5’ Rapid Amplification of cDNA Ends (5’ RACE) of Agrobacterial T-DNA Genes within Transformed Plant Sample

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[Abstract] The T-DNA (transferred-DNA) region of virulent *Agrobacterium tumefaciens* (*A. tumefaciens*) strain is transferred and integrated into the plant genome, and thereby the T-DNA genes are expressed in transformed plant cells. This protocol was used to analyze the transcription start sites (TSSs) of agrobacterial T-DNA genes within plant crown gall tumor. Firstly, the stems of *Arabidopsis thaliana* were inoculated by *A. tumefaciens* strain C58 and developed crown gall tumor. Subsequently, the mRNA was extracted from the crown gall tumor and then used for amplification of 5’ cDNA ends by 5’ Rapid Amplification of cDNA Ends (5’ RACE) assay. The full-length cDNAs were generated in reverse transcription reactions and used to analyze TSSs. Here, TSSs of three oncogenes, *IaaH*, *IaaM* and *Ipt* were analyzed as examples. This protocol also allows for identification of TSSs of the other agrobacterial T-DNA genes that expressed in plant cells.

Materials and Reagents

1. *A. tumefaciens* strain C58 noc^c (nopaline catabolism) (MAX-PLANCK-GESELLSCHAFT, catalog number: 584)
2. *Arabidopsis thaliana* Col-0
3. *Escherichia coli* (*E. coli*) strain MRF’ (Agilent Technologies, catalog number: 200230-41)
4. Dynabeads® Oligo(dT)25 (Thermo Fisher Scientific, Invitrogen™, catalog number: 61005)
5. SMARTer® RACE 5’/3’ Kit (Takara Bio Company, Clontech, catalog number: 634859)
6. DreamTaq DNA Polymerase (5 U/µl) (Thermo Fisher Scientific, catalog number: EP0701)
7. QIAquick PCR Purification Kit (QIAGEN, catalog number: 28106)
8. pGEM®-T Easy Vector Systems (Promega Corporation, catalog number: A1360)
9. Hypodermic Syringes without Needle (5 cc) (Terumo Medical Corporation, catalog number: SS-05L)
10. Hypodermic Needles (Terumo Medical Corporation, catalog number: NN-2138R)
11. KB medium (see Recipes)
12. Agromix buffer (see Recipes)

13. Lysis buffer (see Recipes)
14. Washing buffer A (see Recipes)
15. Washing buffer B (see Recipes)
16. Elution buffer (see Recipes)
17. 2x binding buffer (see Recipes)

Equipment

1. Culture tubes 13 ml (SARSTEDT, catalog number: 62.515.028)
2. NanoDrop 2000c UV-Vis Spectrophotometer (Thermo Fisher Scientific, catalog number: ND-2000c)
3. High-performance, Modular Stereomicroscope for Application Based Customization MZ6 (Leica Microsystems, model: MZ6)
4. Ball mill (RETSCH, model: MM200)
5. Thermal cycler (Eppendorf, catalog number: 950000031)
6. MiniSpin®/MiniSpin® plus Centrifuge (Eppendorf, catalog number: 022620100)
7. MagneSphere® Technology Magnetic Separation Stands (Promega Corporation, catalog number: Z5342)

Procedure

1. Crown gall tumor induction
   a. Overnight culture of *A. tumefaciens* was obtained from colonies grown on KB agar plates. Colonies were transferred into 3-5 ml KB liquid medium in 13 ml culture tubes with assembled two-position ventilation cap (with position 1: Cap lightly pushed on, ventilated) and incubated at 140 rpm in a rotary shaker at 28 °C for overnight.
   b. Overnight *A. tumefaciens* cells were centrifuged at 8,000 rpm for 1 min and suspended in 3 ml agromix buffer. Suspended cells were cultured at 28 °C, 140 rpm again for 2-3 h. OD_{600} was measured by spectrophotometer and adjusted to OD_{600} = 0.5.
   c. Young inflorescence stems (3 to 10 cm) of *Arabidopsis* were injected near the bottom with a 5 ml syringe two times at one direction and two times at another perpendicular direction (Figure 1). The syringe needle was injected completely through the stems. One drop of *A. tumefaciens* cells was used for one injection.

