NORs in Rhoeo (Commelinaeaceae) revisited

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Abstract - Nucleolar organizer regions were localized by silver staining in Rhoeo spathacea (Swartz) Stearn. Our results strongly suggest that interstitially located lightly stained chromosome segments observed in conventional preparations and interpreted by previous authors as secondary constrictions are depleted of active nucleolus organizing regions. There were no Ag-positive intercalary signals within chromosome arms where lightly stained chromosome segments were observed. Ag-NOR loci were found only at telomeric and centromeric chromosome domains, and colocalized with heterochromatin bands.

Key words: Ag-staining, chromosomes, heterochromatin, karyotype, NORs, Rhoeo spathacea.

INTRODUCTION

Rhoeo spathacea (Swartz) Stearn. (= Rhoeo discolor) has been a classical subject of cytological studies for about 70 years since the pioneering works of Belling (1927), Darlington (1929) and Sax (1931). It is widely believed that the permanent structural heterozygosity of this species (the lack of chromosome pairs and unusual mode of meiosis, with chromosome rings or chains at the first division) is conditioned by a series of reciprocal translocations between the distal segments of all chromosomes (for review: Satterfield and Mertens 1972). Most of the data on karyotype structure in this species have been obtained from observations of conventionally stained meiotic chromosomes (Kato 1930; Sax 1931, 1935; Coleman 1941; Lin and Paddock 1973, 1978; Lin 1979). Besides numerous non-interstitially located heterochromatic bands (Natarajan and Natarajan 1972; Stack 1974; Pettenatti 1987; Golczyk and Joachimiak 1999), no other coding and non-coding domains have been localized within the karyotype of this interesting species.

There are some reports on the localization of nucleolar organizers (NORs) in Rhoeo, but only from indirect observations in unrelated studies. Darlington (1929), Bhaduri (1942) and Lin and Paddock (1973) found several interstitially located, weakly stained regions within some conventionally stained meiotic/mitotic chromosomes, and interpreted them as secondary constrictions ("NORs"). However, the constrictions localized by Lin and Paddock (1973) significantly differ from those detected by Bhaduri (1942) and Darlington (1929). On the other hand, Stack (1974), based on the reasonable generalization that nucleolus organizers in plants are usually associated with constitutive heterochromatin, has questioned the interstitial localization of NORs in this species and suggested the presence of only two terminal NOR loci on two submetacentric chromosomes equipped with telomeric heterochromatin on the shorter arms.

Secondary constrictions may be viewed as a state of arrest of condensation of the regions bearing the nucleoli at prophase, that is, they can be treated as chromosomal markers of active NORs within conventionally stained preparations (Bush and Smetana 1970). Although not all interstitially located active NORs are marked by a secondary constriction, all true secondary constrictions should be treated as active NOR sites. Transcriptionally inactive rDNA clusters, if present, do not form secondary constrictions, and satellites are not observed within the chromo-
somes bearing them (Lengerova and Vyskot 2001). The intercalary localization of NORs in Rhoeo suggested by the majority of previous authors (l.c.) seems in disagreement with the lack of intercalary heterochromatin (Stack 1974; Pettenati 1987; Golczyk and Joachimiak 1999), the apparent lack of interstitial CMA-positive signals (Joachimiak and Golczyk, unpubl. results) and also the absence of stable types of satellited chromosomes in conventionally stained mitotic preparations (Golczyk and Joachimiak 1999). Thus it seems probable that the previously described “secondary constrictions” in Rhoeo are artifacts or else represent chromatin segments of unknown structure but lacking active nucleolar organizers. For the first time we use silver staining (Ag-staining), a simple technique specifically detecting functional nucleolus organizers (Cheung et al. 1989) within the Rhoeo karyotype to resolve this problem.

**MATERIALS AND METHODS**

Cytological methods

Roots were taken from cuttings of five different R. spathacea plants we previously analyzed by conventional and C-banding methods (Golczyk and Joachimiak 1999). Floral buds were obtained from the same donor plants. Root tips and anthers were fixed in AA (glacial acetic acid and absolute alcohol, 1:3) for 2 h, stained in aceto-orcein (2% solution of orcein in 45% acetic acid) at room temperature and squashed in a drop of 45% acetic acid with no heating to minimize the risk of chromosome breaks. For Ag-staining the material was fixed overnight refrigerated in FAA (50% alcohol, glacial acetic acid and 37% formaldehyde, 18:1:1), squashed and stained according to Trude Schwarzacher and Karyobiology Group (http://www.le.ac.uk/bl/phh4/methods/agenor.htm). The twenty best mitotic metaphases and ten best meiotic chains were selected and captured in a Lucia G system (Laboratory Imaging Ltd., Czech Republic) or photographed. For C-banding the material was fixed in AA for 2 h and further treated according to the schedule described by Schwarzacher et al. (1980).

Data processing and presentation

Images were captured and processed using a CCD camera and Lucia G. The chromosome lengths were calculated as percentages of total karyotype length. Karyotype analysis was performed on 10 complete meiotic and 25 mitotic metaphases. Chromosome types were identified and classified as previously described (Golczyk and Joachimiak 1999).

Table 1 – *Rhoeo spathacea*, acetoorceine stained chromosome types (1-12) showing different number of LSSs within five chromosome regions (ChR) in mitosis (a) and meiosis (b).

