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COMPARATIVE CYTOGENETIC ANALYSIS OF DIPLOID AND HEXAPLOID *CHENOPODIUM ALBUM* Agg.

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ABSTRACT

Two cytotypes of *Chenopodium album*, diploid ($2n=2x=18$) and hexaploid ($2n=6x=54$), were analysed using flow cytometry and a FISH experiment. The genome size was indicated as 1.795 pg for the diploid and 3.845 pg for the hexaploid plants which suggested genome downsizing in the evolution of hexaploid cytotype. Double FISH with 25S rDNA and 5S rDNA allowed three to five homologue chromosome pairs to be distinguished depending on the cytotype. The variation in size and number of rDNA sites between the polyploid *C. album* and its putative diploid ancestor indicated that rDNA loci underwent rearrangements after polyploidization.

Flow cytometry measurements of the relative nuclear DNA content in the somatic tissue of *C. album* revealed extensive endopolyploidization resulting in tissues comprising a mixture of cells with a different DNA content (from 2C to 32C) in varying proportions. The pattern of endopolyploidy was characteristic for the developmental stage of the plant and for the individual organ. Polysomaty was not observed in the embryo tissues however endopolyploidization had taken place in most tested organs of seedlings. The endopolyploidy in diploid and hexaploid *C. album* was compared to find any relationship between the pattern of polysomaty and polyploidy level in this species. This revealed that polyploid plants showed a decline in the number of endocycles as well as in the frequency of endopolyploidy cells compared to diploid plants.

KEY WORDS: *Chenopodium*, polyploid, genome size, rDNA sequence, flow cytometry, polysomaty.

INTRODUCTION

Polyploidy species are common among angiosperm plants. It is estimated that approximately 80% of flowering plants underwent a polyploidization event during their evolution (Soltis and Soltis 1993; Masterson 1994; Bennett 2004). Polyploids exhibit distinctive morphological, physiological and life history characteristics that may allow plants to colonize new habitats or expand their geographical range (Otto and Whitton 2000; Ramsey and Schemske 2002; Borgen and Hultgard 2003).

As indicated for many species, polyploidization was followed by dramatic genome rearrangements, which often led to genome downsizing and chromosomal changes (Leitch and Bennett 2004; Lim et al. 2004). The mechanisms that are responsible for polyploidy genome rearrangements are still not well known, but most often translocations, elimination of repetitive sequences, activation of transposable elements and homoeologous recombination have been proposed (Wendel 2000; Bennetzen 2002; Kidwell 2002). One of the repetitive sequences often used in genome analysis are rRNA genes. Their tandem organization, high copy number and conserved nature throughout the plant kingdom (Maluszyńska et al. 1998) make them suitable mar-

kers for chromosome identification and karyotyping in many different plant genera such as *Brassica*, *Lupinus*, *Hypochaeris*, *Hordeum* (Kulak et al. 2002; Hajdera et al. 2003; Weiss-Schneeweiss 2003; Takate et al. 2005). The localization and number of the gene loci can change in evolving polyploids as for *Scilla autumnalis* described Vaughan et al. (1993).

Besides generative polyploidization many angiosperm species undergo somatic polyploidization during development. Endopolyploidization of nuclear DNA is a common feature of eucaryots and occurs in over 90% of all angiosperms. The variation of cell ploidy levels designated as somatic polyploidy is a developmental process that involves one or more rounds of nuclear DNA synthesis without chromosomal and cellular division and leads to endopolyploidy (D'Amato 1989; Trass et al. 1998; Małuszyńska and Siwińska 2004). Although somatic polyploidy was found in most angiosperms, in some species like *Crepis capillaries* there are no endopolyploid cells in any tissue (Małuszyńska 1990).

Chenopodium album is a complex species and consists of highly heteromorphic wild and semi-cultivated forms (Wilson 1990; Gangopadhyay et al. 2002). Cytologically, it comprises diploid ($2n=2x=18$), tetraploid ($2n=4x=36$)

and hexaploid ($2n=6x=54$) cytotypes (Tanaka and Tanaka 1980; Partap et al. 1985; Bhargava et al. 2005). This study was undertaken to find relationships between the polyploidy level, genome size and numbers of rDNA loci in a *C. album* complex. We also decided to investigate whether the polyploidy level of the *C. album* plant has an impact on the endopolyploidy pattern during plant development.

