2. Peeled sections are placed peeled side down into 9-cm Petri dishes containing 30 mL of protoplasting solution. Care is taken not to place peeled sections on top of one another (*see* **Note 2**).
3. The Petri dishes are sealed with parafilm to prevent spillage, and incubated overnight on the bench.
4. The following morning the dishes are lightly swirled to loosen any protoplasts still attached to the leaves. They are allowed to sit for a further half hour before being passed through a 64-μm Nitex sieve (*see* **Note 3**).
5. The protoplasts are gently pipeted into a 50-mL screw-cap tube and collected by centrifugation at 300*g* for 5 min (*see* **Note 4**). The supernatant is removed and the protoplasts are gently resuspended in an equal volume of protoplast wash solution. The protoplasts are then collected by centrifugation at 300*g* for 5 min and the protoplasts resuspended in one-third vol (approx 10 mL) of protoplast wash solution.
6. 2.5 mL of 21% sucrose solution is pipeted into the bottom of 10-mL screw-cap tubes. Each tube containing 2.5 mL 21% sucrose solution is gently overlaid with 5 mL of washed protoplasts (*see* **Note 5**). The tubes are spun at 500*g* for 5 min with slow acceleration and deceleration. A distinct layer of protoplasts is seen to be floating at the interface of the solutions. The protoplasts are then removed from the interface by gentle pipeting, and placed in a new tube.
7. Using a measured volume, the number of protoplasts are then counted using a hemocytometer (number of protoplasts/mL = number in five big squares × 100). The general appearance of the protoplasts can also be determined at this stage (*see* **Note 6**).
8. The protoplasts are spun down at 300*g* for 5 min and resuspended in wash solution to approx 5×10^6 to 1×10^7 protoplasts/mL.

3.2. PEG Transformation of Isolated Protoplasts

1. In 10-mL screw-cap tubes, 0.2-mL PEG solution, 20 μg plasmid DNA (in approx 20–40 μL SDW), and 0.2-mL protoplasts are gently mixed and left for 15–20 min at room temperature (*see* **Note 7**).
2. 1 mL 0.275*M* calcium nitrate solution is added dropwise to the mixture, with gentle agitation, followed by another 4 mL of 0.275*M* calcium nitrate solution. The mix is left to stand for 10 min.
3. Protoplasts are collected at 300*g* for 5 min, then resuspended in 5 mL recovery medium and gently poured into 5-cm Petri dishes, and incubated for 6 h to overnight on the bench.
4. The protoplasts are checked for viability by fluorescein diacetate (FDA) staining. FDA, when cleaved by protoplasts or plant cell esterases, releases fluorescein, which is retained only within an intact or viable protoplast. Therefore, viable protoplasts can be visualized under UV light (*see* **Note 8**). FDA stock solution is prepared (2 mg/mL acetone) and stored in the fridge. One drop of this FDA stock solution is added to a small sample of the protoplast solution and the protoplasts are examined after 5 min by fluorescence microscopy. The percentage of proto-