TECHNIQUES IN GENETIC ENGINEERING

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CHAPTER 9

Genetic Manipulation of Plants

“It might seem unfair to reward a person for having so much pleasure over the years, asking the maize plant to solve specific problems and then watching its responses.”

Barbara McClintock

In 2008, over 10 million farmers in 25 countries have planted transgenic plants, the planted area going up from around 44 million hectares in 2000, to 125 million hectares in 2008 (Marshall, 2009). The majority of these crops are herbicide resistant (58.6 %), the rest are either insect-resistant or stacked traits.

Although there is still much debate on the ethics of environmental safety, economics and gene diversity-related issues, transgenic crops are still being considered by many as a source for oral vaccines, biofuels, or improved / high-quality food products, hence genetic engineering is either used to create new products in plants or else to assign plants novel functions, for example to improve crop quality or quantity.

There are quite a number of ethical issues surrounding genetically modified crop plants (a subset of Genetically Modified Organisms, or GMOs). The main target here has long been one of the pioneering companies, Monsanto, who is famous for the **Bt cotton,** which is a trademark genetically modified cotton that produces an insecticide through expression of a bacterial toxin-producing *cry* gene inside cotton.

Bacillus thuringiensis (Bt) *cry* genes code for Cry (Crystal) proteins that are endotoxins, which become activated in the acidic pH of the insect stomach, resulting in the death of the insect. Monsanto had in fact initially produced the Bt potato and obtained approval from the Environmental Protection Agency (EPA) in 1995, and later on went on to insert the same genes into other plants, producing Bt soybean, Bt corn, and the like.

These Bt plants have initially significantly reduced the use of chemical insecticides in the fields, resulting in the big hype towards biotech industry, although in spite of these insect-resistant GM crops the pesticide use has been estimated to be over 1 billion pounds per year in the US alone (Alavanja, 2009).

However, the India example of Bt cotton indicates no significant yield improvement after Bt cotton implantation, and no significant decrease in pesticide use in Bt cotton-planted areas (Coalition for a GM-free India document; http://www.biosafety-info.net/file_dir/551137394f82a8adac3ad.pdf). This may or may not be due to misinformation and mishandling of farmers, or due to the Bt cotton itself.

In fact, global pesticide sales were steadily increasing all over the , except perhaps for North America, where Bt cotton has significantly reduced the use of pesticides, but also the amount of pesticide used per hectare in China, for instance, is almost 5 times more than that used in the US (Plumer, 2013).

Monocotyledons, Dicotyledons and Commercial Crops

Flowering plants are commonly divided into two classes: monocots and dicots.

Monocotyledons (Monocots; Liliopsida) rice (Oryza sativa), maize (Zea mays), barley (Hordeum vulgare), sugar cane (Saccharum officinarum), banana (Musa),	Dicotyledones (Dicots; Magnoliopsida) tobacco (Nicotiana tabacum, Nicotiana rustica), tomato (Lycopersicon esculentum), thale cress (Arabidopsis thaliana), carrot (Daucus carota), potato (Solanum tuberosum), cotton (Gossypium), canola / rapeseed (Brassica)
One cotyledon in the embryo	Two cotyledons in the embryo
One furrow or pore in the pollen	Three furrows or pores in the pollen
Flower petals in multiples of three	Flower petals in multiples of four or five
Scattered stem vascular bundles	Organized stem vascular bundles
Parallel leaf veins	Reticulated leaf veins
No secondary growth	Secondary growth
Roots from nodes in the stem, called prop roots	Root from the lower end, the radicle

Flavr Savr tomato has been the first commercial genetically engineered food product approved for human consumption by the Food and Drug Administration (FDA) in 1992. Produced by the company Calgene, this tomato was rendered more resistant to rotting and softening by transgenic insertion of an antisense gene that suppresses expression from a polygalactouronase gene, which blocks breakdown of pectin in the cell wall, hence reduces softening. However in practice FlavrSavr tomatoes did not stay firm and had to be reaped just like normal wildtype varieties, hence did not provide any significant advantage, and did not survive very long in the market.

Golden Rice has been genetically engineered to synthesize beta-carotene in rice by a research team of Swiss Federal Institute of Technology and University of Freiburg researchers, with the hope of improving nutritional value of this staple crop for a large percentage of the world population that relies on rice for diet, suffering from vitamin A deficiencies (Ye et al, 2000).