MATERIALS AND METHODS

Plant material and DNA probes

Seeds of the diploid *C. album* ($2n=18$) derived from the Botanic Garden in Berlin-Dahlem and the hexaploid *C. album* ($2n=6x=54$) which came from Hortus Botanicus Universitatis "Friedrich Schiller" Jena (Germany) were used. The plants were cultivated in pots in the growth chamber of Silesian University.

The 5S rDNA 410 bp clone isolated from *Triticum aestivum* (Gerlach and Dyer 1980) was amplified and directly labelled using PCR with rhodamine-4-dUTP. The 2.3 kb sub-clone of the 25S rDNA coding region of *Arabidopsis thaliana* (Unfried and Grueler 1990), labelled with digoxigenine-11-dUTP by nick translation, was used for detection of 18S-5.8S-25S rDNA.

Chromosome preparation and FISH

Chromosome preparations according to Maughan et al. (2006) were prepared from young leaves and shoot meristems. Methods of in situ hybridization were adapted from Schwarzacher and Heslop-Harrison (2000) with some modification. The hybridization mixture, consisting of 100 ng labelled DNA probe, 50% formamide, 10% dextran sulphate, 0.1% SDS (sodium dodecyl sulphate) and 10 μ g of sheared salmon sperm DNA, was denatured for 10 min. at 85°C, then chilled for 5 min. on ice and applied to the chromosome preparations. The slides and hybridization mixture were then denatured together at 72°C for 5 min. in an in situ Thermal Cycler (Hybaid) and incubated at 37°C overnight. Stringent washes (20% formamide in $0.1 \times$ SSC at 42°C) were followed by immunodetection of a digoxigenate

DNA probe with FITC-conjugated antibodies (Roche). The preparations were mounted in Vectashield containing 2 μ g/ml DAPI and stored at 4°C. Slides were analysed using an Olympus AX 70 microscope and all pictures were taken using a Hamamatsu (C5810) CCD camera. Pictures were uniformly processed and superimposed using Micrografix Picture Publisher software.

Flow cytometry analysis

Young leaves were used for measuring nuclear DNA content. The samples were prepared according to a two-step protocol (Doležel et Göhde 1995) and nuclei were stained with PI (propidium iodide). For each sample at least 10 000 nuclei were analysed. Samples were measured using a DAKO Galaxy flow cytometer equipped with an air-cooled argon ion laser. The nuclear DNA content of the accessions of *Chenopodium album* was calculated using nuclei isolated from young leaves of *Glycine max* cv. Polanka (2C DNA – 2.50 pg, Doležel et al. 1994) as an internal standard. The value of 2C DNA was estimated as an average of 30 measurements for each cytotype.

The endopolyploidy pattern was obtained for embryos isolated from soaked seeds and for seedlings in the stage of open cotyledons. The diploid plant reached the second stage of development after three days of growth and the hexaploid in the fifth day after germination. Flow cytometry analysis in seedlings was prepared separately for particular parts: radicles, root tips, differentiated root parts, hypocotyls and cotyledons. To release nuclei the tissue sample was chopped with a razor blade in a nuclei extraction buffer (Doležel et Göhde 1995), filtered through a 30 μ m nylon sieve (Partec Cell Trics) and stained with DAPI (4,6-diamidino-2-phenylindole). Each sample consisted of tissue from 10 different plants and for each sample 10 000 nuclei were analysed. At least three replicates were subjected to analysis. The DNA content of the isolated nuclei in the samples were analysed using a DAKO Galaxy flow cytometer equipped with an HBO-100 mercury lamp and the set of filters and mirrors (KG1, UG1, BG38, TK420 and GG435). The FloMax software processed data were plotted on a semilogarithmic scale.

