IN SITU HYBRIDISATION TO CHROMOSOMES OF ARABIDOPSIS
THALIANA

(Karyobiology Group, JI Centre for Plant Science Research, Colney Lane, Norwich NR4 7UH, U.K.)

* DNA-DNA in situ hybridisation can be used for localising highly repetitive and single copy DNA sequences on chromosomes and interphase nuclei of Arabidopsis thaliana. In our laboratory, we routinely use DNA probes labelled with digoxigenin and detected with fluorescent tags. The method is presented in this protocol. Also given is the method for detecting biotin-labelled DNA probes using enzymatic, colorimetric detection.

1. SOLUTIONS
   (i) CITRIC ACID-SODIUM CITRATE BUFFER, pH 4.8
       A:  0.1M citric acid-monohydrate
       B:  0.1M tri-sodium citrate-2-hydrate
       Stock: 40 ml A + 60 ml B
       Use: 1:9 dilution in distilled water.

   (ii) ENZYME SOLUTION
       20% pectinase (Sigma No. P5146, from Aspergillus niger, solution in 40% glycerol)
       2% cellulase (Calbiochem No. 21947)
       made up with 0.01M citric acid-sodium citrate buffer

   (iii) HYBRIDISATION MIX
       * The hybridisation mixture is usually prepared immediately prior to use but can be stored in the freezer for months.

       Amount recommended per slide (Total 20 ul)
       100% formamide (a) 10 ul
       50% (w/v) dextran sulphate (b) 4 ul
       20 x SSC 2 ul
       Probe made up to (c) 2 ul
       Blocking DNA (d) 1 ul
       10% (w/v) SDS in water 1 ul

       (a) High grade formamide is used, it must be stored at -20°C, preferably after passing through an ion exchange resin.
       (b) In water (the solution is difficult to dissolve) and stock solution must be filtered through a millipore filter (0.22 um).
       (c) Total amount of probe typically used is 50-100 ng per slide.
       (d) Blocking DNA, e.g., salmon sperm DNA is used at 4-10 µg per slide.

   (iv) MCILVAINE’S BUFFER (100ml-1)
       0.1M citric acid (18 ml)
       0.2M Na2HPO4.2H2O (82 ml)

   (v) DIAMINO BENZIDINE (DAB)
       5 mg DAB in 0.5 ml water, kept in freezer
       5 ml water
       5 ml 0.1M Tris HCl, pH 7.4
(vi) SILVER STAIN STOCKS
A: 0.2% (w/v) ammonium nitrate
  0.2% (w/v) silver nitrate
  1% (w/v) tungstosilicic acid
  0.5 % (v/v) formaldehyde (diluted from stock 30 % (v/v) formaldehyde in water)
Made up in deionised water
B:  5% (w/v) NaCO3 in deionised water

(vii) GIEMSA COUNTERSTAIN
4% (v/v) Giemsa (Gurr) stain in Sörenson's buffer:
  0.05M Na2HPO4
  0.05M KH2PO4

2. MATERIAL
(i) Seedlings of Arabidopsis growing on agar mineral medium (e.g., Murashige and Skoog, without sugar), 3-4 days old (radicle 3 to 7 mm long) are used.
(ii) Place seedlings into 2mM 8-hydroxyquinoline for 30 min at r.t. and then 30 min at 4oC.
(iii) Transfer material into cold (4oC) fixative (3:1 methanol:glacial acetic acid) for one h at r.t. and then store in the deep freezer (-20oC) until use.

3. CHROMOSOME PREPARATION
(i) Rinse fixed seedlings for 15 minutes in 0.01M citric acid-sodium citrate buffer, pH 4.8
(ii) Digest material in enzyme solution at 37oC for 30 min.
(iii) Remove the enzyme solution carefully with a Pasteur pipette and wash material with citric acid-sodium citrate buffer for 30 min at r.t.
(iv) Isolate root tips and transfer with micro-pipette into 45% acetic acid.
(v) Transfer a few (3-6) root tips with about 10 ul 45% acetic acid to the slide, cover with coverslip (24 x 24) and squash.
(vi) Examine preparation under a phase contrast microscope; check: cell density, metaphase index, and quality of chromosome spread.
(vii) Remove coverslip from the squash after freezing on dry ice, and air dry.

