DNA REPAIR SYNTHESIS IN PLANT PROTOPLASTS IS APHIDICOLIN-RESISTANT

Francesco SALA, Etienne MAGNIEN*, Maria Grazia GALLI+, Xavier DALSCHAERT*, Guido PEDRALI-NOY and Silvio SPADARI

*Istituto di Genetica Biochimica ed Evoluzionistica, CNR, Via S. Epifanio, 14, Pavia, *Biology Group DG XII Joint Research Centre, Ispra* and *+Istituto di Scienze Botaniche, Centro di Studio del CNR per la Biologia Cellulare e Molecolare delle Piante, Università di Milano, Milano, Italy*

Received 27 December 1981

1. Introduction

Animal cells contain 3 distinct DNA polymerases, which, on the basis of studies with isolated organelles and with specific inhibitors [1–9] have been assigned the following main, but possibly not exclusive, functions: α-polymerase is involved in nuclear DNA replication; β-polymerase is involved in nuclear DNA repair synthesis; and γ-polymerase is involved in mitochondrial DNA replication.

In plant cells the presence of an α-like DNA polymerase [10,11], responsible for nuclear DNA replication [12], a chloroplast γ-like [13] and a mitochondrial DNA polymerase [14], possibly involved in organellar DNA replication [12], have been reported. However, there is no clear evidence as to the existence of a β-like DNA polymerase in plants [11].

Since the repair of radiation-induced DNA lesions also occurs in the cells of higher plants [15], we have decided to investigate whether, at variance with animal cells, this DNA repair synthesis is due to the activity of the nuclear α-like DNA polymerase. To this end, we have used aphidicolin [16], a specific inhibitor of the α-like DNA polymerase of higher plant cells [17], as well as that of the DNA polymerase α of animal cells [4,6,9,18].

The results show that the UV light-induced DNA repair synthesis in protoplasts of *Nicotiana sylvestris* is resistant to this drug and thus is not performed by the α-like DNA polymerase.

Contribution no. 1835 of the Biology, Radiation Protection and Medical Research Programme, Directorate General XII of the Commission of the European Communities

2. Materials and methods

2.1. *Chemicals*

[*methyl*-1′,2′-^{3}H]Thymidine (74 Ci/mmol) was obtained from the Radiochemical Centre, Amersham. Aphidicolin was kindly supplied by Imperial Chemical Industries, England. Pancreatic DNase was from Sigma.

2.2. *Plantlet culture and protoplast isolation*

Plantlets of *Nicotiana sylvestris* Spegazzini et Cones were aseptically cultured in vitro by micro-cuttings of nodes with axillary buds. The synthetic substrate was an agar medium [19] supplemented with the vitamins recommended in [20], 200 mg glutamine/l, 5 g sucrose/l and 6 g activated charcoal/l. Controlled growth conditions were 16 h photo-periods at 3000 lux at 24–28°C.

Protoplasts were isolated from 2–3 cm long leaves. This stage of development corresponds in this particular species to a good cytological homogeneity, with >90% of G_1-phase diploid nuclei [21]. The isolation procedure implied carborundum brushing, enzymatic digestion and stationary density gradient purification, as in [19].

2.3. *UV light-induced [^{3}H]thymidine incorporation*

Freshly isolated protoplasts were suspended at 5×10^4/ml culture medium [19] and plated as a thin-layer in Petri dishes. After addition of aphidicolin (10 μg/ml) and [^{3}H]thymidine (30 μCi/ml) the protoplast suspension was exposed for 6 min and 40 s to an irradiation of 10^4 ergs . mm^{-2} from a germicide lamp at 25 ergs . s^{-1} . mm^{-2}. This dose induced total mitotic inactivation and produced an appreciable radiation-induced thymidine incorporation. Lower

Published by Elsevier Biomedical Press
00145793/82/0000–0000/$02.75

UV-light doses were less effective. A supplement of aphidicolin (10 μg/ml) was then added, the protoplasts incubated for 3 h in the dark at 25°C and washed twice in a cold isotonic mineral solution (600 mOsm). After counting the protoplast number with a hemocytometer, each pellet was mixed with an equal volume of 0.2 M NaOH and the resulting lysate precipitated on a fiber glass filter pre-treated with 2% SDS. The filters were repeatedly washed with 5% Na-pyrophosphate, with 5% trichloroacetic acid and ultimately with absolute ethanol. After drying under an infra-red lamp, radioactivity on the filters was counted by scintillation spectrometry (efficiency, 34%).

