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Direct DNA transfer to plant cells

M.R. Davey, E.L. Rech and B.J. Mulligan
Plant Genetic Manipulation Group, Department of Botany, University of Nottingham, University Park, Nottingham NG7 2RD, UK

Abstract

A range of somatic cell and molecular techniques are now available to supplement conventional plant breeding. The introduction and expression of foreign DNA has been used to modify basic aspects of physiology and development, to introduce commercially important characteristics such as herbicide and insect resistance into plants and to insert genes suitable as dominant selectable markers for somatic hybridisation. Several techniques for direct DNA delivery are available, ranging from uptake of DNA into isolated protoplasts mediated by chemical procedures or electroporation, to injection and the use of high-velocity particles to introduce DNA into intact tissues. Direct DNA uptake is applicable to both stable and transient gene expression studies and utilises a range of vectors, including those employed for gene cloning. Although the frequency of stable transformation is low, direct DNA uptake is applicable to those plants not amenable to *Agrobacterium* transformation, particularly monocotyledons.

Transformation of dicotyledonous protoplasts by *Agrobacterium* Ti plasmid

The last decade has witnessed major advances in the genetic manipulation of plants, resulting from a combination of somatic cell and molecular techniques. The impetus for assessing the feasibility of introducing DNA directly into plant cells emanated from reports that cultured animal cells were capable of taking up and expressing cloned genes and genomic DNA. Concurrent with these experiments with animal cells was the demonstration that oncogenic strains of the crown gall-inducing bacterium, *Agrobacterium tumefaciens*, carried a large tumour-inducing (Ti) plasmid. Transfer of part of the Ti plasmid, the T-DNA, from the bacterium to plants, with subsequent integration and expression of the bacterial DNA within recipient plant cells, resulted in tumorous growth. Despite the technical difficulties inherent in the isolation of such a large plasmid (90–150 MDa), it was possible to prepare Ti plasmid in sufficient quantity [23] to mimic those DNA uptake experiments performed with animal cells.

The interaction of viruses with isolated protoplasts demonstrated that poly-L-ornithine (PLO) stimulated infection, while subsequent work confirmed that polyethylene glycol (PEG) also increased uptake of viruses and viral nucleic acids into protoplasts (see [19]). Thus, during the late 1970s, it was appropriate to combine the techniques then available to determine whether isolated Ti plasmid could transform plant protoplasts. Indeed, studies involving the interaction of supercoiled Ti plasmid from an octopine strain of *A. tumefaciens* with cell suspension protoplasts of *Petunia hybrida* in the presence of PLO, resulted in protoplast-derived colonies that expressed the crown gall characteristics of growth on hormone-free medium and the synthesis of octopine, both encoded by Ti T-DNA genes [18]. Work in other laboratories confirmed PEG-mediated transformation of protoplasts from leaves of a streptomycin-resistant line (SR1) of *Nicotiana tabacum* by isolated Ti plasmid [43]. Interestingly, the T-DNA structure and organisation in petunia and tobacco protoplasts transformed with Ti plasmid was different to T-DNA in cells transformed by

intact agrobacteria. Analysis of petunia transformed tissues showed the presence of truncated T-DNA [24], while in tobacco the T-DNA was often fragmented [44]. Overlapping copies of T-DNA of varying size, as well as T-DNA fragments, were also detected. The 23 bp border sequences flanking the T-region in the Ti plasmid were not used as the preferred sequences for integration into the plant genome. Transformation could be achieved using a T-region clone lacking the left border of the TL-DNA and the right border of the TR-DNA. In addition, the use of Ti plasmid carrying a mutated *vir* region, confirmed that the *vir* region was not essential for transformation by direct DNA uptake. Such studies [44, 68] also provided evidence for co-transformation, sequences characteristic of calf thymus DNA used as carrier being detected in transformed plants by Southern blot hybridisation. The transformation frequency in all these experiments with Ti plasmid was of the order of 1 in 10^4 to 10^5 [18, 43]. In an evaluation of chemical methods to deliver pTiACH5 to suspension cell protoplasts of petunia, Draper [22] and Draper and Davey (unpublished) obtained transformation frequencies of 2.9, 6.7, 2.2 and 2.5×10^{-5} after treatment of protoplast-plasmid mixtures with PLO, PEG, high salts and Ca^{2+} at high pH respectively, with concentrations of plasmid delivery agents which maintained acceptable levels of protoplast viability.

