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Agrobacterium-Mediated Plant Transformation: the Biology behind the "Gene-Jockeying" Tool

Stanton B. Gelvin*

Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907-1392

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INTRODUCTION

Twenty-five years ago, the concept of using *Agrobacterium tumefaciens* as a vector to create transgenic plants was viewed as a prospect and a "wish." Today, many agronomically and horticulturally important species are routinely transformed using this bacterium, and the list of species that is susceptible to *Agrobacterium*-mediated transformation seems to grow daily. In some developed countries, a high percentage of the acreage of such economically important crops as corn, soybeans, cotton, canola, potatoes, and tomatoes is transgenic; an increasing number of these transgenic varieties are or will soon be generated by *Agrobacterium*-mediated, as opposed to particle bombardment-mediated transformation. There still remain, however, many challenges for genotype-independent transformation of many economically important crop species, as well as forest species used for lumber, paper, and pulp production. In addition, predictable and stable expression of transgenes remains problematic. Several excellent reviews have appeared recently that describe in detail various aspects of *Agrobacterium* biology (44, 73, 109, 325, 327, 328, 384, 385). In this review, I describe how scientists utilized knowledge of basic *Agrobacterium* biology to develop *Agrobacterium* as a "tool" for plant genetic engineering. I also explore how our increasing understanding of *Agrobacterium* biology may help extend the utility of *Agrobacterium*-mediated transformation. It is my belief that further improvements in transformation technology will necessarily involve the manipulation of these fundamental biological processes.

AGROBACTERIUM "SPECIES" AND HOST RANGE

The genus *Agrobacterium* has been divided into a number of species. However, this division has reflected, for the most part, disease symptomology and host range. Thus, *A. radiobacter* is an "avirulent" species, *A. tumefaciens* causes crown gall disease, *A. rhizogenes* causes hairy root disease, and *A. rubi* causes cane gall disease. More recently, a new species has been proposed, *A. vitis*, which causes galls on grape and a few other plant species (244). Although *Bergey's Manual of Systematic Bacteriology* still reflects this nomenclature, classification is complex and confusing; we now know that symptoms follow, for the most part, the type of tumorigenic plasmid contained within a particular strain. Curing a particular plasmid and

* Mailing address: Department of Biological Sciences, Purdue University, West Lafayette, IN 47907-1392. Phone: (765) 494-4939. Fax: (765) 496-1496. E-mail: gelvin@bilbo.bio.purdue.edu.

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replacing this plasmid with another type of tumorigenic plasmid can alter disease symptoms. For example, infection of plants with *A. tumefaciens* C58, containing the nopaline-type Ti plasmid pTiC58, results in the formation of crown gall teratomas. When this plasmid is cured, the strain becomes nonpathogenic. Introduction of Ri plasmids into the cured strain "converts" the bacterium into a rhizogenic strain (191, 358). Furthermore, one can introduce a Ti (tumor-inducing) plasmid from *A. tumefaciens* into *A. rhizogenes*; the resulting strain incites tumors of altered morphology on *Kalanchoe* plants (53). Thus, because *A. tumefaciens* can be "converted" into *A. rhizogenes* simply by substituting one type of oncogenic plasmid for another, the term "species" becomes meaningless. Perhaps a more meaningful classification system divides the genus *Agrobacterium* into "biovars" based on growth and metabolic characteristics (171). Using this system, most *A. tumefaciens* and *A. rubi* (316) strains belong to biovar I, *A. rhizogenes* strains fit into biovar II, and biovar III is represented by *A. vitis* strains. More recently, yet another taxonomic classification system for the genus *Agrobacterium* has been proposed (374). The recent completion of the DNA sequence of the entire *A. tumefaciens* C58 genome (which is composed of a linear and a circular chromosome, a Ti plasmid, and another large plasmid [114, 115, 363]) may provide a starting point for reclassification of *Agrobacterium* "strains" into true "species."

Regardless of the current confusion in species classification, for the purposes of plant genetic engineering, the most important aspect may be the host range of different *Agrobacterium* strains. As a genus, *Agrobacterium* can transfer DNA to a remarkably broad group of organisms including numerous dicot and monocot angiosperm species (12, 68, 262, 341) and gymnosperms (198, 206, 215, 228, 307, 357, 371). In addition, *Agrobacterium* can transform fungi, including yeasts (32, 33, 260), ascomycetes (1, 71), and basidiomycetes (71). Recently, *Agrobacterium* was reported to transfer DNA to human cells (187).

The molecular and genetic basis for the host range of a given *Agrobacterium* strain remains unclear. Early work indicated that the Ti plasmid, rather than chromosomal genes, was the major genetic determinant of host range (207, 315). Several virulence (*vir*) loci on the Ti plasmid, including *virC* (367, 368) and *virF* (220, 267), were shown to determine the range of plant species that could be transformed to yield crown gall tumors. The *virH* (formerly called *pinF*) locus appeared to be involved in the ability of *Agrobacterium* to transform maize, as established by an assay in which symptoms of maize streak virus infection were determined following agroinoculation of maize plants (153). Other *vir* genes, including *virG*, contribute to the "hypervirulence" of particular strains (41, 146).

However, it is now clear that host range is a much more complex process, which is under the genetic control of multiple factors within both the bacterium and the plant host. The way one assays for transformation can affect the way one views host range. For example, many monocot plant species, including some cultivars of grasses such as maize (152), rice (39, 40, 85, 139, 265, 321), barley (317), and wheat (42), can now be genetically transformed by many *Agrobacterium* strains to the phenotype of antibiotic or herbicide resistance. However, these plant species do not support the growth of crown gall tumors. Host range may further result from an interaction of particular

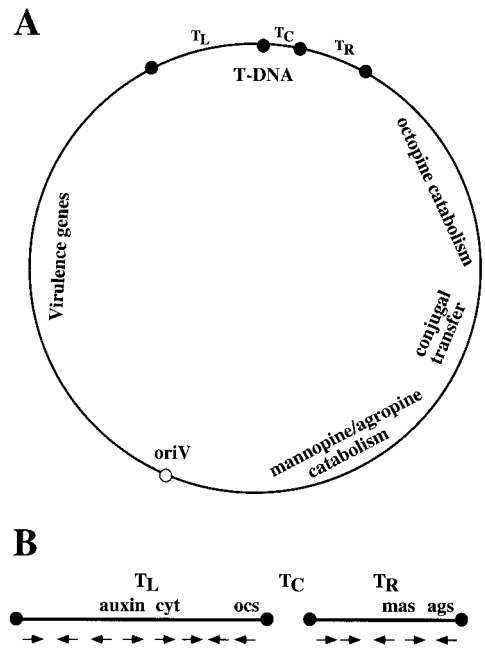

FIG. 1. Schematic representation of a typical octopine-type Ti plasmid (A) and the T-DNA region of a typical octopine-type Ti plasmid (B). (A) The T-DNA is divided into three regions. T_L (T-DNA left), T_C (T-DNA center), and T_R (T-DNA right). The black circles indicate T-DNA border repeat sequences. *oriV*, the vegetative origin of replication of the Ti plasmid, is indicated by a white circle. (B) The various T-DNA-encoded transcripts, and their direction of transcription, are indicated by arrows. Genes encoding functions involved in auxin synthesis (auxin), cytokinin synthesis (cyt), and the synthesis of the opines octopine (ocs), mannopine (mas), and agropine (ags) are indicated.

Ti plasmids with certain bacterial chromosomal backgrounds. For example, the Ti plasmid pTiBo542, when in its natural host strain *A. tumefaciens* Bo542, directs limited tumorigenic potential when assayed on many leguminous plant species. However, when placed in the C58 chromosomal background, pTiBo542 directs strong virulence toward soybeans and other legumes (143). Finally, susceptibility to crown gall disease has a genetic basis in cucurbits (292), peas (272), soybeans (15, 214, 246), and grapevines (312) and even among various ecotypes of *Arabidopsis thaliana* (231). The roles of both bacterial virulence genes and host genes in the transformation process, and the ways in which they may possibly be manipulated for genetic engineering purposes, are discussed below.

MOLECULAR BASIS OF *AGROBACTERIUM*-MEDIATED TRANSFORMATION

What Is T-DNA?

The molecular basis of genetic transformation of plant cells by *Agrobacterium* is transfer from the bacterium and integration into the plant nuclear genome of a region of a large tumor-inducing (Ti) or rhizogenic (Ri) plasmid resident in *Agrobacterium* (Fig. 1A). Ti plasmids are on the order of 200 to 800 kbp in size (81, 100, 111, 114, 145, 166, 175, 177, 245, 250, 251, 261, 311, 332, 342, 363). The transferred DNA (T-DNA) (Fig. 1B) is referred to as the T-region when located on the Ti

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or Ri plasmid. T-regions on native Ti and Ri plasmids are approximately 10 to 30 kbp in size (17, 34, 197, 311, 378). Thus, T-regions generally represent less than 10% of the Ti plasmid. Some Ti plasmids contain one T-region, whereas others contain multiple T-regions (17, 311). The processing of the T-DNA from the Ti plasmid and its subsequent export from the bacterium to the plant cell result in large part from the activity of virulence (*vir*) genes carried by the Ti plasmid (106, 147, 148, 174, 208, 303).

