Atypical nucleoli in *Luzula campestris*

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The nucleolus of *Luzula campestris* is typically a round structure which stains well with aceto-carmine. During examination of unfixed ovary tissue, at a time after all nuclear divisions had ceased, some nuclei were observed which had bipartite or U-shaped structures quite unlike nucleoli in form. After staining with aceto-carmine, many nuclei showed no nucleoli but did possess large chromomeres of much the same shape as the structures seen in the unfixed nuclei. The bipartite and U-shaped structures were thus believed to be especially large, swollen or hydrated chromomeres differentially visible in unfixed tissue (Brown, 1951).

More detailed examinations, however, have revealed that the bipartite and U-shaped structures are nucleoli of atypical form. It is the purpose of this report to describe the observed modifications of nucleolar form, and to offer briefly suggestions as to their possible origin.

Materials and methods

Material for the various methods of preparation was obtained at or about anthesis from plants grown in the lath house at Berkeley. For observations of unfixed cells, the ovary wall was cut into several pieces and mounted in either distilled or tap water and examined immediately.

Tissues studied during acetic acid treatment were mounted first in water. After a suitable nucleus was found, a drop of 45% acetic acid was placed at the edge of the coverslip and allowed to diffuse to the tissue. In this way, a single nucleus could be observed while the concentration of acid gradually increased.

Feulgen and aceto-carmine smears, and crystal violet-safranin sections were made in the usual way.

A recent modification of the aceto-carmine smear method, resulting in weakly stained chromosomes and intensely stained nucleoli, was kindly made available by its originator, Dr. John A. Rattenbury (1952). Following fixation in Carnoy, the material was placed in a 10% solution of formalin for 10 to 14 hours, rinsed in cold 1N HCl, and hydrolyzed in 1N HCl for 5 hours at 60°C. Following a fifteen minute wash in tap water, aceto-carmine smears were made in the usual fashion.

Observations

Unfixed cells. In water mounts, nuclei of the ovary wall appear

to have homogeneous or faintly granular contents. The nucleoli typically appear as spheroidal bodies with no visible internal structure. Occasional nucleoli, however, do possess vacuoles which are strikingly apparent (fig. 1 G & H).

Fig. 1. Photos of atypical and vacuolate nucleoli from unfixed tissue, 6300 $\times$. D & E. - Two focal levels of the same nucleus. I. - Arrow, the smaller nucleolus, slightly out of focus.

Certain nuclei, however, appear to lack typical nucleoli and to have instead structures which are either bipartite (fig. 1 C & F) or U-shaped (fig. 1 B & D). Such U-shaped structures may be much thickened (fig. 1 I)

or their ends may appear to be joined by a delicate strand, thus forming a closed ring (fig. 1 A & E). Only one structure is usually present although two may occur. If two are present, they may be of different sizes, as may be seen by comparing the two in fig. 1 I, and the two in fig. 1 D & E.

Acetic acid treatment. When 45% acetic acid is placed at the edge of the cover-slip, the nuclei in the water mounts maintain their original appearance for a few moments. As the concentration of acid increases, the nuclei first seem to become empty upon the rapid disappearance from sight of the typical nucleoli or the bipartite and U-shaped structures.

The nuclei then acquire a granular aspect attributable to the now highly refractive chromomeres. This second phase takes one or two minutes after which the nuclei do not change in appearance. Similar changes in the refractive indices of nuclear constituents have been reported by Shinke (1937), who worked with the reversible effects of hypo- and hypertonic solutions.

Fig. 2 pictures diagrammatically some of the nuclei observed before and after acetic acid treatment. All the various modifications of the bipartite and U-shaped structures (fig. 1 A, C-E) and the vacuolated nucleoli (fig. 1 B) disappear from sight with equal rapidity. In this respect, these various structures are all identical to the typical, homogeneous nucleoli. None of the disappearing structures is replaced by a differentially enlarged chromomere.

Feulgen smears. The Feulgen preparations are chiefly of

Fig. 2. Diagrammatic sketches of the change in nuclear contents on acetic acid treatment. In fig. D & E, only part of the chromatic granules are shown.

value in their demonstration of the chromosomal constituents of the resting nuclei. The chromosomal threads and smaller chromomeres together make a somewhat indistinct background against which the larger chromomeres show sharply. The larger chromomeres can be

seen to be bipartite in structure, the two elements either lying parallel (fig. 3 A) or forming a V-shape (fig. 3 B). Because the two elements in the larger chromomeres are always the same size, they undoubtedly represent a single divided chromomere rather than the fusion product

Fig. 3. Photos, 6300 $\times$. A & B. - Feulgen smears; arrows, bipartite heterochromatic elements. C-E. - Aceto carmine smears. C-E. - Variation in stainability of nucleoli. F. - Arrows point to two small nucleoli each associated with a chromatic element. Note the unusually chromatic thread.

of two non-homologous chromomeres.