In an attempt to protect the Ti plasmid from degradation during plasmid uptake, Hasezawa *et al.* [34] used *Agrobacterium* spheroplasts and treatment with PEG or polyvinyl alcohol (PVA) to introduce the spheroplasts into protoplasts of *Vinca rosea*, this occurring mainly by endocytosis and to a lesser extent by direct fusion with the plasma membrane of recipient protoplasts. The transformation frequency was higher than with naked Ti plasmid (7.6×10^{-4}). Similar studies with tobacco protoplasts [32] and *Escherichia coli* spheroplasts carrying cloned CaMV DNA [85] confirmed this observation.

Deshayes *et al.* [21] encapsulated pGV23neo, a plasmid carrying the aminoglycoside phosphotransferase type II gene (APHII; neomycin phosphotransferase, NPTII) from Tn5 conferring resistance to kanamycin in plant cells, into negatively charged liposomes and fused such plasmid-containing vesicles with tobacco protoplasts using PEG. Kanamycin-resistant colonies were isolated at 4.0×10^{-5}. The restriction pattern of DNA inserted into transgenic plants indicated a tandem integration of plasmid sequences, implying homologous recombination between the sequences during transformation. Another detailed study [27] compared chemically stimulated uptake of plasmid, the use of liposome-encapsulated DNA and fusion of plasmid-containing *E. coli* spheroplasts to deliver plasmids to protoplasts. Maximum association of plasmid with protoplasts occurred when the protoplasts were interacted with naked DNA or with plasmid-containing liposomes, prepared by the reversed-phase evaporation method, and 15% w/v PEG 6000, or with *E. coli* spheroplasts containing chloramphenicol-amplified plasmid in the presence of 25% w/v PEG 6000.

Transformation of dicotyledonous protoplasts by *E. coli* plasmid vectors

The need to develop small plasmid vectors which could be engineered in *E. coli* and isolated in milligram quantities, unlike the microgram quantities of Ti plasmid normally obtained, was recognised by several workers [27]. Indeed, a major advance in the direct transformation of protoplasts by DNA was achieved when it was demonstrated that T-DNA sequences were unnecessary for stable transformation and expression of foreign DNA in plant cells [66]. A selectable hybrid gene comprising the protein coding region of the Tn5 NPTII gene under the control of the CaMV gene VI promoter was introduced into *N. tabacum* SR1 protoplasts as part of an *E. coli* plasmid (pABD1) by treating protoplast-plasmid mixtures with PEG 6000. Transformed colonies were selected on medium containing 75 μg/ml of kanamycin. The foreign gene was transmitted to the progeny in a Mendelian pattern.

Later work with pABD1, but in linearised form,

showed that the efficiency of gene transfer could be increased more than 1000-fold by heat-shocking the recipient protoplasts (5 min at 45 °C) prior to addition of the plasmid followed by PEG. The resulting mixture was electroporated at 1.0–2.5 kV/cm [80]. Molecular analysis of offspring obtained from transgenic tobacco plants confirmed that one functional copy of the hybrid gene was stably integrated into the chromosomal DNA of transformed protoplasts, that the gene was maintained during clonal proliferation of transgenic plants, transmitted to F1 and F2 offspring and inherited as a single dominant trait [70]. *In situ* hybridisation using ^{3}H-labelled pABD1 localised the low-copy chimaeric kanamycin resistance gene on different homologous chromosome pairs in two transgenic plants [56].

In another attempt to improve the frequency of protoplast transformation using chemically induced plasmid uptake, Hain *et al.* [33] incubated protoplasts with a pLGVneo2103-calcium phosphate coprecipitate, followed by fusion of the protoplasts with PVA and exposure to high pH. Kanamycin-resistant transformants were obtained at 0.01% of the protoplast-derived colonies. Efforts have also been made to improve the vectors used in direct DNA uptake. Balazs *et al.* [2] constructed pKR612B1, the parental plasmid of which, pDOB612, had two unique *Sma* I and *Bam* HI restriction sites for gene insertion into the vector. Turnip and tobacco protoplasts were transformed to kanamycin resistance with pKR612B1 using the PEG procedure of Krens *et al.* [43].

