PLANT CELL MANIPULATION IN CROP IMPROVEMENT

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Modification of crop plants to improve their output has persisted for at least 10,000 years. Early farmers produced better crops simply by saving the seeds of desirable plants. In the past century plant breeding has benefited from the discovery of Mendel's laws of genetics and subsequent developments in genetics. Now there exists a promising method of developing superior crop plants: manipulation of plant cells in the laboratory to produce better combinations of genes and their regeneration to plants.

We shall look at some of these new developments in plant cell manipulation, under 1. Plant Tissue Culture 2. Induction of Haploids and 3. Genetic Engineering.

PLANT TISSUE CULTURE

Introduction

One of the most exciting and important aspects of Plant cell and tissue culture is the ability to regenerate plants by culture of cells and tissues in the laboratory. This is due to a peculiar character of plant cells. As in all other higher living organisms each cell has a complete set of genes packed in chromosomes. This complete set of genes is called a Genome. In contrast to animal cells, plant cells (with a few exceptions) have the ability to go back to an embryonic state, undergo cell division and produce a completely new plant. This remarkable feature is called totipotency. Animal cells do not have this ability.

In practice, plant cells are placed in an artificial culture medium which contains the required nutrients and hormones and incubated under suitable conditions of temperature, light and humidity. This produces an amorphous (without structure) mass of similar cells called a callus. Under suitable growth conditions the callus can develop into a complete plant.

Applications

Some of the applications of this technique in the improvement of crop plants are discussed below.

Meristem culture

In potatoes, disease causing viruses tend to accumulate in the tubers when cultivated year after year resulting in low yields. The technique of meristem culture is useful in virus elimination. The meristem which is 0.1μm in diameter and 0.25μm long, is composed of actively dividing cells at the apices of shoots and roots. This tissue has no vascular system and is less likely to be infected with systemic viruses. Thus removal of the meristem and regeneration of plants in a culture medium gives us plants free from viruses and other disease causing pathogens.

Somaclonal variation

The regeneration of complete plants from single cells can also result in new mutants. Mutant plants are those with new genetic characters. The isolation of single cells from plant tissue and cell multiplication in a culture medium increases the rate of spontaneous mutations in these cells. This effect is called somaclonal variation, since the mutations occur in somatic tissues. This is of importance in the breeding of new ornamental plants.

Micropropagation by Axillary buds

Propagation of selected plants through tissue culture is called micropropagation. The simplest type of *in vitro* plant propagation is the stimulation of axillary bud development. Axillary buds are found in the axils of leaves or branches, which are usually dormant. By treating with hormones, dormancy can be broken and the buds induced to form shoots. These shoots are separated and rooted to produce plants. The shoots may also be used for further propagation.

Plant regeneration from cultured tissues can also be achieved by culturing tissue sections lacking a preformed meristem or from callus and cell cultures. Axillary buds are preformed meristems.

Plant Regeneration by Somatic Embryogenesis

Embryogenesis is the development of embryos from zygotes which result from the fusion of male and female sex cells. Under *in vitro* condition in plants, it is also possible to induce embryo formation from non sexual cells which are called somatic cells. Such embryo formation is called somatic embryogenesis, where somatic cells divide and form complete embryos similar to zygotic embryos in seeds.

Induction of somatic embryogenesis in most species requires a high concentration of the auxin 2,4-D, in the culture medium. Cytokinins are usually not required except in a few monocot species. However, the high auxin concentration is necessary only for induction. For further development of somatic embryos a hormone free medium is use.

Hardening

The recovery of plants from the culture vessel into the soil is an important step in micropropagation. The plantlets are propagated *in vitro* under conditions of 100% relative humidity with all the necessary nutrients. Thus, there is no need to perform photosynthesis or control respiration. Plantlets growing *in vitro* do not develop a waxy cuticle to control transpiration. When transferred to soil conditions the plantlets must now photosynthesise and adapt to lower relative humidity conditions by developing a waxy cuticle and regulating stomatal function. Therefore the transfer from the culture vessel to greenhouse conditions must be gradual. This is called the "hardening process". Also important is a fine balance of water relations: too little water leads to permanent wilt, while too much water may cause rot.

Contamination and Sterilization

The application of plant cell manipulation techniques requires sterile (aseptic) conditions. The cultures also need to be maintained under sterile conditions. This is because the culture medium used for plant tissue culture is also ideal for the growth of micro-organisms such as bacteria and fungi.