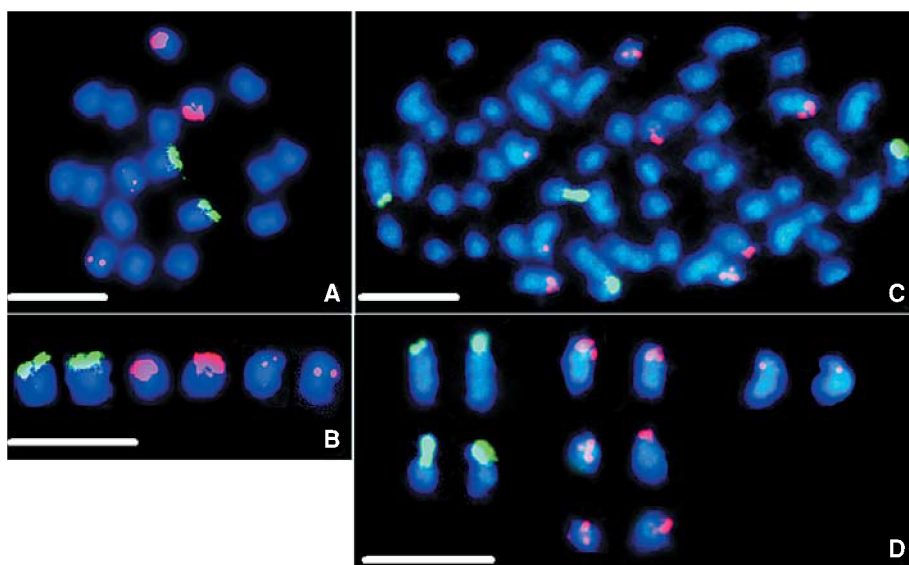


Fig. 1. Fluorescence in situ hybridization of the 25S rDNA (green) and 5S rDNA (red) sequences to diploid (a, b) and hexaploid (c, d) *C. album* chromosomes.

RESULTS

Genome of C. album

The analysed accessions of *C. album* exhibit two different ploidy levels: diploid and hexaploid with the basic chromosome number $x=9$. The somatic metaphase chromosomes of *C. album* cytotypes are shown in Figure 1. Diploid plants possess $2n=2x=18$ chromosomes whose size ranges from 1.4 to 2.7 μm . Hexaploid plants were characterized by $2n=6x=54$ slightly shorter chromosomes and their size was between 1.0 to 2.6 μm .

The genome size of the *C. album* plant was established using a flow cytometry analysis of propidium iodide-stained nuclei. Measurements resulted in histograms with two dominant peaks corresponding to the 2C DNA nuclei of *C. album* and glycine. The 2C DNA content was determined as $1.8 \text{ pg} \pm 0.02$ for the diploid and $3.85 \text{ pg} \pm 0.03$ for the hexaploid.

Fluorescent in situ hybridization with 5S rDNA and 25S rDNA to *C. album* chromosomes reveals hybridization signals only in the terminal parts of chromosomes. The diploid cytotype showed one pair of 25S rDNA sites and two pairs of 5S rDNA sites. The 5S rDNA hybridization signals showed differences in size and intensity. This allowed three pairs of homologue chromosomes to be distinguished (Fig. 1a, b). The hexaploid cytotype displayed two pairs of 25S rDNA loci and four pairs of 5S rDNA loci (Fig. 1c, d). Chromosomes that carry 25S rRNA genes differ in size, which allowed two pairs of homologue chromosomes to be distinguished. In the hexaploid genome, similar to the diploid, 5S rDNA loci differed in fluorescence signal intensity. Six chromosomes exhibited a relatively strong 5S rDNA signal. The chromosomes have a very similar morphology so it was impossible to recognize the homologue pairs. Additionally, one pair of chromosomes carried loci with fewer 5S rDNA repeats and revealed weaker hybridization signals (Fig. 1c, d). Double FISH with rRNA genes to hexaploid *C. album* allowed three pairs of homologue chromosomes to be distinguished and differentiated a group of six chromosomes carrying 5S rDNA sequences.

Endopolyploidy in C. album

Ploidy patterns of *C. album* were obtained for two different stages of plant development: embryos isolated from imbibed seeds and seedlings in the stage of open cotyledons. The patterns of endopolyploidy obtained for embryos of diploid and hexaploid cytotypes were similar. The embryos mainly contained cells with 2C or 4C DNA (Fig. 2). For both cytotypes of *C. album*, 2C DNA picks were large

