Modification of Ti Plasmid which Extracted from *Agrobacteriumtumefaciens* to Construction the dTi Vector for gene cloning

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Abstract

A total of 47 of infected potatoes in different size (*Solanumtuberosum*) were collected from local vegetable and fruit market and from University of Basrah-Collage of Agriculture, Department of Plant Protection in addition to the three soil samples were taken from Basrah city. From which 22 (44%) isolates were isolation and identification including 19(86.4%) isolates from infected potatoes and 3(13.7%) isolates from soil. All isolates were identification and characterization by using biochemical, physiological and biotechinqicalinvestigation.The extracted DNA of *Agrobacteriumtumefaciens* isolates were subjected to PCR for amplifying 16S rRNA and for amplifying T-DNA fragment then subjected to gel electrophoresis. The individual band of the 16S rRNA gene was characterized by 1479bp and of the T-DNA fragment by 1200bp. The products were comparison with the standard molecular DNA ladder (1200 and 1500bp). The purified β-lactamase gene was cut from PGLO plasmid and ligated by use T4DNA ligase enzyme with the Ti plasmid which disarmed T-DNA by the same restriction enzyme. The result was indicated by using the *E.coli K12* for carries the Ti plasmid vector which contain a β-Lactamase gene when put the antibiotic ampicillin in different consternation into the LB, MHA plate only the colonies which that have picked up exogenous DNA(dTi plasmid) can grow that is mean it become resistance to ampicillin by using dTi vector

Keyword: Basrah, *Agrobacteriumtumefaciens*, dTivector, genecloning

Introduction

Agrobacterium tumefaciens is a soil-borne, non-sporulating motile, rod-shaped phytopathogenic bacterium that elicits neoplastic growth at the site of infection in many dicotyledonous plants causing the crown-gall disease. This disease can be traced back to 1850 where it was first reported in grapevines, but remained poorly understood until the early 1900 (Smith and Townsend, 1907). This disease by far represents a unique event, involving the transfer of DNA from prokaryote into the chromosomes of plants, that facilitates its application in modern biology for not only transferring desired genes into plants, but also across other eukaryotic kingdoms including fungi and mammalian cells (Kim *et.al* 2001). *Agrobacterium tumefaciens* which cause crown gall disease in plants (Wood *et. al*., 2001). There are several stages in the process of infection by *Atumefaciens* on dicot plants that requires coordinated response between an individual viable bacterium and the host cell. The molecular basis for genetic transformation of plant cells by Agrobacterium that produces the neoplastic growth at wounded sites, the crown gall is imprinted on large tumor-inducing Ti plasmid residing in the bacterium Ti plasmids in the order of 200 to 800 Kb (Gelvin, 2000). The Ti plasmid encodes nearly 40 genes related to octopine, agaropine and mannopine uptake and use (Zhu *et. al*., 2000). Opines are also involved in conjugal Ti plasmid exchange and chemotaxsis (Yang *et.al*,2001). The disease that produces the crown gall was first described in grapes in 1882 and was subsequently studied in variety of natural host plants (Powell and Gordon, 1989).

Materials and Methods

Genomic DNA was extracted adopting the procedures by

Sambrook*et,al*.(1989),Al-Badran(2003)and Japoni*et,al*(2004).

Concentration of DNA (Sambrook*et. al.*, 1989)

The concentration of DNA was calculated by spectrophotometer method using UV-visible spectrophotometer.

PCR Condation

Table (1-1): Oligonucleotide Primer sequences used for PCR amplification of 16S rRNA gene

Primer	Sequence	TA
Forword primer	FGPS6 5-GGA GAG TTA GAT CTT GGG TCA G- 3	61
Reverse primer	FGPS1509 5- AAG GAG GGG ATC CAG CCG CA-3	61

Table (1-2): PCR condition for 16S rRNA gene:

Step	Temperature	Time	No. of cycle
Initial denaturation	**94°C**	**3 min.**	**1**
Denaturation	**94°C**	**5 sec.**	**35**
Annealing	**61°C**	**15 sec.**	
Elongation	**71°C**	**30sec.**	
Final Elongation	**94°C**	**3 min.**	**1**

The PCR product was electrophoresed in 2% agarose gel (Sambrook*et. al*., 1989), 10 µl of each PCR product was added to each well. 5 µl of molecular marker (100-1500bp ladder) was mixed with 1 µl of loading dye and added at the first

well. Then product was detected by examined under UV. transilluminator.

