Heteromorphic Appearance of Acrocentric Nucleolus Organizer Regions in *Nothoscordum fragrans*

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The somatic chromosome complement of *Nothoscordum fragrans*, Liliaceae, is composed of large metacentric (L) chromosomes and small (S) chromosomes. The number and ratio of these two types of chromosomes has been shown to vary among clones with Köperich (1930) reporting that $2n=16$ L, Levan (1935) reporting that $2n=14$ L + 4 S and Levan and Emsweller (1938) reporting that $2n=13$ L + 6 S. The alteration in number and morphology of the chromosome has been explained by the centric fission of a large metacentric chromosome into two S chromosomes. In the clone with $2n=19$, Levan and Emsweller (1938) reported that all of the six S chromosomes had little satellites at their distal ends. On the other hand, Sato (1942) reported that only four of six S chromosomes had satellites and the two remaining had a terminal centromere. Kurita and Kuroki (1963) found that six S chromosomes had heterochromatic segments adjacent to their distal ends, which sometimes attached to the nucleoli. The present authors observed that these heterochromatic segments sometimes diffused or loosened at metaphase and dispersed into the mesh-like structure of fine fibrils within the nucleoli at interphase (Sato et al. 1979). These results suggested that the short arms of S chromosomes included the nucleolus organizer regions.

It is difficult to determine whether or not all short arms contain the nucleolus organizer regions because the individual S chromosomes cannot be distinguished from each other by their morphology and size. However, we could discriminate S chromosomes from each other by their banding patterns as detected by the C-banding technique, which also enabled us to observe the nucleolus organizer regions in detail.

The present paper describes the results of observations of the nucleolus organizer regions, especially at metaphase and prophase, and discusses these results.

Materials and methods

*Nothoscordum fragrans* Kunth (*Allium fragrans* Vent.) with somatic chromosome number $2n=19$ was used in the present study. Root tips were obtained from plants cultivated in the experimental garden of Ehime University. For the examination of banding patterns, the root tips were pretreated in 0.002 M 8-hydroxyquinoline for 30 min at room temperature followed by 18 h at 4°C. Root tips without pretreatment were used for observing the nucleolus organizer.
regions. They were immediately fixed in acetic alcohol (1:3) for about 1 h at 0–4°C. After they were subjected to an alcohol series (70, 30 and 15%), they were briefly immersed in distilled water. The C-banding technique was carried out according to the slightly modified method of Klášterská and Natarajan (1975). The original Giemsa liquid was prepared for staining as a 5% solution diluted with 1/15 M phosphate buffer (pH 7.4). Staining was carried out overnight in a refrigerator. Finally the slides were placed in absolute xylene for 10 min before they were mounted with coverslips.

The nucleoli of root tips and ovary cells were stained with AgNO₃ after formalin-hydroquinone fixation according to the method of Fernández-Gómez et al. (1969).

Observations

The karyotype of _Nothoscordum fragrans_ used in this study was previously reported by Kurita and Kuroki (1963). The somatic chromosome complement of this plant is comprised of thirteen large metacentric and six short acrocentric chromosomes. The former were referred to as Lⁿ and the latter as Sⁿ (Sato et al. 1979). At metaphase, densely stained segments were situated in all short arms and interstitial regions in the long arms of the Sⁿ (Fig. 1). Several minor bands were also observed in the distal regions in one of the arms of Lⁿ. By the banding patterns in the long arms, the six Sⁿ were easily discriminated and grouped into four types: one Sⁿ without bands, one Sⁿ with a single band, three Sⁿ with double bands, and one Sⁿ with triple bands, and referred to as Sⁿ₀, Sⁿ₁, Sⁿ₂, and Sⁿ₃ respectively.

When the specimens were stained with Giemsa for only a short time, it was difficult to reveal the loosened and diffused chromatin that occurred at the distal ends of the short arms. But staining overnight made it possible to observe such chromatin and also to reveal the nucleoli.

The Sⁿ₀ had the largest short arms of the six Sⁿ, approximately 1.3 μ in length (Fig. 2A). This type of chromosome had two other typical features at metaphase. The distal end of the short arm was partially subjected to fluff-like diffusion, which caused it to be faintly stained, and to puff tandemly (Fig. 2B). Sometimes, amorphously loosened chromatin, which was usually more faintly stained and more dispersedly than the fluff-like diffused chromatin, was seen at the distal end (Fig. 2C). The occurrence of the fluff-like diffused chromatin and the amorphously loosened chromatin slightly reduced the size of the short arms.

The short arms of the Sⁿ₁ had the most stable appearance at metaphase. The majority of the chromosomes of this type examined had strongly condensed short arms about 1 μ in length (Fig. 3A). The fluff-like diffused chromatin was seen in only a few Sⁿ₁ (Fig. 3B) and the amorphously loosened chromatin was never observed in this type of chromosome.

