FUNCTIONAL ROLES OF THE PLANT α-LIKE AND γ-LIKE DNA POLYMERASES

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1. Introduction

Plant cells are endowed with two distinct DNA polymerases [1,2] whose properties closely resemble those of the DNA polymerases α and γ present in animal cells [3,4]. The plant DNA polymerases have consequently been named α-like [1] and γ-like [2].

The α-like DNA polymerase activity is the most abundant in cultured plant cells [1] and responds to changes in the rate of cell multiplication, whereas experiments with spinach leaves have shown that the γ-like DNA polymerase is present in the chloroplast [2].

A DNA polymerase has also been isolated from the mitochondria of wheat embryos [5]. Spinach mitochondria may also contain a DNA polymerase whose properties are partially different from those of the γ-like DNA polymerase isolated from the chloroplasts of the same cells and are similar to those of the wheat embryo enzyme (unpublished).

However, no evidence is available as yet on the existence in plant cells of a DNA repair enzyme similar to the DNA polymerase β of mammalian cells [4,6].

By analogy with animal cells, the assignment of functions to the DNA polymerases in plant cells is hampered by the lack of conditional mutants defective in DNA synthesis. Thus, we approach this problem by exploiting the properties of aphidicolin and of ethidium bromide.

Aphidicolin [7] specifically inhibits the α-like DNA polymerase purified from plant cells [8], while the chloroplast γ-like enzyme [2,8] and the mitochondrial DNA polymerase (unpublished) obtained from spinach leaves are not affected. In vivo experiments with cultured rice cells [8] have also shown that the drug affects the incorporation of precursors into DNA, but not into RNA or proteins.

Ethidium bromide interferes preferentially with ctDNA and mtDNA, thus preventing their replication [9,10].

We now describe the effect of aphidicolin and of ethidium bromide on the synthesis of DNA in the nucleus, chloroplast and mitochondrion of cultured rice cells, as assessed by autoradiography at light and electron microscopy. The results show that aphidicolin specifically prevents the synthesis of nuclear DNA, while it has no effect on the synthesis of the organellar DNA, the latter being specifically affected only by ethidium bromide. This, together with the previous demonstration that only the α-like DNA polymerase is inhibited in vitro by aphidicolin [8], proves that the plant α-like DNA polymerase plays an essential role in the replication of nuclear DNA, and that this enzyme is not involved in the replication of plastid and mitochondrial DNA. Thus, the replication of the plant organellar DNA requires different DNA polymerases, the aphidicolin-resistant DNA polymerases present in the chloroplast [2] and in the plant mitochondrion [5] being the best candidates for this function.

Abbreviations: ctDNA, chloroplast DNA; mtDNA, mitochondrial DNA; EM, electron microscope

2. Materials and methods

2.1. *Chemicals*

[*methyl*-^{3}H]Thymidine (25 Ci/mmol) was from the Radiochemical Centre, Amersham. Aphidicolin

was kindly supplied by Imperial Chemical Industries, England. Ethidium bromide was from Sigma Chemical Co., St Louis, MO.

2.2. *Plant cell growth*

Cell suspension cultures of rice (*Oryza sativa* L. cv Roncarolo) were established and maintained as in [11]. Cells were harvested during the early exponential phase of growth (4 days after inoculation into fresh medium) [11], washed twice with fresh medium and immediately utilized for the experiments on thymidine incorporation.

2.3. *Measurement of the incorporation of thymidine into the acid-insoluble material of suspension cultured rice cells*

For each experimental point 50 mg cells (fresh wt) in 0.5 ml culture medium were incubated at 26°C on a rotatory shaker (120 rev./min). After 30 min, aphidicolin and/or ethidium bromide were added as indicated in the figures and incubation continued for 30 min. Then, [^{3}H]thymidine (2.5 μCi) was added and the complete mixture incubated for 2 h. Control experiments contained no inhibitors. Incubation was stopped by adding 1 ml 10% trichloroacetic acid; then the suspension was homogenized with a motor-driven teflon-in-glass homogenizer and the insoluble material collected and washed 9 times with 5 ml 5% trichloroacetic acid on a Whatman GF/C glass filter. Radioactivity was determined in an Insta-gel liquid scintillation cocktail.

