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Molecular and Biochemical Analysis of *Arabidopsis*

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The standard protocol for *in situ* hybridizations in plants still involves fixing fresh tissue, embedding the tissue in wax, sectioning with a microtome and detection of the transcripts of interest using labeled RNA-probes. The RNA probes are synthesized by *in vitro* transcription, and either radioactive or nonradioactive labels can be used. This protocol concentrates only on nonradioactive methods, as they are easy to perform, very sensitive and even faster than techniques involving ra dioisotope labels.

I. Tissue fixation and embedding

Tissue to be fixed should be small (the smaller the better). Tissue should be placed into ice cold fixative immediately after dissection. If you are fixing stem tissue, cut it into short pieces about 5 mm in length. Root tissue is most easily harvested form plantlets that were grown on hard agar or in liquid culture. This avoids any problems with soil sticking to the roots.

Most plant tissues have a cuticle and will thus simply float on the surface of the fixative. The detergent Tween 20 (alternative: Triton X 100) will aid the penetration of the fixative. The open glass vials with tissue in fixative are placed into a exiccator that is connected to a vacuum pump. Vacuum is applied for about 10 minutes, until the tissue sinks down in the fixative solution at normal air pressure. Leave to fix overnight at 4°C.

1 Preparation of fixative

All solutions should be freshly prepared just before use.

Paraformaldehyde and Formaldehyde solutions and vapour are toxic, so all steps involving these chemicals should be handled in a chemical fume hood. During the vacuum treatments, Formaldehyde vapours are released, so check that the outlet of your vacuum system will not pump toxic vapours into the lab.

- Add a small pellet of NaOH to100 ml PBS, pH 6.5–7.

The pH will increase to about pH 11.

- Heat to 70°C in the microwave.
- Add 4 g Paraformaldehyde and shake vigorously until the Paraformaldehyde is dissolved.
- Cool on ice.
- Add H_2SO_4 to adjust the pH to 7.
- Finally add 30 ml Tween 20.

The fixative is now ready for use. Aliquot the fixative into small glass scintillation vials.

2 Embedding

The tissue is dehydrated through a graded alcohol series, stained with a dye (Eosin Y) to facilitate sectioning and embedded into wax. This procedure will take at least 5 days.

Perform all steps in glass scintillation vials.

Day 1

- Remove the fixative, replace with ice-cold 50% Ethanol, incubate 90 min on ice.
- Ice-cold 70% Ethanol, incubate 90 min on ice.
- 85% Ethanol, incubate 90 min at 4°C.
- 95% Ethanol with 0.1% Eosin Y, incubate 90 min at 4°C.
- 100% Ethanol with 0.1% Eosin Y, incubate *overnight* at 4°C.

Day 2

- 100% Ethanol with 0.1% Eosin Y, incubate 90 min at 4°C.
- 100% Ethanol, incubate 60 min at RT.
- 50% Ethanol:50% Histoclear, incubate 60 min at RT.
- 100% Histoclear, incubate 60 min at RT.
- 100% Histoclear, incubate 60 min at RT.
- 100% Histoclear, incubate 60 min at RT.
- Pour off the last Histoclear, fill the glass vials up to the middle with Histoclear, then add wax pellets to the top. Incubate at 40–50°C *overnight*.

Day 3

- Melt wax pellets at 60°C, replace Histoclear/wax mixture with freshly molten wax and incubate at 60°C.
- Change the wax again in the evening.

The wax (Paraplast, or other brand names) to be used also contains plastic polymers and DMSO that shall facilitate the infiltration and sectioning. These additives are unstable at temperatures higher than 62°C. The Paraplast will solidify at 56–58°C. Be very careful to keep the temperature of the wax always at 60°C. All changes of wax and handling of the embedded materials has to be done quickly.

Day 4 and 5

- For additional 2 days keep changing the wax every morning and evening.

Day 6

Now the tissue blocks are made.

- Place the mould on a heating block at 60°C, pour some wax into the mould and empty a glass scintillation vial with the tissues into the mould.

  Use plastic balance trays as moulds. The tissue can be quickly oriented in the mould using forceps that are preheated in the flame of a gas burner.
- When the tissue is in position, float the mould on cool water, thereby solidifying the wax.
- Store the embedded tissues in the fridge.

