GENE TRANSFER TO PLANTS:
Assessment of Published Approaches and Results

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INTRODUCTION

Since the first transgenic plants were regenerated (30) the area of gene transfer to plants has seen exciting progress. A review on this area could discuss many topics: which genes have been transferred to which plants, how much we have learned about plant genes and their regulation, how elegantly transgenic plants and reporter genes have been applied to study plant development, how efficiently transient expression systems have been used to study gene regulation, how much progress has been achieved in the application of gene technology to the improvement of crop plants, and so on. These and other topics deserve discussion, and many reviews are available (e.g. 15, 22, 25, 30, 42, 47-49, 51, 65, 90). The present chapter focuses on a critical assessment of gene transfer methods, a problem little discussed so far (68, 69).

Numerous laboratories have transferred genes to a wide collection of plants, including not only those considered easy experimental models [such as tobacco (Nicotiana tabacum) and petunia (Petunia hybrida)] but also those considered recalcitrant [such as grain legumes---e.g. soybean (Glycine max)---or cereals---e.g. rice (Oryza sativa) and maize (Zea mays)]. Certainly, for someone interested in studying gene regulation in Arabidopsis thaliana gene transfer is no longer a technical problem. One can choose between several established methods. Either Agrobacterium-mediated gene transfer to root explants and seeds, or direct gene transfer to protoplasts can be expected to function, although one might encounter difficulties with some genotypes or with the seed approach. One could even use the biolistic approach, or microinjection. Someone interested in studying tissue specificity of promoter constructs or the functionality of homologous or heterologous genes in differentiated tissues could apply biolistics to plant tissues with a fair chance of success. In many cases, however, where the experimenter is not free to choose the experimental organism, and where reproducible production of transgenic plants of a given genotype of a given species is required, as is the case with most applied projects or with projects where gene technology should be applied to complex and genetically well-defined marker strains, gene transfer can still be a serious experimental problem. The quest is still open for a method that will allow routine and efficient gene transfer into all desired genotypes of any plant species.

For some time there was good reason to believe that Agrobacterium tumefaciens was the vector system with the capacity for gene transfer to any plant species and variety. As this is not the case (I will propose a hypothesis to explain why), numerous alternative approaches have been tested; some have been successful, including "direct gene transfer to protoplasts" (65), "biolistics" (77), and "microinjection into proembryos" (61). Numerous others have yielded interesting but probably artifactual data. Because Agrobacterium and
the other “successful” methods have inherent limitations it may be unrealistic to hope that a general method will be possible at all; a wide variety of methods might be the solution for future gene transfer problems. Therefore it seems worthwhile to discuss even approaches that have not been successful so far. The present assessment of gene transfer technology focuses on problems. The achievements and perspectives have been highlighted (often too strongly) in the original publications and previous reviews. I focus on cereals as an example of a group of plants difficult to transform. Discussions of cereal problems probably also apply to other plant species difficult to transform.

Because gene transfer problems started with Agrobacterium, I start by discussing why Agrobacterium may not function with cereals (and other plant species). I focus on integrative transformation leading to stable transgenic plants. It will not be possible to include a careful discussion of the exciting achievements of transient expression systems (25, 32).

My assessment here is based on a rigid definition of “proof of integrative transformation.” This is mandatory because many data in the literature have been misinterpreted on the basis of indicative evidence. Those who disagree with the view that indicative evidence can be misleading will not agree with my assessment. This assessment is also based on an interpretation of biological parameters affecting gene transfer, and I make several statements for which no solid experimental data are available. Acceptance or refusal of these statements will not alter the assessment of the available data but may influence interpretation of the future potential of the various approaches. The literature and public presentations in the area of gene transfer methods are confusing; for someone not personally involved, they are probably difficult to understand. I hope my assessment will help to clarify the situation. The assessment rests on two components: It accepts integrative transformation only if definite proof is available, and it tries to understand the biology behind the various gene transfer approaches. It is to be understood as a working hypothesis that can (so far) consistently explain the success or failure of the various approaches. It has also been efficient in predicting failure of novel approaches over many years. Though in agreement with all available data on gene transfer, it is a provocative hypothesis to be falsified or verified by future experimentation.