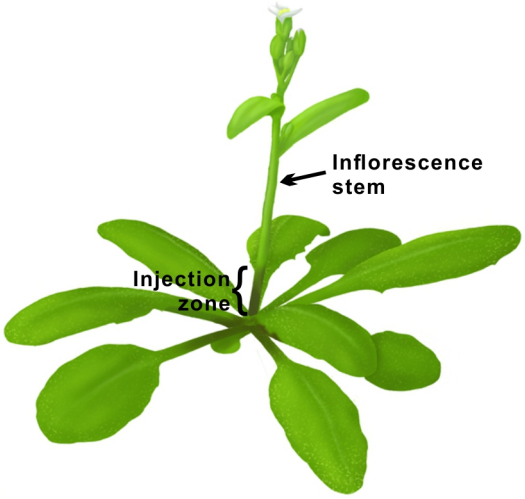

Figure 1. Schematic image of *Arabidopsis* influrecence stem. Young inflorescence stems were injected with a syringe in the injection zone (marked). The original *Arabidopsis* image is from website (http://www.lookfordiagnosis.com/mesh_info.php?term=Arabidopsis&lang=1).

d. The stems were injected eight times in total, so usually eight blocks of tumors were generated at 25 days after inoculation (Figure 2). The tumors were cut from the stems with a scalpel and tweezer under a dissecting microscope Leica MZ6 and weighed in the balance.

Figure 2. *Arabidopsis* crown gall tumors. The crown gall tumors were generated from the *Arabidpsis* stems at 25 days after inoculation. The tumors (marked by red circle) were cut with a scalpel and tweezer and weighed.

2. Poly-A-mRNA extraction

   Approximately 50 mg crown gall tumor was used for mRNA extraction. Poly-A-mRNA was isolated by the oligonucleotide Poly-dTTP (25) bound to polystyrene beads. Dynabeads Oligo-[dT] 25 kit was used.

   a. The plant tissue was frozen in liquid nitrogen and homogenized in a ball mill, and then 1 ml Lysis Buffer was added and the samples were further homogenized for 1-2 min at room temperature until the tissue was completely lysed. The lysate was centrifuged for 30 sec at 10,000 rpm.

   b. The manufacturer's protocol of Dynabeads Oligo-[dT] 25 kit was performed. The supernatant was incubated for 30 min with 20 µl Dynabeads at room temperature in an overhead rotor. The tubes were place on the MagneSphere® Technology Magnetic Separation Stands for 2 min and removed the supernatant. The manufacturer's protocol for the magnetic stand was performed.

   c. The first washing step: The Dynabeads were washed twice with 200 µl Washing Buffer A, and twice with 200 µl Washing Buffer B. The magnet was used to separate beads and supernatant for each washing. The Dynabeads were washed by gently flicking the bottom of the tube until all beads were resuspended, and then the beads were captured by magnetic stand. The supernatant was removed without disturbing the beads.

   d. In order to exclude DNA contaminations, the mRNA was eluted from the beads by 50 µl Elution Buffer and incubation at 75 °C for 2 min.

   e. The second washing step: 50 µl 2x Binding Buffer was added to the eluted mRNA, and the 100 µl of mixture was again incubated 20 min at room temperature in an overhead shaker.

   f. After two washing steps with 200 µl Washing Buffer B, the mRNA was eluted from the magnetic beads with 22 µl Elution Buffer at 75 °C for 2 min. The concentration of mRNA was quantified by NanoDrop 2000c UV-Vis Spectrophotometer. The mRNA was directly used for first-strand cDNA synthesis for 5' RACE assay or stored at -80 °C.

3. 5'- rapid amplification of cDNA ends (5' RACE) assay

   5' ends of the oncogenes cDNA were amplified by SMARTer™ RACE cDNA Amplification Kit. First-Strand cDNA was generated by SMARTScribe™ Reverse Transcriptase and primed using SMARTer II A Oligonucleotide and 5'-CDS Primer A.

   a. The following reagents were added in one tube

      2.75 µl mRNA

      1.0 µl 5'-CDS Primer A

      The reagents were mixed well by pipet and incubated the tubes at 72 °C for 3 min, and then 42 °C for 2 min.

   b. The following reagents were to the mixture from step a for a total volume of 10 µl

      1.0 µl SMARTer II A Oligonucleotide

2.0 µl 5x First-Strand Buffer
1.0 µl DTT (20 mM)
1.0 µl dNTP Mix (10 mM)
0.25 µl RNase Inhibitor (40 U/µl)
1.0 µl SMARTScribe™ Reverse Transcriptase (100 U)

The reagents were mixed well by pipet and incubated the tubes at 42 °C for 90 min.

c. The reaction mixture was heated at 70 °C for 10 min to terminate the reaction and the first-strand reaction product was diluted with 10 folds ddH_2O. Samples were stored at -20 °C or used for next step directly.

