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Rapid Image Analysis Screening Procedure for Identifying Chloroplast Number Mutants in Mesophyll Cells of *Arabidopsis thaliana* (L.) Heynh.[1]

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ABSTRACT

To analyze the genetic control of the process of chloroplast division, a direct image analysis screening procedure has been developed in which mutants of *Arabidopsis thaliana* (L.) Heynh. var Landsberg *erecta* are selected on the basis of abnormal chloroplast number. The selection procedure is based on image analysis thresholding after iodine staining, which facilitates the automatic counting of chloroplasts in isolated mesophyll cells. M2 seedlings are screened for significant deviation from the wild type relationship between mesophyll cell size and chloroplast number. Mutants with both abnormally high and abnormally low chloroplast numbers were identified. Of 3500 individual M2 seedlings screened, 18 mutant lines have been isolated and shown to be stably inherited in three subsequent generations. The most extreme phenotypes show an 80% reduction or a 50% increase in chloroplast number per mesophyll cell.

Chloroplast replication is a ubiquitous characteristic of leaf mesophyll cell development (4, 6). Up to 90% of the chloroplasts in a mature leaf mesophyll cell are products of the division of young green chloroplasts in expanding leaf cells. It has not been possible to identify the essential control mechanisms involved in chloroplast division, and plants in which the process of chloroplast division is modified would be of great potential value. Ideal material for such investigations would be a collection of chloroplast mutants that could be used to investigate the underlying genetic control of the chloroplast division process by comparison with wild-type plants. Because *Arabidopsis thaliana* is currently the only plant species in which a mutant phenotype is all that is required for the isolation of the mutated gene sequence (5), this species offers a unique opportunity to examine the genetic control of chloroplast division in leaf cells. However, identification of suitable chloroplast division mutants in *Arabidopsis* requires a rapid screening procedure capable of identifying subtle changes in cell phenotype. Recent advances in computer-assisted image analysis technology, when linked to light microscopy, have the potential to provide such a suitable procedure. The screening procedure that we describe here has enabled us to identify 18 mutant lines of *Arabidopsis* in which a phenotypic change in chloroplast number is inherited.

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MATERIALS AND METHODS

Plant Material

M2 populations of ethyl methane sulfonate-mutagenized seeds of *Arabidopsis thaliana* (L.) Heynh var Landsberg *erecta* (Lehle Seeds, Tucson, AZ) were sown on trays of moist compost (Levington F2) and covered with plastic lids until they germinated (3–4 d). The trays were placed in a controlled environment cabinet at 20°C with a 5°C night depression, 70% relative humidity and a light intensity of 60 W/m^2 (Philips TLD 58W/35 tubes). The seeds were always sown at 10 AM and the first leaves harvested at 10 AM between 9 and 22 d later for the wild-type and at 20 d for the M2 mutants. So that individual mutant seedlings could be readily identified, the M2 seeds were sown in a numbered grid of 96 compartments produced by placing a grid of 1-inch square wire mesh on the surface of the compost. M2 seeds were sown in each of the 96 squares in each tray with the aid of a blue 1-mL disposable pipette tip (Anachem). Seeds were placed inside the tip; by holding the tip in a horizontal position and tapping gently with a finger, a few seeds at a time were dispensed. Up to five seeds were sown in each square and were thinned to one seedling on germination. Each M2 seedling was identified by a seed tray number and a coordinate number within the tray (*e.g.* YK81 [Y = York]).

Preparation of Mesophyll Cell Suspensions for Chloroplast Counting

Chloroplasts were counted using Nomarski differential interference contrast optics in individual fixed mesophyll cells obtained by the maceration of prepared leaf tissue on a microscope slide (1). The maceration of the leaf tissue to yield intact mesophyll cells was made possible by the pretreatment of the leaf tissue as follows. Entire first leaves of wild type *Arabidopsis* seedlings and M2 seedlings were fixed in 3.5% (v/v) glutaraldehyde for 1 h in the dark. The fixative was removed and replaced by 0.1 M Na_2EDTA (pH 9). Softening of *Arabidopsis* leaf tissue was optimal after the EDTA-treated tissue had been incubated in a shaking water bath at 60°C for 2.5 h. Samples were stored in a refrigerator at 4°C prior to chloroplast counting.

The handling of large numbers of samples was greatly facilitated by the use of 96-well microtiter (ELISA) plates in which the samples were fixed, pretreated and stored. An individual leaf sample could be readily identified at any time

by cross-referencing the position of the leaf sample in the 96 well plates to the seedling position in one of the 96 compartments of the seed tray grid.