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

After incubation with [^{3}H]thymidine and 2 washes with the isotonic mineral solution (section 2.3) the protoplasts were resuspended for 1 h at 4°C in 5% glutaraldehyde in 100 mM phosphate buffer. The protoplasts were then collected by centrifugation, washed 3 times with phosphate buffer and resuspended for 1 h in 2% osmium tetroxide. After washing in water, an aliquot of protoplasts was dried on a slide, stained by the standard Feulgen procedure, autoradiographed as described below and exposed for 13 days for the determination of % of labelled nuclei. Two stained slides were treated with 0.5 mg DNase/ml in 3 mM $MgSO_4$ at pH 6.5 for 4 h at 37°C.

The remaining protoplasts were embedded in 3% agar. Solidified agar was sliced into small blocks which were dehydrated and embedded in epon–araldite [22].

Sections were obtained with an LKB 8802 A ultramicrotome, collected on slides and coated with autoradiographic emulsion (NTB2). After exposure (2 months) the specimens were processed in Kodak D19 solution for 4 min at 20°C and stained with 2% Azur B at pH 9.

3. Results

We have chosen protoplasts for this study on DNA repair synthesis in plants since they offer many of the cultural and experimental advantages of unicellular organisms together with the absence of a cell wall which may interfere with the UV treatment. However, preliminary experiments showed that those protoplasts which are considered optimal for cultural characteristics, such as cell wall regeneration and resumption of mitotic activity, are not convenient for this study.

This was the case with protoplasts from leaves of *N. plumbaginifolia*, where nuclear DNA synthesis resumes within a short time after cell wall digestion [23]. This synthesis is inhibited by aphidicolin, similarly to that shown in cultured plant cells [17] and may therefore be attributed to the activity of the replicative α-like DNA polymerase. However, a 1–2% residual incorporation of the radioactive DNA precursor, observed even in the presence of saturating doses of the drug, hinders the attempts to show a UV-induced [^{3}H]thymidine incorporation into nuclear DNA since the rate of DNA repair synthesis in higher organisms is expected to be ⩾3 orders of magnitude lower than that found in nuclear DNA replication [5].

A similar problem with animal cells was approached by exploiting the unique property of brain neurons which, in adult rats, do not carry out nuclear DNA replication, thus allowing a study of the UV-induced DNA repair synthesis [3,5].

Similarly, we have used plant protoplasts prepared from leaves of *N. sylvestris* which, under the experimental conditions used, do not carry out replicative DNA synthesis and do not enter cell division during the first 3–5 days of culture.

As table 1 shows, with this experimental material, a consistent stimulation of the incorporation of [^{3}H]-thymidine into trichloroacetic acid-insoluble material was detectable following irradiation with UV-light. Both the background and the UV-induced incorporations were resistant to aphidicolin and are thus in no case attributable to nuclear replicative DNA synthesis.

To define the intracellular localization of the incorporation product, an autoradiographic analysis of fixed cells was performed. Freshly prepared protoplasts were irradiated with UV-light, incubated with or without aphidicolin in the presence of [^{3}H]thymidine and subsequently fixed and analyzed by autoradiography for the intracellular distribution of the incorporated radioactivity.

The results show that:

(1) About 40% of the irradiated protoplasts (table 2, fig.1 A,D) where preferentially labelled in the nuclear region. The label was localized in the DNA molecules, since treatment of the fixed slides with pancreatic DNase resulted in disappearance of all nuclear grains.