T-regions are defined by T-DNA border sequences. These borders are 25 bp in length and highly homologous in sequence (167, 366). They flank the T-region in a directly repeated orientation (257, 276, 335, 345, 352). In general, the T-DNA borders delimit the T-DNA (but see below for exceptions), because these sequences are the target of the VirD1/VirD2 border-specific endonuclease that processes the T-DNA from the Ti plasmid. There appears to be a polarity established among T-DNA borders: right borders initially appeared to be more important than left borders (136, 156, 286, 352, 353). We now know that this polarity may be caused by several factors. First, the border sequences not only serve as the target for the VirD1/VirD2 endonuclease but also serve as the covalent attachment site for VirD2 protein. Within the Ti or Ri plasmid (or T-DNA binary vectors [see below]), T-DNA borders are made up of double-stranded DNA. Cleavage of these double-stranded border sequences requires VirD1 and VirD2 proteins, both in vivo (82, 99, 155, 369) and in vitro (281). In vitro, however, VirD2 protein alone can cleave a single-stranded T-DNA border sequence (154, 249). Cleavage of the 25-bp T-DNA border results predominantly from the nicking of the T-DNA "lower strand," as conventionally presented, between nucleotides 3 and 4 of the border sequence (301, 353). However, double-strand cleavage of the T-DNA border has also been noted (155, 305, 344). Nicking of the border is associated with the tight (probably covalent) linkage of the VirD2 protein, through tyrosine 29 (351), to the 5′ end of the resulting single-stranded T-DNA molecule termed the T-strand (91, 99, 137, 150, 355, 373). It is this T-strand, and not a double-stranded T-DNA molecule, that is transferred to the plant cell (318, 375). Thus, it is the VirD2 protein attached to the right border, and not the border sequence per se, that establishes polarity and the importance of right borders relative to left borders. It should be noted, however, that because left-border nicking is also associated with VirD2 attachment to the remaining molecule (the "non-T-DNA" portion of the Ti plasmid or "backbone" region of the T-DNA binary vector [91]), it may be possible to process T-strands from these regions of Ti and Ri plasmids and from T-DNA binary vectors (182, 264, 356). The problem of vector "backbone" sequence transfer to plants is discussed below.

Second, the presence of T-DNA "overdrive" sequences near many T-DNA right borders, but not left borders, may also help establish the functional polarity of right and left borders. Overdrive sequences enhance the transmission of T-strands to plants, although the molecular mechanism of how this occurs remains unknown (131, 156, 256, 291, 336, 337, 345). Early reports suggested that the VirC1 protein binds to the overdrive sequence and may enhance T-DNA border cleavage by the VirD1/VirD2 endonuclease (322). *virC1* and *virC2* functions are important for virulence; mutation of these genes results in loss of virulence on many plant species (299). However, several laboratories have noted that T-strand production in *virC* mutant *Agrobacterium* strains occurs at wild-type levels (301, 344). Thus, any effect of VirC must occur after T-DNA processing.

How Is T-DNA Transferred from *Agrobacterium* to Plant Cells?

As indicated above, many proteins encoded by *vir* genes play essential roles in the *Agrobacterium*-mediated transformation process. Some of these roles have been discussed in several excellent review articles (44, 109, 325, 327, 328, 384), and I shall therefore limit my description to the roles of Vir proteins that may serve as points of manipulation for the improvement of the transformation process.

VirA and VirG proteins function as members of a two-component sensory-signal transduction genetic regulatory system. VirA is a periplasmic antenna that senses the presence of particular plant phenolic compounds that are induced on wounding (3, 87, 162, 195, 303, 324, 359). In coordination with the monosaccharide transporter ChvE and in the presence of the appropriate phenolic and sugar molecules, VirA autophosphorylates and subsequently transphosphorylates the VirG protein (160, 161). VirG in the nonphosphorylated form is inactive; however, on phosphorylation, the protein helps activate or increase the level of transcription of the *vir* genes, most probably by interaction with *vir*-box sequences that form a component of *vir* gene promoters (59, 60, 252). Constitutively active VirA and VirG proteins that do not require phenolic inducers for activity, or VirG proteins that interact more productively with *vir*-box sequences to activate *vir* gene expression, may be useful to increase *Agrobacterium* transformation efficiency or host range. Experiments describing some attempts to manipulate VirA and/or VirG for these purposes are discussed below.

Together with the VirD4 protein, the 11 VirB proteins make up a type IV secretion system necessary for transfer of the T-DNA and several other Vir proteins, including VirE2 and VirF (44, 349). VirD4 may serve as a "linker" to promote the interaction of the processed T-DNA/VirD2 complex with the VirB-encoded secretion apparatus (126). Most VirB proteins either form the membrane channel or serve as ATPases to provide energy for channel assembly or export processes. Several proteins, including VirB2, VirB5, and possibly VirB7, make up the T-pilus (94, 163, 189, 190, 278, 283). VirB2, which is processed and cyclized, is the major pilin protein (94, 163, 189, 190). The function of the pilus in T-DNA transfer remains unclear; it may serve as the conduit for T-DNA and Vir protein transfer, or it may merely function as a "hook" to seize the recipient plant cell and bring the bacterium and plant into close proximity to effect molecular transfer. One aspect of pilus biology that may be important for transformation is its temperature lability. Although *vir* gene induction is maximal at approximately 25 to 27°C (8, 162, 323), the pilus of some, but not all, *Agrobacterium* strains is most stable at lower temperatures (approximately 18 to 20°C) (18, 105, 188). Early experiments by Riker indicated a temperature effect on transformation (268). Thus, one may consider cocultivating *Agrobacterium* with plant cells at lower temperatures during the initial few days of the transformation process.

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The VirD2 and VirE2 proteins play essential and perhaps complementary roles in *Agrobacterium*-mediated transformation. These two proteins have been proposed to constitute, with the T-strand, a "T-complex" that is the transferred form of the T-DNA (149). Whether this complex assembles within the bacterium remains controversial. Citovsky et al. (50) showed that VirE2 could function in a plant cell: transgenic VirE2-expressing tobacco plants could "complement" infection by a *virE2* mutant *Agrobacterium* strain. Several laboratories have shown that VirE2 can transfer to the plant cell in the absence of a T-strand (27, 193, 244, 309, 349), and it is possible that VirE2 complexes with the T-strand either in the bacterial export channel or within the plant cell. A recent report suggests perhaps another role for VirE2 early in the export process: Dumas et al. (90) showed that VirE2 could associate with artificial membranes in vitro and create a channel for the transport of DNA molecules. Thus, it is possible that one function of VirE2 is to form a pore in the plant cytoplasmic membrane to facilitate the passage of the T-strand.

Because of its attachment to the 5′ end of the T-strand, VirD2 may serve as a pilot protein to guide the T-strand to and through the type IV export apparatus. Once in the plant cell, VirD2 may function in additional steps of the transformation process. VirD2 contains nuclear localization signal (NLS) sequences that may help direct it and the attached T-DNA to the plant nucleus. The NLS of VirD2 can direct fused reporter proteins and in vitro-assembled T-complexes to the nuclei of plant, animal, and yeast cells (48, 119, 138, 151, 185, 186, 229, 319, 326, 381, 382). Furthermore, VirD2 can associate with a number of *Arabidopsis* importin-α proteins in an NLS-dependent manner, both in yeast and in vitro (16; S. Bhattacharjee and S. B. Gelvin, unpublished data). Importin-α is a component of one of the protein nuclear transport pathways found in eukaryotes. Recent data, however, suggest that VirD2 may not be sufficient to direct T-strands to the nucleus. Ziemienowicz et al. (382) showed that in permeabilized cells, VirD2 could effect the nuclear targeting of small linked oligonucleotides generated in vitro but could not direct the nuclear transport of larger linked molecules. To achieve nuclear targeting of these larger molecules, VirE2 additionally had to be associated with the T-strands. Finally, VirD2 may play a role in integration of the T-DNA into the plant genome. Various mutations in VirD2 can affect either the efficiency (229) or the "precision" (320) of T-DNA integration.

The role of VirE2 in T-DNA nuclear transport also remains controversial. VirE2 is a non-sequence-specific single-stranded DNA binding protein (45, 46, 49, 112, 286). In *Agrobacterium* cells, VirE2 probably interacts with the VirE1 molecular chaperone and may therefore not be available to bind T-strands (77, 84, 310, 380). However, when bound to single-stranded DNA (perhaps in the plant cell?), VirE2 can alter the DNA from a random-coil conformation to a shape that resembles a coiled telephone cord (47). This elongated shape may help direct the T-strand through the nuclear pore. VirE2 also contains NLS sequences that can direct fused reporter proteins to plant nuclei (48, 50, 326, 383). As with VirD2, VirE2 interacts in yeast with *Arabidopsis* importin-α proteins in an NLS-dependent manner (Bhattacharjee and Gelvin, unpublished). One report indicates that VirE2 bound to single-stranded DNA and microinjected into plant cells can direct the DNA to the nucleus (381). However, other reports demonstrate that VirE2 cannot direct bound single-stranded DNA to the nuclei of either plant or animal cells that are permeabilized in order to effect DNA uptake (380). The cause of these contradictory results remains unclear but may reflect differences in the cell types and DNA delivery systems used by the two groups. When T-DNA is delivered to plant cells from *Agrobacterium* strains that encode a mutant form of VirD2 containing a precise deletion of the NLS, there is at most only a 40% decrease in transformation efficiency (229, 233, 290). Transgenic plants expressing VirE2 can complement a double-mutant *Agrobacterium* strain that lacks *virE2* and contains a deletion in the NLS-encoding region of *virD2* (110). These results suggest that in the absence of NLS sequences in VirD2, some other nuclear targeting mechanism (perhaps involving VirE2) may take place.

When bound to DNA, the NLS motifs of VirE2 may be occluded and inactive. This is because the NLS and DNA binding domains of VirE2 overlap (50). Hohn's group has hypothesized that the primary role of VirE2 in nuclear transport is NLS independent and that VirE2 merely shapes the T-strand so that it can snake through the nuclear pores (274).

Further controversy involves the ability of VirE2 protein to localize to the nuclei of animal cells. Ziemienowicz et al. (381) showed that in permeabilized HeLa cells, octopine-type VirE2 could target to the nucleus, whereas in microinjected *Drosophila* and *Xenopus* cells, the NLS sequence of nopaline-type VirE2 had to be changed in order to effect nuclear localization of the altered protein (50). Although the reason for this discrepancy is not known, it is not likely that it results from the use of octopine- versus nopaline-type VirE2 by the two groups (326).

Finally, VirE2 may protect T-strands from nucleolytic degradation that can occur both in the plant cytoplasm and perhaps in the nucleus (274, 374).

