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Hormone-induced Caulogenesis in Long-Term Tobacco Cell Lines and its Effect on Nuclear DNA Content*

P.L. Traynor and S.M. Flashman
Departments of Botany and Genetics, North Carolina State University, Raleigh, North Carolina (USA)

Summary. Manipulation of exogenous auxin and cytokinin levels during sequential subculture in vitro has been used to induce caulogenesis in several long-term tobacco cell lines. Concurrently, tissue samples at various stages of caulogenesis have been examined for nuclear DNA content. While a variety of hormone regimes permitted caulogenesis, extremely high (122.95 μM) cytokinin levels and extremely low (0.285 μM) auxin levels generally gave optimal response. For three lines, caulogenesis was accompanied by a progressive decrease in nuclear DNA content beyond that due to the loss of polyploid cells. In one line, however, DNA content remained stable during regeneration, perhaps reflecting the acquisition of a stably adapted aneuploid karyotype. Both caulogenic response and amount of nuclear DNA were affected by changes in the culture medium. The progressive nature of the observed changes in DNA content is inconsistent with a single-step selection for euploid competent cells. Alternative models postulating either progressive selection for euploidy, or the regulation of karyotype are proposed to explain the results.

Key words: *Nicotiana tabacum* – Cytogenetics – Tissue culture – Microdensitometry – Regeneration

Introduction

In tobacco callus culture, caulogenesis can normally be induced by manipulation of exogenous phytohormone levels (Skoog and Miller 1957). With continued subculturing, this organogenic response declines and can eventually disappear (Murashige and Nakano 1965). A similar loss of organogenic potential has been observed in other systems and has been attributed to the increased incidence of aneuploidy and polyploidy in older cultures (Murashige and Nakano 1967; Torrey 1967; Smith and Street 1974). On the other hand, highly aneuploid tobacco cell lines have been induced to regenerate both euploid and aneuploid whole plants (Sacristan and Melchers 1969; Zagorska et al. 1974; Ogura 1976). This is believed to involve selection from the general cell population for those cells retaining the genetic information necessary for totipotency (Brossard 1975; Smith and Street 1974).

Rice et al. (1979) have recently succeeded in regenerating whole plants from long-term tobacco cultures which had previously been considered morphogenically incompetent. This reopens the questions of whether the loss of regenerative potential involves genetic or physiological changes, and how reversible the changes are. In this communication we report on conditions for caulogenesis from long-term tobacco cultures and examine the changes in nuclear DNA content accompanying the process.

Materials and Methods

Cell Lines

Four lines of cultured tobacco were used. XDR^{thr} (henceforth abbreviated as XDR) was a threonine resistant line (Heimer and Filner 1969), which had been selected from the XD line derived from diploid *Nicotiana tabacum* L. cv. 'Xanthi' (Filner 1965). It has been maintained in culture for 17 years on a modified White's medium (M-1D) (Filner 1965). SCM7 was selected from XDR in 1976 for selenocystine and selenomethionine resistance (Flashman and Filner 1978) and was also maintained on M-1D medium. Cell line W38 was initiated from a haploid plantlet from anther culture of *N. tabacum* L. cv. 'Wisconsin 38'; it has been maintained for 3 years on Murashige and Skoog's (MS) medium (1962) with 3 mg/l indole-3-acetic acid (IAA) (17.12 μM), 0.3 mg/l γγ-dimethylallylaminopurine (2iP) (1.47 μM), and 0.4 mg/l 2,4 dichlorophenoxyacetic acid (2,4-D) (1.8 μM). A year and a half after the derivation of W38, a subline, W38M, was developed by transfer to M-1D medium for 6 months, after which it was grown again on MS medium for a year prior to the start of the experiments.

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Regeneration

Samples of callus (approx. 1 cm^3) were aseptically transferred from maintenance media to MS media supplemented with IAA at 0.05, 0.1, 0.25 and 1.0 mg/l (0.285, 0.57, 1.425 and 5.71 μM) and 2iP at 5, 10, 15 and 25 mg/l (24.59, 49.19, 73.77 and 122.95 μM) for a total of 16 different formulations. All regeneration experiments were done in duplicate and the cultures maintained at 27°C on a 16 hour day with 40 lux of mixed incandescent and cool white fluorescent light in the Southeastern Plant Environmental Laboratories. Weekly evaluations of calli were made under a 60X dissecting microscope. Subcultures were made every 28 days, visually selecting regions which appeared compact, coherent, or otherwise more organized.

