Chapter 6

Marigold Cell Size and Polyploidy

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Reprinted From: Hunter, K. L. and R. B. Hunter. 2004. Marigold cell size and polyploidy. Pages 125-133, *in* Tested studies for laboratory teaching, Volume 25 (M. A. O'Donnell, Editor). Proceedings of the 25th Workshop/Conference of the Association for Biology Laboratory Education (ABLE), 414 pages.

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Introduction

Most animals are diploid, having one set of chromosomes from the male and one from the female. Polyploid animals, with the exception of some frogs and fish, are usually aborted or die immediately after birth (Gardner et al., 1991). In contrast, estimates are that about 70% of flowering plants and 90% of ferns contain three or more sets of chromosomes (Masterson, 1994; Pichersky et al., 1990). Chromosomes pair at meiosis, therefore most organisms have even sets of chromosomes, such as tetraploids (4 sets), and hexaploids (6 sets). Those with odd numbers have reduced fertility (triploids for example) and often reproduce vegetatively.

Many crop plants are polyploid, including coffee, cotton, potatoes, strawberries, sugar cane, tobacco, wheat and corn. Polyploidy in plants has been investigated since the 1930s to try to understand and perhaps make use of its effects (Stebbins, 1947). The grain crop triticale, for example, is a human-generated hybrid polyploid of wheat (Triticum aestivum) and rye (Secale cereale) formed by scientists containing the complete genomes of both grasses. Plant breeders induce polyploidy to attempt to increase yield, improve qualities like fruit size or vigor, and to adapt crops to particular growing conditions (Dewey, 1980; Zeven, 1980). The seedless watermelon and larger tetraploid grapes are examples. In some instances polyploidy has increased flower, seed or fruit size, increased photosynthetic or respiration rates, or increased tolerance of extreme temperatures, drought or flooding (Tal, 1980). However, there are few consistent effects, the primary one being an increase in cell size (Masterson, 1994; Bennett and Leitch, 1997).

We have developed a lab (Hunter et al., 2002) based on polyploidy and cell size, to introduce middle school, high school, and college students to several important subjects in biology, including genetics (chromosomes, meiosis and mitosis, polyploidy), plant anatomy (stomata, air and water exchange, leaf structure) and cell biology (genome size and cell size). It also allows the use of simple math in data analysis and utilizes quantitative measurements rather than simple observations. The lab involves growing marigolds for about one month from seed, and measuring guard cell (surrounding the stomata) sizes and densities. A modified version of the lab was presented at the 2003 ABLE meeting in Las Vegas.

Materials

- Seeds: diploid – one packet per 20 students of Deep Orange Lady Hybrid marigold – *Tagetes erecta*
  triploid – one packet Nugget Supreme Yellow Marigold (hybrid between *Tagetes erecta* and *Tagetes patula*)
  tetraploid – one packet Jaguar Marigold – *Tagetes patula*
- Potting Soil
- Small paper or plastic cups (three per pair of students)
- Transparent plastic rulers with millimeter marking.
- Microscopes (one for each student or small group) and microscope slides
- Transparent tape
- Clear nail polish, regular or quick-dry
- Fine point permanent markers
- 1.5-ml microcentrifuge tubes (for organizing and storing peels)
- Recommended: For measuring individual guard cells (technique 1), an ocular micrometer for each microscope and at least one calibration slide to calibrate microscopes (available from Carolina Biological Supply Co.)
- Optional: Forceps for handling peels

Notes for the Instructor

For higher-level students we strongly recommend obtaining the ocular micrometers and having the students calibrate their microscopes with the calibration slide. This allows measuring actual guard cell sizes (technique one), as opposed to density (a correlate of cell size).

The data can be profitably analyzed using a standard spreadsheet (e.g. EXCEL, Quattro Pro), with students setting up the columns to calculate stomatal area, averages and some statistical parameters. A sample is shown in Appendix A.

For documentation, students at the ABLE meeting successfully used a standard digital camera placed next to one ocular to photograph the peels, showing the cells and ocular micrometer scale bar.

Growing the plants can be separated from the stomatal observations. They might be grown by the instructor or a technician and supplied to the students just for the guard cell measurements. The techniques also work for measuring random plants found outside. In that case the ploidy of the species will be unknown (some are published in local floras), but one could investigate stomatal sizes in different species, stomatal densities on different surfaces of the leaf, etc.