RESULTS AND DISCUSSION

Rationale and Screening Procedure

In many species, there is an extremely tight correlation ($r^2 > 0.8$) between the number of chloroplasts per mesophyll cell and mesophyll cell size measured as plan area (3, 2, 7). An analogous relationship exists in cells of wild-type *A. thaliana* leaves (Fig. 1). In the wild-type *Arabidopsis* leaf, the number of chloroplasts in each cell is strongly correlated with the mesophyll cell plan area ($r^2 = 0.865$) throughout the period of mesophyll cell expansion (Fig. 1). Using this relationship, we have readily identified chloroplast division mutants by their abnormal chloroplast number to cell size ratio (Fig. 2). It was necessary to show that any change in the relationship between chloroplast number and cell size in an individual mutant held over the whole range of cell sizes within the leaf. This is particularly important during leaf expansion because the rate of development in mutant seedlings frequently differs markedly from the wild type.

Screening of large numbers of *Arabidopsis* plants for chloroplast division mutants by counting chloroplast numbers in mesophyll cells was immensely improved by automation using image analysis. We used an image analysis system provided by Seescan Imaging Ltd (Cambridge, UK) that allows the capture of images from a Nikon Optiphot microscope using a charged coupled device video camera and analysis by a variety of software routines. The screening procedure utilized software routines for the measurements of cell area and the counting of thresholded objects within a preset frame. Because Nomarski images of *Arabidopsis* chloroplasts in mesophyll cells lack sufficient contrast to be thresholded, additional contrast was introduced by iodine staining of the starch by macerating the tissues in a solution of 6% (w/v) KI and 4% (w/v) iodine diluted fourfold before use. (Only 2% of mutant plants had little or no starch and these were screened visually.) In all the mutant lines containing starch, all the chloroplasts within each mesophyll cell contained starch. Using this method, the number of thresholded objects (chloroplasts) on the top surface of each mesophyll cell was automatically recorded and the plan area of the cell was also determined after drawing around the cell perimeter with the mouse.

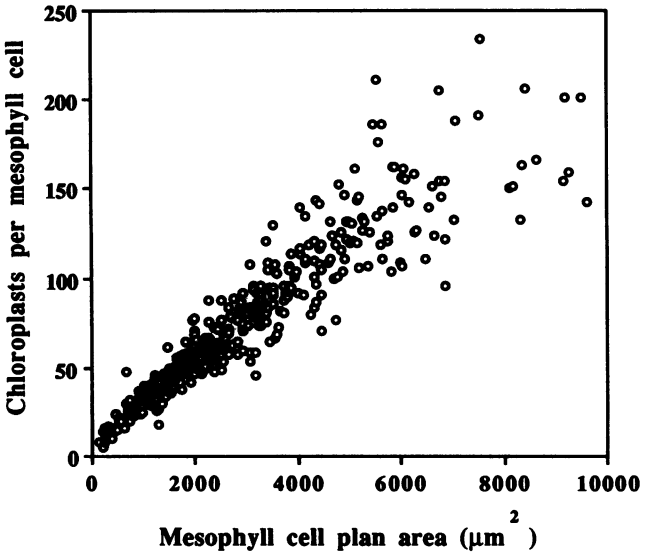

Figure 1. The relationship between chloroplast number per cell and mesophyll cell plan area (μm^2) from leaves of wild-type *A. thaliana* var Landsberg *erecta*. The numbers of chloroplasts were counted in 30 individual mesophyll cells from three whole leaves harvested between 9 and 22 d after sowing. During this period, the mesophyll cells of the first leaf of *Arabidopsis* undergo full expansion from postmitotic cells to fully expanded cells (8). ($r^2 = 0.865$).

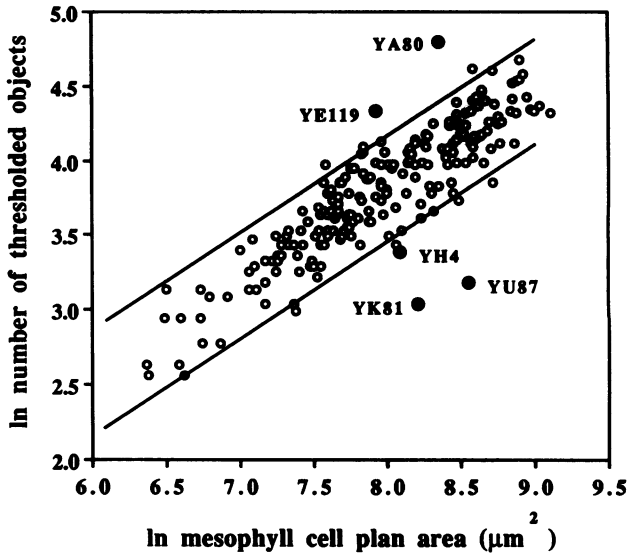

Figure 2. The relationship between ln number of thresholded objects and ln mesophyll cell plan area (μm^2) from leaves of wild-type *A. thaliana* var Landsberg *erecta*. The 95% confidence limits of the relationship are shown ($r^2 = 0.834$). Mean values for five mesophyll cells of five mutant lines selected from an M2 population are shown, all of which are significantly different from the wild-type value.

