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Variable DNA content of *Cyclamen persicum* regenerated via somatic embryogenesis: rethinking the concept of long-term callus and suspension cultures

Thomas Borchert · Jörg Fuchs · Traud Winkelmann · Annette Hohe

Abstract By flow cytometric experiments in *Cyclamen persicum* both with propidium iodide (PI) and DAPI (4′,6-Diamidino-2-phenylindol)-staining we were able to present (1) a new estimation for the absolute DNA content in the range of 3.17 pg DNA/2C and (2) ploidy abnormalities which were detected in the pathway of somatic embryogenesis. These aberrations might have arisen from ploidy mutations or abnormal polyploidization processes. Morphologically normal somatic embryos contained the smallest proportion of individuals with ploidy abnormalities, but also a regenerated plant in the green house displayed a significantly elevated DNA content. The results are discussed in the framework of future application of in vitro propagation techniques based on regeneration from fastly growing undifferentiated cells like long-term callus and suspension cultures.

Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
2-iP	6-(γ,γ-Dimethylallyl-amino)purine
CV	Coefficient of variation
DAPI	4′,6-Diamidino-2-phenylindol
DI	DNA-index
PI	Propidium iodide

T. Borchert
Department of Hospital and Clinical Engineering, Environment and Biotechnology, University of Applied Sciences Giessen, Wiesenstrasse 14, 35390 Giessen, Germany

Present Address:
T. Borchert · A. Hohe (✉)
Department of Plant Propagation, Institute of Vegetable and Ornamental Crops, Kuehnhaeuser Strasse 101, 99189 Erfurt-Kuehnhausen, Germany
e-mail: hohe@erfurt.igzev.de

J. Fuchs
Leibniz-Institute of Plant Genetics and Crop Plant Research, Corrensstrasse 3, 06466 Gatersleben, Germany

T. Winkelmann
Institute of Floriculture and Woody Plant Science, Leibniz Universität Hannover, Herrenhaeuser Str. 2, 30419 Hannover, Germany

Present Address:
T. Winkelmann
University of Applied Sciences Weihenstephan, Am Staudengarten 8, 85354 Freising, Germany

Introduction

Propagation of *Cyclamen persicum* via the pathway of somatic embryogenesis offers the possibility of vegetative cloning with convincing reproduction rates

(Schwenkel and Winkelmann 1998), which is of high practical interest for this ornamental crop that is conventionally propagated exclusively via seeds. Suspension and bioreactor cultures have been established for further optimization as an economically competitive propagation system (Winkelmann et al. 1998a; Hohe et al. 2001). But, major problems concerning the quality of the regenerated plantlets, e.g. low desiccation tolerance of somatic embryos (Seyring and Hohe 2005) and a missing maturation phase (Schmidt et al. 2006) as well as asynchronous development, did prevent a successful industrial scale-up so far. Additional problems might arise from the concept of long-term suspension cultures. It has been reported for a wide range of crops, that in vitro regeneration especially with a prolonged callus phase might result in an enhanced rate of ploidy instability including both polyploidy as well as aneuploidy (e.g. Ezura and Oosawa 1994; Oh et al. 1995; Kubalakova et al. 1996; Nayak and Sen 1997). Recent publications focussing on ploidy stability after regeneration via somatic embryogenesis demonstrated the occurrence of both, ploidy stable as well as instable embryos after prolonged cell culture phases (Endemann et al. 2001; Pinto et al. 2004; Loureiro et al. 2005; Leal et al. 2006). However, in callus cultures of *C. persicum* no ploidy mutations were detected (Winkelmann et al. 1998b). Due to an altered methodological setup, our experiments resulted in considerably high rates of mutations regarding the DNA content of embryos regenerating from long-term suspension cultures of *C. persicum*.

Analyses were performed by flow cytometric measurements, which offer the possibility of fast and large scale analysis of the DNA content of cells for a variety of purposes, e.g. determination of species specific DNA amount, analysis of the cell cycle activity in different tissues and measurement of endopolyploidization levels (Galbraith 1989; Dolezel et al. 2007).

Results are discussed in the context of the recently published plant stem cell concept of Laux (2003). Additionally, the DNA content of *C. persicum* published by Winkelmann et al. (1998b) is corrected.

