LEAF DISC METHOD FOR PLANT REGENERATION

INTRODUCTION

Genetic engineering of plants involves several independent yet closely related steps. First, a gene must be identified and isolated (engineering). Second, a mechanism for the introduction of the gene into plant tissues (cells) must be developed (transformation through vectors). And third, production of whole plants from transformed cells must be performed (regeneration). A great deal of research time and effort has been spent in the engineering of commercially-important, plant genes. Herbicide (glyphosate, Roundup®) resistance and pathogen tolerance have been the most successful to date.

The introduction of these genes through the use of transformation vectors has also been a major research focus. Isolated genes can be introduced into plants several ways. Microinjection, electroporation, ballistic incorporation, *Agrobacterium tumefaciens* co-incubation, and virus infection are potential methods for the introduction of foreign genes into the plant genome. Of these methods, co-incubation of plant cells (or protoplasts) with *Agrobacterium* (the organism responsible for crown gall disease) has been used most effectively for the transformation of plant tissues.

Transformation with *Agrobacterium* was first proposed as a means of incorporating DNA into plants by exposing plant protoplasts (individual cells devoid of their cell wall) to the organism (Figure 2). Disadvantages to this technique included the difficulty in obtaining large numbers of high quality protoplasts and the difficulty in regenerating whole plants from protoplasts. Relatively few plants can be regenerated from protoplasts.

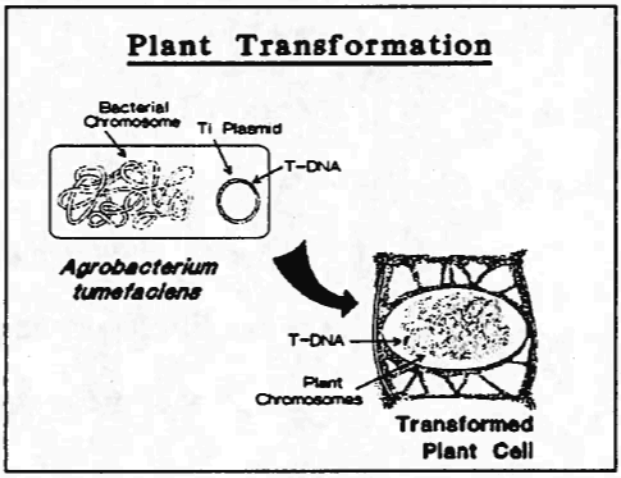

Figure 2. Incorporation of T-DNA from *Agrobacterium tumefaciens* into the chromosomes of a plant cell.

In 1985, scientists at Monsanto developed a leaf disc method for the co-incubation of tobacco, petunia, and tomato leaves with *Agrobacterium* for the transformation of plant tissues. In this method, surface-disinfested leaf discs are incubated with an *Agrobacterium* strain containing a Ti plasmid (Figure 2). The leaf discs are then transferred to a "selective plate" on which only transformed cells will grow. This plate also contains antibiotics that arrest the growth of any bacteria present. The leaf disc technique has been widely accepted and continues to show great promise for the genetic transformation of many plant species.

OBJECTIVES

- to regenerate whole plants (or at least shoots) from leaf discs of petunia
- to learn a technique for the genetic transformation of petunia

MATERIALS

Plant Material - [ ] leaves from *Petunia hybrida* V26

Culture Medium - [ ] Murashige-Skoog salts, full-strength

[ ] Full-strength, MS vitamins

[ ] myo-Inositol - 555 μM (100 mg per liter)

[ ] Sucrose - 87.6 mM (3%)

[ ] BAP - 4.5 μM (1 mg per liter)

[ ] NAA - 0.5 μM (0.1 mg per liter)

[ ] pH 5.7

[ ] Agar - 0.8%

Utensils - [ ] forceps

[ ] paper punch (6 mm diameter hole)

[ ] empty, sterile, plastic petri dishes

[ ] 95% ethanol for flaming, spray bottle of 95% ethanol, cheesecloth

To Be Autoclaved - [ ] Culture medium, in flasks, for distribution into petri dishes after sterilization

[ ] Deionized, wash water (ca. 600 ml) in 1000 ml flasks

[ ] 90 ml of deionized water in 125 ml bottles w/ black lids

[ ] Forceps and paper hole punch wrapped individually in aluminum foil

METHODS

Tissue (leaf) disinfestation

[ ] 1. Carefully select a healthy, vigorous **young** leaf (≈2 cm wide and ≈4 cm long).

[ ] 2. In the laminar flow hood, place the leaf section into a 125 ml bottle containing 100 ml of 10% bleach and 1 drop of Joy® detergent.

[ ] 3. Tightly cap the bottle and agitate every minute or so.

[ ] 4. After 10 minutes, decant the bleach solution and pour in autoclaved rinse water.

[ ] 5. Agitate briefly (10 seconds) and decant the rinse solution.

[ ] 6. Repeat the rinses (Steps 5 & 6) two more times.

[ ] 7. Remove the leaf section from the 125 ml bottle with the forceps and place it in a sterile, plastic petri dish.

[ ] 8. Make several 4-8 leaf discs from the leaf using the paper punch.

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Because of strict federal and state regulations, we cannot actually work with living strains of Agrobacterium tumefaciens. Therefore, the following steps (marked off with asterisks) will not be performed.

9a. Place the leaf discs into a test tube containing a liquid culture of Agrobacterium for 2-5 minutes. Agitate gently.

9b. Remove the leaf discs individually and blot dry on sterile filter paper.

9c. Transfer the leaf discs to a culture medium (as above) containing 100 μM acetosyringone. This treatment will enhance infection.

9d. After 48 hours, transfer the leaf disks to a culture medium containing 200 μg/ml cefotaxime and 10-100 μM kanamycin. The cefotaxime checks the growth of the bacterium and kanamycin selects for transformed cells.

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[ ] 10. Transfer the leaf discs to the culture media prepared in plastic, petri dishes.

[ ] 11. Seal the petri dish with Parafilm® and incubate at room temperature in the light.

OBSERVATIONS AND QUESTIONS

[ ] 1. During the first week, callus will form on the periphery of the leaf discs. In 2-4 weeks, shoots should begin forming from the callus. Fully-developed shoots can be rooted to obtain whole plants.

[ ] 2. When did callus tissue first appear?

[ ] 3. When were shoots first visible?

LITERATURE

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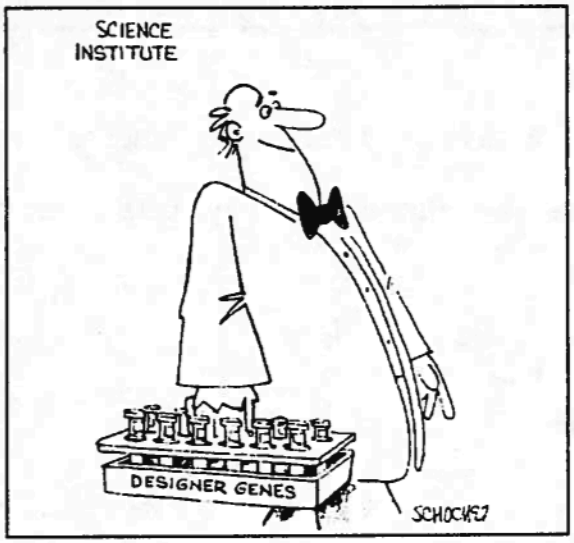

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