The existence of a T-complex composed of a single molecule of VirD2 covalently attached to the 5′ end of the T-strand, which in turn is coated by VirE2 molecules, has generally been accepted by the *Agrobacterium* research community (149). However, the reader should be aware that such a complex has not yet been identified in either *Agrobacterium* or plant cells. It is possible that other proteins, such as importins (16), VIP1 (329), and even VirF (285), may additionally interact, either directly or indirectly, with the T-strand to form larger T-complexes in the plant cell.

Although the role of Ti plasmid-encoded *vir* genes has often been considered of primary importance for transformation, many *Agrobacterium* chromosomal genes are also essential for this process. The role of chromosomal genes was first established by random insertional mutagenesis of the entire *Agrobacterium* genome (106). Further research defined the roles of many of these genes. Included among these functions are exopolysaccharide production, modification, and secretion (*pscA*/*exoC*, *chvA*, and *chvB* [36, 37, 88, 89, 313]) and other roles in bacterial attachment to plant cells (*att* genes [212, 213]), sugar transporters involved in coinduction of *vir* genes (*chvE* [86, 172, 289]), regulation of *vir* gene induction (*chvD* [204]), and T-DNA transport (*acvB* [168, 248, 360, 361, 362]). Other genes, such as *miaA* (116), may also play a more minor role in the transformation process. The recent elucidation of the entire *A. tumefaciens* C58 sequence (114, 363) will surely

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provide fertile ground for the discovery of additional genes involved in *Agrobacterium*-mediated transformation.

MANIPULATION OF *AGROBACTERIUM* FOR GENETIC ENGINEERING PURPOSES

Introduction of Genes into Plants by Using *Agrobacterium*

Years before scientists elucidated the molecular mechanism of *Agrobacterium*-mediated transformation of plants, Armin Braun proposed the concept of a "tumor-inducing principle" that was stably transferred to and propagated in the plant genome (30). Research in the 1970s resulted in the identification of large plasmids in virulent *Agrobacterium* strains (376), although we now know that many strains contain plasmids unrelated to virulence. Genetic experiments indicated that a particular class of plasmids, the Ti (and later Ri) plasmids, were responsible for tumorigenesis (339) and that a portion of these plasmids, the T-DNA, was transferred to plant cells and incorporated into the plant genome (43). It was thus obvious to propose that Ti plasmids be used as a vector to introduce foreign genes into plant cells.

However, Ti plasmids are very large and T-DNA regions do not generally contain unique restriction endonuclease sites not found elsewhere on the Ti plasmid. Therefore, one cannot simply clone a gene of interest into the T-region. Scientists therefore developed a number of strategies to introduce foreign genes into the T-DNA. These strategies involved two different approaches: cloning the gene, by indirect means, into the Ti plasmid such that the new gene was in *cis* with the virulence genes on the same plasmid, or cloning the gene into a T-region that was on a separate replicon from the *vir* genes (T-DNA binary vectors).

Two methods were used for cloning foreign DNA into the Ti plasmid. The first method was based on a strategy developed by Ruvkin and Ausubel (277) (Fig. 2). A region of DNA (either the T-region or any region of DNA targeted for disruption) containing unique restriction endonuclease sites is cloned into a broad-host-range plasmid, such as an IncPα-based vector. These plasmids can replicate both in *Escherichia coli*, in which the initial cloning is performed, and in *Agrobacterium*. The exogenous gene of interest, along with an antibiotic resistance marker, is next cloned into a unique restriction endonuclease site within the target region of DNA. Alternatively, an antibiotic resistance gene can be introduced into the DNA fragment of interest by transposition (107, 297). The resulting plasmid is introduced into *Agrobacterium* by conjugation or transformation. The presence of this plasmid in *Agrobacterium* is confirmed by selection for resistance to antibiotics encoded by both the plasmid vector backbone and the resistance marker near the gene of interest. Next, another plasmid of the same incompatibility group as the first plasmid, but harboring yet another antibiotic resistance marker, is introduced into the *Agrobacterium* strain containing the first plasmid. The resulting bacteria are plated on medium containing antibiotics to select for the second (eviction) plasmid and the resistance marker next to the gene of interest. Because plasmids of the same incompatibility group cannot usually coreside within the same bacterial cell, the bacteria can become resistant to both these antibiotics only if either (i) the first plasmid cointegrates into the Ti plasmid and uses the *oriV* of the Ti plasmid to replicate or (ii) an exchange of DNA on the first plasmid and the Ti plasmid occurs by double homologous recombination (homogenotization) using homologous sequences on the Ti plasmid flanking both sides of the gene of interest plus the resistance marker. In the first case (cointegration of the entire plasmid with the Ti plasmid), the resistance marker of the plasmid backbone would be expressed; these bacteria are screened for and discarded. In the second instance (homogenotization), the resistance marker encoded by the plasmid backbone is lost. Double homologous recombination can be confirmed by DNA blot analysis of total DNA from the resulting strain (107). A variant of this procedure utilizes a *sacB* gene on the plasmid backbone of the first plasmid. Only elimination of the plasmid backbone after homogenotization renders the bacterium resistant to growth on sucrose (194).

Another method to introduce foreign DNA into the T-region of the Ti plasmid involves first introducing a ColE1 replicon, such as pBR322, into the T-region of a Ti plasmid. DNA to be integrated into this T-region is cloned into a separate pBR322-derived molecule containing a second antibiotic resistance marker. This plasmid is introduced into the altered *Agrobacterium* strain, and the resulting strain is selected for resistance to the second antibiotic. Because ColE1 replicons cannot function in *Agrobacterium*, the pBR322-based plasmid would have to cointegrate into the pBR322 segment of the altered T-region for the stable expression of the plasmid-encoded resistance gene (379). A modification of this procedure was used to develop the "split-end vector" system. Using this system, a gene of interest is cloned into a pBR322-based vector that contains a T-DNA right border, a *nos-nptII* chimaeric gene for selection of transgenic plants, a spectinomycin-streptomycin resistance marker to select for the presence of the plasmid in *Agrobacterium*, and a region of homology with a nononcogenic portion of an octopine-type T-region. Cointegration of this plasmid with a Ti-plasmid lacking a right border but containing the T-DNA homologous region restores border activity and localizes the gene of interest and the plant selectable marker within the reconstituted T-region (101).

Each of these *cis*-integration methods has advantages and disadvantages. The first strategy can target the foreign gene to any part of the T-region (or other region in the Ti plasmid). However, it is cumbersome to perform and involves somewhat sophisticated microbial genetic procedures that many laboratories shunned. The second method is technically easier but allows cointegration of the foreign gene only into Ti-plasmid locations where pBR322 had previously been placed. However, a modification of this procedure allows cointegration of a pBR322-based plasmid into any region of the Ti plasmid (338, 377). An advantage of both of these systems is that they maintain the foreign gene at the same low copy number as that of the Ti plasmid in *Agrobacterium*.

Because of the complexity of introducing foreign genes directly into the T-region of a Ti plasmid, several laboratories developed an alternative strategy to use *Agrobacterium* to deliver foreign genes to plants. This strategy was based on seminal findings of Hoekema et al. (140) and de Frammond et al. (70). These authors determined that the T-region and the *vir*

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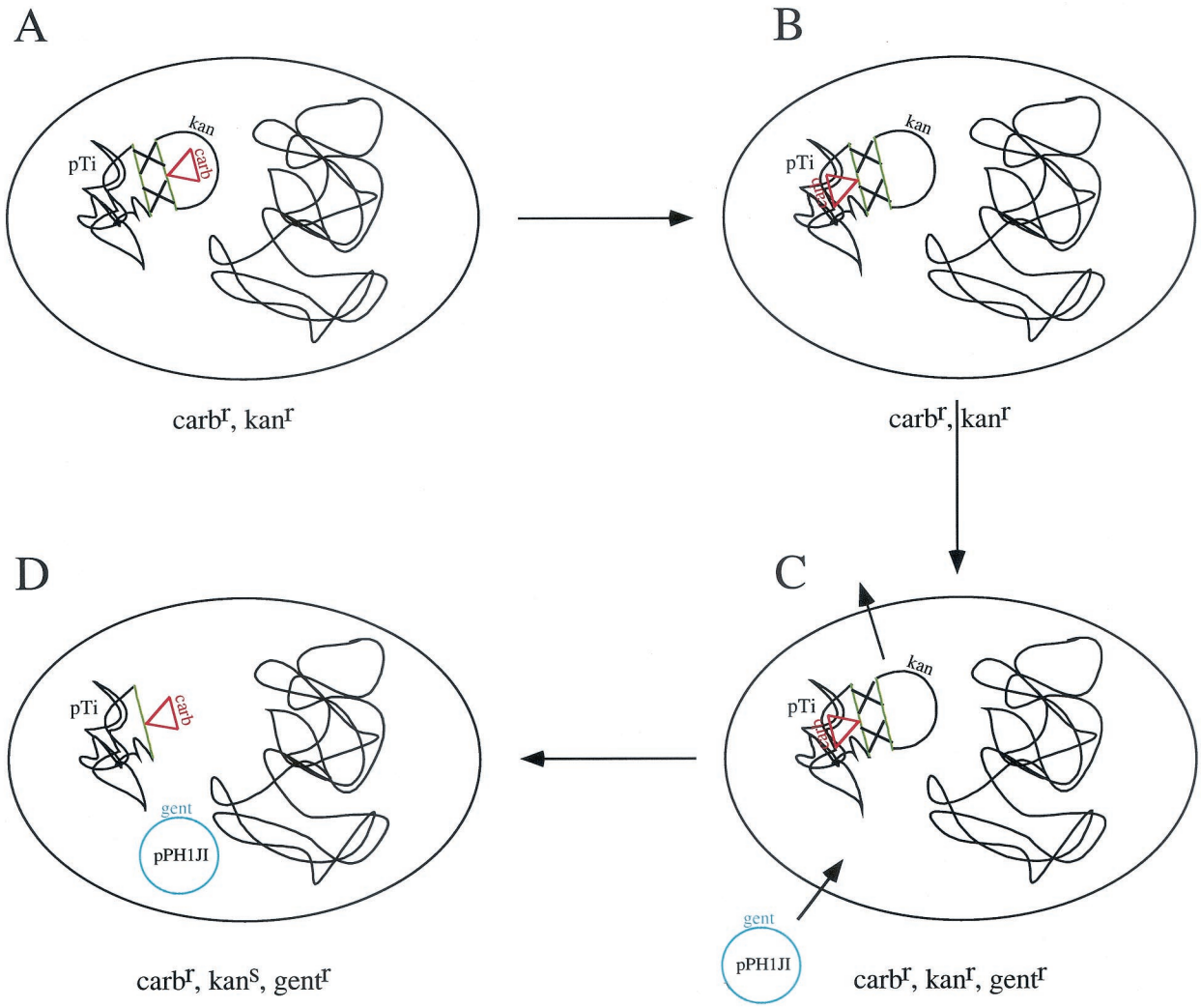

