INTERPHASE STRUCTURE OF ENDOREDUPlicated Nuclei in Diploid and Tetraploid Brassica oleracea L.

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Endoreduplication of nuclei is a common phenomenon in plants. Cells with multiplied DNA content are usually termed endopolyploid or polysomatic. The study was aimed at clarifying the chromosomal status of endoreduplicated nuclei. So far it has been generally assumed that endoreduplicated nuclei represent a form of polyploidy. We propose a distinction between true polyploid nuclei, possessing multiple sets of chromosomes, and chromosomes formed within endoreduplicated nuclei. We supposed that chromosomes within endoreduplicated nuclei are not separated, as in true polyploids, but rather are bound together. To clarify these two alternatives, we subjected interphase nuclei exhibiting different degrees of endoreduplication and ploidy to fluorescent in situ hybridization, using diploid and tetraploid cabbage root tips containing nuclei of four different sizes (2C–16C or 4C–32C, respectively) as revealed by flow cytometry. Nuclei were hybridized with an rDNA probe (pTA71), which showed four rDNA hybridization sites on diploid metaphase chromosomes. The number of hybridization sites was constant for both diploid and tetraploid samples (3–6 per diploid and 7–12 per tetraploid nuclei) irrespective of endoreduplicated nuclear size. The results showed more signals only in the tetraploid compared to the diploid genotype, but not between different nuclear sizes within a genotype of the same ploidy. We suggest that these results indicate that chromosomes within endoreduplicated nuclei are actually bundles of sister chromatids. The consequences of this understanding of the bundled chromosomal status of endoreduplicated nuclei are discussed.

Key words: Endopolyploidy, polysomaty, Brassica oleracea, fluorescent in situ hybridization.

INTRODUCTION

The terms "endopolyploidy" and "polysomaty" refer to a common, well-studied phenomenon in plants, characterized by multiplication of nuclear DNA amounts in various tissues. In addition to nuclei with 2C and 4C content (corresponding to G0/G1 and G2 cell cycle phases), endopolyploid tissues also contain nuclei with 8C, 16C, 32C, 64C and higher content. Endopolyploidy occurs in a wide range of species and is expressed in tissues at varying developmental stages. Commonly, endopolyploidy is expressed during seed germination (Bino et al., 1993), but it has also been detected in leaf and stem cells (Meadagno et al., 1993; Smulders et al., 1994) and in flowers (Kudo and Kimura, 2001a), as well as in developing fruit or seed tissues such as tomato pericarp (Bergervoet et al., 1996) or maize endosperm (Kowles et al., 1997). Several authors have presented observations of plant endopolyploidy (Geitler, 1940), and have also speculated on the function of this phenomenon in plants in relation to improved cold tolerance in maize seedlings (Wilhelm et al., 1995), the morphology of leaf trichomes in Arabidopsis (Traas et al., 1998), senescence phenomena and improvement of assimilative sinks in storage tissues (Bergervoet et al., 1996). The mechanism of endoreduplication has also been investigated at the molecular level (for review: Joubes and Chevalier, 2000). Larkins et al. (2001) reported that the loss of M-phase cyclin-dependent kinase activity and oscillations in S-phase cyclin-dependent kinase are key elements involved in this process.

