5

Genetic Engineering of Plants for Resistance to Viruses

Richard Mundembe[1,2], Richard F. Allison[3] and Idah Sithole-Niang[1]
[1]*Department of Biochemistry, University of Zimbabwe, Mount Pleasant, Harare,*
[2]*Department of Microbiology, School of Molecular and Cell Biology, University of the Witwatersrand, Private Bag 3, Johannesburg,*
[3]*Department of Plant Pathology, Michigan State University, East Lansing,*
[1]*Zimbabwe*
[2]*South Africa*
[3]*USA*

1. Introduction

Genetic engineering has been identified as one key approach to increasing agricultural production and reducing losses due to biotic and abiotic stresses in the field and in storage (Sairam and Prakash, 2005; Yuan *et. al*., 2011). This chapter primarily deals with resistance to viral diseases. It is therefore very important that anyone embarking on a research project to genetically engineer plants fully understands the variety of plant transformation methods that are available, the various forms of (plasmid) constructs that can be used, and their potential implications on the safety of the final product.

The methods that can be used for plant transformation include *Agrobacterium*-mediated transformation, microprojectile bombardment/ biolistics, direct protoplast transformation, electroporation of cells and tissues, electro-transformation, the pollen tube pathway method, and other methods such as infiltration, microinjection, silicon carbide mediated transformation and liposome mediated transformation (Rakoczy-Trojanowska, 2002). Each of these methods, as will be discussed in this chapter, utilizes a different approach to deliver DNA into the vicinity of chromosomes into which the DNA may then integrate. The markers and reporter genes that may be used in conjunction with the different approaches, and additional sequences meant to facilitate integration may have some biosafety implications.

The aim of this chapter is to evaluate the different methods that are used for plant transformation, and to discuss specific results obtained after plant transformation for virus resistance using two of the methods: *Agrobacterium*-mediated transformation and electro-transformation. Implications on biosafety will be discussed as well.

2. Plant transformation

Figure 1 shows the generalized structure of a plant cell. For stable genetic transformation, the desired DNA fragment must be delivered across the cell wall if not removed by pre-treatment, the cell membrane, across the cytoplasm, the nuclear membrane into the nucleus.

Similarly, for organelle transformation, the DNA must be transported across the organelle membrane to reach the organelle's matrix. Once inside the nucleus, the desired DNA fragment must undergo recombination with the host chromosome so that it becomes integrated into the host chromosome, and its inheritance pattern becomes the same as that of the host chromosome. To date, the mechanisms of integration are not well understood, and there is no targeting of particular chromosomes. Also, a lot still needs to be done in terms of organelle transformation. These topics are reviewed in detail in Tinland 1996; Ow, 2002; Tzfira *et al*., 2004; Maliga 2004 and Kumar *et al*., 2006.

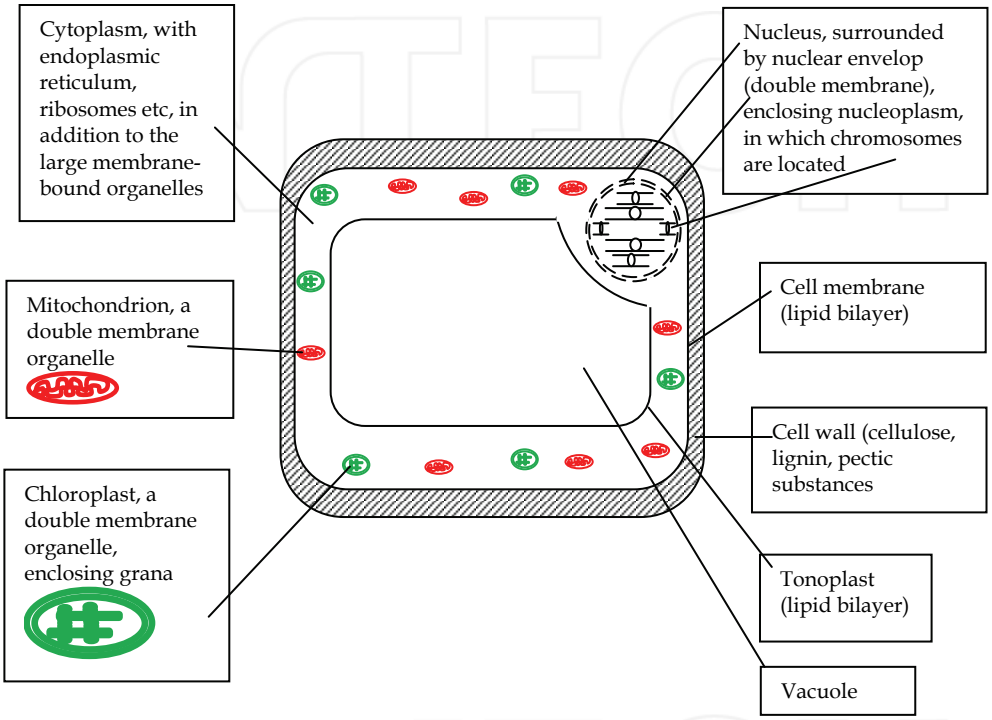


Fig. 1. Diagram to illustrate the structure of a plant cell

Genetic engineering will result in plants that carry additional genes from the same or other species, and are thus referred to as transgenic plants. Such plants may also be referred to as transformed plants, because their genotype and phenotype may have changed from one state to another, for example from disease-susceptible to disease-resistant. The term 'transformed plant' also relates to the original method of *Agrobacterium*-mediated transformation, where, after the bacterium transfers the T-DNA, the recipient plant cells become 'cancerous', and result in cankers that characterize the crown gall disease.

The term 'genetically modified plant' is much broader than 'transformed plant'. While a strict definition of 'plant transformation' may not be practical because of the varying genetics of the plants, it is generally accepted that the plant must be confirmed as transformed based on Southern DNA hybridization evidence of multiple independent transformation events showing different sized fragments correlating to different profiles of

the restriction endonucleases used, and appropriate sustained phenotypic expression of the transgene exclusively in the transformed plants (Potrykus, 1991, Birch 2002).

In plant pathology, the concept of resistance and susceptibility genes is widespread. In the gene-for-gene model of pathogen incompatibility, resistance (R) genes and associated avirulence (Avr) genes have been well studied (reviewed in Belkhadir *et al*., 2004). But one aspect that has not been well elucidated is the concept of susceptible genes. Very few susceptibility genes have been identified. However one example is the Os8N3, a host disease-susceptibility gene for bacterial blight of rice which is a vascular disease caused by *Xanthomonas oryzae* pv. *oryzae* (Yang *et al*., 2006). Deletion of Os8N3 in rice plants by genetic engineering approaches is postulated to result in genetically engineered plants resistant to *Xanthomonas oryzae* pv. *Oryzae*. One may ask if these plants will be considered transgenic. Most susceptibility genes, however, are thought to be essential for plant growth and development, such that their deletion or mutation will result in non-viable plants.

It must be noted that 'transgenic', 'transformed' and 'genetically modified' are not equivalent terms. The definition of transformed plants should be broad enough to encompass deletions. Southern hybridization probes targeting the deletion junctions may be used to confirm the deletion event, and absence of susceptibility gene product can be demonstrated.

Conventional breeding also results in re-assortment of genes from the two genomes that are crossed, and is therefore some form of genetic modification as well. However, no genetic engineering is involved in the process, and the crosses usually involve closely related species. Genetic engineering is particularly useful when the gene/trait of interest is not present in closely related species, making conventional breeding impossible. Furthermore, conventional breeding is not precise, since extensive re-assortment of genes occurs when two species are crossed, and takes a very long time. Genetic engineering therefore becomes the approach of choice especially when there are no Biosafety issues to grapple with. The most common approach in genetic engineering involves excising the gene of interest using restriction enzymes, and cloning it into a plant transformation vector before transfer into the cells of the target species where the gene will integrate into the chromosome. This process is usually more precise and faster. In this case the resulting plants are transgenic, because they carry a gene from another species, introduced by genetic engineering.

Many transgenic plants resistant to diseases have been produced. Collinge and co-workers list the most common genes used for transgenic disease-resistant crops that have been field-tested (Collinge *et al*., 2010). Against fungal diseases, these are the polygalacturonse inhibitor protein (grape, raspberry, tomato), proteinase (soybean), R-gene (Rpg-1, Pi9, RB2, Rps1-k) (barley, festuca, potato, soybean), cell death regulator (wheat), toxin detoxifier (barley, wheat) pathogenesis-related proteins (barley, wheat, grape, cotton, peanut, potato, rice, sweet potato, sorghum, tobacco), chitinases (alfalfa, apple, cotton, melon, onion, papaya, squash, carrot, peanut, rice, tobacco, wheat, tomato), oxalate oxidases (bean, cowpea, lettuce, sunflower, peanut, potato, soybean, tobacco), thionin (barley, potato, rice), antimicrobial peptides (cotton, grape, plum, poplar, tobacco, wheat), cecropin (cotton, maize, papaya), stilbene synthase (potato, tobacco), and antimicrobial metabolites (grape, potato, strawberry, tobacco). Against bacterial diseases, attacin (apple), cecropin (apple, papaya, pear, potato, sugarcane), hordothionin (rice, tomato), indolicidin (tobacco), lysozyme (citrus, potato, sugarcane), megainin (grape), proteinase K (rice, tomato), R-gene

of pepper, tomato, rice (tomato), and transcription factors (tomato) have been field-tested. Against plant viruses, single-stranded DNA binding G5 protein (cassava), viral movement proteins (raspberry, tomato), ribonuclease (pea, potato, wheat), replicase (cassava, papaya, potato, tomato), nuclear inclusion protein (melon, potato, squash, wheat), coat protein (alfalfa, barley, beet, grape, lettuce, maize, melon, papaya, pea, peanut, pepper, pineapple, plum, potato, raspberry, soybean, squash, sugarcane, tobacco, tomato, wheat). Virus resistance will be discussed further in section 2.1.

