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Protocols

Isolation of Plasmid DNA Rescued From Single Colonies of *Agrobacterium tumefaciens* by Means of Rolling Circle Amplification

XIUHUA CHEN, XIAODONG DING and WEN-YUAN SONG*
Department of Plant Pathology, Plant Molecular and Cellular Biology Program, University of Florida, Gainesville, FL 32611, USA

Abstract. We report a simple method to isolate plasmids from single colonies of *Agrobacterium tumefaciens* by means of rolling circle amplification. The amplified DNA can be digested by restriction enzymes for plasmid verification and transformed into *Escherichia coli* for plasmid rescue. Compared with conventional procedures, this method eliminates liquid culturing of *Agrobacterium* cells and subsequent DNA isolation and enables large-scale plasmid analyses.

Key words: *Agrobacterium tumefaciens*, *Escherichia coli*, plasmid DNA, rolling circle amplification

Abbreviations: GUS, beta-glucuronidase; RCA, rolling circle amplification; TAP, tandem affinity purification.

Introduction

Recent advances in plant genome projects have led to the deduction of the existence of a large number of open reading frames. A variety of methods are necessary for systematic investigation of the function of these predicted genes. *Agrobacterium*-mediated plant transformation is a key technique in plant functional genomics studies. For instance, studies of RNA interference–mediated gene silencing and proteome analysis of tandem affinity purification (TAP)–tagged proteins in plants require the production of many transgenic plants expressing distinct constructs. However, culturing *A. tumefaciens* and subsequent isolation of plasmids from the bacterial cells are not suitable for automation.

Rolling circle amplification (RCA) is an increasingly popular method that has been used to rapidly isolate plasmids from single colonies of *E. coli*, phage, and yeast cells (Dean et al., 2001; Ding et al., 2003). RCA is capable of amplifying entire circular DNA ranging in size from fewer than 100 bp to several hundred thousand with high fidelity (Dean et al., 2001; Ding et al., 2003; Fire et al., 1995; Garmendia et al., 1992; Esteban et al., 1993). We have previously shown that the amplified linear concatemeric DNA can be directly transformed into yeast (Ding et al., 2003). However, similar methods are not suited for *E. coli* transformation,

*Author for correspondence. e-mail: wsong@ifas.ufl.edu; fax: 352 392 6532; ph: 352 392 3631 ext. 344.

partly because of the bacterial RecBCD exonuclease activity that degrades linear DNA (Benzinger et al., 1975). In this study, we demonstrate the amplification of plasmid DNA from *A. tumefaciens* for plasmid verification and recovery, and we further describe 2 methods to transform the RCA products into *E. coli*.

Materials and Methods

A. tumefaciens strain LBA4404 was obtained from Invitrogen (Carlsbad, CA, USA), and the *E. coli* strain XL1-Blue was obtained from Stratagene (La Jolla, CA, USA). *E. coli* strain DY329 was a gift from Donald L. Court (National Cancer Institute, MD, USA). TempliPhi 100 Amplification Kit was purchased from Amersham Biosciences (Piscataway, NJ, USA) for RCA amplification. Plasmid pCmPU-GUS was constructed by inserting the β-glucuronidase (GUS) gene into the binary vector pCmPU, a derivative of pCAMBIA1300 (Cambia, Canberra, Australia).

Protocol 1. RCA amplification of plasmid DNA from A. tumefaciens

1. Use toothpicks to pick 1/3-1/2 of single bacterial colonies carrying pCmPU-GUS and suspend the cells into 5 µL of sample buffer.
2. Heat the cells at 95°C for 3 min and then cool to 4°C.
3. Add 5 µL of reaction buffer and 0.2 µL of Phi29 DNA polymerase to the cell suspensions.
4. Incubate at 30°C for 6-18 h for amplification and then incubate at 65°C for 10 min to stop the reactions.
5. Transfer 3 µL of amplified DNA into a new tube and incubate with 10 U of the restriction enzyme *Spe* I in the appropriate buffer at 37°C for 4 h. Stop the reactions by heating the samples at 65°C for 20 min.
6. Fractionate 1 µL of RCA-amplified DNA and 3 µL of *Spe* I–digested RCA products in 0.8% agarose gel.