Amplification of T-DNA fragment:

Table (1-3): Oligonucleotide primer sequences used for PCR amplification of T-DNA fragment (Genbank: cu-462822-pubmed ID-18758448):

Primer	Sequences	TA
Forward primer	LP5¯ - GCG TGG ACC GCT TGC TGC AA CT-3¯	67
Reverse primer	RP5-CCG CAA TTA TAT ACA TTT AAT ACG CG-3¯	65

Table (1-4): PCR condition for T-DNA fragment amplification:

Step	Temperature	Time	No. of cycle
Initial denaturation	94°C	3 min.	1
Denaturation	94°C	5 sec.	35
Annealing	66°C	15 sec.	
Elongation	71°C	30sec.	
Final Elongation	71°C	3 min.	1

The PCR products from amplification of T-DNA fragment was the electrophoresed on an ethidium bromide-stained (1.2%) agarose gel. The presence of band of 1.2 Kb. was indicative of the T-DNA (Left and Right borders) fragment.

Transformation

Transfer of plasmid DNA from its stock to *E.coli* K12 by using pGLO bacterial transformation kit (Bio-rad.com).

Results and Discussion

A total of 22 *Agrobacteriumtumefaciens* isolates were subjected to DNA extraction according to Sambroke*et. al.* (1989) to all isolates and potatoes tumors emphasized the CTAB methods described above works well for many plant species to extraction DNA. Agarose gel electro-phoresiswas performed to detect the extracted Ti plasmid DNA from *Agrobacteriumtumefaciens* isolates figure (1-1) and plant tumor (infected potatoes) isolates with soil isolates figure (1-2A&B) lanes 1-2 show DNA bands viewed under UV. Transilluminator

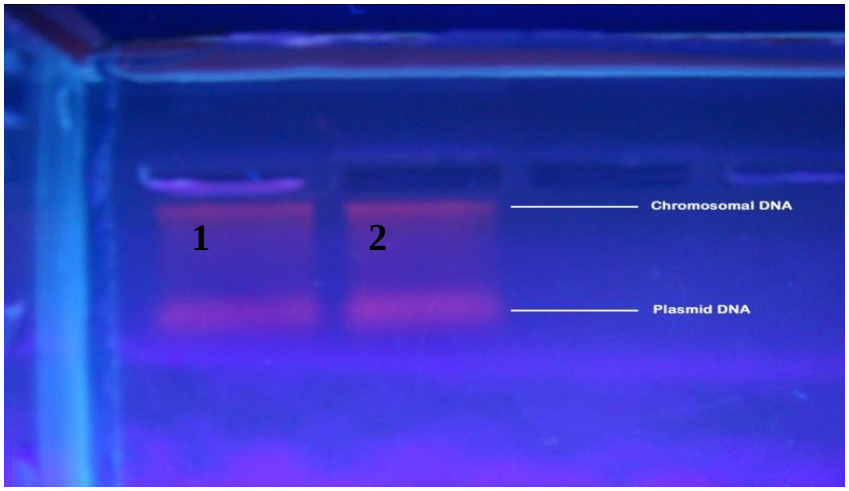


Figure (1-1): agarose (0.8%) gel electrophoresis pattern showing DNA bands of chromosomal and plasmid DNA of *Atumefaciens*.

PCR technique:

Nucleotide sequences of the 16S rRNA for genus Agrobac-terium were concatenated in the length of 1479bp depending on the shorter sequence exhibited from the gene bank http://www.nebi.