Our research focused on the following problem: theoretically, increased nuclear DNA amounts caused by endoreduplication cycles can result in two types of chromosomal rearrangement: chromosomes may exhibit polyploidy (in the narrow sense, meaning sets of individual chromosomes) or may actually remain bundled together. In the case of polyploidy, the number of chromosomes is doubled by each endoreduplication cycle and each chromosome is individually separated, while in the case of chromosome nondisjunction the number of chromosome units remains the same because they are bound together. A similar phenomenon in which chromosomes are bundled together is known...
vulgaris reported by Nagl (1969) in suspensor cells of mosomes in cultured cotyledons of tion of polyploidy (4x to 32x) and giant polytene chro-
tent (up to 174C), the endoaneupolyploid nuclei exhibit
observed that in cells with particularly high DNA con-
term cell suspension cultures of wheat. The authors
(1991) found giant polytene chromosomes formed in
antipodes, tapetal cells of anthers, or endosperm cells
of several plant species (Kress, 1996). Shang and Wang
(1991) found giant polytene chromosomes formed in
embryogenic calli or in root tips regenerated from long-
term cell suspension cultures of wheat. The authors
observed that in cells with particularly high DNA con-
tent (up to 174C), the endoaneupolyploid nuclei exhibit
polyteny. Marks and Davies (1979) reported the induc-
tion of polyplody (4x to 32x) and giant polytene chro-
mosomes in cultured cotyledons of Pisum sativum; they
observed separation of polytene chromosomes into con-
stituent single chromosomes.

From the terminological point of view, endoredup-
plication, endopolyploidy, polysomy, mixoploidy and
polyteny describe phenomena of increased nuclear DNA levels. Endoreduplication is described by Ther-
man (1995) as a form of somatic polyploidy in which the
chromosomes replicate time after time without chromo-
some condensation and no mitoses. As reviewed by
Levan and Mntzing (1991), the term “endopolyploidy”
is associated with polyplody chromosome numbers,
thus causing cells with 4x, 8x, 16x, 32x, etc. The term
“polyteny” normally describes a special type of giant
chromosome, visible in mitosis, that is more or less
suborganized into bands and interbands (Rieger et al.,
1991). The terms “endoreduplication,” “polysomy”
and “mixoploidy” are also related to increased chromo-
some numbers. These terms are used in most recent
papers in which the authors describe the presence of
cells or tissues with endoreduplicated nuclear content.
Whether the condition of chromosomes within nuclei of
increased DNA levels is not polyplody (in the narrow
sense), as generally assumed, but instead represents
chromosomes bound together, the use of these terms
can lead to misunderstanding. This misunderstanding
has consequences related to the response of various
biotechnological manipulations which include plant re-
generation from cells grown in vitro, for example from
intact tissues, calli, cell suspensions, or protoplasts. It
is essential to understand the correct status of the
chromosomes within such nuclei, in order to predict the
success of regeneration and to identify the ploidy of
regenerants.

The phenomenon of endomitosis has been studied
extensively since the 1930s. Early results were sum-
marized by Geitler (1953), the leading researcher in
this field. A particularly interesting scheme of endomi-

tosis was later presented by Tschermak-Woess (1973),
who described the occurrence of chromocenters during
endomitosis and noted that the number of chromocen-
ters remains more or less constant while their size as
well as the nuclear volume increases after each endore-
duplication cycle. A few more recently published papers
contain partial support for bundled structure of chro-
mosomes within endoreduplicated nuclei. Melaragno
et al. (1993) suggested that leaf trichome nuclei with
increased ploidy levels in Arabidopsis represent a form
of polyteny. This suggestion was based on the constant
number of chromocenters (10–13) for all nuclear sizes.
Similarly, Bohdanowicz and Dabrowska (1997) ob-
erved increased nuclear size, independent of an in-
crease in the number of endochromocenters, in papil-
rar tissues of Triglochin maritimum L.

The aim of this study was to present evidence for
either independent or bundled chromosomal arrange-
ment within endoreduplicated nuclei, using flow cyto-
metry and rDNA hybridization, and to discuss the
importance of a correct understanding of chromosome
status within such nuclei.

MATERIALS AND METHODS

PLANT MATERIAL

After screening several potential species using an rDNA
probe (data not shown), we chose white cabbage
(Brassica oleracea var. capitata) as an appropriate
model plant. Roots and root tips of 3–4-day-old seed-
lings were used in the study. Autotetraploid cabbage
plants were induced spontaneously from microspores
obtained in a previous study (Rudolf et al., 1999).

