The current status of plant transformation technologies

K. Veluthambi*,§, Aditya K. Gupta* and Arun Sharma†

*Department of Plant Biotechnology, School of Biotechnology, Madurai Kamaraj University, Madurai 625 021, India
†Department of Plant Molecular Biology, University of Delhi-South Campus, Benito Juarez Road, New Delhi 110 021, India

Plant transformation vectors and methodologies have been improved to increase the efficiency of plant transformation and to achieve stable expression of transgenes in plants. Due to the simplicity of the transformation system and precise integration of transgenes, *Agrobacterium* Ti plasmid-based vectors continue to offer the best system for plant transformation. Binary vectors have been improved by the incorporation of supervirulent *vir* genes, matrix attachment regions (MAR) and the insertion of introns in marker genes and reporter genes. With these improvements and with the use of acetosyringone, transformation of monocotyledonous plants using *Agrobacterium* has almost become a routine process. The green fluorescent protein (GFP) gene has been extensively modified for plant codon preference, ER/plastid targeting and for greater solubility thereby making it a versatile vital reporter for transgenic plants. Significant progress is seen in developing transgenic plants devoid of antibiotic marker genes. Cotransformation of multiple T-DNAs, site-specific recombination strategies and deployment of Ac/Ds-based transposition have helped in the elimination of marker genes in transgenic plants. Positive selection strategies using *ipt*, xylose isomerase and phospho-mannose isomerase have been demonstrated to be useful in many crop plants. The development of BIBAC vectors, a demonstrated capability to transfer multiple genes of a pathway and successful T-DNA tagging in rice, signal the readiness with which transformation technologies can be deployed for the study of 'functional genomics' in plants. The particle bombardment system continues to find use in organelle transformation and transformation of plants that lack efficient regeneration systems. A detailed understanding of gene silencing has led to the design of vectors that minimize transgene silencing while ensuring desired levels of transgene expression. Efforts are underway to understand the mechanism of T-DNA integration in plants so that 'knock out' mutagenesis and homology-based gene replacements can be achieved in plants. We review in this article the current status of transformation technologies. An overview of the status of deployment of plant transformation technologies in India is also presented.

§For correspondence. (e-mail: veluthambi@mrna.tn.nic.in)

Plant transformation is performed using a wide range of tools such as *Agrobacterium* Ti plasmid vectors, microprojectile bombardment, microinjection, chemical (PEG) treatment of protoplasts and electroporation of protoplasts. Though all methods have advantages that are unique to each of them, transformation using *Agrobacterium* and microprojectile bombardment are currently the most extensively used methods[1]. Recent developments in these two technologies have been reviewed together with the phenomenon of 'gene silencing' that has come to centrestage after a large number of transgenic plants have been carefully evaluated for transgene expression in successive generations.

Agrobacterium-mediated gene transfer

The naturally evolved unique ability of *Agrobacterium tumefaciens* to precisely transfer defined DNA sequences to plant cells has been very effectively utilized in the design of a range of Ti plasmid-based vectors. The current status of our understanding of *Agrobacterium* T-DNA transfer process has been reviewed by Gelvin[2] and Zupan *et al.*[3]. Three genetic elements, *Agrobacterium* chromosomal virulence genes (*chv*), T-DNA delimited by a right border and a left border and Ti plasmid virulence genes (*vir*) constitute the T-DNA transfer machinery. Important events of T-DNA transfer and components involved in the process are outlined in Figure 1. The mechanisms governing the transfer of 'T-complex' via the conjugation channel and the roles of plant and *Agrobacterium* proteins in T-DNA integration are being intensely studied.

Agrobacterium-based DNA transfer system offers many unique advantages in plant transformation: (1) The simplicity of *Agrobacterium* gene transfer makes it a 'poor man's vector. (2) A precise transfer and integration of DNA sequences with defined ends. (3) A linked transfer of genes of interest along with the transformation marker. (4) The higher frequency of stable transformation with many single copy insertions. (5) Reasonably low incidence of transgene silencing. (6) The ability to transfer long stretches of T-DNA (> 150 kb).

For long, the inability of *Agrobacterium* to transfer DNA to monocotyledonous plants was considered its major limitation. However, with effective modifications

in Ti plasmid vectors and finer modifications of transformation conditions, a number of monocotyledonous plants including rice, wheat, maize, sorghum and barley have now been transformed. In fact, *Agrobacterium* T-DNA transfer is now viewed as 'universal' based on successful transformation of yeast[4], *Aspergillus*[5] and human cells[6].

Ti plasmid-based vector system

Agrobacterium vir helper strains

A typical binary vector system comprising an octopine-type *vir* helper strain such as LBA4404 (ref. 7) that harbours the disarmed Ach5 Ti plasmid and a binary vector such as pBin19 (ref. 8) is very commonly used for plant transformation. The available range of *vir* helper strains has been expanded with the nopaline-type MP90 (ref. 9) and the L, L-succinamopine-type EHA101 (ref. 10). The bacterial kanamycin resistance gene in EHA101 was deleted to develop the *vir* helper strain EHA105 (ref. 11). EHA101 and EHA105, by virtue of harbouring the 'supervirulent' *vir* genes, exhibit broader host-range and higher transformation efficiency. Many recalcitrant plants such as rice[12], wheat[13] and barley[14] have been transformed using EHA101 and EHA105. A new *vir* helper strain pTiChry5 has been constructed from an *Agrobacterium*