4. PRETREATMENT
* RNase treatment (DNase-free RNase). Chromosome spreads are treated with RNase which reduces non-specific hybridisation.
  (i) Add 100 ul of 100 ugm-1 (w/v) RNase A to the slide, cover with coverslip and incubate for 1 h at 37oC.
* Stock RNase A is prepared by dissolving 10 mgml-1 of DNase-free RNase in 10mM Tris.HCl, pH 7.5, 15mM NaCl. Boil for 15 min and allow to cool. Store frozen in aliquots and dilute when required. Dilute 1:100 in 2 x SSC (20 x SSC = 3M NaCl, 0.3M sodium citrate).
  (ii) Wash 3 x 5 min in 2 x SSC.
  (iii) Dehydrate 5 min in 70%, 90%, 96% and 100% ethanol.

5. DNA PROBE LABELLING
* Standard plasmid or phage DNA extraction protocol are used. Nick translation or oligolabelling of linear DNA also follows standard protocols. After nick translation or oligolabelling, 1 ug DNA probe is resuspended in 20 ul 1 x TE (100 x TE = 1M Tris.HCl, pH 8, 0.1M EDTA). We always test efficiency of labelling with a dot blot.

6. DENATURATION
* Combined denaturation of probe and chromosomal DNA.
* This method is the most satisfactory and seems to leave the chromosomes optimally denatured with minimal DNA loss.

(i) Place paper tissues at the bottom of a chamber and soak the paper with 2 x SSC. This makes the chamber humid and prevents the material from drying out.

(ii) Place the chamber into an oven or a water bath to raise its temperature to 90oC, as measured by a thermometer in the chamber (typically the heater must be set to 95oC). Leave to equilibrate to the temperature.

(iii) Add 20 ul of the hybridisation mix to each slide and cover with a small piece of plastic cut from an autoclavable plastic bag.

(iv) Quickly place slides into preheated humid chamber and incubate at 90oC for 10 min. Typically, after the slides are loaded, the temperature of the chamber drops to 75oC, but returns to 90oC within 4 min.

(v) Transfer the humid chamber with slides to a 37oC water bath or incubator. It is important to transfer quickly so that the material does not cool down too rapidly. This will allow the most similar sequences to reanneal first.

7. HYBRIDISATION
   (i) Hybridise at 37oC in a moist chamber for 12-16 h (o/n).

8. POST-HYBRIDISATION WASHES
   * After hybridisation, it is necessary to remove non-specifically bound and weakly hybridised probe to reduce background levels. We normally wash at a slightly higher stringency than the hybridisation conditions by regulating the concentration of formamide, ionic conditions and temperature.

   (i) Coverslips are removed in 2 x SSC at 37-42oC.
   (ii) A stringent wash is given. Use either:
   (a) 50% formamide in 2 x SSC for 10 min at 40-43oC, or
   (b) 20% formamide in 0.1 x SSC for 10 min at 40oC. The stringent wash removes weakly hybridised sequences with less than to 85% identity.
   (iii) Wash in 2 x SSC, pH 7.0, at 42oC for 10 min. Twice during the 10 min period tip out the old solution and replace with prewarmed fresh. Then take out of water bath and leave to cool.
   (iv) Wash in 2 x SSC, pH 7.0, at r.t. for 10 min, changing the solution twice during this time.

9. FLUORESCENT DETECTION AND AMPLIFICATION

   DETECTION
   * The digoxigenin is detected using a marker, e.g., FITC conjugated to an anti-digoxigenin antibody.

   (i) Place slides in 4 x SSC containing 0.2% Tween 20 (4 x SSC/Tween) at r.t. for 5 min.
   (ii) Bovine serum albumen (BSA) block. Drain slides and add 200 ul of 5% (w/v) BSA in 4 x SSC/Tween to each slide and cover with a plastic coverslip. Incubate for 5 min at r.t.
   (iii) Labelled anti-digoxigenin (e.g., anti-digoxigenin-FITC, raised in sheep). Drain away BSA and add 30 ul labelled anti-digoxigenin diluted 1:100 in BSA block. Incubate for 1h at 37oC.
   (iv) Wash in 4 x SSC/Tween at 37oC for 3 x 8 min.
(v) One can examine the slide here, amplify the signal or counterstain the chromosomes.