Despite performing well in field tests, most of the transgenic plants have not been commercialized. For instance, coat protein transgenic plants make up three quarters of commercialized virus resistant plants. However, the newer and more sophisticated approaches such as RNA interference are set to become more predominant on the market.

There still remain many challenges to plant transformation. Most methods are not effective for all plant species, but are species- or even cultivar specific. Usually the target for transformation is a small group of cells or an organ, which should then grow and regenerate a whole plant. Regeneration of whole plants *in vitro* is not routine for some agriculturally important species. Thus, there are some very important crops for which no routine, reliable reproducible transformation procedure exists. Therefore the efforts to develop more and better transformation methods continue.

The methods that are available for plant transformation include *Agrobacterium*-mediated transformation, microprojectile bombardment/ biolistics, direct protoplast transformation, electroporation of cells and tissues, electro-transformation, and other methods such as microinjection, silicon carbide mediated transformation and liposome mediated transformation. Each of these methods, as will be discussed in this chapter, utilizes a different approach to deliver DNA into the vicinity of chromosomes into which the DNA may then integrate.

2.1 Plant viruses

2.1.1 Plant viral diseases

Biotechnology, through genetic engineering, has the potential to contribute to increased agricultural production by making crops better able to cope with both biotic and abiotic stress. Different research groups are working on different aspects of both biotic and abiotic constraints to increase agricultural production. However, the scope of this chapter will only cover biotic stress and plant viruses in particular. Plant viruses significantly reduce yields in all cultivated crops. By the turn of the millennium, there are as many as 675 plant viruses known and yet annual crop losses due to viruses are valued at US$60 billion (Fields 1996).

There are various ways of controlling viral diseases such as:

- The use of disease-free planting material. Virus-free stocks are obtained by virus elimination through heat therapy and/or meristem tissue culture. This approach is effective for seed-borne viruses, but is ineffective for viral diseases transmitted by vectors.
- Adopting cultural practices that minimize epidemics, for example by crop rotation, quarantine, rouging diseased plants and using clean implements. Pesticides may also be

used to control viral vectors, but the virus may be transmitted to the plant before the vector is killed.

- Classical cross protection, in which a mild strain of the virus is used to infect the crop, and protects the crop from super-infection by a more severe strain of the virus.
- Use of disease resistant planting material. Natural resistance against viruses may be bred into susceptible lines through classical breeding methods or transferred by genetic engineering.
- Engineered cross protection. This involves integration of pathogen-derived or virus-targeted sequences into DNA of potential host plants, and conveys resistance to the virus from which the sequences are derived.

Of all the methods of controlling viral diseases listed above, engineered cross protection seems to have a lot of potential that is only now beginning to be exploited. Before genetic engineering techniques were more widely accepted and applied, natural disease resistance genes bred into target cultivars by classical breeding methods constituted the major focus for introducing disease resistance into plants.

There are 139 monogenic and 40 polygenic virus resistance traits that have been described (Khetapal *et al*., 1998; Hull 2001), but very few have been cloned, and in most cases the mechanism of resistance has not been elucidated (Ellis *et al*., 2000; Dinesh-Kumar *et al*., 2000). Virus-resistant crops that have been obtained by classical breeding include sugarcane resistant to Sugarcane mosaic potyvirus (SCMV) and gerkins (cucumber) resistant to Cucumber mosaic virus (CMV). The N-gene of *Nicotiana glutinosa* that is responsible for the necrotic local lesion reaction of TMV, has also been bred into some *N. tabacum* lines, resulting in the hypersensitive reaction and no systemic infection. Classical breeding has also been used to convey polygenic traits.

2.1.2 Non-viral genes

One approach to protect plants against a viral infection is by the expression of a single chain variable fragment (scFv) antibody directed against that particular virus (Tavladoraki *et al*., 1993; Voss *et al*., 1995). This has been demonstrated for the icosahedral Artichoke mottle crinkled tombusvirus (AMCV) and the rod-shaped Tobacco mosaic tobamovirus (TMV). However, the resistance obtained this way is not broad-spectrum resistance.

An approach that can yield broad-spectrum resistance to viral diseases is to target the inhibition of production of a product that is essential for the establishment of infection in the cell. An example is S-adenosylhomocysteine hydrolase (SAHH), an enzyme involved in the transmethylation reactions that use S- adenosyl methionine as a methyl donor (Masuta *et al*., 1995). Lowering expression of the enzyme suppresses the 5'-capping of mRNA that is required for efficient translation. Overexpression of cytokinin in crops results in stunting. This phenotype may be due to induction of acquired resistance (Masuta *et al*., 1995).

Expression of the pokeweed (*Phytolacca americana)* antiviral protein (PAP), a ribosome inhibiting protein (RIP), in plants protects the plants against infection by viruses (Ready *et al*., 1986; Lodge *et al*., 1993). In this case, expression of this single gene in the plant results in protection against a wide range of plant viruses.

2.1.3 Pathogen-derived resistance

Definition

Pathogen-derived resistance (PDR), also called parasite-derived protection is the resistance conveyed to a host organism as a result of the presence of a transgene of pathogen origin in the target host organism (Sanford & Johnson, 1985). The concept of pathogen-derived resistance predicts that a 'normal' host-pathogen relationship can be disrupted if the host organism expresses essential pathogen-derived genes. The initial hypothesis was that host organisms expressing pathogen gene products at incorrect levels, at the wrong developmental stage or in dysfunctional forms, may disrupt the normal replication cycle of the pathogen and result in an attenuated or aborted infection.

Classical cross protection

Pathogen derived resistance is an extension of the phenomenon of "cross protection" in which inoculation of a host plant with a milder strain of a pathogen can protect the plant from superinfection by more severe strains of the same or a very closely related pathogen (Wilson 1993). An example of cross protection is in tobacco where infecting tobacco plants with the U1 strain of tobacco mosaic tobamovirus (TMV) protects the plants against future infections with a more virulent strain of TMV.

In practice, the protected plants usually become superinfected, and so the definition given above is not practical. For practical purposes, cross protection is still defined by an earlier definition as "the use of a virus to protect against the economic damage by severe strains of the same virus" (Gonsalves & Garnsey, 1989). Classical cross protection, according to this practical definition, has been evaluated in the field in some countries outside Africa for the control of Citrus tristeza closterovirus (CTV), Papaya ringspot potyvirus (PRSV), Zucchini yellow mosaic potyvirus (ZYMV) and Cucumber mosaic cucumovirus (CMV) (ibid).

Engineered protection

The genetic engineering approach to cross protection was first demonstrated by Powell-Abel and co-workers who expressed the TMV coat protein gene in transgenic plants and obtained some degree of resistance against TMV (Powell-Abel *et al.*, 1986). Many viral genes and gene products have since been shown to be effective in conveying engineered PDR. Engineered PDR can be divided into protein-based PDR (coat protein-, replicase- and movement protein-mediated resistances, using these proteins in their wild type or defective forms) and nucleic acid-based PDR (antisense, sense and satellite RNA-mediated resistances, defective interfering RNA or DNA and antiviral ribozymes).

In general, when classical cross protection is incomplete, smaller lesions than in control non-protected plants are formed, indicating reduced movement and maybe reduced replication as well. On the other hand, transgenic plants engineered to confer protection to TMV show no reduction in movement or replication. However, the local lesions for PDR against PVX indicate a reduction in virus replication and movement (Hemenway *et al.*, 1988). This demonstrates the similarity between classical and engineered protection.

The phenotype of PDR varies from delay in symptom development, through partial inhibition of virus replication, to complete immunity to challenge virus or inoculated viral RNA (Wilson, 1993; Baulcombe, 1996). Even a simple delay in symptom development could

be useful if it allows plant biomass, seed or fruit development to outpace disease development.

Coat protein-mediated resistance

Coat protein-mediated resistance (CP-MR) is the phenomenon by which transgenic plants expressing a plant virus coat protein (CP) gene can resist infection by the same or a homologous virus. The level of protection conferred by CP genes in transgenic plants varies from immunity to delay and attenuation of symptoms. CP-MR has been reported for more than 35 viruses representing more than 15 different taxonomic groups including the tobamo-, potex-, cucumo-, tobra-, carla-, poty-, luteo-, and alfamo- virus groups. The resistance requires that the CP transgene be transcribed and translated. Hemenway and co-workers (1998) have demonstrated direct correlation between CP expression level and the level of resistance obtained. The case of CP-MR to TMV is is important because most of the earlier and more detailed work on CP-MR was done with TMV (Bevan *et al*., 1985; Beachy *et al*., 1986; Powell- Abel *et al*., 1986; Register 1988 and Powell *et al*., 1990).

2.1.4 RNA interference (RNAi)

RNA interference is the process that depends on small RNAs (sRNAs) to regulate the expression of the eukaryotic genome (Hohn and Vazquez, 2011). This newly elucidated mechanism opens up many possibilities for genetic engineering interventions due to the simplicity of the molecules involved. Small RNAs regulate many biological processes in plants, including maintenance of genome integrity, development, metabolism, abiotic stress responses and immunity to pathogens (Hohn and Vazquez, 2011; Katiya-Agarwal, 2011). The RNA molecules involved are small and of two types, micro RNAs (miRNAs) and small interfering RNAs (siRNAs). miRNAs are transcribed from miRNA genes by RNA polymerase II, as primary miRNA (pri-miRNA) that then folds into a stem loop structure (imperfectly base-paired) that is then processed in a very specific manner by a number of proteins to result in 22-24mer RNA molecules. These RNA molecules are then incorporated into AGO1 or AGO10 and guide the complex to target mRNA for cleavage or translational inhibition on the basis of sequence complementarity. siRNAs on the other hand, are derived from perfectly paired double stranded RNA (dsRNA) precursors, that are derived either from antisense or are a result of RNA-dependent RNA polymerase (RDR) transcription. Details of types of siRNAs, their origins and processing, and how this approach is used to convey virus resistance in transgenic plants are presented in Hohn and Vazquez (2011) and Katiya-Agarwal (2011).