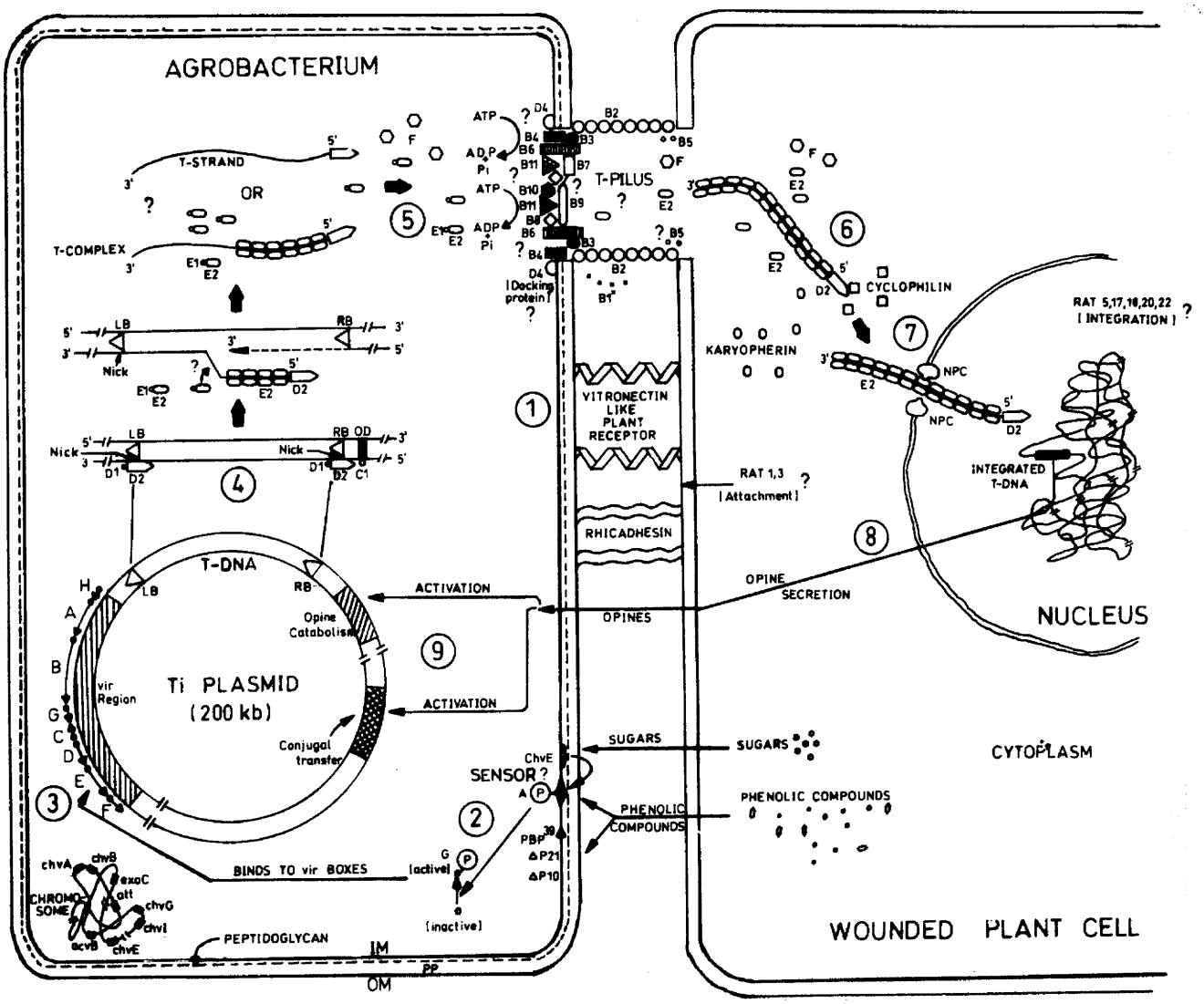

Figure 1. A schematic diagram depicting the cascade of events leading to T-DNA transfer from *Agrobacterium* to the plant genome. Steps 1 through 9 indicate sequential processes that occur during infection of a plant cell with *Agrobacterium*. Step 1, binding of *Agrobacterium* to the host cell surface receptors; step 2, direct or indirect mode of recognition of plant signal molecules by the bacterial Vir A/VirG two-component sensor-transducer system; step 3, induction of bacterial *vir* genes situated on the Ti plasmid; step 4, generation of the T-strand; step 5, formation of the T-complex and its transport into the host plant cell through the T-pilus; step 6, recognition and interaction of T-complex with host cell cytoplasmic proteins; step 7, nuclear transport of T-complex; step 8, T-DNA integration and opine secretion; and step 9, activation of bacterial opine catabolism genes and Ti plasmid conjugal transfer genes. *acvB*, *att*, *chvA*, *chvB*, *chvE*, *chvG*, *chvI* and *exoC* are the chromosome-encoded virulence genes. '?' Indicates that precise steps are yet to be established. P10, P21 and PBP are chromosome-encoded putative phenol-binding proteins. OM, outer membrane; PP, periplasm; IM, inner membrane. Broken lines denote peptidoglycan cell wall.

strain virulent on soybean[15]. This strain has good potential for legume transformation.

Binary vectors

Beginning from the binary vector pBIN19 constructed by Bevan[8] in 1984, many modifications have been made in these vectors to expand the range of their utility and to improve their transformation efficiency. A significant improvement is the construction of the superbinary vector pTOK233 (ref. 16) by cloning the *vir*B, *vir*G and *vir*C genes of pTiBo542 in pGA472. This led to the successful transformation of *japonica* rice. Subsequently, transformation of *indica* rice[17–19], *javanica* rice[20], maize[21], *Sorghum*[22] and *Allium cepa*[23] were performed using the same strategy. A long-held notion that monocots are not amenable to *Agrobacterium*-mediated transformation has been proved wrong. Recently, pTOK233 was used to transform a pulse, mungbean[24].

Many finer improvements have been made in binary vectors. pBIN19 has been completely sequenced[25]. Its improved version, pBIN20, with many additional single restriction sites in the MCS was reported recently[26]. A new series of pPZP vectors have been developed which are small in size and stable in *Agrobacterium*[27]. The pPZP vector backbone was used to construct the pCAMBIA series of vectors with *npt*II, *hpt* or *bar* as selection markers and *gus* or *gfp* as reporters. The pCAMBIA vectors are very widely used for rice transformation. Plant expression vectors of pRT100 series constructed by Topfer *et al.*[28] permit construction of gene cassettes with CaMV 35S promoter and its polyA signal. These cassettes can be excised and placed in the MCS of binary vectors.

Availability of single restriction sites, particularly in the expression cassette with the gene of interest, is a difficulty frequently encountered. Gleave[29] constructed a set of plasmids, pART7 and pART27, to address this problem. The shuttle plasmid pART7 has a MCS placed between CaMV 35S promoter and *ocs* polyA signal. The expression cassette is flanked on either side by *Not*I sites (a rare 8 bp recognition site). The coding sequence of a gene of interest is cloned in the MCS of pART7. The expression cassette is excised by using *Not*I and cloned in the *Not*I site of the binary vector, pART27. pART27 was designed so as to have the *npt*II gene close to the left border to ensure that the selection marker is the last to be transferred into plants. This strategy ensures that all plants selected on kanamycin will have a complete T-DNA inclusive of the gene of interest.

