

11. Adjust the pressure at the helium bottle to 1500 psi and repeat the above process using 1300 psi rupture disks, until all the embryos have also been bombarded twice at 1300 psi.
12. Seal the plates with parafilm and incubate overnight at 26°C in the dark.

### **3.4. Selection and Regeneration**

1. The day after bombardment, the embryos should be transferred to LS2.5 medium supplemented with 4 mg/L bialaphos (*see Note 5*).
2. After incubation for 3 wk (26°C, dark), growth of callus should be apparent on the surface of many of the embryos. The clumps of cells present in these calli should be subcultured to fresh selection plates. During subculture, gently tease the clumps of cells apart, if possible. Also, take care to assure good contact between the tissue and the medium, since crossprotection of bialaphos-sensitive cells is a potential problem (crossprotection can also be a problem when hygromycin is used as a selective agent; *see Note 6*). Also, because of the high frequency of siblings from individual embryos, it is advisable to subculture all calli from each embryo separately.
3. Subculture surviving tissue at least three consecutive times, with approx 3 wk intervals between subcultures. The calli should be exposed to bialaphos for a minimum of 8 wk.
4. Subculture ~10–50 mg (~2–3 mm diameter) pieces of bialaphos-resistant callus onto MSD4 medium. Incubate at 26°C in the dark (*see Note 7*).
5. 10–14 d later, organized structures should be visible on the surface of the calli.
6. After a total of 3–4 wk on regeneration medium, etiolated shoots and possibly roots will develop.
7. Leave cultures in the dark until leaves develop (another 1–2 wk).
8. Transfer to a lighted incubator (26°C, 18 h light:6 h dark).
9. Leaves will green-up in a few days, but do not transfer developing plantlets to Magenta boxes until roots develop (*see Note 8*).
10. Collect leaf samples for PCR analysis during transfer of plantlets to Magenta boxes (*see Note 9*).
11. Transfer each individual plantlet to a Magenta box by picking it up with a pair of forceps and scraping any adhering callus off with another. Insert the base of the plantlet into the medium far enough to submerge at least some of the roots.
12. Once transplanted into Magenta boxes, the plants grow quite rapidly. After 2–3 wk, most plantlets will have developed a vigorous root system, grown leaves that touch the top of the box, and be ready to transplant to soil.
13. Discard plantlet if PCR analysis indicates it lacks the gene of interest.

### **3.5. Potting Plants and Growth Conditions**

1. Prepare potting soil at least 12 h in advance to allow thorough wetting. Mix five parts shredded, pasteurized peat moss with seven parts coarse vermiculite and saturate with Mikkelson's nutrient solution.