ORS.1351T and ORS.2644T. the DNA of all *Agrobacteriumtumefaciens* isolates identified by biochemical tests were extracted and electrophoresed then subjected to PCR for amplifying purified 16S RNA gene figure (1-2) and PCR amplified products of T-DNA fragment (1-3).

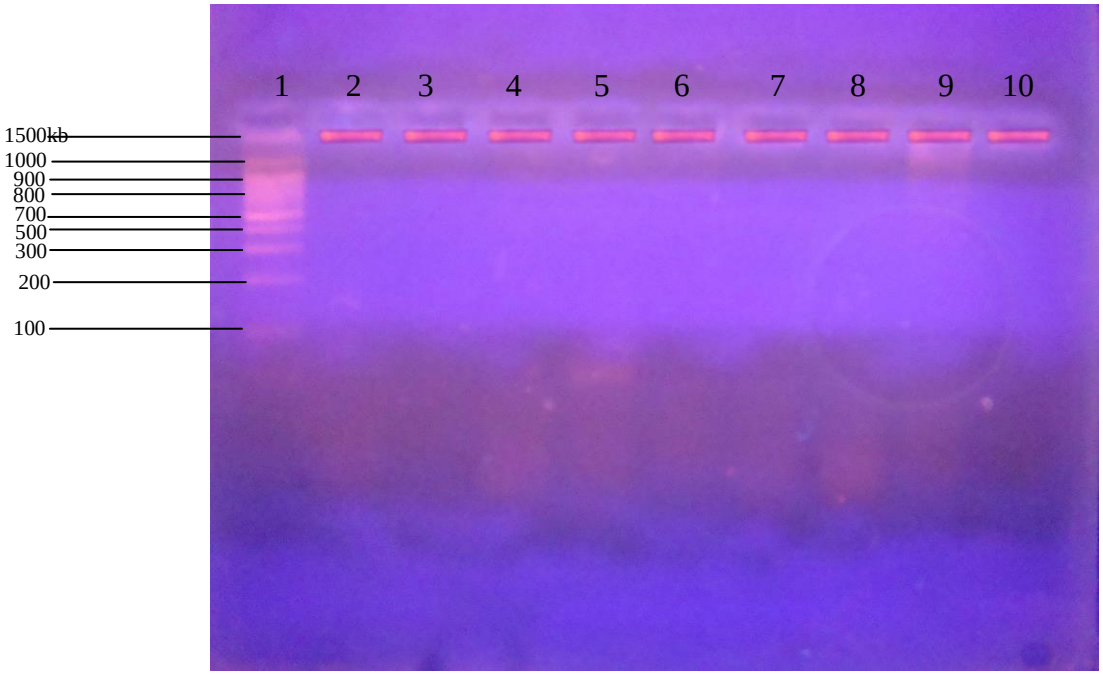


Figure (1-2): agarose (2%) gel electrophoresis patterns show PCR amplified products of 16s rRNA gene Lane 1:1500bp DNA ladder, lanes; (2-10)16s rRNA bands of Agrobacterium isolates No. 2,3,4,5,6,7,8,9,10.

