ISOLATION OF AGROBACTERIUM TUMEFACIENS BY PCR TECHNIQUE

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ABSTRACT

A simple PCR protocol was developed for identifying Agrobacterium as the causal agent of the tumors produced by this bacterium in plant material. The sensitivity of this method was compared with that of bacterial isolation using common and selective media with a previous enrichment step. More than 200 samples from tumors of naturally infected and inoculated plants from several hosts including almond, peach × almond hybrids, apricot, rose, tobacco, tomato, raspberry, grapevine and chrysanthemum, were analyzed by both methods. PCR was the most efficient method for detecting the bacterial aetiology of the plant tumors. Agrobacterium tumefaciens was better detected in crown and root tumors than in aerial tumors with all the methods assayed in inoculated plants. A comparison between the efficiency of the diagnosis by analyzing pieces from the external and internal part of the tumor showed no differences between them.Polymerase chain reaction (PCR) has been used for identification and detection of Agrobacterium in pure culture, soil and infected plants but there is little information on the comparative efficiency of PCR and other techniques for A. tumefaciens diagnosis in tumors of the wide spectrum of hosts of this bacterium. This is particularly important when using PCR for diagnosis in plant material because of the frequent presence of inhibitors of the Taq polymerase in different plant tissues .Three sets of primers were selected for this study because previous experiments had shown that they were appropriate for detection in plant material. Furthermore, several authors have indicated that inside the tumors, viable cells of A. tumefaciens are usually few in number and are confined to the outer cell layers of the gall but as far as is known, there has been no comparative study on the presence of pathogenic bacteria in external and internal tumor tissues.This paper reports on the setting up and evaluation of a new and simple PCR protocol for rapid, sensitive and specific detection of pathogenic Agrobacterium from galled plants and on a comparison with isolation methods, with or without a previous enrichment step.

INTRODUCTION

Agrobacterium tumefaciens causes **crown gall** disease of a wide range of dicotyledonous (broad-leaved) plants, especially members of the rose family such as apple, pear, peach, cherry, almond, raspberry and roses. A separate strain, termed biovar 3, causes crown gall of grapevine.

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marketability of nursery stock, it usually does not cause serious damage to older plants. Nevertheless, this disease is one of the most widely known, because of its remarkable biology. Basically, the bacterium transfers part of its DNA to the plant, and this DNA integrates into the plant's genome, causing the production of tumors and associated changes in plant metabolism.The unique mode of action of *A. tumefaciens* has enabled this bacterium to be used as a tool in plant breeding. Any desired genes, such as insecticidal toxin genes or herbicide-resistance genes, can be engineered into the bacterial DNA and thereby inserted into the plant genome. The use of *Agrobacterium* not only shortens the conventional plant breeding process, but also allows entirely new (non-plant) genes to be engineered into crops. The story of *Agrobacterium* goes even further than this, making it one of the most interesting and significant bacteria for detailed study. For example, there is a highly effective biological control system for this disease - one of the first and most successful examples of biological control of plant disease. Here we consider three major aspects of this intriguing disease:the biology of the bacterium and the infection process,the development of a highly successful biological control system against crown gall disease,the wider use of *A. tumefaciens* as a tool for genetic engineering of plants.The bacterium and its plasmidsA. tumefaciens is a Gram-negative, non-sporing, motile, rod-shaped bacterium, closely related to Rhizobium which forms nitrogen-fixing nodules on clover and other leguminous plants. Strains of Agrobacterium are classified in three biovar based on their utilization of different carbohydrates and other biochemical tests. The differences between biovar are determined by genes on the single circle of chromosomal DNA. Biovar differences are not particularly relevant to the pathogenicity of A. *tumefaciens*, except in one respect: biovar 3 is found worldwide as the pathogen of grapevines. But this is almost certainly because biovar 3 has been spread around the world in vegetative cuttings of vines, not by natural mechanisms.

Most of the genes involved in crown gall disease are not borne on the chromosome of A. *tumefaciens* but on a large plasmid, termed the T_i (tumor-inducing) plasmid. In the same way, most of the genes that enable Rhizobium strains to produce nitrogen-fixing nodules are contained on a large plasmid termed the Sym (symbiotic) plasmid. Thus, the characteristic biology of these two bacteria is a function mainly of their plasmids, not of the bacterial chromosome.A plasmid is a circle of DNA separate from the chromosome, capable of replicating independently in the cell and of being transferred from one bacterial cell to another by conjugation. Plasmids encode non-essential functions, in the sense that a bacterium can grow normally in culture even if the plasmid is lost.