MEASUREMENTS OF NUCLEAR DNA CONTENT

Flow cytometry was used to compare genome sizes in
root tip tissue of diploid and tetraploid cabbage and root
tissue of diploid cabbage. Seeds were germinated on
moistened filter paper for 3–4 days at 25˚C. Nuclear
extraction, staining and measurement were done as
described previously (Bohaneac and Jakše, 1999); brief-
ly, nuclei were released by chopping in 0.1 M citric acid
containing 0.5% Tween 20, and the suspension was
stained using 5.25 µgml-1 4’6-diamidino-2-phenylindol-
de (DAPI) in 0.4 M disodium hydrogen phosphate. DNA
histograms were measured with a Partec PAS III flow
cytometer. For correct interpretation, 2C values obtained
from leaf tissues showing only basic 2C and 4C values
were compared with histograms showing multiple nu-
clear stages obtained from root and root tip tissues.

SLIDE PREPARATION

Seedling root tips were used to make chromosome
preparations. Seeds were germinated on moistened
filter paper for 3–4 days in the dark at 25˚C. When the
roots were 1.5–2 cm long, seedlings were treated with
a saturated solution of monobromonaphthalene for 24 h at 4°C and then fixed in 3:1 ethanol-glacial acetic acid fixative. Root tips were washed in distilled water and partially digested with 4% cellulase (Onozuka) and 1% pectolyase (Y-23) in enzyme buffer (75 mM KCl, 7.5 mM EDTA) at 37°C for 45 min. A few root tips were squashed in a drop of 45% acetic acid. Coverslips were removed after freezing in liquid nitrogen, and the slides were allowed to air-dry. For analysis of isolated nuclei, 3-4-day-old roots of diploid cabbage were chopped in 0.1 M citric acid containing 0.5% Tween 20. The sample was centrifuged at 200 g to increase the concentration of nuclei. After removal of the supernatant, part of the sample was measured by flow cytometry as described before. The other part was put on glass slides and the cover slips were removed after freezing at -80°C. These slides were also used for in situ hybridization. The quality of the slide preparations was microscopically examined after staining with 2 µg/ml DAPI in distilled water. High-quality preparations were stored at +4°C until use.

IN SITU HYBRIDIZATION
A 9 kb EcoRI fragment of the rDNA repeat unit from wheat, pTA71 (Gerlach and Bedbrook, 1979), was labelled with biotin-14-dATP by nick translation (BioNick Labelling System kit; GIBCO-BRL) following the manufacturer’s instructions. Unincorporated nucleotides were removed from the labelled DNA probe by repeated ethanol precipitation.

Following RNase treatment (100 µg/ml, 1 h, 37°C), chromosome preparations were washed in 2 × SSC at room temperature for 3 × 5 min and treated with pepsin (1 mg/ml in 0.01 M HCl) for 10 min at 37°C. They were then washed 2 × 5 min at room temperature and treated with 4% paraformaldehyde for 10 min. After another 3 washes for 5 min each in 2 × SSC at room temperature, the preparations were dehydrated in a graded series of 70%, 90% and absolute ethanol. Chromosomal DNA was denatured in 70% formamide solution in 2 × SSC at 70°C for 2 min. The slides were then dehydrated through a cold ethanol series and air-dried. The hybridization mixture containing 50 ng/slide of labelled DNA probe, 50% formamide, 2 × SSC, dextran sulphate and herring sperm DNA was denatured for 10 min at 80°C and placed on ice for 5 min before being added to the slides. Hybridization was carried out overnight at 37°C in a humid chamber.