3. *Agrobacterium*-mediated transformation

The structure of the Ti plasmid and the requirement for transfer has been established, and the natural host range of the bacterium expanded (Cheng 2004). The first reports of *in vitro* plant transformation utilised the ability of *Agrobacterium tumefaciens* to transfer a specific region of its Ti plasmid DNA into plant cells where they subsequently become integrated into the plant cell genome (Marton *et al*., 1979; Barton *et al*., 1983; Herrera-Estrella *et al*., 1983). This application is based on the observation that in natural diseases of dicotyledonous plants, crown gall disease caused by *Agrobacterium tumefaciens* and hairy root disease caused by *Agrobacterium rhizogenes*, the bacterium transfers part of the DNA of its Ti or Ri plasmid

DNA respectively into the host plant where it becomes integrated into the host genome (Herrera-Estrella *et al*., 1983). The plant host cells are referred to as transformed. The transferred DNA is referred to as the T-DNA and is demarcated by conserved left and right border sequences (ibid). The integrated genes are passed on to the progeny of the initially infected cell, and their expression (using the host's transcription and translation machinery) results in the cancerous growth that characterise the crown gall or hairy root diseases that results. The tumours produce specific amino acid derivatives called opines that are utilized by the *Agrobacterium* as a carbon source (Zupan and Zambryski, 1997). Within the T-DNA is a 35 kb virulence (vir) region that includes the genes virA to virR (Zhu *et al*. 2000), flanked by imperfect 25 bp direct repeat sequences known as the left and right borders. A number of virulence genes (chv) located on the *Agrobacterium* chromosome mediate chemotaxis and attachment of the bacterium to the plant cell wall (Zupan & Zambryski, 1997).

In adapting the *Agrobacterium* system to genetic engineering, only the sequences that are essential for transfer and integration into the host genome have been retained, and DNA sequences of interest are inserted into the transferred DNA region. The first generation plasmids for *Agrobacterium*-mediated plant transformation were the disarmed Ti-plasmids. The oncogenes within the left and right borders of the naturally occurring plasmid pTiC58 were replaced with pBR322 sequences, to give pGV3850 (Zambryski *et al*., 1983), and further improved by the addition of a selectable marker (Bevan *et al*., 1983). Use of intermediate vectors enabled use of smaller plasmids with unique cloning sites for initial cloning experiments in *E. coli* (Matzke & Chilton 1981). The intermediate vector could be transferred from *E. coli* to *Agrobacterium* by conjugation, utilizing a helper plasmid, e.g. RK2013, to supply the requirements for conjugation (ibid). Homologous recombination between the intermediate plasmid and a resident disarmed Ti-plasmid of the *Agrobacterium* (e.g. pGV3850) resulted in a larger plasmid known as a cointegrate disarmed Ti-plasmid.

In a different approach, the virulence genes were placed in a separate plasmid such as pAL4404 where these functions would be provided in *trans* for the transfer of DNA on another smaller plasmid with only the left and right borders, markers and other sequences of interest that need to be transferred such as pBin19 in the same *Agrobacterium* cell (Zupan & Zambryski, 1997). This system is known as the binary vector system. The vectors carry a broad host range replication origin, e.g. *ori* V of pBin 19, which allows replication in *E. coli* and *Agrobacterium*. The *A. tumefaciens* is used most extensively in plant transformation because of the belief that the DNA transfer is discreet, with high proportion of integration events with single or low T-DNA copy number, compared to other methods of plant transformation (Zupan & Zambryski, 1997).

Plasmid origin of replication may encourage rearrangements and recombination, leading to silencing and deletion of transgene in subsequent generations. Gene disruption may occur at the site of insertion, resulting in loss of some essential functions (Birch, 1997). It is therefore important to obtain as many transformants as possible so as to be able to disregard all abnormal regenerants resulting from this or other phenomena. T-DNA transfer occurs sequentially but not always completely from the right border to the left border (Wang *et al*., 1984).

Recently, it has also been realized that some sequences outside the borders also get transferred, and integrate into the host genome (Parmyakova *et al*., 2008). This is undesirable in genetically modified plants for commercial release. Current efforts are to reduce or even

eliminate these undesirable effects through using special vector constructs that prevent integration of vector sequences. It is thought that integration of sequences outside the borders is a result of erroneous recognition of either right or left border sequences, and Vir D proteins are central to this event. However, the transfer always starts at or adjacent to the left right borders. The reduction can be achieved by using vectors that have positive or negative selection markers, or easily identifiable markers, outside the T-DNA, or using vectors with increased numbers of terminal repeats, or with left terminal repeats surrounded by native DNA regions that serve as termination enhancers, or the so-called 'green vectors' in which the sequences outside the T DNA have been removed (Parmyakova *et al.*, 2008) Alternatively, one can use vectors in which the undesirable sequences can be removed by mechanisms such as site-specific recombination, or use vectors with sequences of plant origin only. But there still are problems associated with each approach.

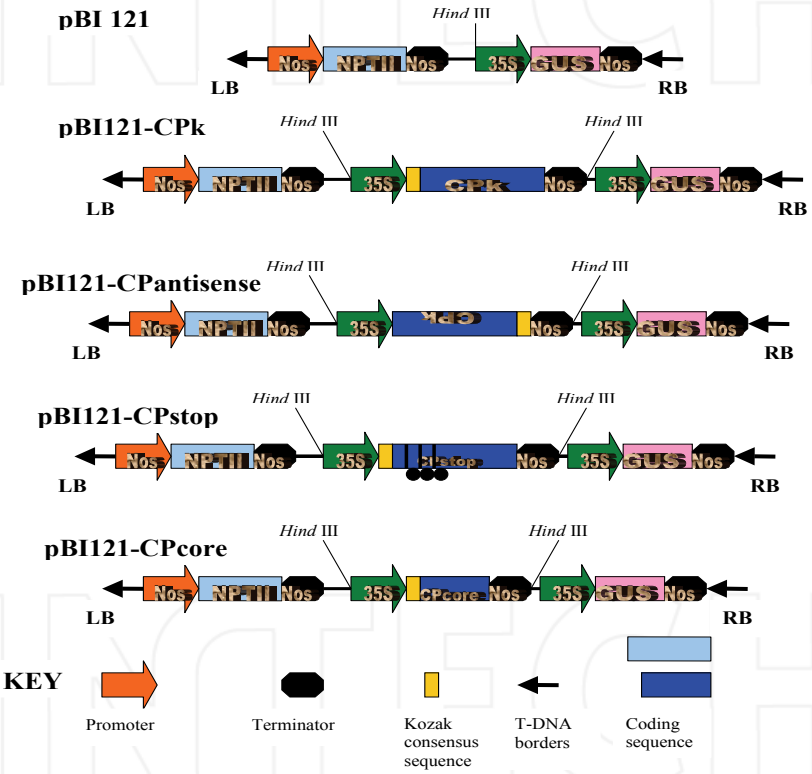


Fig. 2. Illustration of the binary plasmids used for tobacco transformation by *Agrobacterium*-mediated transformation

Despite these limitations, *Agrobacterium*-mediated transformation is still a very useful tool in plant molecular virology. In our laboratory, *Agrobacterium*-mediated transformation was used as a tool to evaluate mechanisms of resistance to Cowpea aphid-borne mosaic virus (CABMV) in *Nicotiana benthamiana*, an experimental host of the virus. CABMV is a positive sense RNA virus that is a member of the genus *Potyvirus* (Sithole-Niang *et al.*, 1996;

Mundembe *et al*., 2009). In an experiment to evaluate the mechanisms of pathogen-derived resistance, *N. benthamiana* was transformed with recombinant pBI 121 carrying various forms of the CABMV coat protein gene, following the method of co-cultivation of leaf explants with *A. tumefaciens* described by An *et al.* (1987). The constructs used were pBI121-CP_k which results in an expressed CABMV coat protein, pBI121-PC which results in antisense CP, pBI121-CP_{stop} which results in a form of the CP mRNA that cannot be translated and CP_{core} which results in only the core region of the CP, together with a pBI121 control.

Evaluation of the responses of transgenic plants obtained indicate that coat protein-mediated resistance only results in delayed symptom development, while RNA mediated approaches may result in recovery or immunity. Out of 68 CP expressing transgenic plants challenged with CABMV, 19 expressed delayed symptom development; and none displayed immunity. Out of 26 CP stop lines, 3 displayed delayed symptom development, 4 tolerance, and 3 recovery phonotypes. Out of 49 antisense lines, 1 displayed delayed symptom delayed symptom development and 3 lines showed modified symptoms.

At the time of carrying out these experiments cowpea could not be transformed in a reliable, reproducible manner, and many research groups were working towards developing a suitable transformation procedure. However, the experiments with transgenic tobacco served the purpose of evaluating the effectiveness of the different approaches. Coat protein mediated resistance would only result in delayed symptom development, RNA mediated approaches are likely to give higher levels of resistance, maybe even immunity.

Therefore, as the method for cowpea transformation become available one would know which particular constructs to use to get the desired levels of resistance.

