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Nucleolar organizer function in developing potato calli

Laas P. Pijnacker* and Margriet A. Ferwerda

Department of Genetics, Centre of Biological Sciences, University of Groningen, P. O. Box 14, NL-9750 AA Haren, The Netherlands

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Summary. The number of transcriptionally active nucleolar organizer regions (NORs) of one monohaploid, one dihaploid and two tetraploids of *Solanum tuberosum* and one diploid *S. phureja* was established by the silver staining of metaphases in root meristems and in in vitro-cultured leaf explants. The maximum number of active NORs per cell was one per haploid set of chromosomes. One or more NORs could be inactive in cells of the tetraploid meristems and in non-polyploidized and polyploidized cells of the dihaploid and tetraploid explants. Inactivation was determined by genotype and tissue and could remain constant during in vitro culture.

Key words: Nucleolar organizer function – Potato – Chromosome – Root meristem – Callus

Introduction

The nucleolar organizer region (NOR) coincides with the secondary constriction of the nucleolar chromosome where large numbers of genes for ribosomal RNA (rRNA) are located. The location and number of NORs in a haploid set of chromosomes are characteristic for the organism (Stahl 1982). In plant cell cultures the genomes are remarkably unstable, and consequently the number of NORs can vary considerably (Bayliss 1980; Krikorian et al. 1983; D'Amato 1985; Lee and Phillips 1988; Pijnacker and Sree Ramulu 1990). The number per nucleus may increase or decrease through numerical (polyploidization, somatic reduction, aneuploidization) and structural (transposition, deletion) changes of the nucleolar chromosomes. Part of this variation may originate from similar mutations in the explants from which the cultures are derived.

At metaphase the chromosomal sites of the NORs stain dark brown or black when treated with silver in the Ag-NOR staining techniques if the rRNA genes were transcribed during the preceding interphase (see Hubbell 1985). The number of active NORs (Ag-NORs) can thus be established. Whether all the NORs function in non-mutated and mutated cells of plant cell cultures has as yet barely been investigated. Burger and Müller (1987) showed that all of the NORs of polyploid cells of *Vicia faba* remained transcriptionally active when stimulated to mitosis by wounding.

During the first days of callus development in leaf explants of monohaploid, dihaploid and tetraploid genotypes of the common potato *Solanum tuberosum* and of the diploid wild potato *S. phureja* metaphases are found that show the normal or a polyploidized number of chromosomes (Pijnacker et al. 1986, 1989b). Polyploidization is caused by endoreduplication in the leaf during its development (polysomaty) and/or in the explant during the in vitro culture period.

In this study leaf explants and, as controls, root meristems of various potato genotypes were stained by silver to investigate the transcriptional activity of the NORs in non-polyploidized and polyploidized cells.

Materials and methods

Leaf segments of one monohaploid clone (H7322, 2n = x = 12), one dihaploid clone (SVP3 = SH78-78-901, 2n = 2x = 24) and two tetraploid cultivars ('Bintje' and 'Astarte', 2n = 4x = 48) of *Solanum tuberosum* and one diploid clone (SVP5 = PH77-1445-2242, 2n = 2x = 24) of *S. phureja* were cultured on callus-inducing medium (Murashige and Skoog medium + 5 mg/l α-naphtalene acetic acid + 1 mg/l benzyl aminopurine), and after 3, 5 and

* To whom correspondence should be addressed

Table 1. Number of Ag-NORs in metaphases with different euploid numbers of chromosomes (x = 12) of root meristems and of leaf explants cultured in vitro for 3, 5 and 7 days of *Solanum tuberosum* H7322, SVP3, Bintje, Astarte and *S. phureja* SVP5

