6. Spread 100 μL of the resulting suspension at dilutions of 10^{-2}, 10^{-4}, and 10^{-6} on LB agar containing the appropriate antibiotics (e.g., for LBA4404 and pROKII, 50 μg/mL rifampicin plus 50 μg/mL kanamycin; *see* **Note 18**). Aliquots of each of the three bacterial cultures should also be plated. Incubate for 48 h at 28°C, to allow single colonies of transconjugants to grow.

7. Confirm the fidelity of the *Agrobacterium* transconjugant using either Southern hybridization or PCR analyses, and store the bacteria in 50% glycerol at –70°C (*see* **Note 19**).

3.3. Analysis of Transconjugants

3.3.1. Using Miniprep and Southern Analyses

1. Prepare the binary vector plasmid DNA using standard miniprep protocols for *E. coli*, except that *Agrobacterium* should be grown for approx 24 h at 28°C (*see* **Note 20**).

2. Digest approx 5 μg of the DNA with restriction enzymes that produce a restriction fragment pattern characteristic of the binary vector construction (*see* **Note 21**).

3. Separate the DNA fragments by agarose gel electrophoresis; analyze using Southern blotting and hybridization to a labeled CP-specific DNA probe (*see* **Note 22**). The techniques are detailed in Chapters 27, 41, and 43.

3.3.2. Using PCR Techniques

The amplification of the CMV-O CP insert using primers that bind to the CaMV 35S promoter and the nos terminator will be described (*see* **Note 23**).

1. Use 1 μL (or 2% of the total volume) of miniprep DNA (e.g., that prepared in **Subheading 3.3.1., step 1**) or a small proportion of the *Agrobacterium* colony under test.

2. Carry out a PCR reaction as described in Chapters 28 and 42, using the following primers:
   a. Primer 1 (CaMV 35S promoter): 5'-ATATCTCCACTGACGTAAGG-3'
   b. Primer 2 (nos terminator): 5'-CGGCAACAGGATTCAATCTT-3'
   and reaction conditions of: Denaturation at 94°C for 7 min, followed by 30 cycles of 94°C for 1 min, 53°C for 2 min, and 73°C for 3 min (*see* **Note 24**).

3. Confirm the presence of the insert within the transconjugant *Agrobacterium* by electrophoresis of 10% of the amplified sample (*see* **Note 24**).

4. Notes

1. Many binary plant transformation vectors are freely available for research; however, for work that is likely to have commercial applications, the use of some vectors produced with industrial funding may be restricted. The vector (pROKII) that we have chosen as an example is freely available and does not suffer from such limitations. Although it has been found that pBIN19 and its derivatives (e.g., pROKII) have a mutation within the *nptII* gene that provides