After hybridization, the slides were washed for 10 min at 42°C in 50% formamide in 2 × SSC followed by 10 min washes in 2 × SSC at 42°C and 2 × SSC at room temperature. Detection of hybridization sites was performed by transferring the slides to detection buffer (4 × SSC, 0.1% Tween-20) for 5 min, treating with 3% bovine serum albumin in detection buffer for 30 min, and then incubating with streptavidine conjugated with CY3 (Sigma) for 30 min at 37°C. The slides were washed in detection buffer at 37°C for 3 × 5 min. For signal amplification, slides were treated for 5 min with 5% normal goat serum and then incubated with biotinylated anti-streptavidin (Vector lab) at 37°C for 30 min, before being washed 3 × 5 min in detection buffer at 37°C.

Fig. 1. Flow cytometric analysis showing DNA histograms (log scale) of DAPI-stained nuclear preparations of cabbage tissues: (a) Diploid leaf tissue, (b) Diploid seedling root tip tissue, (c) Tetraploid seedling root tip tissue.
The slides were mounted in antifade solution containing 6 µM DAPI, 0.2 M 1,4 diazobicyclo(2.2.2.)octane (DABCO), and 0.02 M Tris-HCl (pH 8.0) in 90% glycerol. Slides were examined with a Zeiss Jenalumar 250 epifluorescence microscope using appropriate filter sets. Photographs were taken with a Zeiss MC 80 camera on Fujicolor MS 100/1000 ASA color reversal film.

RESULTS AND DISCUSSION

To analyze the chromosomal status within endoreduplicated nuclei, plant tissues with nuclei of endoreduplication stages up to 16C or 32C were subjected to fluorescent in situ hybridization (FISH) using a probe that is present in only a few loci per genome, to produce signals well visible on chromosomes and within interphase nuclei. According to our preliminary research and published data (Gornik et al., 1997; Kudo and Kimura, 2001b), four endomitotic stages are found in young germinated seedlings of B. oleracea. Maluszynska and Heslop-Harrison (1993) found that rDNA loci in this species exist only as two major and one minor spot per haploid genome. Snowdon and Köhler (1997), however, reported only two clearly distinguishable pairs on metaphase chromosomes. For these reasons, diploid and tetraploid lines of cabbage were chosen as appropriate plant species for which an available rDNA probe (pTA71) matched the defined criteria.

Flow cytometry of root tip tissues revealed four distinct peaks representing 2C, 4C, 8C and 16C or 4C, 8C, 16C and 32C nuclei in diploid or tetraploid genotypes, respectively, in contrast to leaf tissue (Fig. 1). The root tip cells of both genotypes therefore expressed

![Fig. 2. Fluorescence in situ hybridization of root-tip preparations of Brassica oleracea. Blue fluorescence shows DNA counterstaining with DAPI; red CY3 fluorescence indicates in situ hybridization sites of rDNA probe pTA71. (a) Metaphase of diploid genotype with two pairs of rDNA loci, (b) Interphase nuclei of two different sizes from tetraploid genotype, (c-e) Interphase nuclei of different sizes from the same diploid genotype prepared by squash technique, (f-i) Diploid interphase nuclei of different sizes from the same sample as analyzed by flow cytometry shown in Fig. 3. Bar = 20 µm for (a-i).](image)
an additional two peaks together with two basic nu-
clear contents, representing nuclei passed through one
or two endoreduplication cycle(s).

Examination of 40 slides on which diploid or tetra-
ploid root tips had been squashed showed consistently
similar results. Counts of DAPI-stained metaphase
chromosomes of the diploid genotype revealed that the
somatic chromosome number was 2n = 18; no higher
chromosome number was found. Chromosomes sub-
jected to the FISH procedure using a pTA71 probe
showed four distinct rDNA hybridization sites present
at the terminal locations of four chromosomes (Fig. 2a).