Three Sⁿ are included in the type Sⁿ₂. It was difficult to distinguish these chromosomes from each other since both sites at which the bands appeared were the same for all three Sⁿ₂ and the band widths were equal. At metaphase,
the short arms of the $S^a$,2 had the most variable and unstable appearance of the four types of chromosome. The length of the short arms when they were completely condensed so that they stained densely was about 0.7 μ (Fig. 4A). The fluff-like diffused chromatin was frequently observed forming tandem segments (Figs. 4B, C, D). Sometimes, structures of fine fibrils stretching outwards and probably including kinetochores were seen adjacent to the short arms (Fig. 4D). The amorphously loosened chromatin, as seen in the $S^a$,0, was also observed in this type of chromosome (Figs. 4E, F, G). The most distinctive characteristic of this type of chromosome was that the short arms were subjected to loosening
Figs. 3-4. 3, $S^{ac}$-1 chromosomes. The short arms have completely condensed chromatin (A) or fluff-like diffused chromatin (B) at their distal ends. ×2,250. 4, $S^{ac}$-2 chromosomes. The short arms have completely condensed chromatin (A) or fluff-like diffused chromatin (B, C, D) at their distal ends. Some $S^{ac}$-2 have completely loosened chromatin (E, F) and another seems to be uniformly puffed (G). ×2,250.
completely (Figs. 4E, F). In some $S^{ac}$-2, the distal ends seemed to show a puff structure (Fig. 4G). The size of the short arms with the fluff-like diffused or the amorphously loosened chromatin was remarkably diminished as compared with that of the completely condensed short arms. This suggests that the appearance of the short arms reflects a stepwise morphological alteration. In

Figs. 5–6. 5, $S^{ac}$-3 chromosomes. Satellite-like compartments are seen (A, B). They sometimes are subjected to loosening into an amorphous mass of chromatin (C). ×2,250. 6, prophase (A, B) showing the association of the four type of chromosomes with the nucleolus (Nu) at their short arms. Arrows indicate the residual short arms that are reduced in size in comparison with the completely condensed short arms at metaphase. ×2,000.

other words, the diffused or the loosened chromatin is probably condensed gradually and folded into the densely stained segments of the metaphase chromosomes.

The short arms of the $S^{ac}$-3 had some characteristics that differed from those of the other type of chromosome mentioned above. The size of the short arms was the least of the six $S^{ac}$ and the majority had a tendency to be stained
less densely than those of the other types of chromosome. However, in a few $S^{ac}-3$, the short arms were condensed so that they stained densely. Another characteristics was that satellite-like compartments stained faintly were frequently observed (Figs. 5A, B). Although the short arms sometimes showed the amorphous loosening of chromatin, they seemed to be stable in size (Fig. 5C).

The frequency of each characteristic appearance of the short arms, as described above, is summarized in Table 1 for each type of chromosome. The fluff-like diffused chromatin was observed for all types of chromosome but for $S^{ac}-1$ the frequency was conspicuously low (6%). The majority of the chromosomes of

<table>
<thead>
<tr>
<th>Type</th>
<th>Morphological changes of short arms and frequencies</th>
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<td>$S^{ac}-0$</td>
<td><img src="image" alt="Diagram" /> <img src="image" alt="Diagram" /> <img src="image" alt="Diagram" /></td>
<td>$58^a$ (61.7) $29$ (30.9) $7$ (7.4)</td>
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<tr>
<td>$S^{ac}-1$</td>
<td><img src="image" alt="Diagram" /> <img src="image" alt="Diagram" /> <img src="image" alt="Diagram" /></td>
<td>$47$ (94.0) $3$ (6.0)</td>
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<tr>
<td>$S^{ac}-2$</td>
<td><img src="image" alt="Diagram" /> <img src="image" alt="Diagram" /> <img src="image" alt="Diagram" /></td>
<td>$58$ (33.5) $41$ (23.7) $61$ (35.3) $13$ (7.5)</td>
</tr>
<tr>
<td>$S^{ac}-3$</td>
<td><img src="image" alt="Diagram" /> <img src="image" alt="Diagram" /> <img src="image" alt="Diagram" /></td>
<td>$28$ (37.8) $19$ (25.7) $27$ (36.5)</td>
</tr>
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this type retained the completely condensed short arms. Short arms with the amorphously loosened chromatin were observed at high frequencies in $S^{ac}-2$ and $S^{ac}-3$. In a few $S^{ac}-0$, the amorphously loosened chromatin occurred, but it was never seen in any of the $S^{ac}-1$ examined. The complete loosening of the short arms was observed only in the $S^{ac}-2$ chromosomes.

At prophase, all four types of chromosomes were attached to the nucleoli at the distal ends of their short arms. The densely stained segments of the short arms at this stage were apparently reduced in size for all types of chromosome. In the $S^{ac}-0$, the short arm was clearly seen in a nucleolus (Fig. 6A) and it seemed
to be smaller in size than when completely condensed at metaphase. The short arm of the $S^{nc}$-1 also attached to a nucleolus and it was slightly smaller in comparison with the completely condensed short arm at metaphase (Fig. 6A). The minute short arms of some of the $S^{nc}$-2 also attached to nucleoli (Figs. 6A, B). Associations of the $S^{nc}$-3 with the nucleoli were also seen and the short arms had a globular appearance (Figs. 6A, B). The reduction in the size of the short arms at this stage suggests that a portion of the short arms is subjected to diffusing into the nucleoli leaving the residual densely stained segments, and this diffused portion must be the nucleolus organizer region.

