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PLANT TRANSFORMATION: Problems and Strategies for Practical Application

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ABSTRACT

Plant transformation is now a core research tool in plant biology and a practical tool for cultivar improvement. There are verified methods for stable introduction of novel genes into the nuclear genomes of over 120 diverse plant species. This review examines the criteria to verify plant transformation; the biological and practical requirements for transformation systems; the integration of tissue culture, gene transfer, selection, and transgene expression strategies to achieve transformation in recalcitrant species; and other constraints to plant transformation including regulatory environment, public perceptions, intellectual property, and economics. Because the costs of screening populations showing diverse genetic changes can far exceed the costs of transformation, it is important to distinguish absolute and useful transformation efficiencies. The major technical challenge facing plant transformation biology is the development of methods and constructs to produce a high proportion of plants showing predictable transgene expression without collateral genetic damage. This will require answers to a series of biological and technical questions, some of which are defined.

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INTRODUCTION

Plant transformation is at a threshold. Over 3000 field trials of transformed plants are in progress or completed in at least 30 countries. These trials involve over 40 plant species modified for various economic traits (33, and updates in *Genetic Technology News*). We are emerging from a period of plant transformation research dominated by the need to develop proven genetic transformation methods for the major experimental and economic plant species, into the era of application of transformation as a core research tool in plant biology and a practical tool for cultivar improvement. Some of the most important issues (problems and strategies) affecting these uses are very different from those foremost in our thinking in recent years while the discipline focused on the scientific understanding and technical development of reliable systems for genetic transformation of a wide range of plant species.

The capacity to introduce and express diverse foreign genes in plants, first described for tobacco in 1984 (37, 74, 120), has been extended to over 120 species in at least 35 families. Successes include most major economic crops, vegetables, ornamental, medicinal, fruit, tree, and pasture plants. The rapid and simultaneous developments in transformation technology and information technology make tabulations of transformed species quickly out of date (41, 131), and it is advisable to use computer-based searches to locate references to

current transformation methods for species of interest. The process of diversification and refinement of transformation techniques for greater convenience, higher efficiency, broader genotype range, and desired molecular characteristics of transformants will continue to good effect for some time. However, gene transfer and regeneration of transgenic plants are no longer the factors limiting the development and application of practical transformation systems for many plant species. Attention is increasingly being directed to achieving the desired patterns of expression of introduced genes and to solving economic constraints on practical plant molecular improvement.

There are excellent recent reviews of the development of plant transformation systems using *Agrobacterium* (72, 146, 165), direct gene transfer into protoplasts (49, 113, 114), or particle bombardment (14, 25, 26), and the potential for their practical application (30, 73). These topics are now comprehensively addressed in recent texts (12, 54, 61, 90, 110) and methods manuals (39, 53, 57, 127, 153), to which the reader is referred for background information. In this review I aim to identify key problems remaining in the development of plant transformation systems, key issues to be resolved in the practical application of these systems, and strategies by which we may overcome these limitations.

DEFINITION AND VERIFICATION OF TRANSFORMATION

This review is concerned with the stable incorporation and expression of genes introduced into plants by means other than fusion of gametes or other cells. It focuses on transformation involving integration of introduced genes in the plant nuclear genome, although some issues are equally applicable to transformed plants in which introduced genes are expressed from an organelle genome (21), or a replicating viral vector (139).

Ingo Potrykus in 1991 (126) offered a provocative but clarifying assessment of plant transformation technologies based on a rigid definition of proof of integrative transformation, requiring a combination of genetic, phenotypic, and physical data. Unfortunately the combination specified was not useful in practice for verification of transformation in some plants. For example, analysis of sexual offspring populations is problematic in trees that are slow to reproduce sexually, and in some vegetatively propagated crops such as sugarcane with complicated (polyploid, aneuploid) genetics, and many sexually sterile cultivars. Similarly, a “tight” correlation between physical and phenotypic data is not a defining characteristic of gene transfer methods that result in integration of multiple and often rearranged copies of the transferred DNA, because many transformants have unexpressed copies of introduced se-

quences. Integrative transformation has nevertheless been unequivocally verified in such cases.

Most critical researchers would therefore accept a more generally applicable subset of criteria as rigorous proof of integrative transformation:

1. Southern DNA hybridization analysis of multiple independent transformants, using a probe(s) for the introduced gene(s) and restriction enzymes predicted to generate hybridizing fragments of different length at different integration sites (for a graphic representation, see 80). It is important to confirm that sizes of hybridizing fragments including flanking DNA at each integration site are reproducible within a transformed line, and that they differ between independently transformed lines. High molecular weight signals in uncut DNA, PCR-generated bands, or signals from a single putative transformed line are not acceptable substitutes, because it is more difficult to exclude the possibility of artifacts in such data.
2. Phenotypic data showing sustained expression of the introduced gene(s) exclusively in the cells of plant lines positive for the gene(s) by Southern analysis. Unambiguous phenotypic data require: (*a*) negative results from all untransformed controls (the tested control population size must be at least equivalent to that yielding 10 independent transformants from a parallel treated population), and (*b*) an assay revealing the product of transgene expression within plant cells as distinct from contaminating microbial cells, preferably from a transgene shown not to be expressed in bacterial cells. Intron-GUS (149) or anthocyanin regulatory (16, 18) reporter systems are suitable for such assays, as are in situ analyses for gene products without simple visual assays (153). Survival of lines on "escape-free" selection is not sufficient, because of the possibility of cross-protection by secreted products of contaminating microbial cells, or selection of mutants resistant to the selective agent. Enzyme assays on cell extracts are inadequate because of the possibility of contaminating transformed microbial cells.

Of course, more detailed molecular, phenotypic, and genetic characterization is likely to be undertaken on transformed lines produced for practical purposes. In some cases, target gene silencing rather than transgene expression may provide an unambiguous phenotype (15, 103). Data on co-transmission of introduced gene copies and the resulting phenotype in sexual offspring populations, where available, provide compelling confirmation of transformation, given suitable controls (126). Applicability in several independent laboratories is an important practical confirmation, because some techniques with published molecular evidence have never been repeatable.

PURPOSES OF PLANT TRANSFORMATION

An Experimental Tool for Plant Physiology

The capacity to introduce and express (or inactivate) specific genes in plants provides a powerful new experimental tool, allowing direct testing of some hypotheses in plant physiology that have been exceedingly difficult to resolve using other biochemical approaches (31). Exciting examples include the molecular genetic analysis of cellular signals controlling sexual reproduction and plant-microbe interactions (116, 143); the roles of specific enzymes in metabolic processes determining partitioning of photosynthates, and thus harvestable yield (67); and the roles of specific enzymes and hormones in plant developmental processes, including those affecting quality and storage life of marketed plant products (109, 147).

A Practical Tool for Plant Improvement

Much of the support for plant transformation research (and more broadly for plant molecular biology) has been provided because of expectations that this approach could: (*a*) generate plants with useful phenotypes unachievable by conventional plant breeding, (*b*) correct faults in cultivars more efficiently than conventional breeding, or (*c*) allow the commercial value of improved plant lines to be captured by those investing in the research more fully than is possible under intellectual property laws governing conventionally bred plants.

The first of these expectations has been met, with production of the first commercial plant lines expressing foreign genes conferring resistance to viruses, insects, herbicides, or post-harvest deterioration (54, 71, 138, 147), and accumulation of usefully modified storage products (30, 145), including several cases where there was no source of the desired trait in the gene pool for conventional breeding. The future prospects in this respect are also exciting, with preliminary indications that novel genes can be introduced to generate plant lines useful for production of materials ranging from pharmaceuticals (65) to biodegradable plastics (112).

The extent to which the other practical or commercial expectations of plant transformation can be met depends on the efficiency and predictability of production of lines with the desired phenotype, and without undesired side effects of the transformation process. As the sophistication of the physiological hypotheses to be tested by transformation increases, exactly the same factors become limiting. This occurs because of the practical difficulty of screening large numbers of plants for the desired expression pattern and the need to avoid misleading results from unrelated physiological effects of unintended genetic changes during the transformation process.

BIOLOGICAL REQUIREMENTS FOR TRANSFORMATION

The essential requirements in a gene transfer system for production of transgenic plants are: (*a*) availability of a target tissue including cells competent for plant regeneration, (*b*) a method to introduce DNA into those regenerable cells, and (*c*) a procedure to select and regenerate transformed plants at a satisfactory frequency.

One of the simplest available plant transformation systems involves infiltration of *Agrobacterium* cells into *Arabidopsis* plants before flowering, and direct selection for rare transformants in the resulting seedling populations (7, 23). Unfortunately, small plant size, rapid generation time, and high seed yield per plant are prerequisites for this method. These features are not shared by any economically important plant species. Other approaches to transformation via plant gametes have not been successful in practice (126). Therefore, the totipotency of some somatic plant cells underlies most plant transformation systems. The efficiency with which such cells can be prepared as targets for transformation is today the limiting factor in achievement of transformation in recalcitrant plant species.

Using either *Agrobacterium* or particle bombardment, it is now possible to introduce DNA into virtually any regenerable plant cell type. Only a small proportion of target cells typically receive the DNA during these treatments, and only a small proportion of these cells survive the treatment and stably integrate introduced DNA (47, 59). It is therefore generally essential to efficiently detect or select for transformed cells among a large excess of untransformed cells (12), and to establish regeneration conditions allowing recovery of intact plants derived from single transformed cells (156). Alternatively, transformed cells must contribute to the germline so that nonchimeric transformants can be obtained in the progeny from sexual reproduction (28).