II. Sectioning

Sectioning requires a lot of patience and some practice. Use a standard microtome that allows to produce sections 5 to 10 mm thick. Check whether the blade is still unmarked. If possible, use a microtome with cheap, disposable blades that can be exchanged whenever it seems necessary.

Cut the wax in the moulds into small blocks with tissue.

1. Heat a spatula in the flame of a gas burner, press it quickly to the wax block and fix it with some molten wax onto a holder.
   These holders can be bought from companies or made yourself by cutting hard wood into small cubes (2 × 2 × 2 cm).
2. After fixing a number of tissue blocks to the holders, cool the blocks for 20 min.
3. Cut the wax blocks to a rectangular shape, leaving 1 to 2 mm of wax around the tissue.
   Such small wax blocks will allow you to fit more sections on each slide. Mount the holder to the microtome such that the longer side of the block is parallel to the blade.
4. Cut ribbons of sections at 5 to 10 mm thickness.
5. Place ribbons on coated glass slides (Superfrost Plus), using a fine paint brush.
6. Add sterile water so that the wax ribbons float freely.
7. Place the slide onto a hotplate at 42°C until the sections are fully flattened.
8. Drain off the water with a tissue paper (Kleenex).
9. Gently blot the sections dry with a very fine tissue paper.
   You can now check the section under a microscope.
10. Leave the slides on the hotplate overnight, and then store at 4°C until you are ready for the hybridization.

Common problems with sectioning:

The sections break up or appear brittle: material was not properly embedded or the wax was destroyed by overheating.

Sections split along the ribbon: the blade is chipped or dirty – clean or replace the blade.

Ribbons are not straight: the wax block is not rectangular or the long side of the block is not parallel to the blade.

Sections roll up, no ribbon is formed: change the angle of the blade.

Ribbon forms, but the whole ribbon rolls up or sticks to the blade: the blade is electrostatically charged, wipe it with a wet Kleenex.

III. Preparation of probes

The DNA fragment has to be subcloned into a transcription vector, *e.g.*, pBluescript or pGEM. The plasmid DNA to be used for the *in vitro* transcription reaction should be purified using a commercial purification system.

Linearise the plasmid with a restriction enzyme, and check the digest on a minigel.

It is important that the plasmid is cut to completion. Avoid using a restriction enzyme that leaves a 3´ overhang (e.g. Kpn I).
Purify the plasmid after the restriction digest by Phenol/Chloroform extraction and Ethanol precipitation. Be aware that the DNA has to be completely free of RNase!
Resuspend the cut plasmid DNA in autoclaved water at a concentration of 0.5 µg/µl.

In vitro transcription

Use any commercially available T7, T3 or SP6 RNA-Polymerase. The choice of polymerase depends of course on the plasmid vector used and on the orientation of your sequence of interest (Select the appropriate polymerase so that the antisense-transcript is made).

1. Set up the transcription reaction on the bench (not on ice):
   - 5 µl dH_2O
   - 5 µl 5× Transcription buffer
   - 1 µl RNAse Inhibitor (40 U/µl)
   - 2.5 µl 5mM ATP
   - 2.5 µl 5mM GTP
   - 2.5 µl 5mM CTP
   - 1 µl 5mM UTP
   - 2.5 µl 1mM DIG-UTP
   - 2 µl linearized template DNA (= 1 µg)
   - 1 µl RNA-Polymerase (20 U/µl)
2. Incubate at 37°C for 60 to 120 min.
3. Check the transcript on a gel. Therefore take 1 µl from each labeling reaction, add 9 µl TE.
   After electrophoresis a smear of synthesized RNA and a faint plasmid band should be visible.
4. Stop the reaction by adding
   - 75 µl TMS-buffer
   - 2 µl tRNA (100 mg/ml)
   - 1 µl DNase (RNase free!)
5. Incubate 10 min. at 37°C
6. Take 1 µl from each sample, add to 9 µl TE and check on gel whether template DNA has been removed
   - Add
     100 µl 3.8 M NH_4Ac
     600 µl EtOH
   - Incubate at -20°C for 60 min.
   - Spin down for 10 min. at maximum speed in a microcentrifuge.
7. Wash the pellet with ice cold 70% EtOH/0.15 M NaCl.
8. Centrifuge again, take supernatant off and air dry briefly.
9. Resuspend the air dryed pellet in 50 µl dH_2O.