Experiments have demonstrated that co-transformation of protoplasts is possible with *E. coli*-based vectors by mixing two plasmids during the uptake procedure, or by constructing a vector carrying two genes. Schocher *et al.* [76], using a published procedure [80], showed that after transformation of tobacco protoplasts with pABD1 carrying the NPTII gene and pMS1 carrying the zein gene, 88% of the kanamycin-resistant cell colonies contained sequences from the zein genomic clone, while others contained the full zein genomic clone. In experiments with pABD1 and pGV0422 carrying the nopaline synthase (nos) gene, 47% of kanamycin-resistant colonies contained the nos gene. Other workers [84] also used the DNA uptake procedure essentially as described [80], to show that transformation of *Petunia hybrida* leaf protoplasts to kanamycin resistance by the NPTII gene was accompanied by co-transfer, as a non-selectable gene, of phosphoenolpyruvate carboxylase cDNA from *Sorghum* leaves. Cernilofsky *et al.* [15, 16] also studied the integration of two separate plasmids, one containing the NPTII gene, the other containing the maize transposable element Ac.

Expression in transformed protoplast-derived cells of two bacterial antibiotic resistance genes has been reported. One gene coding for NPTII from Tn903 and the other for chloramphenicol acetyltransferase (CAT) from Tn9 were both introduced into tobacco protoplasts using the vector pDH51 carrying the CaMV 35S promoter and polycloning sites. Transformed cells were selected for their resistance to kanamycin or CAT, although CAT selection was difficult and not reproducible [69]. Consequently, the authors suggested that the CAT gene should be considered as an assayable 'reporter' gene. Co-expression has also been achieved by constructing vectors with two marker genes. Thus, Uchimiya *et al.* [90] used the 16 kb pCT1T3 containing the intact nos gene and a chimaeric gene consisting of the promoter and terminator regions from CaMV gene VI and the NPTII structural gene. Eighteen percent of transformed tobacco colonies expressed the two markers, as did plants regenerated from these tissues.

Whilst tobacco protoplasts have been used extensively as a model system for DNA uptake studies, workers have also applied this technique to protoplasts of economically more important crops. Fertile transgenic plants of *Brassica napus* were produced following transfer of pABD1 into mesophyll protoplasts by electroporation [30]. Kanamycin resistance was transmitted to sexual progeny as a single Mendelian trait.

Direct gene transfer proved possible for the grain legume *Vigna aconitifolia*, with kanamycin-resistant calli regenerated from heat-shocked pro-

toplasts treated with PEG and pLGVneo2103 giving rise to transgenic plants [46]. The plant cultivar was important in maximising the transformation frequency, with the highest value (0.58% of resistant colonies) being obtained in cultivar 560. Kanamycin-resistant tissues of three commercially important cultivars of *Glycine max* (Williams 82, Mandarin Ottawa and Hardin) have also been selected following electroporation-induced uptake of pCMC1021. Initially, transient expression studies were carried out with the same plasmid to optimise the electroporation conditions prior to selection of the stably transformed kanamycin-resistant tissues [11]. Roots produced by the calli also exhibited APHII activity.

Influence of the cell cycle and irradiation on transformation

The cell cycle of recipient protoplasts influences transformation. Meyer *et al.* [55] synchronised tobacco leaf protoplasts by culturing them for 2–5 days in the presence of aphidicolin, a mycotoxin that inhibits α-like DNA polymerase, and 2,6-dichlorobenzonitrile, an inhibitor of cell wall synthesis. When transformed with a plasmid derived from pLGVneo11, up to 3% of the surviving cells were transformed to kanamycin resistance, provided transformation was performed at the S or M phase. This frequency was twice that with unsynchronised protoplasts. In similar studies involving aphidicolin, G418-resistant tobacco colonies were obtained with M phase protoplasts at frequencies 2 to 8 times those obtained with protoplasts at other stages of the cell cycle, indicating the absence of a nuclear membrane in mitotic cells favours delivery to the nucleus of exogenous DNA introduced into the cytoplasm [64]. Irradiation of protoplasts prior to treatment with plasmid and PEG has also resulted in a 3–6-fold enhancement of hygromycin- or kanamycin-resistant colonies without affecting colony production and plant regeneration [45]. The higher integration rates of foreign DNA may result from increased recombination in irradiated cells.