The central role of plasmids in these bacteria can be shown easily by "curing" of strains. If the bacterium is grown near its maximum temperature (about 30^oC in the case of *Agrobacterium* or Rhizobium) then the plasmid is lost and pathogenicity (of *Agrobacterium*) or nodule-forming ability (of Rhizobium) also is lost. However, loss of the plasmid does not affect bacterial growth in culture - the plasmid-free strains are entirely functional bacteria.In laboratory conditions it is also possible to cure *Agrobacterium* or Rhizobium and then introduce the plasmid of the other organism. Introduction of the T_i plasmid into Rhizobium causes this to form galls; introduction of the Sym plasmid into *Agrobacterium* causes it to form nodule-like structures, although they are not fully functional.

Agrobacterium tumefaciens is found commonly on and around root surfaces - the region termed the **rhizosphere** - where it seems to survive by using nutrients that leak from the root tissues. But it infects only through wound sites, either naturally occurring or caused by transplanting of seedlings and nursery stock. This requirement for wounds can be demonstrated easily in laboratory conditions. For example, the bases of two young tomato plants where a drop of *A. tumefaciens* bacterial suspension was placed on the stem and a pin prick was then made into the stem at this point. The photograph was taken 5 weeks later. Shows

another laboratory assay, where bacterial suspension was added to the surface of freshly cut carrot disks. After 2 weeks the young galls (green-colored) developed from the meristematic tissues around the central vascular.

MATERIAL AND METHODS

The polymerase chain reaction (PCR) is a technology in molecular biology used to amplify a single copy or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence.Developed in 1983 by Kary Mullis, PCR is now a common and often indispensable technique used in medical and biological research labs for a variety of applications.[3][4] These include DNA cloning for sequencing, DNA-based phylogeny, or functional analysis of genes; the diagnosis of hereditary diseases; the identification of genetic fingerprints (used in forensic sciences and DNA paternity testing); and the detection and diagnosis of infectious diseases. In 1993, Mullis was awarded the Nobel Prize in Chemistry along with Michael Smith for his work on PCR.

Sample Collection:A total of 9 Samples were used in this study. The samples are collected from different plants parts and from different garden soil are taken to study. Samples of Blueberry different plant parts are taken like stem, leaves, roots and soil are taken. Soil from two different garden and grapes plant and rose plant parts are taken for study.

Biosafety level: A biosafety level is a level of the biocontainment precautions required to isolate dangerous biological agents in an enclosed laboratory facility. The levels of containment range from the lowest biosafety level 1(BSL1) to the highest at level 4 (BSL4). In the United States, the Centers for Disease Control and Prevention (CDC) have specified these levels. In the European Union, the same biosafety levels are defined in a directive.

BIOSAFETY LEVEL II

All practices followed in a BSL-1 laboratory should be instituted in a BSL-2 laboratory. Additionally, the following practices taken from Biosafety in Microbiological and Biomedical Labs should be instituted in any laboratory designated BSL-2:

- All persons entering the laboratory must be advised of the potential hazards and meet specific entry/exit requirements.
- Laboratory personnel must be provided medical surveillance and offered appropriate immunizations for agents handled or potentially present in the laboratory.
- Each institution must establish policies and procedures describing the collection and storage of serum samples from at-risk personnel.
- A laboratory-specific biosafety manual must be prepared and adopted as policy. The biosafety manual must be available and accessible.
- The laboratory supervisor must ensure that laboratory personnel demonstrate proficiency in standard and special microbiological practices before working with BSL-2 agents.
- Potentially infectious materials must be placed in a durable, leak proof container during collection, handling, processing, storage, or transport within a facility.
- Laboratory equipment should be routinely decontaminated, as well as, after spills, splashes, or other potential contamination

EXTRACTION OF AGROBACTERIUM TUMEFACIENS DNA BY SILICA COLUMN METHOD

1. Firstly take a MCT for collection of effected part and soil.
2. Now Take 5 µl Effected soil of Rose/Grape of effected area for the extraction of DNA.
3. Now, add Lysis Buffer and 20 µl Proteinase (k) in MCT.

4. Now Vortex the solution in Vortexer.
5. After that incubate the solution at 65°C for One Hour and vortex the sample in every 10 minutes so that it mix well in MCT.
6. Centrifuge the sample at 4000 Rpm for 5 to 10 Min.
7. Centrifuge at 4000 Rpm for 5 to 10 Min and Incubate at 70'C For 5 min.
8. Add chilled Ethanol (400 µl) and vortex it.
9. Transfer 600 µl of sample in silica Column and Centrifuge at 10000 rpm for 2 min
10. Discard Collection tube.
11. Add Washing buffer 1 (500 µl and Centrifuge at 10000 rpm for 2 min.
12. Decant the collection Tube.
13. Add washing buffer 2 (500 µl) and Centrifuge at 13000 rpm for 2 min.
14. Decant the Collection Tube and Dry wash.
15. Now Centrifuge the sample at 13000 Rpm.
16. After that Remove Collection Tube.
17. Now Transfer Silica Column into Fresh Labeled MCT.
18. After that Add Preheated Elution Buffer 70'C (200 µl).
19. Hold the sample for 2 to 3 Min.
20. Now Centrifuge the sample at 13000 rpm for 2 min.
21. Remove silica column of sample.
22. Sample DNA Extracted.
23. Centrifuge at 13000 rpm for 2 min.
24. Remove silica column of sample.
25. DNA is extracted.
26. Transfer DNA Extract to marked PCR tubes.