BASIC CONSIDERATIONS

Proof of Integrative Transformation

Based on indicative evidence, there are numerous methods to produce transgenic plants, including extremely simple ones. Because no transgenic plants could be recovered after use of most of these methods, however, indicative evidence must be untrustworthy. Indeed many researchers have
obviously been misled by artifacts, and it is good advice to demand suitable proof that transgenic plants have been produced. Neither genetic, phenotypic, nor physical data alone are acceptable. Proof of integrative transformation requires: 1. Controls for treatment and analysis; 2. a tight correlation between treatment and predicted results; 3. a tight correlation between physical (e.g. Southern blot) and phenotypic (e.g. enzyme assay) data; 4. complete Southern analysis containing (a) the predicted signals in high-molecular-weight DNA, including hybrid fragments between host DNA and foreign gene, and the presence of the complete gene, and (b) evidence for the absence of contaminating DNA fragments or identification of such fragments; 5. data that allow discrimination between false positives and correct transformants in the evaluation of the phenotypic evidence; 6. correlation of the physical and phenotypic evidence with transmission to sexual offspring; and 7. molecular and genetic analysis of offspring populations.

**Biology of Gene Transfer Protocols**

Certain biological parameters affect the delivery of foreign genes to cells and the fate of these genes in the cells. Consideration of these parameters may help us both to understand the problems of some approaches and to design better experiments in future.

1. Not all plant cells are totipotent.
2. Plants differ in their capacity to respond to triggers: They are competent for specific triggers.
3. Transgenic plants can be regenerated only from cells competent both for regeneration (in a broad sense) and integrative transformation (simultaneously or sequentially).
4. Plant tissues are mixed populations of cells with competence for many different responses. Considering the states of competence essential for recovery of transgenic plants, the following situation must be considered: (a) A very small (and varying) minority of cells in plant tissues will be competent for both transformation and regeneration. (b) Other cells will be competent for transformation or regeneration. (c) A larger fraction of cells will be potentially competent, which means that given the correct treatment they will have the potential to shift to the competent state. (d) A variable portion of the cells will not even be potentially competent but will be noncompetent.
5. The relative composition of cell populations in tissues is determined by the species, the genotype, the type of organ, the developmental state of the organ and tissue areas within the organ, and even by the individual history of the experimental plant.
6. The most effective trigger for shifting cells potentially competent for regeneration into the competent state is mechanical (and enzymatic?) wounding. The "wound response" (45) is probably the biological basis for proliferation and regeneration from somatic cells.

7. Plant species differ in their wound response, as do different cells of the same plant. Graminaceous plant species, especially the cereals including maize (and probably grain legumes), have only a rudimentary wound response.

8. For some genotypes it is possible to cause cells competent for regeneration to proliferate under experimental conditions that maintain this state ("embryogenic suspensions"; 86). Such cell cultures contain cells competent for regeneration and (after protoplasting?) competent for integrative transformation.

9. Plant cell walls are efficient barriers and traps for DNA molecules of the size of a functional gene. A recent publication (16) seems to contradict this statement. However, I present an alternative hypothesis for the interpretation of the data in the section below on free passage of functional genes across cell walls.

10. So far, genes can be transported into walled plant cells only with the help of Agrobacterium, viruses, microinjection, and biolistics.

11. Production of transgenic plants requires efficient gene transfer into cells competent (simultaneous or consecutive) for integrative transformation and regeneration.

12. Competence for integrative transformation has obviously little relation to competence for transient expression.

13. Nonviral DNA can integrate into the host genome; its presence in the cells does not guarantee integration.

14. Nonviral DNA does not travel from cell to cell; it is restricted to the cell to which it has been delivered.

15. Viral DNA does not integrate into the host genome even if present at very high copy numbers.

16. Viral DNA (and RNA) moves from cell to cell and can spread systemically throughout the plant; it is, however, excluded from the meristems and the "germ line."

Of course, statements on biological phenomena never fit 100%.

The above statements include the possibility of rare exceptions. If the reader can agree with the definition of proof and the statements concerning the biological parameters, the assessment of the literature on gene transfer to plants is relatively easy and straightforward, even in the evaluation of the future potential of the various approaches. The present assessment is thus in agreement with the published data and with data presented at public meetings.
It incorporates discussions of an EMBO workshop on gene transfer to plants organized by me in the fall of 1988 (70).

