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ORIGINAL PAPER

Chromosome variations in regenerants of Arabidopsis thaliana derived from 2- and 6-week-old callus detected using flow cytometry and FISH analyses

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Abstract Shoot organogenesis was induced from 2- and 6-week-old callus derived from the leaves of *Arabidopsis thaliana* ecotype Columbia ($2n = 10$). Regenerated plants were evaluated for chromosomal variations by means of flow cytometry and fluorescent in situ hybridization (FISH). Flow cytometric measurements revealed the occurrence of diploid, tetraploid, and octoploid plants among the regenerants of 2-week-old calli, whereas only diploid and tetraploid plants were regenerated from the 6-week-old calli. Chromosome counting showed that plants developed from the 2-week-old calli exhibited mixoploidy and a high frequency of aneuploid cells. These plants were infertile and displayed altered morphology. FISH with 5S and 25S rDNA probes allowed to detect some structural chromosomal rearrangements in regenerated plants. Along with cells which exhibited correct localisation of rDNA loci, also cells bearing chromosomal translocations, deletions or duplications were found. The type of structural aberrations varied between diploid and tetraploid regenerants.

Keywords Organogenesis · Chromosomal aberrations · Flow cytometry · Fluorescent in situ hybridization

Abbreviations

2, 4-D	2, 4-Dichlorophenoxyacetic acid
2iP	6-(γ, γ-Dimethylallylamino) purine
B5	Gamborg medium
DAPI	4′-6-Diamino-2-phenyloindole
FISH	Fluorescent in situ hybridization
IAA	Indole acetic acid
MS	Murashige and Skoog
NAA	α-Naphtalene acetic acid
R1	Plants obtained through organogenesis
R2	Plants obtained from R1 seeds
rDNA	Ribosomal DNA
rRNA	Ribosomal RNA

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Introduction

Plant tissue culture is one of most important tools necessary for successful conducting plant research and broadly used in commercial biotechnology. It is well known that plants regenerated in vitro undergo genetic and epigenetic changes. A variation originating in cell and tissue culture is referred to as somaclonal variation (Larkin and Scowcroft 1981). Although the occurrence of such variation in organisms derived from in vitro culture may provide new variants that possess economically desirable attributes, it is still unpredictable and uncontrolled, which restricts the utility of tissue cultures (Phillips et al. 1994). The phenomenon of variation in the in vitro culture is one of the most frequently studied phenomena, but the causes of the somaclonal variation remain unclear and therefore cannot be manipulated.

Variation in the number of chromosomes (aneuploidy and polyploidy) and/or their structure is one of the crucial problems in plant cell culture and was reviewed as one of the most significant reasons for the somaclonal variation (Karp 1995; Peschke and Phillips 1992). Karyological changes in cells and tissues cultured in vitro occur both in mitotically dividing cells and in interphase nuclei. The most important alterations include chromosomal aberrations, changes in the content of nuclear DNA, somatic crossing-over and cell cycle disorders. The reason for

structural chromosomal rearrangements can be late replicating heterochromatin and nucleotide pool imbalance (Lee and Phillips 1988). Karyotypic instability observed during in vitro culture includes chromosome breakage, deletions, translocations and inversions (Jain 2001).

There are numbers of factors which determine the frequency of variation during in vitro culture. They can either be pre-existing in the plant and/or connected to the tissue culture itself. The first group of factors includes the genotype and the nature of the tissue used as a starting material. In most plants the somatic cell differentiation is associated with genome changes that lead to endopolyploidy. The outcome is defined as polysomaty and involves the repeated cycles of replication which are not followed by cell division (D'Amato 1989). Tissue and organ-specific endoreduplication is observed in the model plant *Arabidopsis thaliana*, where cells with various DNA contents ranging from 2C to 32C are present in differentiated organs (Galbraith et al. 1991). Using non-meristematic tissues of *A. thaliana* as explants resulted in an increased frequency of callus cells with higher ploidy level during the first weeks of in vitro culture (Fras and Maluszynska 2004). Thus the use of non-meristematic mixoploid organs as explants in an in vitro culture can influence from the start the ploidy level of callus cells and the plants regenerated vie callus. The second group of factors which influence the level of somaclonal variation includes culture-dependent conditions. These cover among others the medium composition, the type and concentration of growth regulators, the number and duration of subculture cycles and the method of regeneration (Bairu et al. 2011). A phase of disorganized growth, which occurs during indirect regeneration is one of the fundamental phenomena responsible for the somaclonal variation (Karp 1995).

Detection of structural chromosomal aberrations as well as the changes in chromosome number, including both polyploidy and aneuploidy allow for direct assessment of capital genomic changes and the analyses of variation level in regenerated plants. Flow cytometry allows for rapid and accurate estimation of the nuclear DNA content in thousands of cells and has been widely used for rapid screening of the ploidy levels of regenerated plants like *Centaurea ultreiae* (Mallón et al. 2010), *Vitis vinifera* (Prado et al. 2010) or *Spathiphyllum* (Zhao et al. 2012). Similarly, the analyses of the chromosome numbers and their structural aberrations by means of cytogenetic methods have been successfully employed for detecting the somaclonal variation in plants regenerated in vitro (Do et al. 1999; Molnar-Lang et al. 2000). Fluorescent in situ hybridization (FISH) proved to be particularly useful for these kind of analyses especially in plants characterized by a low number of small chromosomes, such as *A. thaliana*. It is well established in *A. thaliana* for which numerous probes, such as repetitive sequences and chromosome—specific BAC clones, are available (Fransz et al. 1998, Lysak et al. 2001). The rRNA genes are useful chromosomal markers as they are present in each type of cells: they are organized as tandem repeats and their sequence is highly conserved. Two-colour FISH with 25S rDNA and 5S rDNA probes enables the identification of most of the chromosomes in the *A. thaliana* karyotype (Fransz et al. 1998) and can be used to detect large-scale chromosomal rearrangements in plants regenerated from in vitro culture.

