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# Maize Transformation to Obtain Plants Tolerant to Viruses by RNAi Technology

Newton Portilho Carneiro and Andréa Almeida Carneiro  
*Embrapa Maize and Sorghum*  
 Brazil

## 1. Introduction

Plants represent the principal source of human foods and livestock feeds and efforts to improve them in many agronomic aspects have focused on plant breeding. The biotechnology revolution in the past decade made possible for plant breeders access new sources of genetic variability for the development of superior cultivars. It has been possible to define additional strategies for crop improvement through the introduction and stable integration of heterologous genes in plant cells with the knowledge of the regulation of the important agronomic characteristics. The genetic manipulation of plants allows their adaptation to different environmental stresses, whether biotic or abiotic. Currently, the production of genetically modified plants occupies a prominent place in both, basic and applied plant research. Genetically modified crops are generated through a process known as genetic engineering, in which genes of interest are transferred to plants without the need of natural crossing. The most widely used methods for introducing transgenes into the genome of plants are *Agrobacterium* mediated transformation and microprojectile bombardment. In the first case, scientists took advantage of the natural ability of *Agrobacterium* to transfer some of its wild genes to plant cells causing the diseases known as crown gall or hairy roots, and replace them by other genes expressing traits of agronomic interest. However, *Agrobacterium* is not able to infect all plants in a very efficient way, as a consequence, new systems for direct transfer of genes to plants emerged. The microprojectile bombardment system is a direct transfer of genes that involves an equipment known as gene gun. The DNA to be introduced into plant cells is physically attached to metal microparticles that are then propelled against the plant cells, using the gene gun. DNA that penetrates the plant cell can be integrated into the plant genome.

Maize is one of the most cultivated cereals in the world. The main maize producer's countries are the United States, China, and Brazil, followed by Mexico, France, Argentina and India. Among the big losses faced by agriculture are the attacks of pests and diseases. For maize, these problems have worsened since 1990 because of the increase of the cultivated areas in both the normal growing season and the off season, mainly due to intensive cultivation of maize in the irrigated areas, and lack of adoption of crop rotation in certain fields. In recent years, diseases that were not a problem, increased in importance such as the viruses. Among the strains of the virus complexes, potyviruses cause significant losses in grain and forage of maize susceptible genotypes. Plants have different mechanisms for protection against invasion by pathogens, and different genes directly related to

tolerance to viruses have been described in maize. Works have been published using methods of obtaining plants resistant to viruses by antisense, co-suppression and, more recently, RNA interference (RNAi).

This chapter reviews methodologies that have been used to introduce the RNAi construct in maize cells, such as *Agrobacterium* and microprojectile bombardment aiming to produce transgenic maize plants tolerant to SCMV. Topics covered in this chapter include maize regeneration in tissue culture, transformation mediated by *Agrobacterium* and microprojectile bombardment, isolation and cloning of the target DNA into RNAi based vectors, some results already obtained with this technology and its application to crop improvement.

## 2. RNA interference

RNA interference is a natural phenomenon of which double stranded RNA (dsRNA) activates a mechanism that degrades complementary RNA in the cell. This process has been described in many organisms such protozoa, flies, nematodes, insects, mouse and human cells (Napoli et al. 1990; Hammond et al., 2001; Agrawal et al., 2003; Baulcombe, 2004; Tang and Galili, 2004) and has been referred as cellular defense against viruses and post-transcriptional regulation of gene expression. In maize, there are extensive reviews done by McGinnis (2009) describing the application of this process as a reverse genetic tool.

Before the identification of the RNAi phenomenon, there were other methods such as T-DNA insertion, transposon elements and physical and chemical mutagens and antisense suppression to generate gene loss-of-function. These approaches, which have been used until today have allowed scientist study the function of many gene or gene families. The earliest version of gene silence was the process called antisense, which involves the introduction of the antisense strand of RNA to silence an internal RNA homologue (Knee and Murphy, 1997). The antisense strand once inside the cell binds to the target RNA by complementation preventing it to be translated. One possible explanation, on that time, was the inability of the ribosomes bind to the dsRNA. Another possible explanation that came up later, was that the dsRNA might also be a substrate for the DICER/RISC an enzymatic complex responsible for degradation of dsRNA in the RNAi process. The first description of the RNAi phenomenon was done by Fire et al. (1998). This group introduced sense and antisense RNA strands in *Caenorhabditis elegans* and obtained a gene silence ten times greater than with the sense or the antisense strand only. Injecting the sense and antisense strands together, in fact, created the double stranded RNA required for the RNA interference process. Later the same phenomenon was described in trypanosomes (Ngo et al., 1998) and flies (Kennerdell and Carthew, 1998). Due to the great interest in the RNAi technology and its applications, many other works were published trying to elucidate the mechanisms involved. Once the dsRNAs are formed in the cell, they are automatically recognized by an enzyme complex called DICER that cleaves them into small fragments known as small interference or siRNA. The DICER was discovered by Bernstein et al. (2001) in *Drosophila* and, it is an enzyme complex belonging to the RNase III family, which has four domains: a) an N-terminal helicase; b) an double RNase III domain; c) a binding domain to the C terminal dsRNA; d) a PAZ domain (Piwi / Argonaute / Zwill) (Agrawal et al., 2003). The siRNAs are composed of 21-25 base pairs (dsRNA) with a 3' end of an additional base. Wei et al. (2003) found that the hydroxyl group 3' was necessary to direct RNAi process *in vitro*. The PAZ domain of DICER seems

that physically interacts with the PAZ domain of the RISC complex. The RISC complex, also discovered in *Drosophila* by Hammond et al. (2001), is a system component that uses RNAi to trace siRNA and degrade complementary mRNAs. The dsRNA present in the tRNA are highly stable molecule that can not be degraded by DICER / RISC complex. A simplified form of the degradation of dsRNA by DICER / RISC complex is shown in Figure 1.

