3. Shoots should be subcultured routinely every 4–5 wk into fresh MS30 (if the cultures become contaminated, *see* **Note 6**).

3.2. The Introduction of Plasmids into Agrobacterium by Triparental Mating

3.2.1. Setting Up Cultures of Parental Plasmids

1. Two days before the triparental mating, set up 10 mL LB broth + 100 mg/L rifampicin culture of LBA4404. Leave the culture to grow for 48 h at 28–30°C, with constant shaking.
2. The day before the mating, set up a overnight culture of pRK2013 (in *E. coli* strain HB101) and an overnight culture of the *E. coli* strain containing the construct to be transformed into the tobacco, each in 10 mL LB broth + 100 mg/L kanamycin sulfate. In our laboratory, we routinely use the binary vector Bin19 ***(2)*** transformed into XL1 blue cells ***(6)***, supplied by Stratagene (Cambridge, UK). The Bin19 plasmid confers to bacterial and plant tissues resistance to kanamycin sulfate; therefore, add 100 mg/L of kanamycin sulfate to the overnight culture. Grow both cultures at 37°C, with constant shaking.

3.2.2. Mating Procedure

1. Take 100-µL aliquots from each 10-mL culture and mix together in a sterile 1.5-mL Eppendorf tube (store the remaining liquid cultures at 4°C).
2. Spin the cells down in a microcentrifuge (12,000*g* for 5 min).
3. Resuspend the pellet in 10 µL of 10 m*M* $MgSO_4$.
4. Put the 10-µL droplet onto an LB agar plate and incubate overnight at 28–30°C. During this time, mobilization of the plasmid occurs.
5. Using a microbiological loop, streak out a patch of the bacteria onto an LB agar plate containing 100 mg/L kanamycin and 100 mg/L rifampicin. At the same time, as a control, streak out each of the parental strains from **step 3** on a duplicate plate and incubate the plates overnight at 28–30°C. Only the LBA4404 harboring the Bin 19 vector should grow.
6. Restreak for single colonies, if necessary.
7. Check by DNA restriction analysis that no rearrangements have occurred in the gene construct to be transformed into tobacco (*see* **Note 7**).

3.3. Inoculation of Leaf Explants with Agrobacterium

1. Working in a sterile environment, cut fully expanded leaves from 4- to 6-wk-old shoot cultures and place them in a sterile Petri dish.
2. With a scalpel, remove the leaf midrib and the leaf edge and then cut the lamina into small pieces approx 1 cm^2 (*see* **Note 8**). Place 5–6 explants adaxial-side up on a plate of shooting medium 1. Prepare 20–30 explants for each construct.
3. Seal the plates with laboratory sealing film and incubate the explants for 2 d under constant light at 22°C.