PRACTICAL REQUIREMENTS OF TRANSFORMATION SYSTEMS FOR PLANT IMPROVEMENT

Beyond the biological requirements to achieve transformation and the technical requirements for verification of reproducible transformation, desired characteristics to consider in evaluating alternative techniques, or developing new ones for cultivar improvement, include:

1. Ready availability of the target tissue. The resources required to maintain a continuous supply of explants such as immature embryos at the correct developmental stage for transformation can be substantial.

2. Applicability to a range of cultivars. Genotype-specific techniques are of lower value because of the added complexity of extended breeding work to move desired genes into preferred cultivars.
3. High efficiency, economy, and reproducibility, to readily produce many independent transformants for testing.
4. Safety to operators, avoiding procedures, or substances requiring cumbersome precautions to avoid a high hazard to operators (e.g. potential carcinogenicity of silicone carbide whiskers).
5. Technical simplicity, involving a minimum of demanding or inherently variable manipulations, such as protoplast production and regeneration.
6. Frequent cotransformation with multiple genes, so that a high proportion of plant lines selected for marker gene expression will also incorporate cotransformed useful genes.
7. Unequivocal selection or efficient screening to recover transgenic plants from transformed cells.
8. Minimum time in tissue culture, to reduce associated costs and avoid undesired somaclonal variation.
9. Stable, uniform (nonchimeric) transformants for vegetatively propagated species, or fertile germline transformants for sexually propagated species.
10. Capacity to introduce defined DNA sequences without accompanying vector sequences not required for integration or expression of the introduced genes.
11. Capacity to remove reporter genes or other sequences not required following selection of transformed lines.
12. Simple integration patterns and low copy number of introduced genes, to minimize the probability of undesired gene disruption at insertion sites, or multicopy associated transgene silencing.
13. Stable expression of introduced genes in the pattern expected from the chosen gene control sequences, rather than patterns associated with the state of the cells at the time of transformation (34), or the chance site of integration (122).
14. Optionally applicable to transformation of organelle genomes.
15. Absence of valid patent claims on products.

When tested against the above criteria, most published techniques for gene transfer into plant cells must be dismissed as either disproven, unproven, or impractical for use in routine production of transgenic plants. The techniques that have been proven to produce transgenic plants from a range of species, and in many laboratories, are *Agrobacterium*-mediated transformation, bombardment with DNA-coated microprojectiles, and electroporation or PEG

treatment of protoplasts. Techniques requiring protoplasts are generally avoided because of the associated inconvenience and time in culture. As a result virtually all plant transformation work aimed at direct production of improved cultivars currently uses either *Agrobacterium* or microprojectiles for gene transfer. Neither of these approaches is free of patent claims, however, and there is continuing interest in development of alternative techniques (141). The stages and time-courses for typical transformation strategies using *Agrobacterium* or DNA-coated microprojectiles shown in Figure 1.

RECALCITRANT SYSTEMS AND APPARENT CONSTRAINTS

Cereals, legumes, and woody plants are commonly categorized as recalcitrant to transformation, because these groups have included a disproportionate

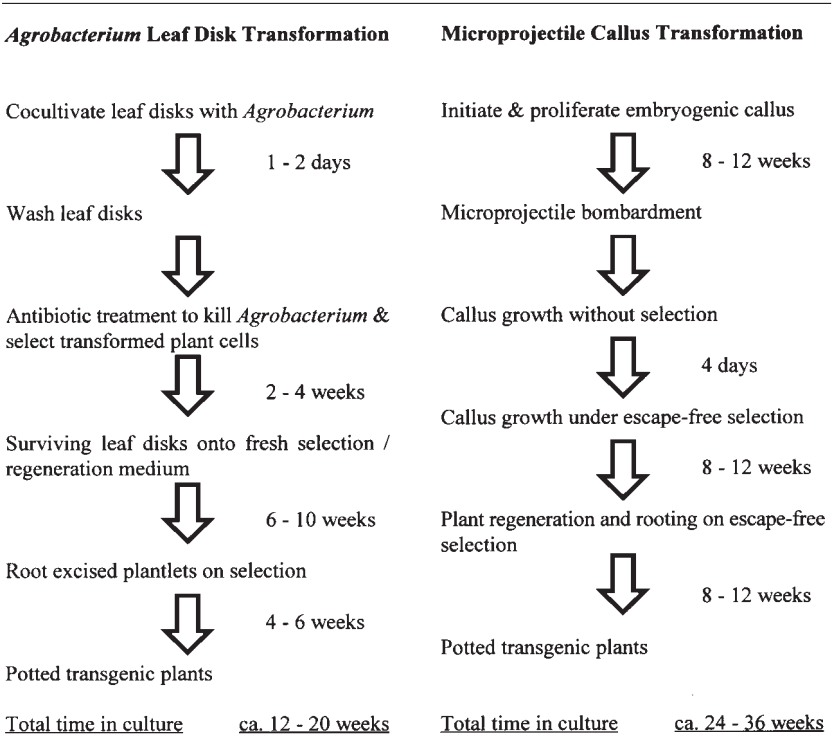

Figure 1 Typical transformation regimens using *Agrobacterium* or DNA-coated microprojectiles.

number of untransformed or difficult to transform species. However, the generalization is becoming less useful as one species after another from these groups joins the list of plants with reliable transformation systems. The hypothesis that some plants lack the biological capacity to respond to essential triggers for integrative transformation, or have cellular mechanisms preventing integrative transformation, can effectively be rejected.

It is a reasonable proposition that transgenic plants can be regenerated only from cells competent for both regeneration and integrative transformation (126). Preliminary evidence indicates that T-DNA integration may be the limiting step in maize transformation (111), but there is no evidence that actively dividing, regenerable cells are not competent to integrate introduced DNA. Where tissue culture systems have been developed to produce proliferating and regenerable cells, into which DNA can be introduced at a high frequency (as indicated by transient gene expression) without interfering with regenerability of the penetrated cells, previously recalcitrant species have become transformable. The transformation efficiency has been proportional to the efficiency of the tissue culture and gene transfer systems (18, 70, 76, 93).

STRATEGIES TO ACHIEVE TRANSFORMATION

It is instructive to consider species such as rice, which was once considered recalcitrant to transformation but can now be transformed via direct gene transfer into protoplasts (140), particle bombardment of immature embryos (27) or cell cultures (20), or *Agrobacterium* treatment of embryogenic callus (70). In each case, success seems to have followed identification (or production through tissue culture) of explants with many regenerable cells, optimization of parameters for gene transfer into those cells, and tailoring selection and regeneration procedures to recover transgenic plants. A generalized approach is illustrated in Figure 2. The nearest transformed relatives of an untransformed species of interest are an obvious reference point in initial work to develop suitable tissue culture, gene transfer, and selection regimens.

Tissue Culture Strategies

Tissue culture is not a theoretical prerequisite for plant transformation, but it is employed in almost all current practical transformation systems to achieve a workable efficiency of gene transfer, selection, and regeneration of transformants. Detailed consideration of the options for and optimization of tissue culture systems useful for plant transformation is beyond the scope of this

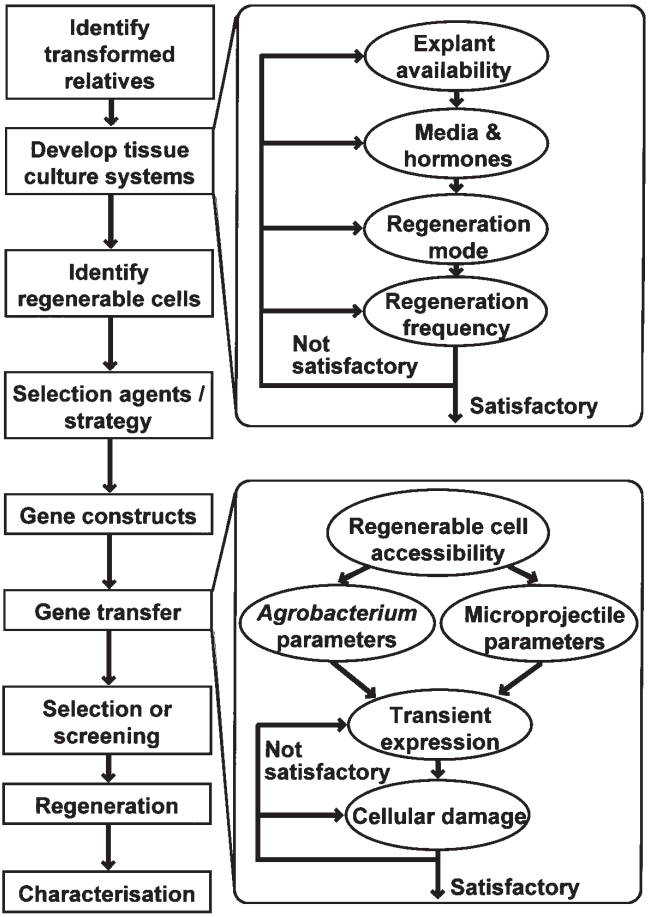

Figure 2 A generalized approach to development of transformation systems for recalcitrant plant species.

review, but the broad technology and detailed protocols are addressed in recent techniques manuals (52, 154).

In tissue culture systems for plant transformation, what is most important is a large number of regenerable cells that are accessible to the gene transfer treatment, and that will retain the capacity for regeneration for the duration of the necessary target preparation, cell proliferation, and selection treatments. A high multiplication ratio from a micropropagation system does not necessarily indicate a large number of regenerable cells accessible to gene transfer (97).

Gene transfer into potentially regenerable cells may not allow recovery of transgenic plants if the capacity for efficient regeneration is short-lived (132).