IV. Hydrolysis

The optimum length for *in situ* probes is about 150 bp. The *in vitro* synthesized RNA probes can be hydrolysed under mild alkaline condition to achieve a mixture of RNA probes whose sizes average around 150 bp.

The following formula is used to calculate the time of hydrolysis:

$$t = \frac{L_i - L_f}{k \times L_i \times L_f}$$

t = time (minutes)
K = rate constant (= 0.11 kb/min)
L_i = initial length (kb)
L_f = final length (kb)

Example: If your cloned DNA fragment to be transcribed is 1.5 kb, the hydrolysis time will be:

$$t = \frac{1.5 - 0.15}{0.11 \times 1.5 \times 0.15} = 54.5$$

Procedure

1. Hydrolyse your *in vitro* transcripts by adding 50 µl 200 mM Carbonate buffer pH 10.2.
2. Incubate at 60°C for the calculated time.
3. Put your sample on ice and add
   10 µl 10% acetic acid
   12 µl 3M NaAcetate
   - Mix briefly, gas bubbles should appear, then add 312 µl Ethanol.
   - Incubate at -20°C, 60 min.
4. Spin down in a microcentrifuge for 10 min,
5. Wash the pellet with ice cold 70% Ethanol/0.15 M NaCl.
6. Centrifuge again, take supernatant off and air dry briefly.
7. Resuspend the air dryed pellet in 50 µl dH_2O and store at -20°C.

Checking the probe with an anti-DIG antibody

1. Spot 1 µl of your probe solution on a small piece of filter.
2. Fix by baking or UV-cross linking.
3. Wet briefly in 100 mM Tris-HCl pH 7.5, 150 mM NaCl.
4. Incubate 30 min. in 0.5% Blocking Reagent.
5. Wash briefly in 100 mM Tris-HCl pH 7.5, 150 mM NaCl.
6. Incubate 30 min. in 5 ml 100 mM Tris-HCl pH 7.5, 150 mM NaCl with 1 µl anti-DIG AP
   - Wash 2 × 15 min in 100 mM Tris-HCl pH 7.5, 150 mM NaCl
   - Wash briefly in 100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$
   - Develop for 10 min in 5 ml buffer 5 with 5 µl NBT and 5 µl BCIP
   - Stop the reaction by washing the filter in water.

   A dark blue spot should be visible where your probe solution was spotted on the filter.

V. *In situ* Hybridization

1 Preparation of cover slips

- Coverslips of the appropriate size (*e.g.* 24 × 50 mm) have to be cleaned and baked before they can be used.
- Wash coverslips in acetone for 15 min
- Drain the slides in a steel test tube rack
- Wrap in aluminum foil and bake at 180°C for 2 h.

2 Tissue pretreatments

On the day that you want to set up the hybridization reactions, the tissue has to be pretreated. These treatments consist of a number of steps in order to make the tissue more accessible for the RNA-probes and to reduce any unspecific binding of the probe to the slide. The slides are put into stainless steel racks (or plastic racks: check first whether they withstand the Histoclear) and passed through the following solutions:

- Dunk the slide rack several times and then leave it for the specified time in each solution.

Solution	Time	Remarks
100% Histoclear (or Rotihistol)	10 min	
100% Histoclear	10 min	
100% Ethanol	1 min	
100% Ethanol	1 min	
95% Ethanol	1 min	
85% Ethanol	1 min	
50% Ethanol	1 min	
30% Ethanol	1 min	
dH_2O	1 min	
0.2 M HCl	10 min	HCl treatment makes the tissue more accessible for the probe.
dH_2O	5 min	
PBS 1	2 min	PBS 1 and 2 are two separate boxes with 1 × PBS buffer.
Pronase (0.125 mg/ml in Pronase buffer)	10 min	Pronase treatment makes the tissue more accessible for the probe.
Formaldehyde (4% in PBS)	10 min	⚠ *Treat in a fume hood!* Formaldehyde has to be prepared freshly from Paraformaldehyde.
Glycine (0.2% in PBS)	2 min	Glycine will stop the Pronase activity.
PBS 1	2 min	
PBS 2	2 min	
Acetic anhydride (1ml in 100ml 0.1 M Triethanolamine pH 8.0)	10 min	⚠ *Treat in a fume hood!* Acetic anhydride is very unstable in water. The slides are incubated in Triethanolamine solution, and acetic anhydride is added while the slides are dunked repeatedly during the 10 min incubation. Acetylation of the tissue (the acetic anhydride step) shall reduce unspecific binding of the probe to the tissue.
PBS 2	2 min	

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- Dehydrate through the Ethanol series up to the second 100% Ethanol
- Wash again in fresh 100% Ethanol.
- Store the slides in a box with a bit of Ethanol at 4°C while you are preparing the hybridization mix.

