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Synchronized tobacco protoplasts are efficiently transformed by DNA

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Summary. A system for the synchronization of tobacco protoplasts was developed using aphidicolin, a mycotoxin which inhibits alpha-like DNA polymerase. Synchronized SR1 tobacco protoplasts were transformed with plasmid-DNA, derived from pLGV neo 11, at different stages of the cell cycle and the frequencies of kanamycin-resistant calli were measured. Compared to unsynchronized protoplasts synchronized cells show a clear increase in transformability provided that the transformation was performed at S- or M-phase. After completion of the M-phase transformation efficiencies dropped to the level of unsychronized cells. The efficiency of transformation for synchronized protoplasts was up to 3% of the surviving cells. This is approximately two orders of magnitude higher than the transformation efficiencies for unsynchronized protoplasts.

Introduction

Transformation experiments with mammalian cells using the coprecipitation method have clearly shown that the DNA-calciumphosphate complex is taken up by nearly all cells, while only a very small amount of DNA can be detected in the nucleus (Loyter et al. 1982). This indicates that the nuclear membrane might be a substantial barrier for the import and incorporation of foreign DNA. The increase in transformation efficiency of up to 1000-fold when DNA is microinjected directly into the nucleus rather than into the cytoplasm (Capecchi 1980; Wong and Capecchi 1985) can also be explained on this basis. As an alternative to microinjection Nagata et al. (1982) proposed the use of M-phase protoplasts because these cells do not possess a nuclear membrane. These authors could also show that aphidicolin, which inhibits alpha-like polymerase in tobacco and therefore blocks cell development at early S-phase (Sala et al. 1982; Petdrali-Noy et al. 1980) can be used to yield a high degree of synchronization in suspension culture cells of N. tabacum (Nagata et al. 1982). Synchronizing agents, however, require relatively long incubation periods during which the cell wall is synthesized, thus inhibiting DNA uptake by the cell. Meyer and Herth (1978) found that the herbicide 2,6-dichlorobenzonitrile (DB) inhibits cell wall formation without effecting nuclear division. Prolonged incubation of protoplasts with DB therefore led to multinuclear cells as cytokinesis was inhibited. Looking for the mechanism of the effect of DB, Galbraith and Shields (1982) reported an inhibition of cellulose production which is reversible and does not prevent the subsequent development of normal, fertile plants even after an incubation in DB for 21 days (Galbraith and Mauch 1980).

In this study, aphidicolin and DB were used in combination in order to synchronize the protoplasts and prevent cell wall formation during incubation. Synchronized cells were transformed with plasmid-DNA at different stages of the cell cycle. The transformation procedure was adapted from a method originally designed for protoplast fusion (Hein et al. 1983). In order to determine the potential susceptibility at different stages of the cell cycle, transformation efficiencies were compared for different stages.

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Materials and methods

Protoplast isolation. Sterile shoot cultures of Nicotiana tabacum cv. Petit Havana SR1 (Maliga et al. 1973) grown on LS medium were used for protoplasts isolation. The basal medium used for isolation and incubation of protoplasts was M3, a K3 medium (Nagy and Maliga 1976) supplemented with vitamins, organic acids, sugars and sugar alcohols and vitamin-free casamino acid according to Kao and Michayluk (1975). As described by Shillito et al. (1983) leaf halves without midribs were soaked in M3 medium containing 0.4 M sucrose and cut into thin sections. 2 g of leaf material were incubated in the dark in 30 ml enzyme solution (1.3% Cellulase "Onozuka R-10" (Serva), Heidelberg, FRG), 0.55% Mazerozyme R-10 (Serva) in M3 medium 0.4 M sucrose) for 20–22 h at room temperature. After 1 h of moderate swirling the digest was filtered through a 100 µm mesh filter. Protoplasts were floated by centrifugation at 100 *g* for 10 min and washed twice with osmoticum (0.16 M $CaCl_2$, 0.5% w/v 2(n-Morpholino)-ethanesulphonic acid (MES), 250 mM mannitol, pH 5.6).

