CITRUS BREEDING BY USING BIOTECHNOLOGY

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Introduction

The term Biotechnology has a loose and versatile meaning to different people in different disciplinary area. Generally it has two meanings according to Ahuya (1989):

1) Classical biotechnology which includes regeneration of plants in vitro cell and tissue culture,

2) New biotechnology, which includes molecular biology and genetic engineering of plants.

Obviously, the knowledge and skills on cell biology, molecular biology, physiology and cell and tissue culture are essential for this complex technology.

Biotechnology has come a long way, at times with some excitement, and at times with some disappointment. There are only two groups of publics and scientists for receiving this relatively new science, one is optimists, and the other pessimists. Despite some road blocks, some major advances have been made in the last decades, namely, in the fields of protoplast culture and somatic hybridization, somatic embryogenesis and gene transformation.

In this paper, I will attempt to summarize the current techniques available for in vitro regeneration of plants and gene transfer system with emphasis on their relative weight to Citrus biotechnology. I will try to address the following questions as to, Why do we need plant cell and tissue culture, Do all the cells have totipotency?, What are the basic requirements for in vitro culture, What kinds of techniques are available for in vitro culture, and finally What kinds of techniques are available for genetic improvements in general or for citrus species in particular.

Micropropagations and Organogenesis

Tissue and cell culture has started as a means for clonal propagation. Assuming that each single cell has a potential to regenerate, the in vitro culture techniques would give a tremendous tool to obtain millions of clones from a single mother plant with an identical genetic make up. The advantages of in vitro culture over the conventional sexual breeding program would be countless. The idea of somatic micropropagation was especially attractive to tree species which takes often years of generation cycles for breeding programs. The asexual propagation becomes an invaluable tool for species that are sexually incompatible, infertile, or otherwise genetically very complex as in citrus species.

Basic steps for in vitro culture

The most important step in starting a in vitro culture for a new material, is the selection of explant source and species. Regeneration ability has been proven to be highly explant (physiological) and species (genetic) dependent. It is best to select explants containing meristematic activities such as stem internodes, hypocotyl, leaf midvein, root tips or axillary buds. Recently, many difficult-to-regenerate-species (recalcitrant) have been successfully regenerated when hypocotyl or cotyledonary tissues were used as explants. Cotyledonary tissue has been particularly successful for pinus and douglas fir species (for review, Ahuya 1989). It is also recommended to the beginners to start with well established model plant systems such as solanaceae (e.g. tobacco species) and protocols, and then move onto the species of your own interest.

Basic medium conditions for in vitro culture

For all in vitro culture, the basic medium requirements are inorganic and organic nutrients. Organic nutrients include vitamins, sucrose and sometimes free amino acids. Murashige and Skoog (MS) medium is the most popular medium, and all other media are mostly the modification of these nutrients. For instance Woody Plant Medium (WPM) frequently used for tree species is the half inorganic strength of

the MS medium. Growth hormones are necessary. Most commonly used auxin is NAA (naphthalene acetic acid) and cytokinin is BA (benzyl adenine). Often, 2,4, D instead of or in addition to NAA, and thiadiazuron instead of, or in addition to BA are supplied for recalcitrant species. For each individual species, the optimal concentrations of NAA and BA must be determined by a quadric block method. For citrus, Murashige and Tucker(MT) is in use with elevated vitamins and adenin sulfate added in the MS medium. Malt extracts has been found useful for citrus culture (Evans et.al. 1981).

Because the cellular, physiological and genetic basis for regeneration is not known, the selection procedure for explant and species is in the stage of trial and error until we have more basic research is done in this field.

For citrus species, somatic embryogenesis of nucellus derived callus is predominant in vitro system, and so far very sporadic data are available for direct organogenesis. When either seedling organs (stem or shoot tips) or in vitro plantlets were used as explants, organogenesis seems possible (see for review, Button and Kochba 1977, George and Sherreyton 1984, Duran-Vila et.al. 1989, 1992). No apparent genetic variations were observed either in plants derived from somatic embryogenesis or from organogenesis, for instance from long term root culture (Bhat et.al. 1992).

Somatic Embryogenesis

Production of embryos from somatic cells is called somatic embryogenesis, and can occur either directly or indirectly. Direct somatic embryogenesis involves the formation of an asexual embryos form a single cell or tissues on an explant without forming callus, such as in citrus. In citrus, pre-existing nucellus tissue cells give rise to nucellar embryos both in vivo and in vitro. Indirect embryogenesis is more common than the direct one, and pro-embryos are formed via proliferation of callus in an artificial environment such as in a high concentration of 2,4, -D (0.45-452 μM). Practically all species are able to form asexual embryogenesis.

