AN ABSTRACT OF THE DISSERTATION OF

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Abstract approved:

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During exploration of imaging photosynthetic fluorimetry of *Arabidopsis thaliana* mutants, we discovered a novel phenomenon wherein photosynthetic efficiency (defined in Ning et al., 1995) is shown to plot in discrete groups. This exploration resulted first 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 and in the wild-type.

Testing the phenomenon, we examined leaves of monocot plants in which the progression of leaf development and greening follows a linear course upwards along the leaf. The monocots chosen were sugarcane and especially *Amaryllis*; data from wheat, *Narcissus*, and other plants are mentioned but not described here. The above results showed that in these plants, chloroplast division phenomenon occurred only where chloroplast division is localized. We found this is also

consistent with the postulate that the biphasic energetics observed correspond to the division of this organelle.

To verify the phenomenon further, we performed preliminary confocal microscopy studies in *Amaryllis*; we saw what seemed to be chloroplast division in the zones where the leaves showed the multiple photosynthetic efficiencies and these results supported the concept that our spectroscopic technique is a real and useful method to observe chloroplast division. Here we also present a novel statistical approach allowing quantification of probability in two- dimensional in vivo fluorescence spectroscopy of these biological samples.

To automate detection of chloroplast division for future use, we develop a digital image processing program we called a software "tool". This tool analyzes photographs of confocal images, identifies chloroplast division and shows statistical information of identified chloroplasts. The statistical information includes distribution of intensity, area and perimeter of each identified chloroplast. We used several image processing techniques to analyze confocal images, including filtering images, object extracting and algorithms in graph theory. We have implemented a "friendly" GUI (Graphical User Interface) that enables a user to perform operations such as correction, addition and deletion of group(s) easily during the execution of the program. By employing an innovative configuration of image analysis techniques, this software tool is able to identify where chloroplast division is occurring and answer related experimental questions.

Imaging Fluorescent Determination of Energetics of Chloroplast Division and Related Matters

by
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APPROVED:

Major Professor, representing Horticulture

Head of the Department of Horticulture

Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Ping Zheng, Author

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CONTRIBUTION OF AUTHORS

Ping Zheng was involved in the design, experimentation, analysis a writing of each manuscript. Dr. Larry S. Daley was involved in overseeing this work from its onset and in the design and analysis of experiments, and proofreading and editing of each manuscript. Although some of the other author's specific contributions are recognized in the acknowledgment section, the synergism, interaction and support of each member of this highly diverse and multidisciplinary group, although difficult to quantify and specifically assign credit, was essential to the production of this body of work. The diversity of the disciplines and the broad sweep of the experimental topics successfully addressed demonstrate the advantages and contributions of such a multidisciplinary group.

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DEDICATION

To my beloved father and mother:

Shi-Shan Zheng and Yan-Ping Zhou

IMAGING FLUORESCENT DETERMINATION OF ENERGETICS OF CHLOROPLAST DIVISION AND RELATED MATTERS

CHAPTER 1

GENERAL INTRODUCTION

Chloroplasts are vitally important because they provide energy to the plant cell by capturing light from the sun and converting it to chemically usable forms of energy, upon which we all ultimately depend (Daniel et al., 1998; Cooper, 2004). The functional leaves of higher plants are packed with them. But, the seeds from which these plants grow commonly have few or no functional chloroplasts. Thus, given their separate DNA, chloroplasts in plants must arise through repeated chloroplast division (e.g. Mullet, 1988).

Plant chloroplasts are large organelles (5 to 10 μm long) that are bound by a double membrane called the chloroplast envelope (e.g. Cooper, 2004). In addition to the inner and outer membranes of the envelope, chloroplasts have an internal membrane system, called the thylakoid membrane. The thylakoid membrane forms a network of flattened discs called thylakoids, which are commonly arranged in stacks called grana. In particular, their three membranes divide chloroplasts into three distinct internal compartments: (1) the intermembrane space between the two membranes of the chloroplast envelope; (2) the stroma, which lies inside the

envelope, but outside the thylakoid membrane; and (3) the thylakoid lumen (e.g. Cooper, 2004).

