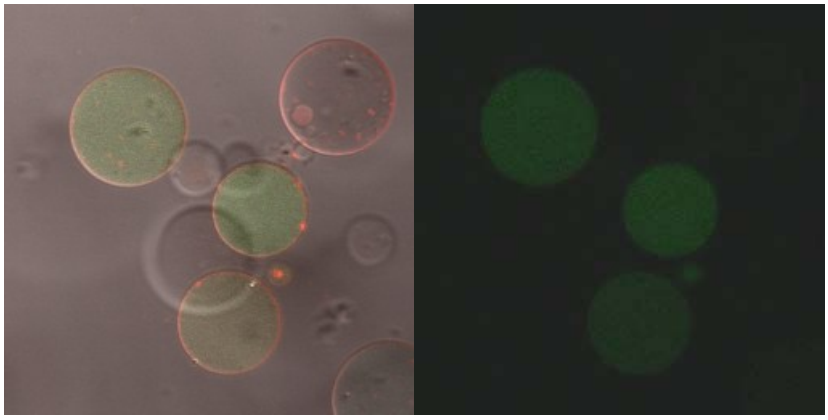
Vacuole isolation protocol

- Preheat lysis buffer at 42°C.
- Transfer the protoplasts in a 50ml tube. Spin at 100g for 10 minutes (brake 0). Remove the dead cells and most of the medium leaving no more than 5 ml. Make up to 50 ml with 250mM NaCl. Mix gently and transfer 5ml in a small falcon (total).
- Spin both tubes 200g for 3 minutes (brake 6). Remove the supernatant leaving as little as possible and place pellets on ice.
- Add 5 ml of prewarmed lysis medium to protoplasts. Sack up and down three times avoiding touching the bottom of the tubes with the pipette.
- Transfer to a small falcon tube and place on ice.
- Layer 3 ml of 1:1 lysis medium/vacuole buffer mix (ice cold) on top of protoplasts.
- On top of this layer 2 ml of vacuole buffer (ice cold).
- Centrifuge at 3000g for 10 minutes at 4°C (brake 0).
- Take off "red vacuole" layer by "hovering" (1 ml).
- Add an equal volume of vacuole buffer 1 ml (ice cold).
- Spin at 1500g for 3 minutes (brake 6).
- Remove the supernatant leaving as little as possible and add 100μl of vacuole buffer and to the cell pellet. Mix gently and transfer 50μl of each one in new eppendorf with 50μl of sample buffer (-80°C).
- Take 50μl of the remaining sample and add 100μl of α-mannisidase extraction buffer. Sonicate per 2 seconds at the quarter of the scale. Spin 5 minutes at max speed at 4°C.

Vacuole buffer 0.6M betain, 10mM HEPES bring to pH 7.5 and add 150μg/ml BSA
Before use add: 0.1μg /ml pepstatin A(stock solution 1000x)

Lysis medium 0.2M mannitol , 10% Ficoll-400, 20mM EDTA, 2mM DTT, 5mM HEPES, bring to pH 8.0 and add 10μg/ml neutral red, 150μg/ml BSA
Before use add: 0.1μg /ml pepstatin A



Vacuoles isolated from tobacco protoplasts transiently expressing a vacuolar-targeted GFP fusion protein.

α-mannosidase assay

- 70μl of extraction buffer (250mM Na-acetate buffer, pH 4,6)
- 20μ of extract (after centrifugation)
- The reaction is started by adding 10μl of the substrate solution (6mM p-Nitrophenol-α-mannopyrannoside, in extraction buffer).
- The mixture is incubated for 30 minutes-2 hours at 30°C (water bath).
- The reaction is terminated by adding 160μl of 1M Na_2CO_3.
- Transfer 200μl to a microtitre plate and read absorbance at 405nm. (the controls are prepare in the same way but adding the 160μl of Na_2CO_3 before the other component).