In marigold and other amphistomatous plants there are stomata on both top and bottom surfaces of leaves. In some species stomata are restricted to the bottom or top surface. We routinely use the upper surface on marigolds.

College level classes might combine this with reading a scientific journal article or writing a report based on the class's results. If measuring stomatal density (technique 1) there is some literature relating stomatal density to climate change and changes in atmospheric CO2 concentrations (e.g., Beerling and Chaloner, 1993; Kürschner et al., 1998; McElwain and Chaloner, 1995; Van de Water et al., 1994).

Student Outline

Growing plants (20 minutes initial setup, @ 1 month for plants to grow.)

Students should work in pairs or groups. Each pair should fill three small cups with potting soil. Bury 3-4 diploid seeds in one cup, 1/8 inch deep. Label the cup with the seed type and its ploidy level (e.g. diploid 2X Orange Lady). Repeat for the triploid and tetraploid varieties, each in its own cup. Thoroughly water the seeds and place the cups in a moderately warm, sunny spot. If the classroom has no windows they may be grown under artificial lights or at the students' homes. Seeds should germinate within two or three days. After germination collect data on growth, such as height and number of leaves, as determined by your instructor.

Questions that might be asked before doing the guard cell measurements might be to determine if one ploidy type grows faster than another, whether one has bigger leaves, and whether the seed sizes vary among the different types.

Painting leaves with fingernail polish (15-20 minutes)

You will use clear nail polish to view the surface cell structures. As the nail polish dries it conforms to the shape of the surface of the leaf, and when peeled off it contains an imprint of each cell. The advantage of looking at the peel rather than the actual leaf surface under the microscope is that you do not have to repeatedly focus up and down through the cell layers and decide where the cell boundaries are.

Each pair should:

1. Collect one leaf lobe from a leaf of the diploid plant.
2. Place the leaflet in a microcentrifuge tube labeled with the partner's initials and ploidy level.
3. Repeat steps 1 and 2 for the triploid and tetraploid plants.
4. Working on a paper towel, paint the top of each leaflet with clear nail polish.
5. Let the polish dry to the touch – about 15 minutes. (If not properly set it will stretch while removing it, distorting the cell impressions.)
6. Firmly apply a piece of tape to one end of the nail polish and carefully pull the polish off the leaf.
7. Place each peel and tape on a separate microscope slide, and carefully label each slide. Place a cover slip over the peel. Discard the leaf.
8. View each slide under a microscope – the surface should look like Figure 1. In marigold the guard cell pairs form an ellipse surrounding the stomatal opening. Swelling and shrinking of the two guard cells controls the size of opening.

