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Ploidy levels in leaf callus and regenerated plants of *Solanum tuberosum* determined by cytophotometric measurements of protoplasts

E. Jacobsen, M. J. Tempelaar and E. W. Bijmolt

Department of Genetics, Biological Centre, University of Groningen, Kerklaan 30, NL-9751 NN Haren, The Netherlands

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Summary. In potato (*Solanum tuberosum*) instabilities in nuclear DNA content in callus cells of a monohaploid and a dihaploid and in tuberprogenies of a regenerated, doubled dihaploid plant were assessed by cytophotometry in isolated, purified protoplasts. Leaf protoplasts of a tetraploid variety have nuclei with 3.6 ± 0.08 pg of DNA. In callus protoplasts a dihaploid showed 3 and a monohaploid 6 ploidy levels. Progeny of a plant, doubled and regenerated from dihaploid callus, showed mixo-euploidy, indicating an instability similar to the dihaploid callus; in addition, there are mixo-aneuploid cells.

These findings confirm and extend earlier observations based on chromosome counts in other genotypes of potato. The experimental approach can be applied to a variety of other purposes.

Key words: Potato – Mixoploidy – Cytophotometry – Tissue culture – Regenerated plants

Introduction

Ploidy levels need to be determined in the course of several lines of investigation. In mutagenesis experiments, the amount of haploid protoplasts (from callus or plant tissues) is decisive for the yield of recessive mutations. Stability during cell culture and regeneration may be estimated by the degree of mixoploidy. In interphase nuclei, DNA content can be measured by cytophotometry. This method has fewer limitations than the counting of chromosomes, as the mitotic index is often very low in cell and callus cultures (Sopory and Tan 1979), and in certain tissues. In addition, it permits characterization of populations of cells by their replicative stage in interphase (G_1; S; G_2), which may be useful in evaluating synchronization procedures of hybridisation experiments.

Cytophotometric studies have been carried out on nuclei in sections (Libbenga and Torrey 1973; Blaschke et al. 1977), squashes (Greilhuber 1978; Dons et al. 1974), suspensions of protoplasts (DeBoucaud 1980), cells (Berlyn et al. 1979) or isolated nuclei (Brunori and Ancora 1977) in various plants but not in potato.

In this paper methods are developed for studying ploidy levels of potato by manipulating protoplasts isolated from callus and leaf tissue. The aim of the present study was to determine ploidy levels in a) leaf callus of a monohaploid and b) in leaf callus of a dihaploid and in leaves of tuber progenies from a plant regenerated from callus of the same dihaploid genotype, which was presumed to have doubled its chromosome number. Earlier studies indicated mixo-aneuploidy in plants after doubling, from root tip chromosome counts of callus regenerated plants (Jacobsen 1978, 1981).

Materials and methods

1 Material

Solanum tuberosum plants needed for leaf callus culture and for leaf protoplast isolation were grown in a temperature-controlled glasshouse with a day length of 16 h, a day temperature of 25 °C, and a night temperature of 20 °C.

Genotypes used were cv Astarte ($4x = 48$), interdihaploid H²578 ($2x = 24$, kindly supplied by MPI, Cologne, BRD), a parthenogenetically induced monohaploid 82.018 (as "PD23-3-2" kindly supplied by Prof. Hermsen, IVP, Wageningen, The Netherlands) and leaf callus regenerated plants of H²578.

2 Leaf callus culture and plant regeneration

From just full-grown top-leaves of about 6 weeks old plants (genotypes H²578 and 82.018) leaf callus was induced. Callus induction and culture were performed according to Behnke

(1975). Callus induction- and callus culture medium was basic MS-medium (Murashige and Skoog 1962) supplemented with 5 mg/l NAA (α-naphthylene acetic acid).

Plant regeneration was according to Behnke (1975) on basic MS-medium supplemented with 2 mg/l zeatin riboside at 25 °C and 14 h light (8,000 lux). For further growth regenerated shoots were transplanted onto basic MS-medium without any phytohormones and containing 20 g/l sucrose. Finally, they were transplanted directly into soil, with R.H. of 100% till rooting.

Ploidy level of 20 different regenerated plants, was tentatively determined by plastid counts in stomatal guard cells and by their habitus (Jacobsen 1981). Fifteen plants were still dihaploid and 5 seemed to be doubled.