2.4. *Autoradiographic analysis of the cells after incorporation of [^{3}H]thymidine*

Cells were incubated as in section 2.3., except that 100 mg cells (fresh wt) in 1.0 culture medium were used and that [^{3}H]thymidine was added at 30 μCi/ml. The complete mixture was incubated for 3 h. When present, aphidicolin and/or ethidium bromide were at the concentrations specified in the figures.

Incubation was stopped by washing the cells 10 times with 5 ml fresh culture medium and labelled DNA was revealed by means of autoradiography of both thick and ultrathin sections. To this purpose, the cell samples were fixed for 1 h at 4°C in a medium containing 4% glutaraldehyde and 100 mM sodium–potassium phosphate (pH 7.0) (buffer A), washed for 12 h with several changes of buffer A, postfixed in 1% osmium tetroxide in buffer A, dehydrated and finally embedded in epon–araldite [12].

Sections (1 μm thick) were prepared from the polymerized samples with an LKB 8802 A ultratome, collected on slides, coated with an autoradiographic emulsion (Kodak NTB2) and dried. After exposure (4 days) the preparations were developed (in Kodak D-19 for 3 min at 20°C) and stained with 1% azur B at pH 9.0.

In the case of EM autoradiography, gold sections were collected on carbon-coated grids and double stained with uranyl acetate and lead citrate. Coating with Ilford L4 emulsion was done as in [13]. After exposure (40 days at 4°C) the grids were developed in Microdol X for 4 min and then fixed (Kodak Quick Finish). Electron micrographs were taken with an Hitachi 11 B electron microscope operating at 75 kV.

3. Results

3.1. *Aphidicolin inhibits partially, but not totally, the incorporation of thymidine into cultured plant cells*

We had noticed that aphidicolin affects DNA synthesis, but not RNA and protein synthesis, in cultured plant cells [8].

As fig.1A shows, 64% of the incorporation of thymidine into the acid-insoluble material in growing rice cells is inhibited by the addition of saturating doses of aphidicolin (>6 μM). A similar inhibition curve was obtained with exponentially growing cells of *Daucus carota* L. (unpublished). This response is similar to, although quantitatively different from that obtained in mammalian cell cultures (HeLa cells) where thymidine incorporation dropped to 2% of control following addition of the drug [14]. In the latter case, the residual thymidine incorporation is due to the aphidicolin-resistant mitochondrial DNA. Ethidium bromide is known to interfere preferentially with both mitochondrial and chloroplast DNA, thus preventing their replication, its action being not quite specific since at high doses it also affects the replication of nuclear DNA [9,10]. We have thus tested the effect of ethidium bromide on the residual 36% aphidicolin-resistant thymidine incorporation. As fig.1B shows, when supplied alone, ethidium bromide affects, although incompletely, the incorporation of the precursor. A possible explanation of the data is that at low doses ($\leq$50 μM) ethidium bromide acts preferentially on the organellar DNA synthesis, while at higher doses it may also partially affect the replication of

