Particle bombardment-Mediated Transformation

Particle bombardment employs high-velocity microprojectiles to deliver substances into cells and tissues. For genetic transformation, DNA is coated onto the surface of micron-sized tungsten or gold particles by precipitation with calcium chloride and spermidine. Once inside the cells, the DNA elutes off the particles. If the foreign DNA reaches the nucleus, then transient expression will likely result and the transgene may be stably incorporated into host chromosomes.

In this technique foreign DNA varies between the binary vector used in Agrobacterium-mediated transformation to a coding region of a certain gene between promoter and terminator and few terminal DNA bases.

Sanford and colleagues at Cornell University developed the original bombardment concept and coined the term “biolistics” (short for “biological ballistics”) for both the process and device. However, several names are now used for the device like “gene guns” or “particle guns” and for the process such as microprojectile bombardment, particle bombardment, particle acceleration, or ballistics. The most widely used device for plant transformation is the Biolistic® PDS-1000/He Particle Delivery System marketed by Bio-Rad Laboratories.
The system employs high-pressure helium released by a rupture disk to propel a macrocarrier sheet loaded with millions of DNA-coated metal particles (microcarriers) toward target cells. A stopping screen halts the macrocarrier, and the microcarriers continue toward the target and penetrate the cells. Because of its physical nature and simple methodology, the biolistic process can be used to deliver substances into a wide range of intact cells and tissues from a diversity of organisms. In plant research, the major applications have been transient gene expression studies, production of genetically transformed plants, and inoculation of plants with viral pathogens.

Many “firsts” were achieved through the application of biolistic technology including chloroplast and mitochondria transformation, as well as nuclear transformation of important monocot species such as wheat, corn, and rice. As with any plant transformation method, several parameters need to be optimized for the process to be maximally effective. In the next section we will discuss these factors briefly.

**Factors affecting efficiency of Particle bombardment-Mediated Transformation**

The efficiency of the process is greatly dependent on type, size and velocity of microcarriers. In addition, number of bombardments and factors concerning the plant tissue (biological factors) affect the success of the process.

**Particle type and size**

Gold particles are often preferred over tungsten ones since they have more round and uniform sizes. They are biologically inert, non-toxic and do not degrade DNA bonds. Tungsten particles, on the other hand, are highly heterogeneous in size and shape, potentially toxic to some cell types and are subjected to surface oxidation. They can also acidify and degrade DNA bonds. However, there are some reports on successful application of tungsten particles in plant cells transformation. But most researchers have
used gold particles for gene delivery to plant tissues. Particle size has a profound effect on the success of bombardment. Too small particles will have low penetration ability that decreases the number of transformed cells. In the same time too large particles will lead to an increase in tissue damage and subsequently affect shoot regeneration.

**Particle velocity**

Velocity of microcarriers also deeply affects the efficiency of the process. The velocity can be controlled, mainly, through optimization of helium pressure. A helium shock wave is used to propel the plastic macrocarrier disk carrying DNA coated with microparticles towards the target tissues. Ability of the microparticles to penetrate the different cell layers or tissue types is greatly dependent on the propelling force of the helium gas (helium pressure). Lower pressure gives poor penetration capability of the microparticles while high pressures might be injurious to the tissues. Another two factors can be manipulated to control velocity. The first is macrocarrier flight distance to stopping screen and the second is the distance from stopping screen to target tissues. The distance that the macrocarrier travels affects the impact of macrocarrier on the stopping screen and consequently the velocity of the released microcarriers towards the target tissues. It was recorded that the greater the distance, the higher is the velocity achieved. Short distance gives insufficient propelling force to penetrate the target tissues while long distance is associated with instability of the macrocarrier resulting in variability and uneven distribution of microcarriers. Finally, it was recorded that long distance from stopping screen to tissue gives reduced penetration force and thereby fewer cells receiving the oncoming DNA. Closed-up range resulted in massive tissue damage as exemplified by a high frequency of tissue dislocation during bombardment.

**Number of bombardments**

Increasing the number of bombardments significantly create mechanical injuries to the tissue. So, by increasing the number of shots would undoubtedly increase the injuries of
the targets thereby decreasing the number of surviving cells. Advantage of multiple bombardments in compensation of the first slight misfire occurs during shooting.