In the present study we analyzed ploidy levels, chromosome numbers and structure in plants regenerated from calli after two different periods of preculture. Fast screening of ploidy levels of all obtained plants was performed by means of flow cytometry. From these plants a group of regenerants was chosen for detailed cytogenetic analyses in order to detect if some of the chromosomes or chromosomal segments are preferentially targets of the variation.

Materials and methods

Plant material and in vitro culture

Diploid plants of *A. thaliana* ecotype Columbia ($2n = 2x = 10$) were used as sources of explants. Axenic plants were obtained from seeds sterilized for 5 min in a mixture of 3 % H_2O_2 (v/v): 70 % (v/v) EtOH (1 : 1), and rinsed five times with sterile distilled water. Seeds were placed in culture vessels containing hormone-free MS medium (Murashige and Skoog 1962) enriched with mineral salts, vitamins, sucrose (3 %, w/v) and agar (0.8 %, w/v). The plants were grown at 21 °C, 16-h photoperiod and 70 μmol m^{-2} s^{-1} irradiance. The same growth-room conditions were used for tissue culture. Leaf explants were obtained by cutting young rosette leaves excised from 3-week-old plants into 1 cm^2 squares, each with a part of a mid vein. Explants were cultured on a B5 callus-inducing medium (Gamborg et al. 1968) containing 0.5 mg/L of 2,4-D and 0.05 mg/L of kinetin (Valvakens et. al 1988). Two and 6-week old calli were transferred on to a shoot-inducing B5 medium supplemented with 0.15 mg/L of IAA and 5 mg/L of 2iP (Valvakens et al. 1988). The regenerated rosettes and shoots were transferred to a root-inducing B5 medium supplemented with 0.125 mg/L of NAA. These procedures produced regenerated plants (R1), and the next generation phase (R2) was obtained from seeds that were collected from the R1 regenerants. The pH of all media was adjusted to 5.8 and autoclaved at 121 °C for 20 min (Jiang et al. 2011).

Control plants were grown on a hormone-free MS medium in the same conditions as the plants used as the source of explants.

Flow cytometry

The ploidy levels of the control and regenerated plants were analyzed by flow cytometry. Rosette leaves were chopped and a suspension of nuclei was isolated with an extraction buffer (Dolezel et al. 1998). The samples were filtered through a 30 μm nylon mesh (Partec Cell Trics) and stained with DAPI (4′-6-diamino-2-phenyloindole). The measurements were performed with the use of a DAKO Galaxy flow cytometer equipped with an HBO-100 mercury lamp and an argon ion laser.

Mitotic chromosome preparations

The chromosome number was analyzed in seedling root apical meristems and flower buds of the control plants as well as in the flower buds of the regenerants. Only a few flower buds were fixed from the regenerants to allow for further growth and seed development (R2 generation). The flower buds and seedlings were pre-treated with 2 mM 8-hydroxyquinoline for 2 h at room temperature and 2 h at 4 °C and then fixed overnight in fresh Carnoy's solution (methanol + glacial acetic acid, 3:1, v/v). The fixed material was stored at −20 °C. The chromosome preparation procedure followed Hasterok et al. (2006), with small modifications. The material was washed in a 0.01 M citrate buffer pH 4.8 and digested with 2 % cellulase "Onozuka" and 20 % pectinase (from *Aspergillus niger*) at 37 °C for 45 min for flower buds and 30 min for roots. After rinsing and incubation in citrate buffer for 20 min the material was placed in 45 % acetic acid. Root tips or young flower buds were then placed on a glass slide, dissected and squashed under coverslips. After checking the quality of the chromosome preparations with the use of a phase contrast microscope, the slides were frozen, the coverslips removed, the material refixed in chilled ethanol: acetic acid (3:1), rinsed in absolute ethanol and air dried.

Chromosome number analysis

The mitotic chromosome preparations were stained with DAPI (4′-6-diamidino-2-phenylindole; 0.5 μg/μL) and analyzed with the use of an epifluorescence microscope (Olympus, Leica). The chromosome number was analyzed in the control plants and individually for each regenerated R1 and R2 plant that had developed flower buds. Several to 88 metaphases were analyzed in each regenerated plant.

DNA probe and labelling

The rDNA probes used for fluorescent in situ hybridization were the following: (i) a 2.3-kb ClaI fragment of the 25S rRNA gene isolated from *A. thaliana* (Unfried and Gruendler 1990), labelled with digoxigenin-11-dUTP (Roche) with the use of a nick translation kit (Roche) according to the manufacturer's protocol and immunodetected; and (ii) 5S rDNA (pTa794): a 410-bp clone isolated from *Triticum aestivum* (Gerlach and Dyer 1980) and labelled with rhodamine-5-dUTP (Roche) in a polymerase chain reaction.