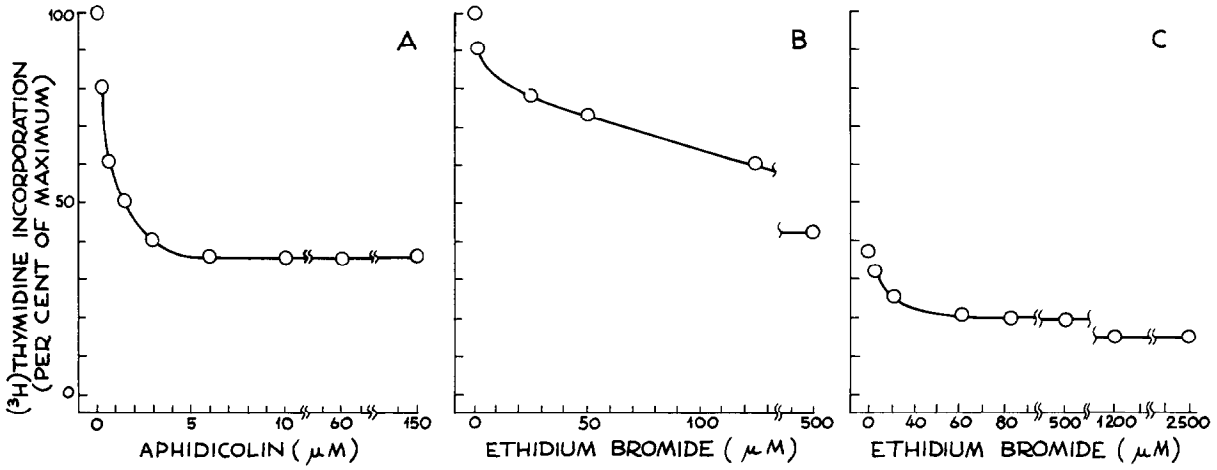

Fig.1. Effects of increasing concentrations of aphidicolin (A), ethidium bromide (B) and ethidium bromide in the presence of 60 μM aphidicolin (C) on the incorporation of [^{3}H]thymidine into the acid-soluble material in cells of *Oryza sativa* L. grown in suspension culture. The experiments were performed as in section 2. The results are expressed as % of the incorporation observed in the absence of the drugs (230 000 dpm).

nuclear DNA. In fact, when supplied at low dose in the presence of a saturating amount of aphidicolin (fig.1C), ethidium bromide inhibits almost 50% of the aphidicolin-resistant thymidine incorporation. The residual acid-precipitable radioactivity (~20% of total incorporation) is insensitive to the simultaneous presence of both drugs and most likely represents the utilization of [^{3}H]thymidine for syntheses other than DNA.

This interpretation is supported by the results of section 3.2. and by the finding that ~15% of the incorporated radioactivity is insensitive to digestion with pancreatic DNase when the enzyme is added to a crude cell extract, while DNA purified from the same cell extract is fully degraded in the same conditions.

3.2. *Aphidicolin specifically inhibits the synthesis of nuclear DNA*

The above experiments have shown that the incorporation of [^{3}H]thymidine in growing rice cells can be split into three classes:

(i) Incorporation sensitive to aphidicolin (64% of total);

(ii) Incorporation insensitive to aphidicolin but sensitive to low doses of ethidium bromide (~16% of total);

(iii) Incorporation insensitive to both drugs (~20% of total).

An autoradiographic analysis of the cells was desirable in order to define the intracellular location and significance of such classes.

To this purpose, exponentially growing rice cells were incubated with aphidicolin and/or ethidium bromide in the presence of [^{3}H]thymidine and subsequently analyzed by light and EM autoradiography for the intracellular distribution of the incorporated radioactivity. The results are shown in fig.2,3. Quantitative data have also been obtained by counting the percentage of labelled nuclei and the number of grains in the cytoplasm per unit area (table 1).

The data show that aphidicolin affects the number of labelled nuclei, but not the label in the organelles. The reverse is true for ethidium bromide. In fact:

(i) 8.9% of nuclei are heavily labelled in the control samples incubated in the absence of inhibitors (fig.2A,B table 1). Evidently, these nuclei were

Fig.2. Autoradiographs at light (A,C) and electron microscopy (B,D) of sectioned rice (*Oryza sativa* L.) cells, after incubation for 3 h in the presence of [^{3}H]thymidine as in section 2.4: (A,B) controls (no additions); (C,D) aphidicolin (66 μM) was added at the beginning of the incubation. Bars: 10 μm in A,C; 1 μm in B,D.