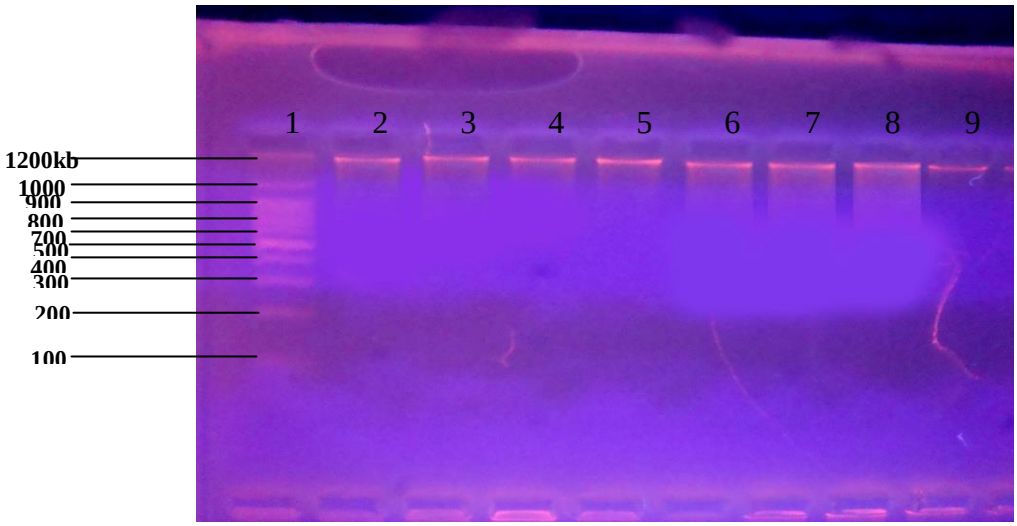


Figure (1-3): agarose (2%) gel electrophoresis patterns show PCR amplified products of T-DNA fragment Lane 1:1200bp DNA ladder, lanes2: 9 T-DNA bands of Ti plasmid Agrobacterium isolates No. 2,3,4,5,6,7,8,9.

Cloning:

The purified genes GFP gene and β-lactamase gene were ligated by use T4 DNA ligase enzyme with the Ti-plasmid which disarmed T-DNA by using the same restriction enzyme. The pGLO digestion product was determined by using 1.5% agarose electrophoresis figure (1-4). The product was purified by the wizard DNA purification system (promega. Com) in purification β-lactamase gene, low melting agarose was used because it melting at 70°C. purification genes of pGLO plasmid was ligated by T4 DNA ligase enzyme. The result was indicated by using the bacteria *EscherichiacoliK12* (Mardigian, 2000). The result of carries the plasmid a β-lactamase gene, when put the antibiotic ampicillin into the LB plate. Only the colonies that have picked up exogenous DNA can

growfigure (1-5). However the other colonies which did not picked the β-lactamase gene by insertion of it with Tiplasmid after cutting with the same restriction enzyme (*HindIII*). Ampicillin negate-vely affects the growth of *E. coli* K12 colonies then there should be fewer on the plate. That's appear in figure (1-5) and that dose agrees with Mardigian (2000).Purified Ti plasmid DNA was sent to the Syria atomic energy lab for sequencing and the result show in figure (1-6).

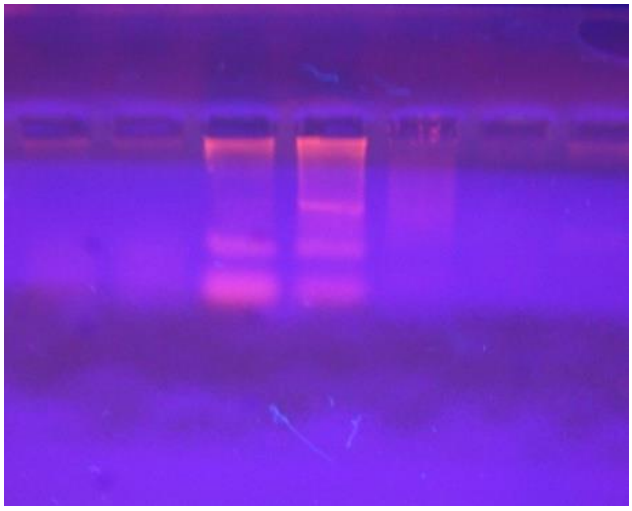


Figure (1-4): Agarose (0.8%) gel electrophoresis pattern show the restriction condition of the pGLO plasmid with Hind III, EcoRI and pstl restriction enzymes Lane 3: β-Lactamase gene and green fluorescent protein gene (GFP), Lane 4: β-Lactamase gene and green fluorescent protein gene and arabinose gene, Lane 5: control restriction enzyme without pGLO plasmid.

