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Development of polysomaty during differentiation in diploid and tetraploid tomato (*Lycopersicon esculentum*) plants

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Abstract

Polysomaty was found in all organs of tomato (*Lycopersicon esculentum* cv. MoneyMaker) tested. The first endoreduplications occurred in cotyledon and hypocotyl during germination (concurrent with cell elongation). Also the stem and leaves became polysomatic during development (cell elongation and expansion). Finally, new rounds of endoreduplications marked the yellowing of the leaves (ageing), during which, in the leaf petiole, levels up to 128C were reached (1C is the DNA content of germ-line cells). Comparing diploid and tetraploid plants, the patterns of polysomaty were similar in four organs tested. The frequency of nuclei at the different C-levels in diploid cotyledons (2C, 51.6% of the nuclei; 4C, 39.0%; 8C, 8.6%; 16C, 0.8%) equalled the corresponding levels in tetraploid cotyledons (2C, 52.1%; 4C, 37.2%; 8C, 9.6%; 16C, 0.9%). Differences in growth and leaf expansion between in vitro- and greenhouse-grown plants were reflected in differences in polysomaty patterns between corresponding cotyledon and leaf tissues. These results indicate that polysomaty is genetically regulated as the number of endoreduplications taking place in the cells, and influenced by the actual growth pattern of the plant. As such, it may be considered as an integral part of the morphogenesis of a plant, from germination to ageing.

Key words: Differentiation; Endopolyploidy; Flow cytometry; *Lycopersicon esculentum* L.; Polysomaty; Tomato

1. Introduction

Repeated cycles of DNA synthesis without intervening cell divisions, called DNA endoreduplication, lead to endopolyploid cells [1]. In

differentiated plant cells, endopolyploidy is the rule rather than the exception, and has been observed in almost all species studied [2]. Organs and tissues are called polysomatic when they contain cells at different levels of endoreduplication.

Flow cytometry enables the study levels of endopolyploidy in virtually all different organs and tissues during the development of a plant. In *Mesembryanthemum crystallinum* [3] and

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Abbreviation: C, unit of DNA content of the genome of germ-line cells.

Arabidopsis thaliana [4], the distribution of nuclei with different ploidy levels was found to be organ-specific and, in leaves, to change during development towards higher ploidy levels, up to 32C in fully expanded leaves. In *Cucumis sativus* (cucumber), cycles of endoreduplication were observed in the transition tissue between hypocotyl and radicle during germination, in the hypocotyl during elongation, in the cotyledon during expansion, and in the flower during maturation. However, both the leaves and roots of cucumber always maintained the initial pattern of polysomaty [5]. In contrast to the attention that has been given to young, developing tissues, nothing is known about endoreduplication patterns during ageing. Nevertheless, all studies thus far indicate that polysomaty develops in a regulated fashion.

The question of how the regulation of endoreduplication cycles takes place at the level of the individual cell in the different tissues remains unanswered. Little is known about relevant signals, although hormones are thought to be involved [1]. Kinetin, in the presence of auxins, induced one DNA endoreduplication in explants of pea root tissues that did not naturally have endopolyploidy [6], and auxin-induced endomitosis in haploid petunia tissue culture [7]. The basic regulatory mechanism at the cellular level can be thought of in two ways: (i) the endoreduplication process may be regulated via negative feedback by the amount of a specific gene product relative to the demand for it; or (ii) the process may be genetically determined based on the number of DNA replication cycles in a certain cell type. A separate question is whether the number of endoreduplication cycles is dependent on the actual level of differentiation of the cell. If so, plant growth conditions would greatly influence the development of polysomaty.

These questions are studied in this report by comparing the patterns of polysomaty in a number of organs or organ parts in diploid and tetraploid tomato plants, by comparing the development of polysomaty in corresponding organs of plants grown under different culture conditions, i.e. in the greenhouse and in vitro, and by following the development of polysomaty in organ parts throughout morphogenesis, from germination to ageing.

2. Methods

2.1. Plant material

Seeds from *Lycopersicon esculentum* cv. Moneymaker were harvested from one parental plant in the summer of 1991. Tetraploid seeds were harvested from tetraploid plants which had been regenerated from tissue culture of (diploid) cv. Moneymaker seedling parts [8].