4. Microprojectile bombardment/ biolistics

Microprojectile bombardment, also known as biolistics, is the most commonly used method falling into the category of direct gene transfer methods. In direct gene transfer methods a plasmid in which the sequences of interest are cloned is delivered across the various plant cell barriers by physical means to enter the cell where integration into the plant genome may occur. The vectors used in direct plant transformation methods usually include the gene of interest cloned between a promoter and a terminator, and the plasmid components of an origin of replication, an antibiotic resistance gene, a selectable marker for use in plants (e.g. herbicide or antibiotic resistance) or reporter gene (e.g. GUS, luciferase genes). The whole plasmid may be transferred into the plant cell and may be integrated into the plant genome as a whole or as fragments. The barriers to be crossed by the DNA in direct DNA transfer methods are the cell wall and the cell membrane before it can cross the cytoplasm and the nuclear envelop to enter the nucleoplasm where the DNA may integrate into the plant genome (Figure 1). Some direct DNA transfer procedures utilize whole plasmids, supercoiled or linear, which may ultimately integrate as a whole, or at least large parts thereof, including the gene of interest (Smith *et al*., 2001).

Direct gene transfer methods were developed in an effort to transform economically important crops that remained recalcitrant to *Agrobacterium*-mediated transformation because of limitations such as genotype and host cell specificity. Some direct gene transfer methods may also circumvent difficult tissue culture methods.

Sanford and co-workers (1987) were the first to report of plant transformation by microprojectile bombardment. Gold or tungsten particles coated with DNA are propelled at high speed toward the plant tissue where they may penetrate the plant cell walls to introduce the DNA into the cytoplasm, vacuoles, nucleus or other structures of intact cells. A modified bullet gun or electric discharge gun is used to propel the particles (Klein *et al*., 1987; Christou *et al*., 1988). Inside the cell, the DNA may be expressed transiently for two or three days before being degraded, or may become integrated into the nuclear or chloroplast genome, and considered stably integrated if it is passed faithfully to subsequent generations. DNA-coated particles delivered into the nucleus are 45 times more likely to be transiently expressed than those delivered to the cytosol, and 900 times more likely to be expressed than those delivered to the vacuole (Yamashita *et al*., 1991). Efficiency of transformation is influenced by the stage of the cell cycle (Iida *et al*., 1991; Kartzke *et al*., 1990). The DNA is also likely to be expressed if it is delivered to the cell close to the time the nuclear membrane disappears at mitosis (Bower & Birch, 1990; Vasil *et al*., 1991).

Direct DNA transfer methods seem to result in transformants with higher copy numbers than *Agrobacterium*-mediated transformation methods (Hadi *et al*., 1996; Christou *et al*., 1989). The multiple copies may be integrated at the same or tightly linked loci, most likely in relation to replication forks or integration hot spots resulting from initial integration events (Cooley *et al*., 1995, Kohli *et al*., 1998). Increasing the amount of DNA entering the cell in bombardment increases the copy number (Smith *et al*., 2001). The DNA may undergo rearrangements (deletions, direct repetitions, inverted repetitions, ligation, concatamerization) prior to, or during integration (Cooley *et al*., 1995). The site of integration is thought to be random. Ninety percent of T-DNA integrations are into random sites within transcriptionally active regions (Lindsey *et al*., 1993).

Like *Agrobacterium*-mediated transformation, microprojectile bombardment also results in integration of vector sequences if they are part of the DNA molecule bombarded into the plant cell (Kohli *et al*., 1999). However, microprojectile bombardment provides an opportunity for the introduction of minimal gene cassettes into the cells. In this approach, only the required gene expression cassettes (promoter, coding region of interest, terminator) is bombarded into the plant cells, or can be co-transformed together with marker genes to be removed before commercialization (Yao *et al*., 2007; Zhao *et al*., 2007). While the screening and selection might be more difficult, probably depending on detection of the gene sequence or gene product of interest, the approach is very attractive since reporter genes and selection markers are completely avoided (Zhao *et al*., 2007).

Marker genes are unnecessary in established transgenic plants, and also limit options when additional transgenes are to be added (stacking) to the original transgenic line. Herbicide resistance genes may potentially be transferred to weeds by outcrossing. Consumers may also worry about the possibility of antibiotic resistance genes spreading to gut microflora, even though there is no scientific evidence for this.

A variation of the microprojectile bombardment method designed to increase the chances of integration is the **Agrolistic transformation method**. In this method, the transforming plasmid is transferred to the plant cell by a direct mechanism together with a second plasmid coding for *A. tumefaciens* proteins involved in the integration process (Zupan & Zambryski, 1997). Transient expression of the *A. tumefaciens* proteins will direct integration of the plasmid into the plant cell genome. As a result, entry of the plasmid into the cell is by

a direct/physical mechanism, but integration into the genome is by a mechanism similar to *Agrobacterium*-mediated transformation. The agrolistic transformation method was expected to address one of the main drawbacks of the microprojectile bombardment method which is that there seem to be a high incidence of high copy number. However, a second drawback that the gene gun accessories are very expensive is still valid.

5. Electroporation and PEG-mediated transformation of protoplasts

Plant cell walls can be removed by enzymatic degradation to produce protoplasts. Polyethylene glycol (PEG) causes permeabilization of the plasma membrane, allowing the passage of macromolecules into the cell. Pazkowski and co-workers were the first to produce transgenic plants after PEG transformation of protoplasts, and many more monocotyledonous and dicotyledonous species have now been transformed using this method (Pazkowski *et al*. 1984). In electroporation, the protoplasts are subjected to an electric pulse that renders the plasma membrane of the protoplasts permeable to macromolecules. The cell wall and whole plants can be regenerated, if procedures exist.

The transgenic plants generated using these methods seem to have characteristics similar to those of plants derived from all other direct transformation methods. However, it is important to note that carrier DNA (usually ~500 bp fragments of calf thymus DNA) is usually included in the transformation mixture to increase transformation efficiency. This may have some consequences in terms of prevalence of transgene rearrangements and integration of superfluous sequences (Smith *et al*., 2001).

The cell cycle stage of the protoplasts at the time of transformation influence the transgene integration pattern. Non-synchronized protoplasts produce predominantly non-rearranged single copy transgenes in contrast to M phase protoplasts that give multiple copies usually at separate loci (Kartzke *et al*., 1990). The S phase protoplasts give high copy numbers, usually with rearrangements. Irradiation of protoplasts shortly before or after addition of DNA in direct transformation procedures increases both the frequency of transformation and number of integration sites (Koehler *et al*., 1989, 1990, Gharti-Chhertri *et al*., 1990). This is consistent with a mechanism of integration that is partly mediated by DNA repair mechanisms.

The main drawbacks of these methods are that protoplast cultures are not easy to establish and maintain, and regeneration of whole plants from the protoplasts is often unreliable for some important species.

6. Electroporation of intact cells and tissues

DNA can be introduced into intact cells and tissues in a manner similar to electroporation of protoplasts. Thus pollen, microspores, leaf fragments, embryos, callus, seeds and buds can be used as targets for transformation (Rakoczy-Trojanowska 2002). Protocols for efficient electroporation of cell suspensions of tobacco, rice and wheat (Abdul-Baki, *et al*., 1990; De la Pena, *et al*., 1987; Zaghmout and Trolinder, 1993), and protocols for regeneration of transgenic plants are available. For maize in particular, the transformation efficiencies are comparable to those obtained by bombardment (Dashayes *et al*., 1985; D'Halluin *et al*., 1992).

7. Electro-transformation

DNA can also be delivered into cells, tissues and organs by electrophoresis (Ahokas 1989; Griesbach and Hammond, 1994; Songstad *et al.*, 1995). This method is known as transformation by electrophoresis or electro-transformation. The tissue to be transformed is placed between the cathode and anode. The anode is placed in a pipette tip containing agarose mixed with the DNA to be used for transformation. The assembly is illustrated in Figure 3.

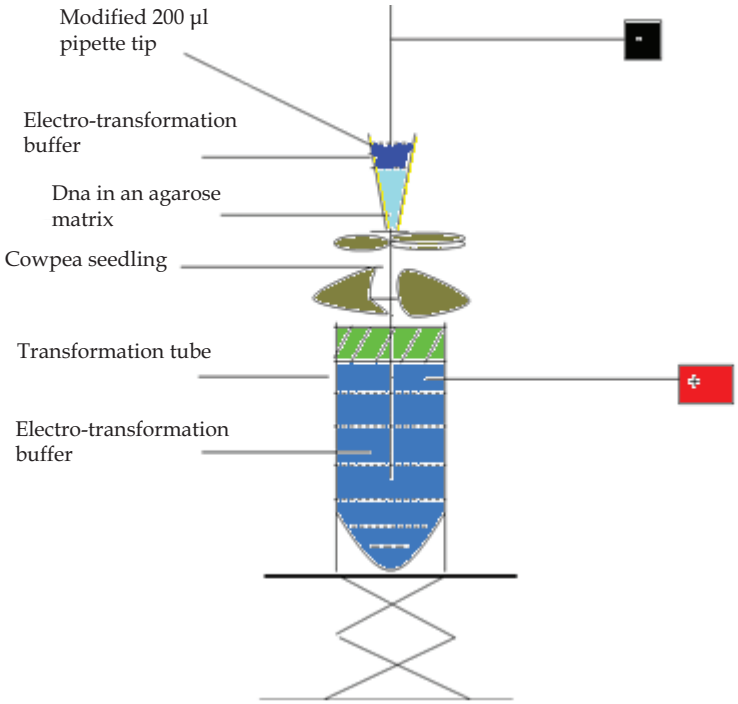


Fig. 3. Diagrammatic illustration of the electro-transformation equipment and experimental set-up

We used this method of transformation on cowpea seedlings, at a time when there was no efficient, reliable, reproducible method for cowpea transformation. The main obstacles to cowpea transformation were that the tissues into which DNA could be introduced failed to regenerate whole plants. We therefore decided to target apical meristems for transformation. In the event of successful transformation, the seeds from transgenic branches of the cowpea plants would be transgenic, and could be screened for desired transformation events.

