Observations on the Cytology and Fine Structure of Mitosis in the Fern, *Ophioglossum petiolatum* Hook.¹

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Received March 20, 1967

Introduction

The adder's tongue, *Ophioglossum petiolatum* Hook., is an Eusporangiate fern, thought to be among the most ancient of extant ferns (Manton 1950). The Ophioglossaceae are of special cytological interest because of their unusually high chromosome number. The chromosome number of *O. petiolatum* may be as high as $n=550$ (Kurita and Nishida 1965), making it among those with the highest chromosome numbers so far found in the plant kingdom. Members of the genus are evidently highly polyploid plants, but lower members of the series are not known. Some evidence indicates a base number of 8 (Manton 1950), but a base number of 15 has also been suggested (Ninan 1956, 1958).

The nuclei are very large and may approach a volume of $20,000\mu^3$. The chromosomes are small, approximately $5\mu \times 0.5\mu$, and are very crowded together during mitosis. Therefore, these plants are not well suited for classical cytological studies. However, these same characteristics offer some advantages for ultrastructural studies of chromosomes, for the chances of getting either entire chromosomes or large segments of chromosomes within a single plane of section are increased. The material should be especially useful for studies of gross chromosome fine structure as well as of the centromere region.

Materials and methods

Apices of shoots and roots of *O. petiolatum* were fixed in Crai fluid and embedded in paraffin. For light microscopy sections were stained in methyl violet-orange G; safranin-fast green, and Johansen's quadruple stain (Johansen 1940). Squash preparations were stained in Feulgen reagent.

For electron microscopy material was fixed in 3% glutaraldehyde in 0.05M cacodylate buffer (pH 6.8) for 24 hr, washed in buffer and post-fixed in 2% cacodylate buffered OsO_4 for 24 hr. After slow dehydration in a cold methanol series the material was embedded in epoxy resin. Sections were doubly stained in uranyl acetate and Reynold's (1963) lead and examined in an RCA-3F electron microscope. Methods for electron microscopy were suggested by Dr. M.C. Ledbetter of this laboratory.

Results

Cytological studies of *Ophioglossum petiolatum* have largely been concerned with the establishment of the chromosome number (Manton and Sledge 1954, Verma 1956,

¹ Research carried out at Brookhaven National Laboratory under the auspices of the U.S. Atomic Energy Commission.

Ninan 1958, Kurita 1965). However, this species was found to have certain features which make the mitotic process especially interesting. It is one of the relatively few organisms in which the nucleolus persists throughout the entire mitotic cycle (Figs. 1-8). We have not encountered previous reports of persisting nucleoli in this species or in other members of the genus. This could be because the nucleoli are not apparent in Feulgen squash preparations of mitotic cells (Fig. 9).

Several nucleoli occur in each nucleus and these often persist through the various stages of mitosis (Figs. 1-8). Figures 1-2 are cells in prophase. In Fig. 2 the spindle formation is advanced but a nucleolus is outside the spindle. Presumably, this represents a case in which the nucleolus has persisted from a preceding cell cycle. In Figs. 3-4 (arrows), the nucleoli are still attached to the chromosomes, where they normally remain at least until the chromosomes are aligned on the metaphase plate. At the beginning of anaphase, the nucleoli break away from the chromosomes (Fig. 5, arrow). After losing contact with the chromosomes they are extruded from the spindle and are displaced poleward in advance of the chromosomes and usually become located near the polar cell walls (Figs. 6-7). Since the nucleoli lie at some distance from the chromosomes at telophase (Fig. 7), there is little chance of their being reincorporated into the daughter nuclei. Following nuclear restitution some nucleoli are visible in the cytoplasm of the interphase cell (Fig. 8). Cells similar to Fig. 2 indicate that nucleoli may persist for a long time in the cytoplasm, but they apparently ultimately disintegrate since there are usually only a few, if any, persisting nucleoli present in the cytoplasm of early prophase cells at any one time.

Because of the high chromosome number it was anticipated that a relatively high frequency of mitotic anomalies might occur. However, only one case of an abnormal mitosis was found. A tripolar spindle occurred in a leaf primordium (Fig. 10). The spindle appears to have a limiting membrane. This appearance is due to an image reinforcement at the edges of the spindle produced as a result of an apparent slight twisting of the spindle.

Interphase and persisting nucleoli were also observed with the electron microscope (Figs. 11-14, arrows). As has been described for other plants, (e.g., Lafontaine and Chouinard 1963, Chouinard 1966), the interphase nucleoli consist of two components, a highly compact, densely staining granular component and a less dense, more fibrous region, but typically the components are loosely organized (Fig. 11). They are, in fact, highly atypical nucleoli compared to nucleoli of several plant species we have examined (Underbrink and Sparrow, unpublished).