At variance from the results obtained in similar experiments with animal cells [7], the remaining fraction of protoplasts did not show preferential nuclear

Table 1
Enhancement of thymidine incorporation in protoplasts of *Nicotiana sylvestris* by UV irradiation (cmp/10^5 protoplasts)

	Unirradiated (a)	Irradiated (b)	Induced incorp. (b−a)
No aphidicolin	1651	2359	708
Aphidicolin (20 μg/ml)	1622	2224	602

Samples of 3×10^5 protoplasts were prepared, exposed to UV-light, and incubated in the presence of [^{3}H]thymidine for 3 h as in sections 2.2 and 2.3. The incorporation of radioactivity into the trichloroacetic acid-insoluble fraction was measured as in section 2.3

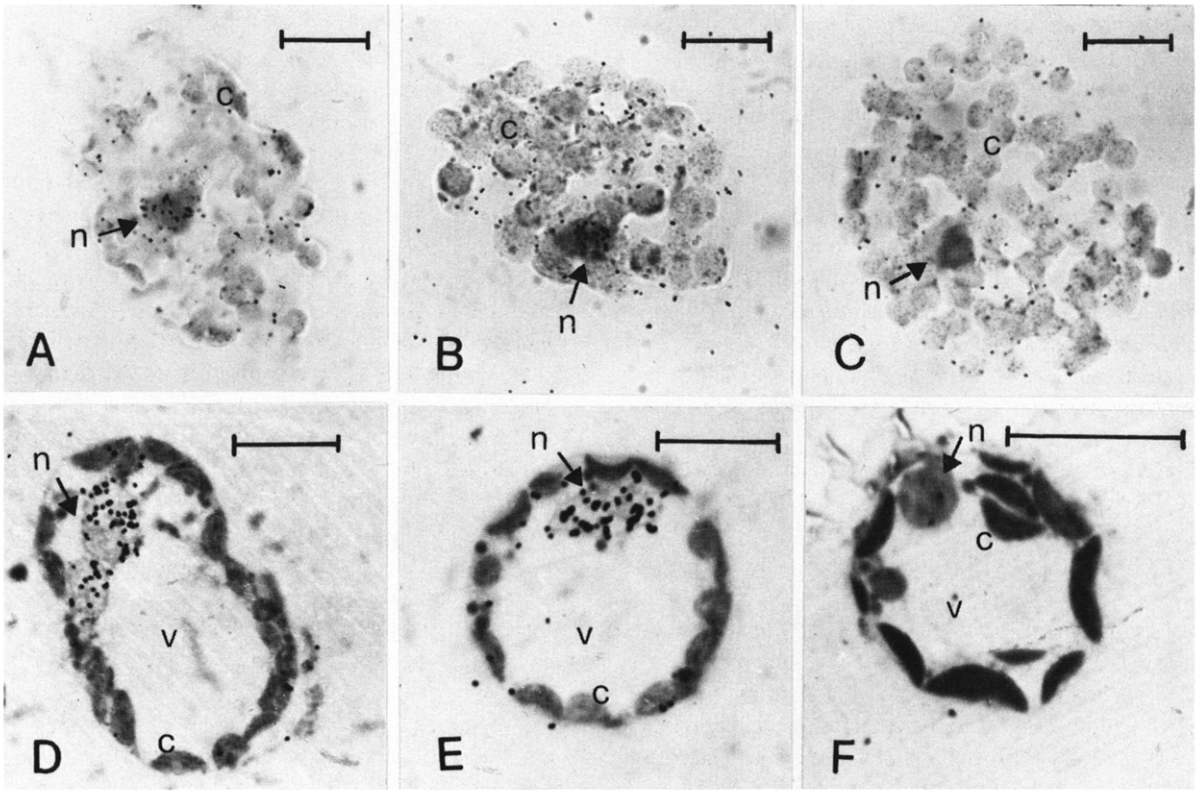

Fig.1. Light microscopic autoradiographs of whole or sectioned *Nicotiana sylvestris* protoplasts, after incubation for 3 h in the dark in the presence of [^{3}H]thymidine (section 2): (A,D) UV-irradiated protoplasts; (B,E) protoplasts treated as in (A) but incubated in the presence of 20 μg/ml aphidicolin; (C,F) unirradiated protoplasts. (c) chloroplast; (n) nucleus; (v) vacuole; bars, 10 μm.