**Biological parameters**

Biological parameters include tissue type, cell size, cell culture age, general cellular health, target tolerance to vacuum, cell density, and cell turgor pressure should be also optimized. Manipulation of osmotic pressure of medium supporting targeted tissues to reduce turgor pressure in cells help in avoiding leakage following the shock wave created during bombardment. In addition, some explants may require a “healing” period after bombardment under special regimens of light, temperature, and humidity. It is worth to mention that amount of DNA used in bombardment should be also optimized to avoid insertion of high copy number of target genes that is responsible for their silencing in next generations.

**Integration of T-DNA into plant genome**

The majority of integration events occurred at telomeric and subtelomeric regions, which are typically gene rich. Following penetration of the cell by the metal particles, the plant’s wound response leads to the presence of DNA repair enzymes (nucleases, topoisomerases, and ligases) which together with large amounts of exogenous DNA would sponsor both degradation of the incoming DNA and joining of free ends. This generates complex and variable arrays containing both intact and rearranged transgenes. Such arrays would then act as the substrates for integration. Integration is proposed to occur at naturally-occurring chromosome breaks. This is supported by the prevalence of chromosome rearrangements, e.g. translocations and deletions, at transgene integration sites.
In addition to food and fiber, plants are exploited for a large variety of commercial chemicals including pharmaceuticals, food colors and flavors, fragrances and sweeteners. Plant tissue culture production methods (phytoproduction) can be developed to profitably manufacture some of these chemicals. Numerous investigators have reported production of useful compounds in both callus and suspension cultures. In some cases production in callus and suspension is much higher than that in whole plant of the same species. Plants produce many of these compounds as inducible chemical defense systems against stress. Understanding the biosynthetic pathways has a great beneficial effect on enhancing production of these compounds. Over production can be obtained through:

1. Over-expressing the key gene(s) involved in the biosynthetic pathway.

2. Blocking the competitive branches of biosynthesizing target compounds.

3. Blocking the degradation pathways or enhancing the transportation of target compounds.

4. Inhibiting the reproductive growth of plants and increase the biomass of vegetation growth, and increase the production of target compounds.

As a source for commercial chemicals, tissue cultures have the following advantages over field grown plants:

1. The culture system doesn’t need much field which can be used for crop growing.

2. The system is not limited by whether and season changes.

3. The secondary metabolism can be regulated to maximize the production of target compounds.

4. No herbicide and insecticide will be used during the maintaining of the system and therefore, the system is eco friendly.

5. Once the system is established, the content of useful compounds will be more stable than harvested herbs from different areas, which will facilitate the quality control.

**Insect Resistance**
The genetic engineering of crop plants to produce functional insecticides makes it possible to develop crops that are intrinsically resistant to insect predators and do not need to be sprayed (often six to eight times during a growing season) with costly and potentially hazardous chemical pesticides. *Bacillus thuringiensis*, commonly known as *Bt*, is a gram-positive bacterium that occurs naturally in the soil around the world. For decades, bacteriologists have known that some strains of *Bt* kill certain insects and that the toxic substance responsible for the insects death is a protein. When certain insects ingest either the bacterium or the protein produced by the bacterium (the protein is called d-endotoxin), the function of their digestive systems is disrupted, eventually resulting in death. When the dose is high, sudden death occurs. The use of *Bt* as a biopesticide was discovered in the first decade of this century when larvae of flour moths died suddenly. Research into their deaths led to the discovery that the presence of *Bt* was responsible for the death.

The *Bt* protein is not harmful to mammals, birds or fish, nor to beneficial insects. Mammals, including humans, do not have d-endotoxin receptors in their guts and all *Bt* proteins tested so far are degraded within 20 seconds in the presence of mammal digestive juices. *Bt* is not effective against all insects; however different *Bt* strains are effective against specific species. The major families of insects that respond to *Bt* are:

- *Lepidoptera* (caterpillars; e.g. European corn borer or cotton bollworm).
- *Coleoptera* (beetles; e.g. Colorado potato beetles).
- *Diptera* (flies and mosquitoes).

This *Bt*-based biopesticides also have several disadvantages. The production of the biopesticide is relatively expensive; its application requires the use of agricultural machinery; most applications need to be repeated several times per season; sunlight breaks down the active ingredient; and water (rain or dew) washes the protein from the plants, thus limiting the time when insects are exposed to it. Biopesticides therefore must be applied where and when the target insects are feeding. Most of these difficulties are overcome with transgenic insect resistant crops.

