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Interaction of Purified DNA with Plant Protoplasts of Different Cell Cycle Stage: The Concept of a Competent Phase for Plant Cell Transformation

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Summary. The polycation mediated attachment of purified tritiated DNA to plant protoplasts has been measured by quantitative microautoradiography. The automated grain counting technique used, also provides information on the cell cycle stage of individual protoplasts, which circumvents the need to synchronize the plant cell population before preparation of protoplasts. With protoplasts from asynchronously dividing suspension cultures of *Nicotiana sylvestris* (NS-1), S-phase protoplasts appear to be inefficient binders of ^{3}H-DNA, as compared with G_1 or G_2 protoplasts. Protoplasts derived from a tumour line of *Crepis capillaris* (CAPT) exhibit ^{3}H-DNA binding at all cell cycle phases, but S-phase protoplasts appear to be preferential binders. These differences are discussed with reference to cell cycle kinetics, membrane charge variation and the possibility of increasing the efficiency of genetic transformation of higher plant cells in culture.

Key words: Protoplasts – DNA binding – Cell cycle – Transformation competence

Introduction

Wigler et al. (1979) have speculated on the existence of a subpopulation of "transformation competent" cells in mammalian cell cultures. They conclude that such competence is not a stably heritable trait but is, rather, a "transient property" of cells. Previous work has demonstrated that the cell cycle phase of plant protoplasts has effects on their capacity to bind tobacco mosaic virus (TMV) virions (Gould 1979; Gould et al. 1981) and on the frequency of protoplast fusion (Ashmore and Gould 1982). These phenomena have been tentatively explained in terms of cyclic variation of the charge on the protoplast membrane; that is, in terms of a "transient property" of the protoplasts.

The charge variation model developed to explain the interaction of negatively charged TMV virions with protoplasts pre-incubated with poly-L-ornithine (Gould et al. 1981), could presumably also be applied to the interaction of purified DNA with such protoplasts, because the same charge considerations would apply to both the polyanionic molecules of DNA and TMV virions. In this report we present results suggesting that the polycation-mediated (i.e. poly-L-ornithine mediated) binding of DNA to plant protoplasts is more efficient at certain cell cycle phases than at others. Such variation in DNA binding is likely to be one of the components which defines the transformation-competence of cultured plant protoplasts.

Materials and Methods

Cell Culture and Protoplast Isolation

Methods for maintenance of suspension cultures of *Nicotiana sylvestris* (NS-1), and production of protoplasts from such cultures during exponential growth have been described previously (Gould et al. 1981). Analogous methods for a tumour derived suspension culture of *Crepis capillaris* (CAPT) have been reported by Ashmore (1981) and Ashmore and Gould (1982).

DNA Labelling and Isolation

DNA was isolated from suspension cultures of *Crepis capillaris* (line CAPT) which had been repeatedly supplied with tritiated thymidine during one, or sometimes two consecutive, seven-day growth cycles. The growth characteristics and aneuploid karyotype of these tumour-derived cultures have been described elsewhere (Ashmore and Gould 1979, 1981). Sterile, tritiated thymidine (Radiochemical Centre, Amersham, U.K. Specific activity (5 Ci/mmol = 185 GBq/mmol) was supplied to the cultures daily (0.5 μCi/ml = 18.5 kBq/ml of culture per day). No retardation of culture growth was apparent at these dosage rates. Cells from seven day old, first or second passage cultures were spun down, washed in fresh medium and rapidly freeze dried. DNA was extracted and purified by the method of Heyn et al. (1974). Purified DNA samples were stored frozen

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until required. For most binding experiments, the highly polymerized DNA samples were passed through a 26 gauge needle to yield sheared fragments of approximately 20,000 to 50,000 base pairs, but in some cases this step was omitted. DNA from line CAPT was used because of the opportunity to study the interaction of DNA and protoplasts in a homologous (CAPT DNA and CAPT protoplasts) and a non-homologous (CAPT DNA and NS-1 protoplasts) system. Also, the tritiated thymidine feeding method described above, produced specific activities of tritium in the final purified DNA which were very suitable for autoradiography (of the order of 700 counts/min/μg DNA).

