materials and methods

Nicotiana tabacum cv. 'Kentucky 14' seeds were sown on two Whatman no. 1 filter papers in 15 cm Petri dishes. The filter papers were moistened with 10 ml distilled water; 100 seeds were present in each dish. After 1 h the seeds were transferred to filter papers moistened with 10 ml of 5, 10, 15 or 20 mM NMU made up in distilled water. The seeds were exposed to NMU for 1 h and then washed three times with distilled water and placed on fresh, moistened filter papers. They were kept moist by adding distilled water, 1 ml at a time, as required. When cotyledons had expanded, seedlings were transferred to individual holders containing sterilized soil moistened with half-strength Hoagland's solution. They were protected with plastic tray covers and grown under GRO-LITE fluorescent tubes. Temperature varied from 22 °C (night) to 28 °C (day).

Seedlings were numbered and scored using a dissecting microscope. This is necessary since the first leaves on tobacco seedlings are only 2–5 mm long after treatment with NMU. Individual leaves were scored, when they had expanded, for the presence of pale green, yellow or white sectors. The percent of leaf area occupied by mutant sectors was also determined for each leaf.

Leaf growth was more rapid after treatment with 5 and 10 mM NMU than after 15–20 mM NMU. After 5–10 mM NMU seedlings had 7–8 leaves at 8 weeks: seeds treated with 15–20 mM NMU did not have 7–8 leaves till 11 weeks. Scoring was stopped at the 8 leaf stage.

Comparisons were made of frequencies of mutant leaves and mean percent of leaf area occupied by mutant sectors. Pairs of values for individual leaves were compared using contingency tables (X^2) or the *t*-test, whichever was appropriate. Comparisons between the response to different treatments were made using analysis of variance: specific analyses are given at appropriate places in the text. Differences were considered to be significant at $P < 0.05$.

Results

The number of seeds that germinated and produced expanded cotyledons or leaves was lower after 10–20 mM NMU than after 5 mM (Table 1). The inhibitory effect of 10–20 mM NMU was already evident when seedlings had reached the 4th leaf stage but was particularly clear when leaves 5–6 had appeared. Of 100 seeds treated with 15–20 mM NMU only 55 and 30, respectively, had produced leaves 5 and 6 even after 11 weeks (Table 1).

Seeds treated with 5 mM NMU produced few mutant seedlings. With 10–20 mM NMU, however, there was an increase in the frequency of leaves with mutant sectors with an increase in the concentration of NMU. This increase is seen whether one compares individual leaves or whole plants. Thus, leaf no. 1 has 6.6% mutant leaves after 10 mM NMU and 39.3% after 20 mM NMU – a 5.95-fold increase in mutant leaf frequency (Table 1). Similarly, the percentage of plants that have at least one mutant leaf increases from 56% after 10 mM NMU to 98.7% after 20 mM NMU. These values are given for

Table 1. *N. tabacum* seeds were treated with 5–20 mM NMU for 1 h. The 100 treated seeds were planted out and leaves No. 1–6 were scored, as they expanded, for mutant sectors. For each leaf, the number scored and the percent leaves with a mutant sector are given. Plants were scored up to 11 weeks after treatment with NMU. After 5 and 10 mM there were 6 expanded leaves by 8 weeks, but after 15 and 20 mM it was 11 weeks before leaves 5 and 6 could be scored

NMU (mM)	Leaf no.																	
	1			2			3			4			5			6		
		Mutant			Mutant			Mutant			Mutant			Mutant			Mutant	
	No.	No.	%	No.	No.	%	No.	No.	%	No.	No.	%	No.	No.	%	No.	No.	%
5	99	0	0	99	4	3.9	99	2	2.0	99	0	0	99	3	3.0	99	2	2.0
10	76	5	6.6	76	13	17.1	75	28	37.3	75	21	28.0	72	21	29.2	66	13	19.7
15	79	24	30.8	77	38	49.4	77	47	61.0	75	43	57.3	67	33	49.3	55	24	43.6
20	89	35	39.9	89	65	73.0	87	66	75.9	80	63	78.8	54	38	70.4	30	13	43.3

Table 2. Cumulative numbers of plants (*N. tabacum*) with mutant sectors in leaves No. 1 through 4. These are the same plants as those in Table 1: the data are from plants scored 5 weeks after treatment and only plants with 4 expanded leaves were scored. Percent mutant plants is the value for plants which had at least one mutant sector on any of the first four leaves

NMU (μM)	No. of plants with mutants				Plants	
	Leaf no. 1	Leaves 1+2	Leaves 1–3	Leaves 1–4	No. scored	% mutant
10	5	17	35	42	75	56
15	19	42	55	61	72	84.7
20	31	63	71	75	76	98.7

Table 3. Numbers of plants with mutant sectors in any one, any two, any three or all four leaves when scored at the 4-leaf stage. The number and percentage of mutant leaves is also given. The data are from the same plants scored for Table 2

NMU (mM)	No. of plants					Mutant leaves	
	0 mutant leaves	1 mutant leaf	2 mutant leaves	3 mutant leaves	4 mutant leaves	No.	%
10	33	21	17	4	0	67	22.3
15	11	16	19	18	8	134	46.5
20	1	11	19	25	20	204	67.1

seedlings that had 4 expanded leaves because the numbers of seedlings are similar (Table 2).