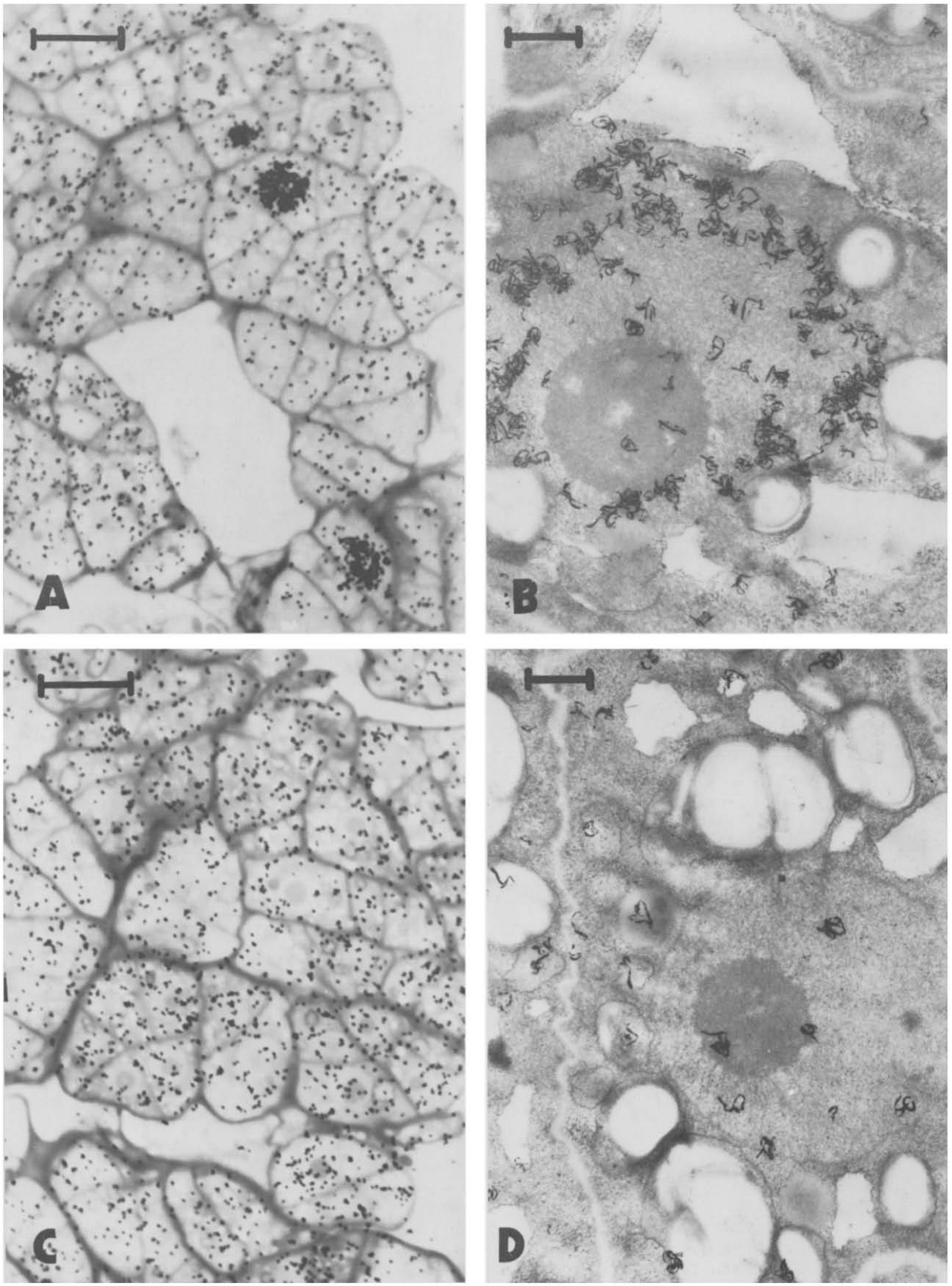

Fig.2

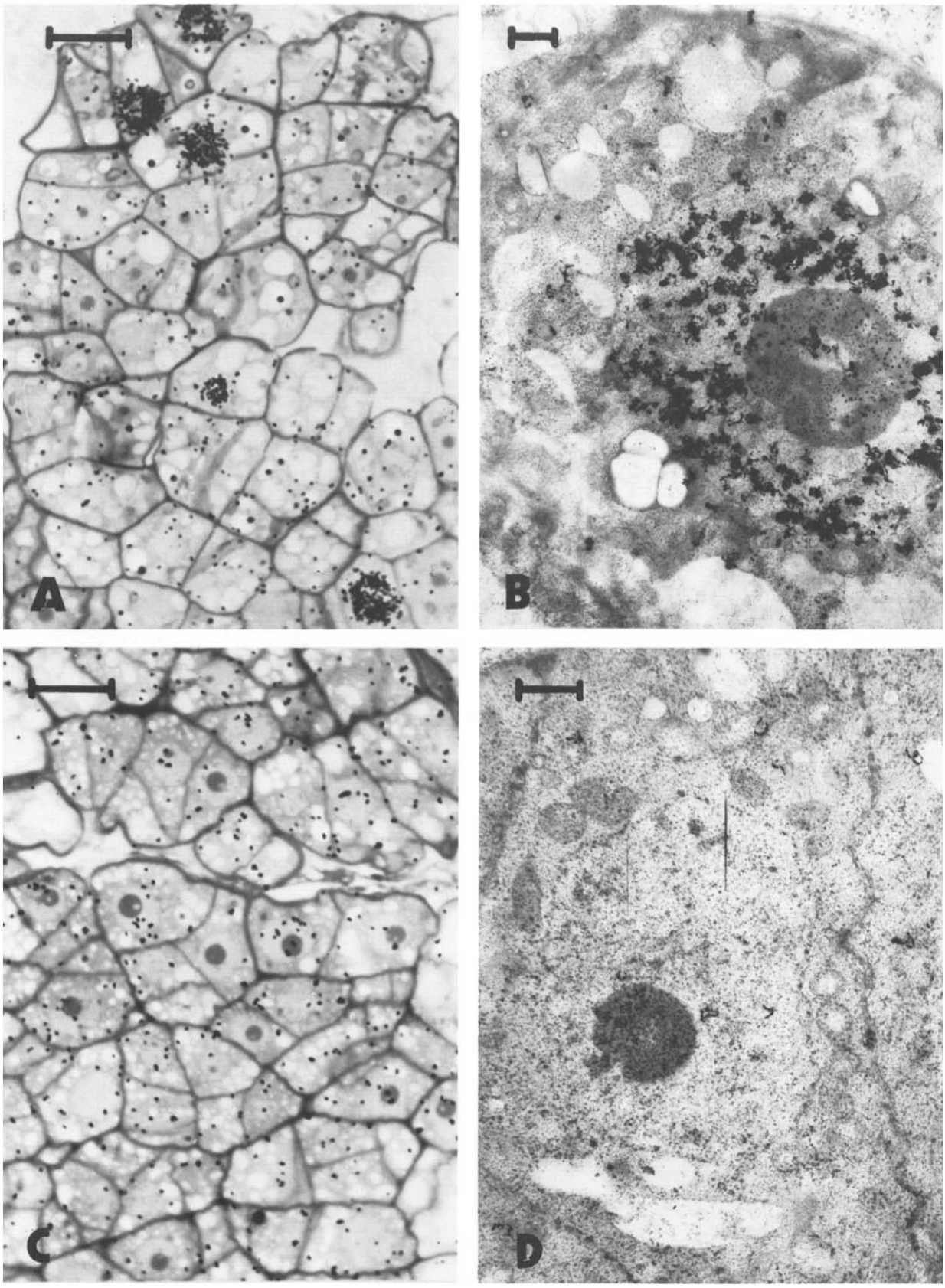

Fig.3

in the S phase of their cell cycle during the incubation with [^{3}H]thymidine. Radioactivity is also observed in the cytoplasmic region with preferential incorporation into plastids and mitochondria, here again suggesting incorporation into the organellar DNA.

(ii) The addition of aphidicolin completely inhibits the incorporation of thymidine into nuclear DNA (fig.2C,D, table 1). The effect is selective: the incorporation of the precursor into plastid and mitochondrial DNA, as well as that in the ground cytoplasm, is not affected by the drug (fig.2D, table 1). About 52–54% of the silver grains found in the cytoplasm are concentrated in the organellar regions, the remaining being scattered over the ground cytoplasm.

(iii) At the tested doses, ethidium bromide selectively inhibits the incorporation into the organellar DNA, while nuclear DNA synthesis proceeds, together with the incorporation in the ground cytoplasm (fig.3A,B, table 1).

(iv) When aphidicolin and ethidium bromide are present at the same time, both nuclear and organellar DNA synthesis are inhibited. The residual [^{3}H]thymidine incorporation is evenly distributed in the cell and is no more concentrated in the DNA bearing organelles (fig.3C,D, table 1). In fact, in this case only 8% of total grains are localized over organelles, the remaining 92% being spread over the ground cytoplasm.

Thus, aphidicolin specifically inhibits the synthesis of nuclear DNA, while the synthesis of plastid and mitochondrial DNA is only affected by ethidium bromide.