Microdensitometry

Measurements of nuclear DNA content, based on Feulgen staining intensity at 550 nm, were made using a Vickers M85 scanning microdensitometer, which uses a flying spot with a variable diameter mask to minimize errors due to nuclear inhomogeneity and non-specific absorbance. Non-specific absorbance was further reduced by restricting measurements to nuclei free from interfering cell wall or cytoplasm. Tissue samples (1-2 mm^3) were prepared according to the method of Berlyn and Miksche (1976), except that squash preparations were made prior to hydrolysis. Two replicate slides were prepared for each tissue type. Leghorn chick erythrocytes (cRBCs) were present on all slides as an internal control for variation in staining efficiency (Dhillon et al. 1977). Hydrolysis curves for W38 callus (Fig. 1) gave an optimal hydrolysis time of 25 minutes. This hydrolysis time was used for all subsequent experiments. For each tissue, density readings were made of 40 tobacco nuclei and 30 cRBC nuclei. Densitometry readings for the tobacco nuclei were normalized against the values obtained for cRBC nuclei on the same slide, and are expressed in picogram equivalents, based on a DNA content of 2.7 pg for the cRBC nuclei (Doerschug et al. 1978). Because of the multi-modal and non-normal nature of the distributions of tobacco nuclear DNA contents, determinations of statistical significance in these distributions were made using the non-parametric Wilcoxon Rank Sum test (Wilcoxon et al. 1963; Snedecor and Cochran 1967). Differences were termed significant at $P < 0.05$.

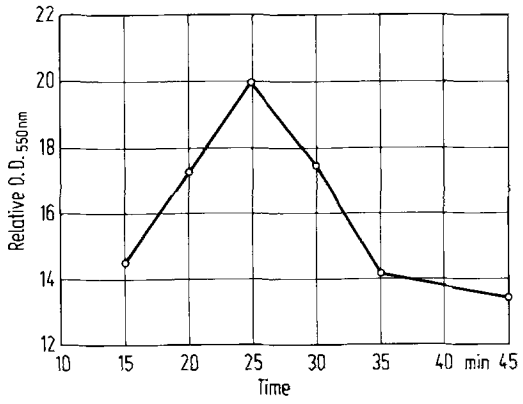

Fig. 1. Hydrolysis curve of W38 callus. Duplicate samples were prepared and hydrolyzed in 5N HCl at 25°C. Samples were then stained and analyzed. Each point is the median of 40 readings, 20 from each slide

Results

Regeneration

The caulogenic response was divided into several developmental stages (Fig. 2a-e). These included meristemoids (Fig. 2c), meristematic domes with primitive leaf primordia (Fig. 2d), and developed shoots (Fig. 2e). The time-course of the responses is summarized in Table 1. In these experiments, SCM7 showed no signs of caulogenesis. However, cultures of SCM7 maintained on 0.3 mg/l IAA and 10 mg/l 2iP for a year developed meristematic domes with leaf primordia. Both W38 and XDR responded best on the highest levels of 2IP, while W38M and SCM7 calli consistently necrosed on these media. Beyond this, caulogenesis was generally both faster and more prolific at higher cytokinin concentrations and lower auxin concentrations. For any specific medium, caulogenesis was significantly slower for XDR than for W38 (by Paired Comparison test). In addition, it was particularly apparent for XDR that some meristematic domes did not continue to develop and did not form leaf primordia by the end of the experiment. W38 and W38M differed in the media giving optimal response, but the optimal responses of W38 were generally more rapid and more shoots were produced per callus piece. Although all tissue was derived from a single small initial callus piece, callus areas could by identified which gave no caulogenesis on the shoot-inducing media. This continued even if subculture was extended an additional twelve weeks (twenty-nine weeks total).

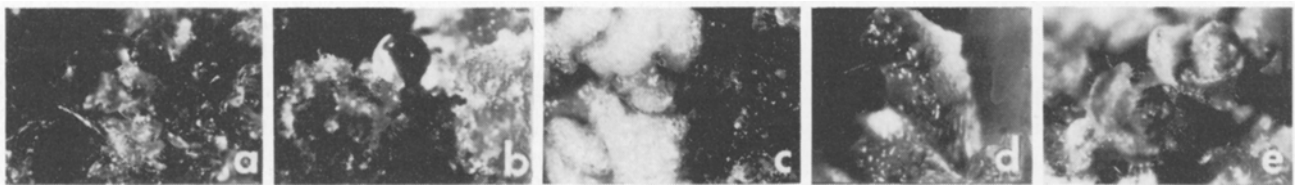

Fig. 2a-e. Tissue morphologies observed in vitro: **a** callus morphology on maintenance media **b** callus morphology on regeneration media **c** meristematic dome tissue **d** leaf primordia **e** early regenerated shoots (magnifications: **a-d**, X 40; **e**, X 12)

