The first installment of this series (1) discussed a novel phenomenon wherein chloroplast efficiency was shown to plot in discrete groups. This exploration resulted in the development of a spectrofluorometric method that apparently allows for in vivo observation of division of chloroplast populations in leaves of Arabidopsis thaliana mutants.

This second installment deals with further testing of the phenomenon in sugarcane and especially Amaryllis; data from wheat, Narcissus, and other plants are mentioned but not described. Also described are initial confocal microscopy studies, apparently confirming that the phenomenon is a result of a decrease in organelle efficiency during chloroplast division.

Chloroplast division is critical in plant development. Thus, such a technology holds promise in applied, as well as theoretical, areas of plant science.

As described in reference 1, chloroplasts are central to life on Earth, and the leaves of higher plants are packed with them. However, the seeds from which these plants grow are small compared with the mature plant, and seeds commonly have few or no functional chloroplasts. Thus, given their separate DNA, chloroplasts in plants must arise through repeated chloroplast division.

Robertson et al. state “it is clear that while chloroplast accumulation in developing leaves enormously affects photosynthetic production, the control of the chloroplast division process itself is one of the least understood areas of chloroplast biology” (2). This situation calls for improved technology to facilitate not only the more rapid unveiling of basic understanding, but also to enable and make practical investigations as to how chloroplast division relates to the myriad species, germplasm, and conditions of crop production.

In addition, a recent technology has arisen in which medically useful proteins are found to be more readily biosynthesized in chloroplasts than in the nuclear-directed part of the plant cell (3). For all these scientific and practical ends, instruments for rapid determination of chloroplast division in vivo are required.

In our laboratory, through a fortuitous exploration of imaging photosynthetic fluorimetry of Arabidopsis mutants, a novel technique arose. When this technique was valuated by biophysical considerations, statistical theory, and preliminary experimental evidence, it appeared to very rapidly measure chloroplast division in vivo (1).

Such an observation needed to be tested further. One simple way to test the method was to acquire a charge-coupled device having more pixels and, thus, more resolution. In this way, we could be sure by observing single chloroplasts in each pixel that we were...
Spectrofluorometry

watching these organelles divide. However, funding for this project was not forthcoming.

The specialty of our laboratory is the biophysics and biochemistry of plant germplasm — that is, the genetically or physiologically determined differences between plant species, cultivars, varieties, and so forth. Therefore, we used a germplasm approach to the problem. We did this by performing a considerable number of experiments with monocot species because these often have chloroplast division localized in one part of the leaf. Thus, here we describe further studies with our method, including a germplasm approach using characteristics of diverse plants and in vivo spectroscopy.

The rationale for this germplasm approach using monocot plants is that leaves of dicotyledonary plants such as those found on Arabidopsis plants, the species with which we had been working so far (1), develop in complex patterns of cell division. In contrast, plants often have an ordered sequential longitudinal development of leaves (4).

In monocots such as grasses, the leaves arise from nodal meristems and the leaf tissue arises from the nodes in subsequent and essentially parallel cell divisions from the base toward what becomes the tip of the leaf. Thus, in these plants, the leaf tissue at the tip of the leaf is the oldest, and that closest to the meristem is the youngest.

In monocot leaves, the youngest tissue is very pale green because the chloroplasts are dividing and developing in this area. In the other parts of the leaf that are dark green, chloroplast division is mostly finished. Thus, we could test our method on areas with and without chloroplast division, simply by selecting the leaf sections used by position and color.

In an additional approach, we performed preliminary confocal microscopy studies, which yielded results that strongly supported the concept that this spectroscopic technique (1) is a real and useful method to observe chloroplast division.

**Experimental**

**Plant materials.**
Growth conditions were described in reference 1. We used Oregon State University (Corvallis, Oregon) soil mix and Osmocote slow-release fertilizer in amounts and release times appropriate for each species; the greenhouse crew waters twice a day and controls any pathogens and pests that might cause problems. We thank Don Powell, owner of Garland Nursery (Corvallis), for his kind donation of numerous Amaryllis bulbs.

