Induction of Crown Gall on Carrot Slices

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Laboratory experiments on the transfer of a plasmid to a bacterium, whether by conjugation, transformation or transduction, are available for use in the teaching laboratory. However, the transfer of plasmid from a bacterium (prokaryote) to a plant cell (eukaryote) has received little attention. The following is an experiment for studying this latter type of genetic transformation.

The Gram negative soil bacterium, *Agrobacterium tumefaciens* L., is the causative agent of crown gall disease, a malignant tumor, occurring on the stems and leaves of infected dicotyledonous plants. Induction of this tumor is mediated by the presence in the bacterium of the large (200 kbp in length) tumor-inducing (Ti) plasmid. Upon bacterial infection of a wounded plant, phenolic defense chemicals released from the plant activate virulence genes on the Ti plasmid, leading to the processing and transferring of a small (20–23 kbp) segment of the Ti plasmid into the host plant cell. The bacterium remains outside the plant cell, living in the intercellular spaces of the plant. By transference through a conjugation tube initiated by the bacterium, this small segment of the Ti plasmid, designated transferred DNA or T-DNA, penetrates the host plant cell and subsequently incorporates into the plant's genome. T-DNA contains genes for the syntheses of opines, chemicals used as sources of carbon and nitrogen by the infecting bacterium, and phytohormones. Overproduction of these phytohormones (i.e. auxin and cytokinin) results in uncontrolled plant cell growth and hence the development of a tumor (Gelvin & Karcher 1996; Russell 1996).

In this exercise, we induce crown gall on carrot slices placed in a petri dish containing agar and maintained for 10–14 days in a humidified atmosphere. Three carrot slices are used. One slice is inoculated with a broth culture of *A. tumefaciens*; crown gall will form on this slice. The two other carrot slices serve as controls. To show that crown gall formation is not due to chemical components in the broth itself, the second carrot slice is inoculated only with sterile broth. To further demonstrate that crown gall formation requires direct bacterium-plant cell contact and that it is not caused by chemicals synthesized and released by the bacterium (e.g. a carcinogen), the third carrot slice is inoculated with filtered bacterial broth medium in which *A. tumefaciens* was grown and subsequently passed through a 0.2 μm syringe filter. The pore size of this filter is small enough to prevent the passage of the bacterium. Thus, this control contains medium (termed, *spent broth*) in which the bacterium grew and produced metabolites but lacks the microbe itself.

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Procedure

First Session

To avoid unnecessary contamination, a knife, forceps, and vegetable peeler are kept in a vessel containing 70% alcohol. The bacterium is grown in tryptic soy broth at 26° C. Because the plasmid does not replicate above 37° C, care should be taken to avoid elevated temperatures throughout the entire experiment.

1. Wash and peel a fresh carrot, preferably one with green leaves at the top.
2. Using a sterilized knife, remove carrot ends to produce a 75-cm carrot segment. This segment should contain enough material to yield approximately six to nine carrot slices and should be sufficient for use by two to three students.
3. Place this carrot segment into a vessel containing 6.4% bleach for about 4 minutes.
4. With sterilized forceps, pick up the carrot segment and let the excess bleach drain into the vessel.
5. To remove any residual bleach, soak the carrot segment in three changes of sterile water (about 4 minutes each rinse).
6. Place the carrot segment into an empty, sterile petri dish and, using a large sterile scalpel, cut a 5-cm slice from the top and bottom of the carrot segment; discard these slices because they were directly exposed to the bleach.
7. Using the scalpel, cut the carrot segment into slices about 0.5 cm in thickness.
8. Prepare a petri dish containing agar (1.5% Bacto-agar) and, with marker pen, divide the bottom of the plate into three equal, triangular sectors. Label one sector "Bacterium," another "Fresh Broth," and the third sector label "Spent Broth."
9. With sterile forceps, transfer a carrot slice to each sector.
10. Using a micropipet, inoculate 10 μl of a broth culture of *A. tumefaciens* onto the center of the carrot slice located in the sector marked "Bacterium." Discard this tip. Using a fresh pipet tip for each subsequent inoculation, transfer similar aliquots of fresh broth and of spent broth to the appropriately labeled carrot slices.
11. Cover the petri dish and place it in a humidified chamber (e.g. a large, partially sealed canister with water on the bottom) housed in the dark within an incubator set at 26° C.

