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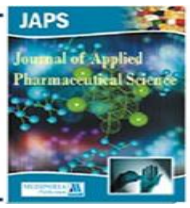

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Agrobacterium mediated biotransformation

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ABSTRACT

Twenty-five years ago, the concept of using *Agrobacterium tumefaciens* (soil gm-ve bacterium) as a vector to create transgenic plants (natural transformation) was viewed as a prospect and a "wish." 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. Genetically engineered corn, cotton and other crop plants have been produced whose genome contains a delta-endotoxin-coding region regulated by sequences. Hence the engineered plants produce the delta-endotoxin protein in their tissues, making them lethal when ingested by insects such as the europian corn borer, (that currently causes crop losses of field corn, popcorn, seedcorn and sweetcorn). From this some people feared that windborne pollen could dust many other plants and potentially harm beneficial insects such as butterflies and bees. *Agrobacterium* mediated transformed plants have economical and medicinal valuable products.

Key words: Agrobacterium, transgenic plants, biotransformation, DNA.

INTRODUCTION

Plants provide human beings with all manner of useful products: food and animal feed, fibers and structural materials, and small molecules that can be used as dyes, scents, and medicines. People have sought to improve plants by breeding and selecting the better-performing and most useful varieties. The one limitation of this approach is that breeders are restricted to the existing gene pool in each species or sexually compatible group of species. 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 (Schroder et al., 1989). Twenty-five years ago, the concept of using *Agrobacterium tumefaciens* (soil gm-ve bacterium) as a vector to create transgenic plants (natural transformation) 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 (Oramas, et al. 1998).

A. tumefaciens involvement in Crown gall disease was viewed by Smith & Townsend (1907). The stable transmission through the germ line was first demonstrated in 1981, when transgenic tobacco plants were generated by transformation using *A. tumefacians*.

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 (Rout, 2001). 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 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. 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 and gymnosperms. In addition, *Agrobacterium* can transform fungi, including yeasts, ascomycetes, and basidiomycetes. Recently, *Agrobacterium* was reported to transfer DNA to human cells (Pelletier, 2000).

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. Several virulence (*vir*) loci on the Ti plasmid, including *virC* and *virF*, 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. Other *vir* genes, including *virG*, contribute to the "hypervirulence" of particular strains (Huffman, 1992).

MOLECULAR BASIS OF *AGROBACTERIUM*–MEDIATED TRANSFORMATION 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*. Ti plasmids are on the order of 200 to 800 kbp in size. The transferred DNA (T-DNA) or Ri plasmid. T-regions on native Ti and Ri plasmids are approximately 10 to 30 kbp in size. 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. 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 (Thomashaw, 1988).

T-regions are defined by T-DNA border sequences. These borders are 25 bp in length and highly homologous in sequence. They flank the T-region in a directly repeated orientation. In general, the T-DNA borders delimit the T-DNA, 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. 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), 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 and in vitro. In vitro, however, VirD2 protein alone can cleave a single-stranded T-DNA border sequence. 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.

However, double-strand cleavage of the T-DNA border has also been noted. Nicking of the border is associated with the tight (probably covalent) linkage of the VirD2 protein, through tyrosine 29, to the 5' end of the resulting single stranded T-DNA molecule termed the T-strand. It is ssT-strand, and not a double-stranded T-DNA molecule, that is transferred to the plant cell. 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), it may be possible to process T-strands from these regions of Ti and Ri plasmids and from T-DNA binary vectors. 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. Early reports suggested that the VirC1 protein binds to the overdrive sequence and may enhance T-DNA border cleavage by the VirD1/VirD2 endonuclease. *VirC1* and *virC2* functions are important for virulence; mutation of these genes results in loss of virulence (Pickardt, 2001 & Stewart, 2003).

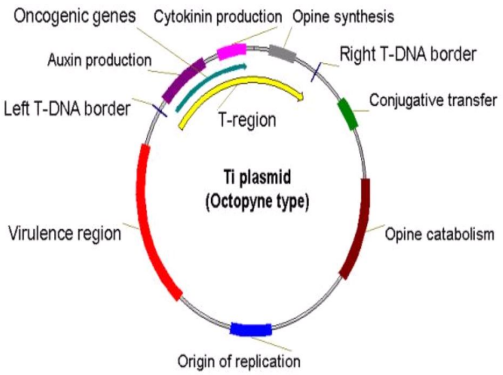

Fig 1: Structure of Ti plasmid.