Transient gene expression studies

Whilst many of the earlier DNA uptake experiments focused attention on the production of stably transformed tissues and transgenic plants, direct DNA uptake into protoplasts has also provided a system for monitoring gene expression within hours of the transformation treatment. Such a rapid procedure is useful in evaluating constructs and gene promoters now that readily assayable reporter genes, such as those for CAT and β-glucuronidase (GUS) [38], are available. Fromm *et al.* [28], using electroporation to induce DNA uptake, reported that gene transfer efficiency into protoplasts increased with DNA concentration and was influenced by the amplitude and duration of the electric pulse and composition of the electroporation medium. Comparison of two plasmids (pNOSCAT and pCaMVCAT), with the nos and CaMV 35S promoters respectively, showed that CAT expression was higher with the CaMV promoter in carrot and maize protoplasts. Both constructs expressed in tobacco protoplasts. Other studies used carrot protoplasts to optimise the electroporation conditions before detecting CAT expression in protoplasts of *Glycine max*, *Petunia hybrida* and those of the monocotyledons *Triticum monococcum, Pennisetum purpureum, Panicum maximum, Saccharum officinarum* and a double-cross, tri-specific hybrid between *Pennisetum purpureum, P. americanum* and *P. squamulatum* [35]. In an extension of the work with *Panicum maximum*, gene expression was evaluated in protoplasts from two cell lines after delivery of pMON563 by PEG, of molecular weights from 1430 to 8000 and concentrations of 20 to 40% w/v, or by electroporation. CAT expression was higher after electroporation, but PEG-treated protoplasts exhibited optimum plating efficiencies [92].

CAT has also been used as a reporter gene in constructs carrying promoters isolated from a developmentally regulated *Zea mays* seed storage protein gene and from the mannopine synthase gene of an octopine Ti plasmid [5]. CAT activity was correlated with DNA concentration, and gene transfer was stimulated by treatment of pro-

toplast-plasmid mixtures with PEG prior to electroporation and by optimisation of the voltage used during electroporation. In carrot and petunia protoplasts, transformation efficiency was dependent on $CaCl_2$ in the system, was stimulated by carrier DNA and was independent of the molecular weight of PEG used [3]. Although most workers have measured CAT expression 24 to 48 hours after DNA uptake, CAT activity has been reported within 30 min of PEG-induced plasmid delivery into tobacco leaf protoplasts, with maximum values 4 to 24 hours after plasmid uptake [73]. The protein synthesis inhibitor cycloheximide influenced CAT expression during the first 4 hours, indicating a rapid decay of biologically active forms of both the DNA transferred and the CAT mRNA synthesised during this time. These workers suggested that CAT protein is stable for up to 2 weeks in tobacco, rather than a continuous synthesis of new enzyme. In addition to influencing stable transformation, the cell cycle also affects transient expression, with M phase protoplasts of tobacco expressing 3 to 4 times higher CAT activity than protoplasts at other stages of the cell cycle [64]. In studies with sugar beet, Lindsey and Jones [50] reported that mesophyll protoplasts were more susceptible to damage by electroporation than cell suspension protoplasts. CAT activity was also demonstrated after electroporation of intact suspension cells with plasmid, gene expression being increased by pectinase treatment of the cells prior to plasmid uptake. CAT expression has also been used to demonstrate that gene expression in plant cells can be inhibited by expression of antisense RNA [26]. It is worthwhile to note that CAT is unsuitable as a reporter gene in *Brassica napus* and *B. juncea*, since plant tissues contain high levels of endogenous CAT activity [9].

Although CAT has been used most extensively as a reporter gene in transient systems, NPTII has also been employed. Maximum NPTII activity was detected 4 days after PEG-induced plasmid uptake into protoplasts of *Triticum monococcum*, with gene expression still detectable after 10 days [94]. The majority of the DNA introduced into protoplasts remained extrachromosomal, with the conformation of the DNA changing from supercoiled to the open circular and linear forms within the plant cells. Other studies involving the NPTII gene fused to four different promoters confirmed that PEG was effective in delivering plasmids to protoplasts of barley, maize, rice and rye [39]. There is little information which compares the levels of sensitivity of CAT, GUS and NPTII as reporter genes. In other experiments using nearly indentical constructs with the CaMV 35S promoter and CAT, GUS and NPTII reporter genes introduced into tobacco SR1 protoplasts by $Ca(NO_3)_2$-PEG treatment, CAT gene expression was the easiest to detect, followed by NPTII and GUS [87]. GUS has been used to study promoter function. Marcotte *et al.* [53] reported the normal regulation of a wheat promoter, inducible by ABA, in a transient expression assay using rice protoplasts. This study provided direct evidence for phytohormone activity to regulate the initiation of gene transcription.