There seems to be no reason to prefer embryogenic or organogenic plant regeneration. In the happy event that several tissue culture systems meeting the primary requirement above are available for a species of interest, the choice can be made based on features affecting convenience or efficiency, including ready availability of explants, and minimal time in tissue culture. Somaclonal variation, once considered a potentially useful source of genetic variation for plant improvement, is now more a bane of gene transfer programs (81). In some circumstances, particularly the direct introduction of genes for desired commercial traits into elite vegetatively propagated cultivars, the need to avoid such random genetic change may become the overriding consideration in the choice of tissue culture and gene transfer systems.

The desire to minimize somaclonal variation is one motivation for eliminating or minimizing the tissue culture phase, by gene transfer into intact tissue explants and regeneration without substantial in vitro culture. In several cereals, it has been possible to dispense with tissue culture for target preparation, by gene transfer into immature zygotic embryos, although embryogenic callus culture is still required to recover transgenic plants (27, 157, 159).

The goal of genetic transformation without tissue culture has been approached in soybean, cotton, bean, and peanut by particle bombardment into meristematic tissue of excised embryonic axes, shoot proliferation to yield some lines with transformation of germline cells, and screening for transformed sexual progeny (26). The limiting factors remain the ability to mechanically prepare the explants, transfer genes into regenerable cells, and select or screen for transformants at an efficiency sufficient for practical use in cultivar improvement. For example, the reported germline transformation rate for bean (0.06% of excised and bombarded apical meristems, 0.03% of assayed shoots) would make the process too expensive for many laboratories (133). There remains unexplained cultivar specificity for transformation via meristem bombardment within some plant species such as bean (133), impracticality of gene transfer into meristems of others such as rice (27), and uncertainty about applicability in vegetatively propagated species such as sugarcane (51).

While tissue culture remains an essential component of practical transformation systems for most plant species, research aimed at minimizing somaclonal variation deserves a high priority. Curiously little of the published research on somaclonal variation has been directed at this goal (81).

Gene Transfer Strategies

Suggested approaches to development or optimization of transformation protocols using particle bombardment (12) or *Agrobacterium* (152) have been published, based on practical experiences in laboratories working on recalcitrant crops. For particle bombardment, it is generally most efficient to first examine the available tissue culture systems, determine the modes of regeneration and the location of the cells involved, optimize tissue culture conditions to increase the number or accessibility of such cells if necessary, and then develop conditions for nonlethal transfer of DNA into large numbers of such cells per bombardment (12).

For *Agrobacterium*, it is considered more efficient to first establish the conditions for gene transfer and then work on conditions for regeneration of transformed cells (152). This contrast may be biologically well-founded, because of the greater complexity and lesser understanding of the biological interaction preceding the gene transfer event from *Agrobacterium.* Unfortunately, there is no guarantee that a transformable plant cell type will prove regenerable, even in the hands of the most successful tissue culturist.

If preliminary transformation experiments using techniques successful in similar plant systems are not successful, my advice is to establish by histological studies the precise cellular origins and timing of events leading to plant regeneration within the explants to be used as targets for gene transfer. This is likely to avoid much wasted time and frustration from optimizing gene transfer and regeneration within the same region of the explant, but potentially in different cells, so that transformed plants are unlikely to result. Work in sunflower is a fine example of the value of this relatively simple check to explain and potentially overcome difficulty in transformation of a recalcitrant species (91).

Assays for transient expression of introduced reporter genes in plant cells can provide unequivocal evidence of gene transfer. A great deal of time can be wasted unless this analysis is focused on regenerable cells, which often comprise a small and inaccessible fraction of the target tissue (12, 91). Exhaustive experiments to maximize transient expression are also futile if they involve conditions harmful to regeneration or molecular characteristics of transformed cells (8, 26). For example, particle bombardment conditions can now be arranged to give highly reproducible results in transient assays, by delivering a large number of copies of potentially transcribable DNA into the nuclei of target cells. However, a different form and concentration of DNA is likely to be optimal for efficient production of low copy number transformants (12).

There is little information on forms of DNA or sequences that may increase the frequency of stable transformation (8, 19, 142).

There is considerable batch-to-batch variation in the frequency of transient expression events following cocultivation with *Agrobacterium* (78). The correlation between transient expression and stable transformation has not been thoroughly tested. *Agrobacterium* employs a highly evolved and still incompletely understood gene transfer and integration system that appears optimized for efficient nuclear targeting and integration of a protein-complexed single-stranded DNA introduced as a small number of copies per cell (165). Therefore, stable transformation may occur at a high frequency in cells without detectable transient expression of the introduced DNA. Positive results from transient assays for *Agrobacterium*-mediated gene transfer into regenerable cells are encouraging (70, 76, 93), but until the parameters affecting such expression are better understood, it is unwise to abandon hope too quickly based on negative results from transient assays.

It may be necessary to introduce a considerable number of genes into plants for some purposes. The limits of available gene transfer techniques have not yet been defined. At least 12 separate plasmids and up to 600 kb of DNA can be introduced at once by particle bombardment (62), but the number of expressed genes has not been tested. There are some indications that large plasmids (>10 kb) may be subject to greater fragmentation during particle bombardment (12). Transposon-derived vectors have been shown to deliver an increased proportion of intact, single-copy inserts of up to 10 kb following direct gene transfer into protoplasts (92). Recent evidence indicates that use of a binary bacterial artificial chromosome vector, with helper plasmids enhancing production of VirG and VirE proteins, can allow efficient *Agrobacterium*-mediated transfer of at least 150 kb of foreign DNA into the plant nuclear genome. Furthermore, the transferred DNA appeared to be present as an intact single copy that was faithfully inherited in the progeny of several of the characterized transformants (64).

Vectors overexpressing *virG* are also a component of the thoroughly verified systems for *Agrobacterium*-mediated transformation of rice (70), maize (76), and cassava (93), and deserve wider testing in recalcitrant plants. Other key variables in *Agrobacterium*-mediated gene transfer include *Agrobacterium* and plant genotype, treatment with *vir* gene inducers such as acetosyringone, wounded cell extracts, feeder cells or sugars; pH, temperature, cell concentration, light conditions, and duration of cocultivation; explant type, quality, preculture (152), hormone treatment (50), wounding, or infiltration (11); and use of appropriate antibacterial agents (95), antioxidants (124), ethylene antagonists (35), and/or methylation inhibitors (117) to reduce damage and/or

gene silencing in treated plant cells. With so many variables, and little evidence of combinations that are broadly applicable across plant species, it is evident why transient expression assays are useful, if imperfect, as indicators of suitable conditions for gene transfer (76), before more expensive studies to optimize stable transformation.

Selection Strategies

For transformation systems that generate substantial numbers of nonchimeric primary transformants, genes conferring resistance to a selective chemical agent (161), genes conferring a phenotype allowing visual or physical screening (16, 18, 129), or even PCR screening to identify plants containing transferred genes (27, 83) can all be used to recover transformants. Transformation systems that generate chimeric primary transformants including transformed germline cells, as intermediates in the production of homogeneously transformed (R_1) progeny plants, generally require screening rather than lethal selection to reveal primary transformants (28, 83, 105).

Screening approaches are expensive unless the transformation efficiency is high, and generally impractical if the proportion of transformants among regenerated lines is below 10^{-2} to 10^{-3}. In our hands, the recovery of transformed plants was 10-fold lower from visual screening compared with antibiotic selection (18). This may occur because antibiotic selection provides a continuous advantage to transformed cells, which may otherwise be overgrown by the far greater numbers of proliferating nontransformed cells (48, 78). Under these circumstances, antibiotic selection may allow a higher proportion of transformed cells to multiply and regenerate, in addition to facilitating the recognition of transformants.

An excellent review has been published on selectable marker genes, assayable reporter genes, and criteria for their use in plant transformation studies (16). Broadly applicable, simple, and robust selection regimens now exist for transgenic plants, requiring little experimentation with the timing and concentration of selective agents to match the target tissue and gene transfer system. However, it is still important to consider the physiology of antibiotic action and resistance mechanisms when choosing or modifying selection protocols (36). There are also reports of interactions between selective agent and subsequent regenerability (137), and interactions between antibiotic and gelling agents (101). Attention is increasingly being directed to introduction of multiple agronomically useful genes into plant lines, without having to pyramid selectable genes in the process (27, 32, 155, 163).

Transgene Expression Strategies

The first attempts to express foreign genes failed because of the inability of the plant transcriptional machinery to recognize some foreign gene control sequences, particularly promoter sequences of many bacterial genes. This initial hurdle was overcome by exploiting control sequences isolated from genes of *Agrobacterium* and cauliflower mosaic virus, which were known to be expressed in plant cells as part of the process of molecular subversion of host cell machinery by these pathogens. Continuing characterization of many plant genes, and analyses of transient and stable expression of foreign gene constructs in plants, have contributed to a growing understanding of features useful for the regulated expression of transgenes in plants. Features typically considered in preparation of such constructs include: (*a*) appropriate transcriptional promoters and enhancers (9); (*b*) introns (98, 100); (*c*) transcriptional terminators and 3′ enhancers (130); (*d*) polyadenylation signals (3, 162); (*e*) untranslated 5′ leader and 3′ trailer sequences (38, 56, 148); (*f*) codon usage (87, 125); (*g*) optimal sequence context around transcription and translation start sites, including absence of spurious start codons (5, 55, 66, 99); (*h*) transit sequences for appropriate subcellular compartmentation and stability of the gene product (58, 68, 82, 115); (*i*) absence of sequences such as cryptic introns (129) or polyadenylation signals resulting in inappropriate RNA processing (87, 125); and (*j*) absence of sequences resulting in undesired glycosylation or lipid anchor sites (24, 46). Recent progress with chloroplast transformation has added the possibility of expression from the plastid or nuclear genome (21, 104).