![Figure 1](Figure 1 (top). Distribution plots of chloroplast efficiencies from the base to the top of sugarcane (Saccharum officinarum L. or S. hybrid) leaves.)

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Fluorometric analysis and data processing are described in reference 1.

Confocal microscopy was done on Oregon State University’s Leica (Hiedelberg, Germany) TCS 4D, using a AgKr laser scanning at 568 nm.

Results and Discussion

We gathered data with this germplasm approach. However, the rigorous statistical analysis often took a few hours per sample, and there were far too many samples. We already knew, from the work described earlier, that the subpopulations observed were statistically sound (1, and manuscript in progress). Thus, in subsequent data processing analysis — because there are a large number of data points (31,680) within each image, which contributes strongly to the statistical validity of the data and generates smooth population curves (Figure 1) — the presence of subpopulations is accepted by simple inspection.

We examined, section by section, the leaves of monocots (sugarcane, wheat, Narcissus, and Amaryllis). In these leaves, where one would expect to observe chloroplast division — judging from the greening of the tissue as measured by in vivo spectroscopy (4–7) — we found multiple populations of chloroplast efficiencies (Figures 1 and 2).

As expected, the most complex patterns were found in leaves of the C-4 plant sugarcane (Figure 1). This pattern was expected because C-4 plants have two kinds of chloroplasts: one in the bundle sheath and one in the mesophyll cells.

Of all the germplasm examined, Amaryllis proved to be the most favorable for our purposes (Figure 2). It was clear in Amaryllis’s rapidly dividing tissues that the dual nature of the subpopulations of chloroplasts was clearly manifested. As the tissues matured toward the leaf tip, these subpopulations were not commonly observed (Figure 2). In addition, the mean $Y'$ (approximation of photosynthetic efficiency) increased as the tissue observed was more mature. This information also followed our working theory that we were measuring chloroplast division.

Trying to test this theory further, we managed to get a little time on a confocal microscope to study the phenomenon in Amaryllis (for botany, see reference 9). And despite the fact that we were at the limit of the lenses of that instrument and working with living tissue, we saw what seemed to be chloroplast division in the zones where the leaves showed these multiple chloroplast efficiencies. Chloroplast division was not often observed by confocal microscopy in the part of the leaves where the imaging fluorometer did not generate different populations of chloroplast efficiencies (Figures 3 and 4).

Figure 3 shows in vivo confocal images of chloroplasts. Lower down on the leaf, in the less mature tissues closer to the meristem (upper right and left), the chloroplasts are smaller (note the larger 20-μm bar) and frequently show the dumbbell shape, especially in the figure at the upper right. In the lower right and left of Figure 3, images from a mature section (tip) of Amaryllis leaf are shown; here the chloroplasts are more uniform and more tidily packed. These chloroplasts are larger (see the 20-μm bar at about the 7:00 position in the lower left figure). These larger chloroplasts do not appear to show the dumbbell-like restrictions characteristic of dividing chloroplasts (10).

Here we found resolution difficulties common with these in vivo measurements. The next day we tried harder and, at the section of the leaf where the chloroplast division takes place — as judged by the incipient greening of the tissue — achieved a little more resolu-
Figure 3 (above left). Upper right and upper left images are from tissues lower down on the leaf, in the less mature tissues closer to the meristem, with smaller, less crowded chloroplasts (see 20-μm bar). Lower left and lower right images show a mature section (tip) of the Amaryllis leaf with larger, more densely packed chloroplasts (see smaller 20-μm bar at about the 7:00 position).

Figure 4 (above right). Confocal images of young tissues where dividing (dumbbell-shaped) chloroplasts are commonly seen.

Conclusion
It seems clear that the phenomenon of the dual peaks of chloroplast efficiency (1) as determined by statistically processing data from our imaging fluorometric device (11) is most readily explained by chloroplast division.

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References