ASSESSMENT OF GENE TRANSFER APPROACHES TO PLANTS

Agrobacterium-Mediated Gene Transfer

Agrobacterium tumefaciens and A. rhizogenes provide excellent vector systems for the production of transgenic plants. We witness an ever-increasing list of novel transgenic species. Numerous excellent reviews (e.g. 22, 42, 48) and laboratory manuals (e.g. 20, 31) have been published. Molecular analysis of the events leading finally to the transfer of the T-DNA to host plant cells is close to completion, and elegant protocols have been worked out for using this biological vector in basic and applied research. The transfer process requires a coordinated interaction between the bacterium and the plant host. Unfortunately, though we know much about the contribution of the bacterial partner, we know little about the interaction from the plant cell—the latter being far more difficult to study. As long as it is possible to work with plant systems that cooperate, this is not a serious problem; but numerous plants (especially economically important ones) do not cooperate. In these cases more knowledge about the biological basis of the plant’s participation would be welcome. Decades ago A. Braun (8) collected valuable information on the importance of a state of competence for tumor transformation in the plant cell. Along the same lines A. Binns (6, 7) is now reviewing our knowledge of the biology of “host range limitations.” Lack of competence in their cells is probably the reason some plants can be transformed (a) only with great difficulty and after screening of numerous plant genotypes and Agrobacterium strains (e.g. 41), (b) during a short developmental window of specific organs, or (c) not at all.

The common denominator may be found in the phenomenon of “wound response” (45). Plants and tissues differ in their wound response. Only plants with a pronounced wound response develop larger populations of wound-adjacent competent cells for regeneration and transformation. Plants that have been recalcitrant to transformation with Agrobacterium probably do not express the appropriate wound response. This is probably the reason for the complete failure to transform cereal plants with Agrobacterium, despite the enormous effort so far invested in this approach. Most experiments have not been published because they failed; some promising data have been presented

1As to the participants of this workshop, I am grateful to my coworkers, Drs. S. K. Datta, G. Neuhaus, J. Paszkowski, M. W. Saul, C. Sautter, and G. Spangenberg, for discussions and for information they collected at meetings and contributed from the literature.
at international meetings, but because no proof has been presented to date, the data are considered artifactual.

Transformation of "monocots" (e.g. 10, 43, 78) is of no importance in this context: It is not because they are monocots that cereals are difficult to transform but because they do not have the proper wound response. Monocots with wound response (e.g. Asparagus) are as easy to transform as dicots with wound response; and dicots without proper wound response (e.g. grain legumes?) are probably as difficult to transform as cereals. The report on transformation of maize seedlings (34) does not present proof and did not lead to transgenic offspring; the data are therefore considered artifacts.

Why is it probably impossible to transform cereal plants with Agrobacterium? Wounding of differentiated cereal tissues does not lead to wound response–induced dedifferentiation of wound-adjacent cells and accumulation of competent cells. Instead wound-adjacent cells accumulate phenols and die. Although Agrobacterium obviously transfers T-DNA into wound-adjacent cells efficiently in cereals (see the section below on agroinfection), even integration of this T-DNA cannot lead to transgenic clones and plants. It is not as easy to understand why even experiments with meristems (e.g. leaf base and split shoot tip), which can form proliferating cultures in vitro, did not yield transgenic clones and plants. A possible explanation may lie in the fact that cereal cell cultures are not the consequence of proliferating wound meristems but rather are based on adventitious or axillary meristems (46). Wounding plus in vitro culture does not lead to many competent cells but to a few meristem initials that proliferate as meristems. And meristematic cells may not be competent for transformation (see the section below on microinjection).

Of the many attempts to transform cereal cell cultures with Agrobacterium, (which should be possible), only one has been reported successful (73). Unfortunately, insufficient data are presented to constitute proof as defined above. From three independent lines of experimentation three different types of indicative evidence are presented, each of which could be considered an artifact. If co-incubation of rice suspension cultures with Agrobacterium could indeed lead to transgenic clones it should be relatively easy to produce conclusive data. The key problem in Agrobacterium-mediated transformation of cereals (and other recalcitrant plants?) probably lies neither with Agrobacterium (it transfers its T-DNA to cereals) nor with the host range (cereals are probably included) but rather with the availability and accessibility of cells competent for integrative transformation and regeneration.

**Agroinfection**

Viral DNA integrated into the T-DNA of the Ti-plasmid can be delivered into plant cells with the normal Agrobacterium T-DNA transfer process (29, 35).
The consequences in plants with wound response are the following: The viral genome enters the plant cell as part of the T-DNA. It is released to form a functional virus that replicates and spreads systemically. It may not be necessary for the T-DNA to integrate in order to release the virus. However, T-DNA can integrate; thus agroinfection can lead to integration of viral DNA in competent wound-adjacent cells and consequently to transgenic plants containing integrated viral DNA. This is similar to normal T-DNA transformation.

