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Analysis of DNA size, content and cell cycle in leaves of Napier grass (*Pennisetum purpureum* Schum.)

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Summary. Mesophyll cell nuclei isolated from leaves of *Pennisetum purpureum* were analysed by flow cytometry to determine the nuclear DNA content and the percentage of cells in different phases of the cell cycle. Samples taken from base, middle and tip regions of leaves 2 to 8 (leaf 1, which was adjacent to the meristem, was too small to sample) showed no significant differences in the amount of DNA per G_1 nucleus due to either age or position. The average amount of DNA per G_1 nucleus was 5.78 pg. Although the majority of cells for each sample were in G_1, samples taken from older leaves had higher percentages of cells in G_2 and S phases. More specifically, base and middle regions of older leaves had a higher percentage of cells in G_2 than all three positions in younger leaves. Electrophoretic analysis of nuclear DNA from leaves 2 to 7 showed no evidence of degradation or difference in fragment size for any sample or position. This study was compared to previous work on the relationship between leaf age and embryogenic competence in *Pennisetum purpureum.* The results suggest that changes in the cell cycle, and/or a loss or fragmentation of the nuclear DNA, are not responsible for loss of embryogenic competence in mature leaf tissue.

Key words: Cell cycle – Embryogenic competence – Nuclear DNA – *Pennisetum purpureum* – Tissue culture

Introduction

The loss of embryogenic (or morphogenic) competence in mature and differentiated tissues of the Gramineae is widely recognized (Vasil and Vasil 1986; Vasil 1987). This has limited the choice of explant material to tissues that are meristematic, such as immature embryos, young inflorescences and the basal region of young leaves. Several studies using leaf explants have shown that temporal and spatial gradients exist within leaves of the Gramineae, such that the youngest (basal) portion of the leaf is the most likely to form embryogenic callus (Wernicke and Brettell 1980; Haydu and Vasil 1981; Lu and Vasil 1981; Wernicke et al. 1981; Hanning and Conger 1982; Ho and Vasil 1983; Wernicke and Milkovits 1984; Linacero and Vazquez 1986; Joarder et al. 1986). Such gradients form the basis of a report by Hesemann and Schröder (1982) which found that the content of DNA per nucleus decreased with increasing leaf age in *Secale cereale* (rye). Furthermore, Beaulieu et al. (1985) have reported that the DNA obtained from mature parts of *Triticum aestivum* (wheat) leaves appears to be fragmented when compared to DNA obtained from immature tissues. They proposed that higher levels of nuclease activity, either spontaneous or induced, were responsible for reduced fragment size. It has been suggested that both fragmentation and/or loss of DNA may be related to the loss of cell division and/or morphogenic competence in mature leaf tissues (Hesemann and Schröder 1982; Beaulieu et al. 1985).

Another factor to be considered regarding the loss of embryogenic competence in mature tissue explants is the cell cycle. Fukuda and Komamine (1981a, b), in their studies of tracheary elements, have shown that the cell cycle is an important factor controlling differentiation. It is also known that the G_1 phase is a major control point for both division and differentiation in cultured cells (Fukuda and Komamine 1981a; Gould 1983). In his review, Gould (1983) has suggested that the cell cycle might play a key role in the regeneration of plants from tissue cultures.

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In an effort to further understand the factors responsible for the loss of embryogenic competence in mature, differentiated tissues of the Gramineae, we have examined the DNA from leaves of *Pennisetum purpureum* (Napier grass) and the percentage of cells in the various phases of the cell cycle. This was done to determine if loss or fragmentation of the DNA and/or cell cycle factors were related to the loss of embryogenic competence in this species.