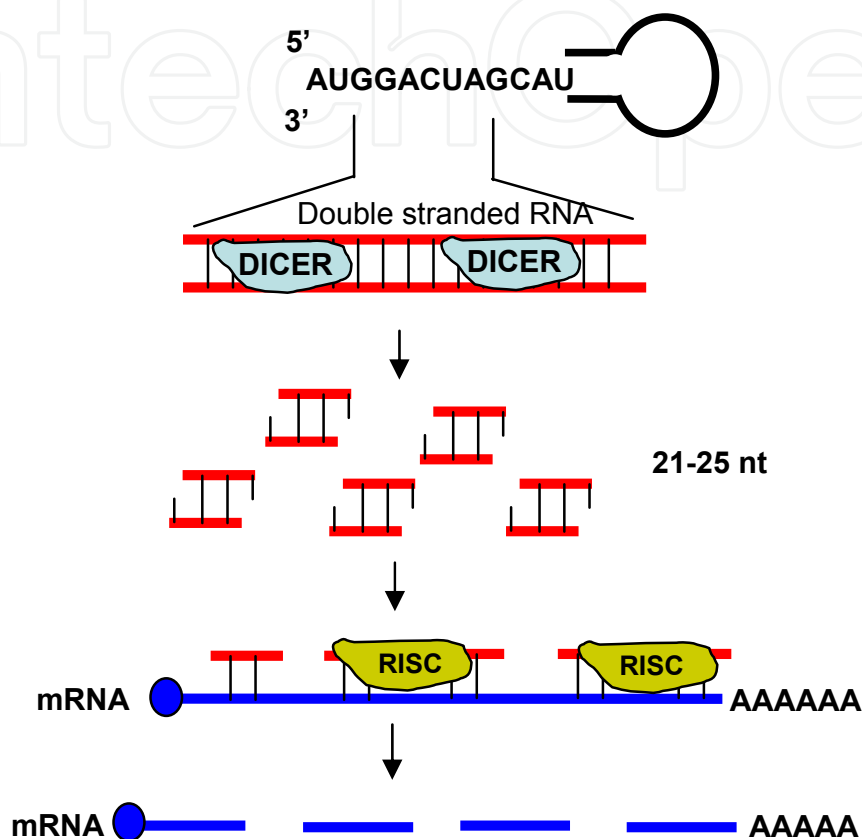


Fig. 1. A simplified diagram of the degradation of dsRNA by DICER / RISC enzyme complex. In the proposed mechanism the DICER RNase III complex identifies the double stranded RNAs and cleaves them into 21 to 25 bp small pieces siRNA. These molecules are then recognized by the RISC complex, that unwound the siRNA, leaving the antisense strand in the RISC, resulting in the complex activation. The activated RISC then targets and cleaves mRNA that is complementary to the antisense strand of the siRNA (Zamore et al., 2000; Kim, 2003; Wall and Shi, 2003).

### 3. Application of RNAi to obtain transgenic maize lines tolerant to the SCMV

Fuchs and Grüntzig (1996) observed that Sugarcane Mosaic Virus (SCMV) and Maize Dwarf Mosaic Virus (MDMV) were the most important potyviruses, causing significant losses in grain and forage of susceptible maize genotypes. In Germany, the maize fields with mosaic symptoms were first found in the early 80's (Fuchs and Kozelska, 1984). Since then, MDMV and SCMV have been regularly observed in maize producing regions of Germany, where epidemiological studies have shown the prevalence of SCMV (Fuchs et al., 1996). For the tropical conditions observed in Brazil, it were described three viruses in maize: (i) mosaic

which can be caused by four distinct potyviruses transmitted mechanically and, by *Rhopalosiphum maidis*; (ii) MRFV (Maize Rayado Fino Virus) , transmitted in a persistent manner by the leafhopper *Dalbulus maidis* and, (iii) MMV (Maize Mosaic Virus), transmitted in a persistent manner by the leafhopper *Peregrinus maidis* (Waquil et al., 1996). The mosaic also attacks sorghum and sugarcane, crops of great economic importance. Besides, numerous species of wild Poaceae constitute reserves of virus inoculum for the cultivated species. Due to non persistent transmission of the potyviruses, control of aphid vectors by chemical is not effective. Therefore, due to ecological and economic reasons, the cultivation of resistant maize varieties is one of the most effective methods of controlling these diseases (Melching, 1998).

The particles of the potyvirus causing the mosaic disease are flexible and have a length of approximately 750 nm and width varying from 13 nm (MDMV and SCMV) to 12 nm for the Johnsongrass mosaic virus (JGMV) (Shukla et al., 1994). Like most plant viruses, the potyviruses have a genome consisting of sense strand RNA-positive, with a length of approximately 10,000 nucleotides and a protein (Vpg) connected to the terminal 5 'genome (Figure 2).