Agrobacterium Ti plasmid vectors have important applications in functional genomics. Random integration of T-DNA in the plant genome causes mutations and simultaneously tags the gene into which insertion occurred. Many *Arabidopsis* genes have been cloned by T-DNA tagging[30]. A similar effort in rice resulted in the generation of 22,090 transgenic plants[31]. Phenotypic analysis of these mutants is expected to lead to the discovery of many new gene functions.

Binary vectors that can transfer T-DNAs that are several hundred kbs are required for genome studies. Hamilton *et al.*[32] constructed binary bacterial artificial chromosome vectors (BIBAC) using which a T-DNA consisting of 150 kb human DNA was precisely transferred and integrated into tobacco genome. These vectors are expected to play a pivotal role in positional cloning of plant genes and in functional genomics.

Engineering of plants with new pathways may require the transfer of multiple genes. Placing multiple genes in one T-DNA is technically difficult due to loss of single restriction sites in successive cloning steps. This limitation was overcome by Ye *et al.*[33] in engineering provitamin A biosynthesis in rice. They placed daffodil *phytoene synthase* gene and *Erwinia phytoene desaturase* gene in one T-DNA. The second T-DNA carried daffodil *lycopene-b-cyclase* and *E. coli hpt* (hygromycin resistance gene). Interestingly, 12 out of 60 plants selected on hygromycin (*hpt* is present in only one T-DNA) carried the genes of the second T-DNA as well. Therefore, 20% of hygromycin-resistant plants carried all the three genes of *b*-carotene biosynthetic pathway. Thus, it is possible to transform multiple genes of a pathway by cotransformation of multiple T-DNAs. Though particle bombardment enables cotransformation, *Agrobacterium* offers the additional advantage of precise integration of complete lengths of multiple T-DNAs.

Matrix attachment regions (MARs) are AT-rich DNA sequences that facilitate the attachment of DNA to the nuclear matrix or inner nuclear membrane thereby producing a series of loops. Two interesting functions are attributed to MARs: (1) Genomic compartmentalization of coexpressed genes in individual loops and (2) Form the boundaries of transcriptionally active DNA loop domains, thereby blocking the inhibitory influence of neighbouring sequences. MARs were recognized to have potential applications in transgenic plants. A transgene flanked on either side by MAR sequences may form an independently expressing loop domain thereby minimizing position effects. Allen *et al.*[34] constructed *gus* gene flanked by tobacco MAR sequences and transformed cell suspension cultures by particle bombardment. The expression of integrated *gus* gene increased by 140-fold. Cheng *et al.*[35] constructed *gus* and *gfp* genes with the tobacco Rb7 MAR sequences and generated transgenic rice plants by particle bombardment. The presence of MAR sequences increased the expression of reporter genes in the range of 3.3-fold to 650-fold. The reduction in reporter gene expression, generally attributed to flanking sequences at the sites of integration, was also minimized.

Improvements in marker genes and reporter genes

Leaky expression of plant selection marker genes such as *npt*II and *hpt* (e.g. under CaMV35S or *nos* promoters) in

Agrobacterium contributes to bacterial overgrowth during selection and to the emergence of false-positive plants. In a strategy to eliminate leaky expression of marker genes in *Agrobacterium*, Maas *et al.*[36] placed the intron 2 of the potato ST-1 gene in the N-terminal part of the *npt*II gene. Lack of mRNA splicing in *Agrobacterium* completely eliminated leakiness in kanamycin selection. In another study, the castor bean catalase 1 intron was placed in the *hpt* gene to overcome leakiness in hygromycin selection[37]. The binary vectors with intron-*hpt* significantly improved rice transformation efficiency by improving *hpt* expression and by limiting *Agrobacterium* overgrowth.

Early detection of transformation events is invariably based on the histochemical analysis of expression of *gus* as a reporter gene. A major limitation of this approach is the expression of this reporter gene in *Agrobacterium* despite the use of plant promoters. This problem was also overcome by inserting introns, which are processed in plants but not in *Agrobacterium*. Vancanneyt *et al.*[38] placed the intron 2 of potato ST-LS1 gene in the coding sequence of *gus* gene. In another study, Ohta *et al.*[39] placed the castor bean catalase gene intron within the N-terminal part of the coding sequence of *gus*. In both cases, GUS activity was limited only to transformed tissues and was not detected in *Agrobacterium*.

Green fluorescent protein gene (*gfp*) from jellyfish finds immense applications in transgenic plants. GFP, unlike GUS, is visualized in living cells. Another reporter gene, luciferase (*luc*), requires an externally added substrate for detection. However, GFP fluorescence occurs with uv/blue light and oxygen[40] without any externally added substrate. The first series of transformation experiments brought to light the following limitations of the application of wild type *gfp* gene in plants: (1) Codon preference was different between jellyfish and plants. (2) Presence of a cryptic intron in *gfp* was recognised and spliced in plants. (3) Low solubility of GFP in cytoplasm. (4) Interference of cytoplasm-accumulated GFP in the regeneration of transgenic shoots. Synthetic versions of *gfp* (e.g. modified *gfp*, *mgfp*) were made[41–43] to avoid the cryptic intron and to change the codon preference to that of flowering plant genes. These changes dramatically increased the intensity of fluorescence in transgenic *Arabidopsis* and tobacco plants and full length GFP was translated. Elevated expression of GFP unfortunately interfered with the regeneration of transgenic shoots. However, fusion of a signal peptide-encoding sequence to *mgfp*4 and consequent targeting of mgfp4–ER protein to ER led to routine regeneration of highly fluorescent shoots[42].

Since reduced solubility of GFP in cytoplasm was viewed as an important reason for reduced fluorescence, the synthetic version, *mgfp*4 was subjected to triple site-directed mutagenesis (F99S, M153T, V164A) to generate a soluble-modified *gfp* (*sm-gfp*)[44]. Expression of *sm-gfp*4 in *Arabidopsis* gave brighter fluorescence compared to *mgfp*4, possibly due to increased solubility of *sm-gfp*. Normally, GFP is excited at 396 nm or 475 nm and emits green fluorescence maximally at 508 nm. Davis and Vierstra[44] carried out two site-directed mutagenesis experiments on *sm-gfp* to generate spectral variants. A soluble-modified red-shifted version of GFP (smRS-GFP) and a soluble-modified blue fluorescent version of GFP (sm-BFP) were generated for use in experiments involving gene expression.

