Endoreduplication is not inhibited but induced by aphidicolin in cultured cells of tobacco

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Abstract
Endoreduplication is a common process in plants that allows cells to increase their DNA content. In the tobacco cell cultures studied in this work it can be induced by simple hormone deprivation. Mesophyll protoplast-derived cells cultured in the presence of NAA (auxin) and BAP (cytokinin) keep on dividing, while elongation and concomitant DNA endoreduplication are induced and maintained in a medium containing only NAA. If aphidicolin is given to the two types of culture, no effect is observed on elongating, endoreduplicating cells. However, the cells programmed for division switch to elongation and DNA endoreduplication. Thus aphidicolin, an inhibitor of the replicative DNA polymerases, α and δ, does not inhibit endoreduplication, and furthermore actually induces it when the mitotic cell cycle is blocked. DNA duplication and cell growth can only be completely blocked if ddTTP, an inhibitor of DNA polymerase-β, is given together with aphidicolin. This result implies that an aphidicolin-resistant DNA polymerase, such as the repair-associated DNA polymerase-β, can mediate DNA synthesis during endoreduplication and can substitute for polymerases-α and -δ when the latter are inhibited. Similar results are obtained in cultures of the BY-2 cell line by withdrawing auxins from the culture medium. In this cell line endoreduplication is induced only in a small proportion of the cells. A greater proportion of the cells are blocked in the G2 phase of the cell cycle.

Key words: Aphidicolin, DNA polymerase, endoreduplication, N. tabacum.

Introduction
DNA endoreduplication occurs widely in plants (Brodsky and Uryvaeva, 1977; D’Amato, 1984; Nagl et al., 1985; Galbraith et al., 1991; Larkins et al., 2001). While the function of the mitotic cell cycle is clear, the reasons why nuclei become polyploid in specific cells are still obscure (D’Amato, 1984; Cavalier-Smith, 1985; Nagl et al., 1985; Larkins et al., 2001). The mechanisms of the normal cell division cycle have been studied more intensely than those of endoreduplication and of the resulting polyploidy. Cyclins and cyclin-dependent kinases (cdks) are the main factors in the regulation of the mitotic cell cycle. Even if they are not exactly the same in different species, a general model for the control of the alternation between mitosis and replication is now accepted, and a unifying nomenclature has been proposed for plant and animal cyclins (Renaudin et al., 1996). Changes in the expression of cdks and cyclins have also been observed in relation to DNA endoreduplication, for example, in Drosophila melanogaster embryos (Sauer et al., 1995), in Arabidopsis thaliana shoot apices (Jacqmard et al., 1999) and in cereal endosperm (Larkins et al., 2001). Nagl suggested that changes in the expression of specific cdks and cyclins allow a switch from a mitotic cycle to an endoreduplication cycle (Nagl, 1995).

The enzymes that actually carry out DNA synthesis in eukaryotes are DNA polymerases-α and -δ (and possibly -ε; see below). The current model proposes that polymerase-α, with its associated primase, initiates synthesis on both leading and lagging strands (and may also carry out the majority of synthesis on the lagging strand) while polymerase-δ takes over from polymerase-α on the leading strand and may also be involved in completion of the Okazaki fragments on the lagging strand (Waga and
Stillman, 1998). The role of polymerase-ε, previously considered to be a repair enzyme, in DNA replication is not clear. It is essential for replication in yeast (Waga and Stillman, 1998) and in the current model for mammalian DNA replication may be involved in gap-filling or maturation, although it is not involved in the replication of SV40 DNA. DNA polymerase-β on the other hand is considered to be exclusively a repair enzyme (Wang, 1996).

It is well established that the structure and properties of the main DNA polymerase-α in plants are similar to those of animals (Misumi and Weissbach, 1982; Bryant et al., 1992; Garcia et al., 1997), and similarities between the plant DNA polymerase-δ and its animal counterpart have been reported (Richard et al., 1991; Matsumoto et al., 1994; Collins et al., 1997). However, there is as yet no proof for the existence of a DNA polymerase-ε-like enzyme in plants. In relation to repair, there is good evidence for the presence in plants of a DNA polymerase-β-like enzyme (Stevens et al., 1978; Chivers and Bryant, 1983; Dunham and Bryant, 1986; Luque et al., 1998) which is similar in properties and structure to the mammalian polymerase-β as far as has been determined.