Chloroplasts develop from proplastids that are self-replicating (e.g. Mullet, 1988). When the leaf is new, there are approximately ten proplastids in each developing leaf cell. As the leaf develops, these proplastids divide by fission, and develop until roughly 65 chloroplasts are present in each mature mesophyll cell (Mullet, 1988). Chloroplast division occurs primarily in developing leaf mesophyll cells (Saurer and Possingham, 1970; Possingham and Smith, 1972; Boffey et al., 1979; Leech et al., 1981). Chloroplast division is essential to mesophyll development (Leech, 1984; Boffey, 1992). Pyke, Ellis and Leech list the environmental factors that influence chloroplast division, such as cell size and nuclear ploidy (Ellis and Leech, 1985; Pyke and Leech, 1987, 1988, 1994, reviewed by Osteryoung and McAndrew, 2001). Possingham and Hashimoto discuss the growth environment of the leaf, especially light quality (Possingham et al., 1975; Hashimoto and Possingham, 1989; reviewed by Hashimoto, 2003).

Robertson et al. (1996) stated "Chloroplast accumulation in developing leaves enormously affects photosynthetic efficiency, yet the control of the chloroplast division process itself is one of the least understood areas of chloroplast biology." This situation calls for improved technology, since technology of this kind could spare the enormous efforts that manual methods hitherto have required. Thus, approaches to in vivo chloroplast measurement, such as the technology presented here, will facilitate not only the more rapid unveiling of basic understanding, but also enable, and make practical, investigations as to

how chloroplast division relates to the myriad species, germplasm, and conditions of crop yield.

In addition, chloroplast transformation by appropriate gene manipulates has produced medically useful proteins (e.g. a secretory protein, human somatotropin). Such proteins were found to be more readily biosynthesized in chloroplasts than in the nuclear-directed part of the plant cell (Staub et al., 2000; e.g. Langbecker et al., 2004). For all these scientific and practical ends, instruments for rapid determination of in vivo chloroplast division are most useful.

Mutants used

In our laboratory, in a fortuitous exploration of imaging photosynthetic fluorometry of *Arabidopsis thaliana* mutants (for botany, see reference Wilson, 2000), a novel spectroscopic technique (Zheng et al., 2002) to observe chloroplast division arose.

In plants, as in animals, normal cellular differentiation depends on coordinated interactions between the nuclear and organelle (chloroplast or mitochondria) genomes (Borograd, 1991). Studies that genetically dissect plant variegation mutants are a powerful means to gain insight into these poorly understood interactions (Pyke and Leech, 1994).

We first worked with *Arabidopsis* variegation mutants (V28, Spotty) having leaves that typically contain green sections, and also have sections of leaves that look white or yellow. Green sections have a full complement of normal chloroplasts with high levels of chlorophyll fluorescence.

The cells in the white or yellow sectors have plastids that for the most part lack chlorophyll. In these sectors there are still, at low density and in small numbers, some plastids with chlorophyll fluorescence. This observation suggests that these cells lack some aspect of nuclear control of chloroplast function that manifests itself, either directly or indirectly, in photosynthetic pigment deficient plastids (Rodermel, 2001).

Energetics of C-3 and C-4 photosynthesis

Our experiments deal with the energetics of photosynthesis.

At a high temperature with high light intensity, the rate of photosynthesis is correspondingly high; this increases the oxygen concentration within cells and causes a high rate of photorespiration. A group of plants including sugar cane, maize, and other commercially important species can avoid a high rate of photorespiration, and increase light use efficiency, by effectively concentrating CO_2 (Reviewed by Leegood, 2002). These are the so-called C-4 plants. The mechanisms involve both additional biochemical pathways and structural changes in green tissue (e.g. Ridge, 2002).

The energetics of C-3 and C-4 photosynthesis are different. "In C-4 plants CO_2 fixation is shared between mesophyll and bundle sheath cells" (e.g. Edwards, 2004). These cells do not function independently of each other. "In C-4 photosynthesis the minimum requirements for energy are the sum of energy

requirements of the C-4 cycle (which costs 2 ATP per CO_2 fixed in the cycle) and the C-3 cycle (which costs 3 ATP and 2 NADPH)" (e.g. Edwards, 2004).