FIG. 2. Schematic representation of the steps involved in gene replacement by double homologous recombination (homogenotization [107, 277]). The green lines represent regions targeted for disruption. (A) An antibiotic resistance gene (in this case, encoding a β-lactamase that confers resistance to carbenicillin) has been inserted into the targeted gene that has been cloned into an IncPα plasmid (containing a kanamycin resistance gene [kan] in its backbone) and introduced into *Agrobacterium.* Double homologous recombination is allowed to take place. (B) Following double homologous recombination, the disrupted gene is exchanged onto the Ti plasmid (pTi). (C) A plasmid of the same incompatibility group as the first plasmid is introduced into *Agrobacterium.* An example is the IncPα plasmid pPH1JI, containing a gentamicin resistance gene (gent). (D) Because plasmids of the same incompatibility group (in this case IncPα) cannot replicate independently in the cell at the same time, selection for gentamicin resistance results in eviction of the other IncPα plasmid, onto which has been exchanged the wild-type gene.

genes could be separated into two different replicons. When these replicons were within the same *Agrobacterium* cell, products of the *vir* genes could act in *trans* on the T-region to effect T-DNA processing and transfer to a plant cell. Hoekema et al. called this a binary-vector system; the replicon harboring the T-region constituted the binary vector, whereas the replicon containing the *vir* genes became known as the *vir* helper. The *vir* helper plasmid generally contained a complete or partial deletion of the T-region, rendering strains containing this plasmid unable to incite tumors. A number of *Agrobacterium* strains containing nononcogenic *vir* helper plasmids have been developed, including LBA4404 (242), GV3101 MP90 (181), AGL0 (192), EHA101 and its derivative strain EHA105 (144, 146), and NT1 (pKPSF2) (247).

T-DNA binary vectors revolutionized the use of *Agrobacterium* to introduce genes into plants. Scientists without specialized training in microbial genetics could now easily manipulate *Agrobacterium* to create transgenic plants. These plasmids are small and easy to manipulate in both *E. coli* and *Agrobacterium* and generally contain multiple unique restriction endonuclease sites within the T-region into which genes of interest could be cloned. Many vectors were designed for specialized purposes, containing different plant selectable markers, promoters, and poly(A) addition signals between which genes of interest could be inserted, translational enhancers to boost the expression of transgenes, and protein-targeting signals to direct the transgene-encoded protein to particular locations within the plant cell (some representative T-DNA binary vector systems are described in references 10, 20, 25, 26, 62, 113, 120, 216, 364, and 386 and at http://www.cambia.org). Hellens et al. (134)

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provide a summary of many *A. tumefaciens* strains and vectors commonly used for plant genetic engineering.

Although the term "binary vector system" is usually used to describe two constituents (a T-DNA component and a *vir* helper component), each located on a separate plasmid, the original definition placed the two modules only on different replicons. These replicons do not necessarily have to be plasmids. Several groups have shown that T-DNA, when located in the *Agrobacterium* chromosome, can be mobilized to plant cells by a *vir* helper plasmid (141, 224).

How Much DNA Can Be Transferred from *Agrobacterium* to Plants?

The T-regions of natural Ti and Ri plasmids can be large enough to encode tens of genes. For example, the T-region of pTiC58 is approximately 23 kbp in size. In addition, some Ti and Ri plasmids contain multiple T-regions, each of which can be transferred to plants individually or in combination (34, 314). For purposes of plant genetic engineering, scientists may wish to introduce into plants large T-DNAs with the capacity to encode multiple gene products in a biosynthetic pathway. Alternatively, the reintroduction of large regions of a plant genome into a mutant plant may be useful to identify, by genetic complementation, genes responsible for a particular phenotype. How large a T-region can be transferred to plants?

Miranda et al. (224) showed that by reversing the orientation of a T-DNA right border, they could mobilize an entire Ti plasmid, approximately 200 kbp, into plants. Although the event was rare, this study showed that very large DNA molecules could be introduced into plants using *Agrobacterium*-mediated transformation. Hamilton et al. (124) first demonstrated the directed transfer of large DNA molecules from *Agrobacterium* to plants by the development of a binary BAC (BIBAC) system. These authors showed that a 150-kbp cloned insert of human DNA could be introduced into plant cells by using this system. However, the efficient transfer of such a large DNA segment required the overexpression of either *virG* or both *virG* and *virE*. VirE2 encodes a single-stranded DNA binding protein that protects the T-DNA from degradation in the plant cell (275). Because *virG* is a transcriptional activator of the *vir* operons (303), expression of additional copies of this regulatory *vir* gene was thought to enhance the expression of VirE2 and other Vir proteins involved in T-DNA transfer. Overexpression of *virE* formed part of the BIBAC system that was used to transform large (30- to 150-kbp) DNA fragments into tobacco and the more recalcitrant tomato and *Brassica* (56, 103, 123, 125). However, the transfer of different-size T-DNAs from various *Agrobacterium* strains had different requirements for overexpression of *virG* and *virE* (103). Liu et al. (203, 204) developed a transformation-competent artificial chromosome vector system based on a P1 origin of replication and used this system to generate libraries of large (40- to 120-kbp) *Arabidopsis* and wheat DNA molecules. This system did not require overexpression of *virG* or *virE* to effect the accurate transfer of large fragments to *Arabidopsis*.

What DNA Is Transferred from *Agrobacterium* to Plants?

T-DNA was initially defined as the portion (the T-region) of the Ti plasmid that was transferred from *Agrobacterium* to plant cells to form crown gall tumors. T-DNA border repeat sequences defined the T-region (366), and regions of the Ti plasmid outside these borders were not initially found in tumor cells (43). However, the transfer of Ti-plasmid sequences outside the conventional T-region may at first have been missed because of a lack of known selectable (e.g., tumorigenesis) or screenable (e.g., opine production) markers. Ooms et al. (241) observed the incorporation into plant DNA of regions of the Ti plasmid later shown to be outside the classical T-DNA borders. Ramanathan and Veluthambi (264) also showed that a *nos-nptII* cassette, placed outside the T-DNA left border, could be transferred to and confer kanamycin resistance on infected tobacco cells.

The use of relatively small T-DNA binary vectors made it easier for scientists to evaluate the transfer of "non-T-DNA" regions to plants. Martineau et al. (211) first reported the transfer of binary vector backbone sequences into transgenic plant DNA and questioned the definition of T-DNA. Wenck et al. (356) found that the entire binary vector, including backbone sequences as well as T-DNA sequences, could frequently be transferred to *Nicotiana plumbaginifolia* and *Arabidopsis thaliana* cells. Kononov et al. (182) carefully examined the structure of binary vector backbone sequences that could be found in up to 75% of transgenic tobacco plants and concluded that such transfer could result from either skipping the left T-DNA border when T-DNA was processed from the binary vector or initiation of T-DNA transfer from the left border to bring vector backbone sequences into plant cells. Considering the previous observation by Durrenberger et al. (91) that VirD2 protein could covalently attach to the 5′ end of the non-T-DNA strand, Kononov et al. suggested that transfer of vector backbone sequences to plants was a natural consequence of the mechanism of VirD2 function. Thus, the definition of T-DNA and vector backbone constitutes a semantic argument. It would thus appear that the transfer of non-T-DNA sequences to plants may be an unavoidable, but frequently unobserved and untested, result of transformation. Indeed, Frary and Hamilton (103) observed incorporation of BIBAC plasmid sequences into 9 to 38% of tested tomato transformants.

Although the transfer of plasmid backbone sequences may be an unavoidable consequence of the mechanism of *Agrobacterium*-mediated transformation, it may be possible to select against transgenic plants containing this unwanted DNA. Hanson et al. (132) showed that the incorporation of a toxic "killer" gene into the binary vector backbone sequences could severely reduce the percentage of transgenic plants containing such extra sequences. Remarkably, the transformation frequency of tobacco, tomato, and grape plants infected using this modified binary vector did not substantially differ from that of plants infected using a binary vector lacking the killer gene. Because the presence of uncharacterized DNA in transgenic plants is important for regulatory concerns, such an approach may be useful in the future for the production of plants (especially

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difficult to transform species) with a more highly defined transgenic composition.

Transfer of Multiple T-DNAs into the Same Plant Cell, and Generation of "Marker-Free" Transgenic Plants

Because of concerns regarding the spread of antibiotic resistance genes in nature or the escape of herbicide resistance genes to wild weedy species, scientists have developed several methods to generate marker-free transgenic plants. These plants would initially be selected for resistance to an antibiotic or herbicide, but the selection marker would be removed on subsequent manipulation and plant growth. Several methods have been proposed to eliminate the selection marker from the primary transformant. These include use of a site-specific recombination system, such as Cre-*lox* or Flp-Frt (2, 19, 57, 209, 235, 347, 348) to remove the marker, transposon-based movement of the selection marker from the initial site of insertion from the plant genome entirely or to another unlinked site from which it can be segregated in subsequent generations (93), or the use of multiple T-DNAs which can insert into unlinked sites for future segregation (reviewed in references 142 and 372). Each of these systems has advantages and disadvantages. For example, excision of marker genes using a site-specific recombination system requires introduction of the site-specific recombinase into plants, either by transformation or by genetic crossing. Segregation of markers can occur only in progeny following the generation of the initial transgenic plant and is limited to species naturally propagated through seed and not those propagated vegetatively.