With the emergence of biotechnology, the development of insect resistant plants by transferring the gene that produces the *Bt* toxin became possible and this procedure is now
well established. The strain of \( Bt \) that is active against a target insect is identified and the gene producing it is isolated and transferred to the crop to be resistant. The most critical component of the process is to use the gene that is effective against the target insect. Many companies and universities have been working on identifying novel \( Bt \) genes and have sought appropriate patent protection.

**Disease Resistance**

- **Resistance to Viral diseases**

Plant viruses often cause considerable crop damage and significantly reduce yields. When transgenic plants express the gene for a coat protein (which usually is the most abundant protein of a virus particle) of a virus that normally infects those plants, the ability of the virus to subsequently infect the plants and spread systemically is often greatly diminished. The mechanism by which the presence of coat protein genes inhibits viral proliferation is thought that it likely works through the generation of RNAi. With this approach, researchers have developed virus-resistant transgenic plants for a number of different crops. Although complete protection is not usually achieved, high levels of virus resistance have been reported. In addition, a coat protein gene from one virus sometimes provides tolerance for a broad spectrum of unrelated viruses.

In both eukaryotes and prokaryotes, an RNA molecule that is complementary to a normal gene transcript (mRNA) is called antisense RNA. The mRNA, being translatable, is considered to be a sense RNA. The presence of antisense RNA can decrease the synthesis of the gene product by forming a duplex molecule with the normal sense mRNA, thereby preventing it from being translated. The antisense RNA–mRNA duplex is also rapidly degraded, a response that diminishes the amount of that particular mRNA in the cell. Theoretically, it should be possible to prevent plant viruses from replicating and subsequently damaging plant tissues by creating transgenic plants that synthesize antisense RNA that is complementary to viral coat protein mRNA.

- **Resistance to fungal and bacterial diseases**
Extensive damage and loss of crop productivity are caused by phytopathogenic fungi and bacteria. At present, the major way of controlling the damage and losses to crop plants that result from infection is through the use of chemical agents that may persist and accumulate in the environment and that are subsequently hazardous to animals or humans. It would therefore be beneficial if a simple, inexpensive, effective, and environmentally friendly nonchemical means of preventing fungal and bacterial damage to crop plants could be found.

Plants often respond to fungal or bacterial pathogen invasion or other environmental stresses by converting a conjugated storage form of salicylic acid (salicylic acid 2-\(O\)-\(\beta\)-d-glycoside) to salicylic acid, which induces a broad systemic defense response in the plant. This “systemic acquired resistance” to pathogens extends to plant tissues that are far from the site of the initial infection and may last for weeks to months. It results from the synthesis of a group of proteins called pathogenesis-related (PR) proteins. Engineering plants with broad-spectrum disease resistance involves overproducing salicylic acid. Theoretically, this can be done by transforming plants with bacterial genes that encode the enzymes isochorismate synthase and isochorismate pyruvate lyase, which catalyze salicylate synthesis.

The \(NPR1\) gene from the plant \(A.\) \(thaliana\) encodes a “master” regulatory protein that controls the expression of the PR proteins, and it can be activated or induced by the addition of salicylic acid. Overexpression of the \(NPR1\) gene can lead to the generation of broad-spectrum disease resistance against both fungal and bacterial pathogens. It was observed that overproduction of this “master switch” is an effective strategy in several plants eg: \(A.\) \(thaliana\), including rice, sugar beet, apple, and corn.

To develop plants resistant to fungal pathogens, researchers have attempted to utilize parts of the systemic acquired resistance system. The PR proteins include chitinases that protect the plant-invading pathogens. Transgenic plants that constitutively express high levels of chitinase, which can hydrolyze the \(\beta\)-1,4 linkages of the \(N\)-acetyl-d- glucosamine polymer chitin, a major component of many fungal cell walls, have been engineered.

**Salinity Resistance**
Considerable efforts have been made to increase salt tolerance of crops through transferring foreign genes into them. Various genes induced by salt stress are grouped into two categories, namely single-function genes and regulatory genes. Genes of first category generally facilitate production of: protective metabolites, which include osmolytes, transporters/channel proteins, antioxidative enzymes,.....etc.

- Protective metabolites, which include osmolytes: These compounds facilitate both water uptake and retention and also protect and stabilize cellular macromolecules from damage by high salt levels. Some well-known osmoprotectants are sugars alcohols, the amino acid proline, and quaternary ammonium compounds (eg: betane).
- Transporters/channel proteins: sequester sodium ions in the large intracellular vacuole (eg: Na\(^+\)/H\(^+\) antiport protein).
- Antioxidative enzymes: Scavengers for Reactive oxygen species (ROS) associated with salt stress.