Table 1. Time of organogenesis in three cell lines at different phytohormone concentrations

Cell line	Auxin (mg/l IAA)	Cytokinin (mg/l 2iP) 5	10	15	25
38	0.05				4: 4,6[a]
	0.1			9: 10,17	5: 6,6
	0.25			11: 12	2: 7,8
	1.0				7: 9,12
38M	0.05	6: 7,9	7: 8,9	4: 5,6	
	0.1			6: 8,10	15:
	0.25	11. 11,13	7:	6: 8,12	5:
	1.0			7:	
XDR	0.05		11: 12	8: 12	6: 8,10
	0.1		10:		6: 10,–[b]
	0.25	12:	9:	9: 13,16	7: 10,11
	1.0			10: 15	9: 11,12

[a] The first number is the number of weeks under regeneration conditions before the first appearance of meristematic domes. The numbers following the colon are the number of weeks under regeneration conditions before the first appearance of leaf primordia on each replicate plates. Absence of numbers indicates lack of development; all lines grown on all media

[b] Lost to contamination

Table 2. Median DNA contents[a]

Cell line	Tissue	Total distribution	Lower peak	Upper peak
W38	haploid root	5.7[b]	——	——
	diploid root	13.4A[c]	12.0A	23.7[d]
	callus	56.6C	——	——
	meristematic dome	27.6B	24.6B	——
	leaf	12.8A	——	——
W38M	callus	53.3C	——	——
	meristematic dome	26.6B	26.3B	——
	leaf	20.6D	20.5D	——
	non-shoot-forming	49.0C	——	——
XDR	callus	24.5E	——	——
	meristematic dome	18.6F	18.2G	——
	leaf	20.1F	18.7G	35.8[d]
	non-shoot-forming	21.3EF	20.9E	——
SCM 7	callus	33.9H	33.0H	——
	meristematic dome	26.7B	26.3B	——

[a] DNA contents given in picogram-equivalents. See text for details

[b] Value significantly less than all other medians; twice median value (11.3) not significantly different from median for diploid root.

[c] Values followed by the same letter were not significantly different (5% level) by the Wilcoxson Rank sum test

[d] Insufficient data for statistical significance test

Microdensitometry

The microdensitometric measurements of nuclear DNA content in the tissue samples are given in figure 3, and the median DNA contents summarized in Table 2. As a measure of staining heterogeneity, variation in the densitometry readings of the cRBC controls was measured. The within-slide coefficient of variation was 4.3%. By comparison with this value, the between-slide variation in cRBC values among simultaneously-stained slides was statistically significant (F test, $P < 0.01$), perhaps indicating unequal staining. Even the total range of variation among slides, however, (4.5%) was not large enough to alter the interpretation of the values for the tobacco cells. The contribution of non-specific absorbance by extranuclear debris was evaluated by measuring the same nuclear DNA contents with differing scanning path size. No significant effect was found. Additional evidence of the lack of artifactual heterogeneity in the readings obtained was the narrow distribution of readings of W38 leaf and root tip nuclei.

The readings for haploid and diploid Wisconsin 38 root tip nuclei were used as standards to indicate 1C, 2C, and 4C DNA contents. By these standards, all callus cultures had median DNA contents of more than 3C. As caulogenesis progressed, there were significant drops in median DNA contents for W38, W38M and SCM7 nuclei. This was true both for the transition from callus to meristematic domes and from meristematic domes to shoots (not done for SCM7). A portion of these decreases can be attributed to the loss of cells with >4C DNA content. However, even if such cells are not considered, the decreases in nuclear DNA content are still significant. No significant differences between cell lines were found for callus or meristematic dome tissues of W38, W38M, and SCM7; but while the median DNA content of W38 leaf was not significantly different from that of 2C root tips, that of W38M leaf was significantly higher (3.4C). The median DNA content of non-shoot forming tissue from W38M did not differ from that of callus, although the shape of the distribution appears different.