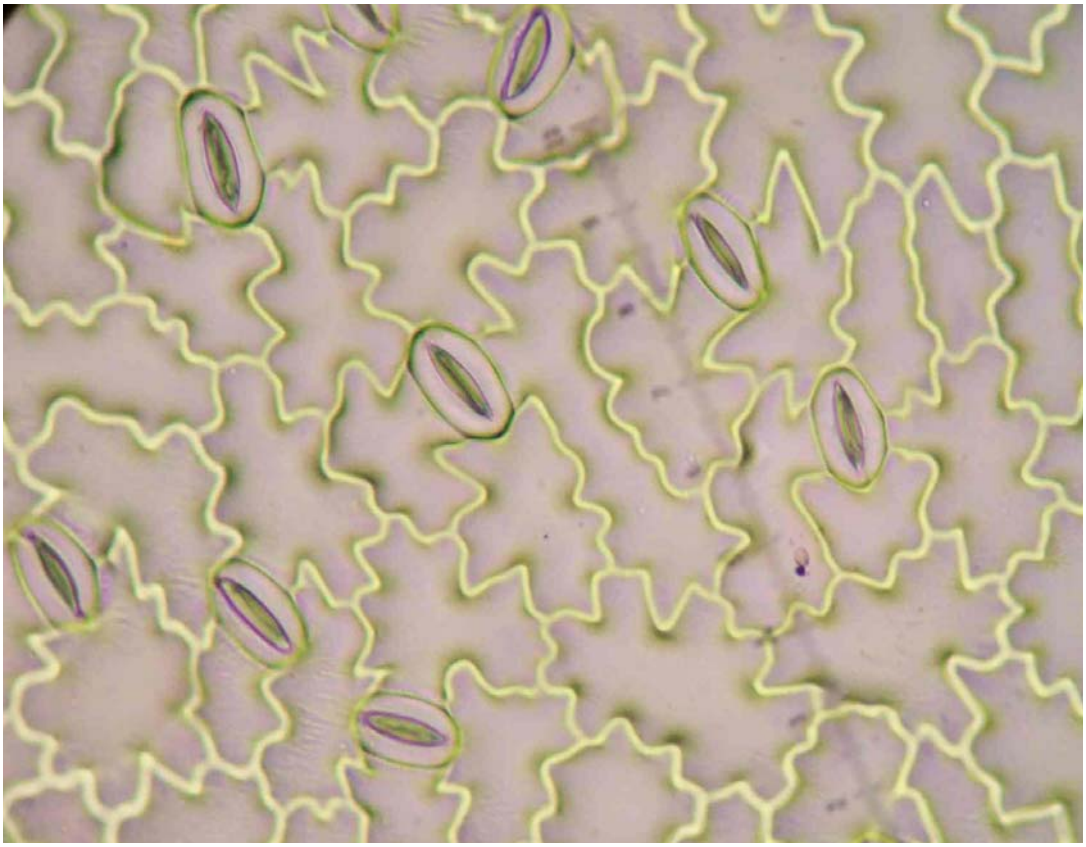

Figure 1. Surface impression of a triploid marigold leaf made in fingernail polish at 400X magnification. Wavy lines are the boundaries of epidermal cells, interspersed with oval pairs of guard cells surrounding the dark stomatal openings. The ocular micrometer is visible in the image, with each guard cell pair approximately 10 units long. This photo was taken with a handheld digital camera held up to the microscope's ocular.

Technique 1 – Measuring guard cell size (30-40 minutes)
Direct measurement of the size of a guard cell or pair of cells is done using an ocular micrometer, essentially a ruler that fits into the eyepiece of the microscope. In order to calibrate the ocular micrometer a slide etched with actual millimeter markings is placed on the stage and measured with the ocular micrometer.

1. Place the diploid peel and slide on the microscope.
2. Looking at the peel with high power (400X, not oil immersion), you will see the cell outlines, including the guard cell pairs, and also the markings from the ocular micrometer. Randomly select a guard cell ellipse and measure its length (L) and width (W) in units of the ocular micrometer. You can rotate the eyepiece (ocular) to reposition the micrometer, or move the slide and/or stage to position the guard cell pair appropriately within the markings.
3. Measure length and width for at least ten pairs of guard cells.

4. Switch slides and measure at least ten pairs of the triploid and tetraploid guard cells.
5. Use the formula for area of an ellipse [area = $\pi(L/2)(W/2)$] to calculate the area of each guard cell pair.
6. Average the areas for each ploidy level. The units are unknown until the ocular micrometer is calibrated with a calibration slide, available from most biological supply companies.

Calibration of the Ocular Micrometer

1. Place the calibration slide on the microscope stage. You will see two rulers, a black one is the ocular micrometer, and the white etched glass one is the calibration slide. The calibration slide units will be magnified ~400X. Line the two rulers up, one next to or on top of the other.
2. Count the number of little (ocular micrometer) lines between two big ones (calibration slide). The lines on the calibration slide are 0.1 mm = 100 µm apart. You might get something like 40 ocular micrometer units in 100 µm. Each ocular micrometer unit is then 100µm divided by the number of lines counted (in our example, 100 µm ÷ 40 = 2.5 µm each).
3. Convert all areas of the guard cell pairs to square micrometers by multiplying the correction factor squared [e.g. $(2.5\ \mu m)^2 = 6.25\ \mu m^2$].

Technique 2 – measuring stomatal density (30-40 minutes)
Students should work in pairs. They will count the pairs of guard cells in each of three fields of view, and then measure the size of a field of view. Stomatal density is the number of guard cell pairs divided by the area (the stomate is the opening, each having two guard cells). Larger cells cause the stomata to be farther apart; hence density is proportional to cell size.