Early research that characterized the integration pattern of T-DNAs in crown gall tumors indicated that each of the two T-DNAs encoded by an octopine-type Ti plasmid could independently integrate into the plant genome, sometimes in multiple copies (43, 63, 314). The molecular analyses suggested that these T-DNAs could be integrated into unlinked sites. These results suggested that cotransformation could be performed to integrate transgenes carried by two different T-DNAs and that perhaps these T-DNAs would segregate in subsequent generations. Three approaches were subsequently used for cotransformation: (i) the introduction of two T-DNAs, each from a different bacterium; (ii) the introduction of two T-DNAs carried by different replicons within the same bacterium; and (iii) the introduction of two T-DNAs located on the same replicon within a bacterium.

Early experiments using these various approaches indicated that cotransformation could be a frequent event. An et al. (9) showed that tobacco cells could be cotransformed to two different phenotypes by a single *Agrobacterium* strain containing both a Ti plasmid (phytohormone-independent growth) and a T-DNA binary vector (kanamycin-resistant growth). This experiment represents a "one-strain, two-replicon" approach to cotransformation. When the cells were first selected for kanamycin resistance, 10 to 20% of them also displayed phytohormone-independent growth; when the cells were first selected for phytohormone-independent growth, 60% of the resulting calli were also kanamycin resistant. The authors credited these differing frequencies to the higher copy number (5 to 10) of the binary vector in the bacterium relative to the single copy Ti plasmid.

These experiments were extended by de Frammond et al. (69), who showed that fertile transgenic plants could be regenerated from cloned tobacco tissue that was cotransformed by T-DNA from a Ti plasmid and from a micro-Ti (the one-strain, two-replicon approach). The two T-DNAs segregated in progeny plants, indicating that the T-DNAs had integrated into genetically separable loci. Other groups have used the one-strain, two-replicon approach to generate transgenic plants which initially expressed both T-DNA markers but could subsequently segregate the markers from each other (58).

Depicker et al. (80) performed a similar experiment in which the selection markers were phytohormone-independent growth and nopaline synthesis (encoded by a Ti plasmid) and kanamycin-resistant growth (encoded by a T-DNA binary vector). They performed the experiment in two ways: either the two T-DNAs were delivered by two different *Agrobacterium* strains (the two-strains, two-replicons approach), or both T-DNAs were delivered from a single replicon in one strain (the one-strain, one-replicon approach). The results of these experiments indicated that cotransfer of T-DNAs from the same plasmid in one strain was considerably more efficient than was transfer from two different strains. The use of a single *Agrobacterium* strain to cotransform plants with two T-DNAs from the same replicon, followed by segregation of the selection gene to generate marker-free transgenic plants, has been described by Komari et al. (178) and Xing et al. (365). In each of these studies, the authors were able to generate marker-free transgenic plants at high frequency.

The use of two *Agrobacterium* strains to deliver different T-DNAs to the same plant cells was studied by a number of groups (65, 66, 67, 76, 217). Although cotransfer of T-DNAs to genetically unlinked sites was reported, some authors also reported close linkage of the two different T-DNAs in many instances. It thus remains unclear which of the three cotransformation protocols will be reproducibly best for the generation of marker-free transgenic plants.

Virulence Gene Expression and Plant Transformation

The processing and transfer of T-DNA from *Agrobacterium* to plant cells is regulated by the activity of the *vir* genes. Virulence gene activity is induced by plant wound-induced phenolic compounds such as acetosyringone and related molecules (28, 74, 75, 92, 228, 293, 295, 298, 300). However, there may be instances in which scientists would like to induce *vir* genes to levels higher than that accomplished by plant extracts. Several groups have therefore identified *virA* and *virG* mutants that function constitutively, in the absence of phenolic inducers. Constitutive *virA* mutants were characterized by several groups (13, 218, 253). However, more emphasis has been placed on inducer-independent *virG* mutants, possibly because *virG* functions downstream of *virA*.

Extensive genetic studies resulted in the identification of a number of mutations that render the VirG protein active in the absence of phenolic inducing compounds (127, 254). These altered proteins contain mutations that converted either asparagine-54 to aspartic acid (*virGN54D*) or isoleucine-106 to leucine (*virGI106L*). Both of these mutant proteins stimulated a high level of *vir* gene expression, especially when expressed from a high-copy-number plasmid (118). When tested in tran-

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sient tobacco and maize transformation assays, strains containing the *virGN54D* mutant effected a higher level of transformation than did strains encoding the wild-type *virG* gene (130). An even greater effect was seen when the *virGN54D* allele was harbored on a high-copy-number plasmid; the presence of this mutant gene in *Agrobacterium* increased the transient transformation of rice and soybean two- to sevenfold (170).

Several laboratories have determined the effect of additional copies of wild-type *virG* genes on *vir* gene induction and plant transformation. Rogowsky et al. (273) showed that additional copies of nopaline-type *virG* resulted in increased *vir* gene expression. Liu et al. (200) showed that multiple copies of *virG* altered the pH response profile for *vir* gene induction. Normally, *vir* gene induction is very poor at neutral or alkaline pH or in rich medium; additional copies of *virG* permitted a substantial degree of induction in rich medium even at pH 8.5. Additional copies of *virG* also increased the transient transformation frequency of rice, celery, and carrot tissues (199).

Given these results in toto, one may conclude that increasing the copy number of *virA* or *virG* or decreasing the dependence of the encoded proteins on phenolic inducers would generally increase the transformation efficiency of the resulting strains. However, the situation is likely to be more complex. Belanger et al. (23) showed that individual *virA* genes may be particularly suited to function in certain genetic backgrounds, and Krishnamohan et al. (183) recently demonstrated that Ti plasmids may have evolved to optimize specific combinations of *virA, virG*, and *vir* boxes. As noted above, the Ti-plasmid pTiBo542 in the C58 chromosomal background is hypervirulent on certain legume species, possibly because of the associated *virG* gene (41, 146, 159), but not in its native Bo542 chromosomal background (143). Recent results from my laboratory indicate that *vir* gene induction and T-strand production by and transformation efficiency of particular *Agrobacterium* strains may not correlate well. *A. tumefaciens* A277, containing the Ti plasmid pTiBo542 within the C58 chromosomal background, is considerably more virulent than are strains A348 and A208, containing the Ti plasmids pTiA6 and pTiT37, respectively, in the same chromosomal background. However, *vir* gene induction by plant exudates and T-strand production are highest in *A. tumefaciens* A208 (L.-Y. Lee and S. B. Gelvin, unpublished data). These data further suggest that increased *vir* gene induction and T-strand production may not necessarily be reliable predictors of transformation efficiency.

T-DNA Integration and Transgene Expression

Plant transformation does not always result in efficient transgene expression. The literature is replete with examples of variable expression levels of transgenes, which frequently did not correlate with transgene copy number (see, for example, reference 255). This lack of correspondence was initially attributed to position effects, i.e., the position within the genome into which the T-DNA integrated was credited with the ability of transgenes to express. T-DNA could integrate near to or far from transcriptional activating elements or enhancers, resulting in the activation (or lack thereof) of T-DNA-carried transgenes (22, 35, 296, 308). T-DNA could also integrate into transcriptionally competent or transcriptionally silent regions of the plant genome. The high percentage (approximately 30%) of T-DNA integration events that resulted in activation of a promoterless reporter transgene positioned near a T-DNA border suggested that T-DNA may preferentially integrate into transcriptionally active regions of the genome. Only integration events that would link the promoterless transgene with an active promoter would result in reporter activity (180). However, a drawback to some of these experiments was that transgenic events may have been biased by the selection of antibiotic resistant plants expressing an antibiotic marker gene carried by the T-DNA. It is not clear whether T-DNA insertions into transcriptionally inert regions of the genome would have gone unnoticed because of lack of expression of the antibiotic resistance marker gene.

An obvious way to circumvent the presumed problems of position effect is to integrate T-DNA into known transcriptionally active regions of the plant genome. However, gene targeting in plants by homologous recombination has been at best extremely inefficient (72, 173, 223, 237, 238, 269, 270). An alternative system for gene targeting is the use of site-specific integration systems such as Cre-*lox*. However, single-copy transgenes introduced into a *lox* site in the same position of the plant genome also showed variable levels of expression in independent transformants. Transgene silencing in these instances may have resulted from transgene DNA methylation (61). Such methylation-associated silencing was reported earlier for naturally occurring T-DNA genes (135, 340). Thus, transcriptional silencing may result from integration of transgenes into regions of the plant genome susceptible to DNA methylation and may be a natural consequence of the process of plant transformation.

We now know not only that transgene silencing results from "transcriptional" mechanisms, usually associated with methylation of the transgene promoter (222), but also that transgene silencing is often "posttranscriptional"; i.e., the transgene is transcribed, but the resulting RNA is unstable (219). Such posttranscriptional gene silencing is frequently associated with multiple transgene copies within a cell. Transgenic plants generated by direct DNA transfer methods (e.g., polyethylene glycol- or liposome-mediated transformation, electroporation, or particle bombardment) often integrate a large number of copies of the transgene in tandem or inverted repeat arrays, in either multiple or single loci (176). Although *Agrobacterium*-mediated transformation usually results in a lower copy number of integrated transgenes, it is common to find tandem copies of a few T-DNAs integrated at a single locus (165). Transgene silencing can occur in plants harboring a single integrated T-DNA (95). However, integration of T-DNA repeats, especially "head-to-head' inverted repeats around the T-DNA right border, frequently results in transgene silencing (51, 164, 304). Thus, a procedure or *Agrobacterium* strain that could be used to generate transgenic plants with a single integrated T-DNA would be a boon to the agricultural biotechnology industry and to plant molecular biology in general. Greveldinge et al. (117) noted that transgenic *Arabidopsis* plants derived from a root transformation procedure tended to have fewer T-DNA insertions than did plants derived from leaf disks. However, it is not clear if this observation can be generally applicable to other plant species. Anecdotal information from several laboratories suggests that *Agrobacterium* strains

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that are less efficient in delivering T-DNA may be more efficient in producing single-copy T-DNA insertions. However, these findings need to be tested rigorously; it is possible that T-DNA copy number may also correlate with the growth state of the bacterium or the plants to be transformed.