For flow cytometric analyses, seedlings were grown aseptically for up to 5 weeks on MS medium [9] supplemented with 3% sucrose and 0.8% agar (BBL) in 720-ml glass jars in a growth room at 24°C and 16 h/day light (Philips fluorescent TL type 50 W 84HF, maximum distance 30 cm). Seeds were also sown in soil and grown in the greenhouse at 16 h/day light. The age of the plant material, as indicated in the experiments, was the time since the start of imbibition.

2.2. Flow cytometry

For flow cytometric analyses, individual plants at different ages were subdivided into several organs or parts of organs. The hypocotyl was divided into three parts, proximal to root, median and distal, which was originally connected to the cotyledon. The leaves were numbered from bottom to top, with the oldest leaf as number one. Ploidy levels in the stem were measured in a part of each internode taken just below each leaf. The separate parts of each plant were measured individually by flow cytometry according to Gilissen et al. [5]. In short, the parts were chopped individually with a sharp razor blade in 2 ml nucleus isolation buffer containing 1 mg/l 4,6-diamino-2-phenylindole (DAPI) for fluorescent DNA staining. After chopping, the samples were passed through a 55-µm nylon filter (Nybolt). The DNA content of the isolated nuclei in the sample was measured using a Partec PAS-II flow cytometer equipped with a HBO-100 mercury lamp, a dichroic mirror TK420, and a long-pass filter GG435. The data were plotted on a semi-logarithmic scale. In this way, the histogram peaks from 2C to 128C are evenly distributed along the abscissa. For calibration, the 2C peak from nuclei of young leaves from in vitro grown diploid tomato plants was adjusted at the channel-100 position. At least 5000 nuclei were analyzed per plant part.

Fig. 1. Frequency of nuclei at various C-values (2C–16C) in different organs of in vitro grown tomato plants 1, 9, 13 and 26 days after start of imbibition. Each panel contains data from one individual plant.

2.3. Analysis of results

The number of nuclei that was present in the peaks of the histogram was analyzed measuring peak height, which is linearly related to the number of nuclei. The data are presented as percentage of the total amount of nuclei in all peaks of the histogram, where 'C' is the unit of DNA content of the genome of germ-line cells, irrespective of whether these are generative polyploids [1]. The resulting percentages do not have a normal distribution; therefore, for statistical analysis the (non-parametric) Mann Whitney *U*-test was used.

3. Results

3.1. Development of polysomaty in seedlings and young diploid plants in vitro

In the dry tomato seed, the embryo contained nearly 100% 2C-nuclei (results not shown; [10]). After 1 day of imbibition, 4C-cells were visible in the radicle [10] and in the cotyledon (Fig. 1). After germination in vitro, which occurred between 5 and 8 days following start of imbibition, 30% of

the cells in the cotyledon already had 4C-nuclei. From the cotyledon through the hypocotyl to the transition zone between the hypocotyl and root, the levels of 2C-nuclei decreased to 15–20%, and both 4C- and 8C-levels increased. The maximum level of both 4C- and 8C-nuclei was approximately 45% (Fig. 1). The pattern of polysomaty in the root was different from the patterns in the other organs: 4C-cells dominated, up to 60–70%, and the frequencies of both 2C- and 8C-cells were relatively low. Once established, the polysomaty patterns appeared to be stable in time; they did not change from 9 to 26 days, even though internodes and leaves were being formed. These new organs initially contained 80–90% 2C-nuclei, but in the first internode, this had diminished to 50% by day 26.

3.2. Polysomaty in diploid and tetraploid plants in vitro

The patterns of polysomaty in different organs of diploid tomato plants were compared with those in tetraploid plants. The tetraploid plants

Fig. 2. Average frequency of nuclei at various C-values (2C–16C) in different organs of either diploid (top) or tetraploid (bottom) tomato plants. Data are the mean of 31–56 (tetraploid) or 20–58 (diploid) 33-day-old plants grown in vitro.

were derived from the diploid plants through tissue culture. Therefore, they supposedly were similar at the genotypic level, except for the chromosome number. Most of the tetraploid seedlings used here developed normally, i.e. comparable in morphology to diploid seedlings.