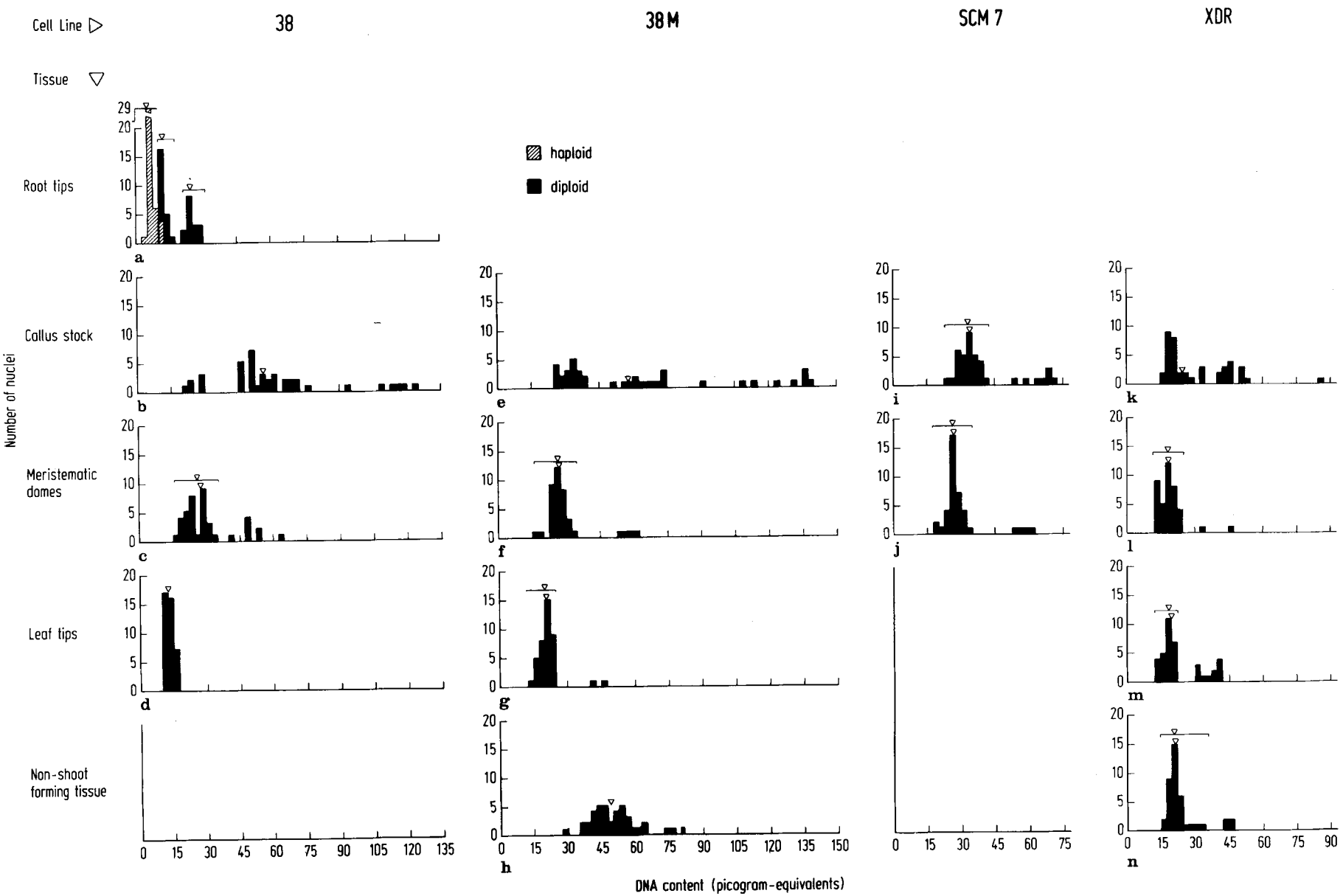

Fig. 3a-n. Distributions of nuclear DNA contents. DNA contents are given in picogram equivalents based on a DNA content of 2.7 pg (Doerschug et al. 1978) for chick erythrocyte internal standards. Larger arrows indicate medians of the total distributions; smaller arrows indicate medians of bracketed regions. a root tips of haploid (▨) and diploid (■) Wisconsin 38 plants. b-d W38 tissues. e-h W38M tissues. i-j SCM7 tissues. k-n XDR tissues

The results of XDR are quite different from those for the other lines. While callus DNA content was lower (median = 4.08C), no major shifts in DNA content were found regardless of morphogenic state. Neither were there any major changes in the shape of the distribution of DNA contents. The median DNA content of the main peak of all distributions was not 2C, however, but approximately 3.2C.

Discussion

In agreement with Rice et al. (1979) we find that caulogenesis can be induced in long-term tobacco cultures. The hormonal responses of such cultures are qualitatively similar to those of short-term cultures, but quantitative differences in response are evident. It is not clear if the cause of these changes is genetic or epigenetic, but they persist long after the initial perturbing influence has been removed.