The regenerated plants are referred to as LC_1 (Leave Callus culture) $H^2578/1-20$ and tuber progenies, derived from these, as LC_2. Further propagation of LC_2 yielded LC_3. LC_2H^2 578/15/1 and $LC_3H^2578/15/1/1$ were used for the experiments.

3 Isolation and fixation of cells and protoplasts

(a) Leaf protoplasts. Isolation of protoplasts was done by incubating 1 mm narrow leaf-strips at 25 °C in the dark floating on enzyme solution in a petri dish (10 cm ∅). The enzyme solution contained per 20 ml: 0.18 g cellulase R_{10}; 25 mg pectolyase; 0.058 g $CaCl_2$; 0.0195 g MES. As osmoticum, 2.2 g mannitol was present. Alternatively, $MgSO_4 \cdot 7\ H_2O$ was used, which is reported to be effective in the mold *Schizophyllum* to get highly purified floating protoplasts (De Vries and Wessels 1975). For leaf protoplasts of potato, the amount of $MgSO_4 \cdot 7\ H_2O$ to get a suitable density of the solution, turned out to be 3.44 g. pH was adjusted to 5.8.

With 20 ml solution, 2 g cut leaf material was treated. After about 17 h the suspension was passed through a sieve (100 µ) and centrifuged at 80 g during 10 min. The floating ($MgSO_4$) or sedimented (mannitol) protoplasts were collected and washed several times with enzyme-free solution. Purified protoplasts were fixed in 70% (m)ethanol-acetic acid (3 : 1) with water added to avoid precipitation and crystallisation of mannitol or $MgSO_4$ (Wichers et al. 1983, submitted).

(b) Callusprotoplasts. The culture medium yielded a soft callus suitable for protoplast isolation. The enzyme solution was the one mentioned above, modified to contain per 20 ml: 2.46 g $MgSO_4 \cdot 7\ H_2O$ and 2 g meicellase instead of cellulase R_{10}. In this way protoplasts always float and can be purified efficiently as described for leaf protoplasts.

Three grams of callus or sieved cell suspension were treated in petri dishes (10 cm ∅) with 20 ml enzyme solution.

(c) Cells from root tips. In order to obtain cells with mitotic stages to be used as reference values, fixed root tips were converted into cell suspensions. They were rinsed in water, treated with pectinase 2% in 0.01 M citrate buffer, pH 4.7 at 28 °C, homogenized with a hand-held Dounce-type homogenizer in 45% acetic acid, and subsequently forced through a hypodermic needle. Callus and leaf-tissue may be treated similarly, if no living protoplasts are required in the course of the experiment.

4 Preparation and staining procedures

Small volumes of the fixed suspensions were dropped onto slides from a height of about 100 cm until sufficient cells were present for efficient microscopic work, as judged from pilot experiments. After drying, preparations were rinsed in H_2O to eliminate any remaining mannitol or $MgSO_4$. The Feulgen staining procedure was applied as described in Tempelaar (1980), with 30 min hydrolysis in 5N HCl at 28 °C.

5 Cytophotometry

The absorbance of the nuclei at 558 nm was determined with a Zeiss MPM 01 microscope-photometer and scanning stage. The equipment was modified as described in Tempelaar (1980). The step-width used was 0.5 µ in the initial experiments ("slow" mode), so as to have a continuous set of measurements over the whole area of the nucleus. To increase the sample size, the step-width was changed to 1 µ in later experiments ("fast" mode). This yields a discontinuous sampling pattern and reduces the number of measurements and, consequently, the time required per nucleus to 25%. The figures for area and absorbance obtained are to be multiplied by 4 to get values comparable to that of the continuous procedure. In order to evaluate this trade-off of accuracy against speed, comparisons between series of measurements were made. In addition, the effect of leaving out the cumbersome hand-operated light limiting diaphragm was determined, which is liable to result in a reduction of the apparent DNA value, because of an increased stray light error. The results (Table 1) indicate, that in both diploid and tetraploid nuclei, the differences in each category of nucleus are small. Increasing the step-width had a decreasing effect of far less than 1% on the apparent DNA value. The effect of omitting to insert the limiting light diaphragm before each measurement was larger, i.e. in the order of 2.5–3.5%. The combined effect of both errors was a systematic error of a magnitude which was not considered objectionable when dealing with determining ploidy levels. Consequently, "fast" and "slow" measurements are not designated as such in the text; "fast" measurements were not corrected to compensate for their slightly lower values.