Analysis of slides revealed the presence of nuclei
that apparently differ in size and could correspond to
the four nuclear classes detected by flow cytometry.
The tetraploid nuclei were larger than diploid nuclei.
Following the FISH procedure, interphase nuclei of all
sizes showed clear red signals, representing sites of
probe hybridization. The number of hybridization sig-
als in interphase nuclei of tetraploid genotypes varied
from 7 to 12. This variation was present in nuclei of all
sizes (Fig. 2b). Analysis of undamaged nuclei of diploid
chromosomes showed results similar to those for tetra-
ploid ones. The number of rDNA sites varied from 4 to
6 and was also more or less constant irrespective of
nuclear size (Fig. 2c–e). The observed variations in
hybridization signals were not associated with nuclear
size, since in some cases we found fewer signals in
larger nuclei than in smaller ones.

An additional experiment was performed, in which
slides were prepared from nuclei of diploid cabbage
roots released by chopping in citric buffer (the same
method as used for flow cytometry) instead of the
standard squash method. This experiment was per-
formed to produce additional information as an indirect
indication for quantification of nuclear size. The prob-
lem with DAPI-stained nuclei is that nuclear size can-
not be quantified exactly, as can be done with nuclei
stained by Schiff reagent (Feulgen staining technique)
using densitometry or image analysis. An exact dis-
tribution into four classes of nuclear sizes is there-
fore not strictly possible, but the size of nuclei can be
assumed on the basis of their appearance. To ensure
that the nuclei observed by microscope are compar-
able to nuclei measured by flow cytometry in this
experiment, one part of the same sample was ana-
yzed using flow cytometry and the other part was
subjected to FISH analysis. The results of flow cyto-
metry showed that four peaks were present (Fig. 3).
The distribution of the four nuclear sizes was not
equal, with 2C and 4C representing 9.3% and 38.6%,
respectively, and 8C and 16C representing 41.0%
and 11.1%, respectively; this means that endoredu-
plicated nuclei represented 52.1% of all measured
nuclei. The other part of the sample, examined by
FISH, gave the same result as obtained using the
squash technique. The number of hybridization sites
was 3–5 per nucleus and was the same in all four
visually determined nuclear classes (Fig. 2f–i).

The observed variation in the number of signals
representing rDNA loci has already been reported in
decondensed nuclei. In fenugreek, for example, Ahmad
et al. (1999) showed an increased number of signals in
decondensed interphase nuclei versus those on meta-
phase chromosomes. Weiss and Maluszinska (1998) re-
ported a decreased number of signals for rDNA loci (1–4
in diploid or 3–8 in tetraploid) in interphase nuclei of
Arabidopsis thaliana. The increased numbers, which
were not observed on condensed metaphase chromo-
somes, can be attributed to the fact that clusters of
rDNA genes are more dispersed in decondensed inter-
phase chromosomes, while the decreased numbers
have been explained by possible binding of rDNA sites
of homologous chromosomes (H. Weiss, personal com-
munication).

Based on the presented data, we concluded that
the chromosomes are organized within endoredupli-
cated nuclei of cabbage in bundled form. The most
convincing evidence was the clear absence of an in-
creased number of signals, even in the largest nuclei.
It should be noted that in the case of polyploidy (narrow
sense), 32 or 64 signals would be expected in the
largest diploid or tetraploid nuclei. Additional confirm-
ation of this hypothesis is provided by the finding that,
in contrast to nuclear size, the number of hybridization
sites in tetraploid nuclei was double that of diploid
nuclei.

Some published data are more understandable if
a bundled chromosomal structure is assumed for en-
doreduplicated nuclei. It is largely assumed that cells
possessing endoreduplicated nuclei represent the end of development. According to Nagl (1981), the endoreduplication cycle irreversibly leads to endopolyploidy. This view is particularly based on study of the endoreduplication cycle irreversibly leads to endopolyploidy. We thank Dr. Michael J. Havey (University of Wisconsin) for kindly providing plasmid pTA71. This work was supported by grants from the Slovene Ministry of Science and Technology (no. S36–486–019 and P4–0077). We wish to thank Dr. J ohann Greilhuber for critical comments on the manuscript.

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Interphase structure of endoreduplicated nuclei