We had previously made constructs based on CABMV coat protein gene designed to confer various levels of resistance to the virus in transgenic plants (Figure 2). Circular or linearised binary plasmid constructs were electrophoresed into the apical meristematic region of cowpea seedling of various ages and lengths, untreated or pre-treated with acid or alkali, under various conditions of current and voltage as summarized in Table 1.

7.1 Electrotransformation of cowpea

Cowpea (*Vigna unguiculata* variety 475/89) seeds were sterilized by shaking in 10% (v/v) bleach for 10 min at room temperature, and washed with double distilled water for 5 min. The seeds were then rolled on a moistened paper towel and placed in a beaker with water and incubated in the growth room at 28˚C until the seeds germinated (7 – 12 d).

For each transformation attempt, a seedling was removed from the paper towel, pre-treated (where applicable) and placed in the transformation tube. About 1 µl of DNA (0.5µg/µl, circular, or linearized by *Nhe*I or *Nhe*I/*Nde*I digestion) was mixed with about 9 µl of 2% (v/v) low melting point agarose (made up in transformation buffer) and allowed to set at the tip of a 200 µl pipette whose tip had been widened by cutting. Both the pipette tip and the transformation tube (Figure 2) were filled with transformation buffer (0.12 M LiCl, 1 mM Hepes, 0.54 mM $MgCl_2$, 0.005% L-ascorbic acid, pH 7.2). The setup (Figure 3) was connected to a power source and allowed to run under the various current and voltage settings.

The aspects of the seedlings that were noted include the height and age of the plant on the day of manipulation, whether the cotyledons were still attached to the plant or had fallen off, and whether the first true leaves were open or closed. The pretreatments were: none, punched meristem, seedling were exposed to temperatures of 35 °C for 1 hour before manipulation, the manipulations were carried out at increased temperatures of >30 °C, meristems and leaves pretreated with 0.1M HCl, or 0.1 M $CaCl_2$, or 2,4-D + kinetin, NAA + BAP. The voltage settings used were DC or AC, at 30, 40, 125 or 250 V; the current was either 1.0 or 0.15 mA), the duration was kept constant at 15 min. The distance between the electrodes varied with the length of the seedling, and was recorded.

Plant ID at screening	DNA construct	Current/ Time/ Distance between electrodes	Age (days)/ Size (cm)	Stem	First true leaves	Cotyledons	Notes
217	pBI121-CP_{core}, circular	0.15 V 15 min 7 cm	7 d 8 cm	Straight	Open	On	No pretreatment
301	pBI121-CP_k, *Nhe*I linearized	0.15 V 15 min 1.5 cm	8 d 6 cm	Straight	Open	On	No pretreatment, AC 30 sec
309	pBI121-CP_k, *Nhe*I linearized	0.15 V 15 min 7 cm	3 d 5 cm	Straight	Open	On	No pretreatment
398	pBI121-CP_k, *Nhe*I linearized	0.15 V 15 min 6 cm	8 d 9 cm	Straight	Open	On	Punched meristem

Table 1. below summarizes the potentially transgenic events that were obtained in the experiment

A common feature of the GUS positive plants in Table 1 is that the manipulations were carried out on plants that had straight stems, first true leaves open and cotyledons still attached to the seedling. No pre-treatment other than maybe punching the meristem appear to be necessary. The pre-treatments except punching the meristem do not seem to increase transformation efficiency. Both DC and AC are effective in delivery DNA to the plant cells.

The leaves of GUS positive plants had a sectored appearance; this was not unexpected since the transformation procedure targets the general apical meristem area of the cowpea seedling. As a result, both meristematic and somatic cells may become transformed, to result in a chimeric plant. Such a chimeric plant appears as a mosaic of transformed and non-transformed sectors, and poses a challenge in terms of sampling especially in this particular case where a destructive GUS assay was used. Since PCR is very sensitive and amplifies any signal present, the CP transgene could be detected in some GUS positive plants. However, the signal detected by both the GUS assay and PCR could be transient, and Southern analysis is the standard way of determining whether integration has occurred. Southern and other analyses of these lines through subsequent generations, if fertile, would be necessary. There is need to ensure that the germline is transformed to enable the transgene to be passed to subsequent generations.

GUS positive sectors were obtained only from plants that had cotyledons attached, open first true leaves and had developed straight stems at the time of manipulation. The electrotransformation procedure stresses the seedling, and only those seedlings that have developed sufficiently will take up exogenous DNA, survive and develop using the food reserve of cotyledons as well as the photosynthate from first true leaves. The pBI121 binary constructs used in this experiment have a gene for kanamycin resistance.

However, kanamycin resistance is not an effective assay against germinating cowpea seedlings since the germinating cowpea seedlings were not affected by kanamycin. This is probably because of the large food reserves of the seedlings.

The various seedling pre-treatments except punching the meristem did not appear to improve transformation efficiency. Punching the meristem wounds the seedling and may make the meristematic cells more accessible to the exogenous DNA since the epidermal cells will have been removed. Acid and calcium chloride pretreatments were expected to make the cell wall and cell membrane respectively more permeable to DNA. Besides chemically weakening the cell wall, acid pretreatment may also induce the production of expansins that may result in further weakening of cell walls (Cosgrove, 2001). The heat and plant growth substance pretreatments were expected to induce other chemical messengers and heat shock proteins that may increase the chances of integration events in the cell (Hong & Verling, 2001). However, no improvement in transformation efficiency was observed.

The mechanism of DNA integration after uptake by electrophoresis is not known, but is likely to occur by non-homologous recombination into sites on the genome that are undergoing repair or replication, as is the case for other direct DNA transfer methods (Smith *et al.*, 2001). Not all GUS-positive lines tested CP-positive possibly because of incomplete transfer. This also means that it is possible that some transformants were GUS-negative but CP-positive, and these would not detected in this screening procedure.

Transformation by electrophoresis, if successful, is a procedure that can be used to avert one of the major concerns of GMOs. The procedure does not necessarily require the use of selectable markers such as antibiotic or herbicide resistance genes, and only the exact sequence required for a particular characteristic in the transgene may be used. It is not understood how integration would occur, but T-DNA borders do not seem to be required. DNA integration by direct transformation methods appears to be random. In this experiment, transformation is not enhanced by pre-treatment with high temperature,

hydrochloric acid, calcium chloride, kinetin, BAP or NAA. Both circular and linearised DNA seemed to be effective. However, the seedling must have developed a straight stem with the first true leaves open, but the cotyledons must be intact. This may be important in ensuring survival of the seedling after the rather harsh handling and subjection to electrophoresis that stresses the plant.

8. Other methods of plant transformation

8.1 Microinjection

DNA can also be delivered to the plant cell nucleus or cytoplasm by microinjection. This approach is more widely used for large animal cells such as frog egg cells or cells of mammalian embryo. Animal cells are usually immobilised with a holding pipette and gentle suction. For plant cells, the cell wall which contains a thick layer of cellulose and lignins is a barrier to the glass microtools. Removal of the cell wall to form protoplasts might allow use of the microtools, but the plant cells might release hydrolases and other toxic compounds from the vacuole, leading to rapid death of the cells (Lorz *et al*., 1981). Protoplasts may also be attached to glass slides by coating with polyL- lysine, or by or agarose. Poly-L-lysine is toxic to some cells. Agarose reduces visibility around the cells to be manipulated. Microinjection has been used for the transformation of tobacco (Schnorf *et al.,* 1991), petunia (Griesbach, 1987), rape (Neuhaus *et al*., 1987) and barley (Holm *et al*., 2000), with the transgenic plants being recovered at very low frequencies. Microinjection therefore remains of limited use for plant transformation, even though it would be very attractive for introduction of whole chromosomes into plant cells.

8.2 Silicon carbide whisker-mediated transformation

In this method of plant transformation, silicon carbide crystals (average dimensions of 0.6 µm diameter, 10 – 80 µm long) are mixed with DNA and plant cells by vortexing, enabling the crystals to pierce the cell walls (Kaeppler *et al*., 1990, Songstad *et al*., 1995). The method appears to be widely adaptable, and can be used with as little as 0.1 µg DNA. It appears as if there is a lot of scope for further development of this method of plant transformation (Thompson *et al*., 1995).

The method is simple and easy to adapt to new crops, but the transformation efficiencies are low, and the fibres must be handled with care since they pose a health risk to the experimenter. Success has however been reported with maize (Bullock *et al*., 2001; Frame *et al*., 1994; Kaepler *et al*., 1992; Petolino *et al*., 2000; Wang *et al*., 1995), rice (Nagatani, 1997), wheat (Brisibe, *et al*., 2000; Serik, *et al*., 1996), tobacco (Kaeppler *et al*., 1990), *Lolium multiflorum*, *L. perenne*, *Festuca arundinacea*, and *Agrostis stolonifera* (Dalton *et al*., 1998).

8.3 The pollen tube pathway

DNA is applied to the cut styles shortly after pollination, and flows down the pollen tube to reach the ovules. This approach has been used to transform rice (Luo an Wa, 1988), wheat (Mu *et al*., 1999), soybean (Hu and Wang 1999), *Petunia hybrida* (Tjokrokusumo *et al*., 2000) and watermelon (Chen *et al*., 1998). Relatively high transformation efficiencies have been reported.