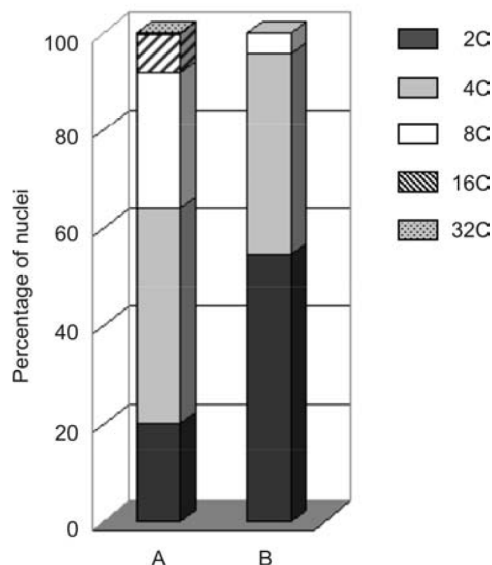


Fig. 3. Average frequency of nuclei with various DNA content in *C. album* seedlings: (A) diploid and (B) hexaploid.

and contained about 90% of the total nuclei. The picks for 4C DNA nuclei were small.

By contrast in the stage of open cotyledons, diploid and hexaploid seedlings showed different endopolyploidy patterns (Fig. 3). Diploid *C. album* revealed a high number of endopolyploid nuclei. Nearly 40% of the cells had DNA content higher than 4C (Fig. 3). A detailed flow cytometry analysis revealed that endopolyploid cells existed in all analysed organs and that particular organs exhibit different patterns of polysomaty (Fig. 4). Differentiated root parts displayed the highest level of endopolyploidization where only 45% of the cells possessed 2C or 4C DNA and only in this part of seedlings were cells with 32C DNA (Fig. 4). Endopolyploid cells were also observed in root tips where nearly 40% of the cells have an 8C or 16C DNA level. The lowest level of polysomaty was observed in cotyledons. These seedling organs revealed only 2C, 4C and 8C DNA nuclei and the number of nuclei with DNA content higher than 4C was 7% (Fig. 4).

A relatively lower level of endopolyploidization was observed in the hexaploid *C. album*. It was revealed that hexaploid plants showed a decline in the number of endocycles as well as in the frequency of endopolyploid cells compared to diploid plants. More than 90% of the cells had a 2C or 4C DNA content and a very small number of cells with 8C DNA were observed (Fig. 3). In the all analysed

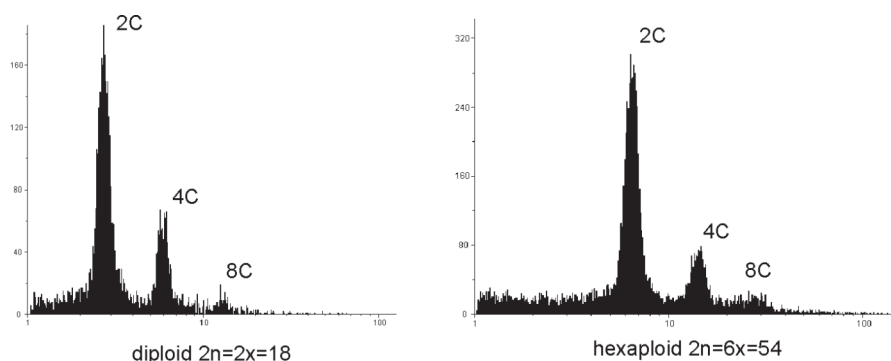


Fig. 2. Typical flow cytometric histograms (log scale) of the nuclei isolated from diploid (A) and hexaploid (B) *C. album* embryos.