Synchronization of protoplasts. Freshly prepared protoplasts were cultured in the dark in 10 mg/l aphidicolin and 10 mg/l DB (kindly supplied by P. Stabel) for 2–5 days. Due to the loss of activity of the aphidicolin after 3–4 days it was necessary to renew the synchronization solution when this period had to be exceeded. To release the aphidicolin block protoplasts were washed and resuspended in 10 mg/l DB in M3. The quality of synchronization was tested by staining the cells with 1% orceine in 50% acetic acid, 22.5% lactic acid in H_2O after fixation in freshly prepared fixative

according to Gould (1984) (13% sorbitol in 9:3:4 ethanol/glacial acitic/H_2O).

Preparation of DNA. As a basic cloning vector pLGV neo 11 was used (L. Herrera Estrella et al., in preparation). This plasmid was a generous gift from Dr. J. Schell. It contains markers allowing selection in bacteria and plants. The latter is a chimeric gene consisting of the nopaline synthase promotor region, the coding region of the neomycin phosphotransferase gene of Tn*5* and the polyadenylation signal of the octopine synthase. Into the unique *Sal*-site of pLGV neo 11 a 1.2 kb fragment from Tn*903* was cloned giving the vector pMP9 which differs from the pLGV 1103 construct described by Hain et al. (1985) only in the altered orientation of the Tn*903* fragment. Into the *Eco*RI cloning site of pMP9 randomly selected 2–10 kb long SR1 DNA pieces were cloned containing repetitive sequences differing in redundancy (Fig. 1). Plasmids were propagated in the *E. coli* strain DH1 (Meselson and Yuan 1968) and prepared according to Birnbaum and Doly (1979). Plasmids were pooled into groups of 5 to 10 and used for protoplast transformation.

Protoplast transformation. The fusion method of Hein et al. (1983) was adapted for DNA uptake. About 250,000 protoplasts were washed with osmoticum and resuspended in a final volume of 120 µl osmoticum. 10–30 µg of DNA dissolved in 20 µl sterile water were added. 140 µl PEG solution (0.5% N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes), 25% PEG 6000, 0.45 M Mannitol, 0.1 M $Ca(NO_3)_2$ pH 9.5) were added slowly. After 20 min 5 ml of wash-solution (0.5% Hepes, 0.29 M $Ca(NO_3)_2$, 0.45 M Mannitol, pH 9) were added dropwise. After 10–15 min cells were pelleted and resuspended in 2 ml M3 (0.4 M sucrose, 1 mg/l NAA, 0.2 mg/l kinetin).

Cultivation and selection of treated protoplasts. Protoplasts were cultured using the bead type technique of Shillito et al. (1983) which was modified as follows: one week after transformation 3 ml protoplast solution was mixed with 3 ml M3 (0.3 M sucrose, 1 mg/l NAA, 0.2 mg/l kinetin) containing 1% Seaplaque LMT agarose (Marine Colloids, Rockland, USA) in a 5 cm petri dish. After the agarose bead had been transferred to a 10 cm petri dish, 5 ml of M3 (0.3 M sucrose, 1 mg/l NAA, 0.2 mg/l kinetin) were added containing 90 mg/l kanamycin acid sulfate to select for kanamycin resistent calli. Media were changed every 3–4 days lowering the sucrose content by 50 mM per step. After 3–4 weeks, colonies were transferred to solid M3 medium (3% sucrose, 1 mg/l NAA, 0.2% kinetin, 100 mg/l kanamycin sulfate). To determine the frequency of transformation calli were grown non selectively, picked at a stage of 2 mm diameter in size and transferred onto solid selection medium containing 100 mg/l kanamycin.

Regeneration of plants. Shoots were induced by incubation of calli on solid M3 medium (3% sucrose, 0.5 mg/l benzylaminopurine (BAP), 0.1 mg/l NAA, 100 mg/l kanamycin acid sulfate (personal communication P. Stabel)). For development of roots, shoots were transferred to solid LS medium Linsmaier and Skoog (1965) (1% sucrose, 100 mg/l kanamycin).

Enzymatic assay of neomycin-phosphotransferase. Resistant calli were assayed for the activity of neomycin phosphotransferase II according to Reiss et al. (1984) with modifications adapted for plant tissue by Schreier et al. (1985).

