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ORIGINAL ARTICLE

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Nuclear fusions contribute to polyploidization of the gigantic nuclei in the chalazal endosperm of *Arabidopsis*

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Abstract Somatic polyploidization is recognized as a means to increase gene expression levels in highly active metabolic cells. The most common mechanisms are endoreplication, endomitosis and cell fusion. In animals and plants the nuclei of multinucleate cells are usually prevented from fusing. Here, we report that the nuclei from the syncytial cyst of the chalazal endosperm of *Arabidopsis thaliana* (L.) Heynh. are polyploid with some intermediate ploidy levels that cannot be attributed to endoreplication, suggesting nuclear fusion. Analysis of isolated nuclei, together with fluorescent in situ hybridization (FISH), revealed that nuclei from the chalazal endosperm are two or three times bigger than the nuclei from the peripheral endosperm and have a corresponding increase in ploidy. Together with the consistent observation of adjoined nuclei, we propose that nuclear fusion contributes, at least in part, to the process of polyploidization in the chalazal endosperm. Confocal analysis of intact seeds further suggested that free nuclei from the peripheral endosperm get incorporated into the chalazal cyst and likely participate in nuclear fusions.

Keywords *Arabidopsis* · Chalazal endosperm · FISH Nuclear fusion · Polyploidization

Abbreviations BAC: Bacterial artificial chromosome · CZE: Chalazal endosperm · DAPI: 4,6-Diamino-2-phenylindole · FISH: Fluorescent in situ hybridization · NOR: Nucleolar organizing region · NCD: Nuclear cytoplasmic domain · PEN: Peripheral endosperm

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Introduction

Somatic polyploidization has been widely reported in plants (Barow and Meister 2003), insects and animals (Edgar and Orr-Weaver 2001). Well-studied examples of obligate polyploid cells are mammalian hepatocytes and megakaryocytes, salivary gland cells in *Drosophila*, as well as the polyploid cells from hypocotyls, leaf epidermis, root tips, trichomes, and the endosperm of plants (for review, see Edgar and Orr-Weaver 2001). Polyploidization was early recognized to correlate with increased growth and metabolic performance of many cell types (Nagl 1976; Brodsky and Uryvaeva 1977). Studies of hepatocytes and megakaryocytes showed that polyploidization coincides with their cellular differentiation accompanied by an increase in the cytoplasmic volume, and a greater protein yield as well as metabolic specialization (Brodsky and Uryvaeva 1977; Gupta 2000; Ravid et al. 2002). A similar relationship is observed in plant tissues such as the elongating cells of the hypocotyls, the differentiating leaf epidermis (Traas et al. 1998) and the proliferating endosperm (Larkins et al. 2001). In addition, polyploidization in trichomes controls cellular morphogenesis, i.e. the degree of trichome branching (reviewed in Schnittger and Hulskamp 2002).

The evolutionary origin of polyploidization has long been discussed and is still a matter of debate (see review by Brodsky and Uryvaeva 1977). One proposal is that polyploidization results from a competition between proliferative and tissue-specific functions, while mitotic components are depleted to the benefit of metabolic syntheses. Alternatively, polyploidization might be viewed as a developmentally regulated process necessary for increasing metabolic performance. The latter option is favoured not only by experimental evidence but also by the observation that polyploidization has been con-

served during evolution (Nagl 1976; Brodsky and Uryvaeva 1977; Larkins et al. 2001). It is generally accepted that genome reproduction by polyploidization is accompanied by a proportional increase in transcription and protein synthesis, which enables the cell to produce a greater metabolic output.

The definition of polyploid cells is not restricted to cells containing a nucleus with a higher genomic content but extends to multinucleate cells (Brodsky and Uryvaeva 1977). The mechanisms of polyploidization are diverse: replication of the chromosomes without mitosis (endoreplication), mitosis without cytokinesis (endomitosis) and cell fusion. Endoreplication is the most common mechanism of somatic polyploidization in plants, insects and animals (Edgar and Orr-Weaver 2001), and can be considered as 'true' nuclear polyploidization. Replication of the genomic DNA without mitosis is enabled by an alternative cell cycle bypassing the M-phase or reiterating the S-phase to different extents depending on the cell type and the organism (Edgar and Orr-Weaver 2001).

Multinucleate cells produced by mitotic division without cell division are not unusual in mammals, insects (Mazumdar and Mazumdar 2002) and plants. In the latter, well-known examples include the syncytial female gametophyte (Grossniklaus and Schneitz 1998), binucleate tapetal cells (Chiavarino et al. 2000), and the endosperm (Berger 2003; Lopes and Larkins 1993). Multinucleate cells are also formed upon cellular fusion, for instance in the formation of certain organs in mammals, but also *Caenorhabditis elegans* (see review by Shemer and Podbilewicz 2000). Some syncytia undergo cellularization to differentiate into a tissue with uninucleate cells. Examples are the cellularization of nuclear blastoderm stage *Drosophila* embryos (Mazumdar and Mazumdar 2002), and the cellularization of endosperm of the nuclear type in the angiosperm seed (Lopes and Larkins 1993). Nuclear fusion seems to be largely prevented in the aforementioned multinucleate structures. However, a few exceptions have been reported, for instance during hepatocyte differentiation (Brodsky and Uryvaeva 1977) and in starving insect larvae of the hemipteran *Rhodnius* (Wigglesworth 1967).

