**Biotechnology in plant Breeding**

**What is biotechnology?**

Etymologically, **biotechnology** is the study of tools from living things. In its current usage, the term is defined either broadly or narrowly. It may be defined broadly **as the use of techniques based on living systems to make products or improve other species**. This would include the use of microbes to make products via fermentation, an age-old practice. **In a narrower** **definition, biotechnology refers to the genetic manipulation of organisms for specific purposes**. The term **genetic engineering** is sometimes used to describe this practice. Some argue that classic plant breeding is genetic engineering, since the genetics (DNA) of plants are manipulated by breeders*.* Consequently, a much narrower definition of genetic engineering is used to describe the manipulation of organisms at the molecular level, *directly* involving the DNA. However, it is the revolutionary technology of **recombinant DNA** (**rDNA**), which enables researchers to transfer genes from any organism to another, that some accept as genetic engineering. The term **molecular breeding** is used to describe the use of a variety of tools for manipulating the DNA of plants (which may or may not involve rDNA) to improve them for specific purposes.

**General steps in rDNA technology**

Even though crossing of two different parents produces new recombinants in the segregating population, the term recombinant DNA is restricted to the product of the union of DNA segments of different biological origins. A cultivar developed by the rDNA procedure is **(GM) cultivar**. Generally, an organism developed by the rDNA procedure is called a **genetically modified organism** (**GMO**).

**Certain basic steps are common to all rDNA projects:**

1. The DNA of interest that is to be transferred (the **transgene**) is extracted from the source organism. The specific DNA sequence of interest is cut out using special enzymes.
2. The transgene is inserted into a special DNA molecule (a **cloning vector**) and joined to produce a new rDNA molecule.
3. The rDNA is transferred into and maintained in a host cell (bacterium) by the process of **transformation**. The vector replicates, producing identical copies (called **clones**) of the insert DNA.
4. The host cells with the cloned transgene are identified and isolated from untransformed cells.
5. The cloned transgene can be manipulated such that the protein product it encodes is expressed by a host cell.

**Gene transfer**

Once the desired gene has been identified from the library, it is ready to be transferred into a host cell, a process called **genetic transformation**. There are two categories of transgene transfer or delivery procedures – **direct** and **mediated transfer**.

**Direct gene transfer**

1. ***By particle acceleration or bombardment***

One of the commonly used direct gene transfer method is **microprojectile bombardment** (or **biolistic**). A biolistic device (called a **gene** or **particle gun**) is used to literally shoot the target DNA into intact cells (hence the nickname of **shotgun transformation**). Small amounts (about 50 μg) of micron-size (1–5 μm diameter) carrier particles (tungsten or gold) are coated with the target DNA and propelled in the barrel of the gene gun at energies high enough to penetrate plant cells. The rate of acceleration may be up to 430 m/s in a partial vacuum. The carrier particles pass through a mesh, hitting biolistic device. A low penetration number of projectiles (1–5 per cell) is desirable. More than 80% of bombarded cells may die if particle penetration reaches 21 projectiles per cell.

1. ***Electroporation***

Callus culture (or explants such as immature embryos of protoplasts) is placed in a cuvette and inserted into a piece of equipment called an electroporator, for electroporation. This procedure widens the pores of the protoplast membrane by means of electrical impulses. The widened pores allow DNA to enter through them to be integrated with nuclear DNA.

1. ***Other methods***

Other direct methods are available, including microinjection and silicon carbide procedures

**In-direct (Biological systems) gene transfer**

*Agrobacterium tumefaciens* mediated transformation

***Agrobacteria*** are soil bacteria. They naturally infect ***dicotyledonous plants*** (Infection of certain monocotyledonous plants has been reported, including yams, asparagus and lily). Because host range is limited, procedure has not been used for some major crops such as corn, wheat, rice, *etc*.

Life cycle of *Agrobacterium* involves living in the soil until it encounters a plant and then infecting the plant. Infection causes a rapid proliferation of plant cells around the infection leading to formation of a **crown gall tumor** (equivalent to cancers in animals). For ***Agrobacterium tumefaciens,*** only the crown gall is produced but for ***Agrobacterium hizogenes***, masses of roots emerge from the gall forming hairy root disease.

**Molecular plant breeding**

**Molecular breeding** may be defined as the use of molecular markers, in conjunction with linkage maps and genomics, to select plants with desirable traits on the basis of genetic assays. The potential of indirect selection in plant breeding was recognized in the 1920s, but indirect selection using markers was first proposed in 1961 by Thoday. The lack of suitable markers slowed the adoption of this concept. Molecular breeding gained new momentum in the 1980s and has since made rapid progress, with the evolution of DNA marker technologies.

Molecular markers are used for several purposes in plant breeding.