Recently, there has been increased interest in protoplasts of woody species, with GUS expression in those of *Alnus incana* [78] and *Picea glauca* [95], and CAT expression in those of *P. glauca* [4, 95]. Gene expression was influenced by the DNA concentration, the amplitude and duration of the electric pulse and, in the case of *Alnus* protoplasts, by the presence of PEG in the electroporation medium. Linearised plasmid constructs gave higher levels of CAT activity than circular plasmid in electroporated *Picea* protoplasts [4], but gene expression was reduced with PEG-mediated DNA delivery to the same protoplasts [95]. However, herring sperm DNA as carrier increased GUS expression by 50% with PEG uptake [95]. Interestingly, electroporated *Picea* protoplasts released a small molecule that mimicked GUS activity in the fluorescence assay, masking GUS gene expression in this system [4].

Luciferase activity is also a suitable 'reporter' to monitor transcriptional regulation of chimaeric genes in plant cells. Using pCV701 with the lux A and lux B genes of *Vibrio harveyi* luciferase, Koncz *et al.* [47] demonstrated lux gene expression in carrot protoplasts 8 to 24 hours after plasmid introduction by electroporation. Luciferase activi-

ty was still detectable 8 days after uptake. Such experiments demonstrated that transgenic plant cells are able to assemble correctly complex heterodimeric bacterial enzymes.

Stable transformation of monocotyledons by direct DNA uptake

Some of the earlier transient gene expression studies, especially those with cereal protoplasts, optimised the DNA uptake conditions for subsequent attempts to produce stable transformants. Although high levels of natural resistance to antibiotics, such as kanamycin, shown by cells of some monocotyledons were believed to preclude the use of kanamycin for selection of transformed cells [91], this has been shown not to be the case. In studies published simultaneously, Potrykus *et al.* [71] and Lörz *et al.* [51] selected kanamycin-resistant tissues of *Lolium multiflorum* and *Triticum monococcum* after PEG-induced uptake of pABD1 and pBL1103-4 carrying the NPTII gene with CaMV and nos promoters respectively.

In an extension of their transient gene expression studies, Fromm *et al.* [29] obtained kanamycin-resistant tissues when protoplast-derived colonies were exposed to 100 μg/ml of kanamycin 2 weeks after electroporation of pCaMVNEO into maize cell suspension protoplasts. Likewise, others [36] assessed the use of vectors conferring resistance to hygromycin, kanamycin or methotrexate with cell suspension protoplasts of *Triticum, Panicum, Pennisetum* and *Saccharum*, and obtained resistant clones at 1.0×10^{-5} to 2.0×10^{-6}. Southern blot hybridisation confirmed stable integration and expression of DNA in *Triticum monococcum* protoplasts using hygromycin vectors and into protoplasts of *Panicum maximum* using the methotrexate vector, with 1 to 10 copies integrated per haploid genome. Other studies with sugar-cane cell suspension protoplasts [10] produced kanamycin-resistant tissues at 8 in 10^7 after PEG-induced uptake of *Sma* I linearised pABD1, the plasmid used extensively in earlier transformation studies with tobacco protoplasts [66]. The time of selection of sugar-cane protoplast-derived colonies on kanamycin-containing medium was critical, 80 μg/ml being used 7 days after DNA uptake. Heat-shocking the protoplasts prior to plasmid uptake did not increase the frequency of transformation, although it had previously increased transformation 5–20-fold for tobacco mesophyll protoplasts by pABD1 [80]. Sugar-cane protoplasts were found to be unstable when electroporated either in the presence or the absence of PEG. The use of agarose droplets during protoplast culture was essential for recovery of transformed tissues in this monocotyledon.