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VI. Hybridization

In general, about 2 µl of the hydrolysed probe solution should be used per slide. However, it is advisable to try hybridizations with larger or smaller amounts of probe to find an optimum probe concentration. The final hybridization mix consists of 1 part "probe mix" and 4 parts of "Hybridization buffer" .

1 Preparation of required solutions

- Probe mix (per slide)
  - 2 µl hydrolysed DIG-labeled RNA probe
  - 2 µl dH_2O
  - 4 µl deionised Formamide
  - Mix, incubate for 2 min. at 80°C
  - Cool on ice.
- Hybridization buffer (for 25 slides):
  - 100 µl 3 M NaCl, 0.1 M Tris-HCl pH 6.8, 0.1 M $NaPO_4$-buffer, 50 mM EDTA
  - 400 µl Formamide (deionised)
  - 200 µl 50% Dextransulphate
  - 10 µl 100 mg/ml tRNA
  - 20 µl 50 × Denhardt´s solution
  - 70 µl dH_2O

2 Procedure

- Add 8 µl probe mix to 32 µl Hybridization buffer, this results in 40 µl Hybridization mix.
- Distribute the 40 µl on the tissue and cover with a 24 × 50 mm cleaned cover slip.

  ! *Avoid any air bubbles.*
- Place the slides on tissue paper soaked in 2× SSC, 50% Formamide in a small box.
- Seal the box with adhesive tape to avoid evaporation, and incubate it overnight in an oven or a waterbath at 50°C.

VII. Washing

1 Put the slides back into slide racks and immerse them in Wash buffer (2× SSC; 50% Formamide, no need to deionise) at 50°C.

! *The cover slips should have slided off the slides after a few minutes. You can also try to lift them off very gently, but be careful not to destroy your sectioned tissue.*

2 Place the slides into fresh Wash buffer (2× SSC, 50% Formamide) and incubate at 50°C, 2 times for 60 min each.

3 Wash in NTE (500 mM NaCl; 10 mM Tris-HCl, pH7.5; 1 mM EDTA) at 37°C, 2 times for 5 min each.

4 Incubate in NTE with 20 mg/ml RNase A at 37°C for 30 min.

! *The RNase A will digest any unspecifically bound single stranded RNA, but will not affect the specifically bound (hybridized) and therefore double stranded probe-RNA.*

5 Wash in NTE at RT, 2 times for 5 min each.

6 Wash in wash buffer at 50°C for 60 min.

7 Wash in PBS at RT for 5 min.

VIII. Detection

The hybridized probe-RNA will now be detected with an anti-DIG antibody that is coupled to alkaline phosphatase.

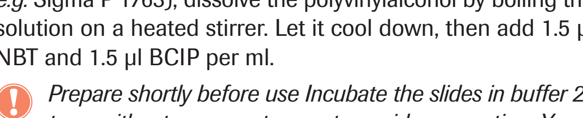
Handling instructions:

The following steps are performed either in slide racks, or in small trays to save solutions. This is recommended for the antibody incubation. Trays should be placed on a shaking platform. Trays should be changed and washed rather than just changing the solutions. All incubations are at Room Temperature.