Fluorescence in situ hybridization (FISH)

Slides used for chromosome number analysis were re-fixed in a mixture of 99.9 % Et-OH : glacial acetic acid (3:1) for 2–3 min to remove the DAPI, rinsed with 99.9 % ethanol, air dried and used for FISH experiments. Slides were incubated in 100 μg/mL RNase in 2xSSC for 1 h at 37 °C and rinsed three times in 2 × SSC at room temperature for 5 min. They were then incubated in 10 mM HCl for 5 min at room temperature, treated with an 0.01 mg/ml pepsin solution for 15 min at 37 °C and washed in 2 × SSC. Subsequently, the slides were fixed in 1 % formaldehyde in 1 × PBS for 10 min and again rinsed three times in 2 × SSC for 5 min; then dehydrated in 70, 90 and 100 % ethanol and air dried. The hybridization mixture containing 1 ng/μL of each DNA probe, 50 % formamide, 10 % dextran sulphate, 0.5 % sodium dodecyl sulphate and 2xSSC was pre-denatured at 75 °C for 10 min and stabilised on ice for 10 min. A 38 μL aliquot of the hybridization mixture was applied to the chromosome preparations, covered with a plastic coverslip and denatured together at 75 °C for 5 min in an Omnislide Thermal Cycler. Hybridization was carried out at 37 °C in a humid chamber for 24 h. Stringent washing of slides at 42 °C involved 2 × SSC for 5 min, two changes of 20 % formamide in 0.1 × SSC for 5 min and three changes of 2 × SSC for 3 min. The Coplin jar was cooled to room temperature and slides were rinsed in 2 × SSC and 4 × SSC/Tween 20. In the case of the digoxygenin-labelled probe, 180 μL of a 5 % milk-blocking reagent was applied to each slide and incubated in a humid chamber at room temperature for 30 min, then 45 μL of fluorescein (FITC) conjugated anti-digoxigenin Fab fragments (Roche), at a concentration 1:11 in the 5 % milk-blocking reagent was added. The slides were then incubated at 37 °C for 1 h. After incubation, slides were washed in three changes of 4 × SSC/Tween 20 at 37 °C, dehydrated and air dried. A Vectashield mounting medium containing 2.5 μg/mL DAPI was applied to the dry slides before adding coverslips. Slides were analyzed with the use of an epifluorescence microscope (OLYMPUS) with the appropriate filters. Pictures were taken with a CCD camera and processed with Adobe Photoshop software using only the functions that are applied equally to all pixels of the image.

Results and discussion

Regeneration

In order to induce callus a young rosette leaves of the *A. thaliana* ecotype Columbia were used as explants. After 2 weeks of culture on callus-inducing medium some of the calli were transferred onto a shoot-inducing medium, and the rest was cultured on callus-inducing medium until the sixth week of culture, after which they were also transferred on to shoot-inducing media. The efficiency of the callus induction, estimated after 2 weeks, was 90 %. In both cases the first shoots were observed 8 weeks after the transfer of the calli and after 12 weeks of culture on shoot-inducing medium about 90 % of them showed signs of regeneration. A higher number of shoots per callus was observed in the 2-week old calli (average 1.55 shoots per callus) than in the 6-week-old ones (1.0 shoot per callus). In both cultures about 80 % of the regenerated rosettes developed roots after transferring them onto a root-inducing medium. The system of indirect organogenesis of *A. thaliana*, which is based on callus, shoot and root induction with the use of specific media allows for a high efficiency of regeneration; although it depends on the ecotype, the type of explant, the culture conditions and the medium and hormones employed (Negrutiu 1976; Akama et al. 1992; Candela et al. 2001). The composition of callus and shoot inducing media used here is recommended as efficient for induction of *A. thaliana* regeneration from leaf explants (Feldmann and Marks 1986, Fras and Maluszynska 2003). The time of preculture on callus inducing media is one of the crucial steps in the regeneration process. The short time of preculture has been shown to induce a generally higher capacity to produce shoots, but the feasibility of organogenesis is also dependent on the explant and ecotype used (Valvekens et al. 1988; Akama et al. 1992). The efficiency of the shoot production was slightly higher in the 2-week-old calli where more than one shoot per callus was observed.

Cytogenetic analysis of starting materials

The starting plant material was analyzed in order to exclude any cytogenetic variation that could influence further analysis. The levels of ploidy and endopolyploidy in rosette leaves from 3 week old plants were estimated. The flow cytometry measurements were conducted on 20 plants, and two samples from each plant were analyzed. All plants were found to be diploids. The young rosette leaves exhibited two cycles of endoreduplication and the maximum DNA content was 16C (Fig. 1a), which is consistent with the organ specific endopolyploidy pattern in *A. thaliana* (Galbraith et al. 1991). The chromosome number analysis was performed on root apical meristems of at least 20 seedlings as well as on flower buds collected individually from 20 plants. In the root apical meristems all metaphases had an exact diploid chromosome number ($2n = 10$), while in flower buds the diploid number occurred in 65 % of the metaphases and the number of chromosomes in the remaining cells varied from 7 to 9 (Table 2). This phenomenon was defined as aneusomaty by Duncan (1945) and has been described for different types of organs and tissues in many species (D'Amato 1995). Although it was suggested that aneusomaty is more frequent in polyploids, for example in *Artemisia czekanowskiana* and *A. macrantha* (Pellicer et al. 2007), it was also noticed in species with a lower chromosome number, e.g. *Deschampsia antarctica* (Cardone et al. 2009). The rDNA sequences are robust chromosome markers for *Arabidopsis thaliana* and after applying FISH with these sequences as probes all chromosomes are easily distinguishable (Fransz et al. 1998). The distribution of 25S and 5S rDNA loci in the karyotype of control plants was analyzed in root tips and in flower buds. The first pair of chromosomes does not possess any rRNA genes, the second pair is characterized by the presence of 45S rDNA site, the chromosomes of the third and the fifth pair bear 5S rRNA genes and the fourth pair has both 45S and 5S rDNA (Fig. 3a). These results confirm that the plants used as the starting material were uniform in the ploidy level, chromosome number and rDNA distribution.