T-DNA TRANSFER FROM *AGROBACTERIUM* TO PLANT CELLS

As indicated above, many proteins encoded by *vir* genes play essential roles in the *Agrobacterium*-mediated transformation process. The role of Vir proteins is that they 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 (Sangwan, 2000).

VirA is a periplasmic antenna that senses the presence of particular plant phenolic compounds that are induced on wounding. 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. 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 (Fry, 2000). 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 transformation efficiency or host range (Wang, 1997).

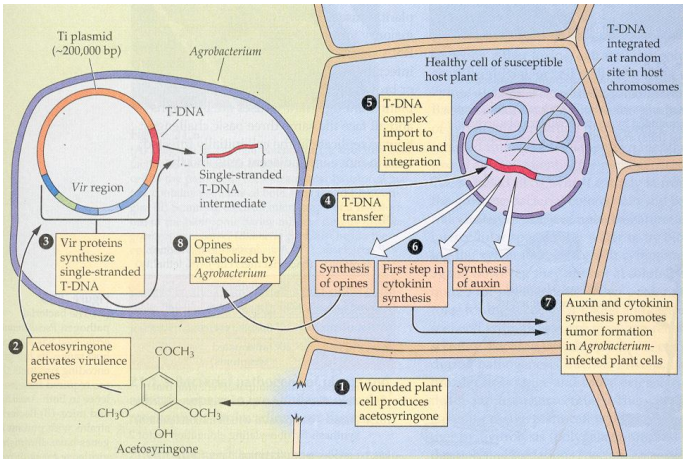

Fig 2: Mechanism of *Agrobacterium* interaction with plant cells.

Together with the VirD4 protein, the 11 VirB proteins make up a secretion system necessary for transfer of the T-DNA and several other Vir proteins, including VirE2 and VirF. VirD4 may serve as a "linker" to promote the interaction of the processed T-DNA/VirD2 complex with the VirB-encoded secretion apparatus. 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. VirB2, which is processed and cyclized, is the major pilin protein. 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 affect 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, the pilus of some, but not all, *Agrobacterium* strains is most stable at lower temperatures (approximately 18 to 20°C). Early experiments indicated a temperature effect on transformation (Fry, 2003). Thus, one may consider cocultivating *Agrobacterium* with plant cells at lower temperatures during the initial few days of the transformation process (Demski, 1996).

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. Whether this complex assembles within the bacterium remains controversial. 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, 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. 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 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. Furthermore, VirD2 can associate with a number of *Arabidopsis* importin- proteins in an NLS-dependent manner, both in yeast and in vitro (Opabode, 2002 & Hinata, 1996). Importin 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. showed that in permeabilized cells,

VirD2 could affect 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 or the "precision" of T-DNA integration (Jelaska, 2000).

The role of VirE2 in T-DNA nuclear transport also remains controversial. VirE2 is a non-sequence-specific single-stranded DNA binding protein. In *Agrobacterium* cells, VirE2 probably interacts with the VirE1 molecular chaperone and may therefore not be available to bind T-strands. 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. 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. As with VirD2, VirE2 interacts in yeast with *Arabidopsis* importin-_ proteins in an NLS-dependent manner. One report indicates that VirE2 bound to single-stranded DNA and microinjected into plant cells can direct the DNA to the nucleus. 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 affect DNA uptake. 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. 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*. 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. 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. Further controversy involves the ability of VirE2 protein to localize to the nuclei of animal cells. It was 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 . 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 (Sim, 1998 & Okkels, 1998).

Finally, VirE2 may protect T-strands from nucleolytic degradation that can occur both in the plant cytoplasm and perhaps in the nucleus. 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. However, such a complex has not yet been identified in either *Agrobacterium* or plant cells. It is possible that other proteins, such as importins, VIP1, and even VirF, 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. 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*) and other roles in bacterial attachment to plant cells (*att* genes), sugar transporters involved in coinduction of *vir* genes (*chvE*), regulation of *vir* gene induction (*chvD*), and T-DNA transport (*acvB*). Other genes, such as *miaA* may also play a more minor role in the transformation process. The recent elucidation of the entire *A. tumefaciens* C58 sequence will surely provide fertile ground for the discovery of additional genes involved in *Agrobacterium*-mediated transformation (Daggard, 2003 & Klee, 2000).