Materials and methods

Somatic embryogenesis

The embryogenic cell line 3738 was established from an in vitro regenerated plant of the cultivar 'Purple Flamed' (originating from plants of Royal Sluis 'Purple Flamed' ($2n = 2x$) used for callus induction in 1990) on semi-solid medium [2 mg/l 2.4-dichlorophenoxyacetic acid (2.4-D), 0.8 mg/l 6-(γ,γ-dimethylallyl-amino)purine (2-iP)] in July 2002 as described in Schwenkel and Winkelmann (1998). Suspension cultures were set up in January 2004 and regular subculturing was carried out as specified in Winkelmann et al. (1998a).

Differentiation and germination as well as classification (globular, torpedo-shaped and cluster) of somatic embryos were performed as described previously (Seyring and Hohe 2005; Schmidt et al. 2006). Somatic embryos of each morphological subclass were analysed 21–49 days after initiation of embryo development by transfer to growth regulator free medium. In addition ten randomly chosen regenerated adult plants (leave pieces of about 0.5 cm^2) already transferred to greenhouse conditions were analysed.

Flow cytometry

Measuring DNA content by DAPI-staining

Flow cytometric estimations of DNA contents were accomplished by UV-excitation of 4′,6-diamidino-2-phenylindol (DAPI)-stained nuclear DNA which was prepared by usage of the Partec 'CyStain UV Precise P' kit ('nuclei extraction buffer', 'staining buffer', Partec GmbH, Münster, Germany). Sample preparations as well as measurement techniques with the Partec Ploidy Analyser (Partec GmbH) are described in Schmidt et al. (2006).

By comparing the DNA content of sample and internal reference standard (leaves of *Solanum lycopersicum* cv. Stupické, kindly supplied by J. Dolezel, http://www.ueb.cas.cz/Olomouc1/LabDol/LabHome/home_labdol_allgrey.htm) the DNA-Index (DI) as defined by Roux et al. (2003) was calculated for quantification of normal and abnormal DNA contents:

$$DI = \frac{\text{mean fluroscence response of G0/G1} - \text{sample} - \text{nuclei}}{\text{mean fluroscence response of G0/G1} - \text{reference} - \text{nuclei}}$$

The nuclear amount of embryos has been measured without internal standard, making it impossible to calculate the DI. Here, we analysed the DNA content of additional peaks by applying a three-point calibration for the absolute DNA content (estimated as described below) and the mean fluorescence response of the corresponding peak via the slope of the regression line. As an essential prerequisite, the G2/G1-ratio as the quotient of mean fluorescence responses of G2 and G0/G1 peaks allowed us to discriminate between additional and normal peaks because the doubled DNA content in the G2 phase should lead to a peak at the position of doubled fluorescence intensity. Peaks resulting in the larger discrepancy to the theoretical value of 2.0 were defined as 'additional'. The additional peak similarity as presented in Table 2 was defined by the position in relation to 2C and 4C peaks.

Estimation of nuclear genome size by propidium iodide (PI) staining

Fresh young leaves of sample and internal standard (0.5 cm^2) were chopped with a sharp razor blade in a Petri dish containing 500 µl Extraction Buffer [CyStain propidium iodide (PI) Absolute P Kit, Partec GmbH], incubated for 30 s, filtered through a 35 µm mesh and stained with 2 ml staining solution including PI and RNase (CyStain PI Absolute P Kit, Partec GmbH). The relative fluorescence intensities of stained nuclei were measured on a FACSAria (BD Biosciences, San Jose, CA, USA) equipped with a 488 nm laser. Two plants of *C. persicum* wild-type and of cultivar 'Sierra Purple Flame' (Goldsmith) were used for absolute DNA content estimation together with *Raphanus sativus* cv. Voran (2C = 1.11 pg, Genebank Gatersleben, accession number: RA 34), *S. lycopersicum* cv. Stupické (2C = 1.95 pg) or *Glycine max* (L.) Merr. convar. *max* var. *max* ('Cina 5202', 2C = 2.23 pg; Genebank Gatersleben, accession number: SOJA 392) as internal standards. The nuclear DNA amounts of the used standards were determined based on the value of 0.32/2C pg for *Arabidopsis thaliana* 'Columbia' (Bennett et al. 2003). Usually 10,000 nuclei per

sample were analysed and each measurement was three times repeated. The absolute DNA amounts of the samples were calculated based on the values of the G1 peak means.