Agroinfection with maize streak virus in maize has led to systemic spread of the virus (37). Showing for the first time that T-DNA transfer to cereals is possible, this finding caused considerable excitement; it was taken as evidence that the well-established Agrobacterium vector system could be used for genetic engineering of cereals. Later it was even demonstrated that the efficiency of such T-DNA transfer is comparable to that in dicot species (36).

Does agroinfection, then, have potential for the production of transgenic cereal? Of course, the virus is released into wound-adjacent cells in cereals, whence it can spread systemically. Unfortunately, the spreading virus does not integrate, even if it reaches one of the rare competent cells in the plant. At the wound site, however, the T-DNA and the virus face the problem discussed above: Even if integration were to occur it would have no consequences because the wound-adjacent cells die. Therefore agroinfection has no better chance of yielding transgenic cereals than does Agrobacterium infection alone. Nevertheless, it does have considerable potential for studies in virus biology, because it can transfer deletion mutants and even single viral genes. It also has the merit of having shown, by its amplifying effect, that Agrobacterium interacts with cereals.

Viral Vectors

In 1984 an experiment was published (9) that we hoped would initiate important contributions of viral vectors to the genetic engineering of plants. A small bacterial antibiotic resistance gene integrated into a deletion mutant of a DNA virus spread systemically throughout infected plants and made them resistant to the antibiotic; but so far hopes that multicopy amplification and systemic spread of engineered viruses could be exploited to produce large quantities of genes or gene products have not been fulfilled. Viral genomes are obviously so compact that they do not easily tolerate foreign genes (27).

The discovery that RNA viruses can be reverse-transcribed to yield cDNA clones that again are infective opened up the possibility of applying genetic engineering technology to the far larger group of RNA viruses (1, 2); but because viral DNA does not integrate into the host genome, and is excluded from the meristems and offspring, it is difficult to envisage how viral vectors could contribute to the production of transgenic plants. The difficulty might
be overcome if a transposable element were part of a systemic virus, and if the
transposable element carried a gene of interest, and if the transposable
element could be induced to excise and integrate.

Protoplasts and Direct Gene Transfer
Protoplasts (isolated plant cells without cell wall) are ideal for gene transfer.

1. The freely accessible plasmalemma guarantees that genes can reach and
enter each and every protoplast at DNA concentrations that can be reg­
ulated experimentally.
2. The enzymatic (or mechanical) isolation procedures, however, induce
wound response; this shifts potentially competent cells into the competent
state, thus increasing the proportion of cells competent for regeneration
and (?) integrative transformation.
3. The foreign genes reach every competent cell, thus increasing the chance
for recovery of transgenic plants from a given population.
4. Gene transfer does not require any biological vector; DNA uptake is a
physical process, thus circumventing any possible hostrange problem.

We developed “direct gene transfer” as an alternative to use of Agrobac­
terium because of the foreseeable difficulties with cereals (67). DNA uptake can
be promoted by various treatments including polyethylene glycol and/or
electroporation (26, 60, 80). Integrative transformation can be very efficient
(60) and leads to stable inheritance of predominantly single-gene loci of the
foreign gene (71). Cotransformation efficiently transfers nonselectable genes
(79). Homologous recombination enables gene targeting (66). A barrier to
gene transfer has so far not been detected: Virtually every protoplast system
has proven transformable, though with different efficiencies.

So, is there no problem with the recovery of transgenic plants from
protoplasts? Unfortunately, there are severe problems, all related to plant
regeneration from protoplasts. Although exciting progress in this respect has
been made (75) and further progress can be foreseen, plant regeneration from
protoplasts will probably always be a delicate process (with exceptions); it
will probably also depend upon parameters not under experimental control
[e.g. species and genotype-dependent competence for wound response and
regeneration (72)]. Transgenic plants have been recovered recently from
protoplasts from important crops that could not be transformed with Agrobac­
terium: Several laboratories reported on transgenic Japonica-type rice (81, 84,
92, 93), one on Indica-type rice (14); following production of sterile transgen­
ic maize (74), a very recent report described recovery of numerous fertile
transgenic maize plants (19). Because plant regeneration from protoplasts in
wheat (87) and plantlet regeneration in barley have also been reported (91),
and because other graminaceous species were regenerated earlier, further transgenic graminaceous monocots will probably follow. However, these successes are far from promising routine application of gene technology to grasses. All these experimental systems depend on the establishment of an embryogenic cell culture system (86) that cannot be established easily for every desired commercial variety.