At metaphase, there was a quantitative difference in the diffused or loosened portions of the short arms, suggesting that the number of ribosomal RNA genes included in the short arms was different for each type of chromosome. This may reflect the bulk of the nucleolus, which urged us to observe this structure. In the root tip cells, the nucleoli developed well and the majority of them fused with each other (Figs. 7A, B). The size of each nucleolus varied greatly and a conspicuously smaller nucleolus was frequently seen in a nucleus. The nucleoli in the ovary cells did not so developed as those of the root tip cells (Figs. 7C, D). The number of nucleoli per nucleus varied from one to six and their size varied within the same nucleus. The previous reports proposed that the maximum number of nucleoli coincided with the number of nucleolus organizer regions (Sato and Asano 1951, Nicolof et al. 1977). This confirms that the number of nucleolus organizer regions of *N. fragrans* is six as proposed in this paper.

**Discussion**

The results of observations at prophase indicate that all of the six acrocentric
chromosomes have nucleolus organizer regions at the distal ends of their short arms. These regions displayed varying appearances at metaphase. The three types of morphology characterizing above presumably result from progressive alterations in the morphology of the short arms. At interphase, a portion of each short arm or the whole short arm, probably loosens and diffuses within a nucleolus. This is also confirmed by the previous suggestion that the short arms, which were densely stained reddish purple with Giemsa liquid, diminished in number at interphase and diffused within the nucleoli where they could be seen as a mesh-like structure of fine fibrils (Sato et al. 1979). It is assumed that this structure is maintained throughout prophase, but at metaphase, the loosened or diffused chromatin is progressively packed into the densely stained segments and forms the completely condensed short arms. It is accordingly conceivable that the three types of morphology, the fluff-like diffusing, the amorphous loosening and the complete condensing of chromatin, are observed during this stage. A similar morphology of the nucleolus organizer regions is seen also in the Iris (Maugini and Maleci 1974).

A quantitative difference in the diffused or the loosened portions compared with the completely condensed short arms was observed for each type of chromosome. For example, the S°-2 underwent complete loosening of the short arms, while only a little fluffing occurred in the S°-1. This suggests that the quantity of genes for ribosomal RNA is different for each type of chromosome. The positive correlation between the size of secondary constrictions determined cytologically and the number of ribosomal RNA was introduced by Maggini and Dominics (1977). Milner and Brown (1969) examined the relationship between the size of secondary constrictions and the number of ribosomal RNA genes per genome of Bufo marinus. They found that some of the animals examined had unequal sized secondary constrictions and produced either one large nucleolus or a large and a small nucleolus, and they proposed that the duplication of ribosomal RNA genes occurred in these nucleolus organizer regions. The same situation was reported also by Macgregor et al. (1977) applying the technique of in situ hybridization to the meiotic chromosomes of Plethodon cinereus. They showed that this animal might be heterozygous with respect to the number of ribosomal RNA genes on each half of the nucleolus bivalent. If the size of the diffused or loosened parts of the short arms at metaphase corresponds with that of the nucleolus organizer regions, the present observations suggest that the size of the nucleolus organizer regions is different for each type of chromosome. This is also supported by the observation that the nucleoli in the same nucleus varied greatly in size both in the root tip and ovary cells. It seems that the conspicuously smaller nucleolus was produced by the S°-1 of which the short arms were most stable in appearance.

However, the four types of chromosome may have characteristic allocyclic diffusion and condensation of their nucleolus organizer regions during the mitotic cell cycle. If this conception is valid, it may be improbable to suppose that the densely stained segments of the residual short arms at metaphase contain no genes for ribosomal RNA. Further investigations such as observations in more
detail during other stages of the cell cycle, N-banding, silver staining method and
in situ hybridization are needed for a precise understanding of the relationship
between the size of the nucleolus organizer regions and the content of ribosomal
RNA genes for each type of chromosome.

Summary

The nucleolus organizer regions were studied in Nothoscordum fragrans,
Liliaceae (2n=19) applying the technique of C-banding. This technique allowed
us to distinguish the six acrocentric chromosomes into four types by the banding
patterns in the long arms and also to observe in detail the nucleolus organizer
regions and nucleoli. The distal ends of the short arms of all six acrocentric
chromosomes had a characteristic appearance at metaphase: completely condensing,
fluff-like diffusing or amorphous loosening of the chromatin. These three charac-
teristic formations seemed to represent a progressive alteration of the morphology
of the short arms during metaphase. These regions were confirmed to be the
nucleolus organizer regions since they were attached to the nucleoli at prophase.
The frequencies of the three characteristic formations were studied for each type
chromosome. There was a quantitative difference in the diffused or loosened
chromatin for each type of chromosome. The possibility that this may reflect
the size of the nucleolus organizer regions is discussed.

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