Ploidy of regenerants

The ploidy of plants regenerated from both types of calli was determined by flow cytometry. Among the plants regenerated from the 2-week-old calli three groups of plants at different ploidy levels were found—diploids (47 %), tetraploids (47 %) and octoploids (6 %) (Fig. 1b–d). The majority of the regenerants from the 6-week-old calli were diploids (70 %) and there were fewer tetraploids (30 %) and no octoploids (Table 1). The ploidy level was also analyzed in 56 R2 plants produced from seeds obtained from diploid and tetraploid R1 plants. The flow cytometry showed that the progeny of diploid R1 plants were diploid and those of the tetraploid R1 plants were also tetraploid (Tab. 2).

Some of the regenerated plants showed differences in morphology. These phenotypic changes were particularly frequent among plants regenerated from the 2-week-old calli (Fig. 2). The type of morphology was often correlated with the ploidy level. Tetraploid and octoploid plants were shorter than diploids and had sturdy stems and leaves. Almost all of the plants, diploids as well as polyploids, regenerated from the 2-week-old calli failed to produce seeds. The flowers were usually small, misshaped and

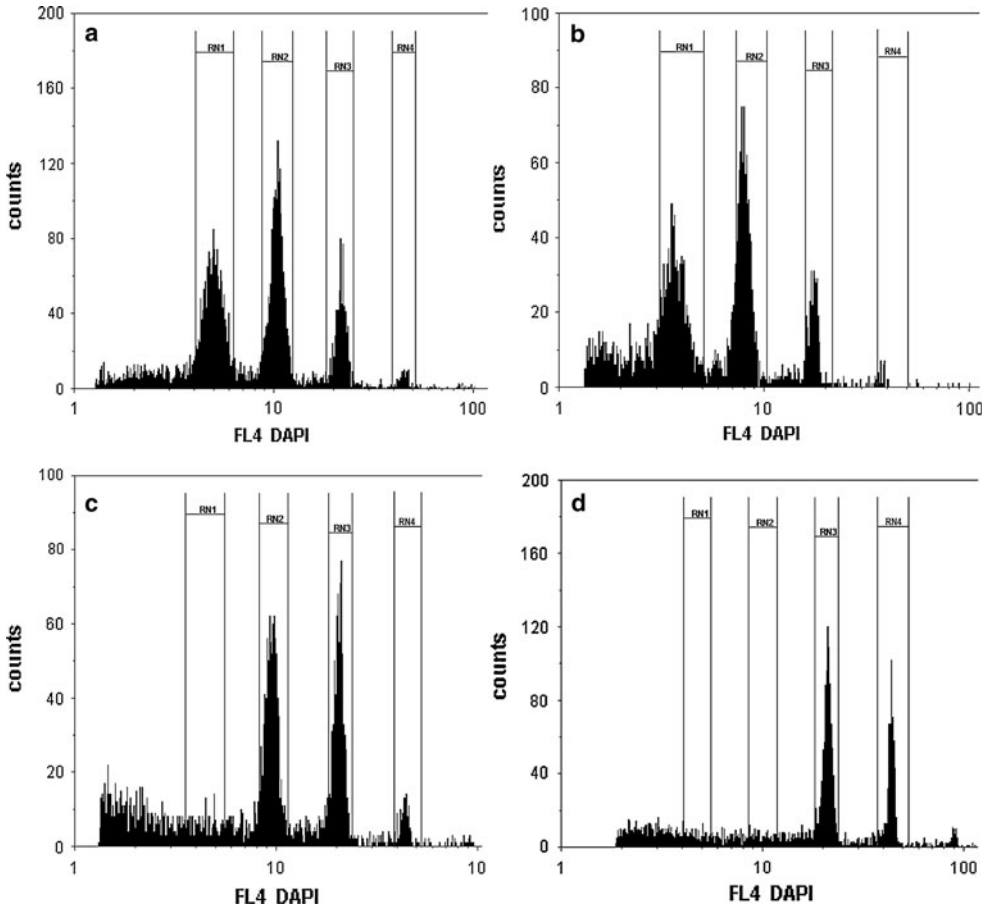

Fig. 1 Flow cytometer histograms showing ploidy level and endopolyploidy patters of control plants (**a**) and regenerants from 2-week-old calli: (**b**) diploids; (**c**) tetraploids; (**d**) octoploids

Table 1 Frequency of diploids and polyploids among regenerants from 2-week-old (R1-II) and 6-week-old calli (R1-VI)

Ploidy of regenerants	R1 from 2-week-old calli (%)	R1 from 6-week-old calli (%)
Diploid	47	70
Tetraploid	47	30
Octoploid	6	–

withered away. Plants obtained from the 6-week-old calli showed similar malformations but only in 20 % of the regenerants. Half of these regenerants had empty pods and 30 % produced seeds, typically 1-23 seeds per regenerant, which were used to produce the next generation (R2). Fifty-five percentage of the seeds produced normal growing R2 plants, while 45 % did not germinate. Interestingly, in one case all of the seeds from one R1 regenerant failed to germinate, which may suggest the occurrence of serious disorders in the seed development.