Much attention has been devoted to the production of transgenic plants in maize and rice, following development of protoplast-to-plant systems. Rhodes *et al.* [75] electroporated protoplasts from embryogenic cell suspensions of *Zea mays* (Black Mexican Sweet) and selected cell colonies exhibiting NPTII activity following growth of protoplasts on filters over feeder layers of maize suspension cells. Unfortunately, the transgenic plants were sterile, but the production of fertile non-transformed plants from protoplasts of elite lines of maize [72, 79] should result in fertile transgenic plants. In rice, Uchimiya *et al.* [89] selected protoplast-derived colonies on medium with 100 μg/ml of kanamycin upon PEG-induced plasmid uptake, such experiments forming the basis for the production of transgenic rice plants by the same group [88]. In the latter experiments, transformed cells were selected by their tolerance to G418 after electroporation-mediated plasmid delivery. In a similar series of experiments with *Oryza sativa* cv. Taipei 309, Yang *et al.* [96] optimised the conditions for transformation of cell suspension protoplasts by pCaMVNEO. Electroporation of heat-shocked protoplasts gave transformation frequencies of 19.9×10^{-5} and was more efficient than PEG or PEG combined with electroporation. Southern blot analysis showed some clones to have a single insert of the NPTII gene; others had a series of bands additional to that of the NPTII gene. These optimum electroporation conditions resulted in transgenic plants when applied to protoplasts of an embryogenic cell suspension [98]. Of 6 regen-

erated plants chosen for analysis, all contained the NPTII gene, but only 2 expressed NPTII activity. One of these NPTII-positive plants was fertile and produced seed. Hygromycin B at 20 μg/ml used 5 weeks after electroporation of *Oryza sativa* cv. Nipponbare cell suspension protoplasts with pGL2 has also been used to select transformed colonies from which fertile transgenic plants have been regenerated [81]. Seeds from transformed plants also expressed GUS activity in their embryos and endosperm, confirming co-transformation of an unlinked non-selectable gene with pGL2.

An alternative to the use of a direct marker to select transformed tissues following plasmid DNA uptake into protoplasts is provided by experiments demonstrating GUS expression in rice varieties Pi-4 and Taipei 309 [97], after PEG-induced uptake of pA_1GusN linearised with *Eco* R1. Out of 378 calli assayed, 61 showed GUS activity, and of 378 regenerated plants, at least 86 were transgenic as confirmed by DNA hybridisation. The plasmid used for transformation included the maize Adh1 gene promoter which is known to be induced by anaerobiosis; GUS activity was increased 5–6-fold in the roots of some transgenic plants under anaerobic conditions.

Evaluation of methods for DNA delivery to plant cells

Although the plasmid delivery procedure is dictated primarily by the protoplast system under investigation, electroporation has become the method of choice in many laboratories. This method has also been employed to introduce viral particles and viral nucleic acids into protoplasts of tobacco [36, 60, 61] and rice [62, 63]. In addition to chemical methods, electroporation, and PEG with electroporation, several other approaches have been reported for delivering DNA to plant cells. The transformation of seeds by exogenous DNA has been a controversial issue since Ledoux *et al.* [49] claimed to have corrected thiamine deficiency in *Arabidopsis thaliana* by soaking seeds in DNA. However, more recent experiments demonstrated NPTII activity in germinating wheat embryos upon uptake of DNA by imbibition into isolated dry and viable embryos, NPTII activity being increased markedly when the NPTII gene was carried by a vector capable of autonomous replication [86]. Transient gene expression, resulting from contamination by microorganisms, was discounted in these experiments.

In an attempt to avoid the need to develop a protoplast-to-plant system for cereals other than maize and rice, de la Pẽna *et al.* [20] obtained transgenic rye plants by injecting pLGVneo1103 into young floral tillers 14 days after meiosis. From 3023 seeds derived by cross-pollinating 98 plants injected with the plasmid, 7 seedlings remained green in the presence of kanamycin, with 2 exhibiting APHII activity.

Other workers have attempted to transform flowering cereal plants. The 'pollen tube pathway' was first reported for rice [25] and later described in detail [52]. DNA applied to the styles of pollinated flowers after excision of the stigma is thought to reach the ovules by flowing down the pollen tubes. Transformation with plasmid containing the NPTII gene was reported to be high, with up to 20% of the rice seeds from treated florets giving plants that contained the foreign gene. However, it remains to be seen whether this method is generally applicable to rice and other cereals.