Procedure

1 Incubate the slides for the specified time in each solution.

Step	Solution/Composition	Time
1	Buffer1: 100 mM Tris-HCl, pH 7.5; 150 mM NaCl	5 min
2	Buffer1 with 0.5% Blocking Reagent	60 min
3	Buffer1 with 1% BSA, 0.3% Triton	60 min
4	Anti-DIG-AP antibody diluted 1:3000 in Buffer 1 with 1% BSA, 0.3% Triton	60 min
5	Buffer 1 with 0.3% Triton	20 min, 4×
6	Buffer 1	5 min
7	Buffer 2: 100 mM Tris-HCl, pH 9.5; 100 mM NaCl; 50 mM $MgCl_2$	5 min
8	Buffer 2 with 10% Polyvinylalcohol (MW 70.000 - 100.000, *e.g.* Sigma P 1763); dissolve the polyvinylalcohol by boiling the solution on a heated stirrer. Let it cool down, then add 1.5 µl NBT and 1.5 µl BCIP per ml.	up to 3 days in the dark



Prepare shortly before use Incubate the slides in buffer 2 in trays with a transparent cover to avoid evaporation. You can then easily check the reaction under a microscope after 12 hours. Incubations for more than three days will result in increased background.

2 Put slides back into slide racks

- Wash in dH_2O, 5 min
- Wash in 70% Ethanol, 5 min.
- Wash in 95% Ethanol, 5 min.
- Wash in fresh 70% Ethanol, 5 min
- Wash in fresh dH_2O, 5 min.

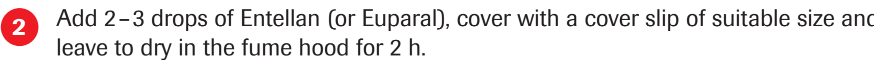
3 The tissue can now be stained with a fluorescent dye (Calcofluor).

- Incubate in 0.1% Calcofluor (in dH_2O), 5 min.
- Wash briefly in dH_2O

The intensity of the staining can be easily checked by holding the slide over a normal UV-transilluminator. The tissue on the slides should show a bright blue intensive fluorescent color.

IX. Mounting

1. Dry the slides by air on the bench.



2. Add 2–3 drops of Entellan (or Euparal), cover with a cover slip of suitable size and leave to dry in the fume hood for 2 h.

   The slides are now ready to view with a light mircoscope. Use a microscope that is equipped with a UV source. Very faint signals can be more easily detected under dark field.

Reagents available from Roche Applied Science for this procedure

Reagent	Cat. No.	Pack size
Anti-Digoxigenin, Fab fragments*	11 093 274 910	150 U (200 µl)
Nylon membrane, positively charged	11 209 272 001 11 209 299 001 11 417 240 001	10 sheets (20 × 30 cm) 20 sheets (10 × 15 cm) 1 roll (0.3 × 3 m)
Blocking Reagent	11 096 176 001	50 g
NBT	11 383 213 001	3 ml (300 mg) dilute prior to use
BCIP	11 383 221 001	3 ml (150 mg)
tRNA, from baker´s yeast	10 109 495 001	100 mg
Set of ATP, CTP, GTP, UTP	11 277 057 001	1 set (4 × 20 µmol)
DIG-11-UTP	11 209 256 910	250 nmol (25 µl)
RNA Polymerase T7	10 881 767 001 10 881 775 001	1000 U 5000 U
RNA Polymerase Sp6	10 810 274 001 11 487 671 001	1000 U 5000 U
RNA Polymerase T3	11 031 163 001 11 031 171 001	1000 U 5000 U
DNase, RNase free	10 776 785 001	10 000 U
DIG RNA Labeling Kit (SP6/T7)*	11 175 025 910	1 kit
DIG RNA Labeling Mix*	11 277 073 910	40 µl (20 reactions)

* The labeling of nucleic acids with DIG is covered by EP patents 0 324 474 and 0 371 262 as well as the following US patents 5.344.757, 5.354.657 and 5.702.888 owned by Roche Diagnostics GmbH.