Edwards continues: "Each turn of the C-4 cycle generates one CO_2 in the bundle sheath compartment where ribulose 1, 5-bisphosphate carboxylase (Rubisco) is located. Thus, the minimum energy requirements per CO_2 fixed in C-4 plants is 5 ATP and 2 NADPH.

However, the real energy costs will be higher because of some leakage of ATP from the bundle sheath cells. C-4 plants benefit by concentrating CO_2 around Rubisco which largely prevents photorespiration. The provision of ATP and NADPH is shared by the mesophyll and bundle sheath chloroplasts, and the way this occurs varies, depending on the type of C-4 photosynthesis" (e.g. Edwards, 2004).

Edwards continues: "In C-3 plants the energy cost per CO_2 fixed is increased due to photorespiration (e.g. about 50% higher than the theoretical minimum of 3 ATP/2 NADPH per CO_2 fixed in the C-3 cycle)" (e.g. Edwards, 2004).

In C-4 plants, as previously mentioned above, carbon dioxide fixation happens in two types of cells: the mesophyll cells and the bundle sheath cells. This fixation is temporary in mesophyll cells and more permanent in bundle sheath cells.

The difference between photosynthetic efficiency (Y') in mesophyll cells and bundle sheath cells is readily attributed to cell-specific amplification of enzymes of C-4 photosynthesis. These also are complementary adjustments of photosystem and electron transport activities (Edwards et al., 2001).

The researchers found that mesophyll cell chloroplasts in *Sorghum bicolor* have a stroma lacking Rubisco, and thylakoids with high activities of both photosystems(Edwards et al., 2001). C-4 chloroplasts have different energetics than C-3. In C-4 photosynthesis, non-cyclic electron transport in mesophyll chloroplasts sustains phosphoenolpryuvate (PEP) synthesis. PEP is the substrate for initial CO_2 fixation of phosphoenolpryuvate carboxylase (PEPCase) in the mesophyll cell cytosol. Here PEPCase catalyses the reaction of CO_2 with PEP; the product of this reaction is oxaloacetate. Oxaloacetate is converted into malate or aspartate (Hibberd and Quick, 2002). Malate is transported to the bundle sheath cell via the plasmodesmata and is decarboxylated in the bundle sheath chloroplast. The released CO_2 is fixed by Rubisco in the Benson-Calvin cycle. PEP is regenerated from pyruvate in the mesophyll chloroplast (Reviewed by Leegood, 2002).

Imaging fluorescence technology

We used an imaging fluorescence technology (Ning et al., 1995). Photosynthetic tissues generate changing patterns of fluorescence (Kautsky transients) that occur immediately after illumination of a dark-adapted leaf (e.g. Ning et al., 1995). The fluorescence under physiological conditions comes from the PSII linked antenna.

Photosystem II (PSII) is contained in a multisubunit protein supercomplex embedded in the thylakoid membranes of higher plants and cyanobacteria. Grana are the principal sites of PSII (Taiz et al., 2002).

PSII uses light energy to catalyze a series of electron transfer reactions resulting in the splitting of water into molecular oxygen, protons and electrons (e.g. Taiz et al., 2002). In otherwords, this process produces oxygen, changes pH by pumping protons (H^+), and produces electrons (e^-).

A chlorophyll molecule excited by absorption of a photon of light may get rid of this energy either by transferring it to another molecule (the route to photosynthetic electron transport), or by releasing the energy partly as heat, and partly by emission of a photon of light of lower energy (and hence longer wavelength) than that of the absorbed photon. Such emission is called fluorescence (e.g. Ridge, 2002). Each quantum of light absorbed by a chlorophyll molecule raises an electron from the ground state to an excited state. Upon de-excitation from an excited state to ground state, a small proportion (3-5% in vivo) of the excitation energy is dissipated as red fluorescence.

PSII appears to be particularly sensitive to a number of stress factors such as high temperatures, chilling, freezing, drought and excessive radiation (Bolhar-Nordenkampf et al., 1993; Fracheboud and Leipner, 2003). Since all these stresses affect the function of PSII, fluorescence yield is highest when photochemistry and heat dissipation are lowest. Therefore, fluorescence is commonly used as a tool to quantify stress response under laboratory and field conditions.