The phenomenon of ploidy variation in plants regenerated in vitro has been observed in *Arabidopsis* as well as in other species (Clarindo et al. 2008; Jin et al. 2008; Lema-Ruminska 2011). There are several factors which can influence the ploidy level of the regenerants. Regeneration via organogenesis was said to induce greater variation than in case of somatic embryogenesis (Pontaroli and Camadro 2005). Gaj (2001) showed that all *A. thaliana* regenerants obtained through somatic embryogenesis were diploids. One of the most important factors which influence the ploidy level in regenerants is the type of explant. A ploidy level analysis of tomato somaclones from leaf, cotyledon and hypocotyl-derived callus confirmed that the mixoploidy of organs from polysomatic plants is the initial source of a ploidy variation in the in vitro culture (van den Bulk et al. 1990). The rosette leaves of *A. thaliana* used as explants in our experiments, were composed of cells with DNA contents from 2C to 16C (Fig. 1a). Moreover, Fras et al. (2007) showed that during the first days of callogenesis the cells of leaf explants from diploid *Arabidopsis* can reach 64C DNA level and cells with a tetraploid chromosome number are most frequent in callus after 4 days of culture. It was suggested that the stressful conditions of in vitro culture may cause endopolyploidization, abnormal microtubule arrangements and DNA damage, which may be responsible for polyploidization during the early stages of callogenesis. The frequency of polyploids among plants regenerated from 2-week-old calli may

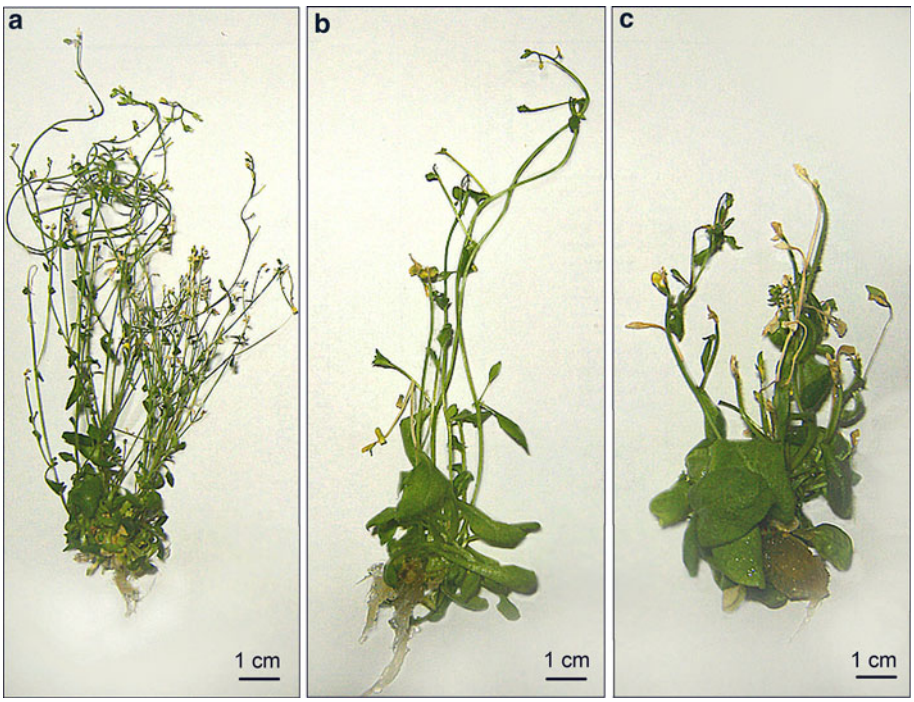

Fig. 2 Morphology of diploid (**a**), tetraploid (**b**) and octoploid (**c**) regenerants from 2-week-old calli

reflect a high level of polyploid cells in short aged calli. A lower frequency of polyploids was observed among regenerants from 6-week-old calli (Table 1). The analysis of regenerants derived from the five-month-old calli from leaf explants of the *A. thaliana* ecotype Wilna (Fras and Maluszynska 2003) showed a similar percentage of diploids as among plants from the 6-week-old calli used in our experiment. This is inconsistent with the common observations that as the period of culture goes on, the level of variation increases (Ziauddin and Kasha 1990; Jain 2001). It may suggest that in polysomatic plants the early preculture is a period of high polyploidization and in the following weeks of culture the ploidy variation is established at a lower level, at least for some time.

Chromosome number analysis

The flow cytometry is an excellent tool to estimate the ploidy level in a large number of specimens. Only a small part of a plant, e.g. leaf, is used for DNA measurements, while other parts can be used for a further analysis. However, while the flow cytometry technique allows for the identification of the ploidy level of the regenerated plants, the presence of aneuploid cells in the analyzed material could not be recognized (Sree Ramulu and Dijkhuis 1986). Therefore additional analyses of chromosome number analysis should be undertaken. In the present study we exploited the floral buds for the analysis of chromosome number. The ploidy level was established for all regenerated plants. Among these chromosome counting was performed for 35 R1 plants which had developed flower buds and for 18 R2 plants. As the analysis of the chromosome number in flower buds of the control plants showed aneusomaty, the variation in the chromosome number in the regenerants was compared with the variation in the control plants. R1 plants from the 2-week-old calli showed the highest variation in the chromosome number. In most diploid regenerants there were about 50 % metaphases with 10 chromosomes and the remaining cells were mainly hypodiploid. Among the tetraploid regenerants only about 30 % of the cells had 20 chromosomes, while 50 % of the cells had 16–19 chromosomes. A wide variation in the chromosome number, ranging from 11 to 76, was observed in the octoploid regenerants, although almost half of the cells had 36-39 chromosomes. The chromosome number in R1 plants from the 6-week-old calli did not vary significantly. On average 89 % of the analyzed metaphases had 10 chromosomes in diploids and in case of tetraploids 79 % of metaphases had 20 chromosomes. The chromosome number in R2 was counted in flower buds, as was the case for R1 plants. In the diploid R2 plants the percentage of cells with $2n = 10$ was even higher than in the control plants and equalled 90 %. In the tetraploids 72 % of the cells had $2n = 4x = 20$ chromosomes (Table 2).