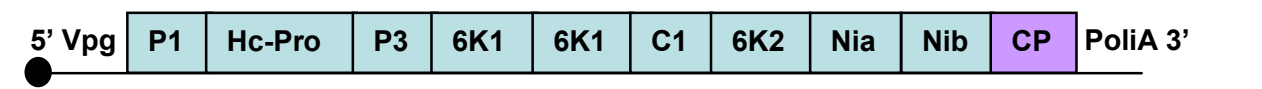


Fig. 2. Schematic representation of genomic organization of potyviruses indicating the proteins encoded by the virus and its possible functions. (P1): first protease; (HC-Pro): helper component - protease; (P3): third protease; (C1): protein with RNA helicase activity; (6K1 and 6K2): peptides; (Nia): nuclear inclusion protease; (Nib): a RNA dependent RNA polymerase; (CP): coat protein. By analogy with other viral systems, it is suggested that Vpg serve as a primer for synthesis of vRNA (Shukla et al., 1994) and stabilization of mRNA against attack by exonucleases.

Plants have different mechanisms for protection against invasion by pathogens such as physical barriers, secondary metabolites and antimicrobial proteins. Once established, elicited molecules produced and released by the pathogen induce new defenses such as cell wall strengthening, phytoalexin production, synthesis of proteins related to plant defense, among others. The identification and application of these mechanisms is one of the most effective manners to rapidly improve crop resistance to diseases. Microarray experiments have shown hundreds of genes regulated by plant-pathogen interactions, most of these are defense-related proteins (PRs) or system acquires resistance (SARs) (van Loon et al., 2006).

An alternative strategy for obtaining materials resistant to pathogens, specifically virus, was published by Grumete et al. (1987), when they over expressed part of the genome of the pathogen in a plant and showed a significant increase in resistance. The explanation given at the time was that the disfunction of the gene products derived from the pathogen could inhibit the pathogen. Similar work has also demonstrated the expression of the coat protein of TMV (Tobacco Mosaic Virus) in the generation of resistant tobacco plants (Abel et al., 1986). These plants in the presence of the virus showed no symptoms or showed a delayed onset of symptoms. Additional experiments showed immediately that the level of transgene expression was correlated with the level of expression of resistance (Fitchen and Beachy, 1993, Powell et al., 1990).



Many different types of viruses in plants have been shown to encode silencing suppressors. Suppressors of silencing of these viruses interfere with different steps of processing the RNA silencing present in plants and are important defense responses (Ratcliff et al., 1999). This process was one of the most evident in plants to identify viruses that have proteins that interfere with the system of the plant RNA silencing. In 1998, Anandalakshmi and collaborators and Brigneti and collaborators shown that HC-Pro protein of TEV and 2b of CMV could have this role. A classic paper demonstrated that the inhibition system of the 5' end corresponding to proteins P1 and Hc-Pro was efficient to obtain plants resistant to Plum pox virus (PPV) in tobacco (Di Nicola-Negri et al., 2005). In this same study were tested four regions of the virus genome: (i) nucleotide (nt) 1-733 of the protein corresponding to P1; (ii) nt 954-1603 corresponding to the end of the protein P1 and protein portion of Hc-Pro; (iii) nt 1680-2386 corresponding to the central part of Hc-Pro/P3 and, (iv) nt 1935 to 2613 corresponding to the end of Hc-Pro protein and part of P3. To access the efficiency of each construct in relation to the resistance of transgenic plants to PPV a large number of transgenics was analyzed by ELISA and, it was shown that 90% of transgenic plants were resistant to PPV. Despite all indicates that the target for this group of RNAi gene constructs are based on the 5' end (mainly the Hc-Pro) there are works based on positive replication region (Guo and Garcia, 1997; Wittner et al., 1998) or in the 3' end of the coat protein (Ravelonandro et al. 1992; Palkovics et al. 1995; Jacquet et al., 1998).

#### 4. Maize transformation

The insertion of sRNAi in the plant can be accomplished by different ways such as electroporation, *Agrobacterium*-mediated transfer, microparticle bombardment or viruses. Most of these methods use an RNA vector that produces stable dsRNA.

Significant progress has been achieved in developing technology for genetic transformation of maize in the last decade. Genetic transformation of maize became nowadays a routine procedure for various genotypes in most public and private laboratories working with this culture. For introducing a siRNA construct in maize is necessary (i) an *in vitro* regeneration protocol for transgenic maize cells and; (ii) methodologies to insert siRNA construct in the genome.

##### 4.1 *In vitro* regeneration of transgenic maize cells

The establishment of maize regeneration systems from somatic cells constitutes a prerequisite of utmost importance within the process of transgenic maize plants production. Regeneration of maize plants in tissue culture can occur via organogenesis (Zhong et al. 1992) or somatic embryogenesis, and the last one is the most used method.

Grasses were considered recalcitrant species with regard to establishing of totipotent cultures *in vitro* (King et al. 1978). The intensification of research in this area enabled rapid progress, especially after 1980 with the discovery of somatic embryogenesis in several grass species (Prioli & Silva, 1989). In maize, plant regeneration from Type I callus cultures was first described in 1975 by Green & Phillips, using immature embryos as explants. For the induction of callus, immature embryos were collected 10-15 days after pollination, with approximately 1.0 to 2.0 mm long and grown with the embryonic axis in contact with the culture medium. This orientation induces better proliferation of the scutellum cells while reduce germination (Green & Phillips, 1975).

Armstrong & Green (1985) introduced the terms of Type I and II callus which are currently used for the classification of embryogenic cultures of maize. Type I callus is composed of hard, compact, yellow or white tissue and usually capable of regenerating plants (Vasil & Vasil, 1981). Type II is soft, friable and highly embryogenic (Armstrong & Green, 1985). Type II callus culture is fast-growing and can be kept for a long period of time without losing their totipotency (Vasil, 1987).