An alternative to the injection of large quantities of DNA into recipient plants is the microinjection of DNA into individual protoplasts, cells and small cell clumps. Steinbiss and Stabel [83] demonstrated that protoplast-derived tobacco cells could survive capillary micro-injection of the dye lucifer yellow, which was followed by the injection of DNA into protoplasts of tobacco [14] and lucerne [74] to produce transgenic colonies. The successful micro-culture of cells [82] laid the foundation for the injection of 12-celled microspore-derived somatic embryos of *Brassica napus* with NPTII gene constructs [59] and their subsequent culture to plants. Of the injected microspores 27–51% gave transgenic plants, the chimaeric nature of these primary regenerants

being demonstrated after their *in vitro* segregation through secondary embryogenesis into pure transformants. Since heart-shaped embryos could also be micro-injected, these workers concluded that this technique might also be suitable for the transformation of zygotic embryos.

A method that has received considerable attention recently has been the use of high-velocity microprojectiles for delivering nucleic acids to intact plant cells [40], negating the requirement to isolate protoplasts for transformation. Tungsten particles (4 μm in diameter) were used to carry p35S-CAT or RNA into epidermal tissue of onion. Extracts from epidermal tissue bombarded with microprojectiles showed high levels of CAT activity. In subsequent work [93], these transient gene expression studies were extended by monitoring GUS expression in rice and wheat cells, followed by detailed studies of CAT expression in cells of rice, wheat and soybean. In studies with soybean, Christou *et al.* [12] used immature embryos as targets for DNA-coated gold particles. Protoplasts prepared from these embryos and cultured under selective conditions for NPTII gene expression produced kanamycin-resistant calli at a frequency of 1 in 10^5. Subsequently, DNA-coated gold particles were introduced into the meristems of immature soybean seeds [54]. Approximately 2% of the shoots derived from the meristems by organogenesis were chimaeric for expression of the introduced gene. R0 and R1 plants expressed the NPTII gene; one R0 plant expressed both NPTII and GUS activity after transformation with a plasmid carrying both of these genes.

Kanamycin-resistant tobacco plants have also been obtained after plant regeneration from cells of leaves and suspension cultures treated with tungsten microprojectiles [41]. Other studies, involving an assessment of factors influencing the efficiency of DNA delivery to suspension cells of *Zea mays* by GUS expression, showed that several parameters were important in maximising transformation, including the nature of the support for the recipient cells in the bombardment apparatus, microprojectile number and velocity, and the concentration of $CaCl_2$ and spermidine used to adsorb DNA to the microprojectiles [42]. These authors suggested that particle bombardment may be useful for the stable transformation of monocotyledonous species. In plants, organelle transformation has been reported using microprojectiles, transformation of three mutants of the chloroplast *atpB* gene of *Chlamydomonas reinhardtii* with tungsten particles coated with cloned chloroplast DNA carrying the wild-type gene restoring the photosynthetic capacity of the mutants [6].

Other vectors for plant transformation; consideration of the size of transferable DNA

Some attention has been directed towards the use of viruses as vectors for introducing genes into plants. Brisson *et al.* [7] replaced the non-essential open reading frame II of CaMV with the dihydrofolate reductase gene and used the construct to transform turnip plants. However, the vector had a restricted host range, limited space for inserting DNA and it was necessary to eliminate 5′ and 3′ non-coding sequences. In order to circumvent these defects, pKR612B1 was constructed containing the NPTII gene under the control of the CaMV gene VI promoter [67]. This construct was similar to, but more versatile than pABD1 [84], and was used to transform protoplasts of *Brassica campestris* var. *rapa* by PEG-induced plasmid uptake. However, transformation was achieved only when the hybrid gene was supplied to protoplasts with wild-type viral DNA.

What is the size limit of DNA constructs above which direct stable transfer to protoplasts becomes inefficient? Several authors [18, 24, 43] have used chemical methods to introduce Ti plasmids into protoplasts, while Langridge *et al.* [48] obtained transgenic somatic embryos of carrot after electroporation with the ca. 194 kb pTiC58. However, while the presence of T-DNA (about 20 kb) was demonstrated in the transformed tissues, these studies gave no clear indication whether large plasmids, other than the 'naturally transferable' plasmids of *Agrobacterium*, might be delivered to protoplasts by such methods.