DNA Binding to Protoplasts

Protoplasts, essentially free of cell wall debris, were isolated from two day old suspension cultures. Suspensions of protoplasts (3 ml), at a final density of about 4×10^5/ml were preincubated with poly-L-ornithine at a range of concentrations (see Results) for 60s with gentle agitation. From a stock solution of 50 μg ml^{-1} tritiated CAPT DNA (^{3}H-DNA), aliquots of 5, 10, 25, 100 or 200 μl were added to the 3 ml suspensions of protoplasts, to give DNA concentrations in the range 0.17 to 6.67 μg DNA per 10^6 protoplasts. Protoplasts were incubated with DNA for 2 m and then fixed in 9:3:4 ethanol:glacial acetic acid:water with sorbitol at a final concentration of 11% for NS-1 or 13% for CAPT. Preparation of autoradiographs with Kodak A.R. 10 stripping film has been described previously (Gould et al. 1981).

Autoradiographic Analysis

Silver grains were counted automatically over microautoradiographs of Feulgen stained, ^{3}H-DNA treated protoplasts, using the silver grain removal method described by Gould (1979). In this method, two measurements are made on each Feulgen stained protoplast, the first with, and the second without, associated silver grains. Silver is removed from autoradiographs by treating slides in a ferricyanide-thiosulphate mixture called Farmer's Reducer. The second measurement (silver grains removed) gives the Feulgen-DNA content of each protoplast and therefore its position in the cell cycle. The first measurement (silver grains present) minus the second measurement gives the silver grain density associated with the protoplast, which in this study is equivalent to the amount of ^{3}H-DNA bound. All measurements were made with a Zeiss photometer system interfaced to a PDP-12 computer using the APAMOS modified programme. This system has a motorized scanning stage which allows rapid relocation of previously measured nuclei.

Results

^{3}H-DNA Binding to NS-1 Protoplasts

Unsheared CAPT ^{3}H-DNA was used in preliminary binding experiments to check the origin of grain appearing in Feulgen stained microautoradiographs. In Fig. 1 A a binucleate protoplast of NS-1 is crossed by a strand which is Feulgen positive and produces high local grain density (i.e. the strand is tritiated DNA, presumably an unsheared association of many molecules). Such binucleates were rare and were excluded from analyses of nuclear DNA content. Figure 1 B shows two NS-1 protoplasts, one of which has light grain associated with fine, Feulgen positive strands over the cytoplasm (some silver grains appear white because they are slightly out of the plane of focus). Microautoradiographs of unsheared ^{3}H-DNA are useful for calculation of exposure times. For all quantitative autoradiographic experiments, sheared ^{3}H-DNA was used, which produced grains over the cytoplasm but no microscopically observable strands.