The numbers of leaf no. 1 that were mutant are significantly different from the numbers of leaves no. 3 or 4 that were mutant; these differences are significant after treatment with 10, 15 or 20 mM NMU ($P < 0.05$). The numbers of leaf no. 1 in comparison to leaf no. 2 that were mutant are also significantly different, but only after treatment with 20 mM NMU ($P < 0.05$); leaf no. 2 relative to leaf no. 3 mutant numbers were significantly different only after treatment with 10 mM NMU ($P < 0.05$). The overall trend, however, is clear and significant: with 10, 15 or 20 mM NMU the frequency of mutant leaves increases from leaf no. 1 to leaves no. 3 or 4. Since leaves no. 1–4 arise from different groups of initial cells, located in different parts of the shoot meristem, the data (Tables 1, 2) suggest the following conclusion: the initial cells destined to give rise to leaf no. 3 or no. 4 are more sensitive to the mutagen than the initial cells of leaves no. 1 or 2. Furthermore, since leaves no. 1–4 arise separately, in different parts of the shoot meristem, ctDNA mutations must be induced in each group of initial cells independently of mutations in any other group of initials. On this basis we would expect that the frequencies of seedlings with three or four leaves having mutant sectors would increase with an increasing dose of NMU. This increase can be shown to occur in two ways. First, there is a cumulative increase in the numbers of seedlings with mutant leaves as the number of leaves scored changes from leaf no. 1 to leaves no. 1–4 (Table 2). Secondly, there is an increase in the number of plants with mutants in all four leaves (Table 3). After treatment with 10 mM NMU only 4 of the 75 plants had 3 or 4 mutant leaves, i.e. 5.3% (Table 2, 3). The comparable values for 15 and 20 mM NMU are 26/72 and 45/76, i.e. 36.1% and 59.2%. Thus, the real mutation rate is actually higher than that suggested by the percentage of plants that are mutant. At the 4-leaf stage 98.7% of seedlings present after 20 mM NMU are mutant (Table 2). Of these, 59.2% have mutant sectors in three or four leaves; there is a total of 204 mutant leaves present on 76 plants (Tables 2, 3). Mutations occurring independently, therefore, in the initial cells for leaves no. 1–4 yield a total of 204 ctDNA mutations in 76 seedlings. This gives a mean value of 2.68 mutations per seedling after treatment with 20 mM NMU. The values for seedlings scored at the 4 leaf stage after treatment with 10 and 15 mM NMU are 0.89 and 1.86 mutations per seedlings, respectively.

Size of mutant sectors

The mean percentage area of leaves occupied by mutant sectors was determined for leaves no. 1–6 (Table 4). In the 10 mM NMU treated seedlings mean values for different leaves were not significantly different, e.g. leaf no. 1 relative to leaf no. 3 etc. After 15 mM NMU, the mean percentage leaf area that was mutant was significantly different for leaf no. 1 relative to leaves no. 3–6 ($P < 0.05$). No other pairs of mean values were significantly different. Similarly, after 20 mM NMU, the mean value for leaf no. 1 was significantly different from the

Table 4. Mean ±SD percentage of leaf area occupied by mutant sectors. Tobacco seeds were treatment with 5–20 mM NMU for 1 h. Data from same seedlings as Table 1

	1	2	3	4	5	6
5	–	8.5± 3.1	5	–	18.3± 7.6	–
10	14.8±20.4	11.5±13.2	11.7±12.2	20.2±21.3	15.5±15.4	10.4± 6.9
15	12.9±11.4	21.4±24.3	25.9±22.5	28.7±25.4	28.0±21.9	31.6±23.9
20	13.4±12.2	28.0±25.8	34.3±25.8	28.9±20.3	33.7±20.7	38.1±20.6

Table 5. Mean percentage area of mutant sectors of leaves of tobacco seedlings following treatment with 10, 15 or 20 mM NMU for 1 h. The SS, MS and F values are given for a one way ANOVA; based on values in Table 4

NMU (mM)	Mean %		Variance	
10	14.02		13.17	
15	24.75		45.21	
20	29.40		75.28	
Source	df	SS	MS	F
total	17	1,415.2		
trials	2	746.9	373.5	8.38
error	15	668.3	44.8	

means for leaves no. 2–6 but no other pairs of means were significantly different ($P=0.05$). The 15 and 20 mM treatments, therefore, affect the relative size of the leaf that consists of mutant cells: this area is relatively greater in leaves no. 3–6 than in leaf no. 1.