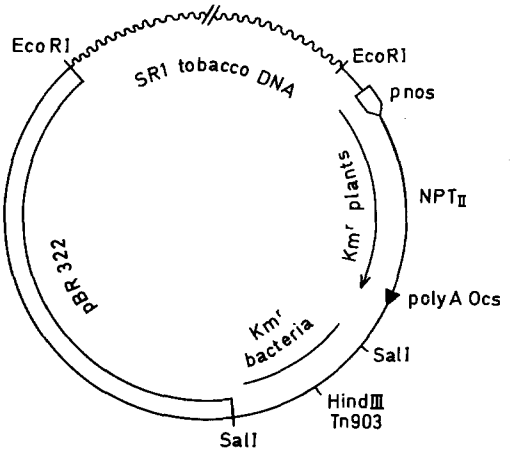


Fig. 1. Plasmid pMP901-pMP920. Randomly selected 2–10 kb long fragments of SR1 tobacco DNA were cloned into the *Eco*RI site of pMP9. Plasmid pMP9 was derived from pLGV neo 11 by cloning a bacterial kanamycin marker, the 1.2 *Sal*-fragment from *Tn*903, into the unique *Sal*-site of pLGV neo 11. Except for the inverse orientation of this fragment pMP9 is identical to pLGV 1103 as described by Hain et al. (1985)

Results

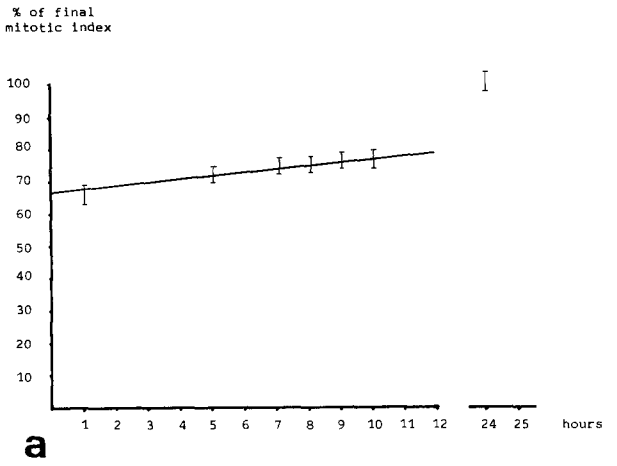
Synchronization of protoplasts

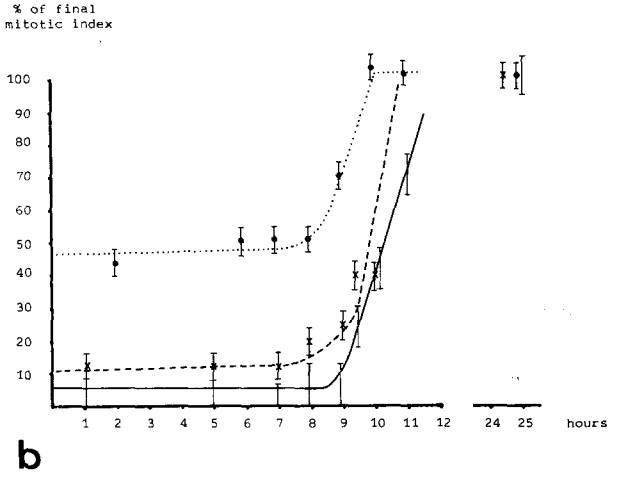
During the synchronization period cells were incubated in 10 mg/l 2.6-dichlorobenzonitrile (DB) in order to prevent cell wall formation. 50–60% of the protoplasts incubated in DB survived the treatment as seen by division of their nuclei within 3 days. This clearly indicated that cell division was prevented by DB while nuclear development was not affected. Concerning the synchrony of the cells, no sudden appearance of dinucleate cells was detected, but rather a gradual increase (Fig. 2a). After the removal of DB, cells developed normally confirming other reports about the reversibility of the cell division block.

When cells were incubated in aphidicolin and DB for 2 or 3 days, more than 90% of the cells contained only one nucleus (Fig. 2b) showing that most nuclear divisions had been blocked in early S-phase preventing nuclear division. When the incubation time was extended to 4 or 5 days the number of dinucleate cells increased, indicating that the blocking of the polymerase was no longer efficient (Fig. 2b). In order to block the nuclear development over longer periods, aphidicolin had to be renewed every 3 days. This effect is seen in Fig. 2c in the decrease of the high basal level of the curve. The rate of survival approached 40% when aphidicolin was added to the DB.