Table 2
Labelling of nuclei in protoplasts of *Nicotiana sylvestris* induced by UV irradiation

	Unirradiated	Irradiated	
	Labelled nuclei	Labelled nuclei	% of total
No aphidicolin	0	202	40.4
Aphidicolin (20 μg/ml)	0	195	39.0

Protoplasts were treated as in table 1 and the autoradiographic analysis was performed on whole protoplasts as in section 2.4. Quantitative data were obtained by screening 500 protoplasts for each experiment. Labelled nuclei were considered those with >15 grains; this value is significantly higher than the background in the cytoplasm

labelling. The reason for this behaviour is not known, although a number of interpretations may be given, among which: partial loss of viability in the protoplast population or shielding of the nuclear region from the UV-light by the cytoplasmic organelles or by whole protoplasts may come into consideration. The UV treatment was performed in an iso-osmotic suspension where some protoplasts float and others sink.

(2) The addition of aphidicolin did not decrease the percentage of UV-stimulated labelled nuclei and the average number of grains per nucleus (fig.1B,E) The inhibitory effect of aphidicolin on replicative DNA synthesis has been shown in plant cells [13] and in protoplasts of *Daucus carota* L. and of *N. plumbaginifolia* (unpublished).

(3) In control unirradiated protoplasts no labelled nuclei were detected (table 2, fig.1C,F). The observed silver grains were diffused all over the cytoplasm (fig. 1C). Part of the label (43% of total) could be removed by pancreatic DNase treatment. Thus, it most probably represents incorporation of [^{3}H]thymidine into the organellar DNA [12]. In fact, the leaf-derived protoplasts utilized in this study were particularly rich in chloroplasts. The DNase-resistant incorporation was most probably due to metabolization of the radioactive precursor and to its utilization for different cellular syntheses, as observed in cultured plant cells [24–26]. We found that the addition of 5-fluorodeoxyuridine during the pulse with [^{3}H]thymidine did not reduce this process, unlike [25].

4. Discussion

DNA repair synthesis is conveniently shown in protoplasts prepared from leaves of *N. sylvestris*, where replication of nuclear DNA does not occur at an appreciable rate. This DNA repair synthesis is not affected by the addition of aphidicolin, an inhibitor of the α-like DNA polymerase.

These results agree with experiments which failed to detect an effect of aphidicolin on DNA repair synthesis in HeLa cells, as measured by autoradiography of UV-irradiated cell [7] or chromosomes [8]. This synthesis has thus been attributed to DNA polymerase β [7–9]. However, contrasting results, suggesting that aphidicolin inhibits DNA repair, have also been reported [27–30]. This discrepancy might be due either to an effective role of DNA polymerase α in DNA repair synthesis under the physiological conditions tested or, possibly, to the use of isolated nuclei [27] or permeabilized cells [28,29] in order to allow the uptake of exogenously supplied labelled deoxyribonucleoside triphosphates.

Thus, although our results do not exclude a role for the α-like DNA polymerase in DNA repair processes in other physiological conditions, they establish that in UV-light-treated plant protoplasts from *N. sylvestris* the observed DNA repair synthesis is not inhibited by aphidicolin and is therefore not attributable to the activity of the α-like DNA polymerase.

A number of investigations have failed to detect a β-like polymerase in various plant cells [11]. However, a minor DNA polymerase activity has been reported in plants which shares some β-polymerase properties [11].

It is important to investigate further the characteristic and function of this enzyme. We have observed that crude extracts from leaves and protoplasts of *N. sylvestris* and *N. plumbaginifolia* contain, in addition to a low amount of α-like DNA polymerase, a consistent fraction of DNA polymerase activity which is resistant to *N*-ethylmaleimide, an inhibitor of both α- and γ-like DNA polymerases, and to aphidicolin (unpublished). We are presently attempting to purify and characterize this enzyme.

Acknowledgements

We thank Dr M. Devreux for helpful discussions and help in the preparation of the manuscript. This investigation was supported by funds of the project Biologia della Riproduzione. (Contract 204121/85/93499.)

References

[1] Bolden, A., Pedrali-Noy, G. and Weissbach, A. (1977) J. Biol. Chem. 252, 3351–3356.