Protocol 2. Transformation of the E. coli *strain XL1-blue with RCA products*

1. Digest 0.5 µL of RCA-amplified DNA with a single-cut enzyme in 15-µL volumes. Stop the reactions by heating the samples at 65°C for 20 min.
2. Self-ligation reactions are performed in 30-µL volumes containing 15 µL digested RCA products, 3 µL of 10 × T4 DNA ligase buffer, and 400 U of T_4 DNA ligase.
3. Incubate overnight at 16°C.
4. Mix 0.2 µL of self-ligated RCA products with 20 µL of XL1-Blue competent cells. Electroporate the cells by using a Cell-Portator (GIBCO, BRL) according to the manufacturer's instructions.

Protocol 3. Transformation of E. coli *strain DY329 with RCA products*

1. Inoculate 5 mL of LB medium containing 20 µg/mL of tetracycline with a fresh DY329 colony. Grow cells overnight at 32°C with vigorous shaking.

2. Dilute 1 mL of overnight culture into 100 mL of LB medium containing 20 μg/mL of tetracycline. Incubate at 32°C with vigorous shaking to an OD600=0.4-0.6.
3. Transfer cells to two 500-mL flasks. Heat shock one for 15 min in a 42°C water bath with shaking (induced). Swirl the flask in an ice-water slurry for 10 min to cool. Cells in the other flask are kept on ice as a negative control (uninduced).
4. Centrifuge for 8 min at 5500 *g* at 4°C. Discard the supernatant.
5. Resuspend the pellets in 1 mL of ice-cold sterile water and transfer the cells into two 1.5-mL Eppendorf tubes.
6. Centrifuge for 20 s at 4°C at maximum speed. Discard the supernatant.
7. Repeat steps 5 and 6 twice. Resuspend the cells in 100 μL of ice-cold sterile water.
8. Mix 0.2 μL of RCA DNA with 20 μL of the induced (Yu et al., 2000) and uninduced competent cells, respectively. Use a Cell-Portator (GIBCO, BRL) to electroporate the cells.

Results and Discussion

Because of low copy numbers of plasmid, verification of the constructs transformed into *A. tumefaciens* is usually achieved by means of Southern blot analysis (Tzfira et al., 1997). Alternatively, the isolated plasmid DNA from *A. tumefaciens* can be transformed into *E. coli* to propagate for subsequent studies. We directly amplified the entire plasmid pCmPU-GUS from single colonies of *A. tumefaciens* by using RCA. Figure 1A shows that DNA with a broad size range was amplified. Most of these DNA products represent distinct concatemeric units of the pCmPU-GUS, which was confirmed by the restriction digestion. Some background could be generated from the genomic DNA of *A. tumefaciens*.

To recover the amplified plasmids, our initial attempts to directly transform the widely used *E. coli* strain XL1-blue with RCA products only resulted in few transformants (data not shown). However, digestion of the RCA products with a single-cut restriction enzyme followed by self-ligation with T_4 DNA ligase dramatically increased the bacterial transformation efficiency. For example, approximately 5000 transformants were obtained from 50 ng of RCA-amplified pCmPU-GUS (1.1×10^5 cfu/μg DNA) by using standard electroporation methods. Thus, RCA products can be efficiently transformed into *E. coli* after circularizing single units of linear concatemeric DNA.