1. Manual DNA extraction to obtain Agrobacterium tumefaciens Bacteria in selected sample.

2. Automated PCR amplification of target DNA using ***Agrobacterium tumefaciens*** **Bacteria** specific complementary primers, which is processed, amplified, and detected simultaneously with the specimen.

SAMPLE PREPARATION FOR GEL ELECTROPHORESIS

Add 5 µl of gel loading buffer to the amplified product and mix well.

The samples are now ready for electrophoresis.

1. Assemble the electrophoresis apparatus. Prepare 2.0% Agarose gel by adding

   0.5gm of Agarose to 100 ml of 1×TAE buffer. Boil the Agarose in a beaker until it becomes clear.

2. Add 10µl of 10 mg/ml Ethidium Bromide dye solution for 100 ml of cool Agarose, and pour it into the gel tank. The volume of the gel will vary according to the size of gel tank. The total thickness of the gel should not be more than 0.8 cm.

3. Once the gel is solidified, add the reservoir buffer
   (1×TAE) and then carefully remove the comb.

4. Load 12µl of the samples (change the pipette tips for each sample) and 5µl ready-to-use DNA Molecular Weight Marker.

5. Electrophoreses at 100-120 volts stop the electrophoresis when the dye reaches around 2/3rd of the gel.

6. Remove the gel and it is now ready for visualization. lay the gel on the mid wave UV-transilluminator to read the final result.

7. After electrophoresis wash the gel tank with plenty of water and any dry to avoid contamination.

DISCUSSION

In natural conditions, the motile cells of A. tumefaciens are attracted to wound sites by chemotaxis. This is partly a response to the release of sugars and other common root components, and it is found even in plasmid-cured strains. However, strains that contain the T_i plasmid respond even more strongly, because they recognize wound phenolic compounds such as acetosyringone which are strongly attractive at even very low concentrations (10^{-7} Molar). Thus, one of the functions of the T_i plasmid is to code for additional, specific chemotactic receptors that are inserted in

the bacterial membrane and enable the bacterium to recognize wound sites.Acetosyringone plays a further role in the infection process, because at higher concentrations (about 10^{-5} to 10^{-4} Molar) than those that cause chemotaxis it activates the virulence genes (Vir genes) on the T_i plasmid. These genes coordinate the infection process and, in particular:lead to the production of proteins (permeases) that are inserted in the bacterial cell membrane for uptake of compounds (opines) that will be produced by the tumors (see later);Cause the production of an endonuclease - a restriction enzyme - that excises part of the T_i plasmid termed the T-DNA (transferred DNA). The excised T-DNA is released by the bacterium and enters the plant cells, where it integrates into the plant chromosomes and dictates the functioning of those cells. The actual mechanism of transfer is still unclear, but it seems to require a conditioning process, perhaps mediated by the production of cytokines (plant hormones) by the bacterium. The tzs (transzeatin) gene on the T_i plasmid codes for the hormone.

RESULT

The current study includes collection of 9 samples from the different soil type and different plant parts and further subjected for different parameters. DNA was isolated by Silica column method for the further detection of *Agrobacterium tumefaciens, PCR* was done with the amplification of gene.

TABLE 1. RESULTS INTERPRETATION

Soil/Plant Part	Host	Symptom of crown gall	Target Band
1	Grape stem	+	-
2	Grape Soil	-	-
3	Garden soil 1	-	-
4	Garden soil 2	-	-
5	Rose Stem	+	-
6	Blueberry Root	+	-
7	Blueberry Soil	-	-
8	Weeping Fig Soil	-	-
9	Rose Soil	-	-

Members of the genus *Agrobacterium* constitute a diverse group of organisms, all of which, when harboring the appropriate plasmids, are capable of causing neoplastic growths on susceptible host plants. The agrobacteria, which are members of the family *Rhizobiaceae*, can be differentiated into at least three biovars, corresponding to species divisions based on differential biochemical and physiological tests. Recently, Young et al. [Int J Syst Evol Microbial 51 (2003), 89–103] proposed to incorporate all members of the genus *Agrobacterium* into the genus Rhizobium. We present evidence from classical and molecular comparisons that support the conclusion that the biovar 1 and biovar 3 agrobacteria are sufficiently different from members of the genus Rhizobium to warrant retention of the genus *Agrobacterium*. The biovar 2 agrobacteria cluster more closely to the genus *Rhizobium*, but some studies suggest that these isolates differ from species of Rhizobium with respect to their capacity to interact with plants. We conclude that there is little scientific support for the

proposal to group the agrobacteria into the genus Rhizobium and consequently recommend retention of the genus *Agrobacterium*.