1. Look into the objective lens of the microscope at about 400X power (10X ocular, 40X objective lens). You will see a circle with cell outlines, some of which will be the elliptical pairs of guard cells. One such circle and everything in it is a "field of view". Count the numbers of guard cell pairs that you see in the circle and record that number. The cells with borders like jigsaw puzzle pieces are epidermal cells.
2. Move the slide to observe different fields of view.
3. Count and record the numbers of guard cells in at least two more fields of view.
4. Change slides, and count the guard cells in at least three fields of view for the other two ploidy levels.

To measure the area of the field of view:

1. Place a clear plastic ruler with millimeter markings on the microscope stage and focus using low power (usually 100X – 10X ocular, 10X objective) and focus on the markings.
2. Count the number of millimeters across the center of the field of view. Estimate fractional parts – e.g., 1.7 mm.
3. Convert this number to micrometers (multiply by 1000 [1 mm = 1000 µm]).

4. Divide the resulting number in micrometers by the ratio of the high power objective magnification (40X) to the low power magnification (10X) to calculate the diameter of the field of view.

$$High\ power\ field of view = \left[\frac{Low\ power\ field\ of\ view}{high\ power\ objective / low power\ objective} \right]$$

5. Plug the radius (diameter divided by 2) into the formula for area of a circle (Area = πr^2).
6. The number of guard cell pairs in a field of view, divided by the area of the field of view, equals the stomatal density (number per square micrometer). Calculate the average for each ploidy level.
7. Repeat for each field of view and for each ploidy level. When they're open, the density of stomata controls the rate of diffusion of water from the leaf and CO_2 into the leaf during photosynthesis.

You should see clear differences among the ploidy levels in the area and/or density of the guard cell pairs. Knowing the ploidy levels, which one has the most DNA per cell? Does cell size correlate with amount of DNA? Does cell density correlate with cell area?

Note that these are observations, not mechanistic explanations, of fundamental marigold cell properties. Questions as to why cell size is larger with more copies of a species' genome have not been addressed. With your knowledge of basic cell structure you might make some hypotheses relating to this question, and try to come up with ways to test those hypotheses.

Acknowledgements

Initial work on this lab was greatly aided by Rebecca S. Leone, Kimberly Kohlhepp, and Starlin Weaver of the Salisbury University Department of Education. Modifications for the ABLE workshop incorporated in this version, and help during the workshop, were provided by Amanda Gordon, Allison Burnett, and Kelsey Glennon. Anonymous participants at the ABLE workshop suggested the transparent tape technique of removing the peels and photographing the cells through the ocular.

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Appendix

Sample Results

In our study the mean areas of a guard cell pair (technique 1) (± standard error of the mean) were: Orange Lady (2X)=472 ± 15.3 μm^2, Yellow Nugget (3X)=570 ± 14.5 μm^2, and Jaguar (4X)=742 ± 13.4 μm^2. The mean densities of guard cell pairs per field of view (technique 2) at 400 power were: Orange Lady (2X)=27.9, Yellow Nugget (3X)=18.5, and Jaguar (4X)=13.4. There was a clear relationship between ploidy and cell size.

The above data can be used to estimate cell volume for one guard cell. Our estimates based on the above results are: diploid=2718 μm^3 (1369 μm^3 per chromosome set), triploid=3619 μm^3 (1206), and tetraploid=5375 μm^3 (1344).

Sample Spreadsheet Setup. Sample format for a spreadsheet (Microsoft EXCEL in this case) to calculate marigold data. (Note the 2.54μm/ ocular micrometer unit and 0.17mm^2 per field of view are for a particular microscope at ~400X.)

A	B	C	D	E	F	G	H	I	J	K
Sample	Cultivar	L	W	L μm	W μm	Ellipse area μm^2	N/field	sto dens	Avg/FOV	Avg dens
JA1	Jaguar	12	6	=C2*2.54	=D2*2.54	=PI()*(E2/2)*(F2/2)	13	=H2/0.17	=AVERAGE(H2:H11)	=AVERAGE(I2:I11)

Seed Sources

Originally all three cultivars were obtained from W. Atlee Burpee & Co., 300 Park Avenue, Warminster PA 18991-0001, USA. In spring 2003 the triploid hybrid (Golden Nugget Supreme Yellow – *Tagetes erecta X T. patula*) was temporarily unavailable from Burpee and was found by searching with the scientific name and ordered from Thompson and Morgan (UK) Ltd., Poplar Lane, Ipswich, England IP3 3BU. The common name for the Thompson and Morgan seeds was Trinity Mixed Marigold.