Chromosome counting

In order to proof the diploid status of the reference *C. persicum* 'Sierra Purple Flame' plants, chromosome countings were carried out on root tip meristems. Seeds were germinated in vitro after surface sterilization applying 70% ethanol for 2 min, 1% NaOCl for 45 min followed by three washing steps in sterile water. After an incubation of 4 weeks at 20°C in the dark the seedlings were transferred to light (16 h, about 20 µmol/m^2/s). Root tips of secondary roots were prepared about 2–3 h after starting of the light period and treated with 2 mM 8-hydroxychinolin for 2.5 h. Thereafter they were fixed in ethanol : glacial acetic acid (3:1) for at least 24 h at 4°C. Following maceration in 1 M HCl for 10 min at 60°C the root tips were stained in 1% carmine acetic acid. Chromosomes were counted under the microscope (ZEISS Axioskop, Oberkochen, Germany) at 1,000-fold magnification.

Results

Estimation of absolute DNA content

For flow cytometric analysis of mutations concerning the DNA content, the use of internal reference standards with a DNA content close to the analysed plant species is crucial (Roux et al. 2003). Preliminary analyses for the selection of a suitable standard, based on the published DNA content of 1.12 pg/2C for *C. persicum* (Winkelmann et al. 1998b), indicated a deviating genome size. Therefore we re-determined the DNA content of *C. persicum*. Two plants of *C. persicum* wild-type and of the cultivar 'Sierra Purple Flame', respectively, were analysed, each with three different internal reference standards (*R. sativus*, *S. lycopersicum* and *G. max*, Fig. 1). Estimated DNA contents calculated from these measurements are
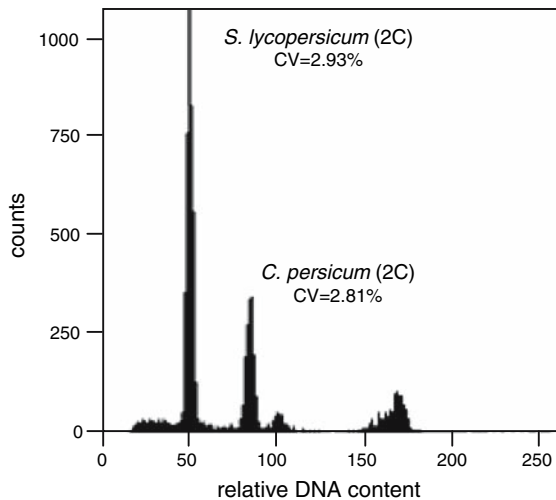
Fig. 1 Estimation of absolute nuclear DNA amount in *Cyclamen persicum*. Histogram of relative fluorescence intensity obtained after analysis of propidium-iodide stained nuclei of *C. persicum* 'Sierra Purple Flame' and the internal standard *Solanum lycopersicum*. The absolute DNA amount of *C. persicum* was calculated based on the values of the 2C peak means

presented in Table 1. The DNA content of *C. persicum* wild-type was determined to be 3.17 ± 0.04 pg DNA/2C. A slightly, but significantly higher ($P = 0.001$, *t*-test) DNA content was calculated for *C. persicum* 'Sierra Purple Flame', 3.21 ± 0.03 pg/2C.