**Biolistics or Particle Gun**

Acceleration of heavy microparticles coated with DNA has been developed into a technique that carries genes into virtually every type of cell and tissue (50, 76). No gene transfer approach since the early *Agrobacterium*-mediated gene transfer experiments has met with so much enthusiasm, and in no other gene transfer approach has there been a comparable investment in experimentation and manpower. Some expected that this technique would solve all gene transfer problems. Indeed the biolistic approach has advantages and potential for general applicability:

1. It is easy to handle.
2. One shot can lead to multiple hits (transfer of genes into many cells).
3. Cells survive the intrusion of one (?) particle.
4. The genes coated on the particle have biological activity.
5. Target cells can be as different as pollen, cell culture cells, cells in differentiated tissues and meristems.
6. They can be located at the surface or in deeper layers of organs.
7. The method depends on physical parameters only, and so on.

Thus the method allows the transport of genes into many cells at nearly any desired position in a plant without too much manual effort. The enormous investment into this technique has paid off, and transgenic plants have been recovered that would have been difficult to produce by other methods. (One wonders whether with a similar investment other methods might not have been made successful, too.)

The first transgenic soybean plants were reported simultaneously via *Agrobacterium* vectoring (41) and biolistics (58); however, biolistics has by now become far more successful than use of *Agrobacterium* in this crop (12). The real breakthrough for biolistics came with the recovery of fertile transgenic maize in three independent laboratories (23, 33; S. Jayne, J. Suttie, M. Koziel, G. Pace et al, personal communication), and other laboratories may be close to a similar success. This success with maize will guarantee further investment in this technique. However, an interesting question remains: Why, given the advantages listed above, is this technique so inefficient in yielding stable integrative events, especially in experimental systems as ideal as those
used (i.e. embryogenic suspensions)? If one compares (a) the number of fertile transgenic maize plants recovered from biolistic treatment in large-scale experiments using embryogenic suspensions with (b) those recovered from a comparable small-scale experiment on direct gene transfer to protoplasts isolated from such embryogenic suspensions, then the low yield from the biolistic experiments is surprising. In my opinion, plants difficult to transform using Agrobacterium probably have very few competent cells; the particle has to reach these rare cells by a random hit, and the DNA has to integrate into the genome of these cells. Considering the low conversion rate of transient events (hits) to stable integrative events in biolistic systems, integrative transformation in recalcitrant transformation systems must be expected to be rare. As I see it, the real advantage of the biolistic technique to date lies in its application in transient gene expression studies in differentiated tissues (32). In this area the technique has little competition.

**Microinjection into Zygotic and Microspore-Derived Proembryos**

Microinjection uses microcapillaries and microscopic devices to deliver DNA into defined cells in such a way that the injected cell survives and can proliferate (61). This technique has produced transgenic clones from protoplasts (59) and transgenic chimeras from microspore-derived proembryos in oilseed rape (62). Like biolistics, microinjection definitely delivers DNA into walled plant cells. In comparison with biolistics, microinjection has disadvantages: Only one cell receives DNA per injection, and handling requires more skill and instrumentation. It also has advantages:

1. The quantity of DNA delivered can be optimized.
2. The experimenter can decide into which cell to deliver DNA.
3. Delivery is precise and predictable, even into the cell nucleus, and is under visual control.
4. Cells of small structures (e.g. microspores and few-celled proembryos, which are not available in the large quantities required for the biolistic technique, can be precisely targeted.
5. Defined microinjected cells can be microcultured.
6. In combination with protocols for the culture of zygotic proembryos, microinjection should offer an approach to transformation open for every species and variety with sexual propagation.

