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

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Abstract

A total of 47 of infected potatoes in different size (*Solanum tuberosum*) 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 biotechnique investigation. The extracted DNA of *Agrobacterium tumefaciens* 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, *Agrobacterium tumefaciens*, dTi vector, 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 chemotaxis (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
The concentration of DNA was calculated by spectrophotometer method using UV-visible spectrophotometer.

Concentration of DNA

(Sambrook et al., 1989)

PCR Condition

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

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>TA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward primer</td>
<td>FGPS6 5-GGA GAG TTA GAT CTT GGG TCA G- 3</td>
<td>61</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>FGPS1509 5- AAG GAG GGG ATC CAG CCG CA-3</td>
<td>61</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
<th>No. of cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>94°C</td>
<td>3 min.</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94°C</td>
<td>5 sec.</td>
<td>35</td>
</tr>
<tr>
<td>Annealing</td>
<td>61°C</td>
<td>15 sec.</td>
<td></td>
</tr>
<tr>
<td>Elongation</td>
<td>71°C</td>
<td>30sec.</td>
<td></td>
</tr>
<tr>
<td>Final Elongation</td>
<td>94°C</td>
<td>3 min.</td>
<td>1</td>
</tr>
</tbody>
</table>

The PCR product was added to each well. 5 µl of molecular marker (100-1500bp ladder) was mixed with loading dye and added at the first
well. Then product was detected by examined under UV.

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):

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequences</th>
<th>TA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward primer</td>
<td>LP5 - GCG TGG ACC GCT TGC TGC AA CT-3</td>
<td>67</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>RP5-CCG CAA TTA TAT ACA TTT AAT ACG CG-3</td>
<td>65</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
<th>No. of cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>94°C</td>
<td>3 min.</td>
<td>1</td>
</tr>
<tr>
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<tr>
<td>Final Elongation</td>
<td>71°C</td>
<td>3 min.</td>
<td>1</td>
</tr>
</tbody>
</table>

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 *Agrobacterium tumefaciens* isolates were subjected to DNA extraction according to Sambrooke *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 electrophoresis was performed to detect the extracted Ti plasmid DNA from *Agrobacterium tumefaciens* 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 *Agrobacterium* 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 (Mardigan, 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 Ti plasmid after cutting with the same restriction enzyme (HindIII). Ampicillin negatively 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).

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Figure (1-4): Agarose (0.8%) gel electrophoresis pattern show the restriction condition of the pGLO plasmid with Hind III, EcoRI and pstI 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:

TTGATTTTATCTCCTGAATATGAACAAAGATACTGATATCTTGCGA
GCATTCCGAAGTAACTCC
TCAACCTGGGAGTTCCACCTGAAGAAGCAGGGGCAGGCTTGATCGTTAC
GAATGTTTCTACTGGGTAC
ATGGACAAACTGGTGACCGATGGAATATCCGCTTGATCGTTAC
AAAGGGCGATGCTACGAA
AATCGAGCCGCTGCCTGGAGAAGAAGTCAATTATGGCTTTATGTA
GCTTACCATTGACCTT
TTTGAAGAAGGTTCTGTCTAACTAAATTTATGTTACTCCATTTGATAGGTA
TTATTTGGTCTCAAAGC
CCTGCAGCTCTACGTCTGGAAGATCTGCGAATCCCTGTTGCTTTATG
TTAAAACTTTCCAAGGGC
CGCCTCATGCATCCAAG

References


تحويل بلازميد Ti المستخلص من بكتريا Agrobacterium tumefacies، والذي المستخدم في التنسل الجيني dTi

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الخلاصة

جمع عينة مختلفة من البذور المصاب من الحبوب مختلفة الأنواع من البذور المحلية للفاكهة والخضروات ومن جامعة البصرة كلية الزراعة، قسم وقتية النبات بالإضافة إلى ثلاثة عينات اخترعت من البذور التي تستخدم البذور المستخلص عن ثلاث عينات (54%) ضُمت 19 عزلة (86.4%) من بذرة مصاب بمرض التورم انفانتي وكذلك 3 عزلات (13.7%) من عينات البذور. جميع العزلات شهدت وصولًا كاملاً على الفحوصات الباثولوجية والإحصائية وتقنية PCR المستخلص لعزلات Agrobacterium tumefaciens

التحليل: DNA المستخلص لعزلة Agrobacterium tumefaciens، عمل له PCR لتضخيم الجين 16S rRNA وكذلك تضخيم قطعة الـ T-DNA بعد ذلك عمل لها ترحيل كهربائي حددت الحزم لكل من 16S rRNA، T-DNA وحرقت مع مقياس قياسي وكانت اطوال الحزم هي 1474 زوج قاعدة لـ 16S rRNA و 1200 زوج قاعدة لـ T-DNA. مقياس متوسط بين 1500 و 1200 زوج قاعدة، نقي الجين المسؤول عن صفة المقاومة للامبيسين، في حين تم قطع قطعة من بلازميد PGLO وتم ربطه بواسطة إنزيم T4DNA بالربط (T) مع بلازميد Ti حيث قطع الأثاث البنفسك انزيماً القاعط

النتائج: باستخدام عوامل E.coli K12 القياسية، نقل الجين المسؤول عن مقاومة مضاد الحيوير الامبيسين المحمل على بلازميد Ti حيث عند إضافة مضاد الحيوي الامبيسين الى الوسط الزراعي LB، ولاحظ أن المستعمرات التي اخترعت هذه الصفة فقط هي التي نمت في الوسط الزراعي الحاوي على المضاد الحيوي الامبيسين مما يعني أنها اكتسبت صفة المقاومة للمضاد الحيوي الامبيسين.