In order to check whether these large discrepancies compared to the results published by Winkelmann et al. (1998b) were caused by polyploidization we determined the chromosome number of the cultivar 'Sierra Purple Flame' by chromosome counting in root tips of young seedlings (Fig. 2). Here, we found 48 chromosomes per nucleus confirming the diploid status of this cultivar.
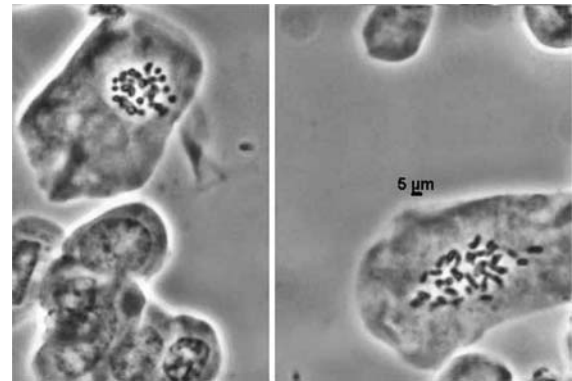
Fig. 2 Chromosomes in root tip cells of diploid Goldsmith *Cyclamen persicum* 'Sierra Purple Flame'

Abnormal DNA contents

In a previous study, we comparatively investigated zygotic and somatic embryo development in *C. persicum* (Schmidt et al. 2006). Flow cytometric analysis of zygotic embryos always resulted in histograms with a clear 2C peak referring to cells in the G0/G1 phase of the cell cycle and a 4C peak probably resulting from cells in the G2 phase of the cell cycle (Fig. 3A) since this peak was completely absent in mitotically inactive cells of mature embryos. 3C and 6C peaks resulted from endosperm cells in G0/G1 phase and G2 phase, respectively. The 4C/2C ratio representing the mitotic activity of a given cell population never exceeded in zygotic embryos a value of 0.15. In contrast, 17.6% of somatic embryos showed 4C/2C ratios above 0.250 and were considered as embryos with increased ploidy level (Schmidt et al. 2006). In the present study, we discovered two groups of altered DNA contents in

Table 1 DNA contents of *C. persicum* wild-type and 'Purple Flame' estimated via measurements with three different internal standards

	Internal standard	DNA content [pg/2C]	CV [%] 2C peak ± SD
C. persicum wild-type	Raphanus sativus	3.18 ± 0.04	2.82 ± 0.30
	Solanum lycopersicum	3.15 ± 0.06	2.8 ± 0.32
	Glycine max	3.18 ± 0.02	2.78 ± 0.33
C. persicum 'Sierra Purple Flame'	Raphanus sativus	3.23 ± 0.05	3.03 ± 0.33
	Solanum lycopersicum	3.21 ± 0.03	2.90 ± 0.25
	Glycine max	3.21 ± 0.02	3.00 ± 0.20