A plastid-targeted version of synthetic GFP (*sgfp*) was constructed with the rbcSSU promoter and transit peptide (rbc-Tp-*sgfp*). Transgenic rice plants generated using *Agrobacterium* with rbcS-Tp-*sgfp* exhibited 20-times higher fluorescence compared to a version without plastid targeting[45]. As discussed earlier for GUS, confirmation of transformation soon after cocultivation requires *gus* that does not exhibit leaky expression in *Agrobacterium*. Two groups[41,46] placed intron-2 of potato ST-LS1 gene in the synthetic *gfp* versions. In the latter report, the intron was placed in *mgfp*4-ER, sm-*gfp*4, smRS-GFP and sm-BFP genes. Intron splicing occurred efficiently in tobacco and in maize cells.

Modifications in cocultivation conditions

Cocultivation of explants with *Agrobacterium* in the presence of acetosyringone, a *vir* gene inducer, has become a routine exercise in the transformation of recalcitrant crops such as rice[16], maize[21], barley[14] and wheat[13]. Aldemita and Hodges[17] reported that preinduction of *Agrobacterium* with 400 μM acetosyringone prior to cocultivation is important in rice transformation. Finer changes such as supplementation of buffers to cocultivation medium[47] and preincubation of explants that increase *vir* gene induction[48] increase transformation efficiency of *Agrobacterium*.

Many physical conditions influence transformation efficiency. Uniform wounding of tobacco leaves and sunflower apical meristem by particle bombardment[49], vacuum infiltration of *Arabidopsis* seedlings[50], rapid proliferation of rice calli at 30°C[51] and cocultivation at a lower (22°C) temperature[52] led to an increase in the efficiency of *Agrobacterium*-mediated transformation.

'In planta' transformation using *Agrobacterium*

The small size of *Arabidopsis* plant and improved efficiency of transformation by vacuum infiltration led to the development of '*in planta*' transformation by floral dips. This method obviates plant tissue culture and eliminates somaclonal variations[53]. In a series of subsequent studies[54,55], it was found that ovules (female gametophytes) of immature flowers were more frequently the targets of floral dip transformation of *Arabidopsis.*

'*In planta*' transformation has recently been demonstrated in *Medicago truncatula*, an emerging model legume plant[56]. Infiltration of flowering plants was superior to infiltration of young seedlings. Binary vectors containing *bar* gene were used for transformation by vacuum infiltration and the T_1 progeny were screened by 'BASTA' spray. This is a potentially useful approach in plants like pulses where regeneration protocols are either not available or regenerating parts of plants are not accessible to *Agrobacterium*-mediated transformation.

Marker-free transgenic plants

Antibiotic resistance markers (e.g. *npt*II, *hpt*) or herbicide resistance markers (e.g. *bar*) are essential for selectively propagating transformed cells and tissues. However, subsequent maintenance of markers in transgenic plants is unnecessary. Elimination of markers is advocated since the antibiotic resistance genes may be transferred to pathogenic bacteria or the herbicide resistance genes may be transferred to weeds. Besides the above concerns, removal of marker genes offers the following research advantages. It enables (i) successive rounds of transformation so that useful transgenes can be stacked without crossing, (ii) retention of promoters along with selection markers which will lead to the presence of multiple copies of a promoter, thereby activating signals for transcriptional gene silencing. Two excellent reviews on marker elimination have been published recently[57,58].

Marker elimination by site-specific recombination

Site-specific recombination systems have been successfully used for the elimination of selection markers. The first example involved the P1 bacteriophage recombinase (*Cre*) and its recognition site (*lox*P). Dale and Ow[59] cloned the luciferase (*luc*) gene and *lox*P/*hpt*/*lox*P cassette in a binary vector and generated transgenic tobacco plants. In the next step, the transgenic plant with *luc*/*lox*P/*hpt*/*lox*P was retransformed with a binary vector with *cre*/*npt*II. In 10 of the 11 kanamycin-resistant plants obtained, excision of *hpt* (by the action of Cre on *lox*P sites) had occurred. There was a good chance that *luc* of the first T-DNA and *cre*/*npt*II of the second T-DNA were present in unlinked loci. Selfing of the T_0 transgenic plants was done and T_1 plants were analysed. In one case, 17 of 104 T_1 plants had luciferase (*luc*) activity but lacked kanamycin resistance. Thus, in a two-step exercise the marker genes (*hpt* and *npt*II) and the recombinase (*cre*) were removed. Instead of retransformation, crossing of *luc*/*lox*P/*hpt*/*lox*P plants with *cre*/*npt*II plants also yielded similar results.

The recombination system of the 2 μm plasmid of *Saccharomyces cereviseae* involving FLP recombinase and FRT recombination site has been successfully deployed for marker elimination in maize cells[60]. A combination of lambda attachment site (*att*P) and negative selection using *tms*2 and napthaleneacetamide (NAM) were used for marker elimination by intrachromosomal recombination[61].

The maize Ac/Ds system was tested for marker elimination in tomato by Goldsbrough *et al*.[62]. A T-DNA with Ds-*gus*-Ds/Ac/*npt*II was used for transformation. Transposition events in T_0 plants would place *gus* and *npt*II genes in unlinked loci thereby segregating them in T_1 plants. As expected, *gus* gene got segregated from *npt*II in 2.3% to 6.6% of T_1 plants. A combination of Ac functions and *ipt* (isopentenyl transferase) gene were used in a multi-auto-transformation vector (MAT) in which elimination of *ipt* (marker gene) could be achieved without selfing and segregation[63].

A single-step *ipt*-MAT vector incorporating the recombinase (R) and recombination sites (RS) of *Zygosaccharomyces rouxii* was reported for rice[58]. The T-DNA constructed by them carried *nptII*/*gus*/*hpt*/RS/*ipt*/35S-R/*gfp*/RS sequences. *Agrobacterium*-mediated transformation of scutellum-derived callus was performed. Selection of transformants was based on the ability to form normal shoots on cytokinin-minus medium. In 25% of the infected calli, regenerated normal shoots carried the *gus* gene without *ipt*. They showed that marker (*ipt*)-free rice plants could be generated in a very short span of one month.

Elimination of marker genes by cotransformation

Cotransformation of two independent T-DNAs, one with selection marker and the other with the gene of interest, offers a simple approach for marker elimination. If the two T-DNAs integrate at different loci in the T_0 plant, they will get segregated in the T_1 progeny leading to the generation of marker-free plants.