Although Type II calli are the most efficient in the production of transgenic maize, Type I calli can also be used. The occurrence of friable embryogenic Type II callus is not so common, only a limited number of maize genotypes are able to express this phenotype in tissue culture, notably the line A188 (Armstrong & Green, 1985) and the hybrid Hill (Armstrong et al. 1991). With the advancement of the *in vitro* culture methodologies, and particularly with changes in the composition of culture media including type and levels of plant growth regulators, it became possible to regenerate a growing number of genotypes (Rapela, 1985, Duncan et al., 1985, Prioli & Silva, 1989). However, most of these genotypes only form compact Type I callus.

It is known that, in maize, the initiation of regenerable callus as well as the frequency of regeneration of plants are affected by a genetic component and depend on the genotype used (Hodges et al. 1986; Prioli & Silva, 1989). Through a diallele involving eight cultivars of maize, Beckert & Qing (1984) found significant heritability for initiation of somatic embryogenesis and plant regeneration. The high heritability indicates that both the initiation of callus and plant regeneration can be improved by crossing genotypes recalcitrant to highly responsive genotypes (Hodges et al., 1986). The formation of somatic embryos and regenerative ability are under control of genes located in the genome of maize cells (Hodges et al. 1986; Vinh, 1989). However, the physiological and developmental stage at the time of explant excision, the time of the year and, the specific interactions between genotypes and growing conditions of the donor plant, may modify the expression of genes that control the induction of somatic embryogenesis and plant regeneration (Prioli & Silva, 1989).

## **4.2 Methods of genetic transformation of maize**

The different methods of genetic transformation of maize can be divided into two major groups: direct and indirect methods. Indirect method of genetic transformation uses a bacterium, *Agrobacterium tumefaciens*, to introduce the gene of interest in the maize genome. In the transformation using direct methods, the gene of interest is introduced into the genome without the intervention of a bacterium. The most used method of direct genetic transformation of maize is the bombardment of cells with microparticles of metal physically covered with the DNA of interest.

### **4.2.1 Transformation of maize cells using microparticle bombardment**

Since most of the monocots are not natural hosts for *Agrobacterium*, initially, the transformation of maize was performed using direct systems.

The particle bombardment of plant cells with DNA of interest is a direct method of transformation designed to introduce nucleic acids into the genome or plastome of cells (Taylor and Fauquet, 2002). It is a methodology commonly used by laboratories working with plant genetic transformation. It was developed in the late 80's to manipulate the genome of plants recalcitrant to *Agrobacterium*-mediated transformation, among which are included cereals (Klein et al. 1988; Taylor and Fauquet, 2002). In the transformation via

particle bombardment or biolistic, microparticles of metal physically coated with the gene of interest are accelerated toward the target cells, using equipment known as "gene gun" (Sanford et al. 1987), with sufficient acceleration to penetrate the cell wall and not cause cell death. Precipitated DNA on the microparticles is released gradually into the cell after the bombardment, and integrated into the genome (Taylor and Fauquet, 2002). The acceleration of microparticles is obtained by a high voltage electrical discharge, or a helium pulse. The particles used are non-toxic, non-reactive, and lower than the diameter of the target cell. Typically, the microparticles used are gold or tungsten and they are propelled toward the target cells by modern devices such as PDS 1000 (BioRad Laboratories, Hercules, CA, USA) or the Accell gene gun (Agracetus, Inc., Middleton, WI, USA). Several physical parameters correlated with the biolistic equipment such as pressure, macrocarrier and microcarrier flight distance, and vacuum, must be optimized for successful transformation. Besides these parameters, the plant material and the gene of interest which will be used should also be tested in preliminary experiments (Sandford et al., 1993). From the 90's the microparticle bombardment was used to transform a wide variety of plants, including maize. Gordon-Kamm et al. (1990) and Fromm et al. (1990) were the first groups to report the production of transgenic maize from the bombardment of embryogenic callus. Then, several reports of transformation of maize showed that the particle bombardment is a successful technique for inserting foreign genes into the genome of maize with high reproducibility of results (Brettschneider et al. 1997; Frame et al. 2000). The main advantages of microparticle bombardment is related to the use of simple vectors and easy handling, plus the possibility of inserting more than one gene of interest into cells efficiently (Wu et al. 2002). Although considered a very efficient method of transforming maize, a possible disadvantage is the occurrence of multiple copies of the gene of interest and complex integration patterns, susceptible to silencing, of gene expression in future generations (Wang and Frame, 2004).

#### **4.2.2 *Agrobacterium tumefaciens* mediated maize transformation**

For several years the transformation of monocots by *Agrobacterium* had a very low efficiency, however, recently this is changing, and this method of gene transfer has become the method of choice for this group of plants. This transformation method uses a natural system of gene transfer developed by *Agrobacterium*. *Agrobacterium* is a soil bacterium capable of causing tumors in the region of plant infection. These tumors result from the presence of the Ti plasmid or plasmid tumor inductor in the bacterial cell. The Ti plasmid is a large circular molecule (200 to 800 kb), double stranded DNA that can replicate independently of the genome of *Agrobacterium tumefaciens* (Gelvin, 2003). Located in the Ti plasmid are two important regions for gene transfer from bacteria to the plant, the T-DNA region and the *Vir* region. The wild T-DNA contains genes that control the production of opines and hormones such as auxin and cytokinin, by the plant cell. Opines are amino acids used by *Agrobacterium* as a source of carbon and nitrogen, while the hormones are responsible for tumor induction in vegetables. The T-DNA is approximately 10 to 30 kb, and its ends are delimited by two 25 bp sequences highly homologous, called right and left ends. Wild *Agrobacterium* transfers its T-DNA across the membranes of plant cells and incorporates it into the plant genome. The T-DNA processing and transfer to the plant cells are largely due to the activity of virulence proteins encoded in the *Vir* region (Gelvin, 2003). To enable the use of *Agrobacterium* in the biotechnology processes of gene transfer to plants is necessary that the endogenous tumor-causing genes of the T-DNA be inactivated, and



that the foreign genes, genes of interest and selection markers, be inserted between the right and left borders of the T-DNA. The resulting recombinant plasmid is again placed in the *Agrobacterium* to be transferred to plant cells (Gelvin, 2003). Transformed tissues or cells can be used for regeneration of transgenic plants (Hiei et al., 1994, Ishida et al., 1996).