Results are presented as mean value ± SD [pg DNA/2C nuclei] of six replications

Fig. 3 Histograms of *C. persicum* embryos. (**A**) Represents a zygotic seed sample 8 days after induction of germination indicating distinct 2C (embryo G0/G1) approximately in channel 50, 3C (endosperm G0/G1), 4C (embryo G2/M) and 6C (endosperm G2/M) nuclei. Polyploidy in somatic embryos (**B**) apparently increased the 4C/2C-ratios (here: 3.792) as a result of G0/G1-stage cells with 4C DNA content. Somatic embryos with abnormal DNA contents (**C–E**) resulted in histograms with additional peaks. The given %-values illustrate the de-/increase in DNA content compared to the 2C-peak, CV-values for each peak are given in the second row. (**F**) Represents a histogram of suspension cells during bioreactor culture
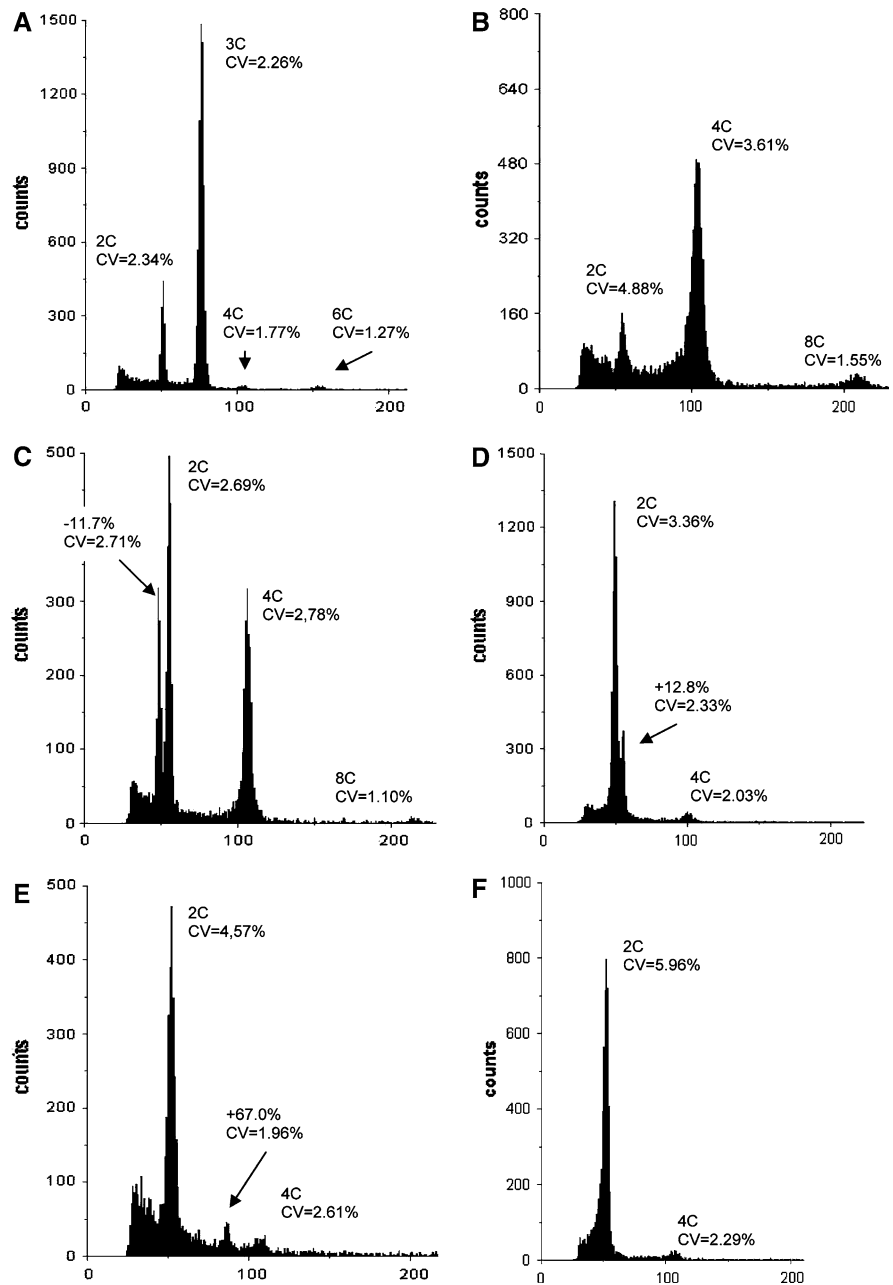
somatic embryos. A clearly increased 4C/2C ratio and/or the appearance of an additional 8C peak (Fig. 3B, maximum value: 3.79), both indicated the presence of polyploid cells. The incidence of additional peaks and/or split peaks which did not belong to the regular G0/G1- and G2-stages of the cell cycle indicated the simultaneous presence of cells with normal and altered DNA content within the same tissue (Fig. 3C–E). Table 2 summarizes the results of ten selected somatic embryos of *C. persicum* with distinctive additional peaks. The higher the probability that both peaks result from the same cell cycle, the smaller is the discrepancy from the predicted value of 2.0 of the G2/G1-ratio. Applying a linear three-point calibration for mean fluorescence responses of 0C and both 2C and 4C peaks, the widespread variation of DNA contents in somatic embryo nuclei given in Table 2 was documented.

Table 2 Estimation of abnormal DNA content for ten somatic embryos of *C. persicum* identified by flow cytometric DAPI measurements