This understanding has greatly improved the ability to tailor transgenes for various strengths and patterns of expression essential for practical plant genetic engineering (10). Levels and patterns of expression generally vary to some extent, even between independent single copy transformants. This reflects the influence of different sequences flanking the integration sites upon expression of the transgene (122, 151). Although this variation may generate transformed lines which by chance have new and useful expression patterns (34), this is unlikely to occur at a frequency that is useful in practice, unless we can bias integration toward regions of the DNA that are preferentially expressed under specified conditions. Otherwise, random variation will greatly increase the expense of the varietal improvement process, because rigorous evaluation of many transformed lines will be needed to identify those with the desired phenotype. One attraction of transformation for cultivar improvement is the theoretical potential for very precise genetic change. If random variation around a desired phenotype in individual transformants necessitates extensive

field characterization to select commercial lines, there may be no advantage in using transformation over conventional breeding. In these circumstances, the value of plant transformation in cultivar improvement would be restricted to traits not achievable by conventional breeding, particularly introduction of new genes to the germplasm available to breeders.

Of even greater concern is unpredictable loss of the improved phenotype in transformed cultivars because of silencing of the transgene. This phenomenon is discussed in several excellent recent reviews (45, 103, 107). In practical terms, it is fortunate that transgene silencing has to date always been detectable soon after transformation, crossing, or field testing of transformants (see 45). The problem would be much more serious if unstable lines could not be reliably detected and eliminated during the routine screening and propagation of transgenic lines before commercial release, because of the potential commercial damage from loss of an essential trait such as disease resistance. There is still much to be learned about the causes of transgene inactivation. For example, multicopy transgenes have been identified as a probable cause (see 45), indicating a disadvantage of direct gene transfer techniques which result in higher average copy numbers than *Agrobacterium.* However, our experience from several years of laboratory and field testing with hundreds of transgenic sugarcane lines is that some promoter-reporter gene constructs are silenced at high frequency, whereas others are almost invariably stably expressed, with no relationship to copy number (13). It will be important to discover what sequences within these constructs trigger or inhibit silencing, and whether matrix attachment regions (142), demethylation sequences (94), or targeted integration systems (2, 118) can be developed to protect susceptible foreign sequences from silencing in transgenic plants.

Integrating Components of Transformation Strategies

Features of representative strategies for production of transgenic plants are compared in Table 1. Other combinations exist, but the table illustrates that the choice of transformation strategy influences many secondary parameters such as time in tissue culture and number of plants processed per transformant, which often determine the practicality of the system.

It is commonly generalized that *Agrobacterium* produces simpler integration patterns than direct gene transfer, but both approaches result in a similar range of integration events, including truncations, rearrangements, and various copy numbers and insertion sites. Furthermore, the frequency distributions of copy number and rearrangements vary with transformation parameters for both gene transfer methods (25, 60, 107, 150). More careful work is required to

Table 1 Features influencing practical application of successful plant transformation strategies

Parameters	Successful Transformation Strategies				
	In planta	Vegetative tissue	Meristem	Embryo	Callus
Gene transfer method	*Agrobacterium*	*Agrobacterium*	Particle bombardment	Particle bombardment	Particle bombardment
Explant	Flowering plant	Any tissue with regenerable cells	Embryonic axes or meristems	Intact or sectioned embryos	Embryogenic or organogenic callus
Target cells for effective gene transfer	Germline cells late in floral development	Any regenerable cells	Germline cells in meristems	Epidermal cells of scutellum	Surface or subsurface cells
Regeneration	Flowering shoot/zygotic embryo	Organogenesis or embryogenesis	Shoot formation/zygotic embryo	Embryogenesis	Embryogenesis or organogenesis
1° regenerant chimeric	Few floral cells transformed	No	Germline transformed	No	No
Sexual reproduction	Essential	Unnecessary	Probably essential	Unnecessary	Unnecessary
Selection	Yes (R_1 progeny)	Yes	Not before R_1 progeny	Yes	Yes
Screenable marker	Optional	Optional	Essential	Optional	Optional
Hormonal treatment	Unnecessary	Auxin +/or cytokinin	Usually cytokinin	Auxin+/– cytokinin	Auxin+/– cytokinin
Transformants per treated explant	1–10	1–10	0.001	0.01	0.01–1
Transformants per tested regenerant	0.001	1	0.0003	1	1
Tissue culture duration	Unnecessary	10 weeks	4–6 weeks	10 weeks	20 weeks
Time from treatment of non-chimeric transformant	7–10 weeks	10 weeks	One generation time	10 weeks	10 weeks
Proven applicability (e.g.)	*Arabidopsis* (7)	Many spp. (59)	Several spp. including legumes (26, 105)	Several cereals (27, 77)	Many spp. (17, 26)

optimize methods for simple integration patterns before any reliable conclusions are drawn about the relative potential of the techniques to deliver such patterns at a satisfactory frequency.

The apparent targeting of T-DNA integration into transcribed regions is useful for gene and promoter tagging, and for transgene insertion into regions favoring subsequent expression (86). However, the observation that over 90% of T-DNA insertions may disrupt transcriptional units (96), with 15–26% of transformants showing visible mutant phenotypes resulting from T-DNA insertions (44), sounds an alarm for direct production of improved cultivars in highly selected crops, where most phenotypic changes from random mutations are likely to be adverse. For such work, integration should ideally be directed to transcribed regions without disruption of existing plant genes. To achieve this will require more research to bring gene targeting technology in plants closer to the level achieved in model animals (79, 118). Whether DNA introduced into plant cells by direct gene transfer is also preferentially integrated into transcribed regions or active genes has not been adequately tested (85).

OTHER CONSTRAINTS TO RESEARCH AND DEVELOPMENT IN PLANT TRANSFORMATION

Regulatory Environment and Public Perceptions

In most countries, planned field releases and commercial development of transgenic plants are first scrutinized and approved by regulatory authorities established by the national government, to ensure that products are safe to the environment and consumers (33, 43, 158). This process can be important to obtain the maximum social benefit from transgenic plant lines. For example, in several countries, release of transgenic insect-resistant plant varieties has been linked to mandatory programs of insect monitoring and industry responses to avoid premature loss of useful insect control genes because of a build-up of resistant insect populations (88).

Scrutiny by regulatory authorities is also an important mechanism to reassure the general public of the safety of a new technology that is not well understood by most people. However, if the process is conducted inefficiently by the regulatory authorities, it can severely slow research. For example, it is essential to characterize a substantial number of independent transformed plant lines in both physiological experiments and in selecting genetically improved cultivars. The availability of sufficient containment greenhouse space rapidly becomes limiting if the process of evaluation before approval of field releases is slow.

The conservative assumption underlying regulations in many countries is that all transgenic plants are potentially hazardous. Scientific theory and practical experience show that this is not the case. The hazards relate to the genes

transferred or the phenotype produced, not to the gene transfer method used. There have been no reports of any harmful environmental effects or other hazardous unforeseen behavior of transgenic plants in the thousands of field trials conducted internationally to date (33). As public experience and understanding of plant transformation increase, it is to be hoped that regulatory processes may be streamlined, with the focus on products rather than on processes of plant genetic modification (108).

Consumer response to transgenic plant products has now been tested with the commercial release of improved varieties in a range of crops (Table 2).

Table 2 Commercial releases of transgenic plant varieties

Trait	Crop	Name	Company	Product Status
Quality (vine-ripened flavor, shelf life	Tomato	Flavr Savr	Calgene	Released 1994
Quality (vine-ripened flavor, shelf life)	Tomato	Endless Summer	DNA Plant Technology	Blocked by patent claims
Quality (paste consistency)	Tomato	—	Zeneca	Released 1995
Oil characteristics	Canola	Laurical	Calgene	Released 1994
Virus resistance	Tobacco Tomato Capsicum	—	(China)	Released 1993–1994
Virus resistance	Squash	Freedom II	Asgrow	Released 1995
Insect resistance	Cotton Potato Maize	Bollgard NewLeaf YieldGuard	Monsanto	Released 1996–1997
Insect resistance	Maize	Maximizer	Ciba Seeds	Released 1996
Herbicide resistance	Flax	Triffid	University of Saskatchewan	Released 1995
Herbicide resistance	Cotton	BXN	Calgene	Released 1995
Herbicide resistance	Canola Corn	Innovator Liberty Link	AgrEvo	Released 1995–1996
Herbicide resistance	Soybean Canola Cotton	Roundup Ready	Monsanto	Released 1995–1996
Herbicide resistance	Soybean Corn	Roundup Ready, STS Liberty Link	Pioneer	Released 1996–1997
Male sterility hybrid system	Canola	—	Plant Genetic Systems	Approved 1996 (USA, FDA)

These releases have coincided with increasing dissemination of information on transgenic plants in forms accessible to the general public (158). In each case, consumers have responded positively to quality or price advantages, and in several cases demand has outstripped supply in the first season. However, it is clear that continued work is important to provide the broad community with information to support considered responses to emerging products (63).

Intellectual Property

As technical limitations are overcome, it is possible that commercial limitations will become more serious barriers to exploitation of genetic transformation. New technologies developed in this area are effectively inventions and are therefore eligible for patent protection (84, 123). For example, patents have already been issued on most established or promising plant genetic transformation strategies (29, 69, 89, 102, 119, 134, 136) and on many isolated genes, promoters, and techniques for plant gene manipulation (see 121 and monthly patent updates in *Genetic Technology News*). The patent literature has become an important source of information in plant transformation research, albeit more difficult and expensive to search than the scientific literature (6, 40).