One drawback to using the above transformation approach is the prerequisite of plasmid sequences to identify a single-cut restriction enzyme site for circularizing RCA products. The digestion and self-ligation steps would also increase workload, particularly when performing high-throughput analyses. To develop more generally applicable methods, we used the recombinogenic *E. coli* strain DY329. This strain was engineered with a λ prophage containing the recombination genes *exo*, *bet*, and *gam* under control of a temperature-sensitive λ cI-repressor (Yu et al., 2000). The bacterial cells become more recombinogenic when treated at 42°C. By using the transformation procedures described in "Materials and Methods", an efficiency of 1.34×10^4 cfu/μg DNA was achieved for the

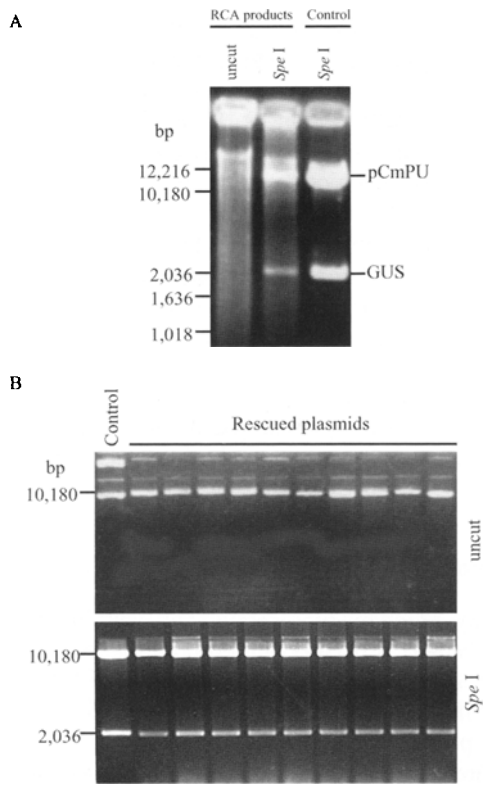

Figure 1. (A) Amplification of the plasmid pCmPU-GUS from *Agrobacterium* cells by using rolling circle amplification. Single bacterial colonies were picked and amplified in 10-μL volumes as described in the text. One microliter of the amplified DNA was digested overnight at 37°C with the restriction enzyme *Spe* I. Restriction digestion of the pCmPU-GUS plasmid purified from *Escherichia coli* is also shown as the control. The samples were electrophoretically separated in an agarose gel. (B) Agarose gel electrophoresis of rescued plasmids from the *E. coli* strain DY329 transformed with rolling circle amplification (RCA) products. Bacterial cells were transformed with the RCA-amplified pCmPU-GUS DNA by using standard electroporation procedures. Plasmids were recovered from 10 independent transformants and analyzed by means of agarose gel electrophoresis. The control (pCmPU-GUS), uncut (upper), and *Spe* I-digested (lower) samples are shown.

linear RCA DNA of pCmPU-GUS. In contrast, no transformants were obtained by using similar amounts of RCA products when the DY329 cells were not treated by the 42°C heat shock, indicating that the engineered recombination activity is required for the transformation. Gel electrophoresis analysis indicated that the plasmids recovered from the transformants are similar in size to the monomeric pCmPU- GUS (Figure 1B). Restriction digestion confirmed that these plasmids contained correct insert (Figure 1B).

The RCA-based methods described in this study provide an alternative for the identification and recovery of transformed constructs from *A. tumefaciens*. Bacterial culturing and subsequent plasmid isolation with conventional methods are eliminated. No specific equipment is required. Amplification of entire plasmids for restriction digestion or even DNA sequencing allows detailed analysis of

the constructs. The experimental steps are amenable to automation, which is critical for processing samples in high-throughput manners.

Transforming DNA from other microorganisms into *E. coli* is a common strategy to purify, propagate, express, and manipulate plasmid DNA. Our methods connect the powerful RCA to the well-established *E. coli* system. Plasmids could be amplified from other microorganisms or transformed cells and introduced into *E. coli* for a variety of applications. For instance, by transforming RCA-amplified plasmids from single yeast colonies into *E. coli*, we have been able to amplify and separate prey and bait constructs in 2-hybrid analyses. The purified prey can then be used to test for protein-protein interactions with other constructs using similar assays. The *E. coli* transformation methods together with our previous yeast transformation procedures provide the necessary tools for RCA-based plasmid analyses and manipulation.

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