Endosperm tissue is devoted to storage of nutrients and their supply to the embryo and/or seedling, to an extent that varies greatly among species (Lopes and Larkins 1993; Baroux et al. 2002; Berger 2003). The primary endosperm nucleus results from the fusion of a sperm nucleus with usually two maternal haploid nuclei, a process that is accompanied by the fertilization of the haploid egg cell by a second sperm during double fertilization (Lopes and Larkins 1993). In *Arabidopsis*, the zygotic endosperm is triploid and rapidly undergoes synchronized acytokinetic mitosis. The resulting syncytium is partitioned into three domains along the anterior–posterior or micropylar–chalazal axis of the seed (for review, see Berger 2003). Each of the three domains, the micropylar, peripheral and chalazal endosperm (abbreviated as MCE, PEN and CZE, respectively, after the nomenclature by Boisnard-Lorig et al. 2001; see Fig. 1a), is multinucleate during early seed development

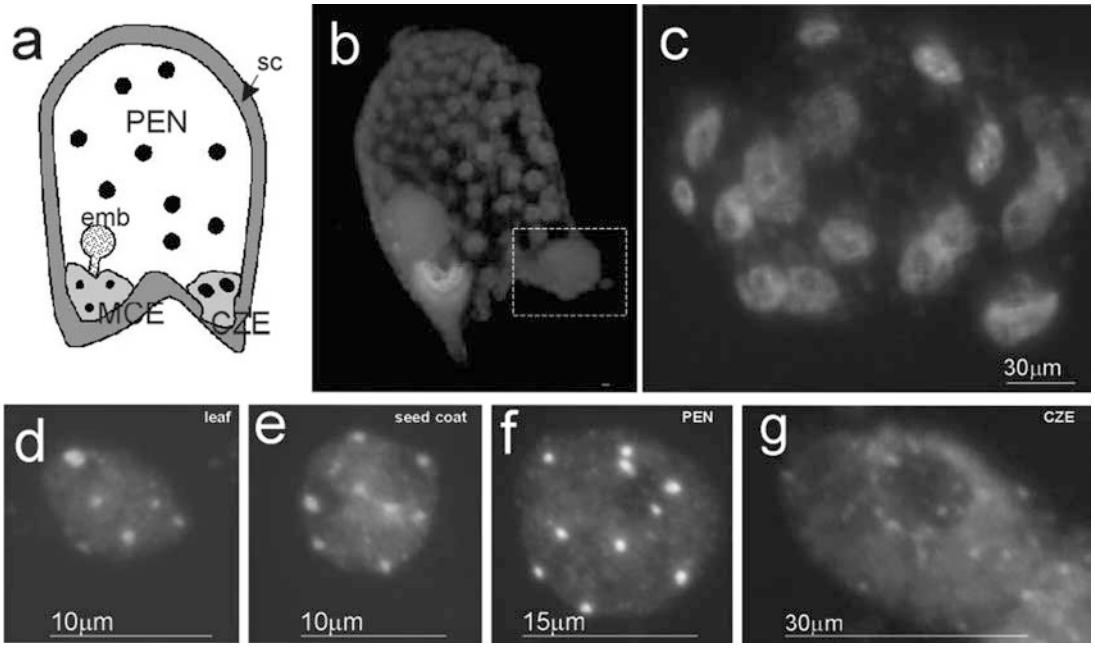

Fig. 1a–g Isolation of PEN and CZE nuclei. **a** Representation of the three endosperm domains in *Arabidopsis*: micropylar endosperm (*MCE*) surrounding the embryo (*emb*), peripheral endosperm (*PEN*), and chalazal endosperm (*CZE*). The maternal seed coat (*sc*) encapsulates the endosperm and embryo. **b** Seed at the globular embryo stage after digestion and dissection of the seed coat, as visualized by reflective light. The *frame* indicates the CZE. **c** The CZE remains as a multinucleate cyst after shearing the endosperm and spreading the nuclei on a slide; counterstained with DAPI. **d–g** Ethanol–acetic acid-fixed nuclei after spreading on a slide and counterstaining with DAPI (gray). Nuclei are from leaf (**d**), seed coat (**e**), PEN (**f**) and CZE (**g**)

and later celullarizes. *Arabidopsis* endosperm undergoes endoreplication to a lesser extent than that of other seed plants (Lopes and Larkins 1993). While in some maize cultivars endosperm nuclei can reach a DNA content of 690C (Lopes and Larkins 1993), the ploidy of *Arabidopsis* endosperm nuclei has not been observed higher than 6C (Matzk et al. 2000; Raz et al. 2001; Kohler et al. 2003). However, these measurements were done by flow cytometry on whole-seed extracts. Cytological observations illustrated the large size of the chalazal nuclei and raised the possibility of their probable polyploid nature (Mansfield and Briarty 1990; Brown et al. 1999; Boisnard-Lorig et al. 2001).