In order to prevent cell wall regeneration, DB had to be present still after removal of the aphidicolin prior to transformation. Synchrony of the cells was observed 8–9 h after the removal of the aphidicolin block, as an increase in the number of dinucleate cells, which reached its maximum within 2–3 hours (Figs. 2b and 3).

DNA transformation of synchronized protoplasts. DNA transformation was performed directly after the release of





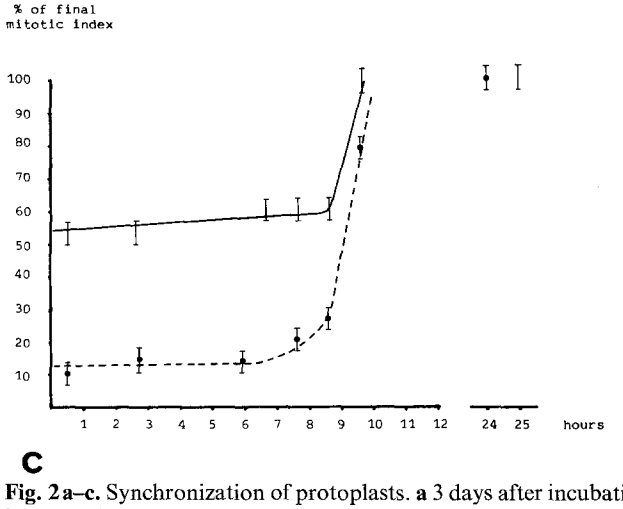


Fig. 2a–c. Synchronization of protoplasts. **a** 3 days after incubation in 10 mg/l DB the mitotic index was determined by counting dinucleate cells. **b** Cells were incubated in 10 mg/l DB and 10 mg/l aphidicolin for 2 days (——), 3 days (– – –) and 4 days (....). After the release of the aphidicolin block cells were further incubated in 10 mg/l DB and the mitotic index was determined. **c** After 5 days incubation in 10 mg/l DB and 10 mg/l aphidicolin cells were further incubated in 10 mg/l DB and the mitotic index was determined. Aphidicolin treatment was done without renewal of the synchronization solution (——) or with a renewal after 3 days (– – –)

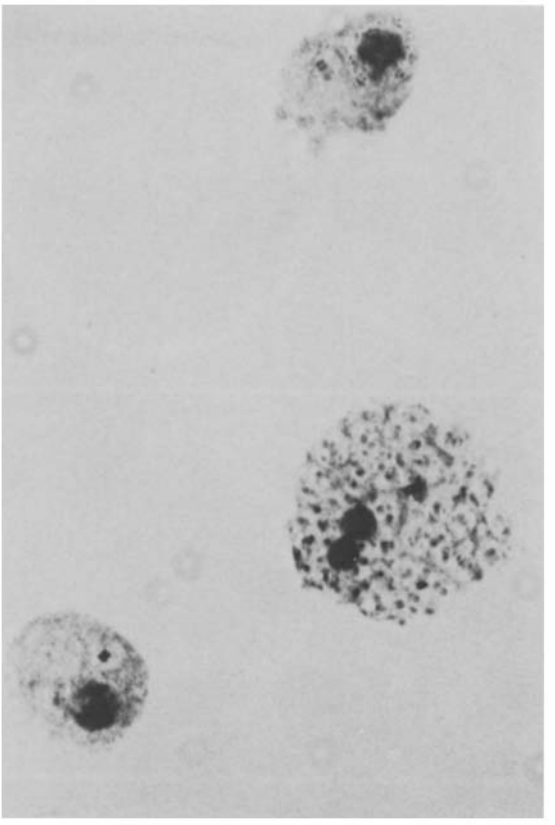


Fig. 3. Orceine staining of cells. Mitotic events were measured from the appearance of dinucleate cells. Such nuclei had gone through mitosis while the cell division was prohibited by the presence of DB. 8–9 h after the release of the aphidicolin block the first dinucleated cells appear

the synchronizing block with cells referred to as S-phase cells and 8–9 h after the release with cells referred to as M-phase cells. For DNA uptake a procedure originally described for protoplast fusion was modified (Hein et al. 1983). 10–30 μg of plasmid DNA were incubated together with 250,000 protoplasts in a PEG solution giving a final concentration of 12.5%, which was slowly diluted by adding a Ca $(NO_3)_2$ solution of pH 9. Transformants were selected by growth on 90 mg/l kanamycin.