A patent provides the inventor or assignee with a period of exclusive ownership, or formally a right to exclude others from making, using, or selling the invention. There is no statutory exclusion for infringment when patented products or methods are used for research purposes. The widespread misconception that disclosure in the patent document allows researchers to practice the invention in order to improve on it (73) possibly arises because patent owners are generally reticent in instituting infringement proceedings until the level of damages that may accrue becomes commercially significant. There is no obligation to license and no constraint on royalty levels provided the patent holder makes active use of the intellectual property. Some patents make extremely broad claims, and patent holders are not required to develop all possible manifestations of an invention to retain broad ownership (144). Penalties for infringement of proprietary rights can be severe, so it is important to determine whether the tools or topics of proposed research are already in the public domain or subject to patent protection (160). This can be difficult to establish without periodic patent searching, or even legal challenges (75).

The position may differ between countries. For example, particle bombardment for gene transfer into plant cells has been patented in the United States (134) but not in Australia. Apparatus for particle bombardment involving a macroprojectile and stop plate has been patented in Australia (135) but not apparatus involving particle acceleration in a gas pulse. Patent coverage for

many genes and promoters is similarly restricted. For example, the maize ubiquitin promoter is the subject of granted patents or applications in Europe, Japan, and the United States (128) but apparently not in many other countries, including Australia.

Under these circumstances, a transgenic plant variety produced and used commercially without infringing any patent rights in one country could infringe certain patent claims if used (even for research or other noncommercial purposes) in another country. To complicate matters further, patent applications are not available publicly in some countries (notably the United States) until the patent is granted, which can be years after the application is filed. There are moves to harmonize international practice, e.g. by providing for ownership for 20 years from the date of application and publishing 18 months after application to eliminate the practice of "submarine" patent applications that only surface after a competitor has independently made the same invention (42). The issues involved are complicated, and some important differences in patent law between countries appear unlikely to be resolved in the near future (4).

Patents are intended to encourage and reward useful invention and technical innovation, and the new technology enters the public domain after a period of 17 to 20 years (42, 84). In the interim, commercial restrictions can appear quite ruthless as patent holders adopt commercialization strategies to capture the value of protected intellectual property (75). In an era of tight public sector research funding and high research and development costs, the benefits of corporate investment to develop transformation technologies outweigh the inconvenience of patent restrictions. Debate continues on mechanisms to balance the competing interests (22).

FUTURE NEEDS AND DIRECTIONS IN PLANT TRANSFORMATION RESEARCH AND DEVELOPMENT

Transformation Efficiency

The methods for gene transfer into plant cells, particularly *Agrobacterium* and particle bombardment, are now sufficiently developed to allow transformation of essentially any plant species in which regenerable cells can be identified. Broadly applicable selection methods are well established. The key to transformation of recalcitrant species appears to be development of methods to expose many regenerable cells to nondestructive gene transfer treatments.

What currently limits the practical transformation of many plant species is the combination of a low frequency of transformation and a high frequency of

undesired genetic change or unpredictable transgene expression. These problems necessitate expensive large-scale transformation and screening programs to produce useful transformants. The first constraint may be addressed by research into tissue culture systems to enrich for regenerable cells accessible to gene transfer. Contract transformation services (106) may implement economies of scale to afford robotic systems (1) for routine large scale target preparation and gene transfer treatments.

A clearer understanding of the events surrounding gene transfer by *Agrobacterium* is also required. Is transient expression a satisfactory test for *Agrobacterium*-mediated gene transfer into plant cells, or can another convenient test be developed to allow rapid detection and optimization of this key event? Does *Agrobacterium* select between cell types, and if so what features determine favored cells for gene transfer? Can these features be imparted to highly regenerable cell types? Direct gene transfer experiments indicate that if naked DNA is transferred into many actively dividing and regenerable cells, a proportion will be stably transformed. Is the same true for cells receiving typically lower doses of T-DNA, or are there additional physiological requirements for efficient T-DNA integration (111)? Is T-DNA integration targeted to potentially expressed regions of the genome, or to regions undergoing active transcription? Can the transcriptional status of target cells be manipulated to achieve a high frequency of integration into regions suitable for subsequent transgene expression, but a low frequency of insertional inactivation of genes influencing the phenotype of regenerated transformants?

There are at least as many relevant questions surrounding direct gene transfer. Is stable transformation efficiency as sensitive as transient expression to decreased DNA concentration? Does DNA concentration affect mean copy number or cotransformation frequency in resulting stable transformants? Is integration targeted to potentially transcribed regions as appears to be the case for T-DNA from *Agrobacterium*? Can artificial T-DNA complexes be manufactured, and will they influence the efficiency or integration patterns available from direct gene transfer?

Useful vs Absolute Transformation Efficiency

In the longer term, a more important goal than increased transformation efficiency is the development of transformation methods and constructs tailored for predictable transgene expression, without collateral genetic damage. We may conclude that much of the current effort in plant transformation directed toward increased transformation frequencies is naive and misdirected. We need to distinguish between absolute and useful transformation frequencies. The limiting process in the application of plant transformation for more so-

phisticated studies of plant physiology or for cultivar improvement is generally not the production of transformants but the screening (or subsequent breeding) required to eliminate transformants with collateral genetic damage that would interfere with meaningful physiological analysis or commercial use. Depending on the ratio of effort required for these processes a large increase in absolute transformation efficiency may be futile if accompanied by even a small decrease in the proportion of useful transformants. Conversely, a large drop in absolute transformation frequency may be more than compensated by a smaller gain in the proportion of useful transformants. These ideas are familiar to most practicing plant breeders but have understandably not been foremost in the minds of most transformation scientists while they struggled to develop reliable and efficient systems for gene transfer into target plant species.

Collateral Genetic Damage

To achieve a high proportion of useful transformants, we need to understand more clearly the factors contributing to undesired genetic change during the transformation process. To what extent is such change associated with the integration of single or multiple copies of foreign DNA, as distinct from the processes of tissue culture, selection, and plant regeneration? Is genetic change induced or selected during such processes, or is it commonly the effect of preexisting mutations in somatic cells that are simply detected when entire plants are regenerated from single (transformed) cells? If change is induced, which are the mutagenic stages in the protocols, and can they be avoided? If mutations are preexisting, can the procedures be tailored to selectively prevent regeneration of mutated cells? Compared to adventitious shoot proliferation, is somatic embryogenesis disadvantageous because of longer duration in culture, or advantageous because the complexity of the embryogenic process acts as a filter to eliminate many cells with mutations? Does the approach of germline transformation of uncultured explants followed by crossing to obtain nonchimeric transgenic progeny reduce the frequency of undesired genetic change, or just mask such change in the background of genetic variation from sexual reproduction? The relative importance of these questions varies between plant species; vegetatively vs sexually propagated crops provide an extreme example. Unfortunately, the answers to many of these questions may also be genotype specific.

Ideal and Model Transformation Systems

As the emphasis on useful transformation frequency increases, we may see a trend toward minimization or elimination of tissue culture stages, targeted

integration of single copy transgenes, and direct (leaf-disc PCR) screening for transformants with useful genes to eliminate the need for reporter sequences. As our understanding of the genetic basis of agronomic traits increases, it is likely that this goal will be extended to the introduction of greater lengths of DNA encoding multiple genes. We will need to determine the capacity of available methods to introduce such lengths of DNA intact.

Although some of these questions may be answered and approaches developed with model plants, the features that make the models attractive for some genetic studies (e.g. small genome, small plant size, rapid generation time for *Arabidopsis*) generally cannot be exploited in practical transformation systems for most economically important plants. We must be prepared to select the models according to the questions, and test the answers for applicability to the practical targets.

Transformation, Breeding, and Genetic Diversity

As with conventional breeding, it is highly undesirable for plant transformation to lead to excessive genetic uniformity in current varieties of any crop. Even a single gene in all varieties can create problems. For example, the United States maize crop in 1970 was devastated because of disease susceptibility accompanying a cytoplasmic male sterility trait used to simplify hybrid seed production (164). This is another reason to aim for the capacity to transform diverse genotypes within a species, to develop diverse genes for desired phenotypes, and to eliminate unnecessary sequences from the transformation process.

When Practical Means Commercial

Plant transformation is already sufficiently developed to allow the testing and even commercialization of plants with novel phenotypes under simple genetic control. For continuing practical benefits, it will be necessary to extend our understanding of the biological basis for efficient plant transformation and develop improved technologies for predictable transgene expression without collateral genetic damage, at a pace matching the exciting scientific advances in gene cloning and characterization. This will require support from industry for the underlying research. As transformation projects are increasingly undertaken with the possibility of generating commercially useful products, transformation scientists in turn must increasingly integrate social, legal, and economic issues as well as technical issues from the earliest stages of project design.