In this work we carried out a cytogenetic examination of individual nuclei from the PEN and the CZE, and using fluorescent in situ hybridization (FISH) analysis we found high ploidy levels in the CZE nuclei. Intermediate levels of ploidy (9n and 15n), which cannot be accounted for by endoreplication, suggest that these giant nuclei arose by nuclear fusion. In addition, we provide cytological evidence supporting nuclear fusion and propose that nuclei from the PEN participate in this nuclear fusion process leading to polyploidization.

Materials and methods

Plant material

The wild-type strain used was *Arabidopsis thaliana* (L.) Heynh. ecotype Landsberg (*erecta* mutant: L *er*). Plants were grown on Type ED73 soil with slow nutrient release (Universalerde, Germany) and lights of type L38 W/72-965 and type L36 W/77 fluora (Osram) in a 1:3 ratio. The indoor growth facility was kept at 70% relative humidity under a day–night cycle of 16 h light at 21°C and 8 h darkness at 18°C.

Isolation of endosperm nuclei

Because the chalazal endosperm is firmly anchored in maternal tissue, simple squashing of the seeds released only the free nuclei from the peripheral endosperm. Therefore, the seeds were fixed and the seed coat was digested before dissection of the endosperm. Siliques containing seeds at a globular to late globular embryo stage were fixed in ice-cold ethanol–acetic acid (3:1, v/v) and kept at −20°C until use. The siliques were opened by two longitudinal cuts, rinsed in water and citrate buffer (10 mM, pH 4.5) and digested 2 h with an enzyme mix [0.3% cellulase (Sigma: C-1794) and 0.3% pectolyase (Sigma: P-5936) in citrate buffer] at 37°C in a moist chamber. The seeds were dissected from the silique in a drop of water. Upon gentle squashing the intact endosperm together with the embryo was released and transferred into a fresh drop of water on a new slide. Six to ten endosperms were collected from each silique for one slide preparation. The endosperm tissue was further sheared into pieces with fine tungsten needles, which allowed the PEN nuclei to be separated from the CZE. Nuclei were then spread on the slide by stirring the suspension with 50% acetic acid at 45°C and fixed with ice-cold ethanol–acetic acid. The slides were air-dried and stored at 4°C until use. The procedure used to prepare and fix the nuclei on slides retains their chromatin organization (Fransz et al. 1996; Hans de Jong et al. 1999).

Fluorescent in situ hybridization (FISH) and immunodetection

Slide preparation was done according to Fransz et al. (1996) with minor modifications. Before the RNAse treatment, spread nuclei were treated for 10 min with 1% enzyme mix (see above) followed by 10 min with 25 μg ml^{-1} pepsin in 0.01 M HCl, both at 37°C. This helped to remove cell wall and cytoplasmic residues, which otherwise impaired immunodetection. FISH probes against the nucleolar organizing region (NOR) from chromosomes 2 and 4, and the centromeres were prepared from the plasmids pTA71 and pAL1 containing the cloned 45S rDNA fragment and the 180-bp centromeric repeat, respectively (Fransz et al. 2002). The respective plasmids were labelled by nick translation with digoxigenin- and biotin-dUTP (Boehringer-Manheim: 1-745-816 and 1-745-824). For bacterial artificial chromosome (BAC)–FISH, two contiguous single-copy BAC clones were arbitrarily chosen and differentially labelled. The 'digoxigenin–biotin'-labelled BAC pairs were: F22D16-T14P4 (Fig. 2a,c), T4B21-T1J1 (Fig. 2b) and F22D16-F10O3 (Figs. 1k, 2e–f). Hybridization and immunodetection of the labelled probes was done as described (Fransz et al. 1996) with minor modifications: post-hybridization washes were done at 45°C instead of 42°C, and washes at 55°C were omitted. Indirect immunodetection was carried out as described by Lysak et al. (2001). Immunolabelling of methylated DNA was carried out after FISH hybridization, together with the immunodetection of DNA probes, using a primary mouse antibody specific for 5-methyl-cytosine (Eurogenetec: MMS-900S-B) and the same secondary and tertiary antibodies as for digoxigenin-dUTP detection. As a result, digoxigenin-labelled probes and methylated DNA were detected as green fluorescent signals [fluorescein isothiocyanate (FITC)/Alexa 488] and biotin-labelled probes as red (Texas Red). The DNA was counterstained with 1 μg ml^{-1} 4,6-diamino-2-phenylindole (DAPI) in Vectashield antifade (Vector Laboratories).