The general procedures for somatic embryogenesis are the establishment of callus from explants of mature or immature embryo or organs in MS medium containing auxin and cytokinins. Most commonly used auxin is 2,4,- D for many species. For inducing somatic embryogenesis, growth hormones are removed, usually additional substances are added (e.g. malt extracts) to increase the number of embryos (Tisserat 1985). The plantlets are formed in growth regulator free medium. Somatic embryogenesis is induced under a stressful culture conditions, and therefore the genetic stability of plants through somatic embryogenesis is considered questionable, and is not ideal system for clonal propagation purpose.

Somatic embryogenesis in woody species has been observed most frequently when embryos (immature) or embryo segments are used as explant. Recently, however, cotyledonary tissue or hypocotyl of some woody species, for instance, pinus (for review, Ahuja 1989) have been reported as successful explant for somatic embryogenesis. Cotyledonary tissues are especially interesting because it is a storage material with terminal growth. Obviously these mature cells can be redifferenciated into meristematic cells, which means there can be other potential tissue types that can be explored.

Detailed and established routine protocols for direct somatic embryogenesis of citrus from polyembryonic nucellus cells of immature ovules have been published (Tisserat 1985, Evans and Sharp 1981)). For citrus embryogenesis, Indole acetic acid and kinetins are used for callus induction of immature fruits as explant, and polyembryonic culture are obtained in MS medium without growth regulators.

Protoplast culture and Somatic Hybridization

Regeneration from a single protoplast into a plantlet was thought to be an ultimate and ideal means for clonal propagation. Last several decades, numerous efforts have been made world wide for numerous species. Those that can be regenerated successfully, however, are disappointingly small, and limited only

to few species, mainly to oolanaceae (e.g. tobacco). Recently additional species are expanded to economic cereal crops such as rice and wheat. Protoplast system can be a powerful tool for somatic hybridization for those species that are impossible by sexual propagation if the protoplast of desired species can be regenerable. For woody species, only few species (pinus, spruce and Populus, cf. Ahuja, 1989) have been reported successful for regeneration from protoplasts, and most of the other species have difficulty to form embryos. Problems accompanying the protoplast isolation and culture are permanent damage in the integridity of membranes during enzymatic digestions. The long and artificial culture period of single protoplasts can be also mutagenic, and therefore, genetically unstable. Regeneration of a whole plantlet or plant from a single isolated protoplast has been proven succesful for citrus species. In fact, the protoplast system is the most prevailing system among all others for citrus species. For citrus, traditional breeding is not desirable because most citrus species are highly heterozygous, reproduces apomixically from seeds or seedlings, long reproductive cycles and nucellar polyembryony. The progress made recently by the Florida research group and Israel group (Grosser et.al. 1990, Tusa et.al.1990; Vardi and Gallun 1988) using protoplasts is impressive, and their success rate for somatic hybridization is noteworthy for their contribution in biotechnology (cf. Grosser et.al. 1989). They find no genetic aberrations in those regenerants from the protoplast culture. They used somatic hybridization by protoplast fusion to obtain scion improvement as a breeding parent having allotetraploids to generate improved seedless triploid scion. It is interesting to note from literature that protoplast system from ovule derived nucellus callus for regeneration is the best, or most predominantly used explant system for citrus (Grosser et.al 1990; George and Sherrington 1984; Tisserat 1985). This is quite intriguing because in most other species, protoplast system is known to have much more culture related problems than organogenesis. Apparently, vegetative organs of citrus seem to be recalcitrant to regeneration. There is a need to explore alternative explant system for direct organogenesis that are abundant and available all year around.

Ploidy Manipulation in vitro for Citrus Breeding

Seedless triploids are desirable for breeding parents from crossing diploid and tetraploid. Tetraploid can be obtained in vitro either spontaneously or by treating the mature axillary buds or embryogenic cells with colchicine (0.01 to 0.1%). Somatic hybridization by protoplast fusion is another source for obtaining tetraploids. Grosser groupd (1989, 1990) used somatic hybridization by protoplast fusion to obtain scion improvement as a breeding parent having allotetraploids to generate improved seedless triploid scion. Triploid plantlets have been recovered from endosperm-derived callus culture (for review, Frederick et.al. 1993). Attempts to culture anthers for obtaining haploid plants (or dihaploid) have resulted in haploid, diploid and mixoploids. Haploid cultures using anthers or pollens through inducing androgensis have been successful for many other herbaceous plants and has been used as a means of getting homogygous plants for breeding program. Haploid cultures for tree species including Citrus, however, have not been very successful.