To determine the DNA-content of the potato-nuclei, chicken erythrocytes obtained from heparinized chicken blood was used as standard. Measurements were performed in the "slow" mode as described above. In order to avoid bias in the comparison between the chicken- and the potato-nuclei due to distributional and stray light error, the apparent DNA values were corrected according to principles developed by Duijndam et al. (1980a, b). Instead of reading back the values from the graphs in their papers, however, they were obtained from equations residing in the statistical program of the calculator associated with the cytophotometer unit, which were developed to generate the corrected values automatically.

6 Squash preparations of root tips

For chromosome counts, squashes were made after 30 min hydrolysis of Feulgen-stained root tips, which were post-treated with pectinase as described in another context for *Vicia faba* (Tempelaar et al. 1982).

Table 1. Comparison of DNA values of nuclei, measured in different ways

Step-width	Limiting light diaphragm	Diploid nuclei[a] Mean ± SEM (%)	Tetraploid nuclei[b] Mean ± SEM (%)
0.5 µ	present	28.124 ± 1.672	55.595 ± 2.540
0.5 µ	absent	27.411 ± 1.644	54.177 ± 2.525
1 µ	present	28.112 ± 1.637	55.948 ± 2.563
1 µ	absent	27.324 ± 1.646	54.114 ± 2.633

[a] $N = 32$, [b] $N = 22$

Results

DNA values in reference material

The mitotic stages of suspended root tip cells (Table 2) contained 24 chromosomes and thus could be considered to represent the diploid number of chromosomes, carrying the 4C-DNA-amount. The absorbance value of these stages was 25.579. Interphase nuclei with half this amount were designated as the 2C-stage with the same ploidy-level, nuclei with the same amount of DNA as the metaphase cells were assigned to the 4C, diploid class of replicated G_2 nuclei. Interclass-values, visible in root-tip-cells (Fig. 1A1), probably indicate DNA-replication in S-phase. In leaves of older flowering plants of both H²578 and cv Astarte mitotic activity had obviously ceased, judging from the single C-levels in protoplasts, shown in Fig. 1A1 and B and enumerated in Table 3. The mean for diploid plants is the same as for the 2C-figure for root tips, while the tetraploids have twice that amount.

In the case of young, diploid plants (Fig. 1A2), there are two populations of nuclear DNA values. The frequency distribution is not unlike that of the root tips (Fig. 1A1), suggesting the presence of 2C and 4C levels.

A comparison was made between the Feulgen-absorbance of chicken erythrocytes, used as a reference because of their well-established DNA-content (2.5 pg) as well as the absence of DNA-replication, and nuclei of potato (Table 2). After extrapolation to the 4C value, the DNA content of normal tetraploid potato was cal-

Table 2. Feulgen-absorbance in interphase and mitotic stages in potato material

Object	C-value	No. of nuclei	Absorbance	
			Mean	Coeff. of variation
Dihaploid H²578				
Root tip cells				
mitotic pro- and meta-phase	4C	14	25.579	11.545
interphase	2C	52	14.577	18.699
	4C	119	25.378	9.119
Leaf protoplasts				
young plant[a]	2C	160	13.144	11.695
	4C	43	25.860	9.716
old plant[b]	2C	60	14.420	11.370
c. v. Astarte				
Leaf protoplasts				
old plant[b]	4C	65	28.050	9.697
Chicken				
Erythrocytes	2C	33	20.268[c]	4.279
Monohaploid 82.018				
Callus	8C	20	59.050[c]	4.645

[a] Full-grown and just full-grown top-leaves collected from 6-weeks old plants
[b] Full-grown top-leaves collected from flowering plants
[c] Corrected for optical errors; erythrocytes on the same slide as callus cells