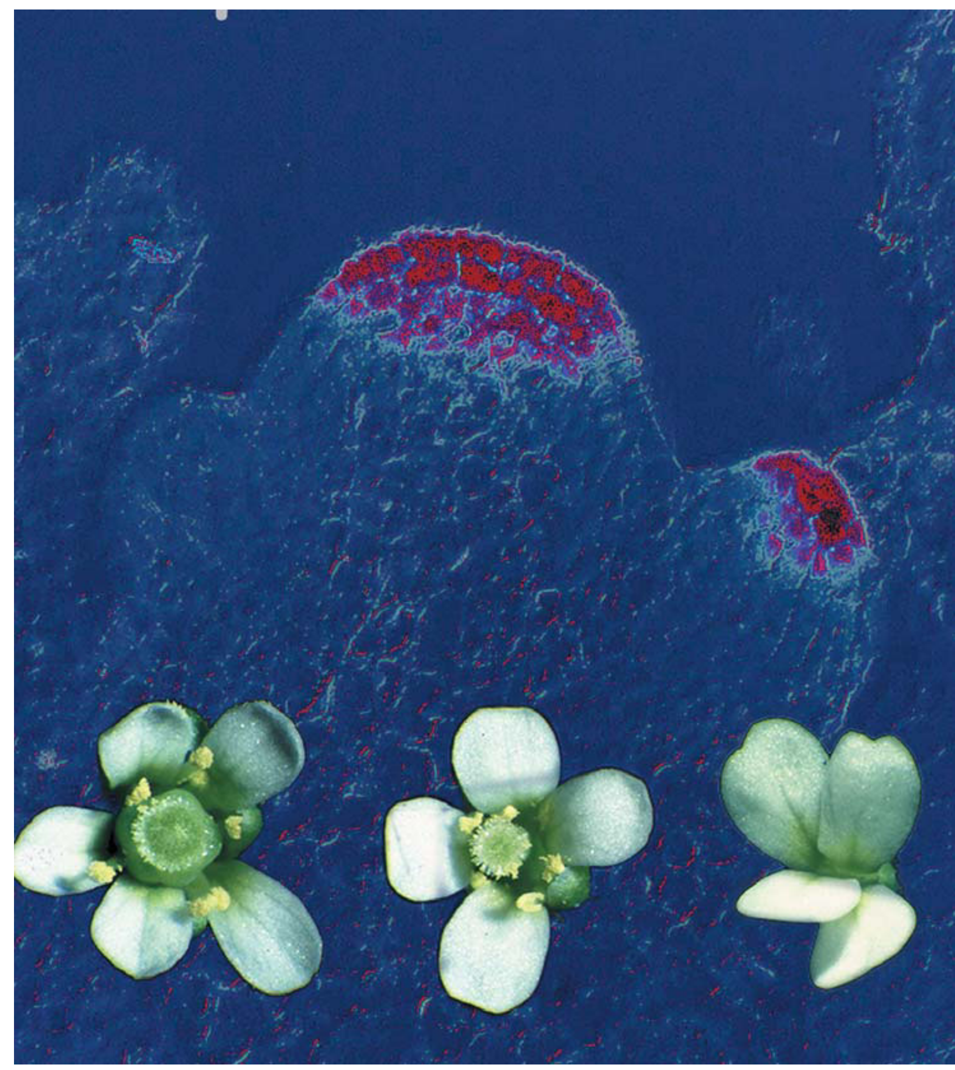
Acknowledgements and Literature

This protocol is a modification of several protocols that were tested out and written up by David Jackson, Enrico Coen, Sabine Hantke and other former colleagues at the John Innes Center, Norwich, U.K. Only a few steps were slightly altered to adapt this method for *Arabidopsis*.

The basic protocols can be found here:

Jackson, D.P. (1991): *in situ* hybridisation in plants. In: Molecular Plant Pathology: A Practical Approach, D.J. Bowles, S.J.Gurr, and M.McPherson, eds. Oxford University Press, England.

Coen, E.S., Romero, J.M., Doyle, S., Elliott, R., Murphy, G., and Carpenter, R. (1990). *floricaula*: A homeotic gene required for flower development in *Antirrhinum majus*. Cell **63**, 1311-1322.



Meristem size in Arabidopsis is controlled by a secreted signalling molecule, *CLV3*. This RNA *in situ* hybridization on a tissue section shows presence of *CLV3* RNA (in red, false colours) in the stem cells at the meristem tip. The tissue used here originated from a *clv1-4* mutant *Arabidopsis* plant that accumulates stem cells in the meristem. The flowers shown are from a *clv3-2* mutant (left), wildtype (middle) and a *CLV3* overexpressing *Arabidopsis* plant (right) (Reference: Brand, U., Fletcher, J.C., Hobe, M., Meyerowitz, E.M. und Simon, R. (2000): Dependence of stem cell fate in *Arabidopsis* on a feedback loop regulated by *CLV3* activity. *Science* **289**, 617-619)

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