Because it is very large, the Ti plasmid is difficult to manipulate, so binary vectors, which are smaller, able to grow both in *Agrobacterium* and *E. coli* and easy to manipulate in the laboratory were created. These vectors have an artificial T-DNA, in which different transgenes can be inserted and an origin of replication compatible with the *Agrobacterium* Ti. The binary vectors are introduced into an *Agrobacterium* that had the T-DNA region removed from its Ti plasmid, called disarmed *Agrobacterium*. The disarmed *Agrobacterium* Ti plasmid still possesses the virulence region (*Vir*) and its expressed proteins can act in trans to transfer the recombinant T-DNA of the binary vector (Gelvin, 2003).

*Agrobacterium tumefaciens* is an excellent system for introducing genes into plant cells because: (i) DNA can be introduced in different plant tissues, (ii) the integration of T-DNA is a relatively accurate process. The region of DNA to be transferred is defined by flanking sequences, right and left ends. Occasionally it produces rearrangements, but in most cases the T-DNA region is inserted into the plant genome intact and, (iii) usually the T-DNA integrated shows genetic maps consistent and adequate segregation. Furthermore, the characters introduced in this way have proven stable over many generations of crosses. This stability is critical for the generation of commercial transgenic plants (Hiei et al., 1994, Ishida et al., 1996).

The first maize transformation protocol mediated by *Agrobacterium* with high efficiency was reported in 1996 by a group of researchers from Japan Tobacco Inc. (Ishida et al., 1996). They were able to infect maize immature embryos of A188 using super-binary vectors (pSB131 or pTOK233) (Ishida et al. 1996). The super-binary plasmid developed by Komari (1990) contains an extra copy of the virulence genes *virB*, *virC* and *virG*. Subsequent work showed that the transformation of maize mediated by *Agrobacterium* was also possible with the use of standard vectors (Frame et al. 2002). For maize the technique of *Agrobacterium* mediated transformation has been reported to result in high efficiency of transgenic plants production, with high number of events with only one or a small number of copies of the transgene in the genome compared to biolistic (Ishida et al., 1996, Zhao et al. 2001; Gordon-Kamm et al 2002, Frame et al. 2002; Lupotto et al. 2004; Huang and Wei 2005, Ishida et al 2007).

## 5. Gene constructs for RNAi target genes

Transgenes or genes that are inserted via molecular biology techniques in plants such as maize, are basically composed of (i) regulatory sequences that control gene expression, (ii) the selection marker gene and, (iii) the gene of interest.

The main sequences controlling gene expression are promoters, enhancers, introns and terminators. Promoters are DNA sequences, normally present in the 5' end of a coding region, used by RNA polymerase and transcription factors to initiate the process of gene transcription (Buchanan et al., 2000). Depending on the ability to control gene expression, the promoters are classified as weak or strong, according to the binding affinity of transcription factors with the promoter sequence (Browning & Busby, 2004). Strong or weak promoters can be further classified as constitutive, tissue and / or organ-specific and inducible. A constitutive promoter directs expression of a gene in all tissues of a plant during the various stages of development. The viral 35S mosaic virus promoter isolated from

cauliflower (*CaMV35S*) is one of the most used to drive high constitutive expression in plants (Odell et al., 1985), however its function in monocots is not as efficient as in dicotyledons. The promoter used to drive the overexpression of a protein constitutively in maize is currently the promoter isolated from maize ubiquitin gene *Ubi1* (Christensen & Quail, 1996). A tissue-specific promoter directs gene expression only in certain tissue, which may or may not be activated during all stages of development. The use of this type of promoter may be advantageous to prevent an unnecessary waste of energy and nutrients by the transgenic plant when the protein of interest is not required throughout the plant. For example, the expression of genes related to absorption of nutrients is required only at the root. An inducible promoter initiates gene expression in response to chemical, physical, or biotic and abiotic stresses (Liu, 2009). Similar to specific promoters, inducible ones avoid the unnecessary consumption of energy and nutrients, since the protein is only produced in response to right stimulus. An example of an inducible promoter is the one isolated from the *AtPHT1*; 4 phosphate transporter gene from *Arabidopsis thaliana*, which was shown to direct expression of the *uidA* reporter gene only in roots of maize subjected to phosphorus stress (Coelho et al. 2010). These features of promoters allow the expression of the transgenic protein be controlled according to the project objectives.

Enhancers are regions of DNA that bind transcription factors responsible for an increase in transcription of a gene, and consequently by an increase in protein expression. Enhancers can be located before or after the coding region. In the genome, sequences of plant enhancers can be located physically distant from the gene which they are controlling, however because of the packaging of DNA in the nucleus, these sequences are geometrically positioned near the promoter. This position allows for an interaction between transcription factors and RNA polymerase II (Arnosti & Kulkarni, 2005).