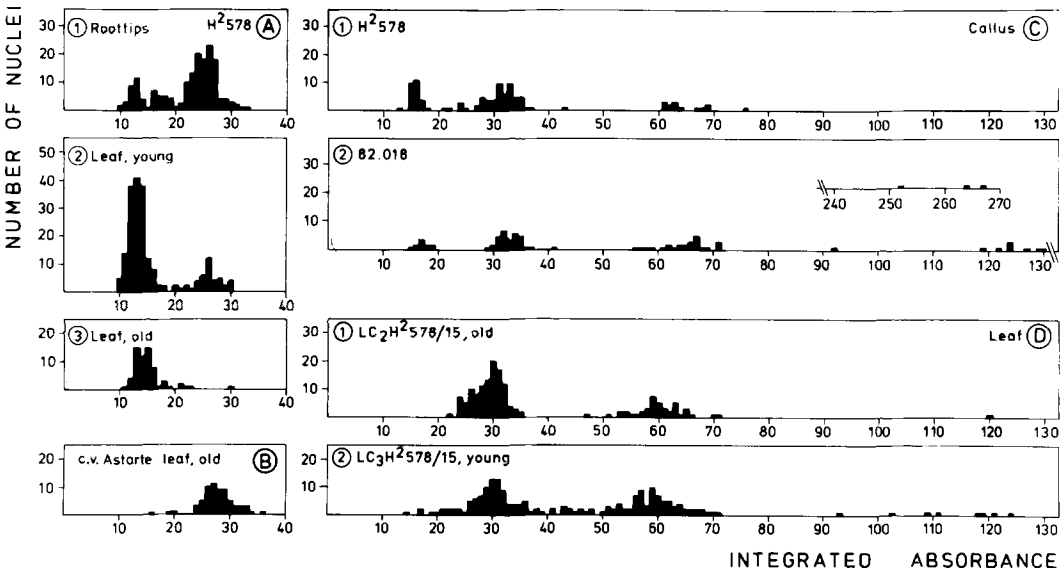

Fig. 1. Frequency distributions of Feulgen-DNA absorbance in nuclei from tissues and callus of *Solanum tuberosum*. *A 1* root tips and *A 2,3* leaves of dihaploid H²578; *B* leaves of cv Astarte (tetraploid); *C 1,2* callus of dihaploid H²578 and monohaploid 82.018; *D 1,2* leaves of first and second generation tuber-propagated plants from callus-regenerated, doubled dihaploid H²578

culated as 3.6 ± 0.08 pg. This amount of DNA yields rather faintly stained nuclei, when flattened as described. For measurements however, this is advantageous, because distributional and diffraction errors are smaller at low local absorptions (Duijndam et al. 1980a). This is borne out by the minor changes in values at fast and slow measuring procedures as described in the "methods" section.

DNA values in callus and in regenerated plants

Inspection of Table 3 reveals the presence of an increased range of DNA values in callus and tuber-propagated plants. Instead of one or two classes, there are at least three. In callus of H²578, the majority of the nuclei has the DNA amount which in plant tissue is associated with the 4C-value and 2x ploidy level. However, as the cells are taken from a culture past its actively growing phase, which is confirmed by the scarcity of interclass-values in Fig. 1C1, it seems reasonable to ascribe the differences in DNA content mainly to the presence of higher chromosome numbers, the more so since 8C values are also present: in this view, the cells of the callus would contain, beside normal diploid nuclei, tetraploid and octoploid ones in relatively large proportions.

Table 3. Feulgen-absorbance of callus protoplasts of dihaploid H²578, monohaploid 82.018, and leaf protoplasts of tuber-propagated plants from a regenerated, doubled plant ($LC_1H^2578/15$)

Object	C-value	No. of nuclei[a]	Absorbance	
			Mean	Coeff. of variation
Callus protoplasts				
H²578	2C	29	16.138	10.951
	4C	54	31.574	8.106
	8C	13	64.077	4.787
82.018	1C	8	6.930	19.484
	2C	11	17.273	6.931
	4C	35	32.657	9.094
	8C	25	64.760	6.495
	16C	13	128.231	4.067
	32C	3	260.300	3.199
Leaf protoplasts				
$LC_2H^2578/15/1$				
old plant	4C	112	28.960	9.220
	8C	39	59.590	6.125
	16C	1	120.000	
$LC_3H^2578/15/1/1$				
young plant	4C	94	30.043	13.115
	8C	78	59.333	8.001
	16C	8	115.125	5.714

[a] Sampled randomly, except for 82.018, 1C values

The same reasoning applies to cells of callus originating from the monohaploid (82.018); in this culture three months after induction, six ploidy-levels are present (Table 3, Fig 1C2). In contrast with callus of H²578, the original (monohaploid) level is present in an extremely low frequency, as indicated by the number of only 8 monohaploid nuclei found among thousands of others.

In leaf protoplasts of the doubled LC_2 H²578/15/1, harvested in flowering stage, three populations of nuclei can be found (Fig. 1D1). The lowest corresponds with the 4C level of tetraploids. The distribution pattern is not comparable with that of a normal tetraploid (Table 2 and Fig. 1B) suggesting the presence of additional 8x-cells (mixoploidy). In tuber-propagated plant $LC_3H^2578/15/1/1$, the distribution pattern again suggests the presence of at least 4x and 8x cells (Fig. 1D2). The shape of the frequency distribution for 4C values is somewhat skewed in Fig. 1D1 and 1D2, which suggests the presence of cells with hypotetraploid chromosome numbers.