Feulgen staining of *Arabidopsis* seeds

A modification of the protocol described by Golubovskaya and Avalkina (1994) was used for Feulgen staining. The detailed protocol was provided by Dr. P. Barrell

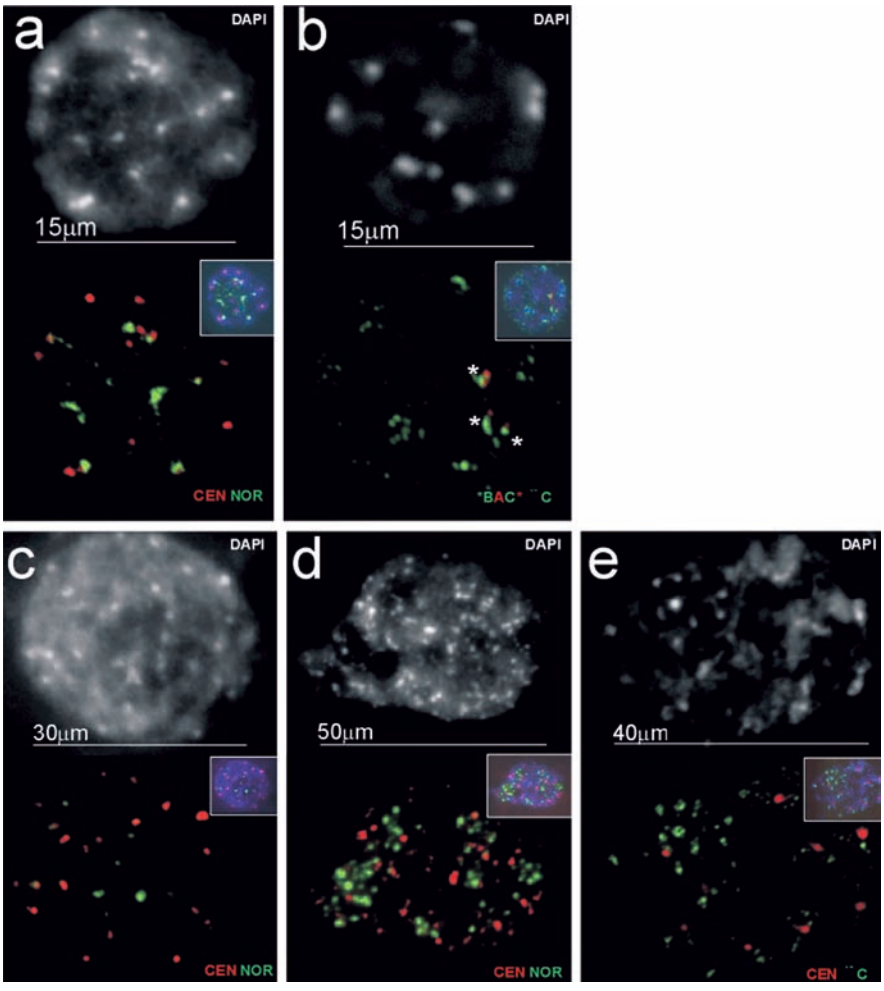

Fig. 2a–e Structures of PEN and CZE nuclei. PEN (**a, b**) and CZE (**c, d**) nuclei were stained with DAPI (*upper panel*) and analyzed by FISH or immunodetection (*lower panel*); overlays are shown in the *insets*. **a,c,d** FISH detection of nucleolar organizing regions (*NOR*, *green*) and centromeres (*CEN*, *red*). **b** BAC–FISH (*BAC red-green*, *stars*) and immunodetection of methylated DNA (^{5m}C, *green*). **e** FISH detection of the centromeres (*CEN*, *red*) and immunodetection of methylated DNA (^{5m}C, *green*)

(University of Zürich, Switzerland, personal communication). Briefly, entire siliques of *A. thaliana* were fixed in the presence of formalin, ethanol and acetic acid, hydrolyzed with 1 N HCl, stained overnight with Schiff's reagent, and dehydrated through an ethanol series before dissection of the seeds and mounting in immersion oil.

Image acquisition and processing

Following FISH, endosperm nuclei were observed under epi-fluorescence using a Zeiss Axioplan microscope. Images were captured with a Magnafire 599802 CCD camera (Optronics) mounted on a 4× TV objective (Zeiss). Each fluorescence signal (DAPI, FITC, Texas Red) was recorded in three independent black and white pictures with the corresponding filters, overlaid (Adobe Photoshop 5.5) and compared with a fourth picture taken with a triple-band-pass detection filter (Zeiss: TBP #488025-0000). When required, the images were corrected with a Gaussian filter (radius 2–4 pixels). Whole seeds stained with Feulgen's reagent were analyzed with a TCS SP2 confocal laser-scanning microscope (Leica). The emitted fluorescence was captured between 640 and 740 nm following excitation at 488 nm, and single-focusing-plane images were recorded.

Results

Structure of PEN and CZE nuclei

To characterize the nuclear organization of PEN and CZE nuclei we carried out cytogenetic analysis of nuclei isolated from dissected endosperms (Fig. 1a,b). While PEN nuclei were easily released, the syncytial cyst of the chalazal endosperm domain resisted shearing. This allowed the identification of CZE nuclei in the preparations (Fig. 1c). Interphase nuclei of the PEN were similar to somatic nuclei but slightly bigger (Fig. 1d–f), typically round, 20–25 μm wide, and displayed regularly shaped heterochromatic domains or chromocenters, which contain centromeric repeats (Fig. 2a) and hypermethylated DNA (Fig. 2b). The size of the nucleolus and the decondensed rDNA signals suggest high activity of the nucleolus (Fig. 2a).