Introns are non-coding sequences within a gene that are removed during transcription. Although the mechanisms underlying the phenomenon are not completely clear, the incorporation of introns in genes can increase or decrease promoter activity and the levels of transcription (Chaubet-Gigot et al., 2001). Typically, the intron is inserted between the 3' end of the promoter and the initial codon of the protein of interest (Liu, 2009). Introns such as the rice actin *Act1* (McElroy et al., 1991), *Ubi1* of ubiquitin from maize (Christensen & Quail, 1996), *SH1* sucrose synthase from maize (Vasil et al., 1989), and *Adh1* corn alcohol dehydrogenase (Rathus et al., 1993) has been used in gene constructs in order to increase the expression of transgenes.

The regions 3' UTRs also known as terminator regions are used to confer greater stability to the mRNA, and to signal the end of the transcript preventing the occurrence of the production of chimeric RNA molecules and consequently the formation of new proteins, if the polymerase complex continues transcribing beyond the end of the gene (Lessard et al. 2002). 3' UTRs sequences used in most gene constructs for transformation of maize include the nopaline synthase gene from *Agrobacterium* (Depicker et al., 1982), the 3' region of *CaMV35S* (Frame et al., 2002), and inhibitor gene proteinase *pinII* from potato (An et al., 1989).

The selection gene is a sequence encoding a protein that when expressed in transgenic cells confer an adaptive advantage. The selection gene is used to identify and select cells that have the heterologous DNA integrated into their genome. Selection genes are fundamental to the development of technologies for plant transformation because the process of transferring a transgene to a recipient cell and its integration into the genome is very inefficient in most experiments, and the chances of recovery transgenic lines without selection are generally very low (Liu, 2009).

Currently, the most used selection markers for the production of transgenic maize are those that confer tolerance to herbicides. Among these, the *bar* gene, isolated from *Streptomyces hygroscopicus* and the *pat* gene, isolated from *Streptomyces viridochromogenes*, both encoding the enzyme phosphinothricin acetyltransferase (PAT) (De Block et al., 1989) are often mentioned.

In majority the gene of interest is a coding sequence or ORF (Open Reading Frame) of a certain protein that when expressed define a characteristic or phenotype of interest. In other cases, is a gene sequence used to silence gene expression, such as the RNAi technology.

An important aspect regarding the use of RNAi for plant biologists is the ability to decide the target region of the gene that should be used to efficiently produce the dsRNA. In 2002 the company Dharmacon ([www.dharmacon.com](http://www.dharmacon.com)) was the first to develop an algorithm as a tool for rational design of a potent silencing, based on data by Reynolds et al., (2004). Today, there are several companies that have developed algorithms for analysis of gene sequence based on a number of parameters that predispose to more effective use of this technology. Many of these softwares are freely accessible on the Internet:

1. [http://www.ambion.com/techlib/misc/sRNAi\\_finder.html](http://www.ambion.com/techlib/misc/sRNAi_finder.html);
2. <http://biotools.idtdna.com/rnai/>;
3. <http://hydra1.wistar.upenn.edu/Projects/sRNAi/sRNAiindex.htm>;
4. <https://rnaidesigner.invitrogen.com/sirna/>;
5. <http://jura.wi.mit.edu/sRNAiext/register.php>;
6. [http://www.protocol-online.org/prot/Molecular\\_Biology/RNA/RNA\\_Interference/sRNAi\\_Design\\_Rules/](http://www.protocol-online.org/prot/Molecular_Biology/RNA/RNA_Interference/sRNAi_Design_Rules/);
7. <http://www.ambion.com/techlib/resources/RNAi/>.

The new synthesized siRNA can target other RNAs on the basis of sequence similarity. Any RNA that possesses sequence similarity with the original trigger dsRNA may be silenced. This fact may limit the use of RNA silencing in plants due to gene family with high sequence similarity (Miki et al., 2005).

One alternative way to express dsRNA in maize is described as followed. The interested cDNA fragment is initially amplified with primers forward containing the *XbaI*-*XhoI*-*Bam*HI and primer reverse containing *Hind*III - *Kpn*I sites on the 5' of each primer. The cDNA fragment (around 450 bp) is cloned in two steps in the multiple cloning site of an RNAi induced transgene in the pKANNIBAL vector (Wesley et al., 2001). In the first step, the cDNA is cloned into the pKANNIBAL *XhoI* - *Kpn*I. In the second step, the original cDNA fragment is cloned again in the inverted direction in the pKANNIBAL *XbaI* - *Hind*III already containing the first copy of the fragment. After the double cloning into the pKANNIBAL, the cassette is excised and cloned into pCAMBIA3301 *Bam*HI site already containing the Ubiquitin promoter and NOS terminator. The transgene expression results in a transcript that terminates within the 3' sequence of the NOS terminator and folds back on itself by virtue of the inverted repeats, thus generating the dsRNA (Fig. 4). The dsRNA is then substrate for the DICER and RISC enzyme complex that cleaves it into siRNA as already explain in the Figure 1. The confirmation of the cloning in an appropriate direction might be either done with restriction mapping or sequencing analysis. However, with the advantage of DNA synthesis today none of the first steps might be required for final cloning. The RNAi cassette in the binary vector pCAMBIA 3301 is then transformed in Hi-II maize genotype by microprojectile bombardment.

