CHLOROPLAST MORPHOLOGY ASSAY PROTOCOL

REAGENTS

1. 3.5% glutaraldehyde (from Sigma 50% photographic grade; dilution stable 1 week 4°C)

2. 0.1 M EDTA, pH 9.0

PROCEDURE – TISSUE FIXATION

If not fixed immediately, good preparations can be obtained from fresh leaf samples kept in closed, chilled tubes (on ice or in 4° chilling rack) for up to one hour after harvest.

1. Scan in the pot barcode.
2. Scan in the barcode of a green 2-ml vial. Harvest an expanded leaf and its petiole, holding the leaf at the side margin. Cut off the petiole at the leaf base and place it in the green vial. Close vial and put in chilling rack.
3. Scan in the barcode of a blue 2-ml vial. Cut a small triangular piece of the mature expanded leaf from Step #2 from its tip and place it in the blue vial. Close vial and put in a chilling rack.
4. Repeat steps 2-3 for all plants (young leaf was also harvested prior to 10/4/2006). Keep samples chilled.
5. *All work with gluteraldehyde must be done in a fume hood*. Transfer the leaf sample tubes to a microfuge tube rack. Add 900 ul 3.5% glutaraldehyde to each sample. Using a nutating mixer, incubate samples at RT 2 hours.
6. Remove all glutaraldehyde from each tube, being careful not to aspirate the leaf sample.
7. Add 1 ml 0.1M EDTA to each tube. Be sure leaf tissue is completely immersed.
8. Incubate at 55°C. for 2 hours.
9. Remove the tubes from the oven and cool at room temperature for 10 minutes. Store fixed samples at 4°C for up to 3 days.

SLIDE PREPARATION AND SAMPLE SCORING

Samples are analyzed using a Leica DMI 3000B microscope with a 40x/0.75 HCX PL FLUOTAR lens; image are captured using a Leica DFC 320 camera.

1. Add a small drop of dH_2O to a slide. Remove a fixed leaf or petiole sample from the tube and place it in the water drop. Cover the sample with a coverslip.
2. Tap the top of the coverslip above the specimen with the eraser-end of a clean, unused pencil to crush the specimen to a fine green haze. Blot excess water.
3. Invert the sample and view at 40x power. Scan the slide specimen and examine the chloroplasts within the cells, locating several to photograph. Focus the image to be photographed using the computer monitor. Photograph two images per sample with one or two representative cells in the fields. If abnormal chloroplasts are seen, capture additional images.

4. Scan the sample tube into database. Score the chloroplasts in each sample for:
    a. Number
    b. Size
    c. Shape
    d. Shape characteristics

   Please see file "chloroplast morphology controlled vocabulary examples." Add any unusual findings or other remarks for clarification purposes to the Comments section.
5. Repeat steps #1-4 for each additional sample.

NORMAL (WT) CHLOROPLAST MORPHOLOGY

Expanded leaf: 80-100 chloroplasts per cell. Chloroplasts are small, round and grass green.

Petiole: cells are very large and rectangular. One petiole cell may extend the entire length of the microscope viewing field at 40x magnification. Chloroplasts are numerous (80-100) as in the expanded leaf, but are in several distinct planes. Focus the sample through several additional planes in order to best quantify the number of chloroplasts.

ABNORMAL CHLOROPLAST MORPHOLOGY

See file "chloroplast morphology controlled vocabulary examples." Cells may appear to be normal, but the chloroplasts can be very large, irregularly shaped, or more/fewer in number. Membranes may not be clearly defined and cells may appear to have one or two giant pale green chloroplasts within them or numerous amorphous and/or heterogeneous-shaped chloroplasts.

LEICA MICROSCOPE – SETTINGS AND SET-UP

The microscope is positioned on three anti-vibration pads. Do NOT move the microscope from its position on the pads or attempt to slide the entire assembly to another place on the counter.

Tilting the microscope: the pillar holding the camera housing may be tilted if needed to add or remove slides to the stage. Always return it to the vertical position. There is a knob to lock the position at the back of the microscope.

Field diaphragm: used to set the condenser. Using the 10x objective, focus the light on the same plane. Close down the condenser to center the light in the field of view. Use the silver knob at back to keep the edges sharp and the "circle of light" centered on screen, then open the condenser all the way so that the field of view fills the monitor screen.

DLF - daylight filter (on microscope pillar): Leave in at all times – this should be in the "up" position.

Condenser: polarizing lens in, analyzer tab pushed in, prism on both sides set to number 7.

Light stop lever: use when changing slide samples so that light does not blind you as you change slides.

D1 = objective lens: 1, 2, 3, and 4 = 10x, 20x, 40x, and 63x (make sure the #3 prism is set when using the 40x objective) to view chloroplasts

For light to pass to the camera vs. eyes: keep the plunger all the way out so that the light goes to camera; specimens are still easily viewed by eye through the ocular lenses.

Aperture diaphragm: As the aperture diaphragm is closed down, resolution is lost. Leave it at "PH" = wide open.

Use ethanol on lens paper to clean ocular and objective lenses. *DO NOT EVER USE KIMWIPES OR OTHER TISSUE TO CLEAN THE LENSES – THEY WILL SCRATCH THE GLASS!!!*

LEICA APPLICATION SOFTWARE PROGRAM:

<u>SET UP</u>

Choose the Acquire tab. Select MIC1: make sure the magnification setting matches the microscope objective lens: 40x. Keep the Mag changer at 1x.

Camera tab: white balance on DIC. At the beginning of each session, the white balance must be calibrated. Find an area of a sample slide having background only (no sample, water droplets, etc.). Left click and drag the mouse to create a box in that area. A drop-down box will appear. Choose white balance; it will be adjusted automatically.

Image section: if "Iris" is highlighted, leave on autofocus
Saturation: 1.45
Gamma: 0.54
Brightness: 60%

Input options: 8 bit as JPEG. Images are saved as JPEG files.

Captured frame: full frame
Captured format: full frame

Calibration Tab: DON'T CHANGE ANYTHING!

Calibration settings: bar can move for um (microns). Make sure objective and screen objectives match (40x) so that the micron determination is accurate.

Processing Tab: Shading - none; Sharpening – low

CAPTURING AND SAVING IMAGES

1. Create a folder on the computer desktop for that session's images.
2. Under Options>>Preferences (or press Control P), highlight the field to name the saved image and scan in the barcode of the sample tube. Make sure the image is saved onto the computer's C drive and on the newly created desktop folder. The computer will automatically add a suffix number of 1, 2, 3, etc., to the filename of each subsequent image from the same sample so that each sample tube need be scanned in only once.
3. Choose the Acquire Tab. Center the cell or cells of interest in the monitor's field of view and focus the image, using the fine focus on the microscope.
4. Press the Acquire Image. This will capture the image and save it to the folder on the desktop as well as storing a thumbnail image at the bottom of the screen.
5. Select the Process tab and check the appropriate boxes to add the date, time, image name and microns labels to the captured image. Select Merge to fuse these items onto the image.
6. Make sure the user length is selected as 20 um for the bar label. Label text should be white, with a gray background for the text. Make sure "Autosize" is checked.
7. Repeat steps #3-5 for each image of that sample.

Burn 3 CDs for each. Roxio: Select all of the images for the chlorophyll folder. Check to make sure the images are satisfactory before deleting from the desktop.

Reference:

Miyagishima, S.Y., Froehlich, J.E., and Osteryoung, K.W. 2006. PDV1 and PDV2 mediate recruitment of the dynamin-related protein ARC5 to the plastid division site. Plant Cell 18 (10): 2517-2530.