Tobacco protoplasts used for transformation showed a high sensitivity to kanamycin, as practically no surviving colonies were found when the culture medium contained 20 mg/l kanamycin. When the transformation procedure was applied to freshly prepared protoplasts, kanamycin resistant clones were obtained at a frequency of 10^{-6}–10^{-5} (Fig. 4a), which is comparable to the results given by Paszkowski et al. (1984), but is roughly 10 times less efficient than the method described by Hain et al. (1985). In the following we will provide evidence that synchronization of protoplasts leads to a considerable increase in transformation efficiency.

Transformation of protoplasts which had been incubated in DB for 3 days yielded resistant calli at about the same frequency as freshly prepared protoplasts (Fig. 4b), which demonstrates that an incubation in DB alone does not lead to a clear improvement. However, synchronization of protoplasts at S-phase or M-phase as described above considerably increased the efficiency of transformation

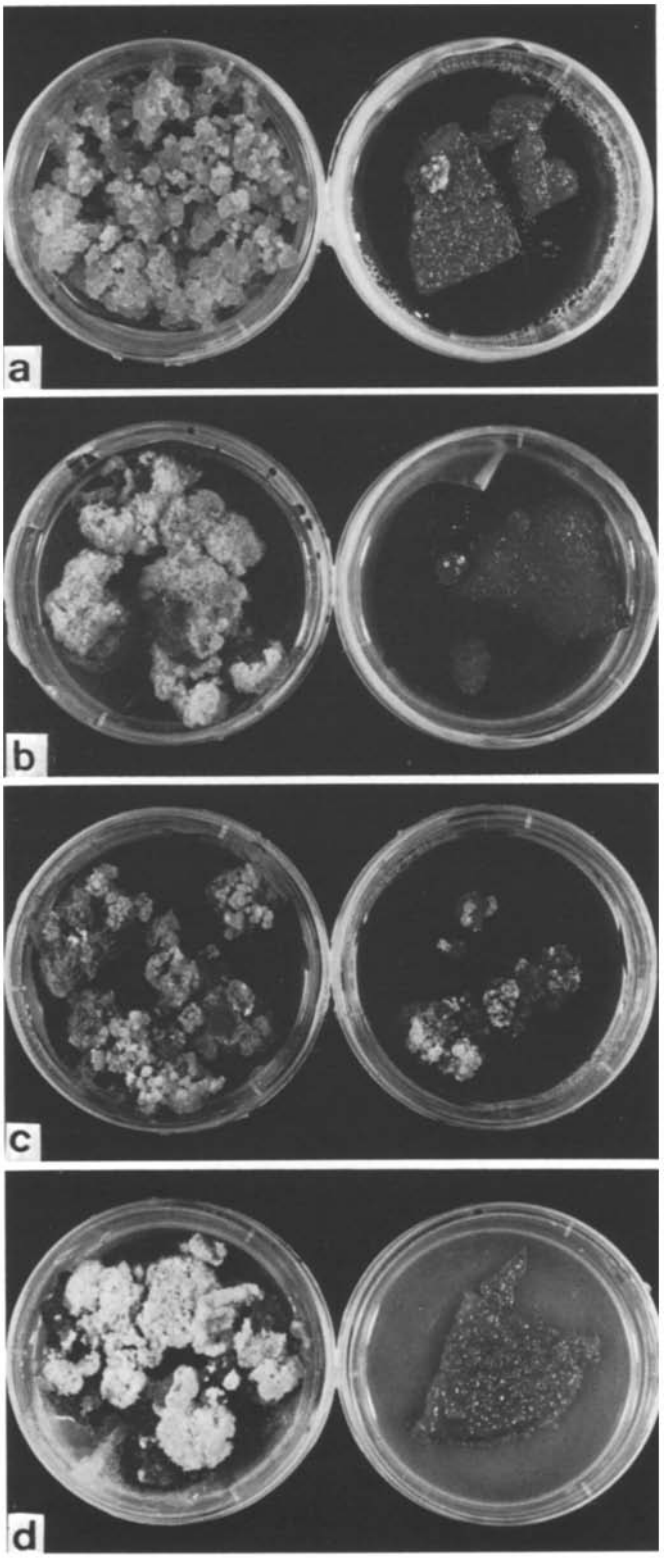


Fig. 4a–d. Comparison of transformation frequencies. SR1 tobacco microcalli grown without selection (*left*) and on 100 mg/l kanamycin sulphate (*right*). Transformations were done with unsynchronized protoplasts (**a**), after an incubation of protoplasts in 10 mg/l DB for 3 days (**b**), after an incubation of protoplasts for 3 days in 10 mg/l DB, 10 mg/l aphidicolin and (after the release of the aphidicolin block) a postincubation in 10 mg/l DB for 8 h (**c**) and for 15 h (**d**)