Gene Transformation

There are several methods available for transferring foreign genes into plant cells, broadly, one is indirect gene transfer using Agrobacterium as vector and the other is direct gene transfer method; direct uptake of DNA into isolated protoplast in the presence of polyethylene glycol, electroporation technique, and most recently developed particle bombardment. Agrobacterium can be directly inoculated onto any explant (e.g. leaf disk), however, its infection is highly genotype specific. One of the biggest obstacle is that they do not infect monocot family. Electroporation transfers genes by an electric shock into a single protoplast, but not to a cell with cell walls. Control of protoplast damage during the poration seems to be a major problem which impairs the regeneration mechanics permanently.
Particle bombardment technique requires no genotype

specificity, nor isolated protoplast system. DNA is coated on the gold particles, accelerated at a high velocity generated by a high pressure where the particles penetrate the individual cell. Therefore genes could be delivered to any cell type without deleterious effects. Most commonly used material for this technique is suspension cells. Reproducibility needs to be worked out each species, and selection procedure, like in any other gene transfer techniques seems to be the major problem yet unsettled.

For many woody species, all of these techniques have been tried. Agrobacterium and particle bombardment technique have been proven most successful, and now even becoming most routine part of the biotechnology. For citrus, Agrobacterium transformation has been tried on the internodal segments of in vitro grown seedlings (Moore et.al 1989) without success, but apprently was successful when embryogenic callus was cocultivated with Agrobacterium (Hidaka et.al 1990). Direct gene transfer into the protoplasts has been tried, but no transformed plants were obtained (Kobayashi and Uchiiya 1989). A successful transformation by direct DNA transfer using PEG has recently been reported by Israel group (Vardi et.al 1990) and the first transgenic plants in woody species by direct gene transfer to protoplasts are confirmed. Though transformation by Agrobacterium and by direct DNA uptake seems apparently possible, their efficiency is extremely low in either techniques. No successful particle bombardment results have been reported for citrus.

According to the experienced scientist (McGown 1989), a success to the bombardment technique is the physiological condition of the tissue, where active meristematic growth is taking place is most receptive to this technique. Unfortunately, not much has been worked out for the biology of gene transfer by bombardment technique. Nerverthless, this technique is rapidly becoming the leading technique in plant genetic engineering area for major species because of its rapid, easy and non-host specificity. One major technical drawback is that the particle can penetrate only 1 to 2 cell layers effectively. Therefore, the experimental system must be either single cells (i.e. suspension cells) or thin section of tissue (i.e. microcross sections, i.e. Lee-Stadelmann et.al 1987, 1989).

The most difficult and remaining task is tackling tissue culture regeneration problems as the regeneration of plants is the prerequisite of the whole biotechnology. Whatever culture system we use, we must make sure that the culture process itself does not induce genetic variability, and the genes introduced are stably expressed in the regenerants.

Perspective and Conclusions

Optimism for citrus biotechnology is not overstated, because citrus is one of the most actively studied fruits crops in the world, and the progress made in the field by the developed countries has proven itself. It seems hopeful that we can achieve through biotechnology, the goal of obtaining desirable traits for citrus such as shortened juvenility, resistance to disease and cold, desirable fruits color and flavor. However, biotechnology is a very broad field, requiring multidisciplinary efforts from cell and tissue culturists, geneticists, physiologists, molecular biologists and horticulturists. Obviously, not a single expertise can solve this vast and complex field alone. It is timely to remind ourselves of the power of collaboration and the power of information.It is utmost important to get all the information we can get through establishing networking at the international level, learning from those who have experience, sharing the experimental material and technology, exchanging the ideas through communications with experts in the field, visiting the leading citrus laboratories of the world, and organizing and participating international seminars and symposium of citrus. Only when we have a grasp of the world trends, we can explore new ideas, make a jump and have a hope to win a world competition. It is my strong personal view that advanced countries became the leader of the field only because they realized early on the power of basic research. Basic research requires knowledge and information. If we stay on copying others, we will remain following the foot steps of others.

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