CZE nuclei exhibited distinct features compared to PEN nuclei. Their shape was more variable (Figs. 1g, 2c–e), mostly oval but frequently of irregular contour,

and the diameter (or length of the longer axis) ranged between 30 and 50 μm (Figs. 1g, 2c–e, 3c–f, 4a). DAPI staining revealed many relatively small chromocenters with an irregular shape, different from PEN or somatic nuclei. More often the chromatin appeared in patches of DAPI staining and the chromocenters were smaller, irregularly dispersed (Fig. 2c–e) , and of variable DAPI staining and methylated-DNA signal intensities (Fig. 2e). The NOR signals were also observed in different patterns: condensed (Fig. 2c) and decondensed (Fig. 2d).

In summary, this cytogenetic analysis showed that the nuclei of the PEN and CZE have clearly distinct nuclear phenotypes in terms of shape, size, chromocenter organization, and the distribution pattern of methylated DNA. The number of chromocenters in interphase nuclei provides a good indication of the ploidy level in somatic nuclei (Ceccarelli et al. 1998). Some CZE nuclei clearly showed more than 30 chromocenters, the maximum number expected for endoreplicated endosperm nuclei with a ploidy level of 6n. However, because chromocenters in CZE nuclei lack sharpness (Fig. 2d,g) and are difficult to count accurately, we found it more reliable to estimate ploidy level using FISH with single-copy BAC probes or centromeric probes.

CZE nuclei are highly polyploid

Since the CZE nuclei are two to three times larger than PEN nuclei, we investigated whether this is reflected by a higher DNA content, as previously suggested by Boisnard-Lorig and co-workers (2001). To address the question we applied FISH with single-copy BAC probes or centromeric probes to CZE nuclei. We estimated the ploidy level from the number of hybridization signals based on one 'red–green' BAC signal and five centromeric signals per haploid genome. Since it is possible that the FISH targets co-localize, our observations may represent underestimations of the ploidy level. Indeed, we observed PEN nuclei showing one or two BAC signals, whereas the presence of 15 chromocenters/centromeres indicated a 3n ploidy (data not shown). The frequency of these associations in endosperm nuclei is not presented here, as our goal was to estimate the maximal ploidy level and not the occurrence of somatic pairing. Although our study remains qualitative, it can provide a comparison of the ploidy levels encountered in PEN and CZE nuclei. We describe nuclei showing the maximum BAC or centromeric signals expected for a ploidy level of three, or multiple of three.

PEN nuclei never displayed more than six BAC signals (Fig. 3a,b), which could correspond to a ploidy level of at least 6n, assuming that no other triploid set of homologues is associated. By contrast, the majority of CZE nuclei showed a greater number of signals. A representative panel is shown (Fig. 3c–f). Beyond six (Fig. 3c), the signal distribution is complex as shown in Fig. 3d–f. This was observed in independent preparations of nuclei and chromosomal locations of the BAC probes, thus excluding locus-specific effects. Such a decondensation was never observed in PEN or somatic nuclei. BAC signals are generally contiguous 'red–green' signals and, hence, are easily distinguished from speckled noise signal (Fig. 3d). In an attempt to count the 'red–green' junctions (indicated by stars in Fig. 3c–f) we found 9, 15 and 24 signals in the nuclei (Fig. 3d, e and f, respectively). Although this is only one interpretation of the complex signal pattern, the range clearly indicates that CZE nuclei reach a ploidy higher than 6n and 12n. This is further illustrated by the centromeric and NOR signals. A few cases are illustrated Fig. 2, with a PEN nucleus showing 14 centromeric and 6 major NOR signals (Fig. 2a), a CZE nucleus showing 22 centromeric and 8 major NOR signals (Fig. 2c) and a gigantic CZE nucleus showing about 65 centromeric signals of variable intensity and 30 major NOR signals (Fig. 2d). The latter case again reflects a ploidy higher than 12n.

The FISH analysis we performed clearly demonstrated that, in contrast to PEN nuclei, CZE nuclei can reach a ploidy greater than 6n and 12n and maybe 15n or 24n for the gigantic specimen we analyzed. The intermediate ploidies of 9n and 15n that we observed cannot be accounted for by endoreplication, suggesting that they arose by nuclear fusion.