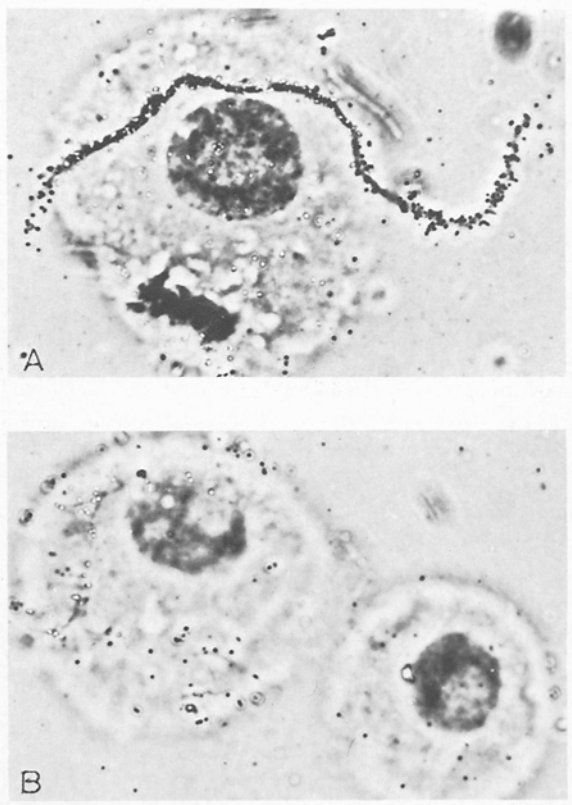
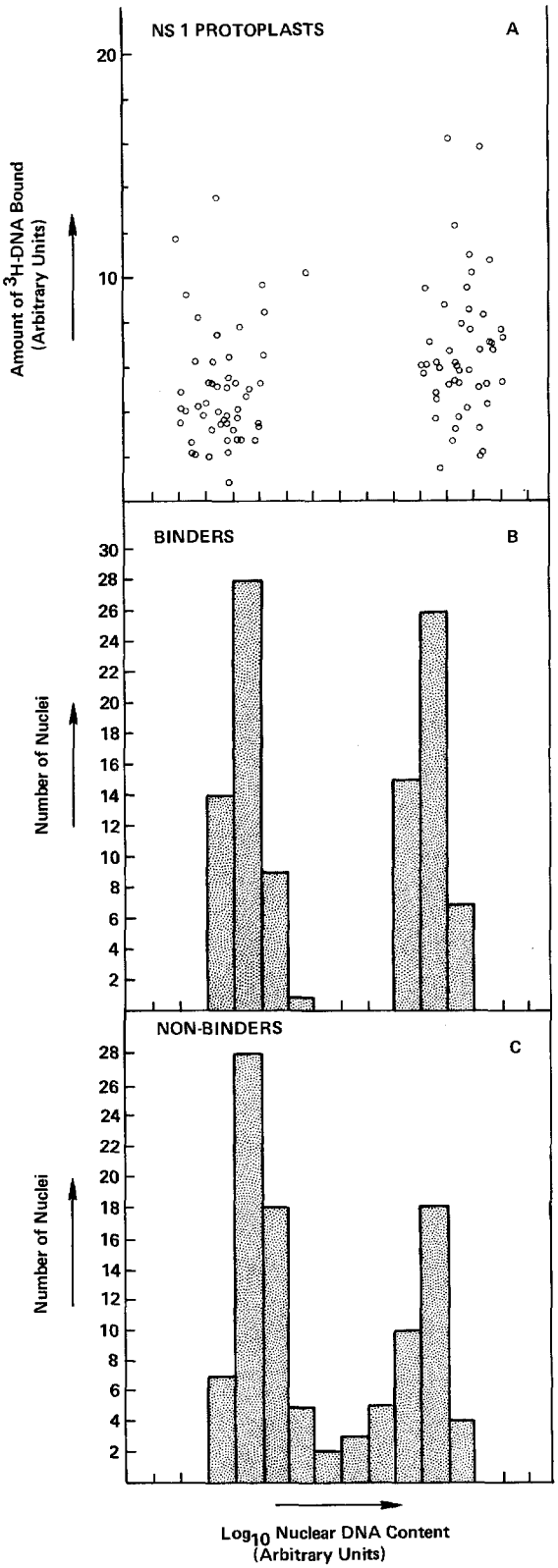


Fig. 1 A and B. Microautoradiographs of Feulgen stained NS-1 protoplasts with associated unsheared CAPT ^{3}H-DNA. Nuclear diameters approx. 10–20 μm

Over the range of poly-L-ornithine and DNA concentrations used, the proportion of NS-1 protoplasts which bound some ^{3}H-DNA varied from about 10% of the population (3 μg ml^{-1} poly-L-ornithine, 0.17 μg ^{3}H-DNA/10^6 protoplasts) to over 95% of the population (100 μg ml^{-1} poly-L-ornithine, 6.67 μg ^{3}H-DNA per 10^6 protoplasts). The former conditions gave very light grain densities over labelled protoplasts, the latter, extremely heavy densities, in autoradiographs exposed for 7 days. Conditions were chosen which gave grain densities previously estimated as suitable for the automated grain counting method (Gould 1979). Gould et al. (1981) have proposed that protoplasts with highest net negative charge bind most poly-L-ornithine,



and subsequently bind most TMV, due to interaction between negatively charged virions and the previously bound polycation. A similar mechanism should apply to the binding of negatively charged DNA molecules.