1. **Gaining a better understanding of breeding materials and breeding system**. The success of a breeding program depends to a large extent on the materials used to initiate it. Molecular markers can be used to characterize germplasm, develop linkage maps, and identify heterotic patterns. An understanding of the breeding material will allow breeders to select the appropriate parents to use in crosses. Usually, breeders select genetically divergent parents for crossing. Molecular characterization will help to select parents that are complementary at the genetic level. Molecular markers can be especially useful in identifying markers that co-segregate with QTLs (quantitative trait loci) to facilitate the breeding of polygenic traits.
2. **Rapid introgression of simply inherited traits**. Introgression of genes into another genetic background involves several rounds of tedious backcrosses. When the source of desirable genes is a wild species, issues of linkage drag becomes more important because the dragged genes are often undesirable, requiring additional backcrosses to accomplish breeding objectives. Using markers and QTL analysis, the genome regions of the wild genotype containing the genes encoding the desirable trait can be identified more precisely, thereby reducing the fragment that needs to be introgressed, and consequently reducing linkage drag.
3. **Early generation testing**. Unlike phenotypic markers that often manifest in the adult stage, molecular markers can be assayed at an early stage in the development of the plant. Breeding for compositional traits such as high lysine and high tryptophan genes in maize can be advanced with early detection and selection of desirable segregants.
4. **Unconventional problem-solving**. The use of molecular markers can bring about novel ways of solving traditional problems, or solving problems traditional breeding could not handle. When linkage drag is recessive and tightly linked, numerous rounds of backcrosses may never detect and remove it. Disease resistance is often a recessive trait. When the could be difficult to remove by traditional backcross procedures. Marker analysis can help to solve the problem, as was done by J. P. A. Jansen when he introgressed resistance to the aphid *Nasonovia ribisnigi* from a wild lettuce *Lactuca virosa* by repeated backcrosses. The result of the breeding was a lettuce plant of highly undesirable quality. The recessive linkage drag was removed by using DNA markers flanking the introgression to preselect for individuals that were recombinant in the vicinity of the gene. The lifespan of new cultivars can be extended through the technique of **gene pyramiding** (i.e., transferring multiple disease-resistance genes into one genotype) for breeding disease-resistant cultivars. Marker-assisted backcross can be used to achieve this rapidly, especially for genes with indistinguishable phenotypes.
5. **Plant cultivar identification**. Molecular markers are effective in cultivar identification for protecting proprietary rights as well as authenticating plant cultivars. The types of molecular markers are discussed next.

**Molecular markers**

Plant breeders use **genetic markers** (or simply markers) to study genomic organization, locate genes of interest, and facilitate the plant breeding process.

**Concept of markers**

Genetic markers are simply landmarks on chromosomes that serve as reference points to the location of other genes of interest when a genetic map is constructed. Breeders are interested in knowing the association (linkage) of markers to genes controlling the traits they are trying to manipulate. The rationale of markers is that an easy-to-observe trait (marker) is tightly linked to a more difficult-to-observe and desirable trait. Hence, breeders select for the trait of interest by indirectly selecting for the marker (that is readily assayed or detected or observed). When a marker is observed or detected, it signals that the trait of interest is present (by association).

Genetic markers can be detected at both the morphological level and the molecular or cellular level – the basis for classification of markers into two general categories as **morphological markers** and **molecular markers**. Morphological markers are manifested on the outside of the organism as a product of the interaction of genes and the environment (i.e., an adult phenotype). On the other hand, molecular markers are detected at the subcellular level and can be assayed before the adult stage in the life cycle of the organism. Molecular markers of necessity are assayed by chemical procedures and are of two basic types – **protein** and **DNA markers**. Markers are indispensable in genetic engineering, being used in selection stages to identify successful transformation events.

**Types of markers**

1. **Morphological markers**
   * Seed color e.g. Kernel color in maize
   * Function based e.g. Plant height associated with salt tolerance in rice

**Limitations**

* 1. Most phenotypic markers are undesirable in the final product (Yellow color in maize).
  2. Dominance of the markers: homozygotes/ heterozygotes not distinguishable
  3. Sometimes dependent on the environment for expression e.g. Height of plants

1. **Molecular markers**
   * Non-DNA such as isozyme markers: Restricted due limited number of enzyme systems available.
   * DNA based markers: Markers based on the differences in the DNA profiles of individuals.

* Some molecular markers are pieces of DNA that have no know function or impact on plant performance (Linked Markers):
  + Detected via mapping.
  + Linked markers are near the gene of interest and are not part of the DNA of the gene.
* Other markers may involve the gene of interest itself (Direct Markers):
  + Based on part of the gene of interest.
  + Hard to get but great once you have it.

**Requirements for a useful molecular marker**

1. Molecular markers must be tightly linked to a target gene. The linkage must be really tight such that the presence of the marker will reliably predict the presence of the target gene.
2. The marker should be able to predict the presence of the target gene in most if not all genetic backgrounds.