Figs. 1-8. Root tip cells. 1, prophase cell. 2, late prophase, showing formation of the spindle. One nucleolus (arrow) presumably has persisted through a previous interphase. 3-4, cell in metaphase shown in two planes of focus. Arrows indicate nucleoli which are still attached to the chromosomes. 5, metaphase. One nucleolus is attached to a chromosome while one has become separated (arrow). 6, late anaphase. Nucleoli have been extruded from the spindle and are located in the peripheral cytoplasm at the polar ends of the cell. 7, persisting nucleoli at telophase. 8, nucleoli persisting in an interphase cell. $\times 975$.

The nucleolar components of most plant species are tightly organized into nearly spherical electron dense masses, though areas of less density may be present.

When nucleoli in dividing cells (Figs. 13–14, arrows) are compared with typical interphase nucleoli (Figs. 11–12), it is apparent that a structural change has occurred. Figure 13 is a highly magnified view of one of the nucleoli in Fig. 14 (lower arrow). During mitosis the granular components characteristic of interphase nucleoli seem to be lost and the nucleoli appear to consist of a fibrous component similar to that of the chromosomes (Fig. 13) but less tightly packed. This renders the persisting nucleoli less electron dense than the chromosomes although nucleoli at interphase are more dense than the chromatin. Neither structural changes nor changes in dye-binding affinities were evident with the light microscope.

The mitotic spindle is relatively broad and the spindle fibers are often strikingly abundant (Figs. 6–7). Thus, *O. petiolatum* also seems well suited for ultrastructural studies of spindle fibers. The spindle fibers were readily observed in thin section (Figs. 15–16) as were long sections through individual chromosomes. The arms of the chromosomes were oriented so that in most cases the centromere region was readily identified. In Figs. 15–16, groups of spindle fibers can be seen attached to the chromosomes at the centromeres (arrows). We have found no evidence to indicate that the centromere is a differentiated structure. Except for the constriction, the centromere appears similar to the rest of the chromosome. Spindle fibers, composed of groups of parallel microtubules, were often observed extending from the centromeres for long distances. The parallel orientation of the microtubules was lost near the periphery of the spindle area. They were not observed to converge on a highly localized pole. Rather, the microtubules appeared to segment and become generally tangled and disoriented over a rather broad peripheral area assumed to represent the polar region (Fig. 16). Spindle fibers in the polar regions were not usually associated with organelles. In some instances, the fibers appeared to terminate near vacuolated regions in the peripheral cytoplasm. However, the cytoplasm at the periphery of the spindles did, in general, consist of many vacuoles (Fig. 16) and no significance can be inferred.

The structure of the chromosomes (Figs. 13, 15–16) was of a fibrous consistency similar to chromosomes of other species (Barnicot and Huxley 1965, Sparvoli *et al.*

Figs. 9–13. 9, root. Feulgen squash preparation of metaphase chromosomes. Persisting nucleoli are not evident. $\times 750$. 10, shoot. Tripolar metaphase. Sharp delineation of spindle appears due to an apparent slight twisting of the spindle $\times 2,200$. 11, shoot. Interphase nucleolus. The dense granular and less dense fibrous components are loosely organized. This loose organization is characteristic of *Ophioglossum* nucleoli. $\times 16,000$. 12, shoot. Interphase nucleolus. Components appear more tightly packed than those in Fig. 11 but not as tightly organized as nucleoli in many plant species. $\times 16,000$. 13, shoot. High magnification view of a persisting nucleolus (N) from Fig. 14 (lower arrow) lying near the cell wall (CW). The nucleolus has lost the densely staining granular components present in interphase nucleoli. It is composed of a fibrous material similar to the chromosomes (C) but less tightly packed. $\times 32,000$.

Fig. 16. shoot. Metaphase chromosomes. Arrows indicate attachment of spindle fibers at the centromeres. Spindle fibers tend to lose their characteristic parallel orientation at the polar areas (P). Some fibers appear to terminate near vacuoles (V). $\times 18,100$.

1965). The surface structure of the chromosome is highly convoluted (Fig. 15, lower arrow). Small sinuses were often observed scattered along the length of the chromosomes. Similar observations in *Tradescantia* (Sparvoli *et al.* 1965) have been interpreted as evidence for a subchromatid structure and coiling within the chromosomes.

Discussion

Persisting nucleoli, although relatively rare, have been reported in several plant species (Frew and Bowen 1929, Jacob 1942, Tjio 1948, Avanzi 1950, Gori 1951, D'Amato-Avanzi 1952). Nucleoli may also persist during mitosis and meiosis in animal cells (Jones 1962, Brinkley 1965, Hsu *et al.* 1965, Wahrman and Friedländer 1966).