Surface sterilization

The surfaces of plant material contain many types of bacteria, fungi or their spores. Since these would rapidly multiply and prevent the growth of plant tissues in a culture medium, it is necessary to sterilize the surface of plant material before culture.

Procedures vary according to the species. A general procedure for sterilizing of shoot buds, leaf and stem sections is outlined below.

1. Immerse the tissues for 1-3 minutes in 70% ethanol. Some tissues are very sensitive to this.
2. Submerge tissues in commercial bleach (calcium hypochlorite) for 5 minutes. Concentration and time can be varied according to the degree of contamination.
3. Rinse thrice in sterile, demineralized water.

Culture Medium

Plant tissue culture media consist of inorganic salts, a carbon source, vitamins and growth regulators. Other components such as organic nitrogen compounds, organic acids etc. may be added for specific purposes. The choice of media components is determined by the purpose of the tissue culture and the plant species. Plants are able to synthesize all required vitamins.

An important component of the culture medium is plant growth regulators. The major classes of plant growth regulators are:

Auxins — required for cell division, cell expansion and sometimes to produce roots. eg. 2,4-D. IAA, NAA.

Cytokinins — Most cytokinins are adenine (aminopurine) derivatives and important for shoot induction and plant regeneration. They may also stimulate cell division. eg. Benzylaminopurine (BAP), Kinetin.

Auxin compounds are very often used in combination with cytokinins, and are the most frequently used growth regulators.

Gibberellins — are used to promote shoot elongation and are applied after the formation of shoot primordia, eg. Gibberellic acid.

Media Sterilization

The culture medium is sterilized by autoclaving. An autoclave is built to withstand high pressure which enables the boiling point of water to be raised to 121°C. At this temperature spores of all micro-organisms are destroyed. The sterilizing process lasts for 20 minutes at a pressure of 105 kpa.

Certain chemicals are broken down by heat. These cannot be autoclaved and should be filter-sterilized. Special filters with a pore size of 0.22μm (diameter) are used.

Glassware are sterilized in a dry oven at 150°-200°C for 1-4 hours or autoclaved.
Utensils such as forceps, scalpels etc. are sterilized by dipping in alcohol and heating over a flame.

Working area for sterile manipulation

A sterile working area is needed to carry out surface sterilizing of the plant material and transfer of the sterile plant material to the culture medium. This is done in a Laminar Flow Sterile Cabinet. These cabinets or work benches are equipped with a pump which forces air through a very fine filter. Thus a steady stream of air flows from the back to the front over the work-top.

INDUCTION OF HAPLOIDS

Introduction

The discovery of inducing microspores to develop into embryos was accidental. In 1964 Guha & Maheswari were studying meiosis *in vitro* in the plant *Datura innoxia*. They found that the microspores developed into embryos. Three years later Bourgin & Nitsch were able to produce entire plants from haploid embryos. Now, nearly 30 years later, microspore derived plants have been reported in more than 200 species in over 30 families. Most of these species are however in 3 families: *Solanaceae, Cruciferae and Graminae.*

Anthers contain pollen, and anther culture involves the culture of these structures *in vitro*. The immature pollen or microspores within the anther either gives rise directly to embryos or to callus tissue, which in turn is induced to regenerate plants in a culture medium. Pollen is haploid and the cells produced from pollen or microspores during culture are haploid. When plants are regenerated from haploid cells, a haploid plant is produced. Haploid plants are sterile and cannot produce seed. However, a spontaneous duplication of chromosomes often occurs to produce fertile double haploid plants.

Haploid - Sex or genetic cells with half the number of chromosomes. egg. pollen, ovules (in plants); sperm, egg - cell (in animals)

Methodology

The most common form of culture to induce haploids is by anther culture on agar solidified medium. The culture medium contains, macro and micronutrients and various combinations of phytohormones, organic supplements and a source of sugar - usually sucrose. Different media have been optimized for different species.

Anthers are also cultured in liquid medium (ie. without agar). The floating anthers may either produce embryoids or callus on the anthers, or they may shed their microspores in the liquid medium. The microspores then undergo development.

The anther wall is of diploid tissue and often undergoes somatic embryogenesis, which is undesired.

In certain species (eg. *Nicotiana* and *Brassica*) it is possible to culture isolated microspores which are isolated by:

- crushing the buds/anthers to release the microspores
- Sieving to filter the debris and microspores.
- Centrifuging to isolate and pellet the microspores and remove the small particles.

The microspores are cultured in liquid medium. The single microspores can be followed through their stages of cell division to form embryos.

Normal Pollen development

The most important factor in the induction of embryogenesis is the stage of pollen development. The critical stage in most species is the late uni-nucleate or early binucleate stage.