The regenerants at each ploidy level exhibited a tendency to lose chromosomes which led to aneuploid cells. The number of cells with missing chromosomes was positively correlated with the ploidy level of the regenerants. The dominance of the hypotetraploid cells in plants regenerated in vitro was observed in potato ($2n = 4x = 48$) and can be

Table 2 Frequency of cells with a different chromosome number in roots and flower buds of control plants (K-roots, K-flowers) and plants regenerated from 2- and 6-week-old calli

Group of plants	Ploidy by flow cytometry	Number of plants (cells) analysed	Mean frequency of metaphases with a different chromosome number (%)							
			Hypo-2x	2x	Hyper-2x	4x	Hyper-4x	Hypo-8x	8x	8x<
Control-roots	Diploids	20 (100)	–	100	–	–	–	–	–	–
Control-flowers	Diploids	20 (200)	35	65	–	–	–	–	–	–
R1 from 2-week callus	Diploids	7 (384)	33.6	50.5	12.2	3.1	0.5	–	–	–
	Tetraploids	4 (178)	1.6	9.5	50.0	29.8	8.9	–	–	–
	Octoploids	2 (60)	–	–	7.8	1.7	16.3	49.4	9.6	25.2
R1 from 6-week callus	Diploids	16 (640)	4.0	89.1	4.1	4.5	–	–	–	–
	Tetraploids	6 (292)	–	13.0	6.2	79.4	1.4	–	–	–
R2	Diploids	2 (100)	7	90.0	3	–	–	–	–	–
	Tetraploids	16 (713)	–	–	23.5	72.0	4.5	–	–	–

explained by a better tolerance of the aneuploid state in cells with more than one genome (Jelenić et al. 2001). An analysis of the chromosome number in *A. thaliana* regenerants shows that plants regenerated from the 2-week-old calli, displayed not only a wider ploidy variation but also a higher instability of the chromosome number. This could explain the altered morphology and infertility of the regenerants.

Apart from the aneusomaty of the flower buds tissue the mixoploidy of somaclones can be a reason for chromosome number variation. It was observed by several authors, for example in *Solanum tuberosum* (Jelenić et al. 2001; Sree Ramulu et al. 1983), *Asparagus officinalis* (Kunitake et al. 1998; Pontaroli and Comadro 2005) and *Carica papaya* (Clarindo et al. 2008). One of the explanations for this phenomenon is the cytological diversity of the cells involved in the organisation of an adventitious bud (Christianson and Warnick 1983). Brutovská et al. (1998) noticed the influence of growth regulators such as high concentrations of BA in a medium on the frequency of mixoploidy among the *Hypericum perforatum* regenerants.

Karyotype analysis with FISH

The next step in our investigations was an examination of the chromosomal distribution of 5S and 45S rDNA loci, which aimed at analyzing the structure of the chromosomes and identify missing or additional chromosomes in aneuploid cells. Plants from the 2-week-old calli had small and deformed flower buds with a low number of dividing cells. For this reason only a small number of cells could be analyzed by FISH for one representative of each ploidy level. In the analyzed plants the rearrangements of rDNA loci was accompanied by a high variation in the chromosome number which hindered the identification of particular chromosomes. It was difficult to determine what types of structural aberrations occurred and which chromosomes were involved in the rearrangements. In plants from the 6-week-old calli the variation in chromosome number was not so high making it possible to analyse the aberrations. The number and localization of 45S and 5S rDNA sequences was studied in 14 diploid and 6 tetraploid regenerants from a total of 496 metaphases. Metaphases with a correct chromosome number and position of rDNA loci were predominant in both diploid and tetraploid regenerants, 59 and 73 %, respectively. In the remaining cells rearrangements of rDNA loci and/or aneuploid chromosome numbers were observed (Fig. 3b–d). Moreover, various types of structural aberrations occurred in the cells from individual plants. For example, one of the diploid regenerants showed different types of structural rearrangements such as rDNA translocation, deletion and duplication. In the tetraploid regenerants, several types of aberrations could also be recognized in one metaphase (Fig. 4a). In all of the analyzed karyotypes no correlation between the type of mutation and a particular pair of chromosomes was found, which indicates that the chromosomes are subjected randomly to the structural rearrangements. The exception were the tetraploid regenerants where deletions of 5S rDNA loci were slightly more common than deletions of 45S rDNA. A comparison of frequency of a particular aberration type in diploids and tetraploids showed interesting results. Among diploid plants the most frequent type of rearrangements were translocations, while in tetraploids deletions, especially of 5S rDNA. Duplications of the rDNA sequences had the lowest frequency and their number was equal in diploids and tetraploids (Fig. 5). One explanation for the higher level of deletions observed in tetraploid regenerants may be a better tolerance to the loss of the DNA sequence in the presence of additional copies in polyploid cells rather than in diploid ones.