A substantial fraction of [^{3}H]thymidine incorporation is resistant to both drugs. This is 15–20% of total incorporation (fig.1C) or 40–46% of the radioactivity incorporated into the cytoplasm. Most likely, this corresponds to the DNase insensitive fraction described in section 3.1. and does not represent DNA synthesis but is rather the consequence of [^{3}H]thymidine metabolization and utilization for different cellular syntheses. In fact, it is already known that plant cells partially degrade exogenously supplied thymidine [17–19].

4. Discussion

These results prove that the α-like DNA polymerase present in plant cells is involved in the replication

Table 1
Effect of aphidicolin and/or ethidium bromide on nuclear and cytoplasmic labelling in rice cells grown in the presence of [^{3}H]thymidine, as examined in light microscope autoradiographs

Additions	% of labelled nuclei	Grains in the cytoplasm per unit area[a]	
		total	% of control
None	8.9	37.20 ± 0.48	100
Aphidicolin (66)	<0.01	38.25 ± 0.32	102.8
Ethidium bromide (50)	10.2	17.35 ± 0.59	46.8
Ethidium bromide (500)	9.9	14.62 ± 0.63	39.5
Aphidicolin (66) and ethidium bromide (50)	<0.01	15.82 ± 0.59	42.5
Aphidicolin (66) and ethidium bromide (500)	<0.01	13.57 ± 0.86	36.5

[a] Unit area was 11 μm^2

The number of labelled nuclei was evaluated on the light microscope autoradiographs obtained in the experiment described in fig.2,3 by examining at least 40 slices, each slice containing ~1000 cell sections. Grain counts were performed on the same experimental material. Each value refers to the average of data obtained from 50 unit areas

Fig.3. Autoradiographs at light (A,C) and electron microscopy (B,D) of sectioned rice (*Oryza sativa* L.) cells, after incubation for 3 h in the presence of [^{3}H]thymidine as in section 2.4. The following additions were made at the beginning of incubation: (A,B) ethidium bromide (50 μM); (C,D) aphidicolin (66 μM) and ethidium bromide (50 μM). Bars: 10 μm in A,C; 1 μm in B,D.

of nuclear DNA. This adds conclusive evidence to our proposal that the plant α-like DNA polymerase [1] is functionally correspondent to the DNA polymerase α of vertebrate cells [4]. In fact, we had observed that, like the animal enzyme, the plant polymerase is the most abundant in exponentially growing cells and responds to changes in the rate of cell multiplication. Furthermore, like the animal enzyme, the α-like DNA polymerase purified from rice cells has a sedimentation coefficient of 7 S, with an app. M_r 180 000, has a neutral pH optimum, prefers Mg^{2+} over Mn^{2+}, is sensitive to *N*-ethylmaleimide, is inhibited at high ionic strength, prefers activated DNA as primer-template and has no or low activity on the synthetic primer template $(dT)_{12-18}$ · poly(rA) [1].

It has been reported that DNA polymerase α, in addition to the γ polymerase, is also involved in the replication of mammalian mitochondrial DNA [20]. Our experiments disprove this suggestion in the case of plant cells and show that the synthesis of mtDNA as well as that of ctDNA is mediated by an enzyme other than the α-like DNA polymerase. This agrees with the results of recent experiments on isolated mammalian mitochondria which show that DNA polymerase α is not involved in mtDNA replication, a process which requires only the γ polymerase [16].

The results also add further strength to the proposal that the aphidicolin-resistant DNA polymerase found in spinach chloroplasts is specifically involved in the replication of ctDNA [2]. This polymerase has been named γ-like since, although some distinctive features are evident, it shares many of the properties of the animal γ polymerase. In fact, like the animal enzyme, it has a sedimentation coefficient of 5.9, corresponding to app. M_r 105 000 and prefers $(dT)_{12-18}$ · poly(rA) as a primer-template. The activity is dependent on Mn^{2+} and on high ionic strength, is resistant to aphidicolin, inhibited by *N*-ethylmaleimide and shows a pH optimum of 8–9.

By analogy with the chloroplast enzyme we expect that the plant mitochondrial DNA polymerase [5] is the replicative enzyme of mtDNA and thus that all DNA polymerases of the γ-type present in eukaryotes are involved in a strand displacement synthesis of the organellar DNAs.

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