Even though the same C-level represents twice as much DNA in the tetraploid plant compared to the diploid plant, the patterns of polysomaty were highly similar, including low levels of endoreduplications in young leaves, a gradient towards higher C-values downward in the plant, and a distinct pattern in the roots (Fig. 2). In a pair-wise comparison of diploid and tetraploid plants, the frequencies of nuclei at corresponding C-values were not significantly different ($P > 0.05$) for the three major C-values (2C, 4C and 8C) in cotyledons and transition zones, and the 2C-values in roots. Apart from the leaf 8C comparison ($P = 0.04$), the remaining comparisons yielded small but significant differences ($P < 0.01$).

3.3. Polysomaty in tissues of diploid plants in the greenhouse

Diploid plants, when grown in vitro, stayed small and did not flower, in contrast to greenhouse-grown plants. For comparison, the patterns of polysomaty were determined in plants grown for 33 days either in vitro or in the greenhouse. The cotyledon and the first leaf of greenhouse-grown plants had nuclei at much higher C-values than the same organs of the in vitro grown plants (Table 1). However, the

polysomaty patterns in young, small and not fully expanded leaves of greenhouse-grown plants (Fig. 3) were not different from the patterns in small in vitro grown leaves.

Figure 3 shows endopolyploidy patterns in a 3-month old tomato plant, which had just started developing its 15th and 16th leaf but had already shed the cotyledons. Typically, the tip of the leaf contained mostly 2C-nuclei (60–80%), both in young and older leaves. The sudden drop of 2C-levels to 10–20% in leaves 1 and 2 coincided with the leaves turning yellow. In the leaf petiole, which contains many vascular bundles, 2C-levels decreased during the development of the young leaf. Once fully expanded (leaf 10 and below), the leaf base contained 2C-nuclei at a frequency of 20–30%, while 4C-nuclei accounted for 40–50% of the nuclei. In yellowing leaves (leaf 1 and 2), 4C-levels decreased a little further, in favour of nuclei at higher C-levels. In this case, 64C-nuclei appeared, and even some traces of 128C-nuclei were found (0.2–0.5%, not visible in Fig. 3).

The development of polysomaty in the stem was similar to that in the leaf petiole. A major difference was that, in the stem area just below the yellowing leaves 1 and 2, no increase in the frequencies of nuclei at higher ploidy levels was found during ageing of the attached leaves.

4. Discussion

In this report, the presence of polysomaty was determined in diploid and tetraploid tomato

Table 1
Comparison of the level of polysomaty in leaves and cotyledons of 33-day-old plants grown in vitro and in vivo

		No. of plants tested	Percentage nuclei at different C-levels			
			2C	4C	8C	16C
<i>Leaf 1</i>						
In vitro	16		82.5 ± 0.8 ^a	14.7 ± 0.8	2.8 ± 0.3	—
In vivo	7		36.4 ± 5.8	58.7 ± 5.4	4.3 ± 0.4	—
<i>Cotyledon</i>						
In vitro	5		51.9 ± 2.7	35.1 ± 2.0	2.3 ± 0.2	2.3 ± 0.2
In vivo	6		9.5 ± 1.0	36.8 ± 2.1	46.4 ± 2.4	7.2 ± 0.5

^aMean ± standard error.

Fig. 3. Frequency of nuclei at different C-values (2C–128C) along a 3-month old tomato plant grown in the greenhouse, measured in the tip of the leaf blade (left), the base of the leaf petiole (centre) and the internode (right). Leaves and internodes were numbered from old to young. In this plant, the leaves were fully expanded from leaf 10 downward. Leaves 1 and 2 were turning yellow and contained traces of 128C nuclei (<0.5%, not visible in the diagram). The cotyledon had already been shed.

plants. Polysomaty was found in all organs at all stages of development of diploid plants; a low level of polyploid nuclei in the small, young organs, and high levels in fully differentiated organs. New cycles of endoreduplication marked the onset of ageing of the leaves, during which levels up to 128C were observed in the leaf petiole.