Figures 2A, B and C illustrate the difference in cell cycle distribution of protoplasts which bind ^{3}H CAPT DNA and those which do not, after preincubation with 20 μg ml^{-1} poly-L-ornithine and inoculation with 10 μl of ^{3}H-DNA (approximately 0.33 μg DNA/10^6 protoplasts). 100 NS-1 protoplasts which showed no DNA binding (i.e. no grain in the microautoradiograph preparation) were scanned on the Zeiss microspectrophotometer, and the Feulgen-density histogram of nuclear DNA contents appears in Fig. 2C. The histogram of nuclear DNA contents for 100 NS-1 protoplasts which had bound ^{3}H-DNA appears in Fig. 2B. The scatter diagram (Fig. 2A) represents the amount of exogenous ^{3}H-DNA bound to each protoplast in the population represented in Fig. 2B. Each value for amount of ^{3}H-DNA bound (grain number) is plotted against the cell cycle position of the protoplast being measured (assessed by nuclear DNA content). Protoplasts with the S-phase (intermediate) DNA content are very obviously missing from the population of protoplasts which bound ^{3}H-DNA in Fig. 2B. These data strongly suggest that NS-1 protoplasts in S-phase are inefficient binders of DNA as compared with G_1 or G_2 protoplasts under these specific experimental conditions.

^{3}H-DNA Binding to CAPT Protoplasts

When low levels of poly-L-ornithine (0–3 μg ml^{-1}) were used for preincubation of CAPT protoplasts, DNA binding did not occur. Preincubation with poly-L-ornithine at 20 or 200 μg ml^{-1} ensured efficient binding of DNA. It is unclear if the high levels of polycation needed to cause measurable binding of DNA to CAPT protoplasts reflect higher or lower surface charge as compared to NS-1 protoplasts. Interpretation depends on whether the binding mechanism involves gross charge neutralization on the whole protoplast or localized displays of excess positive charge due to bound molecules of poly-L-ornithine. Ashmore (1981) has demonstrated that NS-1 protoplasts con-

Fig. 2A–C. DNA binding by NS-1 protoplasts. **A** Scatter diagram showing the Feulgen-DNA (nuclear) content of single NS-1 protoplasts plotted against amount of ^{3}H-DNA associated with them. Quantity of ^{3}H-DNA was assessed by silver grain number in microautoradiographs. **B** Frequency distribution histogram of nuclear DNA contents of NS-1 protoplasts which have associated ^{3}H-DNA. **C** Frequency distribution histogram of nuclear DNA contents of NS-1 protoplasts which have no associated ^{3}H-DNA, (same experiment as for 2B)