Kautsky and Hirsch, in 1934, exposed dark-adapted leaves to light and observed time-dependent fluorescence transients. The fluorescence induction curve is sometimes called the *Kautsky curve*. The different phases are denoted O I D P S M T. The letters correspond to the Origin of curve or initial fluorescence, Initial

rise, Dip, Peak level, and down Slope between the P and secondary Maximum and Terminal level, respectively. The fluorescence rise from O to P occurs during the first second of illumination and is called the 'fast phase'. The 'slow phase' follows after P and it takes several minutes before the terminal phase, T, is reached. The fast phase is related to primary processes of PSII, whereas the slow phase is mainly related to interactions between processes in the thylakoid membranes and metabolic processes in the stroma, primarily carbon metabolism (Bolhar-Nordenkampf et al., 1993).

In the fast phase, there is an immediate rise in fluorescence to a minimal level F_0. Upon illumination with a sufficiently strong light, fluorescence increases from F_0 via I, and often D, to a P. This rise reflects a gradual increase in the yield of chlorophyll fluorescence, as the rate of photochemistry concurrently declines. The reason for this decline is that the first electron acceptor Q_A of PSII becomes fully reduced as the 'traps' close. At I, the electrons start to become transferred via Q_B to plastoquinone pool, so that a transient reoxidation of Q_A occurs. After the dip D, there is a sharp rise in fluoresce towards P (Bolhar-Nordenkampf et al., 1993).

Relationships between time-dependent fluorescence maxima and the time taken to reach quasi-steady-state are very sensitive to changes in plant photosynthetic function. The photosynthetic function of plants, while not as closely related to crop production as was formerly believed, is essential to agricultural production (Bolhar-Nordenkampf et al., 1993). Thus fluorescence parameters are important indicators of photosynthetic function much used in the agricultural and plant sciences (e.g. Ning et al., 1997).

A useful parameter is variable fluorescence (F_v); this is the fluorescence increase from F_0 to Fm. The ratio of $F_v/F_m = (F_m - F_0)/ F_m$ allows the determination of the maximum quantum efficiency of PSII (Y). In healthy leaves, this value is close to 0.8, independent of the plant species studied. A lower value indicates that a proportion of PSII reaction centers is damaged (Bolhar-Nordenkampf et al., 1993; Fuchs et al., 2002) or in rest (this dissertation).

Following the peak P, the fluorescence decreases through the S-shape curve. During the S phase electron transport generates a proton gradient which is gradually built up across the thylakoid membranes causing its energization. The M peak reflects the induction of increased rate of carbon fixation. After the M, the fluorescence yield reaches a steady state value (F_t), once the terminal phase, T, is reached (Bolhar-Nordenkampf et al., 1993).

Occurring in picoseconds, F_0 is hard to calculate from the nonlinear fluorescence transients, and difficult to measure directly by digital imaging technology, and thus, is a source of error in Fv determinations. However, except in some specific cases, such as when examining leaves treated with certain herbicides (Bolhar-Nordenkampf et al., 1993), F_0 can be estimated by F_t, $F_0 \approx F_t$ (Ning et al., 1995). We approximate Y as Y', the empirical estimate of quantum yield [as $Y' = (F_m - F_0)/ (F_m - F_{dark})$] for acquiring fluorescence images by our imaging fluorometer.

Germplasm approach

One simple way to confirm our novel spectrofluorometric method was to acquire a charge-coupled device having more pixels and, thus, better resolution. In this way, we could be sure by observing single chloroplasts in each pixel that we were watching these organelles divide. However, funding for this project was not forthcoming.

Here we describe further studies with our method, including a germplasm approach using amaryllis, wheat, sugarcane, narcissus and other plants (Robertson et al., 1993) and in vivo spectroscopy. 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.