| Sample	Embryogenic stage of sample	Additional peak similarity	Euploid peak data				Additional peak data			
			Fluorescence response		G2 : G1 ratio	Δ	Fluorescence response	G2 : G1 ratio	Δ	Deviating DNA amount (%)
			2C	4C						
1	Globular	2C	48.14	98.07	2.04	0.04	52.38	1.87	−0.13	8.81
2	Globular	2C	55.26	106.48	1.93	−0.07	48.78	2.18	0.18	−11.73
3	Cluster	2C	55.50	104.15	1.88	−0.12	48.99	2.13	0.13	−11.73
4	Cluster	2C	49.10	100.34	2.04	0.04	55.37	1.81	−0.19	12.77
5	Cluster	2C	51.37	104.29	2.03	0.03	44.95	2.32	0.32	−12.50
6	Torpedo-shaped	2C	48.77	99.47	2.04	0.04	41.54	2.39	0.39	−14.82
7	Globular	4C	46.45	99.82	2.15	0.15	107.27	2.31	0.31	130.94
8	Globular	4C	49.34	99.84	2.02	0.02	104.55	2.12	0.12	111.90
9	Torpedo-shaped	4C	50.15	102.98	2.05	0.05	95.62	1.91	−0.09	90.67
10	Globular	None	51.87	107.95	2.08	0.08	86.65	1.67	−0.33	67.05

Classification of cells with regular DNA content was accomplished by calculating the G2 : G1-ratios and comparing them with the prediction value 2.0 $\left(\Delta = DI_{sample} - 2.0 \right)$ though the difference is considerably increased for abnormal DNA contents (the maximum difference is 0.15 from euploid and 0.39 from abnormal samples). DNA contents were subsequently calculated by means of the slope of a three-point regression of 0C (0 pg), 2C (3.21 pg) and 4C (6.42 pg) fluorescence responses. Samples 2, 4 and 10 are shown in Fig. 3C–E

However, samples with abnormal DNA contents were not evenly distributed among the three different embryo classes. Of all tested somatic embryos, globular shaped embryos and cluster showed a considerably higher rate of alteration both for clearly multiplied as well as shifted DNA contents compared to torpedo-shaped embryos (Fig. 4). Due to the lack of an internal standard in our flow cytometric measurements we were only able to detect abnormal DNA contents as additional peak(s) in a histogram if cells with normal DNA content were accessible in adequate amounts in the same sample. Otherwise, no extra peak(s) but only a very small drift along the x-axis due to the slightly altered fluorescence responses would have appeared and could hardly be recognized. This implies the possibility that the mutation rates given in Fig. 4 may even be underestimated.

In order to test whether embryos with abnormal DNA contents developed into mature plants, leaves of ten regenerated plants in the greenhouse were