One of the common properties of the replicative polymerases α and δ (and, in mammalian cells, of polymerase-ε) is their inhibition by aphidicolin (Syvaaja et al., 1990; Bryant et al., 1992; reviewed by Wang, 1996). This inhibitor has been used in many studies for the synchronization of cell cultures by blocking entry of cells into S-phase (e.g. tobacco BY-2 cells: Nagata et al., 1992).

In the experiments described here, this effect was tested on cell cultures of Nicotiana tabacum L. cv. Petite Havana where either division or endoreduplication may be obtained by a simple hormonal control (Tao and Verbelen, 1996). It is reported here that aphidicolin has no inhibitory effect on endoreduplication in this culture system, and furthermore, that it induces endoreduplication when the normal mitotic cell cycle is blocked. A parallel is drawn between the hormonal control and the aphidicolin effect on protoplast cultures and on BY-2 cells. These results are discussed in relation to the roles of DNA polymerases in endoreduplication of DNA and to regulation of the cell cycle.

Materials and methods

Cell cultures

Protoplasts were isolated from leaves of N. tabacum L. cv. Petite Havana grown in vitro, by enzyme treatment (2% cellulase Onozuka R10 and 0.2% macerozyme R10; Yakult Honsha Co.) for 5–6 h at 26 °C. Protoplasts were immobilized on K3A medium containing agarose in Petri dishes. After solidification, 1 ml of K3A, supplemented by α-naphthalene acetic acid (NAA) and 6-benzylaminopurine (BAP), both at a concentration of 1 mg l⁻¹, or supplemented only by NAA (1 mg l⁻¹) was layered on top of the agarose. Details of the protocol can be found in a previous paper (Tao and Verbelen, 1996). To study the effect of inhibitors, 50 μM aphidicolin or 40 μM ddTTP (dideoxy-thymidine triphosphate) were added at the beginning of the culture, and cells were maintained in these conditions until the day of observation.

BY-2 cells were cultured in an MS medium containing 30 g l⁻¹ sucrose and 0.2 g l⁻¹ KH₂PO₄ (Nagata et al., 1992). Cells were transferred every 7 d to a fresh medium and vitamins and 2,4-D (0.2 μg ml⁻¹) were added to the cultures.

CLSM and flow cytometry analysis

Suspensions of nuclei were obtained by chopping plant tissue and cells with a sharp razor blade in nuclei-stabilizing buffer (100 mM TRIS, 50 mM NaCl, 0.1% Triton X-100, pH 7). Propidium iodide (PI, Sigma Chemical Co), and DNase-free RNase (Boehringer Mannheim), at final concentrations of, respectively, 50 μg ml⁻¹ and 25 μg ml⁻¹, were added to the nuclei suspension 30 min before analysis. Nuclear DNA content was measured using FACS-scan flow cytometry. The same staining was used for in situ measurements in individual cells. The Confocal microscope was a Bio-Rad MCR 600 mounted on a Zeiss Axiopt, a 20 × objective (NA 0.4) was used with 1.25 mW laser power at 514 nm wavelength. The total fluorescence of nuclei, proportional to their DNA content, was calculated by multiplying the mean pixel density by the number of relevant pixels and is expressed in fluorescence units (fu). In vitro-grown tobacco leaves were used as a standard for 2C nuclei (for details see Valente et al., 1998).

Results

Cells derived from tobacco mesophyll protoplasts divide extensively in the presence of NAA and BAP while they only elongate in NAA. After 15 d of culture, divided cells had an average diameter of 38 μm and their nuclei had diameters between 6 μm and 12 μm. Elongated cells had an average length of 272 μm and the nuclear diameter reached 40 μm (Fig. 1a, b). Microfluorimetry of the nuclei by CLSM illustrates the relative differences in DNA content. This method was used because of the relatively small number of cells available and the difficulties encountered in preparing isolated nuclei from elongated cells. As the large nuclei were fragmented during isolation, no significant results could be obtained by flow cytometry. The tobacco leaf nuclei used as a standard exhibit a main peak at 15 000 fu (fluorescence units) that corresponds to a 2C DNA content (Fig. 2A). Nuclei from dividing cells after 20 d of culture exhibit 2C and 4C DNA levels (Fig. 2B) and the intermediate levels are indicative of a large proportion of the cells being in the S-phase of the cell cycle. In elongated cells of the same age, the DNA content reaches 8C and 16C levels (60 000 and 120 000 fu; Fig. 2C). This clearly shows that the elongated cells had undergone extensive endoreduplication in the medium containing NAA but lacking BAP.