It may be noted that the heavy chromomere in fig. 3 A is roughly similar in shape to the bipartite bodies shown in fig. 1 C & F while the heavy chromomere in fig. 3 B is similar in shape, although smaller in size, to the U-shaped structure in fig. 1 D.

Aceto-carminic smears. The larger chromomeres in the aceto-carminic preparations can be seen to be bipartite much less readily than those of the Feulgen smears. The smaller chromomeres and threads are also usually much less distinctly differentiated than in the Feulgen preparations although the heavier threads are sometimes clearly apparent. The nucleoli vary in their appearance from deep staining structures (fig. 3 C) to those which are no longer identifiable. In some nucleoli, small vacuoles can be seen; others have a double vacuole identical to that shown for the unfixed nucleoli in fig. 1 C & H. Some of the paler nucleoli may be surrounded by heavy chromomeres (fig. 3 D) while in others, the chromomeres may appear to be in intimate contact with the nucleolar substance (fig. 3 E). A few of the stainable nucleoli are very small, and appear to be associated with but a single large chromomere (fig. 3 F).

Formol-aceto-carminic smears. After the Rattenbury procedure, the nucleoli in the ovary wall stain much more intensely than after the usual aceto-carminic technique. Fig. 4 A & B show nuclei with one and two typical nucleoli. A comparison with fig. 3 C-E demonstrates the much greater contrast achieved by the Rattenbury procedure. A frequently observed phenomenon is the very heavy staining along or near part of the nuclear membrane. This feature is particularly apparent in fig. 4 B. The heavy staining along the nuclear membrane is very frequent in cells containing typical nucleoli, much less frequent in those with atypical nucleoli.

Although the contrast between the remainder of the nuclear contents and the nucleolus is sharply increased by the Rattenbury procedure, many of the heavier chromomeres still stain distinctly. Fig. 4 C & D show nucleoli intimately associated with heavy chromomeres; they may be compared with fig. 3 E, produced by the ordinary aceto-carminic method. The remainder of the atypical forms, fig. 4 E-L, undoubtedly represent those which stain weakly or not at all with the ordinary aceto-carminic method.

In fig. 4 C, the nucleolus has a typical shape even though intimately associated with heavy chromomeres. In fig. 4 D, the nucleolus has a square outline formed in part by chromatic material projecting beyond the nucleolus. What would be the actual shape of the nucleolar material itself cannot be determined.

In fig. 4 E, the nucleolar material is similar in outline to the structures shown in the unfixed nucleus of fig. 1 I. In fig. 4 F, a small mass

Fig. 4. Photos of formol-aceto-carmin smears, 6300 $\times$. A & B.- Typical nucleoli; note the heavy stain at the nuclear membrane. C & D.- Associations of chromomeres and nucleoli. E-J.- Atypical nucleoli. K & L.- Dark and light prints of the same negative; note the disparity in form of the two sets of chromomeres associated with the nucleolus in L.

of nuclear material is bounded by heavy chromomeres which seem to determine, at least in part, the shape of the structure. Fig. 4 G pictures a grossly asymmetric U-shaped structure somewhat similar to that shown in fig. 1 D.

Fig. 4 H-J show bipartite nucleoli, each example made complex by chromomeric associations. In fig. 4 H, the right hand part of the bipartite nucleolus is associated with two heavy chromomeres (arrows). In fig. 4 I & J, a heavy chromomere appears along one side (arrows). Such bipartite structures are thus similar to those observed in the unfixed nuclei (fig. 1 C & F).

It has not been possible to identify any of the chromomeres associated with the nucleolus as the nucleolar-organizing body which is apparent at pachytene as one of the larger chromomeres. The chromomeres associated with nucleolar material have been numerous and of widely varying shape (fig. 4 K-L).

Crystal violet-safranin sections. Except at the vascular bundles, the ovary wall of *L. campestris* is four cells in thickness. By anthesis, the outer layer of cells has enlarged disproportionately. The description which follows, however, is based upon material collected prior to anthesis at a time when a few mitoses were still occurring.

It was not possible to detect any relationship between the occurrence of atypical nucleoli and ovary wall morphology. The positions of atypical nucleoli were plotted on outline diagrams of ovary wall sections. The frequency of atypical nucleoli seems to bear no relationship to the cell layer nor to the vascular bundles. In addition, typical, round nucleoli much smaller in size than many of the bipartite nucleoli are observable in some late telophase nuclei.