Genotype and material	Number of days in culture	Number of chromosomes/number of Ag-NORs											Total number of metaphases
		12/1	24/1	24/2	48/1	48/2	48/3	48/4	72/6	96/3	96/4	96/6	96/8	
Root														
H7322		50												50
SVP3				50										50
Bintje						13	31	43						87
Astarte							15	69						84
SVP5				50										50
Explant														
H7322	3	50		49				4						103
	5	61		65				9						135
	7	60		64				13						137
SVP3	3		14	76		5		6						101
	5		22	73		15		2			2			114
	7		9	79		5	2	10						105
Bintje	3					90	4	9			2			105
	5				1	61	7	33		1	1			104
	7				1	61	24	11			4			101
Astarte	3					5	69	26				2		102
	5					11	57	29			1	2		100
	7					6	50	46					1	103
SVP5	3			90				16						106
	5			84				16					1	101
	7		1	64			2	34	1					102

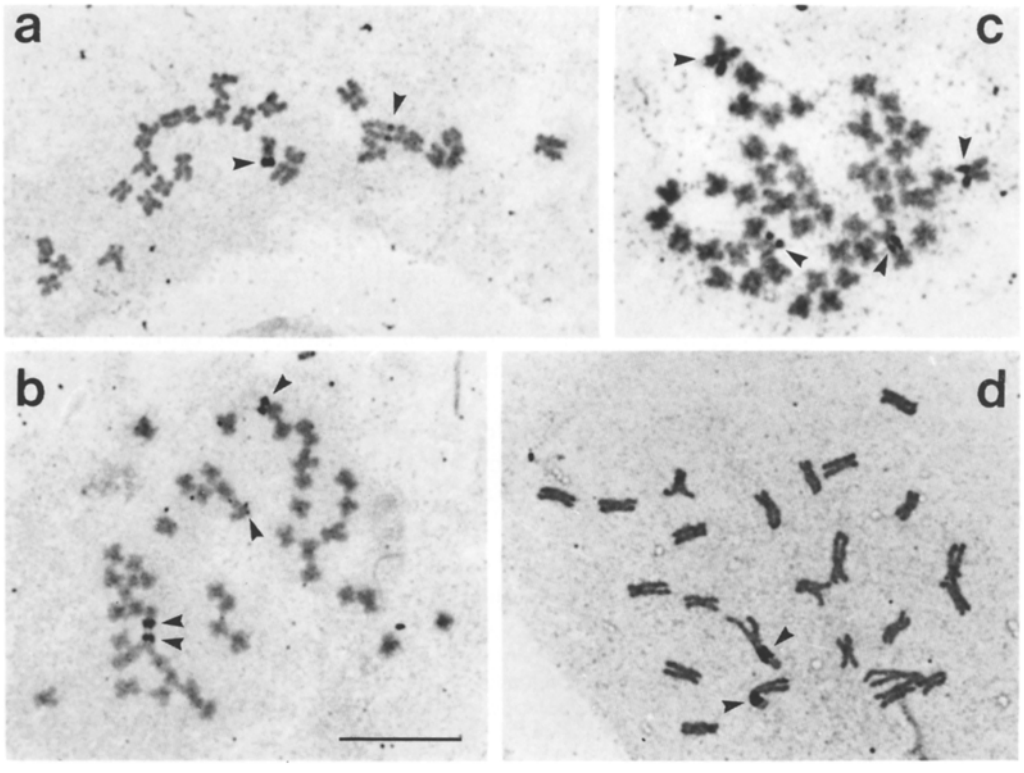

Fig. 1 a–d. Silver-stained metaphase chromosomes and NORs (arrowheads) of root meristems of *Solanum tuberosum* **a** Clone SVP3 (2x = 24); **b** cv 'Bintje' (4x = 48); **c** cv 'Astarte' (4x = 48) and *S. phureja* **d** clone SVP5 (2x = 24). *Bar:* 10 μm