Chloroplast division studies are typically very difficult in the dicots family because the development of the chloroplast is not directly related to the development of the leaf. Conversely, studying chloroplast division in a monocot such as *Amaryllis* is easier because chloroplast development is directly proportional to the temporal stage of leaf development (Yoder, 1990). This relationship means that as the monocot leaf proliferates and matures from the primordial initial, the chloroplasts divide and mature at a rate necessary to meet the energy demands of the developing leaf. Thus, all stages of chloroplast maturation are strictly defined in a single leaf. We verified this by performing a considerable number of experiments with monocot species.

We first used dicotyledonary plants such as those found on *Arabidopsis,* because they were the species with which we began our work (Zheng et al., 2002). These dicots develop in complex patterns of cell division. In contrast, monocot plants as describe above often have an ordered sequential longitudinal development of leaves (Yoder and Daley, 1990). We explored other monocot species to test our method, including *Amaryllis.*

In monocots such as grasses (which can be either C-3 or C-4), the leaf tissue arises from nodal meristems 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 tissue closest to the meristem is the youngest.

In monocot leaves, the youngest tissue is very pale green because the chloroplasts (etioplasts) 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 selecting by position and color the leaf sections used.

In an additional approach, we performed preliminary confocal microscopy studies, which yielded results that strongly supported the concept that this spectroscopic technique (Zheng et al., 2002) is a real and useful method to observe chloroplast division.

How use of C-4 germplasm applies to data

C-4 fluorescence patterns help explain our data, because the two types of cells have different photosynthetic efficiencies(Y'), the bundle sheath cells contain larger chloroplasts with Rubisco replete stroma, but photosystem II deficient thylakoids which lack grana. Specialization of electron transport pathways in the bundle sheath chloroplasts of NADP-malic enzyme (NADP-ME) plants has led to the predominance of photosystem I dependent cyclic electron transport leading to ATP formation. This is accompanied by a low photosystem II activity (Reviewed by Leegood, 2002). C-4 acid decarboxylasese and Rubisco are in bundle sheath cells. Symplastic metabolite exchange between the two cell layers delivers malic acid for decarboxylation by NADP-ME, generating high CO_2 concentrations that minimize the oxygenase activity of Rubisco. This decarboxylase also generates one-half of the reductant needed by 3-phosphoglyceric acid (3-PGA), compensating for the photosystem II deficiency in bundle sheath chloroplasts. The 3-PGA is returned out of these bundle sheath cells for reduction in mesophyll cells.

The concept of Y' difference arising from physiological difference allows us to better understand our data. Since there are differences in function and structure between the chloroplasts of mesophyll cells and the bundle sheath cells. Thus Y' will be different. If Y' in mesophyll cells and Y' in the bundle sheath cells is different, we will see four signal peaks instead of two peaks when we plot the data with respect to Y' on the x axis, with the y axis as the number of pixels at

that Y' range. We will discuss this in relation to our findings later in this dissertation.

Statistical proofs of observation

When doing spectroscopy of chemicals, the number of molecules, atoms, or other moieties -even when observed in complex matrices- is commonly very large and their state relatively homogeneous. During biological in vivo experiments, the moiety units explored are usually far larger in size, less concentrated, and more hetergeneous, because they are found in far fewer numbers than common in chemicals or even biochemical work. Thus, in vivo fluorescent spectroscopy of the relatively small numbers, larger variability and complexity of these biological moieties offers exceptional statistical challenges.

When we were trying to distinguish *Arabidopsis* mutants and wild type (natural or unmutated) plants we found that both types often contained multiple populations of chloroplast efficiencies. We developed novel statistical tools to distinguish between truly divided and spurious segregated populations (Zheng et al., 2003).

An image processing tool

We developed an image processing tool which detects chloroplast division from photographs of confocal microscopy images. Our objective was to automatically

detect chloroplast division. We tried to answer the experimental question: does the distribution of intensity within the large clusters of chloroplasts (where one presumes most chloroplast activity to occur) follow a biphasic curve in each cluster, and does cluster size influence this distribution? In order to test this hypothesis, we group pixels within the edges. Each identified group is represented as a chloroplast. This provides an interactive interface that allows the user to manually correct misdiagnoses made by computer. By utilizing an innovative configuration of image processing techniques, the system is able to detect chloroplast division and test the hypothesis.