Materials and methods

Shoots of *Pennisetum purpureum* Schum. (accession number PP-9, obtained from Dr. S.C. Schank) were collected from field grown plants. Two of each of the eight youngest leaves (except leaf 1, which was too small) were separated and used for the analysis. Samples weighing approximately 60 mg were taken from the base, middle and tip regions of each leaf, except for leaf 2 which was too small to yield a sample from the middle region. Samples were labelled both according to their developmental age and position relative to the meristem (leaf 1 being the youngest and closest to the meristem) and according to the location of the sample along the leaf blade (base = B, middle = M, tip = T). For example, sample 3B would represent the base region of the third leaf out from the meristem. Nuclei were isolated from the leaf tissue and stained for flow cytometry according to Galbraith et al. (1983) and Galbraith (1984). Flow cytometry was used to determine both the average amount of DNA per G_1 nucleus and the percentages of cells in the G_1, S and G_2 phases of the cell cycle. In all, 5,000 nuclei were sampled from every position on each of 14 leaves. Seven leaves from one shoot (ages 2 to 8) were analysed in one experiment. Six leaves from a second shoot (ages 2 to 7) and the eighth leaf from a third shoot were used to form a second data set (see Table 1 for leaf lengths). The cell cycle values were recorded as the percentages of cells in G_1, S and G_2 phases from samples of 5,000 nuclei per leaf position.

Statistical analyses were performed on the data from both experiments, using the percentages as observations. Cochran's test for homogeneity of variance was followed by an analysis of variance (ANOVA) to examine the effect of leaf position and age on the amount of DNA and the percentages of cells in the G_1, S and G_2 phases of the cell cycle. Since no middle region sample could be obtained from leaf 2, no leaf 2 data were included in the statistical analyses. Factors shown to be significant by the F-test (α = 0.05) were further examined using Duncan's multiple range test. Four separate analyses were conducted; one for DNA amount and one for each of the three cell cycle phases (G_1, S and G_2).

The size of the DNA was determined by isolating DNA from leaves 2 to 7 according to the methods of Dellaporta et al. (1983) and Rogers and Bendich (1985). Five to six leaves per age class and 1–4 g of leaf tissue per sample position were used with the Dellaporta method. One leaf per age class and 100 mg per sample position were examined using the method of Rogers and Bendich. The DNA was run out on 0.8% to 1.0% agarose gels with ethidium bromide using a BRL lambda *Hin*dIII marker for size determination.

Results

The analysis of variance showed no significant difference in DNA amount per G_1 nucleus for either sample position (α = 0.05, v = 12, v = 18) or age (α = 0.05, v = 5, v = 18; Fig. 1). For all samples, the average amount of DNA per G_1 nucleus was 5.78 pg. No correlation was seen between DNA amount and leaf length (Fig. 1). Cell cycle data indicated that the majority of cells in all samples were in G_1 (58.2% to 85.2%, Table 2). However, older leaves contained a significantly higher percentage of cells in G_2 and a lower percentage in G_1 than younger leaves (Fig. 2). The data obtained for leaf 2 samples, which were not used in the statistical analyses, fell within the range of the trends based on leaves 3 to 8 (Fig. 2, Table 2). The base and middle regions of older leaves possessed a higher percentage of cells in G_2 when compared to all positions in younger leaves, as well as the tip regions of older leaves. The opposite was true for G_1 phase (Table 2, Fig. 3). The F-test demonstrated that leaf age has a significant effect on the percentage of cells in S phase. This is in contrast to Duncan's multiple range test which failed to detect individual means significantly different from the others. A slight trend showing an increase in the percentage of cells in S phase with increasing leaf age can be seen in Fig. 2. The position of the sample, however, did not appear to have a significant effect on the percentage of

Table 1. Leaf length data for cell cycle analysis grouped by leaf age

Leaf Age	Replicate 1 Leaf Length	Replicate 2 Leaf Length	Average Length
	(cm)	(cm)	(cm)
2	14	18	16.0
3	33	34	33.5
4	54	49	51.5
5	76	68	72.0
6	89	84	86.5
7	98	100	99.0
8	99	110	104.5