Based on the logical idea that conditions which support high efficiency of transformation may lead to a high frequency of cotransformation, Komari *et al*.[64] placed two separate T-DNAs in superbinary vectors that harboured supervirulent *vir*B, *vir*G and *vir*C genes. One T-DNA contained a drug resistance marker (*npt*II for tobacco and *hpt* for rice) and the second T-DNA carried the *gus* gene. In more than 47% of the drug-selected tobacco and rice plants, cotransformation of *gus* was detected. The non-selected *gus* gene segregated from *npt*II or *hpt* genes in T_1 progenies of more than 25% of drug-selected plants. This simple two-step strategy is a convenient method to generate marker-free transgenic plants.

An alternate strategy of cotransformation of two T-DNAs placed on two separate, compatible binary plasmids in one *Agrobacterium* was developed by Daley *et al*.[65]. The selected T-DNA with *npt*II was placed in a

RK2 replicon and the non-selected *gus* gene was placed in a pRiHR1 replicon. Cotransformation in T_0 plants and segregation of *npt*II and *gus* in the T_1 plants occurred at a high frequency. McCormac *et al.*[66] placed two T-DNAs of variable lengths on the binary plasmid. The *npt*II T-DNA was 7 kb long and the *hpt* T-DNA was 3.2 kb long. They found that cotransformation frequency of the non-selected T-DNA was higher when the marker on the longer T-DNA (*npt*II) was used for selection. The selected marker could be genetically separated (segregated) in the T_1 plants. It was concluded that the T-DNA with the selection marker should be twice longer than the non-selected T-DNA to achieve high cotransformation in T_0 plants and to eliminate the marker by segregation in the T_1 plants.

Positive selection of transgenic plants

While efforts were underway to eliminate undesirable markers from transgenic plants, scientists were making a parallel exploration of the use of positive selection markers that are considered environmentally safe. Unlike antibiotic and herbicide markers, which kill the untransformed cells, positive selection markers render metabolic/developmental advantage to transformed cells and promote faster proliferation and regeneration. The problem of untransformed dead tissues being detrimental to the growth of transformed tissues is also avoided. An early example of a positive selection agent is a glucuronide derivative of benzyladenine (BA). *b*-Glucuronidase, expressed in the transformed tissues, released BA and supported tissue proliferation and regeneration in a cytokinin-minus medium[67]. The transformation efficiency obtained via this positive selection procedure was twice than that obtained with kanamycin. Another strategy for positive selection was based on inability of plants to use xylose as carbon source unless it is converted to xylulose by xylose isomerase. *Thermoanaerobacterium thermosulfurogenes* xylose isomerase gene (*xyl*A) was successfully used as a positive selection marker in potato and tomato[68]. The transformation efficiency of positive selection using xylose was comparable to that obtained with kanamycin.

A positive selection method using *E. coli* phosphomannose isomerase gene (*pmi*) is now well established. Mannose is converted to mannose-6-phosphate by plant hexokinases. Plants lack phosphomannose isomerase. Mannose-6-phosphate that accumulates in cells, exerts severe growth inhibition by inhibiting phosphoglucoisomerase and by depleting phosphate required for ATP synthesis. Mannose is not directly toxic. Transformation of sugarbeet[69], maize[70], cassava[71] and rice[72] has been accomplished using mannose selection. Yeast 2-deoxyglucose-6-phosphate phosphatase conferring resistance for 2-deoxyglucose[73] and *Catharanthus roseus* tryptophan decarboxylase that converts the toxic tryptophan analogue 4-methyl-tryptophan to non-toxic 4-methyltryptamine[74] are other tested examples of positive transformation markers.

The problem of 'long transfer' of plasmid backbone

A unique advantage of *Agrobacterium* T-DNA transfer is the accurate processing of the T-DNA between the right and left borders and its precise transfer and integration to the plant genome. However, there are many reports that non T-DNA portions may also get transferred to the plant genome. T-DNA transfer initiates in low frequencies from the left borders as well[75–78]. At fairly high frequencies, T-DNA transfer starting from the right border skips the left border and the entire binary plasmid is transferred to the plant genome in a process termed as 'long transfer'[77–81]. In both these processes, the vector backbone with bacterial antibiotic resistance markers is transferred into the plant genome. This is a potential biohazard. Transgenic plants with such transformation events should be carefully identified and eliminated.

Hanson *et al.*[82] made a simple modification in the binary vector to address the problem of 'long transfer' and to enrich typical transformation events from right to left T-DNA borders. A CaMV35SP-*barnase*-INT cassette was placed to the left of the left T-DNA border as a non T-DNA lethal gene (NTL T-DNA binary vector). Any long transfer event beyond the left T-DNA border will lead to the expression of *barnase*. Such transformed cells will die and get eliminated. By deploying this strategy, Hanson *et al.*[82] could completely eliminate the chances of the transfer of binary vector backbone.

Biolistic gene transfer

Physical DNA delivery methods such as microinjection, PEG-mediated transformation of protoplasts, electroporation of protoplasts and tissues and microprojectile bombardment of tissues have proved useful for transforming plants. Of these methods, microprojectile bombardment is the most widely deployed method for genotype-independent plant transformation. This methodology was first used to deliver DNA and RNA into the epidermal cells of *Allium cepa*[83]. Since then, this technique has been used to transform yeast and filamentous fungi[84], algae[85], cereals[86] and pulses[87]. Chloroplasts of *Chlamydomonas*[88] and mitochondria of yeast and *Chlamydomonas* have also been biolistically transformed[89].

There are basically three systems for particle bombardment with various modifications. Electric discharge particle acceleration device ACCEL^{TM} utilizes an instrument to accelerate DNA-coated gold particles with any desired velocity by varying the input voltage. The biolistic PDS 1000 He device is powered by a burst of helium gas that accelerates the macrocarrier upon which, DNA-coated

gold particles (microprojectiles) are uniformly bombarded over target cells. The advantages of particle bombardment system are: (1) Plants which are not infected by *Agrobacterium* can be transformed. (2) DNA may be transferred without using specialized vectors. (3) The introduction of multiple DNA fragments/plasmids can be accomplished by co-bombardment, thus eliminating the necessity of constructing a single large plasmid containing multiple transforming sequences[90] (4) False positives resulting from reporter gene expression in *Agrobacterium* are avoided. (5) Transformation protocols are applicable to plants which lack good regeneration systems. (6) Organelle transformation is achieved only by particle bombardment.

Though the biolistic gene delivery system has been successfully used to create transgenics, certain drawbacks of the technique have been observed, for example, high copy number and rearrangements of transgene(s), thus causing gene silencing or genomic rearrangements. However, many recent improvements have been made to overcome problems relating to gene silencing[91] and genetic integrity[92]. The overall transformation efficiency of biolistics has been improved by osmotic conditioning of cells[93].