4. Amplification and sequencing of 5' ends of the oncogenes cDNA

a. The fragments of 5' ends of the oncogenes cDNA were amplified by DreamTaq DNA Polymerase and primed using Universal Primer A Mix (UPM) and specific target primer (Table 1) as shown in the following reaction mixture.

PCR reaction (a total volume of 50 µl):

cDNA from step 3.3.1	1 µl
5x Buffer	10 µl
10x Universal Primer A Mix	5 µl
Specific target reverse primer (10 µM)	1 µl
dNTP (10 mM)	1 µl
DreamTaq DNA Polymerase	0.5 µl
ddH_2O	to a final volume of 50 µl

PCR program:

Step 1: 95 °C	1 min
Step 2: 95 °C	30 sec
Step 3: 55 °C	30 sec
Step 4: 72 °C	15 sec
Step 5: repeat step 2-4 for 30 cycles	
Step 6: 72 °C	5 min
Step 7: 4 °C	Store

Table 1. Specific oncogene reverse primer

Gene name (Accession No.)	Primer sequence (5' ➔ 3')
IaaH (pTiC58, AE007871)	CCCCGATTGCTAACAGACG
IaaM (pTiC58, AE007871)	CAAGAGTGTTCGAGAGG
Ipt (pTiC58, AE007871)	TCCCATGAATCAACTTAT

b. The PCR products were purified by QIAquick PCR Purification Kit.

c. The purified PCR products were inserted into pGEM-T Easy Vector by TA cloning.

2x Rapid Ligation Buffer 5 µl

pGEM-T Easy Vector (50 ng/ µl) 0.5 µl
PCR product 3.5 µl
T4 DNA Ligase 1 µl
The reagents were mixed by pipet and incubated for 1 h at room temperature.

d. The resulting recombinant vectors from step 4c were transformed into *E. coli* strain MRF' using the heat shock method.
e. More than three independent colonies were sequenced for 5' end of the PCR products, which were the transcription start sites of oncogene.

Representative data

Table 2. *Cis*-regulatory sequence elements within the oncogene promoters

Regulatory sequences	*IaaH*	*IaaM*	*Ipt*
Inr box (YY**A**NWYY)	−2 CC**A**AACC +5	−2 CT**A**CACA +5	−2 CT**A**ATCC +5
Start coden ATG	+12 ATG	+26 ATG	+44 ATG
TATA box (TATAAA)	−36 TATATT −31 [1]	−32 TAAATA −27 [2]	−29 TATAAC −24 [3,4,5,6]

This data was published on PLOS Pathogens (Zhang *et al.*, 2015). Positive numbers indicate the positions downstream and negative numbers the positions upstream of the TSSs (+1). TSS is underlined. Y = C/T, W = A/T, N = A/G/C/T. 1. (Klee *et al.*, 1984); 2. (Nester *et al.*, 1984); 3. (Goldberg *et al.*, 1984); 4. (Heidekamp *et al.*, 1983); 5. (de Pater *et al.*, 1987); 6. (Lichtenstein *et al.*, 1984)

Recipes

1. KB medium
   20 g protease peptone
   1.5 g K_2HPO_4
   0.87% glycerol
   600 µM $MgSO_4$
   15 g agar (plate only)
   Sterilized by autoclave
2. Agromix buffer
   0.01 M $MgCl_2$
   0.01 M MES
   pH 5.6
3. Lysis buffer

100 mM Tris-HCl (pH 7.5)
500 mM LiCl
10 mM EDTA
1% LiDS
5 mM dithiothreitol (DTT)
If any precipitation is observed, warm the buffer to room temperature and shake until all the components are fully resuspended.

4. Washing buffer A
   10 mM Tris-HCl (pH 7.5)
   0.15 M LiCl
   1 mM EDTA
   0.1% LiDS
5. Washing buffer B
   10 mM Tris-HCl (pH 7.5)
   0.15 M LiCl
   1 mM EDTA
6. Elution buffer
   10 mM Tris-HCl (pH 7.5)
7. 2x binding buffer
   20 mM Tris-HCl (pH 7.5)
   1.0 M LiCl
   2 mM EDTA

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