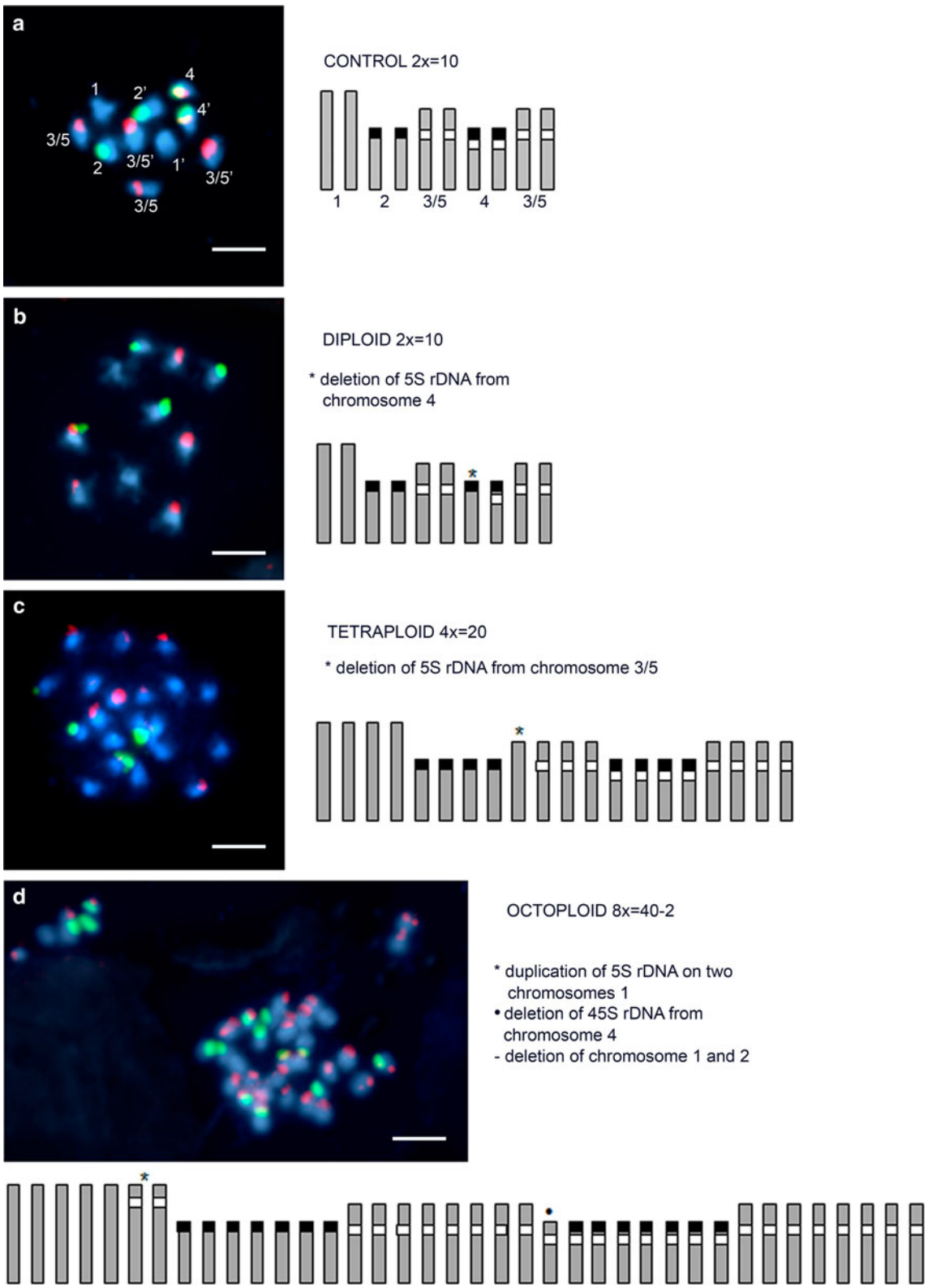

Fig. 3 FISH with 45S rDNA and 5S rDNA on chromosomes of *A. thaliana* plants. (**a**) Control plant 2x = 10; (**b–d**)—regenerants from 2-week-old calli; (**b**) diploid regenerant; (**c**) tetraploid regenerant; (**d**) octoploid regenerant. Bar 10 μm