The continued caulogenic response of all four cell lines indicates that they retain the genetic information necessary for organogenesis. Although genetic changes may be involved in the decline of morphogenic response, such changes cannot involve the irreversible loss of morphogenic genes.

Three different patterns of DNA content were found accompanying caulogenesis. In W38 tissue, the callus DNA contents were broadly distributed with a median value much greater than 2C. During caulogenesis, the distribution narrowed and the median decreased, reaching a value of 2C in the regenerated leaves. A qualitatively similar response was seen in W38M and SCM7 lines, but DNA contents never returned to the 2C level, even in fully developed W38M leaves. For XDR cells, although the initial distribution was narrower and its median closer to 2C, no appreciable changes in the distribution accompanied caulogenesis. Conceivably, since DNA measurements are not fixed to one part of the cell cycle, some of the observed variation could reflect changes in DNA content associated with genome replication. The slow growth rate of the tissues and the absence ($< 1\%$) of mitotic figures suggest to us that most of our cell populations were in G0 (i.e. G1 arrest) and hence genome replication should not be a factor. Further, even in actively dividing tissues, many of the cells would be found in the G1 stage, when DNA content is at its minimum. The decreased DNA content of the cell subpopulation with lowest DNA content (presumably G1 cells) cannot be explained by changes associated with the cell cycle. In addition to the difference in DNA content between callus and leaf tissue, our data also shows an intermediate decrease in DNA content for meristematic dome tissue. This indicates that the change in DNA content associated with caulogenesis is progressive, rather than a single-step selection for organogenic competence.

The lack of a shift in DNA content during caulogenesis of XDR cells is in contrast to the other cell lines examined, and to the chromosomal changes observed by most investigators (Murashige and Nakano 1967; Sacristan and Melchers 1969; Orton 1980). Particularly striking is the difference between XDR and SCM7 since the latter line was derived directly from XDR. We would like to propose that the relatively undisturbed culture conditions (no media alterations, continued selection for threonine resistance) under which XDR had grown for the previous ten years may have resulted in the selection of a stable, adapted genome. Apparently this non-euploid genome is totipotent and stable during the caulogenic process. The selection of SCM7 may then have disrupted this adapted genome.

The recovery of euploid plants from aneuploid callus is not uncommon. Melchers and co-workers (Melchers and Sacristan 1977; Melchers 1977; Wenzel et al. 1979) have also found an increased frequency of aneuploid regenerated plants when cells are maintained as callus for longer periods. Shifts in karyotype during regeneration have been explained as resulting from selection for totipotent genotypes (Sacristan and Melchers 1969; Smith and Street 1974; Brossard 1975; Orton 1980). This is a possible explanation for our observations, although any selection would have to be multiphasic and gradual (i.e. analogous to selection for a quantitative trait) to account for the intermediate distribution of DNA contents in meristematic domes. An alternative explanation which we feel is also compatible with all available evidence is to postulate that karyotype is strictly conserved in organized tissues and that this regulation is impaired or lost in unorganized callus. The restoration of organization during caulogenesis would also progressively restore the karyotypic regulation. As a result, a gradual return to euploidy would ensue. The increasing karyotypic heterogeneity during long-term callus culture could be expected to result in the loss or alteration of genes regulating karyotype in some cells. This in turn would result in an increased frequency of aneuploid regenerated plants. The partial and complete inactivation of the genes responsible for karyotypic regulation in W38M and XDR lines, respectively, would explain their observed DNA distributions during caulogenesis. This hypothesis has the advantage of explaining why W38 returned to a DNA content of 2C during caulogenesis while W38M and XDR did not. Likewise, since regeneration would induce the DNA changes rather than the reverse, it explains why in W38M and SCM7, two lines with very different rates of caulogenesis, identical changes in DNA content accompanied the achievement of the meristematic dome stage.

At present, we have no experimental evidence to distinguish between the above mechanisms. Further, data on

DNA contents is certainly less than definitive. We do not know if leaves with a 2C DNA content actually have 48 normal chromosomes or whether there are offsetting duplications and deficiencies or chromosomal rearrangements. This can only be decided by examination of meiotic metaphase chromosomes and their pairing patterns. Likewise, the apparent uniformity of XDR cell DNA contents may hide heterogeneity in genome compositions in the cell population. Experiments are now in progress to try to answer some of these important questions.

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Ms. P. Traynor
MSU-DOE Plant Research Laboratory
Michigan State University
East Lansing, Mich. 48824 (USA)

Dr. S. Flashman
Stauffer Chemical Company
De Guigne Technical Center
Western Research
1200 S.47th St.
Richmond, Calif. 94804 (USA)