Results of this dissertation

The specific results of this dissertation are:

1. A fortuitous exploration of imaging photosynthetic fluorometry of *Arabidopsis* mutants 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.

2. Further testing of the phenomenon is described in result 1 using sugarcane, and most especially *Amaryllis*. Data from wheat, *Narcissus*, and other plants are mentioned but not described. We noted that sugar cane (C-4) has multiple signals. Because there is difference between photosynthetic efficiency (Y') in mesophyll cells and bundle sheath cells.

3. We also performed preliminary confocal microscopy studies which yielded results that strongly supported the concept that this spectroscopic technique (Zheng et al., 2002) is a real and useful method to observe chloroplast division. This apparently confirms that the phenomenon is a result of a decrease in organelle efficiency during chloroplast division.

4. We provide a statistical approach allowing quantification of probability in two-dimensional in vivo fluorescence spectroscopy of biological samples. We also provide novel statistical tools to distinguish between really divided and spurious by divided population.

5. We have developed an image processing tool which detects chloroplast division from photographs of confocal microscopy images.

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CHAPTER 2

TEST OF AN IN VIVO METHOD TO DETECT CHLOROPLAST DIVISION IN CROP PLANTS PART I: DISCOVERY OF THE PHENOMENON

Ping Zheng, Carolyn Wetzel, Karim Ammar, Anne-Marie Michelle Girard, Steve Rodermel, David R. Thomas, Li Ning, James B. Callis, Gerry E. Edwards, and Larry Daley

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TEST OF AN IN VIVO METHOD TO DETECT CHLOROPLAST DIVISION IN CROP PLANTS PART I: DISCOVERY OF THE PHENOMENON

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This article describes a novel spectrofluorometric method that apparently allows for in vivo observation of division of chloroplast populations in leaves of *Arabidopsis thaliana*.

INTRODUCTION

Chloroplasts, the green organelles found in plant cells, are central to life on Earth because of their photosynthetic functions. The leaves of higher plants are packed with them. However, the seeds from which these plants grow commonly have few or no functional chloroplasts. Chloroplasts have a separate genome that codes much of this organelle's function. Thus, chloroplasts in plants must arise through repeated chloroplast division.

Chloroplast division occurs primarily in developing leaf mesophyll cells (references 1 to 4). Here chloroplast division is essential to mesophyll development (5, 6). References 7-10 list the environmental factors that influence

chloroplast division, such as cell size and nuclear ploidy. References 11 and 12 discuss the environment of the leaf, especially light quality.

Although it is clear that 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 (13). This situation calls for improved technology to facilitate not only more rapid unveiling of basic understanding, but also to enable and make practical investigations of how chloroplast division relates to the myriad species, germplasm, and conditions of crop production.

In addition, chloroplast transformation method has arisen in which medically useful proteins are found to be more readily biosynthesized in chloroplast than in the nuclear-directed part of the plant cell (14). Thus, for all these scientific and practical ends, instruments for rapid determination of chloroplast division in vivo are required.

In our laboratory, in a fortuitous exploration of imaging photosynthetic fluorometry of *Arabidopsis* mutants, a novel technique arose that appears to measure chloroplast division in vivo. Preliminary studies employing diverse methods, including a germplasm approach using characteristics of diverse plants, in vivo spectroscopy, and confocal microscopy, suggested that this approach is "doable". Biophysical considerations, statistical theory, and preliminary experimental evidence support this hypothesis.

However, all this was not on our minds when we stumbled on our new technology. We were investigating variegation mutants of *Arabidopsis thaliana*.

In plants, as in animals, normal cellular differentiation depends on coordinated interactions between the nuclear and organelle (chloroplast or mitochondria) genomes (23). Genetic dissection of plant variegation mutants is a powerful means to gain insight into these poorly understood interactions (7).

We worked with *Arabidopsis* mutants with leaves that typically contain green sections having a full complement of normal chloroplasts with high levels of chlorophyll fluorescence. Most notably, these leaves also have sections of leaves that look white or yellow.