In the anther, pollen develop from pollen mother cells(2n) which undergo mitosis and meiosis to give a tetrad of haploid microspores. The tetrads are released from the pollen mother cells. Initially the microspores are thin walled and vacuolate with a central nucleus. As the microspores increase in volume, a vacuole develops and the nucleus is pushed to the periphery by this vacuole. At this stage, the first pollen mitosis occurs to produce a large vegetative nucleus and a small generative nucleus. This is an asymmetrical division. The vacuole disappears and a second pollen mitosis of the generative cell produces 2 sperm cells one of which ultimately fertilizes an ovule.

GENETIC ENGINEERING

Introduction

A dramatic discovery made in the 1980s, in plant biology was the introduction of foreign genes into the plant genome (the complete chromosome set in the nucleus). Such gene transfer is popularly called Genetic Engineering and plants with foreign genes are called transgenic plants. The introduction of genes into plants requires two important steps.

1. Methods to introduce the gene into the plant cell or tissues.
2. Produce or regenerate fertile plants from the plant cells or tissues.

Methods for introducing genes into plants.

Agrobacterium mediated gene transfer.

Several methods are available to transform plant cells and tissues. The most common is using the bacterium, *Agrobacterium tumefaciens*. This is useful as a gene transfer system because upon infection of the plant tissue, genes are transferred from the bacterium into the plant genome.

In nature, plant cells infected by this soil bacterium integrate segments of a large plasmid into the nuclear genome of the plant cell. This produces a disease called crown gall.

For the bacterium to be effective, its disease causing genes should be removed. This was achieved in 1983. Thus *Agrobacterium tumefaciens* strains were identified which did not cause disease but had the ability to transfer DNA.

To utilize Agrobacterium to transfer genes or genetically modify crops the following are essential.

1. T-DNA vectors into which genes can be inserted adjacent to a selectable marker gene.
   The first engineered gene, constructed with Agrobacterium in the early 1980s made plant cells resistant to the antibiotic Kanamycin, a compound that inhibits plant growth. This breakthrough was important for two reasons. (a) It showed that foreign genes could be expressed in plants and (b) Kanamycin resistance is useful as a "Marker" gene to help us to identify those cells into which genes have successfully been introduced.

2. Plant cells be capable of accepting and integrating the T-DNA.
   Because plant cells are totipotent, complete plants can emerge from the transferred cells. Most methods rely on cells of explants, or pieces of plant tissue for transformation.

3. Transformed cells are capable of regeneration into plants which express the transgene. To identify cells expressing the foreign DNA, reporter genes such as the gene for β- glucuronidase (GUS) enzyme activity are used.

However, many important cereal species such as rice, wheat and maize are not natural hosts for *Agrobacterium* and so cannot be transferred by this method. Thus alternative systems of transferring DNA into plant cell had to be developed.

Direct delivery of DNA into protoplast

Protoplasts are plant cells whose cell walls have been removed by enzymes. The pores of cell walls are too small to allow DNA through. The only barrier in Protoplasts is the plasma membrane. Poly-ethylene glycol (a thick organic polymer) can penetrate the plasma membrane to transport DNA.

Electroporation

Short high voltage pulses are briefly given to plant cells in a liquid culture medium. This produces holes in the protoplast membrane. The DNA molecules can enter through these spaces.

Once DNA enters a cell it should combine with the plant genome, which does not always occur. Thus many attempts have to be made to ensure that the foreign DNA has entered the plant genome.

The genetic engineer does this by assembling genes from its components such that it is able to function in the new host plant.

What is the use of plant genetic engineering?

One of the most promising characters that gene transfer offers is disease resistance. Virus diseases in plants are important because there is no direct way to treat virus-infected crops. A virus is made up of a protein coat which encloses a piece of RNA. It is the RNA that causes the disease. Scientists have introduced the genes for the coat protein of a virus into tobacco plants and found that such plants were resistant to the virus. This is called "cross protection" in plants. The principle is similar to the vaccinations we receive as children.

Other examples of the use of transgenic plants are:

- resistance to insect attack in crops: in cotton, genes toxic only to insects have been transferred. These genetically engineered plants reduce the use of insecticides.

- besides viruses and insects, crops also face a problem from weeds which compete for moisture, nutrients and sunlight. Herbicides are used to control weeds. Since crop plants can also be affected by the herbicides, genetic engineering offers a partial alternative to weed control. The strategy is to create plants that can tolerate the chemicals in a herbicide.