The mean percentage of mutant areas for all leaves was 14.02%, 24.75% and 29.40% in seedlings that had been treated with 10, 15 and 20 mM NMU respectively (Table 5). These values are significantly different (Table 5) and they confirm that an increase in the relative area of a leaf occupied by mutant cells accompanies an increase in the concentration of NMU used to treat seeds. We suggest that the initial cells for leaves no. 3–5 are differently sensitive to NMU not only in terms of the number of mutations induced but also in terms of the time in seedling growth when homoplasmons for mutant plastids are formed and of the proliferative ability of the mutant homoplasmons. These changes in the relative sizes of mutant sectors provide evidence that cell responses to NMU are complex and that meristem organization and growth may be affected by NMU: this may, in turn, alter the formation and distribution within a meristem of mutant homoplasmons.

Discussion

NMU and ethylmethanesulphonate (EMS) induce mutations in ctDNA (Chia et al. 1986; Davidson and Whitaker 1986; Hagemann 1982; Hosticka and Hanson 1984; Fluhr et al. 1985; Miller et al. 1980, 1984). The most frequent type of mutation so far reported results in a reduction in chlorophyll content of plastids and the presence of pale green, yellow or white sectors in leaves: this is the class of mutations described here. The mutants we have analysed show maternal inheritance (data to be reported elsewhere).

The induction and detection of plastid mutants in tobacco have been analysed in this study with regard to the developmental biology of the seedling. The relevant aspects of the structure and early growth of tobacco seeds are these: 1. the shoot apex of the seed consists of approximately 70 cells (Davidson and Whitaker 1986); 2. the first four leaf primordia are initiated in different areas of the shoot meristem and so are produced from different cells (Hannam 1968; Williams 1975; Davidson and Whitaker 1986); 3. after leaf no. 4 all subsequent leaves are initiated in a pattern of spiral phyllotaxis from the shoot meristem (Williams 1975). The important consequence of the discrete origin of leaves no. 1–4, i.e. from different groups of cells, is that mutations seen in any one of the first four leaves must arise independently of a mutation in any other of these leaves (Davidson and Whitaker 1986). In that study we presented evidence that the initial cells for leaves no. 1–3 are not only spatially separate but that they also show a differential response to NMU; the results reported here extend that observation. After treatment with 10, 15 or 20 mM NMU there is an increase, from leaf no. 1 to leaf no. 3 or 4, in the percentage of mutant leaves (Table 1). The increase is greatest after 10 mM NMU, from 6.6% in leaf no. 1 to 37.3% in leaf no. 3 but it is also seen after treatment with 15 and 20 mM NMU, both of which show a doubling in percentage mutant leaves from leaf no. 1 to leaf no. 3 or 4 (Table 1).

This increase in percentage of mutant leaves could result from an increase in the rate of formation of mutant homoplasmons, i.e. leaf cells containing only mutant plastids. With this in mind, the increase in mutant leaf frequency would indicate an increase only in formation of mutant homoplasmons, not in frequency of induced mutations. The results, however, contradict this suggestion. After treatment with 10 mM NMU, for example, the mean percentage of leaf area that is mutant in leaf no. 1, 14.8±20.4%, is not significantly dif-

ferent from that in leaf no. 3, 11.7 ± 12.2% (Table 4) yet the percentages of mutants in leaves no. 1 and 3, 6.6% and 37.3% (Table 1), are significantly different ($P < 0.05$). We conclude that the increase in percentage of leaves with mutant sectors results from an increase in the number of mutations induced in the initial cells for leaves no. 3–4 in comparison to leaf no. 1.

The evidence that mutations are induced independently in the initial cells for each of the first four leaves means that values reported for percent mutant plants underestimate the real number of mutations induced. As we show here 98.7% of seedlings with 4 leaves are mutant (Table 2) but 45/76 seedlings had mutants in 3/4 or 4/4 leaves. This means that the high frequency of mutant plants is the result of multiple mutations. We estimate that the 204 mutant leaves seen after treatment with 20 mM NMU (Table 3) are, in fact, products of 204 plastid mutations in 76 seeds. With an average number of shoot meristem cells of 70 (Davidson and Whitaker 1986) this indicates 204 mutations in approximately 5,320 cells, i.e. 76 plants each with approximately 70 cells, or 0.038 mutants per cell.

More accurate estimates of the frequency of plastid mutations will be sought because of the need for a precise definition, in studies of induced plastid mutation, of ". . . the minimum population to be examined. The task is now to establish the size of the population and to screen the requisite number of plants" (Somerville and Ogren 1982). The evidence presented here shows that plastid mutations occur in high frequency in the first leaves formed in tobacco seedlings. It may be that a wider range of mutant types is present in the first leaves than occurs in more mature leaves or influorescences: this could be explored by tissue culture of seedling leaves. In irradiated soybeans an analysis of the spectrum and frequency of mutants was carried out by determining mutation yield in successive individual branches. The frequency of mutants decreased from 31.7% in branch no. 1 to 14.6% in branch no. 4 and to 2.43% in branch no. 7 (Upadhyaya et al. 1985). The results suggest that nuclear mutations show, at least in soybean, a distribution pattern within the seedlings that parallels the data for plastid mutations in tobacco. The mutations induced in cells that give rise to the first leaves or branches produced by seedlings may constitute a source of novel mutations that have not been fully exploited.

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