An interesting feature of persisting nucleoli in plants is their mode of transport poleward since they evidently precede the chromosomes to and past the poles even

Figs. 14–15. 14, shoot. Cell at metaphase showing the numerous chromosomes and two persisting nucleoli (arrows). Vacuoles (V) and starch grains (S) are abundant in the peripheral cytoplasm. $\times 4,200$ 15, shoot. Metaphase chromosomes. Spindle fibers are attached to chromosomes (upper arrows) but the centromeres do not show structural differentiation. Undulations and sinuses along the length of the chromosome (lower arrow) suggest coiling and a subchromatid structure. $\times 17,300$.

though ejected from the spindle. While we have observed no structural changes with light microscopy, other workers (Tjio 1948, Avanzi 1950, Gori 1951) have observed an elongation of the nucleoli in a poleward direction shortly before losing contact with the chromosomes. Tjio (1948) and later Avanzi (1950) and Gori (1951) have suggested that this stretching effect was related to forces within the spindle which tend to move the nucleoli towards the poles. Since their proposal, electron microscopy has resolved many questions concerning the nature of the mitotic spindle, but we have not found any indications that the spindle fibers themselves are concerned with nucleolar movements. Obviously, improved knowledge of the movement of these structures would be valuable in understanding mitotic movements.

The persisting components of the nucleoli are Feulgen negative but we can draw no conclusions as to their composition until cytochemical tests are performed.

The persisting nucleoli in *O. petiolatum* appear to undergo changes in structure similar to those found in Chinese hamster tissue culture cells (Brinkley 1965) where the compact granular appearance is lost and the bulk of the nucleolus is characterized as being predominantly fibrous. However, the Chinese hamster nucleoli apparently remain attached to the chromosomes throughout mitosis and at telophase are reorganized into their interphase structure whereas the fibrous component of the nucleoli in *O. petiolatum* does not remain attached to the chromosomes and it apparently ultimately disintegrates. Thus, it seems likely that this fibrous component must be synthesized anew at the completion of mitosis since it does not become reincorporated into the daughter nucleoli.

A study of the nuclei in a homozygous mutant of the toad, *Xenopus laevis*, which does not synthesize ribosomes revealed that the 150–300Å granules normally present in nucleolate nuclei were absent although electron-dense masses composed of a finely granular substance containing fine 40–100Å threads occurred in place of normal nucleoli (Jones 1965). These dense fibrous masses are morphologically not unlike persisting nucleoli. Jones (1965) concluded that the ribosomes and the nucleolar 150–300Å granules share the same pathway, although a direct precursor-product relationship has not been proven. From observations of persisting nucleoli in meiotic cells of the insect, *Dielocroce baudii*, Wahrman and Friedländer (1966) have suggested the possibility that it is the ribosome-producing nucleolar component which disintegrates.

Because of the numerous chromosomes in *O. petiolatum* it was frequently possible to observe long regions of the same chromosome in a single plane of section. Some evidence of chromosome coiling could thus be observed. However, it seems apparent that the most informative data concerning chromosome structure will come from whole mount studies of isolated chromosomes (Wolfe 1965, DuPraw 1966, Govaerts and Dekegel 1966, Rae 1966, Wolfe and Hewitt 1966) rather than from sectioned material. Sectioned material, however, does have the advantage of showing the achromatic figure in relation to the chromosomes. In *O. petiolatum* the

microtubules comprising the spindle fibers are similar to those previously described for plant spindles (Ledbetter and Porter 1963, 1964, Pickett-Heaps and Northcote 1966).

The high chromosome number has greatly facilitated observation of centromere regions. Some appear in nearly every section through mitotic figures. We were unable to observe any differentiation of this structure, although in animal cells the centromere may exhibit staining characteristics which differ from the rest of the chromosome (Harris 1962, Mota 1962, Luykx 1965, Murray *et al.* 1965, Brinkley and Stubblefield 1966). Pickett-Heaps and Northcote (1966) also have observed no particular centromere structure in wheat, but Dietrich (1966) has observed that the centromere in *Lilium candidum* definitely stains lighter than the rest of the chromosome. Thus the centromere structure in plant cells is not clear and requires further investigation.

Summary

Persisting nucleoli were found in the fern, *Ophioglossum petiolatum* Hook. The nucleoli remain attached to the chromosomes until meta-anaphase at which time they are extruded from the spindle and are displaced poleward to become situated in the peripheral cytoplasm. They may persist through interphase and into the next mitosis. The nucleoli undergo structural changes during mitosis. They apparently lose the dense granular components present in interphase nucleoli and appear as loosely organized fibrous masses. A tripolar spindle was observed in which the spindle appeared to be slightly twisted. The high chromosome number ($2n \approx 1020$) facilitated the study of the centromere region, although no fine structural differentiation of this structure was found. Spindle fibers do not converge on highly localized poles but tend to become disorganized in the polar regions. The presence of undulations and sinuses along the length of the chromosomes suggests coiling of the chromosomes and a subchromatid structure.

Acknowledgements

The authors thank Mr. W.J. Geisbusch and Mr. R.N. Ruffing for technical assistance, Mr. R.F. Smith for the light micrographs and Dr. M.C. Ledbetter for suggestions concerning the manuscript. They also express their indebtedness to Dr. W.H. Wagner for providing a plant of *Ophioglossum petiolatum* from which a clone was established by vegetative propagation.

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