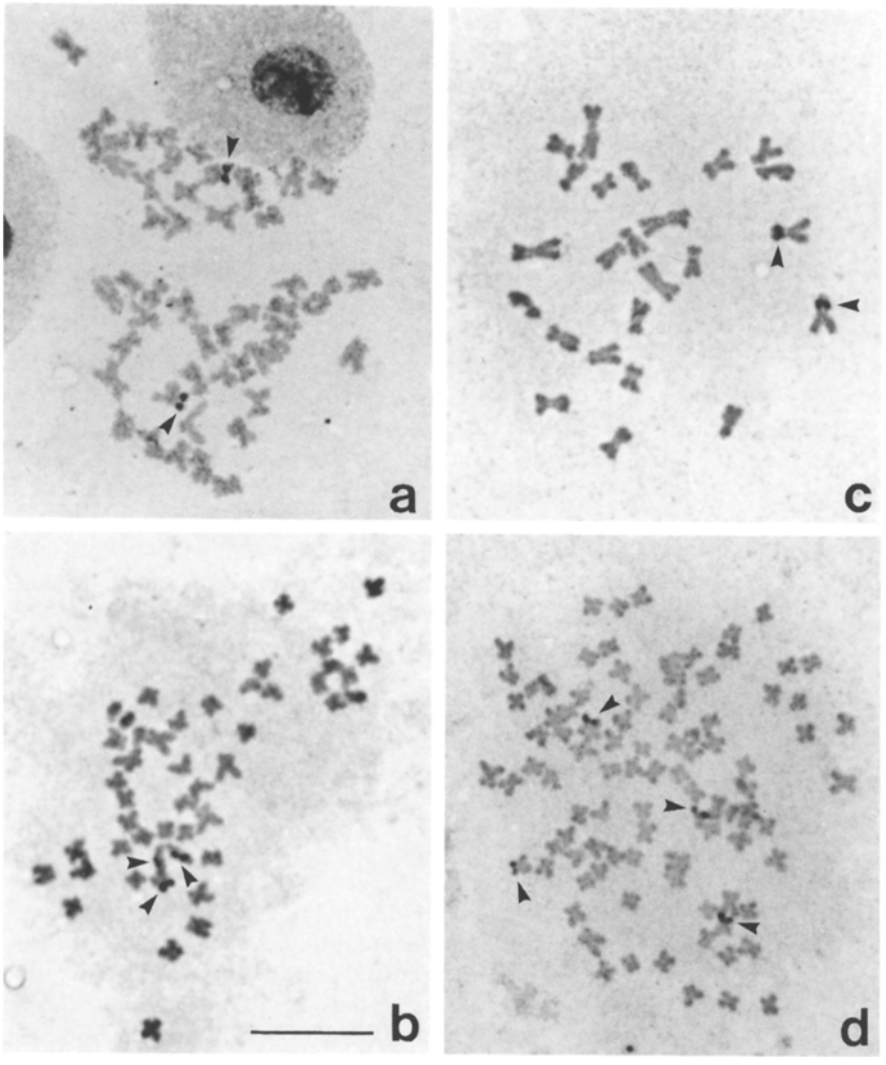

Fig. 2a–d. Silver-stained metaphase chromosomes and NORs (*arrowheads*) of in vitro-cultured leaf explants of *Solanum tuberosum*. **a** cv 'Bintje' (4x=48); **b** cv 'Astarte' (4x=48); **c** clone H7322 (2x=24); **d** clone SVP3 (8x=96). *Bar:* 10 μm

7 days the explants were fixed and processed to microscopic slides as described by Pijnacker et al. (1986). Leaf segments do not contain any mitotic cells or only contain a few (day 0). Root tips of potted plants of the same genotypes were used for comparison.

The silver-staining procedure of Kodama et al. (1980) was used with minor modifications: the silver nitrate was dissolved in the sodium citrate solution used by Lacadena et al. (1984), and the slides were stained for about 20 min at 37 °C. To obtain the total number of metaphases mentioned in Table 1 at least 20 slides of roots or 30 slides of explants were scored.

Chi-square contingency tests were used for the statistical analysis.