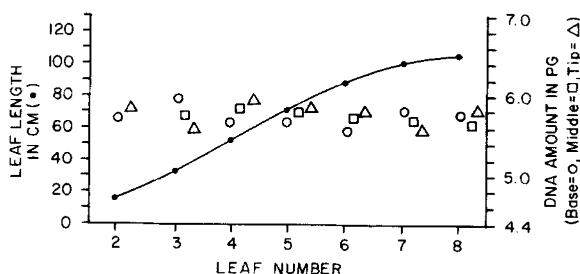

Fig. 1. Leaf length and DNA content grouped by leaf age. For DNA content (given in picograms) each sample point is the mean DNA content taken from the percentage of G_1 nuclei in a sample of 30,000 (15,000 from each of 2 experiments). Leaf age numbers refer to the position of the leaf with respect to the meristem. Note that no samples were significantly different with respect to DNA amount

Table 2. Results of Duncan's multiple range test on cell cycle data grouped by sample position. Means followed by the same lower case letters are not significantly different at $\alpha = 0.05$, $n = 2$ observations/mean. Asterisks indicate the rankings with which leaf 2 base (*) and tip (**) data are most closely associated. S Phase data grouped by sample position were not significant by the F-test, and were not tested using Duncan's multiple range test. Each of the 2 observations/mean represents a percentage of 5,000 sampled nuclei. B = base, M = middle and T = tip regions of the leaf. Numbers 3 to 8 refer to the position of the leaf away from the meristem

G 1 Phase							G 2 Phase			
Sample Position	Ave. % of Cells	Groups of Means					Sample Position	Ave. % of Cells	Groups of Means	
4T	85.2	a					7M	21.2	a	
3M	83.8	a					8B	19.8	a	
4M***	83.2	a					7B	19.4	a	
3T	81.6	a					8M	18.8	a	
5M	81.4	a	b				6B	11.9	a	b
5T	81.2	a	b				4B	9.6		b
6T	80.1	a	b				6M	9.3		b
3B	78.7	a	b				5B	8.5		b
5B	77.6	a	b				3B	8.3		b
6M	76.1	a	b				7T	8.1		b
8T	75.6	a	b				4M*	6.3		b
7T	75.2	a	b				8T	5.6		b
4B	72.2		b	c			3T	4.5		b
6B	67.1			c	d		5T	4.5		b
8M	64.0				d	e	5M	4.5		b
7B	63.0				d	e	3M**	2.6		b
8B	59.0					e	6T	2.2		b
7M	58.2					e	4T	1.7		b

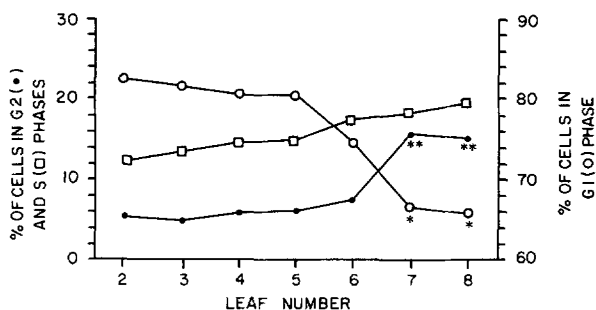

Fig. 2. Cell cycle percentage grouped by leaf age. Each point represents the percentage of 30,000 sampled nuclei (base, middle and tip regions combined), except for leaf 2 data, which are based on 20,000 nuclei since no middle sample positions were available. Leaf 2 data were not used in the analysis, but follow the trends shown by leaves 3 to 8. $\alpha = 0.05$, $n = 6$ observations/mean for each of the cell cycle phases. Each of the 6 observations/mean represents a percentage from a sample of 5,000 nuclei. Results of Duncan's multiple range test on leaf age data: * leaves 7 and 8 had a significantly lower percentage of cells in G1 than leaves of all other ages; ** leaves 7 and 8 had a significantly higher percentage of cells in G2 than leaves of all other ages

cells in S phase (Fig. 3). Note that with respect to leaf age and sample position, leaves 7 and 8 form a group that is separate from leaves 2 to 5, with leaf 6 cell cycle values being intermediate to those of leaves 2 to 5 and 7 to 8 (Figs. 2 and 3).