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Fig. 1 – Somatic *Rhoeo* metaphase chromosomes with clearly visible LSSs. Acetoorceine staining. Bar 10µm.

Fig. 2 – Karyotype of *Rhoeo spathacea*. Chromosomes ordered according to their relative position within meiotic rings/chains. Black – chromosome regions with the highest frequency of LSSs (above 0.4).
Fig. 3 – Silver-stained chromosomes (A-D) and nuclei (E-H) of *Rhoeo spathacea*. A-C, mitotic metaphase plates with two (A), three (B), and five (C) distinct telomeric Ag-NOR signals. Note the large silver positive centromeric signal in B (arrowed) and other centric signals appearing as tiny double dots. D, C-banded mitotic metaphase plate. E-G, interphase nuclei from the root meristem showing different numbers of silver-stained nucleoli. H, mitotic prophase nucleus with large, collective nucleolus. Bars 10μm.
The lightly stained chromosome segments (LSSs) we observed (see Introduction) were found in most aceto-orcein stained metaphases. The LSS positions within each analyzed chromosome were calculated as the distance from the end of the longer chromosome arm. Each chromosome type was divided into five regions of equal length, the first region beginning at the end of the longer arm (Fig. 2, Table 1). The expected vs. observed distribution of LSSs along the distinguished regions of chromosomes was tested with the chi-square test for goodness of fit (SOKAL and ROHLF 1969). The expected distribution was an even one, that is, an equal chance that an LSS would appear in a given chromosome region.

RESULTS

We observed LSSs (Fig. 1) within all conventionally stained mitotic metaphases and in six meiotic rings/chains. Within meiotic chromosomes these structures were not easy for distinction and they were less frequently observed (Table 1); thus mitotic metaphases seem more suitable for analysis of their distribution. Mitotic LSSs were clearly unstable as to their number and localization within chromosomes. Their chromosome positions are highly variable, so finally we distinguished only some chromosome regions characterized by relatively high (above 0.4) LSS frequency (Fig. 2). The number of chromosomes with LSSs in a given metaphase varied from 3 to 11, and the number of these structures within a particular chromosome was from 1 to 4. All chromosome variants with “secondary constrictions” described by previous authors (DARLINGTON 1929; BHADURI 1942; LIN and PADDOCK 1973) were observed in our study, but some of them at a very low frequency. The observed distribution of LSSs differed significantly from the expected even distribution (chi-square test = 176, s.s. = 4, \( p < 0.0001 \)). The pattern of departure from the expected distribution points to the central domains of the longer chromosome arms, where an excess of LSSs is manifested.

All the Ag-positive signals were located at chromosome termini and/or within centromeric clusters of chromatin (Figs. 3A, B, C). There were no positive interstitially located signals within arms, where LSSs were frequently observed in orcein-stained preparations. In mitotic chromosomes large signals were located mainly at telomeres, whereas centric signals were weak and detected chiefly in the form of tiny double dots (Figs. 3A, B, C). The number of potentially active nucleolus organizers in Rhoeo (identified within different chromosome positions) seems relatively high; this may be additionally supported by the observations of interphase nuclei (Figs. 3E, F, G). In the majority of root-tip interphase cells the number of silver-stained nucleoli was 1-6, markedly exceeding STACK’S (1974) estimates but in accordance with BHADURI (1942), who stated that the maximum number of separate nucleoli observed in Rhoeo root meristem is 6. In fact, the strong tendency to nucleolar fusion in Rhoeo (Figs. 3G, H) obscures the situation; nucleoli could be even more abundant (BHADURI 1942).

Centromerically located Ag-NOR signals colocalized with previously described (GOLCZYK and JOACHIMIAK 1999) centric blocks of heterochromatin, but in C-banded mitotic chromosomes such heterochromatin clusters are markedly larger than the majority of centric Ag-positive signals detected here (Fig. 3D). Thus the possibility that heterochromatin in Rhoeo is agyrophilic per se can be ruled out. Terminal Ag-NOR signals could also be traced to the heterochromatin because five terminally located segments of heterochromatin have been carefully localized within the Rhoeo karyotype (GOLCZYK and JOACHIMIAK 1999). Unfortunately, exact identification of NOR-bearing Rhoeo chromosomes by morphology is very difficult in our silver-stained preparations, and further studies are needed.

DISCUSSION

It is broadly accepted that Ag-NOR-positive chromosome signals correspond to the locations of active rRNA genes (CHEUNG et al. 1989), and that the size and intensity of Ag-stained areas are related to their transcriptional activity (MILLER et al. 1976; HOFGARTNER et al. 1979). The observed exclusively proximal and distal localization of Ag-stained regions strongly suggests that interstitially located LSSs are not true secondary constrictions (structures organizing the nucleolus). These lightly stained bands observed by us and by previous authors (DARLINGTON 1929; BHADURI 1942; LIN and PADDOCK 1973) proved surprisingly unstable as to their number and localization within different metaphase plates. The nature of these bands remains unknown; their distribution differs significantly from the expected random distribu-
tion (see Results). Even if the LSSs are breaks caused by the method used, their non-random distribution and abundant occurrence within definite chromosome segments suggest increased susceptibility of these segments to acidic treatment.

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