Use of Matrix Attachment Regions To Ameliorate Transgene Silencing

At present, the generation of single-copy transgenic plants is still somewhat hit and miss. Scientists usually produce a relatively large number of independent transformants and screen them for plants containing a single-copy T-DNA insertion. At best, this can be a time-consuming nuisance. However, for agronomically important species, elite cultivars, or lines that are recalcitrant to transformation, it can become a rate-limiting step. An alternative to this approach may be to generate transgenic plants containing a few copies of T-DNA that are insulated from each other. One proposed mechanism to accomplish this is to flank transgenes within the T-DNA with matrix attachment regions (MARs). MARs are DNA sequences that either are associated with chromosome "matrices" as isolated or can associate with these matrices in vitro (121, 122, 294, 350). Among other properties, they have been ascribed the role of insulating genes within a looped chromatin domain from transcription-activating or -silencing effects of neighboring domains. In animal cells, such insulating effects may render transgene expression proportional to transgene copy number (306). However, some of the MARs initially used in animal experiments may also have contained enhancer elements, confounding the interpretation of the original experiments (29, 259).

When they flank transgenes delivered to plants via *Agrobacterium*-mediated transformation, MARs appear to have only a small effect on transgene expression (128, 198, 201, 225, 226, 227, 334). Larger increases in transgene expression have been observed using particle bombardment-mediated transformation (5, 6, 236). However, this increase is generally associated with expression of transgenes in plant cells rather than in whole plants (330, 333). It is possible that the higher transgene expression effects of MARs using particle bombardment reflects the higher integrated transgene copy number resulting from this technique as opposed to the relatively lower copy number of integrated T-DNAs delivered by *Agrobacterium* (7). As such, it is not clear whether MARs will be, on their own, highly useful for decreasing the silencing of transgenes delivered to plants by *Agrobacterium*-mediated transformation.

Use of Viral Suppressors of Gene Silencing To Increase Transgene Expression

Recent data from a number of laboratories indicates that some plant viruses, both DNA and RNA viruses, contain genes that suppress gene silencing (4, 11, 21, 31, 38, 55, 169, 210). Several investigators have speculated that viral antisilencing has evolved as a mechanism for viruses to evade a plant's defense through viral gene silencing (55, 266). Regardless of the reason for and mechanism of antisilencing, viral suppressors of silencing may be useful to mitigate the silencing of transgenes.

As indicated in some of the references cited above, viral suppressors of gene silencing can activate a previously silenced stable transgene. One would then wonder whether such silencing suppressors could prevent the silencing of transgenes stably introduced into plants by *Agrobacterium*-mediated transformation. Although this hypothesis has not yet been tested and possible negative consequences (such as increased viral susceptibility) may ensue from the stable incorporation of antisilencing genes into a plant genome, experiments in which viral silencing suppressors have been used to increase the levels of transient expression of *Agrobacterium*-introduced transgenes appear promising. O. Voinnet and colleagues (unpublished data) have recently demonstrated that when cotransformed with various transgenes encoding green fluorescent protein, the potato virus Y Nia protein, or tomato Cf-9 and Cf-4 disease resistance proteins, various viral silencing suppressors dramatically increased the expression of these other proteins. Expression levels up to 50-fold higher than those achieved in control transformations (lacking the viral silencing suppressor genes) were obtained. Several different viral silencing suppressors, including the p25 protein of PVX, the P1-HcPro protein of tobacco etch virus, and the p19 protein of tomato bushy stunt virus, were able to enhance transient transgene expression from both the cauliflower mosaic virus 35S promoter and from native transgene promoters. Of these, the p19 protein was most effective in both increasing transient transgene expression and decreasing the levels of small (21- to 25-bp) RNA molecules associated with posttranscriptional gene silencing. The authors concluded that viral suppressors of gene silencing could be useful for the production of large amounts of proteins in plants.

When Transgene Expression Is Not Forever

Experiments to express transgenes in plants initially used elements, such as the cauliflower mosaic virus 35S and 19S promoters or opine synthase promoters, that would express the transgene in a relatively constitutive manner (24, 133, 179, 196, 234, 280, 343). However, as plant genetic engineering experiments became more refined, scientists turned to regulated promoters that would express a transgene in a particular developmental, environmental, or tissue-specific pattern. Systems were also developed that would allow scientists to induce transgene expression at will, allowing for the overexpression of a particular product or expression of a product that may be toxic during certain stages of plant development. Such inducible systems included those regulated by tetracycline (108), alcohol (279), copper (221), heat shock (284), and steroid hormones (14, 282) (see reference 158 for a recent review of chemically inducible gene induction systems). Many of these systems were leaky, permitting the expression of transgenes under noninduced conditions.

There may be instances, however, when one would not want a transgene or its product to be present after the initial few hours or days following transformation. Such traits include those that would aid in the transformation process itself or would effect plant DNA rearrangements desired only during the initial transformation event (e.g., gene targeting using site-specific recombinase systems). Two strategies are currently being developed to permit only transient expression of gene

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products in plants. These are the use of "nonintegrating" T-DNA systems and the transfer of proteins, rather than DNA molecules, from *Agrobacterium* to plant cells.

Nonintegrating T-DNA systems include the use of mutant *Agrobacterium* strains and/or plant cells that are proficient in T-DNA nuclear transfer but deficient in T-DNA integration. During a search for domains of VirD2 necessary for nuclear targeting of the T-DNA, Shurvinton et al. (290) defined a C-terminal domain, termed ω, that showed high amino acid sequence homology among *virD2* genes. Although this domain was not required for either VirD2 endonuclease activity or nuclear targeting of the T-DNA, replacement of four conserved amino acids by two serine residues resulted in a mutant protein that rendered the encoding *Agrobacterium* strain highly attenuated in virulence. Narasimhulu et al. (233) and Mysore et al. (229) further showed that *Agrobacterium* strains harboring this VirD2 ω substitution were highly deficient in stable transformation (with 2% of the efficiency of wild-type strains) but were still able to transform plant cells transiently at 20% of the efficiency of wild-type strains. Thus, this mutation rendered *Agrobacterium* strains highly deficient in T-DNA integration but still relatively proficient in delivering T-DNA to the plant nucleus. This mutant VirD2 protein can therefore be used to target T-strands to the nucleus, where they can transiently express but not efficiently integrate.

Nam et al. (231) used a root assay to screen almost 40 *A. thaliana* ecotypes for their ability to be transformed by *Agrobacterium.* Among these ecotypes, UE-1 was easily transiently transformed but poorly stably transformed. Genetic and molecular characterization of this ecotype indicated that the block in transformation occurred at the T-DNA integration step. Nam et al. (232) further identified a large number of *Arabidopsis* T-DNA insertion mutants that were resistant to *Agrobacterium* transformation (*rat* mutants). Of the initial 21 mutants, 5 were efficiently transiently transformed but were highly recalcitrant to stable transformation, a phenotype associated with a deficiency in T-DNA integration. Mysore et al. (230) characterized one of these mutants, the *rat5* mutant, in greater detail. This mutant was generated by the insertion of T-DNA into the 3′ untranslated region of a histone H2A gene (*HTA1*). Biochemical and molecular data indicated that this mutant could be transiently transformed efficiently but that T-DNA integration was disrupted. Although the precise role of the *HTA1* gene in T-DNA integration has yet to be elucidated, root transformation of this mutant (and perhaps other T-DNA integration-deficient mutants) can be used for the transient delivery of T-DNA without efficient subsequent T-DNA integration. The use of the *HTA1* gene to improve the transformation efficiency of wild-type plants is discussed below.

Vergunst et al. (349) recently described a novel procedure to transfer proteins directly from *Agrobacterium* to plant cells. This system relies on the ability of the type IV protein secretion system encoded by the *Agrobacterium virB* and *virD4* genes to transfer certain Vir proteins to plant cells. VirD2, VirE2, and VirF are the three proteins identified to date that can be transferred by this system. These authors showed that translational fusions of the Cre recombinase protein to the N terminus of either VirE2 or VirF could be transferred to plant cells and effect recombination at *lox* sites. They further showed that the C-terminal 37 amino acids of VirF were sufficient to transfer the fusion protein. These experiments lead to the possibility of using Vir proteins as carriers to introduce other proteins transiently into plant cells.

MANIPULATION OF PLANT GENES TO IMPROVE TRANSFORMATION

Plant Response to *Agrobacterium* Infection

Although great advances have been made over the past decade to increase the number of plant species that can be transformed and regenerated using *Agrobacterium*, many important species or inbred lines remain highly recalcitrant to *Agrobacterium*-mediated transformation. The question has often arisen, "Who has the problem with transformation, *Agrobacterium* or the researcher?" The very wide host range of *Agrobacterium*, including gymnosperms and perhaps lower plant phyla, a variety of fungi, and even animal cells, suggests that T-DNA transfer to the recipient (i.e., entry exclusion) may not be the problem. That *Agrobacterium* can transiently transform a number of these species efficiently, including agronomically important species such as maize and soybean (170, 271, 288), suggests that in many instances T-DNA integration may remain the limiting step. Alteration of the tissue culture conditions, for example by the use of antioxidants during the transformation of grape, rice, maize, and soybean, has increased the probability of stably transforming cell types that can be regenerated (96, 102, 239, 240, 258). However, such manipulations of the transformation conditions may have limitations.