The cells in the white or yellow sectors have plastids that for the most part lack chlorophyll. In these sectors there are, at low density and in small number, some plastids with chlorophyll fluorescence. This suggests that these cells lack some aspect of nuclear chloroplast function in a way that manifests itself, either directly or indirectly, in pigment-deficient plastids.

The method we used is an imaging fluorescence technology (24). Photosynthetic tissues generate changing patterns of fluorescence (Kautsky transients) that occur immediately after illumination of a dark-adapted leaf.

Relationships between time dependent fluorescence maxima and time taken to reach quasi-steady-state are very sensitive to changes in plant photosynthetic function. The photosynthetic function of plants, while not as closely related to crop production as was formerly believed, is without doubt essential to agricultural production. Thus, fluorescence parameters are important indicators photosynthetic function in the agricultural and plant sciences.

EXPERIMENTAL

Plant materials. The material was (grown in the greenhouses adjacent to the laboratory, sampled with permission from some of the material grown by other researchers at Oregon State University (OSU, Corvallis), or sent by express mail from other researchers across the United States.

The *Arabidopsis thaliana* used for these experiments were prepared and grown by Steve Rodermel at Iowa State University of Science and Technology (Ames). These materials consisted of grown *Arabidopsis* plants. The *Arabidopsis thaliana* germplasm used was V28, spotty and wild type. Plants were shipped, carefully protected and by express mail service, from the Iowa laboratory site to the OSU laboratory site. Once the plants arrived at OSU, they were carefully unpacked and allowed to grow overnight to permit recovery of photosynthetic function from shipping stresses.

Leaves were sampled and then analyzed by the imaging fluorometer. These *Arabidopsis thaliana* leaves were taken at zero, four, and eight days after arrival from Iowa. Mutants and their growing conditions have been described previously (references 25 to 33).

When we grew our own plants we used OSU soil mix [which is, by volume, two parts pumice, one of peat (Canadian medium horticultural grade), and one of sandy loam soil] (Ning et al., 1995) and Osmocote (Sierra Chemical, Milpitas, CA) slow-release fertilizer (14-14-14) in amounts and release times

appropriate for each species. The greenhouse crew watered each plant twice daily or as needs and controlled any pathogens and pests that could cause problems.

Fluorometric analysis followed Ning et al. (24). *Our* laboratory developed an imaging fluorometer/spectrophotometer (24, references 34 to 39) that can quantify, in two-dimensional space, the fluorescence characteristics of leaves. This instrument detects changes related to the photosynthetic function and pathology of plants. Our imaging fluorometer uses a charge coupled device (CCD) as detector element, acquiring spectra for 31,680 positions per sample (24).

This instrument has had a good number of applications. These applications include using in vivo fluorescent imaging for detection of damage to leaves by fungal phytotoxins (40); the recovery of digital information stored in living plant leaf photosynthetic apparatus as fluorescence signals (41); and imaging of water in living leaves (38, 39, 42). The instrument has been featured in a number of places, such as front covers for scientific journals (43-45). It was discussed in reference 46.

Data processing. In addition to the expected result characterizing the mutants, we found discrete photosynthetic populations with diverse mean levels of photosynthetic functionality in the chloroplasts of both wild-type and mutant plants. Our data demonstrate the "statistical reality" of this previously undescribed phenomenon using a statistical deconvolution program specifically developed for this purpose.

Each image contains 31,680 data points (pixels). In their original distribution, these data points generated the image of the chosen leaf, showing the

distribution of photosynthetic efficiency (Y'). Here the data points are reorganized for analysis.

First, sample leaf data points with value zero (data points having no photosynthetic activity) were removed. The remaining data were measured only the photosynthetic active surface of the leaf sample. Data from each image were placed in order of rank of Y' along the *x* axis to evaluate statistically the distribution of Y' data. That is, the density of data points at that value of Y' (*y* axis) was plotted against rank of Y' (*x* axis) of that data point.

All this generated curves with Y' as the *y* axis and rank of Y' as the *x* axis. The resulting curves were deconvoluted using the expectation maximization (EM) method (see second paragraph following and [47]) to seek the number of normal curves found in each population distribution curve of each leaf.