Gigantic CZE nuclei are likely products of nuclear fusion

To investigate the hypothesis that the giant CZE nuclei arose by nuclear fusion, we performed additional cytological investigations. In all preparations, we observed that the gigantic CZE nuclei had an irregular contour and a discontinuity in the DAPI staining (Figs. 2e, 4a), which is reminiscent of several adjoined nuclei. In addition, we frequently observed contacts between nuclei (Fig. 4b, characterized with 3 and 6 BAC–FISH signals), sometimes with clear fusion between the chromatins (Fig. 4c, arrow). In order to confirm these observations with a method preserving the nuclei within their endosperm domain we used confocal laser-scanning microscopy to analyze intact seeds that were fixed and stained with Feulgen's reagent (Fig. 4d–g). With this dye, the cytoplasm is slightly stained and the nuclear DNA appears as a brightly staining structure. In the peripheral endosperm, the nuclei are surrounded by dense cytoplasm and form nuclear cytoplasmic domains (NCDs; Fig. 4d,e; Brown et al. 1999; Boisnard-Lorig et al. 2001). The CZE appears as a multinucleate cyst anchored at the posterior pole of the seed. In the vicinity of the CZE, at the transition zone with the peripheral endosperm, multinuclear nodules (Fig. 4e–g) can be observed, consisting of dense patches of cytoplasm containing one or more nuclei (Mansfield and Briarty

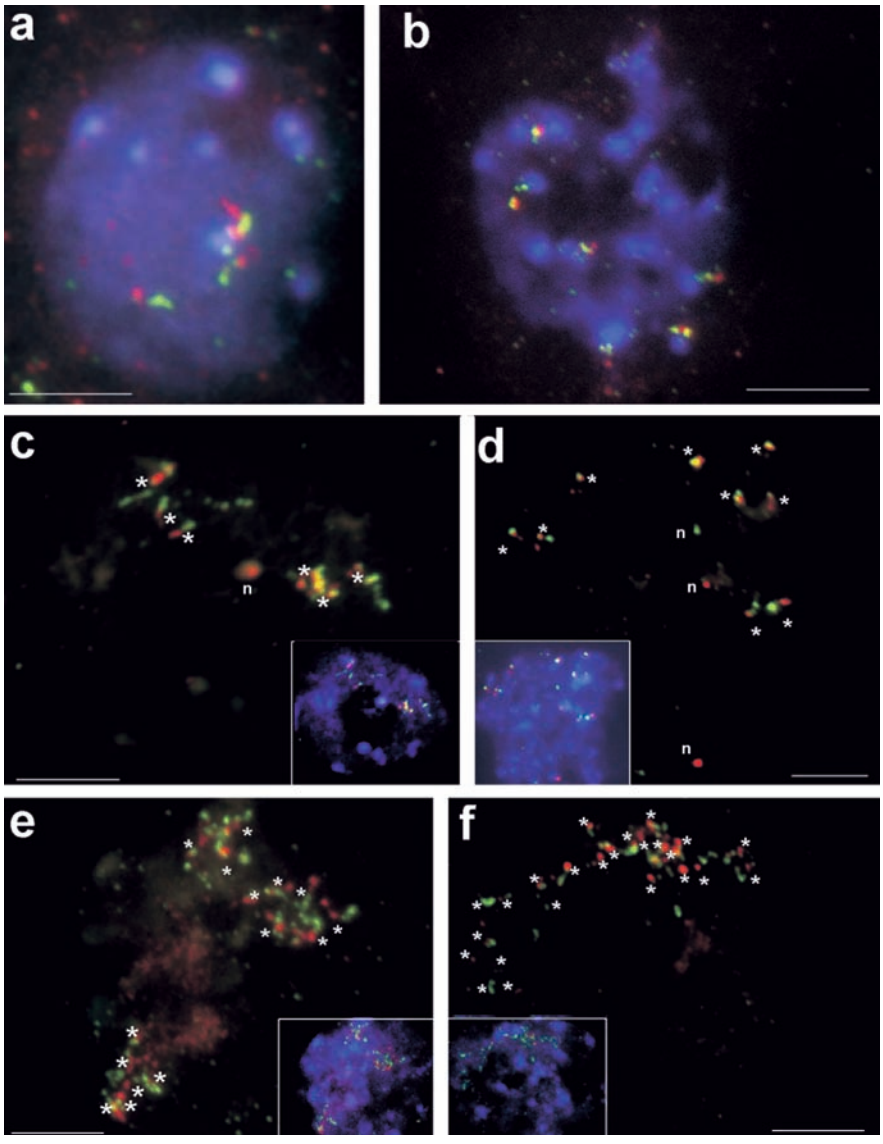

Fig. 3a–f Ploidy levels of PEN and CZE nuclei assessed with BAC–FISH. One pair of 'red–green' signals corresponds to two contiguous, single-copy BAC clones (see Materials and methods). BAC–FISH was applied to PEN nuclei (**a,b**) and CZE nuclei (**c–f**). Overlay with DAPI is shown in **a** and **b**, and in the insets of **c–f**. Each BAC-pair signal is indicated with a *star* (**e–f**) and is distinguished from the speckle noise (*n*). Bars = 10 μm

1990; Brown et al. 1999; Boisnard-Lorig et al. 2001; Fig. 4d–f). The characteristics of Feulgen's-stained endosperm nuclei were consistent with our previous observations. Typically, CZE nuclei were larger than PEN nuclei (Fig. 4d, inset) and showed variable heterochromatin (chromocenter) formation [Fig. 4d (inset), g]. Along with embryo and endosperm development the CZE shows a greater number of nuclei (Fig. 4d–f). Strikingly, at the chalazal pole several multinuclear nodules are in contact with each other and with the chalazal cyst (Fig. 4e,f, insets). Within the CZE we clearly observed contact between nuclei (Fig. 4f).