Gene silencing in transgenic plants

One of the common problems faced by those involved in making transgenic plants with increased level of endogenous gene expression by the use of heterologous strong promoters or a foreign gene of some utility, is silencing of transgenes in a vast population of transgenic plants[94–97]. This silencing at transcription level is referred to as transcriptional gene silencing (TGS); whereas silencing at post-transcriptional level is referred to as post-transcriptional gene silencing (PTGS). TGS involves inhibition of transcription and association with methylation of promoter region. In cases of PTGS, though the genes are transcribed, their mRNA is degraded. PTGS is associated with methylation of the coding region of the transgenes.

TGS has been found to be associated with multiple copies of transgenes and inverted repeats (IRs) in transgenes, which can act in *cis* as well as in *trans*[98,99], suggesting a homology-dependent mechanism of gene silencing. It is possible that plants have also evolved TGS as a mechanism to safeguard themselves from the activities of endogenous transposable elements, which are present in multiple copies. However, PTGS has been observed even when unrelated foreign genes have been transferred to plants. PTGS has been associated with high copy number of transgenes, strength of the promoter or the stability of the transcripts. It seems that there is a threshold level for accumulation of transcripts and above that PTGS sets in. Viruses are known to infect plants and accumulate their transcripts at very high level. PTGS may be a mechanism to stop transcription of viral genes. Recent studies indicate that some plants use PTGS to recover from viral infection and to stay free from future infections[100]. As organisms compete with each other for their survival, some plant viruses have evolved mechanisms to counter or suppress PTGS[101].

Though DNA methylation is involved in both TGS and PTGS, whether this is the cause or the effect of gene silencing is not very clear. However, attempts have been made to answer this question. Though common sites of methylation are CG and CNG symmetric sequences, some methylation at asymmetric sites has also been observed[102]. In an attempt to determine the involvement of DNA methylation in gene silencing, a mutant form of 35S promoter that lacked all symmetrical methylation acceptor sites was synthesized. This promoter was used to drive the *bar* gene. When resistant lines obtained were crossed with plants carrying the 271-silencer locus (which had the ability of silencing other 35S promoters) a significant reduction in the number of phosphinothricin (PPT) resistant progeny was observed. The silenced lines were found to be methylated at only asymmetric methylation acceptor sites. When the 271-silencer locus was outcrossed, PPT resistance was restored[103]. Other studies on similar lines, but using wild type 35S promoter concluded that even after outcrossing of the silencer locus the silenced state was maintained. These studies suggest that for initiation of silencing, methylation at symmetric methylation sites is not required. However this is essential for maintenance of silencing through generations. Mutation in *MET* gene, which encodes a DNA methyltransferase, causes drastic reduction of DNA methylation and results in the release of silencing at most loci; however some loci still remain silenced[104]. This could be because of the presence of other functional methyltransferases. Interestingly the mutation in *DDM1* locus which encodes a plant homologue of SW12/SNF2 protein, a component of yeast chromatin remodelling complex, also results in loss of methylation and release of silencing[104]. This study suggests that normal chromatin structure is required for maintenance of normal methylation status as well as gene silencing. Studies with mammalian systems suggest that a methylated DNA-binding protein MeCP2, which can bind to even a single methylated CpG, recruits a repressor complex which includes histone deacetylase[105]. If a similar situation exists in plants, mutation in histone deacetylase (HADC) gene should be able to relieve silencing without affecting methylation status. Transgenic inhibition of HADC in *Arabidopsis* has been found to be associated with pleiotropic effects similar to those observed in case of *MET/ DDMI* mutation, without any significant decrease in DNA methylation[106]. Mutation of *MOM* locus, which stands for 'morpheus molecule' also causes release of silencing without affecting DNA methylation[107]. This gene could be another component of chromatin remodelling complex.

Is there any linkage between TGS and PTGS? TGS and PTGS operate differently but both involve DNA methylation, albeit of different regions. It has been proved that expressed RNA can trigger methylation of coding region as well as PTGS. The potato spindle tuber viroid, which has a small RNA genome and does not code for any protein can direct methylation of its transgene, integrated into plant genome, upon infection[108]. A recent report shows that production of double-stranded RNA complementary to coding sequence from a single transcript, which can pair to itself can induce PTGS of genes having homologous sequence[109]. Such double-stranded RNA can also be produced *in vivo* from promoter sequences having inverted repeats or other complex structures by read-through transcription from nearby promoters. This possibility has been supported by recent experimental evidence. Mette and co-workers[110] expressed double-stranded RNA corresponding to the promoter sequence and observed TGS. These studies suggest that TGS and PTGS may have some features in common.

It has been suggested that aberrant RNAs are synthesized during PTGS. RNA-dependent RNA polymerase uses these RNAs as substrates and synthesizes antisense RNAs. The pairing of both sense and antisense RNA leads to formation of double-stranded RNAs, which are targeted for degradation[111]. Experimental evidence for the presence of RNA-dependent RNA polymerase in tomato has been obtained[112]. The degradation products of about 25 bases corresponding to the silenced transgene have been observed during PTGS[96]. Recently, Bernstein *et al.*[113] have found a RNAase from *Drosophila* which they named 'Dicer' because it chops RNA into small pieces of uniform size. In this process, first the RNA is chopped into 22 nucleotide fragments and then these small fragments are incorporated into a multisubunit complex and act as guide RNA for targeting degradation of single-stranded mRNA. Such a mechanism could be operative in plants also.

Since transgene silencing is an unpredictable phenomenon, researchers have tended to discard plants showing silencing of the desired phenotype and use those plants which do not show silencing. Attempts to overexpress endogenous genes have often resulted in silencing of transgenes as well as endogenous genes, a phenomenon known as co-suppression. Since expression of RNA above a threshold has been observed to lead to PTGS, attempts to overexpress genes of utility have suffered. Though some researchers have been successful in achieving high expression levels of the genes of interest, others have faced problems. De Neve *et al.*[114] produced five different homozygous lines of *Arabidopsis* for production of antibodies, and found gene silencing to occur in all the lines. Gene silencing was associated with methylation of promoter alone, promoter and coding sequence, coding sequence alone or coding region and downstream sequences. Though there is correlation between presence of duplicated sequences, high copy number of foreign genes, methylation acceptor sites, strength of promoter with silencing, attempts to overcome silencing by addressing these features are not well documented. The common occurrence of multiple copies of transgenes in case of biolistic method of transformation has made *Agrobacterium*-mediated method of transformation more popular. However, there are reports of silencing of even single copy genes[115]. Some attempts have been made to avoid silencing of transgenes. One of the approaches has been to utilize matrix attachment region (MAR) in transformation vectors to prevent the influence of heterochromatin on the integrated genes[34]. Insulation elements have been used in vertebrate systems to protect transgenes from effects of nearby *cis*-acting elements[116]. Plant viruses have evolved strategies of avoiding PTGS that plants use to avert viral infections. HC-protease (HC Pro) of potato virus Y (PVY) and 2b protein of cucumber mosaic virus (CMV) have been found to interfere with PTGS in tobacco plants infected by these viruses. Whereas HC Pro acts by blocking the maintenance of gene silencing in tissues where silencing has already occurred, 2b protein prevents initiation of gene silencing at growing points of plants[117]. Recently, a calmodulin-like protein, named rgs-CaM has been identified from tobacco. This protein has the ability to suppress PTGS (ref. 118). These proteins can be exploited to prevent gene silencing. However, whether the expression of such proteins would render these plants vulnerable to viral infections is yet to be investigated.