Agrobacterium infection of plant tissues may in some instances result in plant tissue necrosis. Several groups have described a slow, spreading necrosis in grape infected by particular *Agrobacterium* strains (79, 263). More recently, Hansen (129) described an apoptotic response of maize to *Agrobacterium* infection. The response included both rapid tissue necrosis and cleavage of nuclear DNA into oligonucleosome-sized fragments by endogenous nucleases and is characteristic of a caspase-protease cascade. The expression of two baculovirus cell death suppressor genes, *p35* and *iap*, greatly inhibited both tissue necrosis and endogenous DNA cleavage. Manipulation of these genes during the *Agrobacterium*-mediated transformation process may thus be useful to increase both plant cell viability and transformation efficiency in plant species with an apoptotic response to *Agrobacterium*.

Several groups have recently begun to identify plant genes and protein products involved in the transformation process. One of the rationales for these experiments is the hope that identification of these genes may eventually result in their manipulation either to improve transformation or to make plants resistant to crown gall disease. A number of approaches have been employed to identify these plant genes, including (i) use of yeast two-hybrid systems to identify plant proteins that may interact with virulence proteins, (ii) direct "forward genetic" screening to identify plant mutants that cannot be transformed, (iii) "reverse genetic" screening to test whether particular genes of interest may be involved in transformation, and (iv) various genomics approaches to identify plant genes that may be induced or repressed soon after infection by *Agrobacterium.*

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Identification of Plant Genes Encoding Proteins That Interact with *Agrobacterium* Virulence Proteins

Several *Agrobacterium* virulence proteins would be expected to interact with plant proteins. These include the processed form of VirB2, the major component of the T-pilus that is required for transformation; VirD2, the protein that caps the 5′ end of the transferred T-strand; VirE2, the single-stranded DNA binding protein that presumably coats the T-strand; and VirF, which is transferred to plant cells but whose function remains unknown. Several other Vir proteins that are on the bacterial cell surface, such as VirB5 and VirB7 (minor components of the T-pilus), and VirB1* (a processed product of VirB1 that can be found in the extracellular medium), may also interact with proteins on the surface of plant cells.

Early work (16) utilized VirD2 as the bait protein in a yeast two-hybrid system to identify an *A. thaliana* importin-α (AtKAP, now known as importin-α1) as an interacting partner. Importin-α proteins are involved in the nuclear translocation of many proteins harboring NLS sequences, and *Arabidopsis* encodes at least nine of these proteins (Bhattacharjee and Gelvin, unpublished). Ballas and Citovsky (16) showed that interaction of VirD2 with importin-α AtKAP was NLS dependent both in yeast and in vitro. The importance of importin-α proteins in the *Agrobacterium* transformation process has recently been suggested by demonstrating that a T-DNA insertion into the importin-α7 gene, or antisense inhibition of expression of the importin-α1 (AtKAP) gene, results in a highly attenuated transformation phenotype (Bhattacharjee and Gelvin, unpublished).

VirD2 also interacts with at least two other plant proteins by using a yeast two-hybrid system. Deng et al. (78) identified three VirD2- and two VirE2-interacting proteins. They characterized more fully one of the VirD2 interactors, an *Arabidopsis* cyclophilin. This protein, as a glutathione *S*-transferase fusion, interacted strongly with VirD2 in vitro. The authors further showed that the interaction domain of VirD2 was in the central portion of the protein, a region to which no previous function had been ascribed. Cyclosporin A, an inhibitor of cyclophilins, inhibited *Agrobacterium*-mediated transformation of *Arabidopsis* roots and tobacco suspension cell cultures. The authors suggested that this plant protein may serve as a chaperone to help in T-complex trafficking within the plant cell. Experiments in my laboratory identified a tomato type 2C protein phosphatase as an interacting partner with VirD2. This phosphatase may be involved in the phosphorylation and dephosphorylation of a serine residue near the C-terminal NLS motif in VirD2. Overexpression of this phosphatase in transfected tobacco BY-2 cells resulted in decreased nuclear targeting of a GUS-VirD2-NLS fusion protein, suggesting that phosphorylation of the VirD2 NLS region may be involved in nuclear targeting of the VirD2/T-strand complex (Y. Tao, P. Rao, and S. B. Gelvin, submitted for publication).

Using VirE2 as the bait protein in a yeast two-hybrid system, Tzfira et al. (329) identified two interacting proteins from *Arabidopsis*, VIP1 and VIP2. VIP1 may be involved in nuclear targeting of the T-complex because antisense inhibition of VIP1 expression resulted in a deficiency in nuclear targeting of VirE2. Tobacco VIP1 antisense plants were also highly recalcitrant to *Agrobacterium* infection. Recent results further suggest a use for VIP1 in improving plant transformation: transgenic plants that overexpress VIP1 are hypersusceptible to *Agrobacterium* transformation (327, 330). VirE2 also interacts in yeast with several of the *Arabidopsis* importin-α proteins, suggesting that VirD2 and VirE2 may have a common mechanism of nuclear import (Bhattacharjee and Gelvin, unpublished).

Schrammeijer et al. (285) recently identified an *Arabidopsis* Skp1 protein as an interactor with the F-box domain of VirF. Skp1 proteins may be involved in targeting proteins such as cyclins to the 26S proteosome, suggesting that VirF may function in setting the plant cell cycle to effect better transformation. The interaction of VirF with VIP1, but not VirE2, may also suggest a mechanism for Vir protein turnover: if VirF is targeted to the proteosome, it may help target other Vir proteins for proteolysis.

The T-pilus is essential for *Agrobacterium*-mediated transformation. Mutations in various VirB proteins disrupt transformation but not T-DNA processing (301, 344). As mentioned above, the major T-pilus component is the processed and cyclized VirB2 protein (189), although other virulence proteins, including VirB5 and possibly VirB7, also are minor T-pilus constituents (278, 283). Although the precise role of the T-pilus remains controversial, it is expected that the T-pilus would interact with the plant cell wall or membrane. Experiments in my laboratory have begun to address possible plant-interacting partners with T-pilus components. Using a yeast two-hybrid system and the processed, but not cyclized, form of VirB2 as a bait protein, we have identified two classes of *Arabidopsis* proteins that strongly and specifically interact with this major T-pilus constituent (H.-H. Hwang and S. B. Gelvin, unpublished data). One of these classes of plant proteins is encoded by a three-member gene family. Although the identity of these three related proteins is not currently known, their hydropathy profiles suggest that they contain membrane-spanning domains. The other interacting protein is a Rab-type GTPase. Each of these four plant proteins interacts in yeast with itself and with each other but not with other tested virulence proteins, including VirB1, VirB1*, VirB5, VirD2, VirE2, and VirF. In vivo data indicate that each of these proteins is involved in *Agrobacterium*-mediated transformation. Antisense or RNAi inhibition of expression of the genes encoding these proteins results in a transformation-deficient phenotype. In addition, an *Arabidopsis* mutant line containing a T-DNA insertion into the promoter region of one of the "unknown protein" genes also is highly recalcitrant to *Agrobacterium*-mediated transformation.

Forward Genetic Screening To Identify Plant Genes Involved in *Agrobacterium*-Mediated Transformation

Scientists have shown a genetic basis for susceptibility to crown gall disease in some plant species (15, 214, 231, 246, 272, 292, 312). In an effort to identify plant genes involved in *Agrobacterium*-mediated transformation, my laboratory embarked on a major project to identify *Arabidopsis* T-DNA insertion mutants that are resistant to *Agrobacterium* transformation (*rat* mutants [232]). These studies have resulted in the identification of more than 70 such mutants to date. The roles of many of the mutated genes in the transformation process

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have been revealed by various assays. Thus, *rat1* (encoding an arabinogalactan protein) and *rat3* (probably encoding a plant cell wall protein) are involved in bacterial attachment to roots (23). Other *rat* genes that may affect cell wall structure include a xylan synthase (*rat4* [232]) and a β-expansin (A. Kaiser, A. Kopecki, Y. Zhu, and S. B. Gelvin, unpublished data). Because bacterial attachment to the roots of the *rat4* mutant appears nearly normal (A. Matthysse, unpublished data), *RAT4* may be involved in T-DNA transfer to the cytoplasm.

Using this forward-genetics approach, we have identified a number of other *rat* mutants in later stages of the transformation process. T-DNA insertions into genes encoding α- and β-importins are probably blocked in the T-DNA nuclear targeting process (S. Bhattacharjee, H. Cao, J. Humara, Y. Zhu, and S. B. Gelvin, unpublished data). Other mutants, including the *rat5* (a histone H2A mutant [230, 232]), *rat17*, *rat18*, *rat20*, and *rat22* mutants, are probably involved in T-DNA integration (232). A T-DNA insertion between two closely spaced replacement histone H3 genes (histone *H3-4* and *H3-5*) also results in the rat phenotype (J. Li, Y. Zhu, and S. B. Gelvin, unpublished data).

The finding that the histone *H2A-1* gene affects T-DNA integration has led to a more extensive characterization of this gene. The *Arabidopsis* histone H2A gene family includes 13 members. We have initiated a study of the expression pattern of each of these genes and an examination of the role that each of these genes may play in *Agrobacterium*-mediated transformation (370; H. Yi, T. Fujinuma, and S. B. Gelvin, unpublished data). The histone *H2A-1* gene, encoded by *RAT5*, is expressed in numerous cell types, including cells that are not undergoing rapid division. This expression pattern is characteristic of a "replacement" histone gene. In roots, the gene is expressed in lateral root primordia, the meristem region, and the elongation zone. Interestingly, the root elongation zone is the region most highly susceptible to *Agrobacterium*-mediated transformation (370). Other experiments indicate that histone *H2A-1* gene expression and susceptibility to *Agrobacterium*-mediated transformation are highly correlated. Thus, expression of this gene may be predictive of cell types that are most sensitive to transformation. Knowledge of plant cell transformation competency may be important for the genetic engineering of recalcitrant plant species and cultivars.