Second Session

(about 14–21 days later, depending in the strain of *A. tumefaciens*):

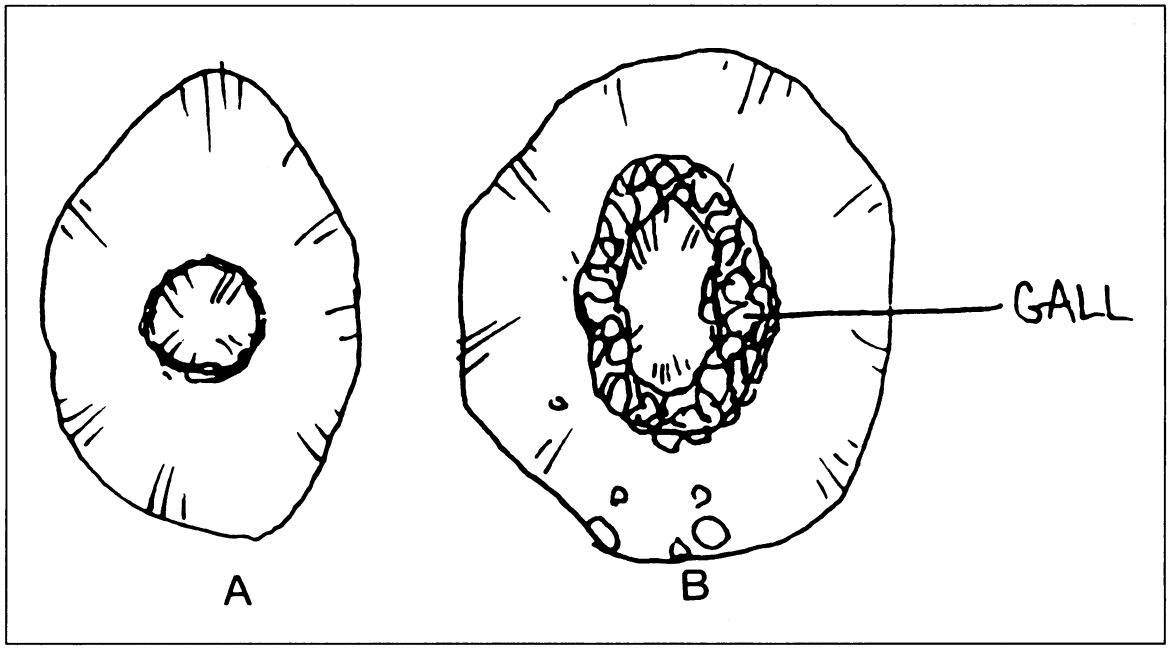

Figure 1. Crown gall formation on a carrot slice. (A) Control: inoculated only with bacterial growth medium. (B) Experimental: inoculated with a broth culture of *Agrobacterium tumefaciens*.

1. Examine the slices. The crown gall appears as a concentric ring of a creamy-to-yellowish lumpy growth surrounding the pith of the carrot slice (Figure 1).
2. Using a sterile single-edged razor or scalpel, make a longitudinal cut through the gall and the plant tissue. From this, prepare a thin slice for microscopy.
3. Transfer this thin slice to a microscope slide and view, initially, with the scanning (4X) objective and then with the low power (10X) objective. There is no need to add a coverslip.
4. For better contrast, add a few drops of methylene blue to the