As already discussed, the recent trend has been to improve transformation efficiency by reducing the size of vector DNA. Most groups have confined their attention to plasmids of less than 20 kb in size when transforming protoplasts by direct DNA uptake, the 16 kb pCT1T3 [90] being one of the largest plasmids to have been used in such studies. By comparison, using *Agrobacterium*-mediated delivery, somewhat larger fragments of foreign DNA may be introduced into plant cells by employing suitable cosmid shuttle vectors. For example, such a vector, pOCA18, was used to deliver 20 kb fragments of *Arabidopsis* DNA into tobacco [65]. Some (10/16) transformants produced in these experiments contained full-length fragments of *Arabidopsis* DNA, while others (6/16) possessed partially deleted forms of the foreign DNA sequences. It would be of interest to determine whether such recombinant cosmids could be introduced into protoplasts using direct methods. Obviously, to be of general use, chemical methods or electroporation would have to deliver the cosmids efficiently, such that transformants could be obtained with a frequency as good as or better than obtained upon *Agrobacterium* delivery.

The question of the upper size limit for stable DNA transfer and integration by chemical or electrical methods is particularly topical in the light of recent developments in DNA cloning and electrophoresis methodology. Burke *et al.* [8] have developed a cloning system based on the construction of yeast artificial chromosomes (YACs), which can accept foreign DNA inserts of more than 100 kb at a suitable restriction site; the foreign DNA-containing YACs can be maintained in *Saccharomyces cerevisiae* after PEG-induced transformation of spheroplasts. The YAC vectors themselves can be maintained as circular molecules in *E. coli*, which permits the isolation of milligram quantities for cloning procedures. YACs containing 100 kb inserts can be distinguished and separated from native yeast chromosomes by pulsed field gel electrophoresis [13, 77]. A preliminary investigation of the use of the YAC system in constructing libraries of large (greater than 100 kb) fragments of *Arabidopsis* and carrot DNA has been described [31]. If such libraries can be made truly representative of the entire genome of plants such as *Arabidopsis*, this YAC methodology should be a stimulus in attempts to isolate genes by chromosome walking. In addition, it will be of interest to determine whether such plant DNA-containing YACs can be introduced into protoplasts by chemical methods or electroporation and maintained without deletion or rearrangement. Although at present somewhat speculative, success in this technical challenge would greatly facilitate the development of strategies for isolating genes by complementation of genetic mutants, especially mutants of species with relatively small genomes such as *Arabidopsis*, by direct transformation with plant DNA-YACs. An intriguing report [1] indicates that large pieces of DNA can be taken up and expressed in animal cells, since a chromosome of the fission yeast *Schizosaccharomyces pombe* can replicate autonomously in cultured mouse cells and confer resistance to the antibiotic G418.

One area where direct methods for delivery to protoplasts may be a valuable alternative to current procedures, lies in the generation of developmental mutants by insertional mutagenesis. Assuming for the species under study that plant regeneration from protoplasts is efficient and somaclonal variation is negligible, it should prove possible to produce insertional mutants by, for example, electroporation of protoplasts with small plasmids. T-DNA insertion mutagenesis is effective in *Arabidopsis* when *Agrobacterium* is used to deliver transforming DNA with reasonably high efficiency. Thus, the recent report of a direct DNA transfer method for this species [17] may therefore prove valuable in mutagenesis studies.

Concluding remarks

Direct DNA uptake is used routinely for introducing genetic information into plants, with the merits and limitations of this transformation approach being discussed [57]. Chemical proce-

dures and electroporation are used extensively for species in which a protoplast-to-plant system is available, while injection and ballistic approaches are being assessed for several crop plants. Extensive transient and stable gene expression studies using protoplasts as recipient cells have shown that gene delivery and expression are influenced by a number of parameters, including plasmid configuration, carrier DNA, the concentration and molecular weight of PEG used and, in the case of electroporation, the voltage, pulse duration and buffer solution. The nature of the protoplasts themselves also influences transformation [58]. Although each of these parameters must be optimised for the protoplast-DNA combination under investigation, this approach will continue to be applicable to the transformation of a range of crop species, especially monocotyledons.

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