On the assumption that few-celled zygotic proembryos contain competent cells, our group has established plant regeneration from isolated zygotic proembryos of maize (*Zea mays*), wheat (*Triticum aestivum*), rice (*Oryza sativa*), barley (*Hordeum vulgare*), soybean (*Glycine max*), cotton (*Gossy-
pium hybrid), sunflower (Helianthus annuus), tobacco (Nicotiana tabacum), and Arabidopsis thaliana. Following multiple microinjections with proven marker genes, putative primary transgenic chimeras and sexual offspring have been analyzed for the presence of the foreign genes (G. Neuhaus, G. Spangenberg, S. K. Datta, personal communication). So far we have only indicative evidence for transgenic chimeras. As we have no proof yet for transgenic offspring, these may be artifacts. Gene transfer to structures consisting of more than one cell can at best produce transgenic chimeras. Therefore two interpretations of the data are possible to date: (a) Larger experiments will increase the chance for transmission of the transgene to the offspring (as exemplified with the biolistic approach), or (b) meristematic cells have little competence for integrative transformation. To test the second hypothesis an experiment has been performed using the well-established gene transfer system of Agrobacterium and zygotic proembryos of N. tabacum var SR1, a well-documented model for Agrobacterium-mediated transformation. No transgenic tissue could be detected in the primary regenerants and in the sexual offspring (G. Neuhaus, A. Matzke, M. Matzke, personal communication). The microinjection data from proembryos indicated above, the data from the experiment just described, the difficulties in transforming embryogenic cultures and meristems with the biolistic approach [transgenic soybeans (58) are not from meristematic cells but from differentiated cells below the meristem developing to adventitious shoot buds], the negative experience using Agrobacterium with meristematic cells in general, and the well known phenomenon of virus exclusion from meristems—all this may point to a biological problem well worth studying: Do meristematic (embryonic) cells have a mechanism that prevents integration of incoming nucleic acids?

Four of the approaches discussed so far have provided proof for the production of transgenic plants (Agrobacterium vectoring, direct gene transfer to protoplasts, biolistics, and microinjection); two have not (agroinfection and viral vectors). One key difference between the former group of approaches and the following one is the concern about the cell wall. Agrobacterium uses a still-unexplained biological mechanism to overcome the cell wall; viral vectors use amplification of rare events; protoplasts have stripped off their cell wall; and biolistics and microinjection apply brute force. All of the following (and so far unsuccessful) approaches hope (with the exception of the microlaser technique) for a free movement of genes across the plant cell wall.

**Free Passage of Functional Genes across Cell Walls?**

The experimental design of the gene transfer approaches discussed below requires free movement of large DNA molecules across one, several, or many cell walls to achieve transformation of target cells. The feasibility of this
movement will determine the potential of most of these following approaches, of which none has yet yielded a single proven transgenic plant. In this context a recent publication (16) on transient expression of marker genes in rice (Oryza sativa) tissues following electroporation of tissue slices from seedlings is of crucial importance. Using GUS and NPTII genes under constitutive and tissue-specific expression signals and electroporation with cross sections of seedlings, the authors describe data on transient expression of foreign genes within the rice tissue that convince even the critical reader. If the interpretation of the data in this publication is correct, then functional genes actively travel, without problems, across many cell walls. It is especially striking how evenly GUS gene expression was distributed over the entire cross section of leaf whorls and how well organ-specific expression fits the expectation. The assumption is that the genes reached every cell in the tissue. However, owing to (a) hundreds of experiments that had failed to transport genes across cell walls (including electroporation with other experimental systems), (b) the theoretical problem of envisaging how entire genes might cross the physical barrier of the cell walls, (c) the fact that cellulose fibrils are efficient adsorbers of DNA, (d) the knowledge that electroporation does not move DNA but just opens pores in membranes, and (e) the fact that there is not even a gradient in the experimental data, the interpretation of the data is difficult to understand; the data may fit an alternative hypothesis better.

I propose the following interpretation, which to my understanding fits all the data presented and agrees with the previous experience with DNA and cell walls. In the experimental system used, DNA was transferred not into walled cells but into protoplasts within opened cells at the cut surface of the tissue. (Cutting of plasmolyzed tissues with elongated cells was a standard technique in the late 19th and early 20th century to collect protoplasts.) If this were the case, the GUS stain would diffuse to neighboring cells and would be transported over longer distances along vascular bundles. Histological examination at different distances from the treated surface would mimic constitutive or organ-specific expression. The response of the system to the optimization of the electroporation protocol would be (and was) identical to the response of protoplast populations. At present, without further experimentation, it cannot be decided which interpretation of the data is correct. At present I continue in the view that passage of functional genes across plant cell walls does not normally occur.