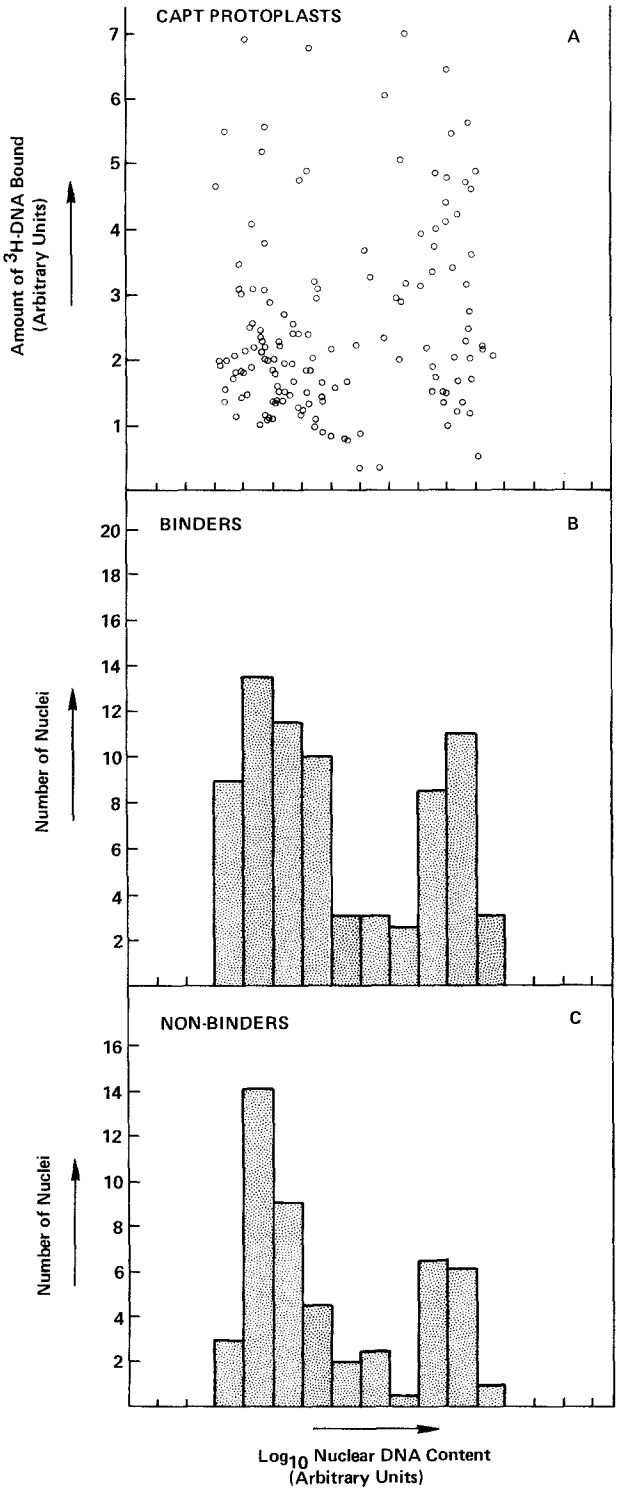


Fig. 3A–C. DNA binding by CAPT protoplasts. **A** Scatter diagram showing the Feulgen-DNA (nuclear) content of single CAPT protoplasts plotted against amount of ^{3}H-DNA associated with them. **B** Frequency distribution histogram of nuclear DNA contents of CAPT protoplasts which have associated ^{3}H-DNA. **C** Frequency distribution histogram of nuclear DNA contents of CAPT protoplasts which have no associated ^{3}H-DNA, (same experiment as for 3 B)

sistently bind greater quantities (about double) of negatively charged tritiated liposomes as compared to CAPT protoplasts when similar concentrations of poly-L-ornithine are used to achieve binding.

For CAPT protoplast samples preincubated with 20 μg ml^{-1} poly-L-ornithine, and then mixed with 100 μl of tritiated DNA the mean fraction of S-phase protoplasts binding DNA was 0.52 ± 0.013 (500 protoplasts counted), and the fraction of total protoplasts which showed DNA binding was 0.30 ± 0.019 (500 protoplasts counted). This suggests that CAPT protoplasts are preferential DNA binders during their S-phase.

Quantitative autoradiographic and microspectrophotometric analysis of Feulgen stained CAPT protoplasts with bound ^{3}H-DNA has revealed that G_2 protoplasts bind more DNA than G_1 protoplasts, whilst S-phase protoplasts, although they appear to be preferential binders, are very variable in the quantity of DNA with which they are associated. Figures 3 A, B and C demonstrate the cell cycle distribution of protoplasts which bind ^{3}H-DNA (Fig. 3 B) and those which do not (Fig. 3 C). The scatter diagram (Fig. 3 A) indicates that on average G_2 protoplasts bind higher levels of ^{3}H-DNA than do G_1 protoplasts. The mean value obtained from the Zeiss photometry system for grain densities (bound ^{3}H-DNA) over G_1 protoplasts was $2{,}287 \pm 128$ (arbitrary units) and for G_2 protoplasts the mean was $3{,}025 \pm 222$. Note that the S-phase difference in histograms 3 B and 3 C do not appear significant because of the low proportion of S-phase protoplasts in the population. For the estimates of mean fraction of S-phase protoplasts which bind DNA (quoted above), 500 S-phases were identified by nuclear incorporation of ^{3}H-thymidine and assessed as binders or nonbinders according to cytoplasmic grain (i.e. labelling due to exogenously supplied ^{3}H-DNA).