Roundup Ready Soybean, also from Monsanto, has been genetically engineered for increased resistance to the trademark herbicide of Monsanto, glyphosate, which interferes with synthesis of essential amino acids, and hence is detrimental to not only herbs but also crops. This chemical inhibits an enzyme, 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), which catalyzes a critical step in the synthesis of these essential amino acids. The Roundup Ready variety is genetically engineered to synthesize a variant of this EPSPS enzyme that is not responsive to glyphosate, which protects the commercial crop from the herbicide. The major problem with the Roundup Ready use has been that although in the early times the system worked well and herbs were effectively removed with relatively less amount of glyphosate, herbs (similar to bacteria and antibiotics) have gained resistance to this herbicide over the years, and the use of herbicide has gradually gone up, effectively outweighing the advantages of the genetic modification (ie less use of herbicides – no more).

Plant manipulation methods

i. Plant Cell and Tissue Culture

Unlike animal cells, plant cells possess a unique property, they are totipotent – meaning any part of the plant can in theory grow vegetatively and generate an entire plant, which makes both cell and tissue culture as well as manipulation of plants relatively easier than mammalian cells.

Many different parts of the plant may be cultured – be it cells from the embryo, specialized organs, callus or dispersed cells. A **callus** is a mass of rapidly proliferating cells at the site where plant is cut or injured, in a way similar to proliferating precursor cells or fibroblasts at the site of wounds in animals. Thus, callus can develop into root, shoot and stem structures in tissue culture (Dodds and Roberts, 1985).

The same asceptic culture conditions as in mammalian cell culture also applies to plant cell culture; culture media, however, are based on the nutritional requirements of plant cells and follow a few basic rules, although it should be optimized for each plant cells. In general, inorganic salts including nitrogen, magnesium and phosphorus, sugar (mostly sucrose of D-glucose), amino acids, nicotinic acid, glycine and pyridoxine, plant growth hormones (also known as plant hormone regulators, PHR) such as auxins and cytokinins, and vitamins such as vitamin C, biotin and riboflavin are added to the culture medium (Dodds and Roberts, 1985). Culture medium can either be aqueous or matrix-based (including starch of sucrose polymers); **monocots and dicots may have different culture requirements and detailed protocols should be obtained and optimized in the laboratory.**

Plant cells can be genetically manipulated with polycationic chemicals, such as calcium, or liposomes, or electroporation etc. However, the plant cell wall, with its rigid architecture, is one challenge in genetic manipulation of plants cells, significantly reducing the efficiency of many of the above-mentioned methods of gene delivery. Therefore several methods have been devised to penetrate the cell wall, thereby increasing the efficiency of transformation.

Gene Gun, also known as **biolistic particle delivery system** or **particle bombardment method**, is a direct method of nucleic acid delivery into cells that are otherwise difficult to transfer, such as bacteria, yeast or plant cells. Usually helium-based or other source accelerates gold or tungsten particles coated with nucleic acids, which can then penetrate both cell membrane and cell wall in such organisms. Once inside the cell, the nucleic acid is removed from the particle and either transient expression or stable expression (if integrated into host chromosome, with very low probability) will take place.

The highly structured and rigid cell wall of plants is a serious obstacle for the delivery of DNA; therefore in some strategies the cell is stripped of this cell wall, and is left with only its plasma membrane – this is called the **protoplast**. The first protoplasts were cultured in 1892 from an onion bulb scales by Klercker, however it was not until 1960s that the method was refined for sterile tissue culture applications (Trigiano and Gray, 1996).

A protoplast can be obtained by two major protocols –

(a) either plant tissues can be mechanically sliced or chopped, which would damage many of the cell walls (the original method which will also result in the loss of many cells, hence not very popular), or

(b) a milder version that uses hydrolytic enzymes to get rid of the cell wall, while maintaining the cell membrane (the preferred and more common version).

A protoplast, devoid of its cell wall and left with only a cell membrane, can then be transfected with DNA the same way as a typical mammalian cell – for example by electroporation or polycationic chemicals, liposomes etc. Protoplasts can also be co-cultivated with *Agrobacterium tomafeciens*, as described below (Trigiano and Gray, 1996).

Agrobacterium

Agrobacterium tumfaciens is a plant pathogen that causes **crown gall disease**, a type of plant tumor. These bacteria transfer a tumor-inducing (Ti) plasmid to a range of monocot or dicot plants; this Ti plasmid contains a so-called T-DNA (transferred DNA), with genes for tumor induction and nopaline synthesis. These T regions on Ti plasmid, defined by left and right T-DNA borders, are usually 10 to 30 kbp in size, and there may be multiple T regions on some Ti plasmids (Gelvin, 2003). The virulence endonucleases recognize and cut these border sequences, releasing the T region, and the single stranded T strand is than coated with VirD2 protein and gets transferred to the plant with the help of a combination of other virulence proteins