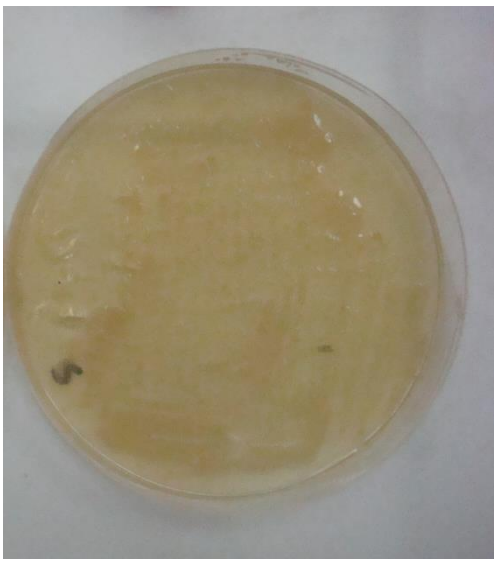
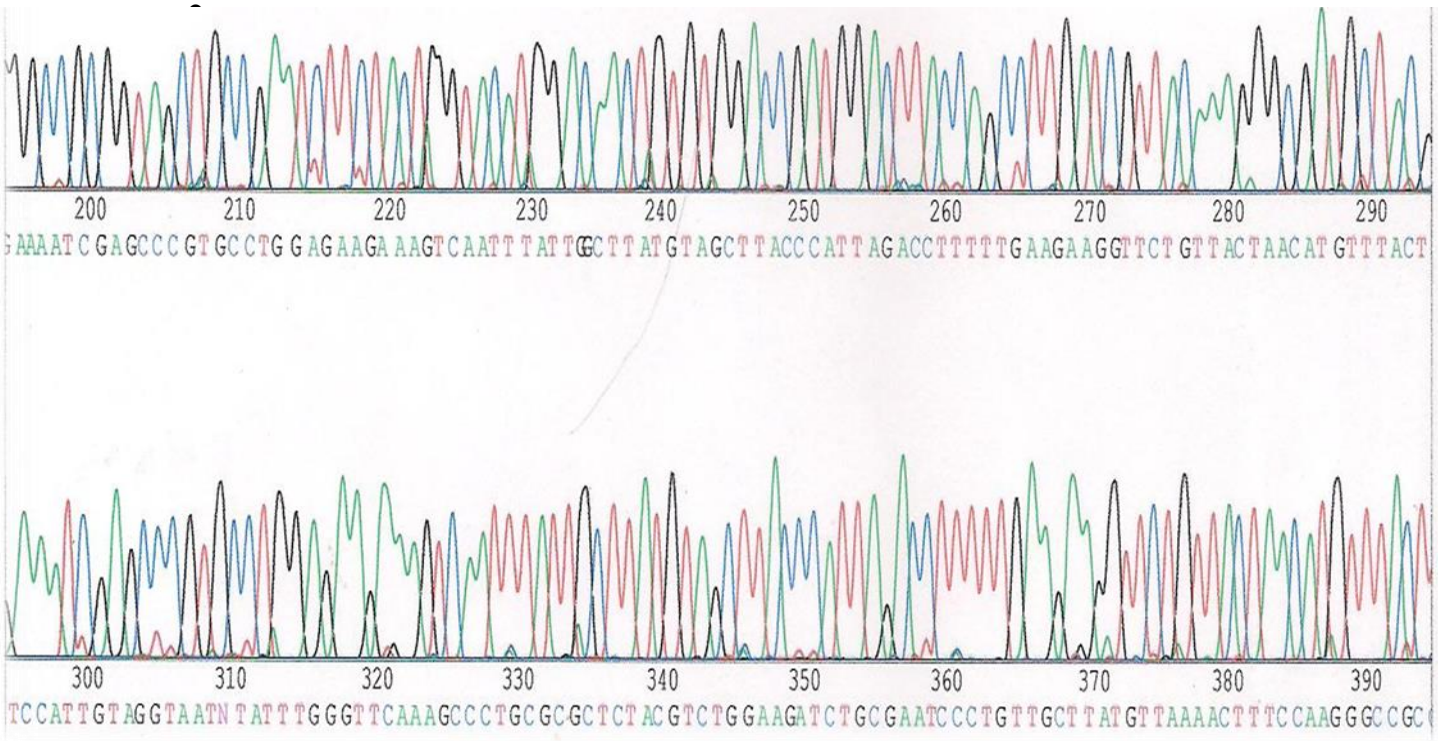