Discussion

Efficient genetic transformation of plant protoplasts with purified DNA is a goal which might be brought nearer by a consideration of experience with bacterial and mammalian systems (Lacks 1978). Certainly, the primary requirement for such transformation is binding of donor DNA to the recipient cell, and this first, essential step, has been the focus of this study. In the highly transformable bacteria, efficient DNA binding requires the development of a competent state, which arises at a specific stage of the bacterial culture growth cycle. Similarly, with mammalian cells in culture, it has been recognized that successful transformation requires that the cell populations are cycling, and the involvement of a specific cell cycle stage in "competence" or

susceptibility has been suggested (Sompayrac and Danna 1981).

The binding of ^{3}H-DNA to protoplasts of *Nicotiana sylvestris* line NS-1 reported here, follows a pattern similar to that observed for binding of tritiated tobacco mosaic virus (^{3}H-TMV) virions (Gould et. al. 1981). Under the conditions used, S-phase protoplasts bind little or no ^{3}H-DNA, whilst G_1 and G_2 protoplasts bind similar and significant amounts as assessed by micro-autoradiography. This pattern of binding is considered a reflection of a transient low negative surface charge associated with S-phase protoplasts of NS-1. Electrophoretic and two phase separation studies on NS-1 protoplasts yield data consistent with this idea (Ashmore 1981; Gould et al. 1981). Such cyclic fluctuations in surface charge are not limited to plant protoplasts. In a study of the polyanionic regions on the surface of human fibroblasts, Noronha-Blob and Pitha (1982) also report a decline in charge-mediated binding of poly-uridylate as synchronized cell populations enter S-phase.

In contrast to the results obtained with NS-1 protoplasts, protoplasts isolated from tumour-derived cultures of *Crepis capillaris* (CAPT) bind ^{3}H-DNA at all cell cycle phases. On average, G_2 protoplasts bind more ^{3}H-DNA than G_1 and S-phase protoplasts, but S-phase protoplasts bind ^{3}H-DNA more frequently than protoplasts of other cell cycle phases.

In a previous study the cell population kinetics of the CAPT culture were found to be distinctly unusual in that cells in the dividing fraction appeared to lack a G_1 phase. Cells in CAPT populations which contained the G_1 nuclear DNA content were thought to be in a non-proliferative compartment (Ashmore and Gould 1979). Thus, the difference in pattern of ^{3}H-DNA binding through the cell cycle for protoplasts of the NS-1 and CAPT cultures seems likely to be related to the radical difference in cell cycle kinetics between the two lines (Gould et al., unpublished). This idea is especially attractive in view of the anionic changes induced on cell membranes due to transformation to the tumour phenotype in cultured animal cells (Borysenko and Woods 1979).

Susceptibility to DNA binding should be only one component of a whole array of requirements which define a cell as physiologically predisposed to genetic transformation by exogenous DNA. Consider for example the case of a late G_1 and a late G_2 NS-1 protoplast, both of which according to data presented in this paper, can bind high levels of donor DNA. Although both protoplasts are "competent" in terms of DNA binding, the anatomy and immediate fate of their chromatin is very different. The chromatin of the G_1 cell is relaxed and diffuse, and will shortly undergo DNA replication during S-phase. The chromatin of the G_2 cell is soon to be committed to early prophase and the subsequent full contraction of mitosis proper. If DNA transformation (in terms of stable integration) relies on some form of illegitimate genetic recombination, it seems likely that the presence of replication forks during S-phase would be more conducive to a transformation event (i.e. to illegitimate recombination) than would the relatively inert and contracted chromatin of mitotic cells. Thus, the late G_1 cell would have greater competence for transformation than the late G_2 cell, although both are initially competent to bind DNA. However, protoplasts released from CAPT cells with the G_1 DNA content could not be expected to be efficiently transformed, no matter how much DNA they bind, because they are derived from a non-cycling population.