	Transformation Method	Short Description	Pros	Cons	Main Results Achieved
Indirect transfer methods	*Agrobacterium*-mediated	T-DNA mobilized from *Agrobacterium* into the plant cell under the direction of *Agrobacterium*-encoded virulence proteins	Based on a naturally occurring process	Marker and reporter genes required Vector back-borne often integrated into the plant genome	Mono- and dicotyledonous plants Field-tested and commercialized. Very successful
Direct transfer methods	Microprojectile bombardment/ Biolistics	Tungsten or gold microprojectiles coated with DNA are propelled at high speed across the cell barriers into the nucleus	Not cultivar or genotype dependent	Multiple copies often reported	Non-homologous recombination. Also organelle transformation
	Direct protoplast transformation – electroporation or PEG-mediated	With cell wall removed, DNA can be moved into the cell by methods similar to those used on bacteria	Introduction of DNA into protoplasts is easy	Dependent on ability to regenerate whole plants from protoplast	Can also be used for organelle transformation
	Electroporation of cells and tissues	High voltage discharge is used to open pores on the cell membrane and carry DNA into the cell	Higher regeneration success than with protoplasts	Protocol for regeneration required	Maize, rice, tobacco and wheat
	Electro-transformation	Electric current is used to carry DNA cells or tissues of intact plants	Circumvents problems associated with regeneration,	Low success rates. Needs further investigation of factors to improve success	Experimental
	Microinjection	DNA delivered through a needle into cells immobilized by microtools	Can potentially be used for the introduction of whole chromosomes	Practical only for protoplasts.	Tobacco, *Petunia*, rape and barley
	Silicon carbide mediated transformation	Silicon carbide whiskers coated with DNA pierce and enter the cells	The method is widely adaptable, and requires little DNA	Low transformation efficiencies. Silicon carbide whiskers are a health risk to the experimenter.	Tobacco, maize, rice, other grasses.
	The pollen tube pathway	DNA delivered to ovule via cut end of pollen tube	Apparently widely applicable.	Apparently widely applicable, but particular protocols need to be developed	Successful for rice, wheat, soybean, water melon and *Petunia hybrida*
	Liposome mediated transformation	Liposomes loaded with DNA are made to fuse with protoplast membrane	Uptake depends on the natural process of endocytosis	Effective only for protoplasts	Success for tobacco and wheat
	Infiltration	A suspension of *Agrobacterium* cells habouring the DNA construct of interest is vacuum-infiltrated into inflorescences	Simple procedure	Not generally applicable to most species	Very efficient for *Arabidopsis*

Table 2. Summary of plant transformation methods

A modification of the procedure is to inject plasmid DNA or *A. tumefaciens* carrying the plasmid DNA into inflorescences in the premeiotic stage, without removing the stigma, as was done for rye (De la Pena *et al*., 1987), to result in high transformation efficiencies.

8.4 Liposome mediated transformation

Liposomes are microscopic spherical vesicles that form when phospholipids are hydrated. They can be loaded with a variety of molecules, including DNA. Liposomes loaded with DNA can be made to fuse with protoplast membrane and deliver their contents into the cytoplasm by endocytosis. Liposomes can also be carried through the pores of pollen grains to fuse with the membrane of the pollen grain. Transgenic plants have been reported by liposome-mediated transformation only from tobacco (Dekeyser *et al*., 1990) and wheat (Zhu *et al*., 1993). The process is inexpensive, but is laborious and inefficient, and so has not been widely adopted. It might be worthwhile to consider delivering the liposomes through the pollen tube pathway.

8.5 Infiltration

Infiltration (vacuum infiltration) is a method for plant transformation almost exclusively used for the transformation of *Arabidopsis*. Inflorescences of plants in early generative phase (5 – 15 cm) are immersed in *A. tumefaciens* and 5% sucrose. The inflorescences are then placed under vacuum for several minutes. Typically 0.5 to 4% of the seeds harvested from the inflorescences will be transgenic (Chung *et al*., 2000; Clough *et al*., 1998; Ye *et al*., 1999). This method is highly optimized and works well for *Arabidopsis*.

9. Summary and conclusions

There now exists a wide variety of methods of plant transformation that can be used to produce virus-resistant plants (Table 2). *Agrobacterium*-mediated transformation and microprojectile bombardment have been used to produce virus resistant plants that have been field-tested, or even been commercialized. These transgenic plants are also important as study material to further understand the methods of plant transformation. However, consumer demands require continuous improvement of these methods, and it is hoped that some of these methods will evolve to become marker-free, vector-free plant transformation methods.

10. Acknowledgements

We acknowledge The French Ministry of Foreign Affairs for funding the tobacco transformation experiments and The Rockefeller Foundation for funding the cowpea transformation experiments.

11. References

Abdul-Baki, A.A., Saunders, J.A., Matthews, B.F. and Pittarelli, G.W. (1990). DNA uptake during electroporation of germinating pollen grains. Plant Sci. 70:181-190.

Ahokas, H. (1989). Transfection of germinating barley seed electrophoretically with exogenous DNA. *Theor. Appl. Genet*. 77: 469-472.

An, G., Watson, B.D., Stachel, S., Gordon, M.P. and Nester, E.W. (1987). New cloning vehicles for transformation of higher plants. *EMBO Journal*, 4: 277 – 284.

Baulcombe, D.C. (1996). Mechanisms of pathogen-derived resistance to viruses in transgenic plants. *The Plant Cell*, 8: 1833 – 1844.

Barton, K.A., Binns, A.N., Matzke, A.J.M. and Chilton, M.D. (1983). Regeneration of intact tobacco plants containing full-length copies od genetically engineered T-DNA, and transmission of T-DNA to R1 progeny. *Cell*, 32: 1033 – 1043.

Beachy, R.N., Abel, P, Oliver, M.J., (1986). Potential for applying genetic information to studies of virus pathogenesis and cross protection. In: M. Zaitlin, P. Day and A. Hollaender (eds). *Biotechnology in Plant Science: Relevance to Agriculture in the Eighties*, pp 265 – 275.

Beavan, M.W., Flavell, R.B., and Chilton, M.D. (1983). A chimaeric antibiotic resistance gene as a selectable marker for plant cell transformation. Nature, 304: 184 – 187.

Bevan, M.W., Mason, S.E. and Goelet, P. (1985). Expression of tobacco mosaic virus coat protein by a cauliflower mosaic virus promoter in plants transformed by *Agrobacterium*. *EMBO J.*, 4: 1921 – 1926

Belkhadir, Y., Subramaniam, R. and Dangl, J.L. (2004). *Current Opinions in Plant Biology* 7: 391 – 399.

Birch, R.G. (1997). Plant transformation: Problems and strategies for practical application. *Annual Reviews of Plant Physiology Plant Molecular Biology*, 48: 297 – 326.

Brisibe, E.A, Gajdosava, A., Olsen, A. and Andersen, S.B. (2000). Cytodifferentiation and transformation of embryogenic callus lines derived from anther culture of wheat. *J. Exp. Bot*. 51:187-196.

Bower, R. and Birch, R.G. (1990). Competence for gene transfer by electroporation in a subpopulation of protoplasts from a uniform carrot cell suspensions. *Plant Cell Reports*, 9: 386 – 389

Bullock, W., Dias, D., Bagnal, S., Cook, K., Teronde, S., Ritland, J., Spielbauer, D., Abbaraju, R., Christensen, J. and Heideman, N. A high effuciency maize "whisker" transformation system. *Plant and Animal Genomes IX Conference*, San Diego, CA, Jan 13-17, 2001. Abstr. 148.

Chen, W.S., Chiu, C.C., Liu, H.Y., Lee, T.L., Cheng, J.T., Lin, C.C., Wu, Y.Y. and Chang, H.Y. (2002). Gene transfer via pollen-tube pathway for anti-fusarium wilt in watermelon. *Bioch. Mol. Biol. Intern.* (46) 1201-1209.

Cheng, M., Lowe, B.A., Spenser, T.M, Ye, X.D. and Armstrong, C.L. (2004). Factors influencing Agrobacterium-mediated transformation of monocotyledonous species. *In Vitro Cellular & Developmental Biology – Plant* (40): 31 – 45.

Chung, M.H., Chen, M.K. and Pan, S.M. (2000). Floral spray transformation can efficiently generate *Arabidopsis* transgenic plants. *Transgen. Res*. 9: 471-476.

Christou, P., McCabe, D.E., and Swain, W.F. (1988). Stable transformation of soybean callus by DNA-coated gold particles. *Plant Physiology*, 87: 671 – 674.

Christou, P., Swain, W.F., Yang, N-S., and McCabe, D.E. (1989). Inheritance and expression of foreign genes in transgenic soybean plants. *Proceeding of the National Academy of the Sciences*, U.S.A. 86: 7500 – 7504.

Clough, S.J. and Bent, A.F. Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J*. 16 (1998) 735-743.

Cooley, J., Ford, T., and Christou, P. (1995). Molecular and genetic characterisation of elite transgenic rice plants produced by electric discharge particle acceleration. *Theoretical and Applied Genetics*, 90: 744 – 104.

Cosgrove, D.J. (2001). Loosening plant cell walls by expansins. *Nature*, 407: 321 – 326.

Dalton, S.J., Bettany, A.J.E., Timms, E., and Morris, P. (1998). Transgenic plants of *Lolium multiflorum, Lolium perenne, Festuca arundinacea*, and *Agrostis solonifera* by silicon carbide whisker-mediated transformation of cell suspension cultures. *Plant Science*, 132: 31 – 43.