REFERENCES

1. Smith, E. F.; Townsend, C. O. (1907). "A Plant-Tumor of Bacterial Origin". Science 25 (643): 671–673. Doi:10.1126/science.25.643.671. PMID 17746161.
2. "Rhizobium radiobacter (Agrobacterium tumefaciens) (Agrobacterium radiobacter)". UniProt Taxonomy. Retrieved 2010-06-30.
3. Young, J.M.; Kuykendall, L.D.; Martinez-Romero, E.; Kerr, A.; Sawada, H.; et al. (2001). "A revision of Rhizobium Frank 1889, with an emended description of the genus, and the inclusion of all species of Agrobacterium Conn 1942 and Allorhizobium undicola de Lajudie et al. 1998 as new combinations: Rhizobium radiobacter, R. rhizogenes, R. rubi, R. undicola and R. vitis". International Journal of Systematic and Evolutionary Microbiology 51 (Pt 1): 89–103. doi:10.1099/00207713-51-1-89. PMID 11211278.
4. "Taxonomy browser (Agrobacterium radiobacter K84)". National Center for Biotechnology Information. Retrieved 7 December 2015.
5. Chilton, MD; Drummond, MH; Merio, DJ; Sciaky, D; Montoya, AL; Gordon, MP; Nester, EW. (Jun 1977). "Stable incorporation of plasmid DNA into higher plant cells: the molecular basis of crown gall tumorigenesis". Cell 11 (2): 263–71. doi:10.1016/0092-8674(77)90043-5. PMID 890735.
6. Moore, LW; Chilton, WS; Canfield, ML. (1997). "Diversity of Opines and Opine-Catabolizing Bacteria Isolated from Naturally Occurring Crown Gall Tumors". Appl. Environ. Microbial. 63: 201–207.
7. Stanton B. Gelvin,Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907-1392, Agrobacterium-Mediated Plant Transformation: the Biology behind the "Gene-Jockeying" Tool,
8. Zupan, J; Muth, TR; Draper, O; Zambryski, P. (2000). "The transfer of DNA from Agrobacterium tumefaciens into plants: a feast of fundamental insights". Plant J. 23 (1): 11–28. Doi:10.1046/j.1365-313x.2000.00808.x.
9. Goodner, B; Hinkle, G; Gattung, S; Miller, N; et al. (2001). "Genome Sequence of the Plant Pathogen and Biotechnology Agent Agrobacterium tumefaciens C58". Science 294 (5550): 2323–2328. Doi:10.1126/science.1066803. PMID 11743194.
10. Wood, DW; Setubal, JC; Kaul, R; Monks, DE; et al. (2001). "The Genome of the Natural Genetic Engineer *Agrobacterium tumefaciens* C58". Science 294 (5550): 2317–2323. Doi:10.1126/science.1066804. PMID 11743193.
11. Vaudequin-Dransart, V; Petit, A; Chilton, WS; Dessaux, Y. (1998). "The cryptic plasmid of Agrobacterium tumefaciens cointegrates with the Ti plasmid and cooperates for opine degradation". Molec. Plant-microbe Interact 11 (7): 583–591. Doi:10.1094/mpmi.1998.11.7.583.
12. Schell, J; Van Montagu, M. (1977). "The Ti-plasmid of Agrobacterium tumefaciens, a natural vector for the introduction of nif genes in plants?". Basic Life Sci. 9: 159–79. Doi:10.1007/978-1-4684-0880-5_12. PMID 336023.
13. Zambryski, P.; et al. (1983). "Ti plasmid vector for introduction of DNA into plant

cells without alteration of their normal regeneration capacity". EMBO J 2 (12): 2143–2150. PMC 555426. PMID 16453482.

14. Root, M (1988). "Glow in the dark biotechnology". Bioscience 38 (11): 745–747. Doi:10.2307/1310781.
15. Kunik, T.; Tzfira, T.; Kapulnik, Y.; Gafni, Y.; Dingwall, C.; Citovsky, V. (February 2001). "Genetic transformation of HeLa cells by *Agrobacterium*". Proceedings of the National Academy of Sciences 98 (4): 1871–1876. Doi:10.1073/pnas.041327598. PMC 29349. PMID 11172043.