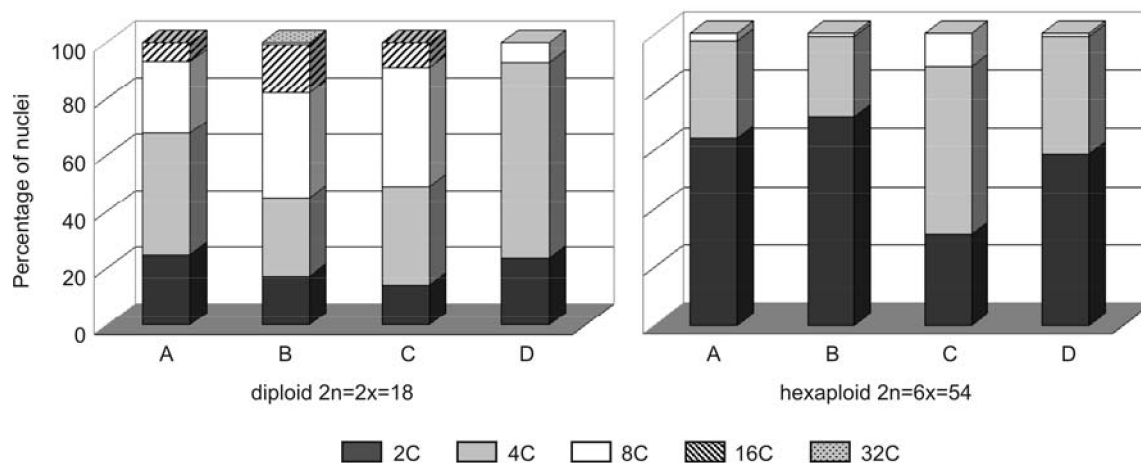


Fig. 4. Frequency of the nuclei with various DNA content in diploid and hexaploid *C. album* seedlings in stage of open cotyledons. (A) root tip, (B) differentiated part of root, (C) hypocotyl, (D) cotyledons.

hexaploid organs, cells with 2C and 4C DNA dominated but the endopolyploidy pattern was different for the every organ (Fig. 4). The lowest level of polysomaty was found in cotyledons and the differentiated part of a root where only 1% of the cells have DNA content higher than 4C. More endopolyploid cells were observed in root tips (2.5%) and hypocotyls. The hypocotyls, where 11% of the cells have an 8C DNA content, showed the highest level of polysomaty (Fig. 4). The hypocotyl was also the seedling part with the lowest level of cells with a 2C DNA content. Additionally, we checked the endopolyploidy level in mature leaves. In both diploid and hexaploid plants a lack of endopolyploidy was observed in these organs (data not shown).

DISCUSSION

Genome of *C. album*

The analysed *C. album* accessions represent two different ploidy levels and possess $2n = 18$ (diploid) or $2n = 54$ (hexaploid) chromosomes. Our results are in agreement with earlier data that described the *C. album* plant with diploid, tetraploid and hexaploid chromosome number (Tanaka and Tanaka 1980; Partap and Kapoor 1985) and support the hypothesis that *C. album* is a polyploidy complex. The origin of the polyploidy *C. album* is still unclear and there are reports suggesting that hexaploid *C. album* could be an allo- not autopolyploid plant (Gangopadhyay et al. 2002; Kolano et al. 2006). The genome size of diploid and hexaploid *C. album* was assumed to be 1.795 pg and 3.846 pg respectively. Our results differ from data obtained earlier. The diploid *C. album* plant analyzed by Ohrii (2002) had a smaller genome size 1.54 pg/2C DNA and the hexaploid had a larger 2C DNA value (4.66 pg). *C. album* is a widespread and variable species so the revealed differences could represent karyotypical and genetic differences among different populations. Intraspecific variations in genome size (not connected with different ploidy levels) have been reported earlier for some species like *Zea mays* or *Glycine max* (Rayburn et al. 1997; Rayburn et al. 2004). The variations were connected with different geographical

localizations as for *Arabidopsis thaliana* (Schmuths et al. 2004) or different altitudes as for *Dactylis glomerata* (Reeves et al. 1998).

The hexaploid *C. album* analyzed in this study has a genome that is twice as large as that of a diploid. Polyploids often have a somewhat lower genome size than expected from their diploid relatives (Song et al. 1995; Leitch and Bennett 2004; Cavalier-Smith 2005). The mechanisms, which lead to genome downsizing in polyploids are still not understood although there are many analyses which have shown that the elimination of duplicated genes and loss of repetitive sequences could be the reasons. The elimination of DNA sequences from polyploid genomes was reported for a few species, for example *Ranunculus*, the *Aegilops-Triticum* group and *Nicotiana* (Horandl and Greilhuber 2002; Ozkan et al. 2003; Narayan 1998).