**Incubation in DNA of Dry Seeds or Embryos**

Whereas in the previous experimental protocol one could envisage even recovery of transgenic cell clones (from protoplasts within opened cells), this is difficult to conceive in the following approach (82, 83) unless DNA moves freely into and between cells. The authors describe experiments in which
every precaution was taken to avoid the experimental pitfalls of earlier reports (52, 53). Incubation of dry seeds and embryos (from cereals and grain legumes) in viral or nonviral DNA yielded interesting evidence for gene expression and recombination. Although the experiments included convincing controls and clearly demonstrated the presence and expression of defined marker genes as well as the replication of engineered viral DNA, they do not provide proof of integrative transformation. The authors' conclusion that the data demonstrate uptake of foreign DNA into the cells of the embryo, and that this approach therefore has potential for the recovery of transgenic cereals and grain legumes, is probably too optimistic. On the assumption that the cell wall is no problem, these experiments should (but do not) yield transgenic plants. On the assumption that the cell wall is a problem, it is difficult to envisage how the DNA might reach those meristematic cells that will form the new plant. Alternatively, the data could be explained without the necessity of massive DNA transport across cell walls: The dry embryos were split off from the endosperm, creating a giant wound across the scutellum. Because the tissues are dry, the cell contents do not leak out. Incubation in DNA solutions could create a micro-environment in the open cells that enables in vitro transcription, translation, and replication. Further experiments will have to show which of the possible hypotheses is correct. I do not see, however, much potential here for the production of transgenic cereals.

**Incubation in DNA of Turgescent Tissue or Cells**

Over a period of more than 20 years, seedlings, organs, tissue explants, cell cultures, and cells have been brought in direct contact with DNA and defined marker genes with the hope of transformation. Included have been experimental designs involving open plasmodesmata or loosening of cell wall structures, as well as treatments assuring that potentially competent cells were present at high frequencies. However, even in experiments that would have detected extremely rare events, not a single case of integrative transformation has been proven. Experiments relying on the passage of functional genes across plant cell walls obviously have little chance for success. Plant cell walls are not only efficient barriers, but they are also efficient traps for DNA molecules. It would be very surprising if DNA can cross cell walls efficiently (16).

**Pollen Transformation**

This approach, under experimental challenge since the early 1970s, is based on the hope that DNA can be taken up into germinating pollen and can either integrate into the sperm nuclei or reach the zygote with the pollen tube (39). Indeed, this would be the ideal method for gene transfer into plants. Although surprising phenotypes have been recovered that could be interpreted as in-
dicative evidence for gene transfer (18, 39, 63), in no case so far has proof been provided. As numerous large-scale experiments in experienced laboratories and with defined marker genes have only given negative results, this approach does not seem a promising one. Not only the cell wall but also external and internal nucleases and the heterochromatic state of the acceptor DNA present problems for this technique. The latter problems may be overcome with the approach of “in vitro maturation” (5), where immature microspores are treated with DNA, matured to pollen, and used for pollination.

**Pollen Tube Pathway**

If it were possible to deliver DNA to the zygote via open pollen tubes, this approach would be very attractive. Unfortunately, a recent publication (56) providing phenotypic and molecular data on transgenic rice plants does not present proof. The Southern data show neither integration into high-molecular-weight DNA nor defined hybrid fragments, and can easily be understood as artifacts; the dot blot technique used is prone to artifacts, and the enzyme data are not reliable because cereals have a rich record in false positives with the assay used. Data from a recent poster presentation (38) do not clarify the situation but require complicated additional assumptions for explanation. It is also difficult to understand how the DNA applied to the cut pistil might reach the zygote: The pollen tubes are not open pipes but are sealed off with callose plugs; the DNA will be trapped in cell wall material; there are probably nucleases in the pollen tube and also in the synergids. However, the approach is attractive enough to be worthy of rigorous testing. Considering the possibility of transformation of contaminating endophytes might help to exclude this possible source of artifacts in future experiments. So far, transgenic plants have not been recovered from this approach.

**Macroinjection**

Use of injection needles with diameters greater than cell diameters destroys the cells into which DNA is delivered. DNA integration into cells would, therefore, require the DNA to move into wound-adjacent cells. This is not possible via plasmodesmata because of the size of the DNA molecules and because plasmodesmata are sealed off immediately upon wounding; nor is it possible across the cell walls. In the experiment that produced not only phenotypic changes in the offspring but also exciting molecular data (17), the DNA would have had to travel through many cell layers. A marker gene was injected into the stem below the immature floral meristem of rye (*Secale cereale*) to reach the sporogenic tissue. Hybridization with the marker gene and enzyme assays with sexual offspring having survived selection yielded strong indicative evidence, but no proof of transformation. Unfortunately, it has so far not been possible either to reproduce these data in several large-
scale experiments with other cereals or to establish proof with the original material. This approach probably has little chance of success.