The continuous presence of aphidicolin (50 μM) in the cultures gave surprizing results. In the NAA-only medium cell elongation was slightly affected (the average cell size...
of 214.1 μm was less than the control), but their nuclei enlarged in the same way as in the control (Fig. 1d). Microfluorimetry shows that 4C–8C–16C nuclei also predominate in the aphidicolin-treated cells (Fig. 2E). These unexpected results suggest that aphidicolin does not block DNA endoreduplication and calls into question the role of the replicative polymerases in this process.

When aphidicolin was added to the medium that supports cell division (i.e. containing NAA and BAP) cell division was inhibited as expected, but very unexpectedly the cells switched to DNA endoreduplication and cell elongation. Indeed, cell size, nuclear size and DNA content were similar to those observed in the cells endoreduplicating under cytokinin deprivation (Figs 1c, 2D). Thus aphidicolin, a specific inhibitor of the polymerase-α and -δ, not only fails to block endoreduplication, but it can actually induce it.

The failure of aphidicolin to block endoreduplication suggests that a DNA polymerase other than polymerases-α-δ (and -ε) can mediate this process. Therefore, 40 μM ddTTP (2,3-dideoxythymidine triphosphate), an inhibitor of the repair enzyme DNA polymerase-β was added to the cultures. In the absence of aphidicolin no effect was observed on dividing cells, nor on elongated cells (Fig. 1e–f), suggesting that polymerase-β did not participate in either normal DNA replication or endoreduplication. However, if ddTTP was added to cells together with aphidicolin then cells did not elongate any more

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**Fig. 1.** Protoplasts derived cells after 15 d of culture in different conditions. Nuclei are stained with PI (propidium iodide). Pictures on the left side show cells cultured in a medium supplemented with auxin N, and cytokinin B, and pictures on the right side show cells cultured in the presence of N only. (a, b) Control, (c, d) with aphidicolin (50 μM), (e, f) with dTTP (40 μM), (g, h) with aphidicolin and dTTP. The scale bar represents 200 μm.
(the average size was 122 μm compared with 214 μm with aphidicolin only) (Fig. 1g–h), and the endoreduplication that previously took place in the presence of aphidicolin did not occur (data not shown). The simplest conclusion from these data is that, normally, endoreduplication is mediated by polymerase-α and -δ and thus ddTTP, an inhibitor of polymerase-β, has no effect. However, when aphidicolin has blocked polymerases-α and -δ, then endoreduplication can be mediated by polymerase-β. When all three of these polymerases are blocked by adding both aphidicolin and ddTTP, endoreduplication does not occur. A further conclusion is that cells programmed for cell division enter a polymerase-β-mediated endoreduplication programme when DNA replication is blocked by aphidicolin.

Both the hormonal change and the aphidicolin induction of endoreduplication were also investigated in BY-2 cells. This cell line is only auxin (2,4-D)-dependent, therefore it was transferred to a fresh culture medium without 2,4-D. After 6 d of culture, cells showed extensive enlargement with an average size of 176.8 μm compared to 62.0 μm for the dividing cells (Fig. 3A, B). The measurement of DNA content by flow cytometry was straightforward for dividing BY-2 cells, but the preparation of

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**Fig. 2.** DNA content measurements made on 20-d-old protoplast-derived cells using CLSM, and expressed in fluorescence units (fu) measured at 514 nm. The values of 15,000, 30,000, 60,000, and 120,000 fu correspond to a DNA content of 2C, 4C, 8C, and 16C, respectively. For each sample the fluorescence of 300 nuclei was measured. (A) The standard: isolated nuclei from *in vitro* tobacco leaves have 2C nuclei. (B) Divided cells in NAA+BAP. (C) Elongated cells in NAA. (D) Elongated cells in NAA+BAP+Aphidicolin. (E) Elongated cells in NAA+Aphidicolin.
isolated nuclei from the elongated cells was more difficult, which is reflected by the noise in the dot plot (Fig. 3B). It would be expected that cells transferred from the stationary phase to a medium without 2,4-D would remain in G1 phase with a 2C DNA content (Fig. 3A). However, they did not: 34% of the cells went through one round of replication (4C) and 7% went through two rounds (8C) (Fig. 3B). Although the percentage of endoreduplicating cells was small (7%) compared with the protoplast culture, the effect of hormonal deprivation was similar in the two culture systems.