**Marker-assisted breeding**

Molecular markers may be used in several ways to make the plant breeding process more efficient. The adoption of a **marker-assisted selection** (MAS) or **marker-aided selection** in a breeding program hinges on the availability of useful molecular markers. Fortunately, this resource is becoming increasingly available to many species, thanks to the advances in biotechnology. This breeding approach is applicable to improving both simple and complex traits, as a means of evaluation of a trait that is difficult or expensive to evaluate by conventional methods. The basic requirement is to identify a marker that co-segregates with a major gene of the target trait. MAS is more beneficial to breeding quantitative traits with low heritability.

**Conditions under which MAS is valuable**

1. Low heritability traits
2. Traits too expensive to score: Soybean Cyst Nematode (SCN) resistance. Young (1999)
3. Recessive genes: Pyramiding of dominant and recessive genes conferring resistance to important crop diseases which would otherwise be very difficult

1. Multiple genes (Quantitative traits): QTLs underlying phenotypic and physiological traits can be traced using markers. Although QTL mapping is tedious, markers once identified can be used fast and accurately to detect the QTLs of interest.
2. Quarantine: No need to grow plants to screen for viral diseases that can not be visually detected, and small tissues can be used for DNA typing.

**Advantages of MAS**

1. Improvement of response to selection (Rs)
2. Assays require small amount of tissue, therefore no destructive sampling.
3. Use of codominant markers allows accurate identification of individuals for scoring without ambiguity
4. Multiple sampling for various QTLs is possible from same DNA prep
5. Can assay for traits before they are expressed, e.g. before flowering
6. Time saving.

**Limitations of MAS**

1. Cost of equipment, reagents and personnel.
2. Data collected in the field is assumed to be normally distributed, but usually is not.
3. Integration of the DNA information into existing systems is difficult.
4. Linkage drag. As the marker distance from the target gene increases, more of the donor DNA is retained in the desired background resulting in need for more backcrosses.

**The Role of PCR in MAS**

Once a direct or linked marker has been located, characterized, and sequenced, a method called *polymerase chain reaction (PCR)* can be used to make copies of a specific region of DNA to produce enough DNA to conduct a test.

**DNA replication in natural systems requires:**

1. A source of the nucleotides adenine (A), cytosine (C), thymine (T), and guanine (G);
2. The DNA polymerase (DNA synthesis enzyme);
3. A short RNA molecule (primer);
4. A DNA strand to be copied;
5. Proper reaction conditions (pH, temperature).

The DNA is unwound enzymatically, the RNA molecule is synthesized, the DNA polymerase attaches to the RNA, and a complementary DNA strand is synthesized. Use of PCR in the laboratory involves the same components and mechanisms of the natural system, but there are three primary differences:

1. DNA primers are used instead of the RNA primer found in the natural system. DNA primers are usually 18-25 nucleotide bases long and are designed so that they attach to both sides of the region of DNA to be copied.
2. Magnesium ions that play a role in DNA replication are added to the reaction mixture.
3. A DNA polymerase enzyme that can withstand high temperatures, such as *Taq*, is used.
4. A reaction buffer is used to establish the correct conditions for the DNA polymerase to work.

The DNA primers are complementary (match up) to opposite strands of the DNA to be copied, so that both strands can be synthesized at the same time. A and T match, and C and G match. Because the reaction mixture contains primers complementary to both strands of DNA, the products of the DNA synthesis can themselves be copied with the opposite primer. The length of the DNA to be copied is determined by the position of the two primers relative to the targeted DNA region. The DNA copies are a defined length and at a specific location on the original DNA. Because DNA replication starts from the primers, the new strands of DNA include the sequence of the primers. This provides a sequence on the new strands to which the primers can attach to make additional DNA copies. Over the years, the PCR procedure has been simplified and the results made uniform as a result of two important developments. The first was the isolation of a heatstable DNA polymerase, *Taq* polymerase. This enzyme gets its name from the bacteria from which it was isolated, *Thermus aquaticus*. This bacteria was discovered living in the boiling water of hot springs. Until *Taq* polymerase was discovered, the DNA polymerases available to researchers were destroyed at 65ºC. The *Taq* enzyme is not destroyed by the high temperature required to denature the DNA template (pattern). Therefore, using this enzyme eliminates the need to add new enzyme to the tube for each new cycle of copying, commonly done before *Taq’s* discovery.

The PCR procedure involves three steps that make up a cycle of copying. Each step allows the temperature of the mixture to change to optimize the reaction. The cycles are repeated as many times as necessary to obtain the desired amount of DNA.

**Step 1: Denaturation**

The double-stranded DNA that is to be copied is heated to ~95ºC so that the hydrogen bonds between the complementary bases are broken. This creates two, single stranded pieces of DNA.

**Step 2: Annealing or hybridization**

The temperature is lowered to ~58ºC so the DNA primers can bind to the complementary sequence on the single-stranded DNA by forming hydrogen bonds between the bases of the template and the primers.

**Step 3: DNA synthesis or extension**

During the replication step, the reaction solution is heated to ~72ºC so the DNA polymerase incorporates the nucleotide bases A, C, T, and G into the new copy of DNA. The new DNA strand is formed by connecting bases that are complementary to the template until it comes to the end of the region to be copied.