The elimination of rRNA genes from polyploid genomes is well-documented. A comparative analysis of the number, localization and intensity of rDNA-FISH signals in polyploid and their diploid ancestors allowed researchers to follow the chromosome evolution in polyploids in many cases (Maluszynska and Heslop-Harrison 1993; Vaughan et al. 1993; Hasterok and Maluszynska 2000). The comparative study showed a non-additive distribution pattern of 25S and 5S rDNA loci in the polyploid *C. album*. FISH results indicated that there was a loss of one locus of 25S rDNA in the hexaploid (Fig. 1). Additionally the chromosome pairs that bear the 25S rDNA loci had a different morphology (Fig. 1c). This suggested that chromosome rearrangement involving 25S rDNA locus happened after polyploidization. In the case of 5S rRNA genes the loss of site number concerned only the locus with weak hybridization signals. The number of strong 5S rDNA signals in the hexaploid was as expected when compared with a diploid plant. The rearrangement of rDNA sites has been reported in the evolution of other polyploids, for example *Arabidopsis*, *Camellia*, *Brassica* (Weiss and Maluszynska 2001; Gu et Xiao 2003; Hasterok et al. 2006). Polyploidy genome downsizing caused by the loss of repetitive sequences could be an adaptative response to the genomic stress caused by polyploidization, which serves to stabilize the polyploidy genome (Ozkan et al. 2003).

*Comparison of polysomaty
in diploid and hexaploid C. album plants*

The connection between generative and somatic polyploidy is still unclear. In some species such as *Beta vulgaris* and *Zea mays*, polyploid plants have a lower level of endopolyploid cells than diploids, but on the other hand, diploid and tetraploid tomato plants exhibit the same pattern of polysomaty (Biradar et al. 1993; Smulders et al. 1994; Sliwiska and Lukaszewska 2005).

In this study we compared polysomaty patterns in diploid and hexaploid *C. album*. The plants used for this study were at a comparable stage of development and were grown in the same conditions. The results obtained for embryos isolated from imbibed seeds were similar for diploid and hexaploid cytotypes. The embryos mainly contained cells with a 2C and 4C DNA content although a small peak for 8C DNA was observed. Earlier researches also showed that embryo tissues consist mostly of cells with 2C DNA (*Fagus sylvatica*, *Pinus nigra*, *Cichorium endive*) or 2C and 4C DNA (*Rahanus sativus*) although there are species whose embryos contain some endopolyploid cells (*Spinacia oleracea*, *Cucumis sativus*) (Bino et al. 1993; Gilissen et al. 1993). The additional 8C DNA peak for *C. album* embryos indicated that endopolyploidization took place in a small number of cells at the early stages of tissue differentiation during embryo development. Then during seedling development, the number of endopolyploid cells rose. An increase of endopolyploidy often occurs with age and it is believed that a higher DNA content might be needed to sustain the larger cell volume (Galbraith et al. 1991; Kondrosi et al. 2000). Remarkably, there was a lack of endopolyploidization in both diploid and hexaploid *C. album* leaves which is in contrast with the observation of *Arabidopsis*, *Lycopersicon*, and *Brassica* (Galbraith et al. 1991; Smulders et al. 1994; Kudo and Kimura 2001). The results obtained in this study showed that although endopolyploidy occurs in diploid and hexaploid plant, the level of the occurrence differs. The diploid *C. album* seedlings had a pattern of polysomaty that consisted of 2C-32C DNA cells and existed in most analysed organs, even in the root tip. Meristematic tissue usually contains cells with 2C or 4C DNA. However, some reports have described endopolyploid cells in root meristems of other *Chenopodium* species (Wang et al. 1993). The presence of endopolyploid cells in the root tip could be connected with methaxylem cell differentiation (Baluska 1990).

The polysomaty pattern in the hexaploid plant was different than in the diploid. The highest level of endopolyploidy observed in hexaploid seedlings was 8C DNA and the hexaploid *C. album* contained fewer cells with a DNA content higher than 4C. In the hexaploid the average endopolyploid cell content was lower than 5% whereas in the diploid it was approximately 40%. This is in agreement with data obtained for diploid, triploid and tetraploid accessions of *Beta vulgaris* (Sliwiska and Lukaszewska 2005). A comparison of polysomaty development between *B. vulgaris* plants of different ploidy level showed that the number of endocycles is negative correlated with the plant ploidy level. A similar tendency was observed in diploid and tetraploid *Zea mays* and *Portulaca grandiflora*. However, in these two species the number of endocycles was the same but the level of endopolyploid cells was higher in the

diploid than in the polyploidy plant (Biradar et al. 1993; Mishiba and Mii 2000).

Our results indicated that endopolyploidization is an integral part of the differentiation process in *C. album* but there is negative correlation between genome size and the number of endocycles. In a hexaploid plant most of the cells have a 2C or 4C DNA content and the level of endopolyploidization is much lower than in diploids. This suggests that in this species a certain maximum nuclear DNA content is programmed rather than the number of endocycles.

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