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Literature Cited

1. Aitken CJ, Kozai T, Smith MAL. 1995. *Automation and Environmental Control in Plant Tissue Culture*. Dordrecht: Kluwer
2. Albert H, Dale EC, Lee E, Ow DW. 1995. Site-specific integration of DNA into wild-type and mutant *lox* sites placed in the plant genome. *Plant J.* 7:649–59
3. An GH, Mitra A, Choi HK, Costa MA, An KS, et al. 1989. Functional analysis of the 3′ control region of the potato wound-inducible proteinase inhibitor II gene. *Plant Cell* 1:115–22
4. Ardley J, Hoptroff CGM. 1996. Protecting plant 'invention': the role of plant variety rights and patents. *Trends Biotechnol.* 14: 67–69
5. Baker BF. 1993. The 5′ cap of mRNA: biosynthesis, function and structure as related to antisense drugs. In *Antisense Research and Applications*, ed. ST Crooke, B Lebleu, pp. 37–53. Boca Raton, FL: CRC Press
6. Barks AH. 1994. Patent information in bio technology. *Trends Biotechnol.* 12:352– 64
7. Bechtold N, Ellis J, Pelletier G. 1993. *In planta Agrobacterium* mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. *C. R. Acad. Sci. III* 316: 1194–99
8. Benediktsson I, Spampinato CP, Schieder O. 1995. Studies of the mechanism of transgene integration into plant protoplasts: improvement of the transformation rate. *Euphytica* 85:53–61
9. Benfey PN, Ren L, Chua N-H. 1990. Combinational and synergistic properties of CaMV 35S enhancer subdomains. *EMBO J.* 9:1685–96
10. Bennett J. 1993. Genes for crop improvement. In *Genetic Engineering*, ed. JK Setlow, 15:165–89. New York: Plenum
11. Bidney D, Scelonge C, Martich J, Burrus M, Sims L, et al. 1992. Microprojectile bombardment of plant tissues increases transformation frequency by *Agrobacterium tumefaciens. Plant Mol. Biol.* 18: 301–13
12. Birch RG, Bower R. 1994. Principles of gene transfer using particle bombardment. In *Particle Bombardment Technology for Gene Transfer*, ed. N-S Yang, P Christou, pp. 3–37. New York: Oxford Univ. Press
13. Birch RG, Bower R, Elliott AR, Potier BAM, Franks T, et al. 1996. Expression of foreign genes in sugarcane. In *Proc. Int. Soc. Sugarcane Technol. Congr., Cartegena, Sept. 1995, 22nd*, ed. JH Cock, T Brekelbaum, 2:368–73. Cali, Colombia: Tecnicana
14. Birch RG, Franks T. 1991. Development and optimization of microprojectile systems for plant genetic transformation. *Aust. J. Plant Physiol.* 18:453–69
15. Bourque JE. 1995. Antisense strategies for genetic manipulations in plants. *Plant Sci.* 105:125–49
16. Bowen B. 1993. Markers for plant gene transfer. See Ref. 90, 1:89–123
17. Bower R, Birch RG. 1992. Transgenic sugarcane plants via microprojectile bombardment. *Plant J.* 2:409–16
18. Bower R, Elliott AR, Potier BAM, Birch RG. 1996. High-efficiency, microprojectile-mediated cotransformation of sugarcane, using visible or selectable markers. *Mol. Breed.* 2:239–49
19. Buising CM, Benbow RM. 1994. Molecular analysis of transgenic plants generated by microprojectile bombardment: Effect of petunia transformation booster sequence. *Mol. Gen. Genet.* 243:71–81
20. Cao J, Duan X, McElroy D, Wu R. 1992. Regeneration of herbicide resistant transgenic rice plants following microprojectile-

mediated transformation of suspension culture cells. *Plant Cell Rep.* 11:586–91
21. Carrer H, Maliga P. 1995. Targeted insertion of foreign genes into the tobacco plastid genome without physical linkage to the selectable marker gene. *BioTechnology* 13: 791–94
22. Caskey CT. 1996. Gene patents: a time to balance access and incentives. *Trends Biotechnol.* 14:298–302
23. Chang SS, Park SK, Kim BC, Kang BJ, Kim DU, et al. 1994. Stable genetic transformation of *Arabidopsis thaliana* by *Agrobacterium* inoculation *in planta. Plant J.* 5:551–58
24. Chow M, Der CJ, Buss JE. 1992. Structure and biological effects of lipid modifications on proteins. *Curr. Opin. Cell Biol.* 4: 629–36
25. Christou P. 1992. Genetic transformation of crop plants using microprojectile bombardment. *Plant J.* 2:275–81
26. Christou P. 1995. Strategies for variety-independent genetic transformation of important cereals, legumes and woody species utilizing particle bombardment. *Euphytica* 85:13–27
27. Christou P, Ford TL, Kofron M. 1992. The development of a variety-independent gene-transfer method for rice. *Trends Biotechnol.* 10:239–46
28. Christou P, McCabe DE. 1992. Prediction of germ-line transformation events in chimeric R0 transgenic soybean plantlets using tissue-specific expression patterns. *Plant J.* 2:283–90
29. Coffee RA, Dunwel JM. 1995. Transformation of plant cells (to Zeneca Ltd.). *US Patent No. 5,464,765*
30. Collins GB, Shepherd RJ, eds. 1996. *Engineering Plants for Commercial Products and Applications. Ann. NY Acad. Sci.*, Vol. 792
31. Coruzzi G, Puigdomenech P, eds. 1994. *Plant Molecular Biology: Molecular Genetic Analysis of Plant Development and Metabolism.* Berlin: Springer-Verlag
32. Dale EC, Ow DW. 1991. Gene transfer with subsequent removal of the selection gene from the host genome. *Proc. Natl. Acad. Sci. USA* 88:10558–62
33. Dale PJ. 1995. R & D regulation and field trialling of transgenic crops. *Trends Biotechnol.* 13:398–403
34. De Block M. 1993. The cell biology of plant transformation: current state, problems, prospects and the implications for plant breeding. *Euphytica* 71:1–14
35. De Block M, De Brouwer D, Tenning P. 1989. Transformation of *Brassica napus* and *Brassica oleracea* using *Agrobacterium tumefaciens* and the expression of the *bar* and *neo* genes in transgenic plants. *Plant Physiol.* 91:694–701
36. De Block M, De Sonville A, Debrouwer D. 1995. The selection mechanism of phosphinothricin is influenced by the metabolic state of the tissue. *Planta* 197: 619–26
37. De Block M, Herrera-Estrella L, van Montagu M, Schell J, Zambryski P. 1984. Expression of foreign genes in regenerated plants and their progeny. *EMBO J.* 3: 1681–89
38. De Loose M, Danthine X, Van Bockstaele E, Van Montagu M, Depicker A. 1995. Different 5′ leader sequences modulate -glucuronidase accumulation levels in transgenic *Nicotiana tabacum* plants. *Euphytica* 85:209–16
39. Draper J, Scot R, Armitage P, Walden R, eds. 1988. *Plant Genetic Transformation and Gene Expression: A Laboratory Manual*. Oxford: Blackwell
40. Electronic Data Systems Corporation. 1995. *Shadow Patent Off., http://www.spo.eds.com/spo*
41. Ellis JR. 1993. Plant tissue culture and genetic transformation. In *Plant Molecular Biology Labfax*, ed. RRD Croy, pp. 253–85. Oxford: BIOS Sci.
42. Enayati E. 1995. Intellectual property under GATT. *BioTechnology* 13:460–62
43. Engel K-H, Takeoka GR, eds. 1995. *Genetically Modified Foods: Safety Issues.* Washington, DC: Am. Chem. Soc.
44. Feldmann KA. 1991. T-DNA insertion mutagenesis in *Arabidopsis*: mutational spectrum. *Plant J.* 1:71–82
45. Finnegan J, McElroy D. 1994. Transgene inactivation: plants fight back! *BioTechnology* 12:883–88
46. Firek S, Whitelam GC, Draper J. 1994. Endoplasmic reticulum targeting of active modified beta-glucuronidase (GUS) in transgenic tobacco plants. *Transgenic Res.* 3:326–31
47. Franks T, Birch RG. 1991. Microprojectile techniques for direct gene transfer into intact plant cells. See Ref. 110, pp. 103–27
48. Fromm ME, Morrish F, Armstrong C, Williams R, Thomas J, et al. 1990. Inheritance and expression of chimaeric genes in the progeny of transgenic maize plants. *BioTechnology* 8:833–39
49. Gad AE, Rosenberg N, Altman A. 1990. Liposome-mediated gene delivery into plant cells. *Physiol. Plant.* 79:177–83
50. Gafni Y, Icht M, Rubinfeld BZ. 1995. Stimulation of *Agrobacterium tumefaciens*