Taken together, these observations are consistent with the hypothesis that the giant nuclei of the CZE are the product of nuclear fusion. The observation of multinuclear nodules in contact with the CZE may also suggest that they contribute to the increase of nuclei in the CZE.

Discussion

Endoreplication, endomitosis and cell fusion are the three main processes for somatic polyploidization in plants, animals and insects (Edgar and Orr-Weaver 2001), although nuclear fusion is relatively exceptional (Wigglesworth 1967; Fowke et al. 1975; Brodsky and Uryvaeva 1977). In plants, the endosperm is an example of a highly polyploid tissue that undergoes endoreplication, endomitosis or both (Lopes and Larkins 1993). Our study provides evidence that nuclear fusion may also contribute to polyploidization of the chalazal domain of the endosperm. In *Arabidopsis* endosperm, ontogeny is characterized by a syncytial phase before cellularization. Three endosperm domains differentiate along the anterior–posterior or micropylar–chalazal axis of the seed and mitotic activity is independently regulated in each domain (Boisnard-Lorig et al. 2001; Berger

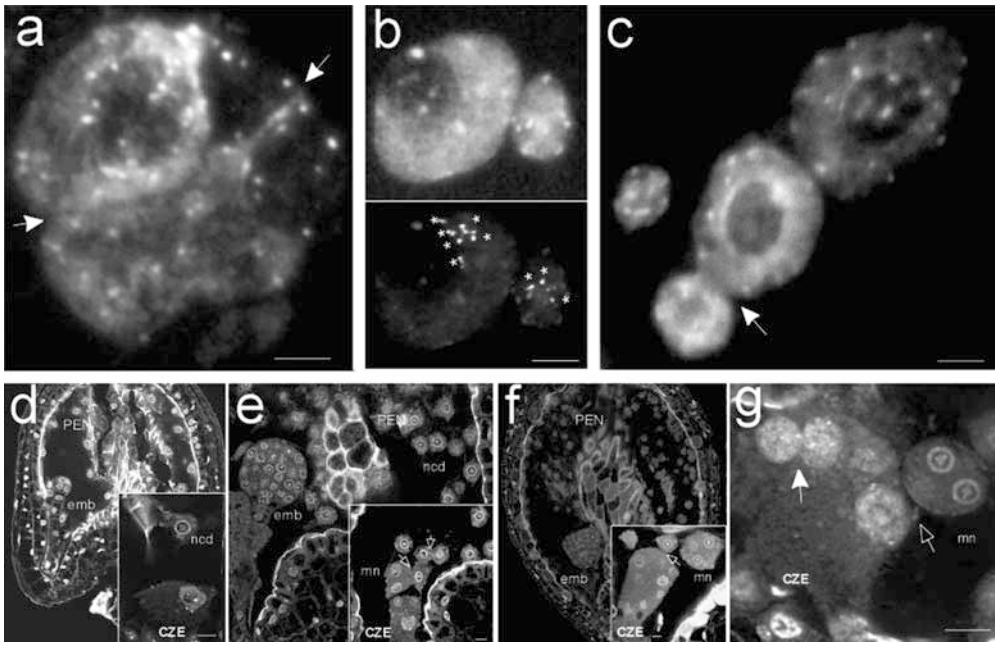

Fig. 4a–g Contact between CZE nuclei in preparations of nuclei and whole mounts of seeds suggests nuclear fusion. **a–c** DAPI-stained isolated nuclei. **a** The *arrows* indicate the trace remnants of adjoined nuclei. **b** (*bottom panel*) The nuclei are overlaid with BAC–FISH signals (*stars*). **c** The *arrow* indicates contact between the chromatin of two nuclei. **d–g** Confocal sections of whole seeds at the octant (**d**), late globular (**e**), and triangular (**f,g**) embryo stages, stained with Feulgen's reagent. *emb* Embryo, *PEN* peripheral endosperm, *CZE* chalazal endosperm, *ncd* nuclear cytoplasmic domain, *mn* multinuclear nodule. The *insets* show a different section of the same seed, across the chalazal endosperm (*CZE*) and the transition zone with the peripheral endosperm. **g** A confocal section of the CZE from another seed. The *open arrows* (**e,f** insets and **g**) indicate contacts of *ncd* and *mn* with each other or with the chalazal cyst; the *closed arrow* (**g**) shows nuclear fusion in the CZE. Bars = 10 μm

2003). In the peripheral endosperm (PEN) and the micropylar endosperm (MCE), nuclei undergo a series of synchronized acytokinetic mitoses, which coincides with mitotic cyclin cycB1;1 expression. In contrast, no mitotic division has been observed in the chalazal endosperm (CZE). In addition, the nuclei are very large and seem to have a higher DNA content, which was suggested to be the result of endoreplication because the expression of the mitotic cyclin cycB1;1 was barely detectable (Boisnard-Lorig et al. 2001). Our study confirms that CZE nuclei exhibit a much larger size than PEN nuclei, and FISH analysis provided an estimation of their ploidy level.