Results

Ag-NORs in metaphases of root meristems

The metaphases of root meristems of the five potato genotypes showed the expected number of chromosomes; polyploidized metaphases were not found (Table 1). *S. tuberosum* and *S. phureja* have one nucleolar chromosome (=chromosome 2) per haploid set of chromosomes. The secondary constriction of the NOR is situated close to the centromere in the short arm and delimits a terminal satellite. All secondary constrictions of a metaphase of all the genotypes can be silver-stained, which means that all the NORs can be active (Table 1). The dark-brown or black deposit appears on each of the two chromatids or forms one band on each chromosome. The satellite(s) of *S. tuberosum* H7322 and *S. phureja* SVP5 remain(s) visible after Ag-NOR staining, but those of the other *S. tuberosum* genotypes are generally totally covered by silver (Figs. 1, 2). The absolute size of the Ag-NORs vary from metaphase to metaphase, but the relative sizes remain rather constant. The two Ag-NORs of clone SVP3 differ considerably in size, one Ag-NOR of cv 'Bintje' and cv 'Astarte' is smaller than the other three and one Ag-NOR of cv 'Astarte' may be larger than the other three. The two Ag-NORs of clone SVP5 rarely differ (<1%) (Fig. 1).

Cultivar 'Bintje' and cv 'Astarte' showed metaphases with fewer Ag-NORs (Table 1). Metaphases with different numbers were found within one meristem. Three Ag-NORs occurred in both cultivars, and one was always smaller than the other two. Metaphases with two Ag-NORs, uniform in size, were only found in cv 'Bintje'. Significantly more metaphases, of which all four NORs had remained active, were observed in cv 'Astarte' than in cv 'Bintje' ($P<0.001$).

It may be mentioned that at the end of telophase the nucleoli of the chromosome.complements of the diploid and tetraploid genotypes fuse in all of the nuclei of the root meristems. As a result the number of active NORs could not be determined by counting the number of interphase nucleoli.

Ag-NORs of non-polyploidized metaphases in leaf explants

In the non-polyploidized metaphases of the leaf explants of all genotypes numbers of Ag-NORs could be found as in root meristems during the whole culture period (Table 1). All NORs of a cell of an explant could remain active. Their silver-staining pattern was similar to that observed in the metaphases of root meristems.

On days 3, 5 and 7 of culture metaphases of *S. tuberosum* SVP3 appeared with only one Ag-NOR. The metaphases of cv 'Bintje' and cv 'Astarte' showed significantly fewer Ag-NORs than the metaphases of root meristems ($P<0.05$ and $P<0.001$, respectively). In cv 'Bintje' the mode was two Ag-NORs (Fig. 2a), and two metaphases with one Ag-NOR were found. In cv 'Astarte' the mode was three (Fig. 2b), and two, generally similarly sized, Ag-NORs occurred in 5–11% of the metaphases.

During the culture period the frequency of metaphases with one Ag-NOR of clone SVP3 fluctuated. A significant decrease took place between 5 and 7 days of culture ($0.05>P>0.02$), but over the whole period the numbers did not change significantly ($P>0.05$). The frequency of metaphases with one to three Ag-NORs of cv 'Bintje' and with two to three Ag-NORs of cv 'Astarte' underwent significant changes ($P<0.001$ and $P=0.01$, respectively). In cv 'Bintje' this was mainly caused by a decrease on day 5 ($P<0.001$) and in cv 'Astarte' by a decrease on day 7 ($0.05>P>0.02$). The number of Ag-NORs of *S. phureja* SVP5 remained stable during the culture period, except for one metaphase with one Ag-NOR found on day 7.

Metaphases with different numbers of Ag-NORs occurred within the same explant.