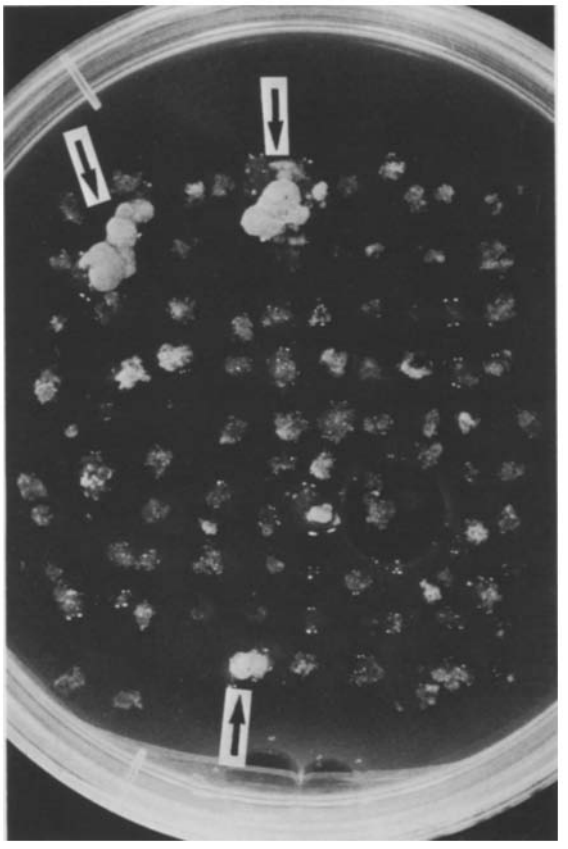


Fig. 5. Determination of transformation frequencies. Microcalli were grown without selection until they reached a diameter of about 2 mm. 100 calli were transferred onto selective medium where 3 continued to grow (indicated by *arrows*)

(Fig. 4c). This increase, however, was not observed when the treated protoplasts had passed the S-phase by more than 15 h. Under these conditions only very few cells are expected to be in S-phase or M-phase (Fig. 4d). Synchronized cultures not treated with DNA did not yield any resistant calli under selection pressure.

Determination of transformation frequencies. The selection of resistant calli using the bead type culture technique made it difficult to determine the number of resistant calli due to the high density of developing calli. Therefore microcalli were grown in the absence of selection pressure. 200 developing calli were transferred onto solid medium containing kanamycin and 7 resistant calli were obtained (Fig. 5) indicating a transformation frequency of a few percent.

Analysis of resistant calli. Five of the kanamycin-resistant calli were assayed for neomycin phosphotransferase II-activity using the procedure of Reiss et al. (1984), modified by Schreier et al. (1985). One callus was obtained from transformation of unsynchronized protoplasts (Fig. 6a), the others were selected after transformation of synchronized cells which were either selected directly from the bead type culture (Fig. 6b, c) or which were selected from microcalli grown without kanamycin (Fig. 6d, e). All of the candidates examined showed NPT II activity though at different levels. In control-calli no NPT II activity could be detected. Due to low amounts of cell material DNA analysis of the transformants could not as yet be conducted.