The numbers of binucleolate nuclei and atypical nucleoli were determined in one complete locular segment in four successive sections. Each locular segments possessed an estimated 300 nuclei, or a total of 1200 for the four sections. The number of nuclei in division was less than one per cent. Of the approximately 1200 resting nuclei, 36 had two typical nucleoli, 39 had a single atypical nucleolus, while 40 were binucleolate with at least one of the nucleoli atypical. In some binucleolate nuclei, one or both nucleoli were obscured by chromomeres and could not be classified as to shape; such nucleoli were counted as typical. If the occurrence of the binucleolate condition is not related to the occurrence of atypical nucleoli, then only 0.1% of nuclei would be expected to show both conditions simultaneously. The observed frequency of 3.3% is thus significantly different ($\chi^2=230.0$) from that which might be expected on the basis of chance. It may therefore be concluded that atypical nucleoli are more likely to be present when the nucleus possesses two rather than one nucleolus.

Discussion

The observations described above have bearing on several distinct features of nucleolar organization. Vacuoles have been reported in several instances in living plant material (Dermen, 1933; Linsbauer, 1932; Zirkle, 1931). The vacuoles observable in *Luzula* nucleoli are visible in unfixed tissue and are therefore not artifacts.

For both plants and animals, the nucleolus has been reported to arise first as two tiny droplets, each formed independently on a separate sister strand of a split chromosome (Dearing, 1934; Heitz, 1931). The droplets soon fuse to give one nucleolus on each organizer chromosome. In *Hordeum vulgare* Heitz (1931) found that the nucleolus was first composed of four "Teilnukleolen." In view of McClintock's (1934) demonstration of the compound nature of the maize organizer, it seems likely that the four "Teilnukleolen" could represent the primary activity of two parts of the organizing zone on each of two sister strands.

Raghavan (1938) has reported for plant nucleoli an outer nucleolar layer composed of chromatic materials staining similarly to the chromocenters. At another time, a stage which he believed to be early prophase, the chromocenters became associated with the nucleolar material giving it angular or distorted shapes. In later prophase stages, the nucleoli returned again to the round form. In formol-aceto-carmin smears, the nucleoli of *Luzula* frequently show chromatic hulls; such hulls are not uniform and are possibly composed of small chromomeres. Nucleolar shape may be distorted in *Luzula* by the adhesion of large chromomeres. These distortions occur, however, only in the resting nuclei and at very late prophase just prior to the disintegration of the nucleolus. All other prophase stages show typical, round nucleoli.

Because of the abundance of matrix material, it has not been possible to observe at late telophase the very early stages of nucleolus formation in *Luzula*. Thus the stages in the development of either the typical or atypical nucleoli remain unknown. It is believed, however, that the bipartite forms of the atypical nucleoli may be explained most readily on the assumption that the normal sequence of nucleolar fusion has not taken place. This sequence may be summarized as follows: two primary nucleoli on sister strands fuse to give a nucleolus associated with a single chromosome; the "single-chromosome" nucleoli next fuse to give a single nucleolus per nucleus. The bipartite forms of the atypical nucleoli would thus be produced by a failure of fusions during the increase in amount of nucleolar material at late telophase.

The idea of a relationship between the fusion process and the occurrence of atypical nucleoli receives some support from the unexpectedly high frequency of binucleolate nuclei in which one or both of the nucleoli is atypical.

At pachytene in *Luzula* (Brown, 1951, and unpub.) there may be seen two separate rows of granules leading out from the paired organizers. This observation indicates that organizers may act independently even though closely paired and in association with a single nucleolar mass. Therefore, they might be expected to act independently if side by side on a split chromosome.

Adhesions of large chromomeres to the nucleolar material may also be considered to be a possible factor in delaying or preventing fusions. The smaller sizes of the atypical nucleoli may indicate that the nucleolar material itself is different, perhaps less highly hydrated, and therefore less capable of fusion. Ehrenberg (1946) has discussed the coacervate nature of nucleoli.

Summary

In unfixed tissues of the nucleolar wall of *L. campestris*, the nucleoli are occasionally bipartite or U-shaped. When bipartite, their appearance may be similar to that of heterochromatic elements visible in smear preparations.

When treated with acetic acid, the atypical nucleoli react similarly to normal nucleoli and unlike the chromosomal substance. The atypical nucleoli are usually not stained by the customary aceto-carmine technique but can be stained intensely following Rattenbury's formol modification.

Atypical nucleoli are much more frequent in nuclei with two nucleoli than would be expected if their occurrence were not related to the binucleolate condition. The idea is offered that the atypical nucleoli are formed in part by a failure of the usual fusions of material collected by the organizers.

A further observation of vacuoles in unfixed nucleoli is noted.

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