virulence with indole-3-acetic acid. *Lett. Appl. Microbiol.* 20:98–101
51. Gambley RL, Bryant JD, Masel NP, Smith GR. 1994. Cytokinin-enhanced regeneration of plants from microprojectile bombarded sugarcane meristematic tissue. *Aust. J. Plant Physiol.* 21:603–12
52. Gamborg OL, Phillips GC, eds. 1995. *Plant Cell, Tissue and Organ Culture. Fundamental Methods*. Berlin: Springer-Verlag
53. Gartland KMA, Davey MR, eds. 1995. *Agrobacterium Protocols: Methods in Molecular Biology*, Vol. 44. Totowa, NJ: Humana Press
54. Gatehouse AMR, Hilder VA, Boulter D, eds. 1992. *Plant Genetic Manipulation for Crop Protection*. Wallingford, Engl: Cent. Agric. Biosci. Int.
55. Geballe AP, Morris DR. 1994. Initiation codons within 5′-leaders of mRNAs as regulators of translation. *Trends Biochem. Sci.* 19:159–64
56. Gil P, Green PJ. 1996. Multiple regions of the *Arabidopsis* SAUR-AC1 gene control transcript abundance: the 3′ untranslated region functions as an mRNA instability determinant. *EMBO J.* 15:1678–86
57. Glick BR, Thompson JE, eds. 1993. *Methods in Plant Molecular Biology and Biotechnology*. Boca Raton, FL: CRC Press
58. Gomord V, Faye L. 1996. Signals and mechanisms involved in intracellular transport of secreted proteins in plants. *Plant Physiol. Biochem.* 34:165–81
59. Grant JE, Dommisse EM, Christey MC, Conner AJ. 1991. Gene transfer to plants using *Agrobacterium*. See Ref. 110, pp. 50–73
60. Grevelding C, Fantes V, Kemper E, Schell J, Masterson R. 1993. Single-copy T-DNA insertions in *Arabidopsis* are the predominant form of integration in root-derived transgenics, whereas multiple insertions are found in leaf discs. *Plant Mol. Biol.* 23:847–60
61. Grierson D, ed. 1991. *Plant Genetic Engineering*. Glasgow: Blackie
62. Hadi MZ, McMullen MD, Finer JJ. 1996. Transformation of 12 different plasmids into soybean via particle bombardment. *Plant Cell Rep.* 15:500–5
63. Hallman WK. 1996. Public perceptions of biotechnology: another look. *BioTechnology* 14:35–38
64. Hamilton CM, Frary A, Lewis C, Tanksley SD. 1996. Stable transfer of intact high molecular weight DNA into plant chromosomes. *Proc. Natl. Acad. Sci. USA* 93: 9975–79
65. Haq TA, Mason HS, Clements JD, Arntzen CJ. 1995. Oral immunization with recombinant bacterial antigen produced in transgenic plants. *Science* 268:714–16
66. Hensgens LAM, Fornerod MWJ, Rueb S, Winkler AA, van der Veen S, et al. 1992. Translation controls the expression level of a chimaeric reporter gene. *Plant Mol. Biol.* 20:921–38
67. Herbers K, Sonnewald U. 1996. Manipulating metabolic partitioning in transgenic plants. *Trends Biotechnol.* 14:198–205
68. Hicks GR, Smith HMS, Shieh M, Raikhel NV. 1995. Three classes of nuclear import signals bind to plant nuclei. *Plant Physiol.* 107:1055–58
69. Hiei Y, Komari T. 1994. Transformation of monocotyledons using *Agrobacterium*. *Int. Patent WO 94/00977*
70. Hiei Y, Ohta S, Komari T, Kumashiro T. 1994. Efficient transformation of rice (Oryza sativa L.) mediated by Agrobacterium and sequence analysis of the boundaries of the T-DNA. *Plant J.* 6: 271–82
71. Hinchee MAW, Padgette SR, Kishore GM, Delannay X, Fraley RT. 1993. Herbicide tolerant crops. See Ref. 90, 1:243–64
72. Hooykaas PJJ, Schilperoort RA. 1992. Agrobacterium and plant genetic engineering. *Plant Mol. Biol.* 19:15–38
73. Horsch RB. 1993. Commercialization of genetically engineered crops. *Philos. Trans. R. Soc. London Ser. B* 342:287–91
74. Horsch RB, Fraley RT, Rogers SG, Sanders PR, Lloyd A, et al. 1984. Inheritance of functional foreign genes in plants. *Science* 223:496–98
75. Hoyle R. 1996. Another salvo in the patent wars. *Nat. Biotechnol.* 14:680–82
76. Ishida Y, Saito H, Ohta S, Hiei Y, Komari T, et al. 1996. High efficiency transformation of maize (*Zea mays* L.) mediated by *Agrobacterium tumefaciens*. *Nat. Biotechnol.* 14:745–50
77. Jähne A, Becker D, Lörz H. 1995. Genetic engineering of cereal crop plants: a review. *Euphytica* 85:35–44
78. Janssen B-J, Gardner RC. 1989. Localized transient expression of GUS in leaf discs following cocultivation with *Agrobacterium*. *Plant Mol. Biol.* 14:61–72
79. Jasin M, Moynahan ME, Richardson C. 1996. Targeted transgenesis. *Proc. Natl. Acad. Sci. USA* 93:8804–8
80. Jenes B, Moore H, Cao J, Zhang W, Wu R. 1993. Techniques for gene transfer. See Ref. 90, 1:125–46
81. Karp A. 1995. Somaclonal variation as a tool for crop improvement. *Euphytica* 85: 295–302

82. Keegstra K, Bruce B, Hurley M, Li HM, Perry S. 1995. Targeting of proteins into chloroplasts. *Physiol. Plant*. 93:157–62
83. Kim JW, Minamikawa T. 1996. Transformation and regeneration of french bean plants by the particle bombardment process. *Plant Sci*. 117:131–38
84. Kjeldgaard RH, Marsh DR. 1994. Intellectual property rights for plants. *Plant Cell* 6:1524–28
85. Klein B, Töpfer R, Sohn A, Schell J, Steinbi H-H. 1990. Promoterless reporter genes and their use in plant gene transformation. In *Progress in Plant Cellular and Molecular Biology*, ed. HJJ Nijkamp, LHW Van Der Plas, J Van Aartrijk, pp. 79–84. Dordrecht: Kluwer
86. Koncz C, Martini N, Mayerhofer R, Koncz-Kalman Z, Körber H, et al. 1989. High-frequency T-DNA mediated gene tagging in plants. *Proc. Natl. Acad. Sci. USA* 86:8467–71
87. Koziel MG, Beland GL, Bowman C, Carozzi NB, Crenshaw R, et al. 1993. Field performance of elite transgenic maize plants expressing an insecticidal protein derived from *Bacillus thuringiensis*. *BioTechnology* 11:194–200
88. Koziel MG, Carozzi NB, Desai N, Warren GW, Dawson J, et al. 1996. Transgenic maize for the control of European corn borer and other maize insect pests. See Ref. 30, pp. 164–71
89. Kryzyzek R, Laursen CRM, Anderson PC. 1995. Stable transformation of maize cells by electroporation. *US Patent 5,472,869*
90. Kung S, Wu R, eds. 1993. *Transgenic Plants*, Vols. 1–2. San Diego: Academic
91. Laparra H, Burrus M, Hunold R, Damm B, Bravo-Angel A-M, et al. 1995. Expression of foreign genes in sunflower (*Helianthus anuus* L.): evaluation of three gene transfer methods. *Euphytica* 85:63–74
92. Lebel EG, Masson J, Bogucki A, Paszkowski J. 1995. Transposable elements as plant transformation vectors for long stretches of foreign DNA. *Theor. Appl. Genet.* 91:899–906
93. Li H-Q, Sautter C, Potrykus I, Puonti-Kaerlas J. 1996. Genetic transformation of cassava (*Manihot esculenta* Crantz). *Nat. Biotechnol*. 14:736–40
94. Lichtenstein M, Keini G, Cedar H, Bergman Y. 1994. B-cell-specific demethylation: a novel role for the intronic kappa-chain enhancer sequence. *Cell* 76:913–23
95. Lin JJ, Assadgarcia N, Kuo J. 1995. Plant hormone effect of antibiotics on the transformation efficiency by *Agrobacterium tumefaciens* cells. *Plant Sci*. 109:171–77
96. Lindsey K, Wei W, Clarke MC, McArdale HF, Rooke LM, et al. 1993. Tagging genomic sequences that direct transgene expression by activation of a promoter trap in plants. *Transgenic Res*. 2:33–47
97. Livingstone DM, Birch RG. 1995. Plant regeneration and microprojectile-mediated gene transfer in embryonic leaflets of peanut (*Arachis hypogaea* L.). *Aust. J. Plant Physiol*. 22:585–91
98. Luehrsen KR, Walbot V. 1991. Intron enhancement of gene expression and the splicing efficiency of introns in maize cells. *Mol. Gen. Genet*. 225:81–93
99. Luehrsen KR, Walbot V. 1994. The impact of AUG start codon context on maize gene expression in vivo. *Plant Cell Rep*. 13: 454–58
100. Maas C, Laufs J, Grant S, Korfhage C, Werr W. 1991. The combination of a novel stimulatory element in the first exon of the maize Shrunken-1 gene with the following intron 1 enhances reporter gene expression up to 1000-fold. *Plant Mol. Biol*. 16: 199–207
101. Maheswaran G, Welander M, Hutchinson JF, Graham MW, Richards D. 1992. Transformation of apple rootstock M26 with *Agrobacterium tumefaciens*. *J. Plant. Physiol*. 139:560–68
102. Maliga P, Maliga ZS. 1995. Method for stably transforming plastids of multicellular plants. *US Patent 5,451,513*
103. Matzke MA, Matzke AJM. 1995. How and why do plants inactivate homologous (trans)genes? *Plant Physiol*. 107:679–85
104. McBride KE, Svab Z, Schaaf DJ, Hogan PS, Stalker DM, et al. 1995. Amplification of a chimeric *Bacillus* gene in chloroplasts leads to an extraordinary level of an insecticidal protein in tobacco. *BioTechnology* 13:362–65
105. McCabe DE, Martinell BJ. 1993. Transformation of elite cotton cultivars by particle bombardment of meristems. *BioTechnology* 11:596–98
106. McElroy D. 1996. The industrialization of plant transformation. *Nat. Biotechnol*. 14: 715–16
107. Meyer P. 1995. Variation of transgene expression in plants. *Euphytica* 85:359–66
108. Miller HI. 1995. Unscientific regulation of agricultural biotechnology: time to hold the policymakers accountable? *Trends Biotechnol*. 13:123–25
109. Mol JNM, Holton TA, Koes RE. 1995. Floriculture: genetic engineering of commercial traits. *Trends Biotechnol*. 13: 350–55
110. Murray DR, ed. 1991. *Advanced Methods*