Because mutation of the histone *H2A-1* gene resulted in decreased *Arabidopsis* root transformation, we examined whether overexpression of this gene would increase the efficiency of *Agrobacterium*-mediated transformation. Transgenic *Arabidopsis* plants containing additional genomic (230) or cDNA (H. Yi and S. B. Gelvin, unpublished data) *H2A-1* copies are two- to sixfold more transformation competent than are plants containing the normal histone *H2A-1* gene complement. In addition, transient expression of the histone *H2A-1* gene from an incoming T-DNA both complements the *rat5* mutant (230) and increases the transformation efficiency of normally susceptible and highly recalcitrant *Arabidopsis* ecotypes (L.-Y. Lee and S. B. Gelvin, unpublished data). Finally, overexpression of the *RAT5* histone *H2A-1* gene in various *rat* mutants (other than the *rat5* mutant) also restores transformation competency (L.-Y. Lee, S. Davis, X. Sui, and S. B. Gelvin, unpublished data). Expression of the *RAT5* gene is therefore epistatic over the rat phenotype of other *rat* mutants and thus may sensitize plant cells to *Agrobacterium*-mediated transformation. We suggest that overexpression of the *RAT5* histone *H2A-1* gene may improve the transformation efficiency of recalcitrant plants.

Reverse Genetic Screening for Plant Genes Involved in *Agrobacterium*-Mediated Transformation

Plant genes encoding several proteins that interact with virulence proteins have been identified using a yeast two-hybrid system. Such interactions are at best suggestive of a role for these genes in plant transformation. Their roles must be shown directly. One way to accomplish this is to inhibit gene expression in planta using techniques such as antisense RNA, RNAi, or mutagenesis. I have discussed above the use of antisense RNA and RNAi to show that VIP1 (a VirE2 interactor), a Rab GTPase, and several proteins of unidentified function (VirB2 interactors) are involved in *Agrobacterium*-mediated transformation. Suppression of expression of these genes may be one method to generate plants resistant to crown gall disease.

Another method to test the role of a particular gene in transformation would be to mutate that gene and then assay the plant for transformation susceptibility. However, at present site-directed mutagenesis is not an efficient method for use in plants. An alternative reverse genetic approach is to identify mutant plants containing transposon or T-DNA insertions in genes of interest. Several PCR-based strategies have been described to identify such knockout mutations in *Arabidopsis* (98, 104, 184), tomato (52), and rice (157). Using one such strategy, my laboratory has identified *Arabidopsis* mutant lines containing disruptions in various importin-α and importin-β genes (putatively involved in nuclear transport of the T-complex) and various genes involved in plant chromatin structure (putatively involved in T-DNA integration into the plant genome). Some of these mutants are either moderately or highly resistant to *Agrobacterium*-mediated transformation (S. Bhattacharjee, H. Cao, H. Humara, A. Kaiser, A. Kopecki, J. Li, X. Zhao, and S. B. Gelvin, unpublished data), and contain T-DNA insertions in or near genes encoding importin-α7 or importin-β3, various histones (including histones H2A1, H2A3, H2B5, H2B6, H3-4, H3-5, and H4-1), histone acetyltransferases (including HAC4, HAC6, HAC9, HAC10, and HAC11), and a histone deacetylase (HDA1). We have not yet, however, established the precise roles of these plant genes in the *Agrobacterium*-mediated transformation process.

Escobar et al. (97) have recently described a novel reverse genetic strategy to produce crown gall-resistant plants. They generated transgenic *Arabidopsis* and tomato plants expressing double-stranded RNA constructions targeted to T-DNA-encoded auxin and cytokinin biosynthetic oncogenes. These genes are highly homologous among a large variety of different *Agrobacterium* strains. Many transgenic plants expressing these RNAi constructions were highly resistant to crown gall disease directed by a broad range of oncogenic strains, although they were not generally resistant to *Agrobacterium*-mediated transformation per se. A similar approach has been used to generate crown gall disease-resistant tobacco and apple plants (W. Ream, unpublished data).

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Genomics Approaches To Identify Plant Genes That Respond to *Agrobacterium* Infection

As described above (129), plants may respond to infection by *Agrobacterium*, and this response may involve differential plant gene expression. Genes that are induced or repressed during the early stages of *Agrobacterium*-mediated transformation may provide targets for manipulation of the host to improve the efficiency of transformation of recalcitrant plant species. Several laboratories have consequently begun investigations to identify these differentially expressed plant genes.

Ditt et al. (83) recently investigated the response of *Ageratum conyzoides* suspension cell cultures to infection by a non-tumorigenic supervirulent *A. tumefaciens* strain (233). Using cDNA-amplification fragment length polymorphism (AFLP) to amplify 16,000 fragments, they identified 251 bands that were differentially regulated 48 h after infection. Reverse transcription-PCR analysis of some of these genes confirmed the results of the cDNA-AFLP analysis. Some of these bands were also induced or repressed 24 h after inoculation. Whereas most of the bands investigated (encoding, e.g., an RNase, a putative recpetor kinase, a peroxidase, and a pathogenesis-related protein) were also differentially regulated following incubation of plant cells with *E. coli*, four genes, including one encoding a nodulin-like protein, responded specifically to *Agrobacterium* infection. The authors speculated that this nodulin gene may respond to signals from the bacterium to regulate plant cell division or differentiation.

Our laboratory has conducted a similar study, using tobacco BY-2 suspension cell cultures inoculated with five different non-tumorigenic *Agrobacterium* strains (Veena, H. Jiang, R. W. Doerge, and S. B. Gelvin, submitted for publication). One strain could transfer T-DNA but not VirE2 protein, one could transfer virulence proteins but not T-DNA, one could transfer neither, and two could transfer both. Using suppressive subtractive hybridization followed by DNA and RNA macroarray analyses of RNA samples from eight different time points following inoculation (from 0 to 36 h), we identified more than 400 genes that were differentially regulated after various periods of infection. Most of these genes showed a general differential response to *Agrobacterium* inoculation; however, some genes responded specifically to a T-DNA and Vir protein transfer-competent strain. A few genes responded specifically to T-DNA or Vir protein transfer only. Among the genes that were induced (or whose expression was maintained at a high level) were those encoding histones and ribosomal proteins. The activity of several plant defense and stress response genes was repressed by *Agrobacterium* infection. Because of the importance of histones in the T-DNA integration process (230), we propose that *Agrobacterium* infection induces the expression of plant genes necessary for transformation while simultaneously repressing the host defense response. Further analysis of these differentially expressed genes will indicate whether they play a direct or indirect role in *Agrobacterium*-mediated plant transformation.

PROSPECTS

In less than 20 years, the use of *Agrobacterium* to genetically transform plants has advanced from a dream to a reality. Modern agricultural biotechnology is heavily dependent on using *Agrobacterium* to create transgenic plants, and it is difficult to think of an area of plant science research that has not benefited from this technology. However, there remain many challenges. Many economically important plant species, or elite varieties of particular species, remain highly recalcitrant to *Agrobacterium*-mediated transformation, and the day has not yet arrived when flowers will be the only things seen coming from the barrels of gene guns. However, I feel that such a day is not too far in the distant future. I also feel that *Agrobacterium* evolved millions of years ago to genetically transform a very wide range of organisms; it is now up to the scientist to harness the natural ability of this bacterium. In addition to extending the host range and transformation efficiency of plants by *Agrobacterium*, some of the remaining challenges to the scientific biotechnology community are summarized below.

(i) The first is the use of *Agrobacterium* for homologous or site-directed recombination. Many scientists consider homologous recombination to be one of the remaining "holy grails" of plant molecular biology. The ability to perform gene replacement experiments has become a staple of bacterial, fungal, and even animal cell and molecular biology research. However, homologous recombination in plants generally occurs at 10^{-5} the frequency of illegitimate recombination (71, 173, 223, 237, 238, 269, 270). We need an *Agrobacterium*-mediated transformation system that delivers T-DNA to the plant nucleus efficiently, but is deficient in random T-DNA integration.

(ii) The second involves stable and predictable transgene expression in plants. Too often, the level of transgene expression in plants is highly variable. Often, lines of transgenic plants that are "good expressers" lose this characteristic after several generations of growth under field conditions. We need to understand the roles of position effects, chromatin effects, and T-DNA integration patterns in transcriptional and post-transcriptional gene silencing in order to develop strategies to enhance the extent and stability of transgene expression.

(iii) The third is manipulation of the *Agrobacterium* genome. The availability of the complete *A. tumefaciens* C58 genomic sequence (114, 363) presents us with an unparalleled opportunity to investigate *Agrobacterium* gene expression patterns and the ways in which they may be altered during cocultivation of the bacterium with various plant species. Such information may provide clues to methods to further manipulate *Agrobacterium* in order to effect higher levels of transformation of recalcitrant plant species.

(iv) The fourth is plastid genetic transformation by *Agrobacterium.* Although a few scattered references to chloroplast transformation by *Agrobacterium* exist in the literature (see, e.g., references 64 and 346), these reports have not been confirmed by the scientific community. The existence of NLS sequences in VirD2 and VirE2 proteins may ensure T-DNA targeting to the nucleus. Even if these NLS sequences could be removed without altering other essential functions of these proteins, the recent finding that the plant actin cytoskeleton is involved in *Agrobacterium*-mediated transformation (P. Rao and S. B. Gelvin, unpublished data) may preclude redirection of the T-DNA from the nucleus to plastids.

(v) The fifth is genetic transformation of animal and plant pathogenic fungi. Many medically or agronomically important pathogenic fungi remain highly recalcitrant to genetic trans-

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formation. Recent reports of *Agrobacterium*-mediated transformation of several filamentous fungal species (1, 71) suggest that *Agrobacterium* may be a useful "gene-jockeying tool" for more than just plant species.

(vi) The final challenge involves genetic transformation of human and animal cells. The recent report of *Agrobacterium*-mediated genetic transformation of human cells (187) suggests the exciting possibility of using *Agrobacterium*, or *Agrobacterium*-like processes, for human and animal gene therapy.

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