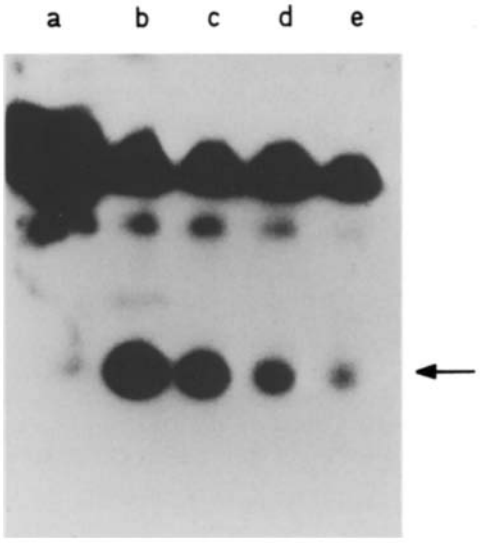


Fig. 6a–e. NPT assay of KM-resistant calli. *Arrow* indicating spots representing NPT II-activity. **a** Callus selected after transformation of non-synchronized protoplasts. **b, c** Calli selected directly after transformation of synchronized protoplasts. **d, e** Calli selected out of calli which had been grown before without selection after transformation of synchronized protoplasts

Discussion

It has been shown in animal cells that the frequency of transformation can be raised by up to a factor of 1000, when foreign DNA is injected directly into the nucleus (Wong and Capecchi 1985). This indicates that the nuclear membrane might be a considerable barrier for the introduction of DNA. This apparently is also true for plant cells as shown in this report. For synchronization of protoplasts, a combination of aphidicolin and DB was used. At a concentration of 10 mg/l DB blocks cell division very efficiently without affecting the nuclear development. It was reported that more than 90% of mesophyll protoplasts are in G1-phase (Sala et al. 1982). When we incubated protoplasts in DB for 3 days and counted the increase of dinucleate cells, we observed a gradual increase which indicates that the cells enter S- and M-phase more or less randomly after they have been prepared. An aphidicolin concentration of 10 mg/l is sufficient to block chromosomal development at early S-phase, preventing duplication of the nucleus.

For DNA-transformation several methods have been published which differ mainly in conditions used for the actual uptake of DNA by the protoplasts (Krens et al. 1982; Paszkowski et al. 1984; Hain et al. 1985). With these methods transformation efficiencies up to 2.3×10^{-4} have been achieved (Hain et al. 1985). In our hands transformation of a non-synchronized protoplast preparation is roughly 10 times less effective, but we observe a high degree of variability due to the quality of the individual protoplast preparation and treatment. When synchronized protoplasts are used, transformation efficiencies are increased to a level of 3% for cells which have not yet passed through mitosis. S- and M-phase protoplasts showed similar results. It is significant that the transformation frequency decreases rapidly as soon as the cells have passed mitosis. This seems to indicate that cells in the S- and M-phases of development are particularly susceptible for DNA transformation.

By detailed DNA analysis of the transformants, it should be possible to differentiate between DNA uptake in S-phase or M-phase. Chimeric plants might arise from a transformation in M-phase with the foreign DNA incorporated in different loci, while DNA-uptake in S-phase should lead to a similar position of integration for all cells of the transformed plant. Efforts can now be directed towards studying the mechanism of cycle-dependent DNA uptake, as well as towards analysis of the DNA integration mode. It remains to be seen whether plasmid DNA is integrated randomly or by recombination between genomic DNA and the homologous SR1 sequences on the plasmids. Likewise copy numbers must also be determined. The transformants described so far (Potrykus et al. 1985a; Hain et al. 1985) did not use homologous DNA on the vectors. However, in these experiments the transformed DNA was integrated at single positions in the genome together with modified and non-functional copies.

It is conceivable that the SR1 DNA carried by the vector may contain sequences sufficient for autonomous replication. This would allow the plasmid to be maintained extrachromosomally.

A DNA transformation procedure with an efficiency in the percent-range is of general interest provided other plant protoplasts can also be synchronized and regenerated. It remains to be tested whether this method can be adapted for other plant systems with the same efficiency. An adaptation of the method might be especially usefull for cereals where cocultivation with *Agrobacterium tumefaciens* is not applicable. DNA transformation has successfully been performed for several species (Lörz et al. 1985; Potrykus et al. 1985b). Transformation of synchronized protoplasts might be helpful in increasing transformation efficiencies.

The synchronization system needs to be optimized further in order to reach the 10% range which has been reported for cocultivation (Hain et al. 1985) and which might make shotgun cloning experiments feasible. Efficiencies could possibly be increased by improving DNA uptake by the protoplast. Therefore we will combine synchronization of the nucleus and treatment of the cell by electroporation, heat shock, DMSO and other chemicals thought to increase the transport of DNA into the protoplast.

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