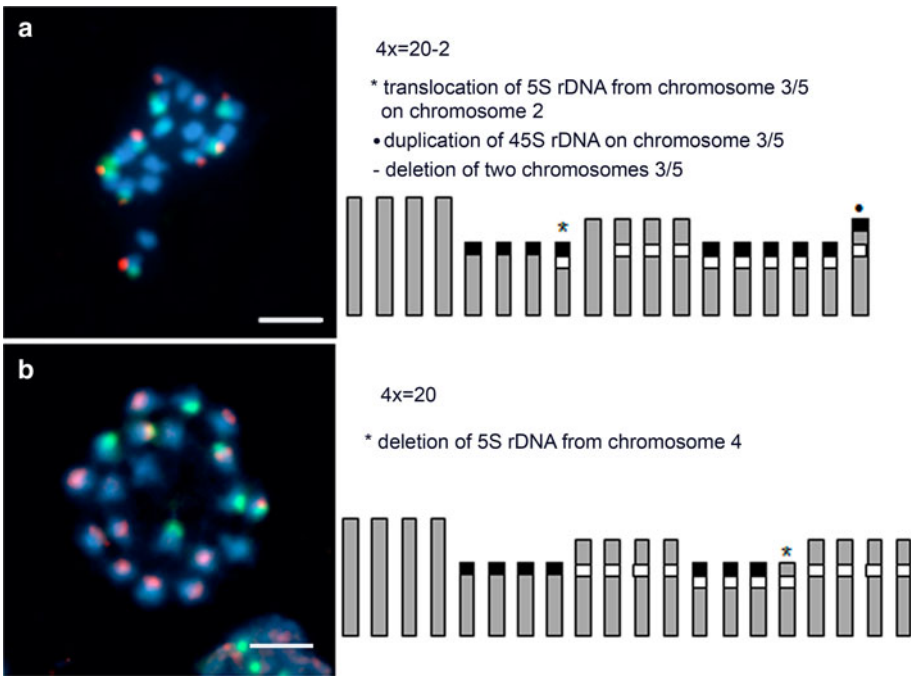

Fig. 4 FISH with 45S rDNA and 5S rDNA on chromosomes of *A. thaliana* regenerants. (**a**) tetraploid regenerant from 6-week-old calli; (**b**) tetraploid plant from R2 generation of regenerants. Bar 10 μm

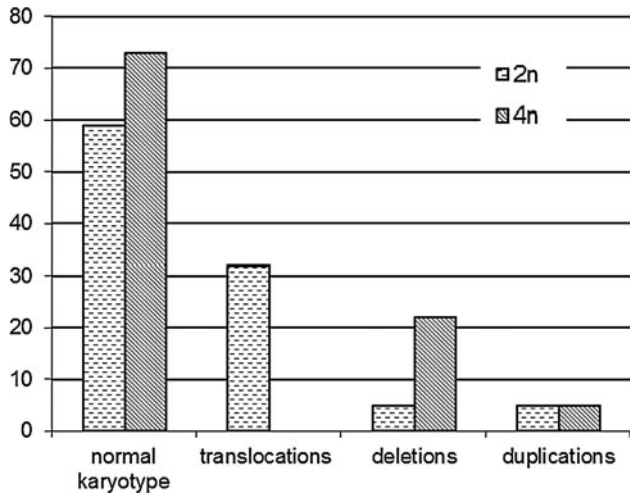

Fig. 5 Frequency of different types of structural aberrations among diploid and tetraploid plants from 6-week-old calli

The localization of rDNA loci was studied in 5 plants from the R2 generation, two diploids and three tetraploids, which were the progeny of two R1 regenerants. The diploids exhibited a normal karyotype, while in all the tetraploid plants deletion of 45S rDNA from one chromosome 4 was observed (Fig. 4b).

An analysis of FISH signals in the aneuploid cells of regenerants from the 6-week-old calli enabled identification of additional and missing chromosomes in these cells (Figs. 3d, 4a), but among the 34 cells analyzed no preference was noticed. FISH with rDNA probes has proved to be useful in karyotype examination in plants regenerated in vitro. In the tetraploid species *A. tuberosum* (2n = 4x = 32) about 40 % of somaclones were hypotetraploid, and FISH with rDNA probes confirmed the identification of missing chromosomes in variants with 29, 30 and 31 chromosomes. In each of these variants, different chromosomes of the karyotype were lost (Do et al. 1999, 2000).

Many cytological studies have reported alterations in chromosome number in plants regenerated in vitro, although there are fewer reports on structural rearrangements, generally because such analyses are time-consuming and often tedious. Kharabian and Darabi (2005) analyzed aberrations in regenerants of two *Oryza sativa* cultivars in prometaphase mitotic and meiotic chromosomes stained with Giemsa. Changes in chromosomal structure like deficiencies, duplications and translocations were detected. It is relevant that these aberrations involved various chromosomes among particular regenerated plants. These data, together with our present results, suggest that the structural rearrangements are random as it was observed for numerical aberrations. The level and type of cytogenetic changes depends on a number of factors. In our research the influence of the preculture period was evaluated, but also using of different type of explant or ecotype can result in a different response. For instance, structural chromosome abnormalities have not been detected during molecular analysis of *A. thaliana* regenerants obtained from root explants after 1 week of preculture on callus inducing medium (Jiang et al. 2011).

Conclusions

Ploidy and chromosome number analysis of plants regenerated from calli after different periods of preculture suggests that the 2-week-old calli displays a higher frequency of polyploid cells which influence the ploidy of regenerants. Diploids, tetraploids and octoploids were observed among plants produced from these calli, and a high variation in the chromosome number in these regenerants was found. Variation in chromosome number, particularly a high frequency of aneuploid cells, probably influence the morphology and fertility of regenerants derived through organogenesis from short-aged calli. Only diploid and tetraploid plants were regenerated from the 6-week-old calli. In these plants a low frequency of aneuploid cells was detected and they were able to produce seeds.

FISH with rDNA probes revealed structural as well as numerical aberrations in the cells of the regenerated plants. Significantly, no preference of any type of aberrations to a particular chromosome or chromosome segment was distinguished. The correlation between the structural aberration type and the ploidy of regenerants is noticeable. In diploid regenerants translocations of rDNA loci were the most frequent type of mutation, while deletions occurred most often in tetraploids. The progeny of R1 regenerants exhibited the same chromosome number as their parents, and structural rearrangements were transmitted.

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