Isolation of vacuoles from *Arabidopsis*（Leaves）

Preparation of protoplasts

For 1.6 g of fresh leaves:

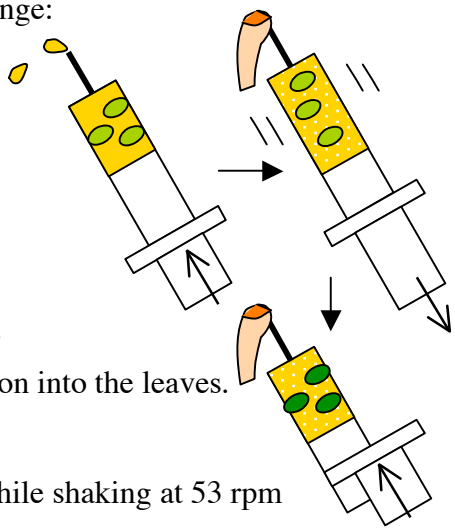
Enzyme solution 50ml

1 % Cellulase R-10

2 % Macerozyme R-10

P sor (0.6 M) pH 6.0

Infiltration of the enzyme solution into the leaves with a 50-100 ml syringe:



Put the Arabidopsis leaves in a syringe with the enzyme solution and force the air inside of the syringe out.

Pull the piston to create negative pressure inside of the syringe while sealing the tip of the syringe with a finger.

Shake the syringe while maintaining the pressure, and push the piston back to its original position. The step removes the air cellular vacancy.

The additional pressure completes the infiltration of the enzyme solution into the leaves.

Transfer the leaves and enzyme solution into a 200 ml flask.

Incubate the leaves in the enzyme solution for about 3 hours at 32℃ while shaking at 53 rpm

Purification of vacuoles

Remove the undigested tissues from protoplasts with a filter

Divide the released protoplasts into four glass tubes underlayed with 0.5 ml Vc suc (0.4 M)

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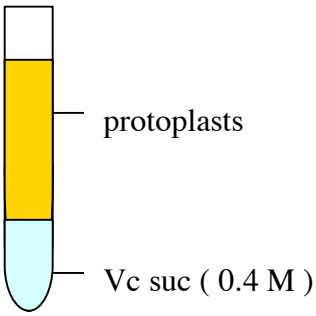
Centrifugation (200 ×g, 10 min)



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Remove the supernatant

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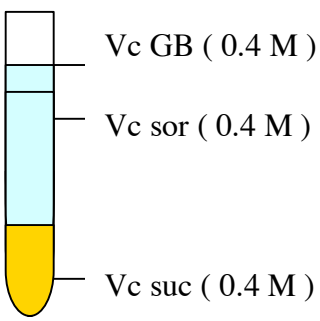
Collect the protoplasts into two tubes

Add 2 ml Vc suc (0.4 M) and mix well

Form a gradient by overlaying

3 ml Vc sor (0.4 M)

0.5 ml Vc GB (0.4 M)



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Centrifugation（200 ×g, 1-2 min, and then 400 ×g, 8 min）

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Check the vacuoles present under the VcGB with a microscope

Remove the solution over the purified protoplasts

Transfer the protoplasts to a beaker

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Prepare new four glass tubes and add 4 ml Vc suc

Squeeze the protoplasts gently through a syringe 19 G（1.10 × 90 mm）

Check the released vacuoles with a microscope (with Neutral Red)

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Divide the solution into two glass tubes

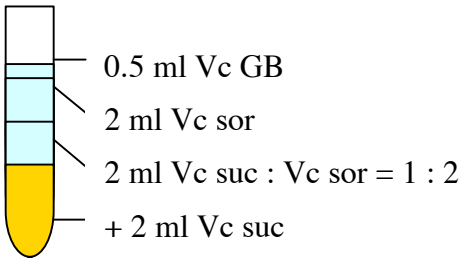
Add 2 ml Vc suc and mix

Form a gradient by overlaying

2 ml Vc suc : Vc sor = 1 : 2

2 ml Vc sor

0.5 ml Vc GB



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Centrifugation（200 ×g, 1-2 min, and then 400 ×g, 8 min）

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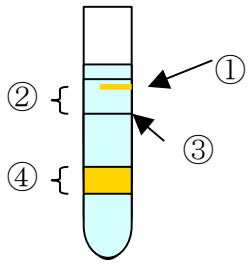
Vacuoles are obtained under the VcGB (①)

Collect solution (②)

Confirm vacuoles in the interphase (③),

Collect vacuoles



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Remove the layers over ④ and move the protoplast（④）into a beaker

Squeeze through a syringe 19 G (1.10 × 90 mm)

↓

As described above, form a gradient

↓

Centrifugation（200 ×g, 1-2 min, and then 400 ×g, 8 min）

↓

Collect vacuoles

↓

As described above, remove the layers over ④ and move the protoplast（④）into a beaker

Add equal volume of Vc med 0 and vortex

Incubate on ice for 5 min

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Squeeze through a syringe

↓ ↓

As described above, form a gradient

↓

Centrifugation（200 ×g, 1-2 min, and then 400 ×g, 8 min）

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Collect vacuoles

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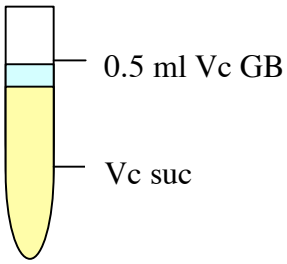
In order to concentrate the vacuoles,
add Vc suc to the solution of vacuoles,
and overlay Vc GB



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Centrifugation（200 ×g, 1-2 min, and then 400 ×g, 8 min）

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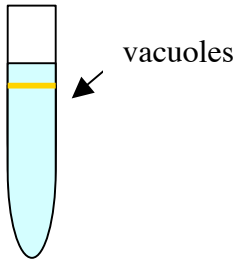
Collect vacuoles



Solutions

P sor (0.6 M) pH 6.0

10 mM Mes

1 mM $CaCl_2 \cdot 2H_2O$

0.6 M sorbitol

Vc med 0

30 mM HEPES

2 mM EGTA

30 mM Potassium gluconate

2 mM $MgCl_2$

Tris pH 7.2

Vc suc (0.4 M)	200 ml
Vc med 10 x	20 ml
2M sucrose	40 ml

Vc sor (0.4 M)

Vc med 10 x	20 ml
2M sorbitol	40 ml

Vc GB (0.4 M)

Vc med 10 x	20 ml
2M Betain Monohydrate	40 ml

Vc suc : Vc sor = 1 : 2