In order to avoid transgene silencing the following important guidelines should be followed while designing a transgene. Depending upon the correlation of various features of vectors and gene silencing observed by various researchers, it can be stated that attempts should be made to avoid the use of potential methylation-acceptor sites. DNA repeats can be avoided by using different promoters and termination signals to drive expression of different genes of the vector. Vectors can be designed to incorporate MARs or insulators and even the genes for suppression of gene silencing could be incorporated.

Rice: A paradigm system for rapid integration of emerging technologies of transformation

As one traces the evolution of developments in transformation, it is interesting to note that all improvements made in transformation systems were readily exploited for rice transformation. In the early stages, electroporation of protoplasts[119] and PEG-mediated transformation of protoplasts[120] were deployed to generate fertile transgenic rice plants. Christou *et al.*[121] used an electric discharge particle acceleration device to bombard immature rice embryos and generated fertile transgenic rice plants. Until 1994, it was strongly believed that rice is not amenable to *Agrobacterium*-mediated transformation.

There was only one isolated report of Chan *et al.*[122] on the generation of a few transgenic rice plants using *Agrobacterium*. However, the situation completely changed with the construction and deployment of the superbinary vector pTOK233 for rice transformation[16]. Now, *Agrobacterium*-mediated rice transformation has become routine in many laboratories[123].

Agrobacterium-mediated cotransformation, a very simple and elegant approach for marker elimination, was simultaneously evaluated in tobacco and rice[64]. The cotransformation strategy was successfully deployed in rice for engineering provitamin A biosynthesis[33]. A total of four genes contained in two separate T-DNA cassettes were transferred together.

The usefulness of MARs in compartmentalizing the expression of integrated transgenes was successfully demonstrated in rice using tobacco MAR sequences, which brought about a 140-fold increase in *gus* expression[35]. As soon as the usefulness of intron-containing marker genes was realized, a *hpt*-intron gene was successfully evaluated for rice transformation[37]. As *gfp* was emerging as a useful, vital reporter of plants, a synthetic *gfp* version fused with *rbc*S promoter and transit peptide was used for the first time to target the GFP protein to plastids to overcome its tendency to form insoluble complexes in cytoplasm[45]. At a time when shoot apices were becoming attractive transformation targets, Park *et al.*[124] successfully used isolated rice shoot apices for *Agrobacterium*-mediated transformation. As discussed earlier in this review, Ebinuma *et al.*[58] successfully used an *ipt*-MAT strategy coupled to R/RS-based excision to obtain marker-free transgenic rice plants in one step. The positive selection strategy using the *pmi* system has already been demonstrated in rice[72].

Transformation technologies in rice are evolving rapidly to meet the requirements of functional genomics. A shotgun transformation approach (particle gun) involving many rice genomic fragments was performed to identify the clone with the *Xa21* gene for bacterial blight resistance[125]. T-DNA tagging has emerged as a powerful tool in *Arabidopsis* to clone new genes and to identify their functions. With a similar objective, Jeon *et al.*[31] have generated 22,090 independent transgenic rice plants using *Agrobacterium*. It is estimated that as many as 25,700 T-DNA tagged loci will be available for functional genomics analysis. Thus, with parallel developments in transformation technologies and genome analysis, rice has emerged as the first crop plant targeted for complete functional genomics analysis.

An overview of the deployment of transformation technologies in India

Many Indian laboratories have succeeded in effectively using *Agrobacterium* Ti plasmid vectors and microprojectile bombardment for plant transformation. An attempt is made here to present a spectrum of research groups that have successfully raised transgenic plants.

The early success of *Agrobacterium*-mediated transformation of *Brassica juncea* was reported by Pental's group[126] from TERI, New Delhi. The transformation work of this group is now continued in University of Delhi-South Campus. The *barnase/barstar*-based genetic engineering approach for introducing male sterility and its restoration has been successfully deployed by this group in *B. juncea*[127]. A complete account of *Agrobacterium*-mediated transformation of rice was reported by Tyagi's group[18]. Rajam's group, in collaboration with Grover[128], demonstrated transient transformation of *indica* rice by particle bombardment. Improved conditions standardized for regeneration of rice enabled Rajam's group in transforming Pusa Basmati 1 using both superbinary and binary vectors[19]. They have also developed transgenic brinjal for salinity and drought tolerance by introducing *mannitol phosphodehydrogenase* gene[129]. In an effort to develop abiotic stress resistance, Grover's group is introducing *Arabidopsis* heat shock protein gene (*hsp*101) and rice pyruvate decarboxylase 1 (*pdc*1) into rice using *Agrobacterium* Ti plasmid vectors. *Rice tungro virus* genes cloned from RTBV and RTSV genomes by Dasgupta's group are being introduced into rice to develop virus resistance. Khurana's group[130] reported efficient permeabilization of DNA into wheat zygotic embryos using membrane interactive agents like saponin and toluene. This group is currently introducing into wheat, the *bar* gene for herbicide resistance, *pin*III for pest resistance and barley *HVA1* gene for abiotic stress resistance.