Screening of Mutants

The image analysis procedure described above was used to establish the relationship between mesophyll cell plan area and the number of thresholded objects in individual wild-type mesophyll cells. Figure 2 shows a plot of ln number of thresholded objects against ln mesophyll cell plan area and shows there is a significant linear relationship between these two parameters in wild type mesophyll cells. Natural log transformation was used to equalize the variation in y at differing values of x so that the 95% confidence limits for the regression could be plotted; the limits are shown as the two parallel black lines in Figure 2.

The M2 seedlings were then screened in a similar manner to the wild-type plants and every M2 seedling for which the relationship of mean value of ln thresholded objects to ln mesophyll cell plan area fell outside the 95% confidence limits

was retained. Examples of mean values for five mutants which fell outside these confidence limits are plotted in Figure 2.

To confirm that these mutant lines had significantly different chloroplast numbers for a given cell size, the chloroplast complement was counted visually under Nomarski interference optics and these mean values for mesophyll cell plan area and chloroplast number as directly determined were compared to the values for wild-type cells previously determined by counting and shown in Figure 1. In addition, the values in Figure 1 were also ln transformed and compared with similarly transformed direct visual measurements from the mutants, thus providing a further check that the mutants were significantly different from the wild-type *Arabidopsis*.

Using this rapid screening procedure, two workers have been able to screen 3500 individual M2 seedlings in about 4 months, *i.e.* on average it was possible to screen about 50 seedlings per day. Thirty-nine M2 seedlings were retained for further analysis. In the self-pollinated progeny (M3), the chloroplast complements were counted visually and 18 mutant lines were retained. (Any lines showing premature plant death, poor seed set, or reversion to the wild-type cell phenotype were discarded.) The M3 progeny were also selfed and M4 seedlings in which the altered phenotype had been inherited through three sexual generations were also analyzed.

The relationship between chloroplast number and mesophyll cell plan area for three M4 mutants is compared in Figure 3 to the wild-type relationship derived from the values in Figure 1. It can be seen that YA80 has a significantly

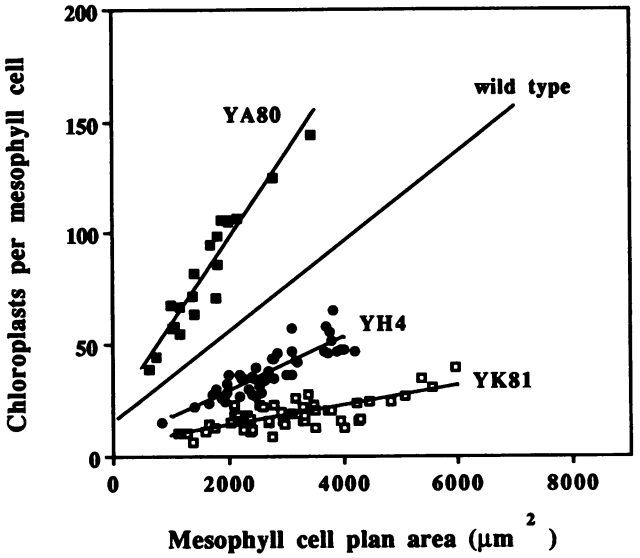

Figure 3. The relationship between chloroplast number per mesophyll cell and mesophyll cell plan area (μm^2) for three M4 lines originally selected in the M2 generation (see Fig. 2). The regression line for the wild-type relationship is derived from the values in Figure 1. For clarity, the data points for the wild-type relationship have been omitted.

greater number of chloroplasts per mesophyll cell for all cell plan areas than does the wild type. Interestingly, the chloroplasts in mutant YA80 were also significantly smaller than the wild type. By contrast, the mutants YH4 and YK81 have fewer chloroplasts per mesophyll cell over a wide range of cell size than the wild type: the chloroplasts in both of these latter mutants are larger in size than wild-type chloroplasts. The most extreme phenotypes showed an 80% reduction or a 50% increase in chloroplast number per mesophyll cell. The chloroplast division mutants we have selected show no consistent whole plant phenotype that would have allowed their selection without more detailed analysis.

The array of *Arabidopsis* mutants that we have isolated will provide excellent material for the further analysis of the genetic control of chloroplast division. Using genetic mapping methods and chromosome walking, it should be possible to identify the nature of the underlying genetic lesions giving rise to these mutant phenotypes.

As far as we know, this is the first identification of *Arabidopsis* leaf cell mutants by direct observation. The isolation of the mutants has been greatly facilitated by image analysis procedures that can be readily adapted to detect other subtle cell phenotypes. The method is rapid and provides a numerical output that can be subjected to statistical analysis. Large numbers of plants can be screened for subtle changes in cell phenotype with great rapidity and accuracy and several different structural features can be screened for simultaneously.

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