Diploid and tetraploid plants grown under the same environmental conditions were found to have similar patterns of polysomaty in corresponding organs (Fig. 2), even though the tetraploid plants have twice as much DNA per C-level compared to the diploid plants. This indicates that cells in organs with corresponding development and differentiation undergo the same number of endocycles. This conclusion is also supported by flow cytometric data from Sree Ramulu and Dijkhuis [11] and Pijnacker et al. [12] on *Solanum* genotypes and species, and data from Birader et al. [13] on diploid and tetraploid maize genotypes. In

1965, Titz [14] used nuclear volume (calculated from nuclear area) to determine the amount of DNA per nucleus. He found that the maximum level of polysomaty in some cell types (e.g. epidermal cells), measured in this way, was similar in diploid and tetraploid accessions of five out of seven species tested. Thus, the development of polysomaty is regulated genetically. However, the growth conditions of the plant also influenced the polysomaty patterns, as is clear from the differences in the patterns of polysomaty in cotyledons and leaves between plants grown *in vitro* and plants grown in the greenhouse (Table 1). The greenhouse plants had much higher levels of polyploid nuclei in their full-grown leaves. This could be a response to, for instance, different light conditions. Pea epicotyls contained higher levels of 8C- and 16C-nuclei when grown in the dark compared to the levels in light [15]. Alternatively, it could be due to differences in cell elongation and differ-

entiation [16], since the young, not fully developed leaves of greenhouse plants (Fig. 3) had polysomaty patterns similar to the small, not well developed *in vitro* leaves. This raises the question how intimately the development of polysomaty is related to the process of differentiation.

For the answer to this question, it is important first to notice that the development of polysomaty is regulated beyond the organ level. For instance, parts of different organs composed of similar tissues showed the same patterns of polysomaty (compare the leaf petiole with the stem internode in Fig. 3). Also, different parts of one organ showed different patterns of polysomaty (compare the leaf petiole with the leaf tip in Fig. 3), presumably because they are mainly composed of different tissues.

Two results indicate that the endoreduplication process is regulated not only beyond the organ, but also beyond the tissue level. First, a differentiation gradient from one organ to the other was observed, as in the cotyledon, hypocotyl and transition zone (Fig. 1), whose polysomaty patterns are related. These patterns appear to reflect a difference in duration of the process of endoreduplication, and this, in turn, may reflect the time during which the same developmental program has continued. A good candidate for this developmental program is cell elongation. In cucumber, Gilissen et al. [5] showed that transition zone, hypocotyl and cotyledon elongate successively after germination, and cycles of endoreduplication occurred during tissue elongation in these organs. In pea, endoreduplications took place during the first part of epicotyl elongation [15].

Second, when the tomato leaf started ageing, the petiole and leaf blade turned yellow simultaneously. This coincided with a strong increase in the frequency of nuclei with higher C-values in both petiole and leaf blade (Fig. 3), presumably by rounds of DNA synthesis in 2C- and 4C-nuclei. An alternative explanation is that the 2C-nuclei were depleted because of selective death. This is not as likely, however, since traces of 64C- and 128C-nuclei were absent in the other leaves, even when an excess of nuclei was measured. To our knowledge, DNA replication in tissues destined for self-destruction has not been reported before.

The reason for this process is unknown, but it took place in organ parts composed of very different tissues, as can be seen in the polysomaty patterns before ageing discussed above. In this case, the process of endoreduplication is coupled very clearly to a developmental program, rather than to any specific cell, tissue or organ.

Therefore, we may conclude that in tomato, the development of polysomaty is an integral part of differentiation, throughout the life cycle of a plant. This conclusion about the relationship between endoreduplication and cell differentiation is similar to the conclusion of Barlow [17] on the occurrence of endoreduplications as part of the morphological and biochemical differentiation of maize root cap cells. It has been found in many species that the differentiation and cell elongation of roots were accompanied by endopolyploidization [1]. However, using flow cytometry, we now have been able to extend this to other differentiation processes and other organs, which have not been so amenable to studies of DNA replication cycles in the past, and, most notably, also to the differentiation processes leading to programmed ageing and death.

Knowledge of the regulation of the phenomenon of polysomaty is relevant for many purposes. It may be useful in forcing back the occurrence of somaclonal variation at the level of unwanted polyploidization in cell and tissue cultures (11,12,18). It is important to understand its involvement in the cellular competence for regeneration and transformation [5]. Increased knowledge may also contribute to the identification of the genes involved, which might open possibilities for manipulation of differentiation processes.

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