Figure (1-5): resistance *E.coli* K12 to ampicillin on LB plate ampicillin agar



Figure(1-6)The watching monitor of Ti nucleotide sequences

nucleotide sequences:

TTGATTTTATCTCCTGAATATGAACCAAAGATACTGATATCTTGGCA
GCATTCCGAAGTAACTCC
TCAACCTGGGAGTTCCACCTGAAGAAGCAGGGGCCGCGGTAGCTGCC
GAATGTTCTACTGGGTAC
ATGGACAACTGGTGTGGACCGATGGACTTACCAGCCTTGATCGTTAC
AAAGGGCGATGCTACGAA
AATCGAGCCCGTGCCTGGAGAAGAAAGTCAATTTATTGGCTTATGTA
GCTTACCCATTAGACCTT
TTTGAAGAAGGTTCTGTTACTAACATGTTTACTTCCATTGTAGGTAA
TGTATTTGGGTTCAAAGC
CCTGCGCGCTCTACGTCTGGAAGATCTGCGAATCCCTGTTGCTTATG
TTAAAACTTTCCAAGGGC
CGCCTCATGCATCCAAG

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تحوير بلازميدTi المستخلص من بكتريا *Agrobacterium tumefacies* لتصنيع الناقل dTi والمستخدم في التنسيل الجيني

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الخلاصة

جمعت 47 عينة مختلفة الاحجام لبطاطا مصابة بمرض التورم التاجي من الاسواق المحلية للخضر والفواكة ومن جامعة البصرة كلية الزراعة ، قسم وقاية النبات بالاضافة الى ثلاثة عينات اخذت من التربة لمدينة البصرة. عزلت منها 22 عزلة شكلت (44%) شملت 19 عزلة (86.4%) من بطاطا مصابة بمرض التورم التاجي وكذلك 3 عزلات (13.7%) من عينات التربة. جميع العزلات شخصت وصنفت اعتماداً على الفحوصات البايوكيمياويةوالفسلجية والتقنية الاحيائية. الـ DNA المستخلص لعزلات*Agrobacteriumtumefaciens* عمل له (PCR) لتضخيم الجين 16S rRNA وكذلك تضخيم قطعة الـ T-DNA بعد ذلك عمل لها ترحيل كهربائي حددت الحزم لكل من T-DNAو16S rRNA وقورنت مع مقياس قياسي وكانت اطوال الحزم هي 1474 زوج قاعدة لـ16S rRNA و 1200 زوج قاعدة لـ T-DNA مع مقياس يتراوح بين 1500و 1200 زوج قاعدة ، نقي الجين المسؤول عن صفة المقاومة للامبسلينβ-lactamase gene حيث تم قطعة من بلازميد PGLO وتم ربطة بواسطة انزيم الرابط T4DNA مع بلازميد(Ti) حيث قطع الاثنان بنفس الانزيم القاطع .

وظهرت النتائج باستخدام عترة *E.coli* K12 القياسية لنقل الجين المسؤول عن مقاومة المضاد الحيوي الامبسلين المحمول على بلازميد(Ti) حيث عند اضافة المضاد الحيوي الامبسلين الى الوسط الزرعي (LB) لوحظ ان المستعمرات التي اخذت هذه الصفة فقط هي التي نمت في الوسط الزرعي الحاوي على المضاد الحيوي الامبسلين مما يعني انها اكتسبت صفة المقاومة للمضاد الحيوي الامبسلين.