The authors did not succeed in obtaining flow cytometry data for aphidicolin-treated cells. For this reason the DNA content was measured by microfluorimetry. Aphidicolin is commonly used in BY-2 cells for cell cycle synchronization. The cells are blocked in G1 for a maximum of 24 h, and after an intensive wash a high percentage of the cells enter the S-phase synchronously. In the experiments described here the effect of aphidicolin was studied over a longer period of time: 24 h after their transfer to fresh medium with aphidicolin (50 μM) the cells were still blocked in the G1 phase (2C DNA content corresponding to 15 000 fu) as expected (Fig. 4B). After 48 h the G1 peak had disappeared and the DNA content values were spread between 10 000 and 30 000 fu (Fig. 4C). At 72 h a 4C peak (30 000 fu) appeared corresponding to cells in the G2 phase (Fig. 4D), and this situation remained until after 6 d of culture (Fig. 4E). At that time the control cells in division had already entered the stationary phase and the majority of them had a 2C DNA content (Fig. 3A). Cells were obviously blocked by aphidicolin during the first 24 h of culture, but after a period of 48 h they overrode the aphidicolin block and underwent at least one round of replication. They did not divide at any time and they reached a size comparable to the size of cells cultured without 2,4-D.

**Discussion**

Current ideas on the role of cytokinin in the regulation of the plant cell cycle involve regulation of entry into the S-phase and into mitosis (Soni et al., 1995; Frank and Schmülling, 1999; Laureys et al., 1998). In contrast to the expectations (Frank and Schmülling, 1999) omission of cytokinin from the medium for protoplast culture not only prevented normal cell division in mesophyll protoplast-derived cell cultures, but it also led to cell elongation and DNA endoreduplication (Valente et al., 1998, and results presented here).

This suggests that different controls (cdks, cyclins) act on the entry into S-phase during endoreduplication and during normal replication. Lines of support come from
the findings that in *Drosophila melanogaster* (Sauer et al., 1995), *Arabidopsis thaliana* (Jacqmard et al., 1999) and *Zea mays* (Larkins et al., 2001) there are indeed differences in the populations of cyclins and cdk's between replicating and endoreduplicating cells.

It is well known that aphidicolin inhibits the replicative DNA polymerases of plants (Bryant et al., 1992), and the synchronization of cultures of tobacco cells by aphidicolin relies on this. However, the data presented here show that DNA endoreduplication can occur in the presence of aphidicolin. The most obvious explanation of these data is that DNA endoreduplication may be mediated by another polymerase, such as DNA polymerase-β. Indeed, only the presence of both ddTTP and aphidicolin in the culture medium blocks the entry of the cells in endoreduplication. There are precedents for this. For example, in rat giant trophoblast cells, DNA endoreduplication is not inhibited by aphidicolin, but is inhibited by ddTTP, an inhibitor of DNA polymerase-β (Siegel and Kalf, 1982). However, in the results presented here, ddTTP does not inhibit endoreduplication in the absence of aphidicolin, which suggests that polymerases-α and -δ are certainly able to mediate endoreduplication and presumably normally do so.

Most surprisingly the inhibition of the replicative polymerases in cells maintained in the auxin plus cytokinin medium also leads to entry into the elongation/endoreduplication programme, as if the cells had been maintained under cytokinin deprivation.

BY-2 cells are only auxin dependent as they produce endogenously the cytokinins necessary for their development. When 2,4-D was omitted from the culture medium, cells did not go into division but enlarged considerably. Their DNA was replicated at least once, and 7% of the cells went through two cycles of replication. The auxin 2,4-D is thus necessary for the entry of BY-2 cells into mitosis, but not for their progression through S-phase.

Also in BY-2 cells, aphidicolin inhibited M-phase, but failed to inhibit the replication process. Replication could not occur during the first 24 h of the aphidicolin block presumably because of the inhibition of the polymerases α and δ. But then BY-2 cells managed to override this block, entered S-phase and reached a 4C DNA content. The mitotic process was, however, blocked. Exceptionally, a cell went through a second round of DNA replication. This suggests that BY-2 cells can also use another enzyme to replicate their DNA, such as polymerase β, and that aphidicolin has an effect on the G(2)/M checkpoint and does not allow cells to enter mitosis.

The two tobacco cell cultures used in this research have different requirements, they are cultured in different conditions and under different hormonal controls. However, they show similarities in their reaction to hormonal deprivation and to aphidicolin. In both cases there is a change in the regulation of the cell cycle that does not allow cells to go into mitosis, but allows DNA replication that eventually drives them into endoreduplication. The hormone deprivation acts directly on the G(2)/M transition (Soni et al., 1995; Frank and Schmülling, 1999; Laureys et al., 1998), but the explanation of the dual aphidicolin
Further experiments on the process of replication and on the control of the cell cycle will help in the understanding of the mechanisms of this complex but interesting switch from division to endoreduplication.

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