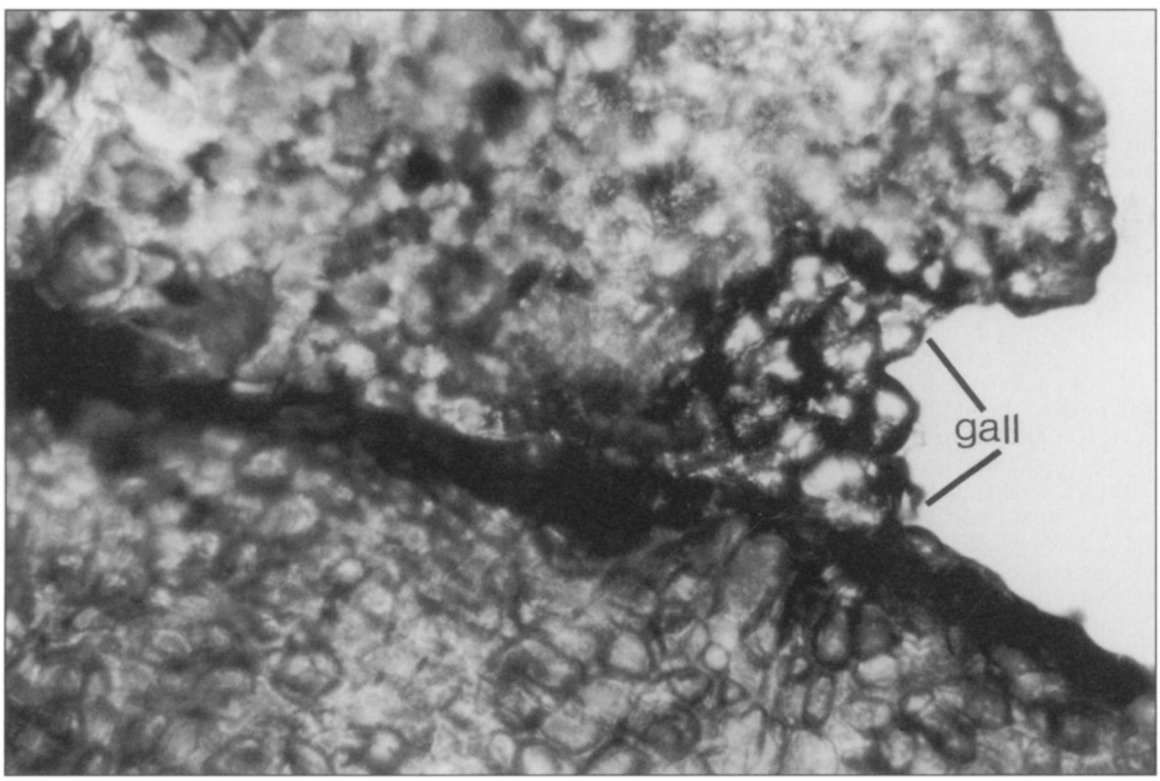

Figure 2. Microscopic appearance of cells from crown gall and from uninfected carrot tissue. Original magnification: 100X; unstained.

thin slice of carrot. After one minute, add a few drops of water to remove the excess stain and draw off the excess fluid with an absorbent towel.

5. Observe under scanning and lower power objectives. The cells comprising the gall appear smaller and disorganized; whereas, the cells of uninfected, nonmalignant carrot tissue are organized with a definitive morphology. Xylem tissue will be noted in the uninfected carrot tissue (Figure 2).

Discussion

A meaningful discussion can be generated on the use of the Ti plasmid to establish transgenic plants. By removing nonessential genes from the T-DNA region, especially those controlling malignancy and tumor formation, researchers have constructed genetic vectors for foreign DNA, including genes from a variety of plant species as well as animals and bacteria, that effectively transfer desirable genes to the transfected plant genome (Russell 1996).

This experiment has several applications depending on the needs of the class. The following procedure can be followed to demonstrate that the bacterium is still associated with the gall:

1. Remove some of the crown gall from the carrot slice.
2. Place it in a sterile mortar and add sterile water.
3. Mash with a sterile pestle.
4. Streak the resultant slurry on a tryptic soy agar plate.

After appearance of bacterial growth in about 24 to 48 hours, a smear can be prepared and stained for microscopic examination (Johnson & Case 1992).

To demonstrate crown gall development in the intact plant, a potted dicotyledon plant (e.g. sunflower; tomato) can be inoculated with *A. tumefaciens* (Johnson & Case 1992). To emphasize that the induction of plant cancer is due to genes on the plasmid, rather than to those on the bacterial chromosome, carrot slices or intact plants can be inoculated with a plasmidless strain of *A. tumefaciens*. [A plasmidless strain of *A. tumefaciens* (ATCC 51350) can be obtained from the American Type Culture Collection (Rockville, MD).] To add a biotechnology component to the exercise, gel electrophoresis can be used comparing the DNA isolated from plasmid and plasmidless strains of *A. tumefaciens* (Lebowitz & Sanderfoot 1991).

The experiment even in its simplest form, i.e. tumor induction on slices of carrot, is unique in that it involves gene transfer from a bacterium to a plant. Furthermore, the induction of malignancy in plants is both exciting and novel to most students. A Plant Cancer Study Kit (P7-15-4741) is available from the Carolina Biological Supply Company (Burlington, NC).

Acknowledgments

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