The second category includes genes for regulatory proteins that regulate gene expression and signal transduction in the stress response eg: transcription factors. The complexity and multigenic nature of salt tolerance trait makes it better to manipulate more than one gene simultaneously. Fortunately, most controls of stress responses appear to be through the transcriptional regulation of genes via an array of transcription factors. Consequently, genetic engineering of plants for a multigenic trait like salt tolerance could be better to achieve through regulating the expression of genes encoding stress-inducible transcription factors that in turn would regulate a set of stress tolerance genes.

Transcriptional factors are important regulatory proteins that are able to regulate the expression of target genes by specifically binding to the cis-acting elements of interactional genes. Transcriptional factors are regarded as master switch that regulate stress-response genes and function in establishing stress tolerance. Based on the structure of their DNA binding domains, these transcriptional factors can be classified into various families. DREB (Dehydration responsive element-binding protein) are an important class of transcription factors that activate the expression of a number of stress responsive genes by binding to cis acting dehydration responsive elements (DRE) with a core motif of A/GCCGAC in their
promoters. They participate in stress response to drought, salinity and freezing, and improve stress resistance in plants. DREBs were found to activate many genes involved in production of antioxidant enzymes eg SOD and catalase and accumulation of compatible solutes eg proline and soluble carbohydrates. Thus different DREBs may perform different functions in plants and may be involved in several pathways and/or participate in crosstalk between pathways during the course of abiotic stress.

Transformation with different DREBs may lead to various abiotic tolerances in transgenic plants. For example over-expression of OsDREB2A improved salinity and drought tolerance in rice, DREB2A from Pennisetum glaucum was used to enhance salt and dehydration tolerance in tobacco. Over-expression of CKDREB from Caragana korshinski enhanced salt tolerance in tobacco.

Yield Improvement
In general, potential yield may be raised by improving the efficiency of photosynthesis, improving the efficiency of resource use by the plant, or enlarging resource allocations to the food/feed components of the crop, all of which have been targets of research for many years. Some of genes that have been explored for their ability to increase yield and that have wide-ranging effects on plant metabolism include fasciated ear2 (fea2), which controls branching and seed number; Phytochrome B which regulates plant light responses; and CDK (proteins controlling cell cycle) inhibitor-like proteins in corn. Some trials are recorded for genes involved in nitrogen assimilation but with little success.

Yield Quality
Ex: Rice grains containing pro-vitamin A
Upwards of 3 billion people depend upon rice as their main staple. β-carotene (provitamin A), a natural plant pigment, provides the chemical precursor for the body to produce Vitamin A. In rice, beta-carotene is present only in the outer grain layers. Unfortunately, in order to keep the grain from rotting, these layers are removed during milling and polishing.
The kernel that most people eat—the starchy interior called the endosperm—does not contain betacarotene.

Since no species in the entire Oryza family produces beta-carotene in its endosperm, hybridization of Oryza lines—the traditional crop improvement approach—is not considered feasible. In the year 2000, an international group of scientists reported using *Agrobacterium*-mediated transformation to introduce the entire β-carotene biosynthetic pathway into rice. Thus genes for phytoene synthase, phytoene desaturase and lycopene β-cyclase were introduced. The frequency of insertion of all three genes into the rice genome and their subsequent expression were quite high. Thus, the engineered rice produces β-carotene, which, after ingestion, is converted to vitamin A. The transgenic rice that produces β-carotene has a yellow or golden color and has been called “golden rice” by the scientists involved in its development. Unfortunately, the initial version of golden rice, now called golden rice 1, synthesized only 1.6 μg of β-carotene per gram of rice, so that individuals would have had to consume around 3 kg of golden rice 1 each day to reach the recommended minimal daily requirement of vitamin A. However, in 2005, scientists reported replacing the daffodil phytoene synthase gene with a similar gene from corn that produces an enzyme with a higher level of activity, resulting in a variety called golden rice 2 that produces a 23-fold-higher level of β-carotene than golden rice 1.

Generally, plants can be used as bioreactors to produce vitamins, pigments, lipids, proteins…etc. Unlike recombinant bacteria, which are grown in large bioreactors, a process that requires highly trained personnel and expensive equipment, crops can be produced relatively inexpensively by less-skilled workers. In addition, when proteins that are intended for human use are produced in transgenic plants, there is a significantly reduced risk of mammalian virus contamination in comparison to proteins that are produced in animal cells grown in culture.