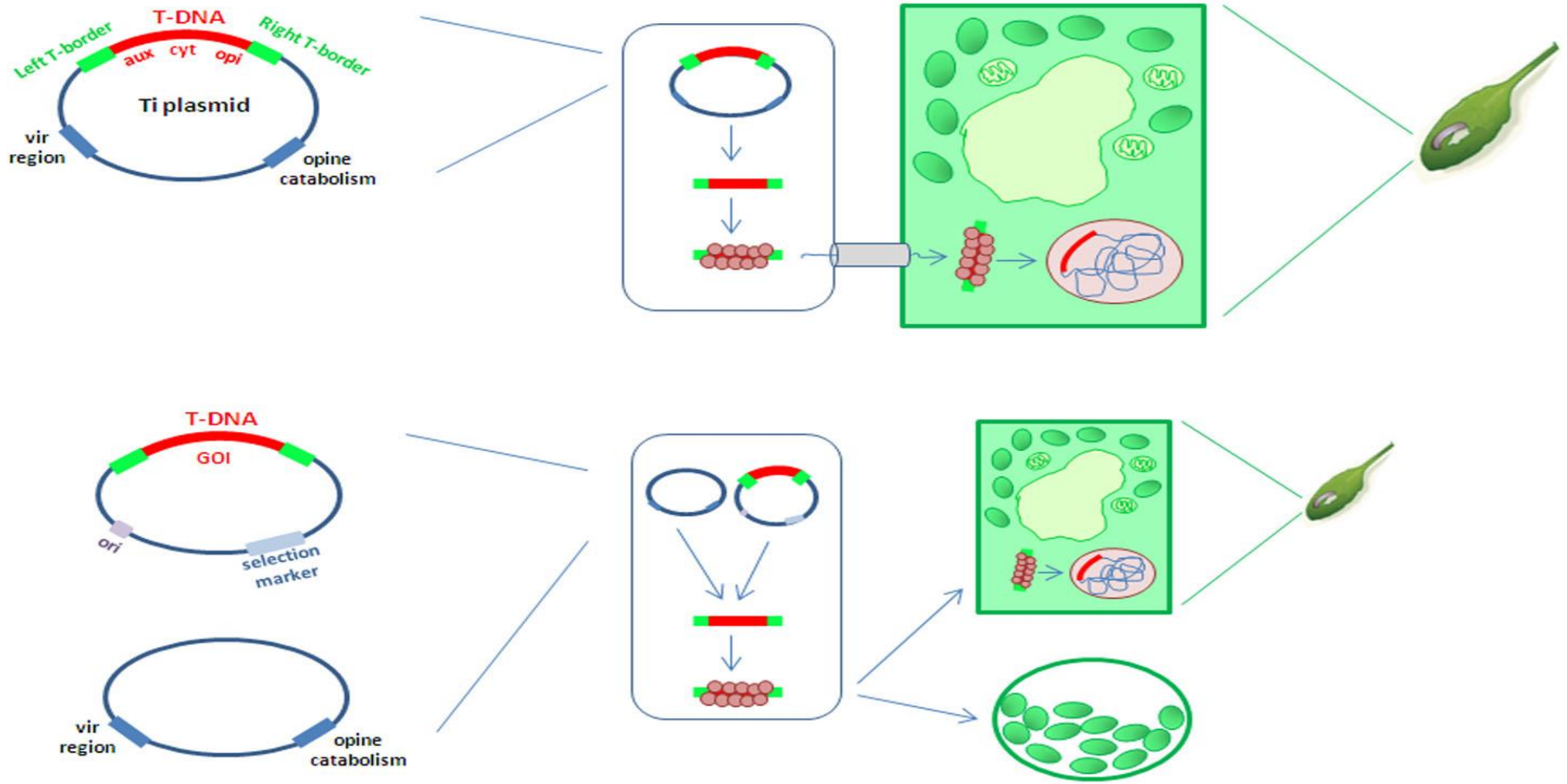

Figure 9.1. Simple schematic of *Agrobacterium*-mediated plant transformation. *Agrobacterium* typically transfers its T-DNA region (coated with proteins encoded by *vir* region) to the wounded leaf, causing over-proliferation, hence "tumor-induction" (upper panel). This feature is exploited in plant genetic manipulation: a disarmed Ti plasmid, which contains virulence region but no T-DNA (hence cannot "infect" the plant) and a binary vector consisting of the T-DNA left and right borders with gene of interest (GOI) cloned in between, a bacterial replication origin (ori) and a bacterial selection marker (for easy cloning in *E. coli)* are co-transformed into the same *Agrobacterium*, which then uses the proteins coded by *vir* region *in trans* to coat the transgene flanked by left and right borders – this mechanism can be used either on wounded plants as above, or on protoplast cultures (lower panel).

Plant Expression and Reporter Vectors

(a) A **promoter** that is strongly active in plant cells. Many of the plant expression plasmids use promoters from plant viruses, such as those of the Cauliflower Mosaic Virus (CaMV) genes. One may also use tissue-specific promoters as before, such as fruit-specific, leaf-specific or seed-specific promoter, depending on where the transgene expression is required.