The research groups of Kumar and Sharma at IARI, New Delhi have successfully expressed[131] cryIAb in brinjal plants to develop resistance against the larvae of fruit borer. Transgenic tomato plants, which they engineered with cryIAc in collaboration with Reddy of ICGEB, exhibited resistance to the larvae of *Helicoverpa armigera*[132]. The transgenic brinjal plants and tomato plants from the above experiments have been successfully evaluated in field trials. The collaborative efforts of Bhatnagar and Reddy in ICGEB and Kumar in IARI resulted in the generation of transgenic tobacco plants expressing cryIIa5 (ref. 133), that conferred complete protection against *H. armigera*. Reddy's group is developing a chloroplast transformation system in ICGEB to achieve a high level of expression of a wide range of useful proteins. Raina's group at IARI reported transformation of Basmati and IR64 cultivars of rice using both binary and superbinary vectors[134]. Bansal's group at IARI is working towards developing tolerance for UV-B radiation by introducing the *cod*A gene from *Arthobacter globiformis*.

At Jawaharlal Nehru University, New Delhi, Guha-Mukherjee's group is working on desensitized aspartate kinase for regulating amino acid biosynthesis in trans-

genic plants[135]. Datta's group[136] has raised transgenic potato plants with increased nutritive value by engineering tuber-specific expression of *Amaranthus* albumin gene *AmA1*. Sopory's groups working in Jawaharlal Nehru University and in ICGEB, expressed *glyoxalaseI* gene of *Brassica* in tobacco and demonstrated its usefulness in developing salt tolerance[137].

Saradhi's group at Jamia Millia Islamia, New Delhi developed *B. juncea* expressing bacterial *cod*A[138]. The transgenic plants exhibited enhanced salt resistance. Jaiwal's group at M.D. University, Rohtak, collaborated with Sautter's group in Switzerland to demonstrate successful transformation in mungbean[24]. Kothari's group at University of Rajasthan, Jaipur in collaboration with Hodges in Purdue University, has transformed rice using *Agrobacterium*[139].

The group of S.K. Sen at the Bose Institute, Kolkata has carried out pioneering work in India using the particle bombardment system. A significant achievement of the group is the generation of *CryIAc*-expressing IR64 transgenic rice that is highly toxic to the larvae of yellow stem borer[140]. This group also reported transformation of embryo axis of chickpea by particle bombardment with cryIAc[141]. At National Chemical Laboratory, Pune, Krishnamurthy's group (in collaboration with O. Schieder's group in Germany) performed *Agrobacterium*-mediated transformation of chickpea and generated transgenic plants with marker genes[142]. The Plant Biotechnology Division in BARC, Mumbai which has carried out extensive tissue culture work on legumes, has reported successful transformation of groundnut using *Agrobacterium*[143].

Rao's group at Osmania University, Hyderabad, has carried out collaborative research with Hodges in Purdue University, USA and Gatehouse in UK and used particle bombardment to engineer the *Galanthus nivalis* agglutinin (*GNA*) gene in rice to develop resistance against sap-sucking insects like brown plant hopper[144]. Reddy's group at the Central University of Hyderabad, in collaboration with C. Fauquet in USA, introduced the maize anthocyanin biosynthesis genes by particle bombardment into rice to develop blast disease resistance[145]. Sharma's group at ICRISAT, Hyderabad, succeeded in raising transgenic groundnut plants with the coat protein gene of *Indian peanut clump virus*[146].

The Indian Institute of Science, Bangalore, has two active plant transformation groups. Sankara Rao's group has performed *Agrobacterium*-mediated transformation of groundnut to introduce tobacco chitinase and reported the development of resistance against fungi[147]. Lakshmi Sita's group, which has extensive tissue culture experience, is working on the transformation of a wide range of crop plants. They recently succeeded in transforming pigeonpea, a pulse considered very difficult for transformation[148]. The collaborative efforts of the groups of Savithri and Lakshmi Sita led to the generation of transgenic tobacco[149] and tomato[150] plants that exhibited resistance to physalis mottle virus by expressing the viral coat protein.

The groups of Sudhakar and Balasubramanian in Tamil Nadu Agricultural University, Coimbatore, are collaborating with Christou's group in the UK to engineer a number of useful traits in rice. A representative example is the report on the phloem-specific expression of the snowdrop lectin, GNA in transgenic rice[151]. Basic studies on *Agrobacterium*-mediated transformation are being carried out by the group of Veluthambi at Madurai Kamaraj University. They have demonstrated the feasibility of blackgram transformation using *Agrobacterium*[152].

Plant genetic engineering work in various Indian laboratories is being carried out as per the guidelines evolved and implemented by the Review Committee on Genetic Manipulation (RCGM) of the Department of Biotechnology, Govt of India. The guidelines carry clear descriptions of greenhouse, net house and field evaluation of transgenic experiments. An account of the transgenic plants developed in Indian research laboratories that have reached the 'field trial' level is presented by Ghosh and Ramanaiah[153].

Indian groups have made impressive progress in the transformation of crops like *Brassica*, rice, potato, tomato and brinjal. However, we still face hurdles in transforming other important crops such as legumes, cotton and sugarcane, in which farmers face many problems that can be effectively addressed by genetic engineering approaches. In both legumes and cotton the basic limitation is the non-availability of a good regeneration system. Standardization of conditions to regenerate plants via somatic embryogenesis can immensely help in achieving *Agrobacterium*-mediated transformation. In parallel, we should work towards improving transformation efficiencies by using various approaches such as (i) evaluation of different Ti plasmid backgrounds in *vir* helper strains, (ii) use of multiple copies of supervirulent *vir* genes, (iii) application of vacuum infiltration, (iv) use of marker genes with introns, (v) evaluating multiple markers including positive selection markers, (vi) performing co-cultivation at lower temperatures, and (vii) the use of matrix attachment regions in the T-DNA. *In planta* transformation and *bar* gene-based screening in T_1 generation are attractive alternatives.

Conclusion

The traditional strength of many Indian laboratories in plant tissue culture has facilitated a successful transition to plant genetic engineering. Any general improvement in transformation methods reported at the international level, whether in the form of vector development or in the form of methodology, is immediately adopted and improved upon in Indian laboratories. This success is

reflected by the fact that more than 10 Indian laboratories routinely perform rice transformation now. The successful approaches that we applied to rice should now be extended to other challenging crops such as legumes, cotton, sugarcane and groundnut, in which we encounter several problems that can be addressed by genetic engineering.

The approval given by the Genetic Engineering Approval Committee, Govt of India in March 2002 for the commercial release of *Bt* cotton sets the stage for the cultivation of genetically engineered crops in India. This sets a high demand for scientifically strong and cost-effective transformation technologies in Indian crops. The scientific expertise that many Indian laboratories have been developing over the years in genetic engineering must now be converted into 'technologies' so that many problems faced by Indian farmers can be solved by plant genetic engineering approaches.

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