Dekeyser, R.A., Claes, B., De Rycke, R.M.U., Habets, M.E., Van Montagu,

M.C. and Caplan, A.B. (1990). Transient gene expression in intact and organized rice tissues. *The Plant Cell* 2: 591-601.

Deshayes, A., Herrera-Estrella, L. and Caboche, M. (1985). Liposome-mediated transformation of tobacco mesophyll protoplasts by an Escherichia coli plasmids. *EMBO J.* 4:2731-2737.

Dinesh-Kumar, S.P., Tham, W-H. and Baker, B.J. (2000). Structure-function analysis of the tobacco mosaic virus resistance gene N. *Proceedings of the National Academy of the Sciences, USA*, 97 (26): 14789 – 14794.

Ellis, J., Dodds, P. and Pryor, T. (2000). The generation of plant disease resistance gene specificities. *Trends in Plant Sciences*, 5: 373 – 379.

Ellis, J., Dodds, P. and Pryor, T. (2000). Structure, function and evolution of plant disease resistance genes. *Current Opinions in Plant Biology*, 3: 278 – 284.

Frame, B.R., Drayton, P.R., Bagnall, S.V., Lewnau, C.J., Bullock, W.P., Wilson, H.M., Dunwell, J.M., Thompson, J.A. and Wang, K. (1994). Production of fertile transgenic maize plants by silicon carbide whisker-mediated transformation. *Plant J.* 6 941-948.

Frame, B.R., Drayton, P.R., Bagnall, S.V., Lewnau, C.J., Bullock, P., Wilson, H.M., Dunwell, J.M., Thompson, J.A., and Wang, K. (1994). Production of fertile transgenic maize plants by silicon carbide whisker-mediated transformation. *Plant Journal*, 6: 941 – 948.

Gharti-Chhetri, G.B., Cherdshewasart, W., Dewulf, J., Paszkowski, J., Jocabs, M. and Negrutiu, I. (1990). Hybrid genes in the analysis of transformation conditions. III. Temporal-spatial fate of NPTII gene integration, its inheritance and actors affecting these processes in *Nicotiana plumbaginifolia*. Plant Molecular Biology, 14: 687 – 696.

Gonsalves, D and Gansey, S.M. (1989). Cross protection techniques for control of plant virus diseases in the tropics. *Plant Diseases*, 73: 592 - 597.

Griesbach, R.J. (1987). Chromosome-mediated transformation via microinjection. *Plant Sci*. 50:69-77.

Griesbach, R.J. and Hammond, J. An improved method for transforming plants through electrophoresis. *Plant Sci*. 102 (1994) 81-89.

Hadi, M.Z., McMullen, M.D., and Finer, J.J. (1996). Transformation of 12 different plasmids into soybean via particle bombardment. *Plant Cell Reports*, 15: 500 – 505.

Hemmenway, C., Fang, R.-X., Kaniewski, W.K., Chua, N.-H. and Turner, N.E. (1988). Analysis of the mechanism of protection in transgenic plants expressing the potato virus X coat protein or its antisense RNA. *EMBO J.*, 7: 1273 – 1280.

Herrera-Estrella, L., Depicker, A., Van Montagu, M. and Schell, J. (1983). Expression of chimeric genes transferred into plant cells using a Ti-plasmid-derived vector. *Nature*, 303: 209 – 213.

Hohn, T. and Vasquez, F. (2011). RNA silencing pathway of plants: Silencing and its suppression by plant DNA viruses. *Biochemica et Biophysica Acta* (xx): x – xx. Doi: 10:1016/j.bbagrm.2011.06.002

Holm, P.B., Olsen, O., Schnorf, M., Brinch-Pederse, H. and Knudsen, S. Transformation of barley by microinjection into isolated zygote protoplasts. *Transgen. Res*. 9 (2000) 21-32.

Hong, S.W. and Verling, E. (2001). Hsp101 is necessary for heat tolerance but dispensable for development and germination in the absence of stress. *Plant Journal*, 27: 25 – 35

Hu, C.Y. and Wang, L. (1999). In planta soybean transformation technologies developed in China: procedure, confirmation and field performance. *In Vitro Cell. Dev. Biol.-Plant* 35: 417-420.

Hull, R. (2001). Matthews' Plant Virology (Fourth Edition). Academic Press, San Diego.

Kaeppler, H.F., Gu, W., Somers, D.A., Rines, H.W. and Cockburn, A.F. (1990). Silicon carbide fiber-mediated DNA delivery into plant cells. *Plant Cell Reports*, 9: 415 – 418.

Kaeppler, H.F., Somers, D.A., Rines, H.W. and Cockburn, A.F. (1992). Silicon carbide fibre mediated stable transformation of plant cells. *Theoretical and Applied Genetics*, 84: 560 – 566.

Kartzke, S., Saedler, H., and Meyer, P. (1990). Molecular analysis of transgenic plants derived from transformations of protoplasts at various stages of the cell cycle. *Plant Science*, 67: 63 –72.

Katiyar-Agarwal, S. and Jin, H. (2010). Role of small RNAs in host-microbe interactions. *Annual Review of Phytopathology*, 48: 225 – 246.

Khetarpal , R.K., Maisonneuve, B., Maury *et al*., (1998). Breeding for resistance to plant viruses. In: A. Hadidi, R.K. Khetarpal and H. Koganezawa (eds) *Plant Virus Disease Control.* pp. 14 – 32. APS Press, St Paul, MN.

Klein, T.M., Wolf, E.D. and Wu, R. (1987). High velocity microprojectiles for delivering nucleic acids into living cells. *Nature*, 327: 70 –73.

Kohli, A., Ghareyazie, B., Kim, H.S., Khush, G.S., Bennett, J. and Khush, G.S. (1996). Cytosine methylation implicated in silencing of β-glucuronidase gene in transgenic rice. *Rice Genetics Symposium-III*, pages 825 – 828. IRRI, Manilla, Philippines.

Koehler, F., Cardon, G., Poehlman, M., Gill, R., and Schieder, O. (1989). Enhancement of transformation rate in higher plants by low-dose irradiation – Are DNA repair systems involved in the incorporation of exogenous DNA into the plant genome? *Plant Molecular Biology*, 12: 189 – 200.

Koehler, F., Benediktsson, I., cardon, G., Andreo, C.S., and Schieder, O.(1990). Effect of various irradiation treatments of plant protoplasts on the transformation rates after direct gene transfer. *Theoretical and Applied Genetics*, 79: 679 – 685.

Kohli, A., Leech, M., Vain, P., Laurie, D.A. and Christou, P. (1998). Transgene organisation in rice engineered though direct DNA transfer supports a two-phase integration mechanism mediated by the establishment of integration hot spots. *Proceedings of the National Academy of the Sciences*, USA, 95: 7203 – 7208.

Kumar, S., Allen, G.C. and William, F.T. (2006). Gene targeting in plants: fingers on the move. Trends in Plant Science, 11(4): 159 – 161.

Lindsey, K., Wei, W., Clarke, M.C., McArdale, H.F. and Rooke, L.M. (1993). Tagging genomic sequences that direct transgene expression by activation of a promoter trap in plants. *Transgenic Research*, 2: 33 – 47.

Lodge, J.K., Silva-Rosales, L., Proebsting, W.M. and Dougherty, W.G. (1993). Induction of a highly specific antiviral state in transgenic plants: Implications for regulation of gene expression and virus resistance. *Plant Cell*, 5 (12): 1749 - 1759.

Lörz, H., Paszkowski, J., Dierks-Ventling, C. and Potrykus, I. (.1981) Isolation and characterization of cytoplasts and miniprotoplasts derived from protoplasts of cultured cells. *Physiol. Plant* 53:385-391.

Luo, Z.X. and Wa, R. (1998). A simple method for the transformation of rice via pollen-tube pathway. *Plant Mol. Biol. Report* 6 :165-174.

Marton, L., Wullems, G.J., Molendijk, L. and Schilperoort, R.A. (1979). *In vitro* transformation of cultured cells from *Nicotiana tabacum* by *Agrobacterium tumefaciens*. *Nature*, 277: 129 – 131.

Masuta, C., Tanaka, H., Vehara, K., Kuwata, S., Koiwai, A. and Noma, I. (1995). Broad resistance to plant viruses in transgenic plants conferred by antisense inhibition of a host gene essential in S-adenosyl methionine-dependent transmethlation reactions. *Proceedings of the National Academy of the Sciences*, USA, 92 (13): 6117 - 6121.

Matzke, A.J.M. and Chilton, M. (1981). Site specific insertion of genes into T-DNA of the *Agrobacterium* tumour-inducing plasmid: an approach to genetic engineering of higher plant cells. *Journal of Molecular and Applied Genetics*, 1: 39 – 49.

Maliga, P. (2004). Plastid transformation in Higher Plants. *Annual Reviews of Plant Biology* 55: 289 – 313.

Mundembe, R., Matibiri, A. and Sithole-Niang, I. (2009). Transgenic plants expressing the coat protein gene of cowpea aphid-borne mosaic potyvirus predominantly convey the delayed symptom development phenotype. African Journal of Biotechnology, 8(12): 2682 – 2690.

Nagatani, N., Honda, H., Shimada, T. and Kobayashi, T. (1997). DNA delivery into rice cells and transformation using silicon carbide whiskers. *Biotechnol. Techniq.* 11:781-786.

Ow, D.W. (2002). Recombinase-directed plant transformation for the post-genomic era. *Plant Molecular Biology* 48: 183 – 200.

Parmyakova, N.V., Shumnyi, V.K. and Deineko, E.V. (2008). Agrobacterium-mediated transformation of plants: Transfer of vector DNA fragments in the plant genome. *Russian Journal of Genetics* 45 (3): 266 – 275.