Ag-NORs of polyploidized metaphases in leaf explants

Metaphases of polyploidized cells were observed in all the genotypes. In *S. tuberosum* H7322 diploid (Fig. 2c) and tetraploid metaphases showed without exception two and four, equally sized, Ag-NORs respectively during the 7 days of in vitro culture. Tetraploid metaphases of clone SVP3 had either four Ag-NORs, of which two were smaller than the other two, or two similarly sized Ag-NORs on days 3, 5 and 7 and two octoploid metaphases, found on day 5, had four almost equally sized Ag-NORs (Fig. 2d). These numbers reflect a doubling series of functional NORs from those in the diploid cells. On day 7, however, two metaphases showed three Ag-NORs of which one was smaller in size. During the culture period the frequency of tetraploid metaphases with two Ag-NORs changed significantly ($0.05>P>0.01$), and the percentage was highest on day 5. Tetraploid metaphases with a lower number of Ag-NORs were relatively more abundant than diploid metaphases with a lower number. The numbers of Ag-NORs observed in the octoploid metaphases of cv 'Bintje' and cv 'Astarte' also demonstrated a doubling series from the numbers of the tetraploid metaphases. One exception was found, i.e. a metaphase of cv 'Bintje' with three equally sized Ag-NORs on day 5.

The tetraploid, hexaploid (origin unknown) and octoploid metaphases of *S. phureja* SVP5 showed the expected numbers of Ag-NORs. Two tetraploid metaphases had one Ag-NOR less after 7 days of culture.

Diplochromosomes

In *S. tuberosum* H7322 and *S. phureja* SVP5 metaphases with 12 or 24 diplochromosomes, i.e. chromosomes consisting of four chromatids, were found (see Pijnacker and Ferwerda 1990). They have not been incorporated in the above results. The nucleolar diplochromosomes showed two silver-stained NORs, one Ag-NOR per chromosome covering both chromatids.

Discussion

Ag-NOR-staining of the metaphases of the root meristems revealed that all the NORs of the investigated genotypes can be active. Differences in the amount of silver deposit between the NORs of a metaphase are presumed to be dependent on the state of condensation of the NOR, the number of rRNA-genes and the level of transcription (Von Kalm and Smyth 1984; Medina et al. 1986; Sánchez et al. 1989). Which of these parameters determines the differences in size among the Ag-NORs of *S. tuberosum* clone SVP3, cv 'Bintje' and cv 'Astarte' is not revealed by silver staining. In cv 'Astarte' differences may be due to differences in the number of rRNA-genes because this cultivar originated from a cross with *S. vernei*. Visser et al. (1988) showed by in situ hybridization with radioactive rDNA probes that the two NORs

of *S. phureja* SVP5 became similarly labelled (though exact measurements were not carried out) and thus likely contain the same number of rRNA-genes. The Ag-NORs of clone SVP5 were uniform in size, and consequently both NORs are likely active at the same level.

Fewer Ag-NORs than expected appeared in the tetraploid cv 'Bintje' and cv 'Astarte'. It is unlikely that the lower numbers are due to deletion of NORs because elimination is not expected to occur in root meristems. Amphiplasty, i.e. the suppression of the function of a NOR of one genome by the function of a NOR of another genome, occurs in all the cells of intergeneric hybrids (Rieger et al. 1979; Lacadena et al. 1988) and could have taken place in cv 'Astarte'. However, in the root meristems of cv 'Astarte' 82% of the metaphases still had four Ag-NORs. It may thus be concluded that in the cells of root meristems of both tetraploid cultivars not all the NORs need to be active in transcription. Which NOR becomes suppressed, could not be established. As mentioned above, the size of the Ag-NOR is determined by more than one parameter, and these are not discerned by Ag-NOR staining. So a small or large Ag-NOR in a metaphase with the maximum number of Ag-NORs may be larger or smaller, respectively, in a metaphase with a lower number of Ag-NORs because of changes in transcriptional activity. In cv 'Astarte' significantly more metaphases with four Ag-NORs occurred than in cv 'Bintje', which indicates a genotypic influence. Whether suppression is total is not known because it may not be excluded that staining remains beyond visualization in the case of a residual level of transcription.