in Plant Breeding and Biotechnology. Wallingford, Engl: CAB Int.
111. Narasimhulu SB, Deng X, Sarria R, Gelvin SB. 1996. Early transcription of *Agrobacterium* T-DNA genes in tobacco and maize. *Plant Cell* 8:873–86
112. Nawrath C, Poirier Y, Somerville C. 1995. Plant polymers for biodegradable plastics: cellulose, starch and polyhydroxyalkanoates. *Mol. Breed*. 1:105–22
113. Negrutiu I, Dewulf J, Pietrzak M, Botterman J, Rietveld E, et al. 1990. Hybrid genes in the analysis of transformation conditions. II. Transient expression vs stable transformation: analysis of parameters influencing gene expression levels and transformation efficiency. *Physiol. Plant*. 79: 197–205
114. Neuhaus G, Spangenberg G. 1990. Plant transformation by microinjection techniques. *Physiol. Plant*. 79:213–17
115. Neuhaus JM. 1996. Protein targeting to the plant vacuole. *Plant Physiol. Biochem*. 34: 217–21
116. Newbigen E, Smyth DR, Clarke AE. 1995. Understanding and controlling plant development. *Trends Biotechnol*. 13:338–43
117. Palmgren G, Mattson O, Okkels FT. 1993. Treatment of *Agrobacterium* or leaf discs with 5-azacytidine increases transgene expression in tobacco. *Plant Mol. Biol*. 21: 429–35
118. Paszkowski J, ed. 1994. *Homologous Recombination and Gene Silencing in Plants*. Amsterdam: Kluwer
119. Paszkowski J, Potrykus I, Hohn B, Shillito RD, Hohn T, et al. 1995. Transformation of hereditary material in plants. *US Patent 5,453,367*
120. Paszkowski J, Shillito RD, Saul M, Mandak V, Hohn T, et al. 1984. Direct gene transfer to plants. *EMBO J*. 3:2717–22
121. Patent bibliography: plant biotechnology. 1993. *Curr. Opin. Biotechnol*. 4:253–55
122. Peach C, Velten J. 1991. Transgene expression variability (position effect) of CAT and GUS reporter genes driven by linked divergent T-DNA promoters. *Plant Mol. Biol*. 17:49–60
123. Peet RC. 1995. *Protection of plant-related inventions in the United States*. http://biotechlaw.ari.net/plnt-ovr.html
124. Peri A, Lotan O, Abu-Abied M, Holland D. 1996. Establishment of an *Agrobacterium*-mediated transformation system for grape (*Vitis vinifera* L.): the role of antioxidants during grape-*Agrobacterium* interactions. *Nat. Biotechnol*. 14:624–28
125. Perlak FJ, Fuchs RL, Dean DA, McPherson SL, Fischhoff DA. 1991. Modification of the coding sequence enhances plant expression of insect control genes. *Proc. Natl. Acad. Sci. USA* 88:3324–28
126. Potrykus I. 1991. Gene transfer to plants: Assessment of published approaches and results. *Annu. Rev. Plant Physiol. Plant Mol. Biol*. 42:205–25
127. Potrykus I, Spangenberg G, eds. 1995. *Gene Transfer to Plants*. Berlin: Springer-Verlag
128. Quail PH, Christiansen AH, Hershey HP, Sharrock RA, Sullivan TD. 1994. Plant ubiquitin promoter system. *Eur. Patent EP 342926*
129. Reichel C, Mathur J, Eckes P, Langenkemper K, Koncz C, et al. 1996. Enhanced green fluorescence by the expression of an *Aequorea victoria* green fluorescent protein mutant in mono- and dicotyledonous plant cells. *Proc. Natl. Acad. Sci. USA* 93: 5888–93
130. Richardson JP. 1993. Transcription termination. *Crit. Rev. Biochem. Mol. Biol*. 28: 1–30
131. Ritchie SW, Hodges TK. 1993. Cell culture and regeneration of transgenic plants. See Ref. 90, 1:147–78
132. Ross AH, Manners JM, Birch RG. 1995. Embryogenic callus production, plant regeneration, and transient gene expression following particle bombardment, in the pasture grass *Cenchrus ciliarus* (Gramineae). *Aust. J. Bot*. 43:193–99
133. Russell DR, Wallace KM, Bathe JH, Martinell BJ, McCabe DE. 1993. Stable transformation of *Phaseolus vulgaris* via electric-discharge mediated particle acceleration. *Plant Cell Rep*. 12:165–69
134. Sanford JC, Wolf ED, Allen NK. 1990. Method for transporting substances into living cells and tissues and apparatus therefor. *US Patent 4,954,050*
135. Sanford JC, Wolf ED, Allen NK. 1992. Biolistic apparatus for delivering substances into cells and tissues in a nonlethal manner. *Aust. Patent AU 621561*
136. Schilperoort RA, Hoekema A, Hooykaas PJJ. 1990. Process for the incorporation of foreign DNA into the genome of dicotyledonous plants. *US Patent 4,940,838*
137. Schöpke C, Taylor N, Carcamo R, Konan NK, Marmey P, et al. 1996. Regeneration of transgenic cassava plants (Manihot esculenta Crantz) from microbombarded embryogenic suspension cultures. *Nat. Biotechnol*. 14:731–35
138. Shah DM, Rommens CMT, Beachy RN. 1995. Resistance to diseases and insects in transgenic plants: progress and applica-

tions to agriculture. *Trends Biotechnol*. 13: 362–68
139. Shen WH, Hohn B. 1994. Amplification and expression of the beta-glucuronidase gene in maize plants by vectors based on maize streak virus. *Plant J*. 5:227–36
140. Shimamoto K, Terada R, Izawa T, Fujimoto H. 1989. Fertile transgenic rice plants regenerated from transformed protoplasts. *Nature* 338:274–76
141. Songstad DD, Somers DA, Griesbach RJ. 1995. Advances in alternative DNA delivery techniques. *Plant Cell Tissue Organ Cult*. 40:1–15
142. Spiker S, Thompson WF. 1996. Nuclear matrix attachment regions and transgene expression in plants. *Plant Physiol*. 110: 15–21
143. Staskawicz BJ, Ausubel FM, Baker BJ, Ellis JG, Jones JDG. 1995. Molecular genetics of plant disease resistance. *Science* 268:661–67
144. Stone R. 1995. Sweeping patents put biotech companies on the warpath. *Science* 268:656–58
145. Sun SSM, Larkins BA. 1993. Transgenic plants for improving seed storage proteins. See Ref. 90, pp. 1:339–72
146. Tepfer D. 1990. Genetic transformation using *Agrobacterium* rhizogenes. *Physiol. Plant*. 79:140–46
147. Theologis A. 1994. Control of ripening. *Curr. Opin. Biotechnol*. 5:152–57
148. Turner R, Foster GD. 1995. The potential of plant viral translational enhancers in biotechnology for increased gene expression. *Mol. Biotechnol*. 3:225–36
149. Vancanneyt G, Schmidt R, O'Connor-Sanchez A, Willmitzer L, Rocha-Sosa M. 1990. Construction of an intron-containing marker gene: splicing of the intron in transgenic plants and its use in monitoring early events in *Agrobacterium*-mediated plant transformation. *Mol. Gen. Genet*. 220: 245–50
150. van der Graaff E, den Dulk-Ras A, Hooykaas PJJ. 1996. Deviating T-DNA transfer from *Agrobacterium tumefaciens* to plants. *Plant Mol. Biol*. 31:677–81
151. Van-der-Hoeven C, Dietz A, Landsmann J. 1994. Variability of organ-specific gene expression in transgenic tobacco plants. *Transgenic Res*. 3:159–66
152. van Wordragen MF, Dons JJM. 1992. Agrobacterium tumefaciens-mediated transformation of recalcitrant crops. *Plant Mol. Biol. Rep*. 10:12–36
153. Varner JE, Ye Z-H. 1995. Tissue printing to detect proteins and RNA in plant tissues. In *Methods in Plant Molecular Biology: A Laboratory Course Manual*, ed. P Maliga, DF Klessig, AR Cashmore, W Gruissem, JE Varner, pp. 79–94. Plainview, NY: Cold Spring Harbor Lab. Press
154. Vasil IK, Thorpe TA, eds. 1994. *Plant Cell and Tissue Culture*. Dordrecht: Kluwer
155. Von Bodman SB, Domier LL, Farrand SK. 1995. Expression of multiple eukaryote genes from a single promoter in *Nicotiana*. *BioTechnology* 13:587–91
156. Walden R, Wingender R. 1995. Gene-transfer and plant-regeneration techniques. *Trends Biotechnol*. 13:324–31
157. Wan Y, Lemaux PG. 1994. Generation of large numbers of independently transformed fertile barley plants. *Plant Physiol*. 104:37–48
158. Webber GD. 1995. *Biotechnology Information Series* (NCR# 483, 487, 551, 553, 557). Ames: Iowa State Univ.
159. Weeks TJ, Anderson OD, Blechl AE. 1993. Rapid production of multiple independent lines of fertile transgenic wheat (*Triticum aestivum*). *Plant Physiol*. 102:1077–84
160. Williams KM. 1994. How to avoid patent infringement. *BioTechnology* 12:297–98
161. Wilmink A, Dons JJM. 1993. Selective agents and marker genes for use in transformation of monocotyledonous plants. *Plant Mol. Biol. Rep*. 11:165–85
162. Wu L, Ueda T, Messing J. 1995. The formation of mRNA 3′-ends in plants. *Plant J*. 8:323–29
163. Yoder JI, Goldsbrough AP. 1994. Transformation systems for generating marker-free transgenic plants. *BioTechnology* 12:883–88
164. Zadoks JC, Schein RD. 1979. Southern corn leaf blight. In *Epidemiology and Plant Disease Management*, pp. 331–35. New York: Oxford Univ. Press
165. Zupan JR, Zambryski P. 1995. Transfer of T-DNA from Agrobacterium to the plant cell. *Plant Physiol*. 107:1041–47