[2] Falaschi, A. and Spadari, S. (1978) in: DNA synthesis: Present and Future (Molineux, I. and Kohiyama, M. eds) pp. 487–511, Plenum, New York.

[3] Hubscher, U., Kuenzle, C. C., Limaker, W., Scherrer, R. and Spadari, S. (1978) Cold Spring Harbor Symp. Quant. Biol. 43, 625–629.

[4] Ikegami, S., Taguchi, T. and Ohashi, M. (1978) Nature 275, 458–460.

[5] Hubscher, H., Kuenzle, C. C. and Spadari, S. (1979) Proc. Natl. Acad. Sci. USA 76, 2316–2320.

[6] Geuskens, M., Hardt, N., Pedrali-Noy, G. and Spadari, S. (1981) Nucleic Acids Res. 9, 1599–1613.

[7] Hardt, N., Pedrali-Noy, G., Focher, F. and Spadari, S. (1981) Biochem. J. 199, 453–455.

[8] Giulotto, E. and Mondello, C. (1981) Biochem. Biophys. Res. Commun. 99, 1287–1294.

[9] Spadari, S., Sala, F. and Pedrali-Noy, G. (1982) Trends Biochem. Sci. in press.

[10] Amileni, A., Sala, F., Cella, R. and Spadari, S. (1979) Planta 146, 521–527.

[11] Bryant, J. A. (1980) Biol. Rev. 55, 237–284.

[12] Sala, F., Galli, M. G., Levi, M., Burroni, D., Parisi, B., Pedrali-Noy, G. and Spadari, S. (1981) FEBS Lett. 124, 112–118.

[13] Sala, F., Amileni, A. R., Parisi, B. and Spadari, S. (1980) Eur. J. Biochem. 112, 211–217.

[14] Castroviejo, M., Tharand, M., Tarrago-Litvak, L. and Litvak, S. (1979) Biochem. J. 181, 183–191.

[15] Howland, G. P. (1975) Nature 254, 160–161.

[16] Bucknall, R. A., Moores, H., Simms, R. and Hesp, B. (1973) Antimicrob. Agents Chemother. 4, 294–298.

[17] Sala, F., Parisi, B., Burroni, D., Amileni, A. R., Pedrali-Noy, G. and Spadari, S. (1980) FEBS Lett. 117, 93–98.

[18] Pedrali-Noy, G. and Spadari, S. (1979) Biochim. Biophys. Res. Commun. 88, 1194–2002.

[19] Magnien, E., Dalschaert, X., Roumengous, M. and Devreux, M. (1980) Acta Acad. Sinica 7, 231–240.

[20] Morel, G. and Wetmore, R. H. (1951) Am. J. Bot. 38, 138–140.

[21] Magnien, E., Dalschaert, X. and Faraoni-Sciamanna, P. (1982) Plant Sci. Lett. in press.

[22] Mollenhauer, H. A. (1964) Stain Technol. 39, 111–114.

[23] Magnien, E., Dalschaert, X. and Devreux, M. (1980) Plant Sci. Lett. 19, 231–241.

[24] Takats, S. T. and Smellie, R. M. S. (1963) J. Cell Biol. 17, 59–66.

[25] Howland, G. P. and Yette, M. L. (1975) Plant Sci. Lett. 5, 157–162.

[26] Lesley, S. M., Maretzky, A. and Nickell, G. (1980) Plant Physiol. 65, 1224–1228.

[27] Hanaoka, F., Kato, H., Ikegami, S., Ohashi, M. and Yamada, M. (1979) Biochem. Biophys. Res. Commun. 87, 575–580.

[28] Berger, N. A., Kurahara, K. K., Petzold, S. T. and Sikorski, G. W. (1979) Biochem. Biophys. Res. Commun. 89, 218–225.

[29] Ciarrocchi, G., Jose, J. G. and Linn, S. (1979) Nucleic Acids Res. 7, 1205–1219.

[30] Snyder, R. D. and Regan, J. D. (1981) Biochem. Biophys. Res. Commun. 99, 1088–1094.