Non-polyploidized metaphases in the explants of clone SVP3, cv 'Bintje' and cv 'Astarte' could show one Ag-NOR less than the metaphases of root meristems, and the frequency of metaphases with a lower number of Ag-NORs was higher during the whole culture period. In the explants of *S. phureja* SVP5 only one metaphase with a deviating number was found (on day 7). Pijnacker et al. (1989 b) showed that on day 3 the metaphases of clones SVP3 and SVP5 and cv 'Bintje' could have passed at least one complete cell cycle. It is rather unlikely that the lower numbers were caused by deletion of complete NORs during the first cell cycle in the explant. In studies on similar explants with Giemsa-staining the nucleolar chromosomes still showed their secondary constrictions, and elimination by structural rearrangements was not observed (Pijnacker et al. 1986, 1989 b and unpublished). Therefore, it is hardly conceivable that elimination could have taken place during leaf development. However, elimination cannot be completely excluded because NORs can be eliminated in cell cultures of intergeneric somatic hybrids of potatoes (Pijnacker et al. 1987, 1989 a), and the number of rRNA genes can be reduced in cell cultures of potato (Landsmann and Uhrig 1985). These cells thus reveal suppression of rDNA transcription in one or more NORs when compared with root meristems. This suppression is under control of the genotype since at the tetraploid level more NORs remain active in cv 'Astarte' than in cv 'Bintje', like in the root meristems, and at the diploid and species level more NORs remain active in clone SVP5 than in clone SVP3. Whether suppression was already present in the leaves or whether it took place during the G1- or G2-phase of the first cell cycle in the explants cannot be concluded from the present study. Cell divisions require rRNA, and therefore it is unlikely that suppression took place during in vitro culture but rather during cell differentiation in the leaf.

During the culture period the frequency of metaphases with a lower number of Ag-NORs did not change when diploidy was involved (clone SVP3), but it did at the tetraploid level (clone SVP3, cv 'Bintje', cv 'Astarte'). This variation cannot be explained, but there is no evidence of a regular increase or decrease in the number of Ag-NORs during the culture period. It is unlikely that a constant reactivation or a constant (further) suppression of NORs takes place in the early callusing cells. This is substantiated by the fact that the numbers and size of the Ag-NORs in polyploidized metaphases of all the genotypes reflect doubling series. After chromosome doubling a similar number of NORs thus remains active per chromosome complement. It may be mentioned that polyploidization could have taken place before as well as after the start of the in vitro culture (Pijnacker et al. 1986, 1989 b). Exceptions, like three Ag-NORs in two tetraploid metaphases of clone SVP3, three Ag-NORs in one octoploid metaphase of cv 'Bintje' and one Ag-NOR in one diploid metaphase of *S. phureja* SVP5, could still have evolved during leaf development.

All NORs of the diploid and tetraploid metaphases in the explants of *S. tuberosum* H7322, without exception, show silver deposits. Compared with the heterozygous diploid and tetraploid metaphases of the other genotypes, homozygosity does not involve suppression of the transcription of complete NORs.

The polyploidized cells arise through endoreduplication during interphase as evidenced by the presence of diplochromosomes in the following mitosis (Pijnacker and Ferwerda 1990). The newly formed chromatids have Ag-NORs. The endoreduplication process thus does not interfere with transcription of the rRNA-genes and confirms the observations of Burger and Müller (1987) on polyploidized cells of *Vicia faba*.

Transcription of rRNA as evidenced by functional NORs is of great importance for a healthy cell culture. Our results show that in potato explants, dependent on the genotype, the non-polyploidized mitotic cells consist of two types: i.e. cells with the same number of functional NORs as found in root meristems and cells with a lower number of functional NORs. It is important to know

whether both types have the same amount of transcription potential or whether they differ in rRNA production. In the latter case it must be questioned whether both types of cells have an equal chance to survive during establishment of a cell culture or later on during or after regeneration to plants, and so contribute to somaclonal variation. How differences in the functioning of the NORs are determined, for instance by methylation of the ribosomal DNA (Flavell 1986; Quemada et al. 1987; Anderson et al. 1990), also needs to be investigated for an understanding of the mechanisms involved in the genetic (in)stability of cell cultures (see Phillips et al. 1990).

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