Evidence that cell cycle phase is an important factor in efficiency of DNA mediated transformation is implicit but rarely expressed in the literature. An exception is the recent interesting work of Cress (1981) on the uptake of plasmid DNA by synchronized cell suspensions of soybean. Unfortunately, the possibility that the observation of increased plasmid uptake during S-phase, is due to non-specific endonuclease and exonuclease degradation of plasmids and resynthesis into soybean nuclear DNA has not been eliminated.

In the classic case of tumour induction by *Agrobacterium tumefaciens,* susceptibility has long been considered linked to a particular stage in the preparations for cell division, after recruitment of quiescent cells into a cycling population by the wounding response (Braun 1962). Clearly, a consideration of plant cell population kinetics, and more especially of cell cycle distribution, will be essential to the improvement of efficiency of DNA mediated transformation of higher plant cells in culture.

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Literature

Ashmore, S.E. (1981): Kinetic and cytological analyses of plant tissue cultures. Ph.D. Thesis. Canberra (Australia): Australian National University

Ashmore, S.E.; Gould, A.R. (1979): Cell cycle analysis of tumour-derived cultures of *Crepis capillaris:* a kinetic analogy with proliferation of animal tumour cells. Protoplasma **101,** 217–230

Ashmore, S.E.; Gould, A.R. (1981): Karyotype evolution in a tumour-derived plant tissue culture analysed by Giemsa C-banding. Protoplasma **106,** 297–308

Ashmore, S.E.; Gould, A.R. (1982): Plant protoplast fusion and the cell cycle. Plant Cell Rep. (In press)

Borysenko, J.Z.; Woods, W. (1979): Density distribution and mobility of surface anions on a normal/transformed cell pair. Exp. Cell Res. **118,** 215–227

Braun, A.C. (1962): Tumour inception and development in the crown gall disease. Ann. Rev. Plant Physiol. **13,** 533–558

Cress, D.E. (1981): Uptake of plasmid DNA by protoplasts from synchronized soybean cell suspension cultures. Z. Pflanzenphysiol. **105,** 467–470

Gould, A.R. (1979): Combined microspectrophotometry and automated quantitative autoradiography applied to the analysis of the plant cell cycle. J. Cell Sci. **39,** 235–245

Gould, A.R.; Ashmore, S.E.; Gibbs, A.J. (1981): Cell cycle related changes in the quantity of TMV virions bound to protoplasts of *Nicotiana sylvestris.* Protoplasma **108,** 211–223

Heyn, R.F.; Hermans, A.K.; Schilperoort, A. (1974): Rapid and efficient isolation of highly polymerised plant DNA. Plant Sci. Lett. **2,** 73–78

Lacks, S.A. (1978): Mechanisms of DNA uptake by cells. In: Genetic interaction and gene transfer. Brookhaven Symp. Biol. **29**

Noronha-Blob, L.; Pitha, J. (1982): Probe for polyanionic regions on the cell surface. In Vitro **18,** 122–128

Sompayrac, L.M.; Danna, K.J. (1981): Efficient infection of monkey cells with DNA of simian virus 40. Proc. Natl. Acad. Sci. USA **78,** 7575–7578

Wigler, M.; Sweet, R.; Sim, G.K.; Wold, B.; Pellicer, A.; Lacy, E.; Maniatis, T.; Silverstein, S.; Axel, R. (1979): Transformation of mammalian cells with genes from procaryotes and eucaryotes. Cell **16,** 777–785

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