High molecular weight DNA isolated using either the Dellaporta et al. (1983) or the Rogers and Bendich (1985) procedures showed no evidence of DNA degradation from any sample position or age (Fig. 4).

Discussion

The fragmentation of wheat DNA that results in the appearance of 1 to 10 kb fragments (Beaulieu et al. 1985; Jones and Boffey 1984) was not seen in *Pennisetum purpureum*. The method of isolating DNA was the same as that used in the report on wheat. An alternative explanation for the lack of morphogenic competence of differentiated tissues could be the reported loss of DNA in older leaf tissues of rye (Hesemann and Schröder 1982). However, these differences in nuclear DNA content do

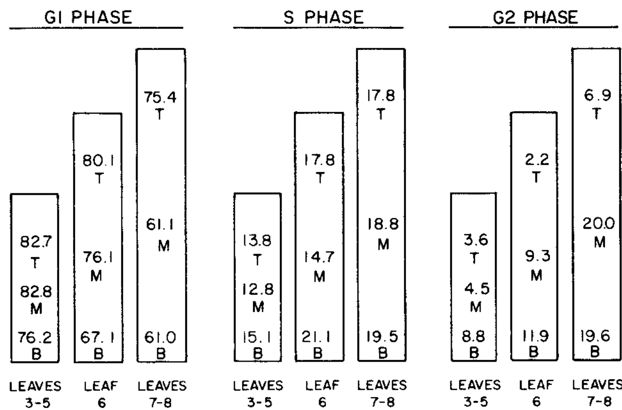

Fig. 3. Summary of the percentage of cells in G_1, S and G_2 phases of the cell cycle by sample position and age. B = base, M = middle and T = tip regions of the leaves. Numbers 3 to 8 refer to the position of the leaf away from the meristem. This is a diagramatic representation; see Table 1 for actual leaf lengths

not appear to have been subjected to a thorough statistical analysis. The present study found no evidence for the loss or fragmentation of nuclear DNA in leaf tissues of *P. purpureum* which could be related to the observed temporal and spatial gradients of embryogenic competence.

The cell cycle data compliments work by Rajasekaran et al. (1987a). In that study four regions of leaf tissue were examined for embryogenic competence and endogenous levels of plant growth regulators. The basal region (defined as the first 30 mm of the four leaves closest to the meristem) had the highest levels of IAA and ABA and the highest percentage of embryogenic callus formation (45%). The middle region (30 to 60 mm from the meristem) had the next highest levels of IAA, ABA and embryogenic callus formation (28%), followed by the distal region (60 to 90 mm from the meristem; 5% embryogenic callus formation). The mature tissues (consisting of leaves 5 and 6 and the ex-