Fig. 4 Total distribution of alterations of DNA contents in somatic *C. persicum* single embryos detected by flow cytometric measurements of DAPI-stained nuclear DNA. Torpedo-shaped (*n* = 235) embryos showed a noticeably lower rate of changes than globular embryos (*n* = 149) and cluster (*n* = 189)
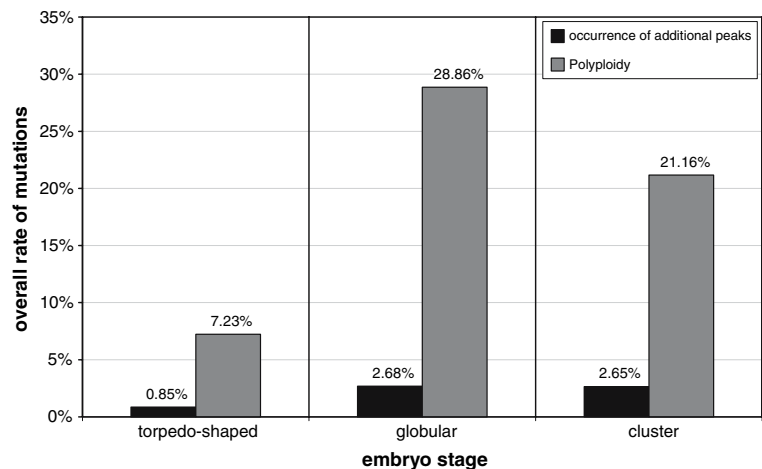
Fig. 5 DNA-Index of adult *C. persicum* plants (**A**) ten plants from the greenhouse regenerated from hybrid seeds, (**B**) ten plants regenerated from cell line 3738, *n* = 6 each. Mean DI of seed samples was 1.553 ± 0.008 (mean value ± SD). Measurements including leaves of *S. lycopersicum* as internal reference standard identified one regenerated plant for abnormal DNA content by its increased DI (sample 5 in Fig. 5b). Significant differences according to ANOVA with subsequent Tukey-test are marked by different letters (*a*, *ab*, *b*), *bars* represent SD
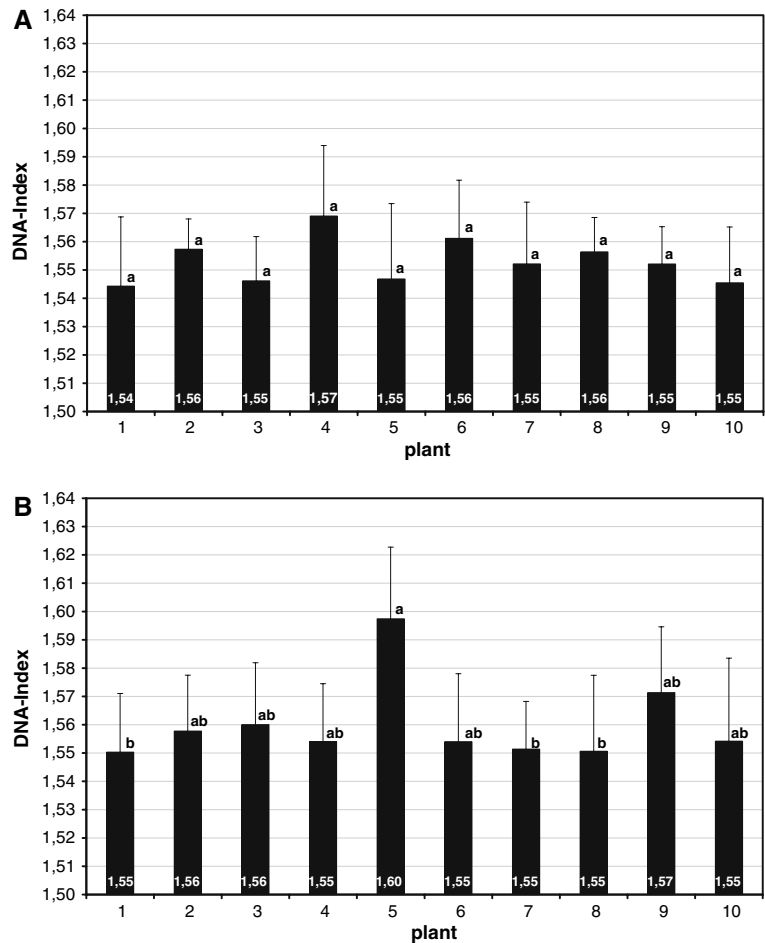
subjected to flow cytometric measurements with tomato leaves as internal standard (Fig. 5B). As a control ten plants grown from seeds were tested (Fig. 5A). Here, highly constant values were obtained which showed no significant differences. In contrast to this, one plant regenerated from cell line 3738 (plant 5 in Fig. 5B) displayed significantly (ANOVA, Tukey-HSD) increased values for the DI indicating an abnormal (higher) DNA content in comparison to other regenerants (plants 1, 7 and 8).

Discussion

We determined the genome size of *C. persicum* (wild-type) to be 3.17 pg DNA/2C. This contradicts the results published by Winkelmann et al. (1998b), who reported a DNA content of 1.12 pg DNA/2C. To exclude variations between different cultivars we

confirmed our results by measuring the DNA content of plants of the same cultivar 'Purple Flamed' used by Winkelmann et al. (1998b). The obtained value was only slightly deviating (3.21 pg) from that of *C. persicum* wild-type, which might be a consequence of the breeding process. Moreover, the diploid nature of this cultivar was confirmed by chromosome counting. Thus, the DNA amount of *C. persicum* should be corrected to be 3.17 pg DNA/2C. The DNA content of *C. persicum* is still significantly lower compared to those published for other *Cyclamen* species, ranging from 6.0 pg for *C. hederifolium* up to 27.5 pg for *C. trochopteranthum* (Bennett and Leitch 2005).