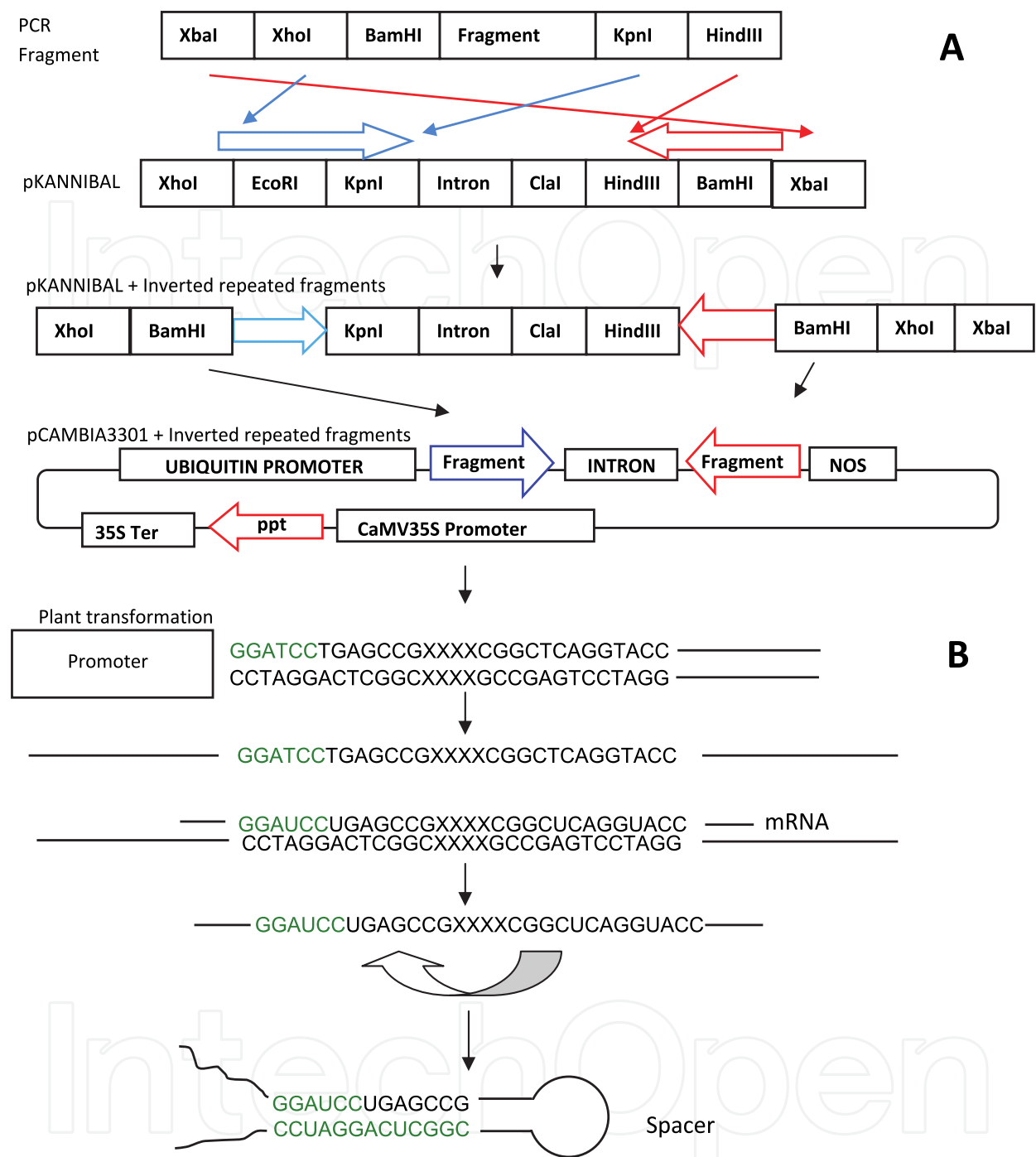


Fig. 4. (A) Diagram of the cloning of a gene fragment in an inverted repeat direction into de pKANNIBAL vector and the transfer of the cassette to the pCAMBIA3301 which carries the selection marker phosphinothricin acetyl transferase (*bar*) (B) Diagram of hpRNA prior to folding into the characteristic hairpin structure. It has two inverted oriented repeated sequences between a spacer.

The transgenic T1 plants arise in the frequency around 1% relative to the original number of explants. The first confirmation of the transgenic is done by spraying leaves with 3 mg/L Finale herbicide (ammonium glyfosinate - AgrEvo Environmental Health, Montvale, NJ). The *bar* gene present in the pCAMBIA3301 plasmid confers resistance to this herbicide.



Transgenic plants that express this selectable marker gene survive herbicide spraying whereas the nontransgenic plants die (Figure 5).

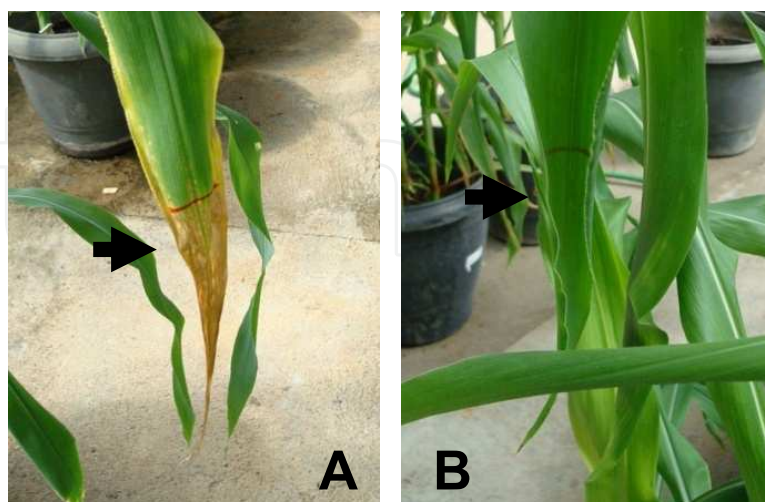


Fig. 5. Test with the Finale herbicide (ammonium glyfosinate) in maize leaves. (A) Sample sensitive and (B) insensitive to the herbicide.