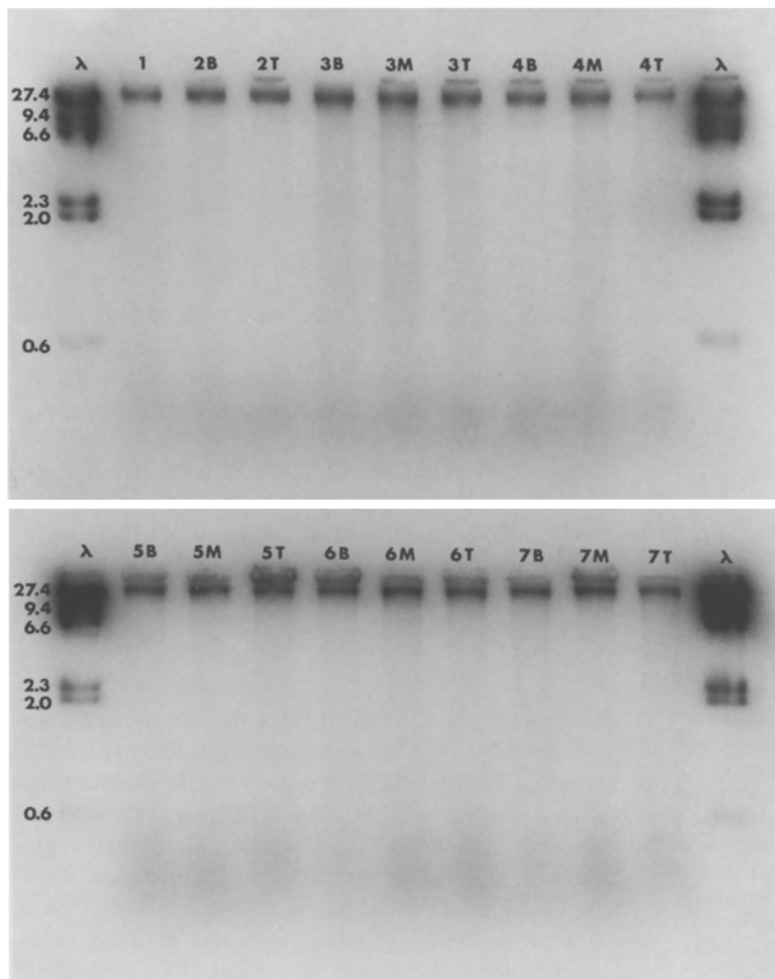

Fig. 4. DNA sample size. Agarose gel showing no evidence of genomic DNA degradation for any sample position or age. See "Materials and methods" for explanation of sample codes. A BRL *Hin*dIII marker was used for size determination

panded tip of leaf 4) had low levels of IAA and ABA, and formed no embryogenic callus. The gradient of response which is obvious just 60 mm from the meristem indicates that the factors affecting embryogenic competence are doing so at a very young stage. In our study, all of the samples from middle and tip regions were located more than 90 mm from the base of the leaf, yet distinct cell cycle changes were not seen in these samples until the sixth leaf. This strongly suggests that there is no correlation between the observed cell cycle changes and embryogenic competence in *P. purpureum*.

In another study, Karlsson and Vasil (1986) observed fully mature leaves that were older than the eighth leaf used in this study. These leaves had 100% of their cells in G_1 (similar results were reported in rye by Hesemann and Schröder, 1982). This differs from the lower G_1 values observed in the seventh and eighth leaves of the present study. It is possible that the cells of the seventh and eighth leaves had not completed their last cell division. If so, this would explain why they had a higher percentage of cells in G_2 and S phases than the older leaf cells examined by Karlsson and Vasil (1986). This also suggests that leaf cells of *Pennisetum purpureum* lose their morphogenic competence before they have completed their last cell division, and idea similar to that put forth by Joarder et al. (1986) in their study on *Lolium*. It is clear that the cell cycle changes and the completion of cell divisions which result in 100% of the cells being in G_1 are not critical to embryogenesis since embryogenic competence is lost long before a leaf reaches the seventh or eighth position out from the meristem.

The association between endogenous plant growth regulator concentrations and the age of the leaf tissue is still the most likely explanation for embryogenic competence (Rajasekaran et al. 1987a, b). Recently, Wernicke and Milkovits (1987a, b) have provided evidence that the sensitivity of wheat leaf tissue to auxin could be as important as the concentration. Other cell cycle factors, such as the need for cells to be in a competent phase (most likely G_1) are not ruled out by the results of the present study.

In summary, the results suggest that changes in the percentages of cells in a particular cell cycle phase and/or a loss or fragmentation of the nuclear DNA are not responsible for the loss of embryogenic competence in mature leaf tissues of *Pennisetum purpureum*.

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