As presented in Fig. 4, we detected a high rate of regenerants with aberrant DNA contents especially for phenotypic aberrant somatic embryo stages (i.e. large globular embryos and cluster). These results are contradictory to those of Winkelmann et al. (1998b) who reported a high degree of ploidy stability for

embryogenic and non-embryogenic callus cultures of *C. persicum* using the same culture protocol and the same DNA staining dye as in the current study. However, Winkelmann et al. (1998b) did not study single regenerated structures, but undifferentiated cells. This might be a reason for their inability to discover aberrant DNA contents: even a high number of mutated single cells would have been hidden in histograms in comparison to a total count of 5,000 or more. Also the cell line used in our study displayed 'normal' looking histograms when the total cell population in growth regulator containing or free medium has been analysed (Fig. 3F).

Endopolyploidy, i.e. the regular multiplication of the DNA content during cell differentiation, frequently occurs in plants especially in angiosperms (Barow 2006). Therefore, besides mutations, this process must be considered as a possible explanation of our results. However, we never observed this phenomenon in zygotic embryos from the globular stage up to germinating seedlings (Schmidt et al. 2006). Moreover, endopolyploidy is a strictly controlled process with regard to the level multiplications as well as to the tissue. This is in strong contrast to our results that differ from embryo to embryo indicating a highly irregular process which seems not to be based on true amplification. Thus, we prefer that our results are better not interpreted in the context of endopolyploidy. However, for a discrete decision chromosome countings would have been necessary. We were not able to accomplish such an examination because (1) it is hard to find sufficient numbers of cells in the metaphase stage of the cell cycle and (2) embryos are completely destroyed by our flow cytometric methodology.

The occurrence of additional peaks and split peaks in the histogram of embryo individuals might be interpreted as mixoploidy und thus be a hint on multicellular origin of the respective embryo.

According to the developmental class of the embryos the proportions of individuals with aberrant DNA contents varied and were lowest for torpedo-shaped embryos, the developmentally most advanced class of embryos (Fig. 4). This might be interpreted as a hint that embryos with altered DNA content are mutated in a way impeding embryo development. However, our data show, that also embryos with altered DNA content might be able to develop into adult plants. Thus, the regeneration process by itself cannot be taken as an equally stringent selection system against mutated cells as it has been reported, e.g. for regeneration of *Euphorbia pulcherrima* (Geier et al. 1992).

Altered DNA contents are common mutations reported as a consequence of in vitro regeneration (Kubalakova et al. 1996, Nayak and Sen 1997, Ezura and Oosawa 1994). In *Musa* (Shepherd 1996) aneuploidy has been reported for plants propagated by in vitro techniques. In recent investigations on ploidy stability after regeneration via somatic embryogenesis both, stability (Loureiro et al. 2005; Leal et al. 2006) as well as aberrations after long-term cell culture (Endemann et al. 2001) are described. A longstanding hypothesis is that mutations are the result of the application of plant growth regulators. Phillips et al. (1994) considered tissue cultures as a key origin for chromosome instabilities although the molecular mode of action is still unknown. More recently, Grossmann (2000) and Wei et al. (2000) demonstrated that 2.4-D and 2-iP interact with ethylene and ABA synthesis/function and thereby increase the total intracellular 'stress' hormone levels.

Regarding the recently discussed 'plant stem cell concept' (Laux 2003), one might question whether looking for the 'right' plant growth regulator combination for avoiding mutations might be successful at all. If meristem organization with so-called founder cells in a less rapidly dividing central zone in the shoot meristem developed as a superior system during evolution because rapid cell proliferation in undifferentiated status inevitably results in high-mutation rates, plants need these 'stem cells' in order to maintain their genetic integrity. Exactly this principle is circumvented in micropropagation systems based on regeneration from callus and suspension cultures. This means that the problem of mutation induction could be not a precarious combination of plant growth regulators but rapid cell proliferation in undifferentiated status itself.

Against this background the idea of long-term callus and suspension cultures as a readily available pool for plant regeneration as suggested, e.g. by Ammirato and Styer (1985) should be critically re-evaluated. Possibly the well-known practice of regular new establishment of in vitro cultures from elite plant material can be regarded as mimicking nature's stem cell concept. Our data suggest that this laboratory practice is essential for propagation of *C. persicum* via somatic embryogenesis.

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