While PEN nuclei did not show more than an apparent 6n ploidy, CZE nuclei showed a ploidy higher than 6n and 12n, and a few cases of 9n,15n and 24n were suggested by our observation. Previous flow-cytometry analyses of *Arabidopsis* seeds only reported major peaks of nuclei with 3C and 6C contents (Matzk et al. 2000; Raz et al. 2001; Kohler et al. 2003) most probably corresponding to the ploidy of PEN nuclei. It is possible that few or no intact CZE nuclei are present in the seed extract in those avorementioned studies because of the difficulty of shearing the multinucleate chalazal cyst, and the latter might be retained on the 35- to 90-μm filters usually employed when preparing extracts for the ploidy analyzer.

The origin of the high ploidy of the CZE nuclei could be explained by endoreplication, for which there is indirect evidence (Boisnard-Lorig et al. 2001). In addition, endoreplication is common in many plant species (Barow and Meister 2003) and particularly in the endosperm (Lopes and Larkins 1993; Larkins et al. 2001). However, endoreplication is expected to yield nuclei with a ploidy level of 6n, 12n, 24n, or higher. Our results suggest that intermediate ploidy levels of 9n and 15n are produced, which cannot be accounted for by endoreplication and are likely due to nuclear fusion. It is, however, noteworthy that we analyzed seeds at a later stage than Boisnard-Lorig et al. (2001). Therefore, endoreplication and nuclear fusion may both be responsible for the polyploidization of CZE nuclei but these processes might occur at different developmental stages of endosperm development.

In addition and importantly, we observed numerous contacts of the chalazal cyst with multinuclear nodules present at the transition zone between the peripheral endosperm and the chalazal endosperm. Although a dynamic study remains to be done, these observation suggest that the multinuclear nodules fuse into the CZE, and this could provide an explanation for the increasing number of CZE nuclei observed during seed development despite the fact that mitotic division has not been reported (Boisnard-Lorig et al. 2001).

The significance of nuclear fusion in the CZE is intriguing. It is not clear whether polyploidization of the CZE nuclei is a passive mechanism resulting from the prolonged syncytial state of the CZE or whether this

process is actively regulated and plays an important role for the biological function of the CZE. The *spätzle* mutant, in which cellularization during endosperm development is affected, may provide some hints (Sorensen et al. 2002). Mutant seeds exhibit numerous fusions of the NCDs in the PEN, concomitant with the formation of enlarged nuclei. The latter might be formed by nuclear fusion as a result of a loss of boundaries between the NCDs. Cytoplasmic boundaries and cellularization are orchestrated by the cytoskeleton and are absent in the syncytial CZE (Otegui and Staehelin 2000; Brown and Lemmon 2001). Therefore, nuclear fusion in the CZE might result from the close vicinity of the increasing number of nuclei in the cyst.

An alternative, although not mutually exclusive explanation for nuclear fusion, is that the nuclei fuse because of the proposed metabolic benefit for the CZE. Polyploidization is believed to be beneficial for metabolically highly active cells, not only by saving the energetic cost of cytokinesis but also by providing extra copies of DNA templates allowing a proportional increase in transcription and translation that permits a high metabolic output (Brodsky and Uryvaeva 1977; Larkins et al. 2001). Mutant analyses in maize provide thorough evidence that the posterior endosperm, the basal endosperm transfer layer (BETL), participates in the storage and transfer of nutrients to the endosperm. Likewise, the CZE in *Arabidopsis* seeds presents several features supporting a role of this tissue in nutrient transfer to the seed. Typically, the CZE has a dense cytoplasm and is rich in mitochondria, ER cysternae, vesicles, and vacuoles (Mansfield and Briarty 1990; Nguyen et al. 2000). These ultrastructural features are associated with the capacity to transiently sequester minerals until their translocation to the embryo (Otegui et al. 2002). In this context, polyploidization of the nuclei in the CZE can be seen as a process for the benefit of a high metabolic activity required during the development of the young seed.

Interestingly, however, the chalazal cyst is already multinucleate and therefore nuclear fusion does not change the overall ploidy level of the syncytium. One possibility is that nuclear polyploidization might facilitate coordination of transcribing the multiple copies of the genes for which a high expression is required, thereby providing a better molecular control of the required metabolic performance. Another possibility, as mentioned above, is that nuclear fusion arises passively as increasing numbers of nuclei come into close contact. Certainly, it would be interesting to investigate the CZE or the corresponding endosperm region of other species to evaluate whether nuclear fusion has been conserved during evolution.

Note added in proof Guitton et al. (2004) report that, during seed development, nuclei of the peripheral endosperm undergo oriented migration towards the posterior endosperm and NCDs eventually fuse to the chalazal cyst.

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