Pazkowski, J., Shillito, R.D., Saul, M., Mandak, V., Holn, T., Holn, B., and Potrykus, I. (1984). Direct gene transfer to plants. *EMBO Journal*, 3: 2717 – 2722.

Petolino, J.F., Hopkins, N.L., Kosegi, B.D. and Skokut, M. (2000). Whisker mediated transformation of embryogenic callus of maize. *Plant Cell Rep*. 19 (2000) 781-786.

Powell-Abel, P., Nelson, R.S., De B., Hoffmann, N., Rodger, S.G., Fraley, R.T. and Beachy, R.N. (1986). Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. *Science*, 232: 738 - 743.

Powell-Abel, P., Saunders, P.R., Turner, N., Frayley, R.T. and Beachy, R.N. (1990). Protection against tobacco mosaic virus infection in transgenic plants requires accumulation of coat protein rather than coat protein RNA sequences. *Virology*, 175: 124 – 130.

Rakoczy-Trojanowska, M. (2002). Alternative methods of plant transformation – a short review. *Cellular and Molecular Biology Letters* 7: 849 – 858.

Ready, M.P., Brown, D.T. and Robertus, J.D. (1986). Extracellular localisation of pokeweed antiviral protein. *Proceedings of the National Academy of the Sciences*, (USA), 83: 5053 – 5056.

Register, J.C. and Beachy, R.N. (1988). Resistance to TMV in transgenic plants results from interference with an early event in infection. *Virology*, 166: 524 – 532.

Sairam, R.V. and Prakash, C.S. (2005). OBPC Symposium: Maize 2004 and Beyond – Can agricultural biotechnology contribute to food security? *In Vitro Cellular & Developmental Biology – Plant* 41: 424 – 430.

Sanford, J.C. and Johnson, S.A. (1985). The concept of parasite-derived resistance: deriving resistance genes from the parasite's own genome. *Journal of Theoretical Biology*, 115: 395 – 405.

Sanford, J.C., Klein, T.M., Wolf, E.D., and Allen, N. (1987). Delivery of substances into cells and tissues using a particle bombardment process. *Journal of Particulate Science and Technology*, 5: 27 – 37.

Sithole-Niang, I., Nyathi, T., Maxwell, D.P. and Candresse, T. (1996). Sequence of the 3'-terminal region of Cowpea aphid-borne mosaic virus (CABMV). *Archieves of Virology,* 141: 935 – 943

Smith, N., Kilpatrick, J.B. and Whitelam, G.C. (2001). Superfluous transgene integration in plants. *Critical Reviews in Plant Sciences*, 20(3): 215 – 249.

Songstad, D.D., Somers, D.A., and Griesbach, R.J. (1995). Advances in alternative DNA delivery techniques. *Plant Cell Tissue and Organ Culture*, 40: 1 –15.

Tavladoraki, P., Benvenuto, E., Trinca, S., De Martinis, D., Cattanco, A. and Galeffi, P. (1993). Transgenic plants expressing a functional single chain Fv antibody are specifically protected from virus attack. *Nature* (London), 366 (6454): 469 – 472).

Thompson, J.A., Drayton, P.R., Frame, B.R., Wang, K. and Dunwell, J.M. (1995). Maize transformation utilizing silicon carbide whiskers: a review. *Euphytica*, 85: 75 – 80.

Thottappilly, G. and Rossel, H.W. (1992). Virus diseases of cowpea in tropical Africa. *Tropical Pest Management* 38(4): 337 – 348.

Tinland, B. (1996). The integration of T-DNA into plant genomes. *Trends in Plant Sciences* 1 (6): 178 – 183.

Tjokrokusumo, D., Heinrich, T., Wylie, S., Potter, R. and McComb, J. (2000). Vacuum infiltration of Petunia hybrida pollen with *Agrobacterium tumefaciens* to achieve plant transformation. *Plant Cell Rep*. 19:792-797.

Tzfira, T, Jianxiong, L., Lacroix, B. and Citovsky, V. (2004). *Agrobacterium* T-DNA integration: molecules and models. *Trends in Genetics* 20 (8): 375 - 378

Vasil, V., Brown, S.M., Re, D., Fromm, M.E., and Vasil, I.K. (1991). Stably transformed callus lines from microprojectile bombardment of cell suspension cultures of wheat. *Bio/Technology*, 9: 743 – 747.

Voss, A., Niersbach, M., Hain, R., Hirsch, H.J., Liao, Y.C., Kreuzaler, F. and Fischer, R. (1995). Reduced virus infectivity in *N. tabacum* secreting a TMV-specific full size antibody. *Molecular Breeding*, 1 (1): 39 - 50.

Wang, K., Herrera-Estrella, L., Van Montagu, M. and Zambryski, P. (1984). Right 25 bp terminus sequence of the nopaline T-DNA is essential for and determines direction of DNA transfer from *Agrobacterium* to the plant genome. *Cell* 38: 455 – 462.

Wang, H.L., Yeh, S.D., Chiu, R.J. and Gonsalves, D. (1987). Effectiveness of cross-protection by mild mutants of papaya ringspot virus for control of ringspot disease of papaya in Taiwan. *Plant Diseases*, 71: 491 – 497.

Wang, K., Drayton, P., Frame, B., Dunwell, J., Thompson, J.A. (1995). Whisker mediated plant transformation: an alternative technology. *In Vitro Cell Dev. Biol*. 31 (1995) 101-104.

Wilson, T.M.A. (1993). Strategies to protect crop plants against viruses – pathogen derived resistance blossoms. *Proceedings of the National Academy of the Sciences*, USA, 90: 3134 – 3141.

Yamashita, T., Iida, A. and Morikawa, H. (1991). Evidence that more than 90% of β-glucuronidase-expressing cells after particle bombardment directly receive the foreign gene in their nucleus. Plant Physiology, 97: 829 – 831.

Yang, B., Sugio, A. and White, F.F. (2006). Os8N3 is a host disease-susceptibility gene for bacterial blight of rice. *Proceedings of the National Academy of the Sciences*, USA, 103 (27): 10503 -10508.

Ye, G.N., Stone, D., Pang, S.Z., Creely, W., Gonzalez, K. and Hinchee, M.(1999), *Arabidopsis* ovule is the target for *Agrobacterium in planta* vacuum infiltration transformation. *Plant J*. 19:249-257.

Yuan, D., Bassie, L., Sabalza, M., Miralpeix, B. *et. al* (2011). The potential impact of plant biotechnology on Millenium Development Goals. *Plant Cell Reports* 30: 249 – 265.

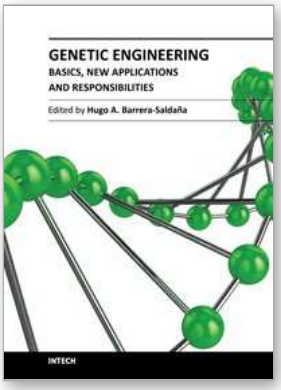
Zaghmout, O.M.F. and Trolinder, N. (1993). Simple and efficient method for directly electroporating plasmid DNA into wheat callus cells. *Nucl. Acid Res*. 21:1048.

Zambryski, P., Joos, H., Genetello, C., Leemans, J., Van Montagu, M., and Schell, J. (1983). Ti plasmid vector for the introduction of DNA into plant cells without alteration of normal regeneration capacity. *EMBO Journal*, 2: 2143 – 2150.

Zhu, J., Oger, P.M., Schrammeiler, B., Hooykaas, P.J.J., Farrand, S.K. and Winans, S.C. (2000). The basis of crown gall tumerigenesis. *Journal of Bacteriology*, 182: 3885 – 3895

Zhu, Z., Sun, B., Liu, C., Xiao, G. and Li, X. (1993). Transformation of wheat protoplasts mediated by cationic liposome and regeneration of transgenic plantlets. *Chin. J. Biotech*. 9:257-261.

Zupan, J. and Zambryski, P. (1997). The Agrobacterium DNA transfer complex. *Critical Reviews in Plant Science*, 16: 279 – 295.



Genetic Engineering - Basics, New Applications and Responsibilities

Edited by Prof. Hugo A. Barrera-Saldaña

ISBN 978-953-307-790-1
Hard cover, 256 pages
Publisher InTech
Published online 18, January, 2012
Published in print edition January, 2012

Leading scientists from different countries around the world contributed valuable essays on the basic applications and safety, as well as the ethical and moral considerations, of the powerful genetic engineering tools now available for modifying the molecules, pathways, and phenotypes of species of agricultural, industrial and even medical importance. After three decades of perfecting such tools, we now see a refined technology, surprisingly unexpected applications, and matured guidelines to avoid unintentional damage to our and other species, as well as the environment, while trying to contribute to solve the biological, medical and technical challenges of society and industry. Chapters on thermo-stabilization of luciferase, engineering of the phenylpropanoid pathway in a species of high demand for the paper industry, more efficient regeneration of transgenic soybean, viral resistant plants, and a novel approach for rapidly screening properties of newly discovered animal growth hormones, illustrate the state-of-the-art science and technology of genetic engineering, but also serve to raise public awareness of the pros and cons that this young scientific discipline has to offer to mankind.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Richard Mundembe, Richard F. Allison and Idah Sithole-Niang (2012). Genetic Engineering of Plants for Resistance to Viruses, Genetic Engineering - Basics, New Applications and Responsibilities, Prof. Hugo A. Barrera-Saldaña (Ed.), ISBN: 978-953-307-790-1, InTech, Available from: http://www.intechopen.com/books/genetic-engineering-basics-new-applications-and-responsibilities/genetic-engineering-of-plants-for-resistance-to-viruses

INTECH
open science | open minds

InTech Europe
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821