In an attempt to support the cytophotometric data, a number of mitotic metaphases was scored in squashes of root tips of $LC_3H^2578/15/1/1$. The number of chromosomes observed confirmed the assumptions about the presence of 4C and 8C levels, as cells with 48 and 96 chromosomes were scored. In addition, a number of cells indeed showed aneuploid numbers, e.g. 30, 38, 42, 43. These cells may stem from abnormal reductional mitosis, indicated by the observation of separate groups of chromosomes in metaphases of polyploid cells.

Discussion

Remarks on the technique

Application of enzymatic tissue digestion to obtain separate cells and protoplasts and cytophotometric measurement of their nuclei turns out to be feasable in *Solanum tuberosum*.

Sacrificing topological relations, one can obtain perfectly flattened, whole nuclei without much background, in any concentration required. In the present paper, these were used to assess stability in callus cells and regenerated plants from the degree of mixo-euploidy and mixo-aneuploidy. The data are conclusive for determining presence and frequency of ploidy classes in protoplast suspensions. It can also be applied as a positive test for the presence of additional ploidy levels in callus and plants. When used to determine low frequency or absence of a class in a tissue, however, caution is warranted and additional verification may be needed to eliminate the possibility that the absence of certain cell types is due to selection in the procedures. It is legitimate however to compare frequency distri-

butions from a variety of sources, as reference plant material yielded consistent results: bimodal frequency distributions from growing material, unimodal 2C and 4C distributions from diploid and tetraploid older plants.

As for mixo-aneuploids, chromosome counts in root tips indicated extensive loss of chromosomes in tuber progenies of regenerated plants. Accordingly, the shape of the frequency distribution of DNA values of nuclei in leaves at the 4C level from the same plants suggests loss of chromosomes, resulting in hypotetraploidy.

A point deserving further attention is the difference in DNA values of similar ploidy levels between callus and plants (Table 3), the figures of callus being 5–15% higher in value. Although differences of 10–20% in DNA content is claimed in plants under different conditions (early development, senescence, wounding) (Nagl 1979; De Cleene et al. 1980; Dhillon and Miksche 1982), a similar assertion is not made here at this point, as this would require additional information to quantify the amount of variation within and between experiments evaluating the effects of pretreatments, density of the chromatin, shape of the hydrolysis curve.

DNA content and ploidy levels in Solanum tuberosum

A genome of 48 chromosomes has a 4C DNA-amount of 3.6 ± 0.08 pg which is uniformly present in nuclei from leaves of old plants according to measurements in protoplast suspensions. Likewise, leaves of dihaploids have only one predominant ploidy level.

A doubled plant, regenerated from dihaploid callus, showed extra ploidy levels in leaves, when judged from cytophotometric data (Table 3); in root tips chromosome counts also suggested instabilities before, during and after regeneration. As the callus itself yields several ploidy levels, instability in this phase is clearly indicated. This is confirmed by the result, mentioned in the "methods" section, that after regeneration, dihaploid as well as tetraploid plants have been found. In addition to the presence of mixo-euploidy, the earlier conclusions of Jacobsen (1981) on the occurrence of mixo-aneuploidy are confirmed. These abnormalities appear not only in regenerated plants, but also in their tuber-propagated descendants. Chromosomal changes of this nature have also been observed in plants regenerated from leaf protoplasts of dihaploids (Wenzel et al. 1979) and tetraploids such as cv Bintje (Roest, pers. commun.) and cv Maris Bard and cv Fortifold (Karp et al. 1982).

In contrast to the situation in callus protoplasts from dihaploid H^2578, callus from the monohaploid seemed more unstable, as more ploidy levels could be observed in protoplasts whereas the original (monohaploid) ploidy level was infrequently found.

This is another indication that monohaploid cells are in the minority in the callus: the failure of regeneration of monohaploids from a similar experiment (not shown) may be taken to support this assumption, and also the fact that Sopory and Tan (1979) could observe some monohaploid cells only in the initial stages of calli originating from microspores during anther culture of dihaploids.

Research efforts now focus on 1) selection of monohaploid genotypes (derived from parthenogenesis and anther culture) with improved stability in cell culture and 2) separation of material from different ploidy levels before DNA measurement by means 1-g sedimentation.

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