AMPLIFICATION
* The digoxigenin signal can be amplified.
  (i) Goat or rabbit serum block. Drain slides and add 200 ul of 5% (v/v) normal goat (rabbit) serum in 4 x SSC/Tween and cover with a plastic coverslip. Incubate at r.t. for 5 min.
  (ii) Labelled antibody raised against sheep (e.g., Rabbit-anti-sheep-FITC). Drain away normal goat serum block and add 30 ul labelled antibody diluted 1:40 in goat serum block. Incubate for 1 h at 37°C.
  (ii) Wash 2 x 5 min in 4 x SSC/Tween at 37°C.
  (iii) One can examine the slides here or counterstain the chromosomes.

DAPI COUNTERSTAINING
* DAPI (4'-6-diamidino-2-phenylindole) counterstains chromatin by binding to A-T nucleotide pairs.
  (i) Use about 50 ul of 2 ugml-1 (w/v) DAPI solution in McIlvaine's buffer for 10-30 min at r.t.
Stock 100 ugml-1 DAPI in water (stored in freezer) is diluted to use in McIlvaine's buffer pH 7.
  (ii) Wash off with distilled water and air dry.
  (iii) Mount in Citifluor antifade. Apply about 50 ul Citifluor AF1 antifade solution per slide. Place a thin coverslip (preferably UV transparent and of high quality; No.0, 24 x 40) over material and gently squeeze excess antifade from the slide with filter paper.

VISUALISATION
* The in situ signal should be visualised using high performance objectives. For high resolution studies the immersion oil should not have fluorescent properties (use Leitz immersion oil).

10. AVIDIN-HORSE Radish Peroxidase/Diamino Benzidine (HRPO/DAB) DETECTION
* This is an alternative, non-fluorescent detection method.
* Horseradish peroxidase is used to reduce diamino benzidine (DAB, a dangerous carcinogen) to produce a brown precipitate at the site of in situ hybridisation. It is visualised by transmitted or reflection contrast microscopy.

  (i) Place slides in 1 x PBS containing 0.2% Tween 20 (1 x PBS/Tween) at r.t. for 5 min.
  (ii) Bovine serum albumen (BSA) block. Add 200 ul of 5% BSA in 1 x PBS Tween 20 to each slide and cover with a coverslip. Incubate for 5 min at r.t.
  (iii) Avidin-horseradish peroxidase (avidin-HRPO). Drain away BSA and add 40 ul per slide avidin-HRPO, diluted 1:1000 in BSA block. Cover and incubate for 1h at 37°C.
  (iv) Wash 3 x 10 min in 1 x PBS/Tween.
  (v) Drain slides and add 200 ul DAB detection reagent without H2O2, cover and incubate in dark for 20 min at 40°C.
  (vi) Drain slides and add 200 ul DAB detection reagent with H2O2 (1 ul 30% fresh H2O2 to 2 ml DAB detection reagent). Incubate at 40°C for 20 min.
  (vii) Stop reaction with an excess amount of water.
* The slide can be counterstained (below) or the DAB precipitate amplified with silver.
SILVER AMPLIFICATION
(i) Mix an equal volume of silver stain solution A into solution B. Immediately add 500 ul to the material, cover and monitor silver deposition under the microscope.
(ii) Stop the reaction with water followed by 1% (v/v) acetic acid in water for 2 min.

COUNTERSTAINING
(i) Incubate slides in a Coplin jar containing Giemsa counterstain solution for 10 min at r.t.
(ii) Wash material with distilled water and air dry.
(iii) Mount in standard microscope mountant e.g., DPX or Euparal

11. SUPPLIERS OF NON-STANDARD REAGENTS

Anti-digoxigenin-FITC:
Boehringer Mannheim GmbH, Biochemica, P.O. Box 310 120, D-6800 Mannheim 31, Germany.
Avidin-horseradish peroxidase, Normal Goat Serum:
Vector Laboratories Ltd., 16 Wulfric Square, Bretton, Peterborough PE3 8RF, U.K.
Rabbit-antisheep-FITC:
Dakopatts, 42, Produktionsvej, Postbox 1359, 2600 Glostrup, Denmark.
Citifluor AF1:
Citifluor Ltd., Connaught Building, City University, Northampton Square, London EC1V 0HB.

* All other reagents from Sigma, BRL or companies listed above.

12. REFERENCES