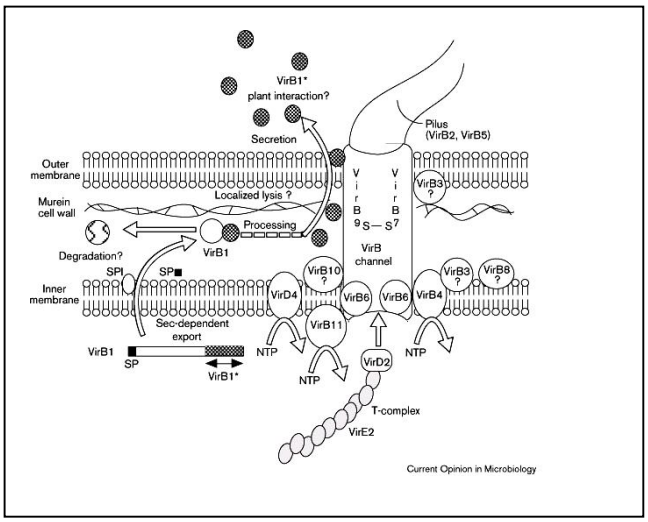

Fig 3: Mechanism of interaction with pilus in plant cell wall and bacterium.

MANIPULATION OF *AGROBACTERIUM* FOR GENETIC ENGINEERING PURPOSES

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. 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. The T-region and the *vir* 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 (Kumashiro T, 1996 & Suzuki M, 2000). A number of *Agrobacterium* strains containing non-oncogenic *vir* helper plasmids have been developed, including LBA4404, GV3101 MP90, AGL0, EHA101 and its derivative strain EHA105, and NT1 (pKPSF2). 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 (Loerz H, 2006). 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 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, and 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 (Filipowcz W, 1996).

Amount of DNA that 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. 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. 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. 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. Because *virG* is a transcriptional activator of the *vir* operons, 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*. However, the transfer of different-size T-DNAs from various *Agrobacterium* strains had different requirements for overexpression of *virG* and *virE*. Liu et al. 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 affect the accurate transfer of large fragments to *Arabidopsis* (Towsend, 1907 & Rathore, 2001).

T-DNA Integration and Transgene Expression

Plant transformation does not always result in efficient transgene expression. The variable expression levels of transgenes, which frequently did not correlate with transgene copy number due to 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 of T-DNA-carried transgenes . 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 (Wang, 2000 & Wakasa, 2001).

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. 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. Such methylation-associated silencing was reported earlier for naturally occurring T-DNA genes. 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, but also that transgene silencing is often "posttranscriptional"; i.e., the transgene is transcribed, but the resulting RNA is unstable. Such posttranscriptional gene silencing is frequently associated with multiple transgene copies within a cell. 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. Transgene silencing can occur in plants harboring a single integrated T-DNA. However, integration of T-DNA repeats, especially head-to-head' inverted repeats around the T-DNA right border, frequently results in transgene silencing. 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 (Waterhouse, 2001 & Brettel, 1997).

CONCLUSION

Transgenic plants for example, the bacterium *Bacillus thuringiensis* produces a variety of insecticidal protein called delta-endotoxins that form crystals in the bacterial spores. These proteins are toxic to insects (moths, butterflies, beetles). When the bacterial spores are injested by an insect, the crystals dissolve and are activated by proteases in the gut, where they bind to specific receptors and create leakage channels that kill the insect. Genetically ingineered corn, cotton and other crop plants have been produced whose genome contains a delta-endotoxin-coding region regulated by sequences. Hence the engineered plants produce the delta-endotoxin protein in their tissues, making them lethal when ingested by insects such as the europian corn borer, (that currently causes crop losses of field corn, popcorn, seedcorn and sweetcorn). From this some people feared that windborne pollen could dust many other plants and potentially harm beneficial insects such as butterflies and bees. *Agrobacterium* mediated transformed plants have economical and medicinal valuable products.

FUTURE 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. The day is not too far in the distant future, 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 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. 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 posttranscriptional 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 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 affect 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, 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 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-formation. Recent reports of *Agrobacterium*-mediated transformation of several filamentous fungal species 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 suggests the exciting possibility of using *Agrobacterium*, or *Agrobacterium*-like processes, for human and animal gene therapy.

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