**Electroporation**

Discharge of a capacitor across cell populations leads to transient openings in the plasmalemma. This electroporation facilitates entry of DNA molecules into cells if the DNA is in direct contact with the membrane. For protoplast systems, electroporation is one of several standard techniques for routine and efficient transformation (24–26, 80). Since in numerous important plant species regeneration is possible from cell cultures and tissue explants but not (yet) from protoplasts, it has been important to test whether electroporation could transfer genes into walled cells (54). A great variety of experimental systems have been challenged, including germinating pollen tubes, suspension cultures, and tissue explants. There were interesting phenotypic changes, but no proof of transformation was obtained. The most interesting case (16), transient expression in rice, has been discussed above.

**Electrophoresis**

In contrast to electroporation, electrophoresis can be expected to transport DNA. A series of experiments has been performed using shoot meristems of barley seeds to test whether electrophoresis of DNA across tissues could transport genes into cells (4). The data obtained included radioactively labeled cell walls (after use of radiolabeled DNA), positive GUS assays, and a protein band on SDS-PAGE with GUS mobility. There was no proof for integrative transformation and the data can be interpreted as artifacts. It might be worthwhile to test this idea with a simpler experimental system that can provide clearcut answers. One assumes, however, that even electrophoresis cannot overcome the cell wall barrier.

**Liposome Fusion**

Fusion of DNA-containing liposomes is an established technique for the production of transgenic plants (11). It has no obvious advantage over simpler methods of direct gene transfer and is not much in use. DNA-containing liposomes have also been applied to various tissues, cell cultures (28), and pollen tubes (3) with the rationale that liposomes might help in transporting the DNA via plasmodesmata or directly across cell walls. It has been shown that liposomes can carry small dye molecules into cells within tissues via fusion with the plasmalemma, but there is no proof of transport and integration of marker genes. As plasmodesmata are sealed off immediately upon wounding, this route is not open to even very small liposomes; impregnation of cell walls with phospholipids does not seem to change their barrier function.
Liposome Injection

Microinjection into differentiated cells can easily deposit the DNA into the vacuole, where it is degraded. Microinjection of liposomes into the vacuole, however, can lead to fusion with the tonoplast, thus releasing the content of the liposome into the cytoplasm, as demonstrated with cytoplasm-activated fluorescent dyes (57). It was an elegant idea to exploit this situation for transformation of vacuolated cells (55). Unfortunately, activity of DNA delivered by this method has yet to be shown. Though elegant, this method probably has no advantage over straightforward microinjection, especially for the transformation of recalcitrant plants, because those probably have to be regenerated from meristematic cells which do not contain a large central vacuole.

Microlaser

A microlaser beam focused into the light path of a microscope can be used to burn holes into cell walls and membranes (89). It was hoped that incubation of perforated cells in DNA solutions could serve as a basis for vector-independent gene transfer into walled cells (88). There are no conclusive data available on DNA uptake, and there are problems with DNA adsorption to cell wall material even before it could be taken up. As microinjection and biolistics definitely transport DNA into walled plant cells, the microlaser could offer advantages only in very specific cases where those techniques would not be applicable.

SUMMARY

This review is selective; it does not attempt an encyclopedic overview of the many publications and presentations in the area. However, I hope it is complete in discussing all the various approaches to integrative transformation. I here interpret the available data on the basis of a rigorous definition of proof and on the assumption that DNA does not freely cross cell walls.

Of the numerous approaches to integrative transformation Agrobacterium-mediated gene transfer and direct gene transfer to protoplasts are routine and efficient methods; further optimization may lead to better efficiency in integrative transformation for the biolistic process; microinjection probably has more potential than has been realized so far. Compared to the enormous investment in biolistics, microinjection has had only marginal support; experiments have focused exclusively on meristematic cells, which may not even be competent for integrative transformation.

Future developments in gene transfer will result from protocols effective with meristematic cells. Transformation of meristematic cells is probably the bottleneck through which production of transgenic plants from recalcitrant
species and varieties (those lacking a proper wound response) must proceed. There is a series of important questions to be studied, if we are interested in establishing a solid ground for further progress: What constitutes the type of wound response that provides the basis for the formation of wound meristems? What makes a cell competent for dedifferentiation, proliferation, and regeneration? What makes a cell competent for integrative transformation? Are viruses "excluded" from meristems because they cannot enter or because they cannot replicate? Development of more efficient protocols for routine transformation of recalcitrant species and varieties is less a technical than a biological problem. Efficient methods to transfer genes into plant cell already exist. Agrobacterium use is probably as efficient as biolistics, microinjection, and direct gene transfer. (I cannot share the optimism of those who hope for a free passage of DNA across plant cell walls.) First of all, however, we must learn into which cells to deliver the genes.

**Literature Cited**

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GENE TRANSFER METHODS