The second confirmation of the transgenic is done by PCR using primers specific to the gene construct. To produce high-quality, stable transgenic lines it is necessary to define individuals with a single copy insertion and in homozygosity. This decision is based on the premise that expression of one copy is more stable and reliable than multicopy in the following generations. DNA purified from a single leaves (~100 mg of tissue) of T1 transformed plants is screening in a Southern blot analysis to identify events that possess single copy insertion. DNA is digested with restriction enzyme and subjected to gel electrophoresis. After the transfer of the DNA to the nylon membrane it is hybridized either with the *bar* gene or any other fragment present on the genetic cassette. The choice of the enzyme depends on the way the cassette was prepared. If there is no site in the cassette of the restriction enzyme used for the initial digestion of the DNA, the number of bands reflects the number of copies of the fragment integrated into the genome. Even for the self pollinated T0 plants many of the T1 generation are still heterozygous specially if there is more than one insertion. In this case, the test of herbicide and PCR in a sample of the following generation will help identify the one that are homozygous. If 100% of the T2 progeny of a single T1 plant are resistance to the herbicide (or show positive for the PCR) it indicates that the T1 parent (as well as all the T2 sibs) is homozygous for the transgene.

Recent works at Embrapa Maize and Sorghum (Brazil) obtained SCMV resistant transgenic maize plants by transforming friable callus of maize HiII using a construction based on the RNAi technology (data not published). Previous study on the SCMV gene family identified the region of the coat protein as a conserved region that might be used to produce the cassette to silence the expression of the SCMV virus in maize. Once this fragment from the SCMV genome was choose and isolated, it was cloned twice, in inverted position, into the vector pKANNIBAL containing a spacer, transferred to a binary vector pCAMBIA 3301



containing the ubiquitin promoter and NOS terminator and used to transform maize by particle bombardment as explained above.

The phenotypic evaluation of the transgenic plants was done by inoculation of the SCMV virus complex every week for three consecutive weeks starting in a maize V5 stage. The inoculation was confirmed by PCR and microscopy. From the 20 events obtained 30% of the plants did not show any viruses symptoms and in approximately 46% the symptoms reduces along the plant life cycle. These results indicated that the technique of RNAi based on the Coat protein sequence was capable of generating transgenic maize resistant to the SCMV virus (Figure 6).



Fig. 6. Transgenic and non-transgenic plants inoculated with SCMV in the greenhouse. (A) Plant with symptoms and (B) transgenic plants with no symptoms; Black arrow indicates the symptoms.

Other groups also got similar results, in maize, by induced RNAi-mediated transgenic virus resistance. Bai et al. (2008) transformed maize with an hpRNA expression vector p3301 containing the inverted-repeat sequence of the SCMV Nib gene, and obtained transgenic resistant lines. Also, Zhang et al. (2001) constructed an hpRNA expression vector containing reverted-repeat sense and antisense arms to target the MDMV gene encoding the P1 protein (protease) and used this cassette to transform maize embryonic calli and obtain plants tolerant to MDMV viruses.

## 6. Conclusions

In the 60's and 70's the world experienced a vast increase in the agricultural productivity based on conventional breeding techniques, intensive use of industrial inputs (fertilizers and pesticides), mechanization and cost reduction of management. In the 21st century, molecular biology techniques have been coupled with the conventional breeding techniques to boost up crops productivity. In the mid 90's the discovery of the RNAi added a new perspective to the gene regulation. This technology became a powerful tool to understand gene function and to the breeders improve crop varieties such as the development of barley varieties resistant to BYDV (Barley Yellow Dwarf Virus) (Wang et al., 2000), reduce the level

of glutenin in rice which is important for patients that are incapable to digest it (Kusaba et al., 2003) and among others, to obtain varieties of banana resistant to BBrMV (Banana Bract Mosaic Virus), a virus that has devastated the Southeast of Asia and Indian .

Some applications of RNAi in plants have relied in non *Agrobacterium* mediated methods to induce dsRNA into the cells. This chapter described the potential use of RNAi to knock out gene in plants and obtain tolerant transgenic maize lines using a vector capable to form dsRNA. The results implicate in the creation of an improved maize cultivar resistance to SCMV. This approach might be a very interesting alternative and innovation to narrow the gap between productivity and disease, insects and virus resistance, nutritionally rich and toxic-free crops and abiotic stresses.

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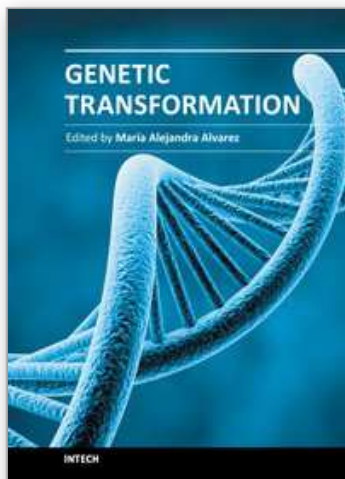
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## **Genetic Transformation**

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Genetic transformation of plants has revolutionized both basic and applied plant research. Plant molecular biology and physiology benefit from this power tool, as well as biotechnology. This book is a review of some of the most significant achievements that plant transformation has brought to the fields of Agrobacterium biology, crop improvement and, flower, fruit and tree amelioration. Also, it examines their impact on molecular farming, phytoremediation and RNAi tools.

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Unit 405, Office Block, Hotel Equatorial Shanghai  
No.65, Yan An Road (West), Shanghai, 200040, China  
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Phone: +86-21-62489820  
Fax: +86-21-62489821

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