(b) **Selectable markers** such as **nptII** (encoding resistance to kanamycin), **hptII** (encoding resistance to hygromycin) or **pat** (encoding phosphinothricin N-acetyltransferase, which detoxifies phosphinothricin (ppt), an inhibitor of plant growth). In many of the food crops, selectable markers are removed (mostly through Cre-LoxP, Flp-FRT or similar site-specific recombinase-mediated cleavage of the marker gene and/or the transgene) prior to market release due to biosafety concerns, but this also enables the producer to not label its product as "GMO".

(c) **Reporter genes** such as **gusA** (β-glucuronidase, which will cleave the X-gluc, 5-bromo-4-chloro-3-indolyl glucuronide which is a colorless substrate, into 5,5'-dibromo-4,4'-dichloro-indigo, a blue colored insoluble product), **smgfp** (soluble version of the codon-modified green fluorescent protein, the so-called "soluble-modified GFP"; Davis and Vierstra, 1998), and **luc** (encoding the enzyme luciferase).

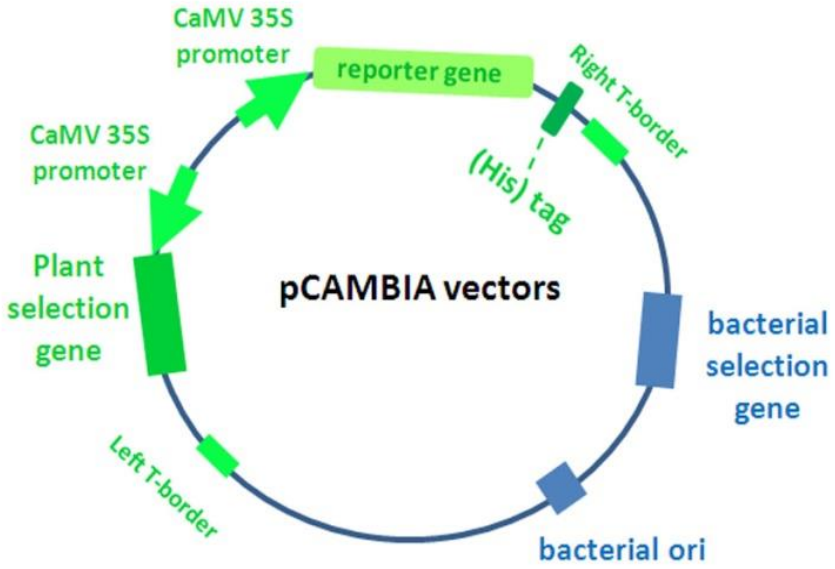

Figure 9.2. Cartoon diagram of a generic pCAMBIA vector, which expresses a reporter gene and a plant selection gene from their respective CaMV 35S promoters, flanked by left and right T-borders. The His tag helps affinity purification of expressed protein, if required. Bacterial *ori* and bacterial selection gene are used for cloning in bacteria

GATEWAY™ system for plant expression uses the classical GATEWAY™ technology (Chapter 2c) for effective and easy cloning of large DNA sequences into T-DNA binary vectors, which can otherwise be time-consuming (Karimi et al, 2002). Various versions of GATEWAY™ system vectors are possible for overexpression, GFP fusion synthesis, gene silencing, marker expression, promoter analysis etc in plants (Karimi et al, 2002).

Manipulation of the chloroplast genome has been realized in a limited number of plant species, starting from tobacco, and has attracted some attention due to its many advantages, including exclusively homologous recombination-mediated integration, high number of chloroplasts (hence high levels of stable expression in green tissues, although there are problems with expression in non-green tissues such as roots or seeds), lack of gene silencing mechanisms, and maternal mode of inheritance that significantly reduces transgene transmission through pollen (Bock, 2014). Gene gun is the common method used, since the transgene has to penetrate several layers of membrane, however plastid transformation methods are still not well established (Bock, 2014).

One point to remember is that codon usage may change from bacteria to fungi to humans to plants, hence for optimum translational efficiency of the transgene in plants, codon optimization is recommended.

If, for example, a human gene is to be expressed in plants, one must analyze the codon frequencies coding for the same amino acid in humans and plants: supposing the protein product that has the amino acid Glycine is coded by GGU in humans, with a codon frequency of 14.5 %, but in plants this codon has a frequency of 2 %. This would interfere with the translational efficiency. H

owever, the codon GGC, again coding for Glycine, has a frequency of 30 % in plants. Then replacing GGU with GGC would improve translational efficiency significantly in plants.

This is particularly true for organelle genomes, where codon frequencies tend to show greater variation than nuclear genomes.

“The science of today is the technology of tomorrow.”

Edward Teller