In this circumstance, one expects that only one population of Y' in each leaf in the resulting curve will follow a normal distribution. If the resulting curve contains a number of normal curves, then there is more than one population of Y'. Although technically speaking, what we are following are data points, not chloroplasts, we are, in essence, sampling the Y' of groups of chloroplasts across the leaf, seeking differences among them. When we find such differences, it is reasonable to imply that there are different groups of chloroplasts with different properties.

Program for data analysis. One of the most computation intensive problems is to calculate a maxim likelihood * estimate for a mixture of normal distributions.

This can be thought of as parametric estimations of likelihood functions. Maximizing these likelihood functions is complicated by singularities and numerous spurious maximizers. Here we used the EM algorithm, which is a technique for finding maximizers of likelihood functions (47). The program running the EM algorithm was written using a numerical software package called MATLAB (The Math Works, Natick, MA).

This iterative EM algorithm is extremely reliable and usually finds the "good" maximizer from most reasonable initial guesses. However, it is very slow in cases where there is considerable overlap between component normal distributions.

We set up a model in which the resultant distribution is considered as a mixture of normal distributions. To do this we wrote a novel program in MATLAB using the established formulas and mathematical conventions. In these formulas we consider the unknown distribution as a mixture of a number of subdistributions from 1 to m, where m is the number of normal components in the mixture (Ping Zheng, David R. Thomas, and Larry Daley; manuscript in preparation).

Confocal microscopy was done on the OSU instrument, a Leica (Hiedelberg, Germany) TCS 4D, using a AgKr laser scanning at 568 nm,

* Statistics terminology (47)

RESULTS AND DISCUSSION

With the objectives and intent previously described, we estimated photosynthetic efficiency measuring various points of the transient spectra of fluorescence released on the onset of photosynthesis. These data were collected with a CCD with 31,680 data collecting units, or pixels. The data gathered were then processed through a patented algorithm, generating an image estimating the photosynthetic efficiency across a section of leaf (24, 34, 35, 40, 41, 42, 48).

Photosynthesis occurs at numerous sites in each active chloroplast. However, what our instrument detects is an aggregate image of the summed function of each set of chloroplasts that impinge on each pixel.

The data are presented as a false-color image. The intensity of function is color coded as an essentially continuous light spectra function, in which the deepest red represents the most efficient range, the diverse oranges, yellows, greens, and blues represent are progressively less efficient Y'; and black (no color) is background. In this image, nonfunctioning chloroplasts and non-photosynthetic tissues are perceived as low efficiency or background colors (24, 34, 35, 37, 40, 41, 42, 48).

For this particular series of experiments; seeking differences in photosynthetic efficiency between the mutant and the normal part of these *Arabidopsis*, we processed the efficiency data. We ranked the data with respect to efficiency on the x axis, with the y axis as the number of pixels at that efficiency

range. This approach generated normal curves of the efficiency of the chloroplasts within each image. Our original intent was, in this way, to measure the proportion of mutated tissue and the amount of normal tissue.

Initially, we tested the data by rigorous statistical analysis for subpopulations. This statistical approach verified the presence of these subpopulations. In these mutants we often, but not always, saw two populations with different photosynthetic efficiencies. This observation seemed to explain the differences between the dark green and the much lighter white or yellow sectors of the mutation plant's leaves (for example, spotty mutant data presented in Figure 2.1).

Figures 2.1a and 2.1b are the curves and histograms for raw data, and Figures 2.1 c and 2.1d are for statistically transformed data. If the data were random, Figure 2.1c and especially Figure 2.1d would not be expected to show this binary distribution, this seemed to confirm our initial working hypothesis because, at least at first, most of the wild type seemed to have merely one peak (Figure 2.2).

In Figure 2.2d, application of the deconvolution program to these data shows that, except for a hint of a low peak at low density, the data support a single normally distributed population of Y' (estimated photosynthetic efficiency) (35) data. This suggests that one major population of chloroplast has a common photosynthetic efficiency. Because this distribution was most common in wild type, we were happy to forget this little hint of something else*.

. At this point we expected the wild type to have a single, normal curve and mutants to have two. Nice, tidy, and logical or so it seemed then

Thus, at first it seemed safe to ignore the small suggestion of a second *Pm* (statistically transformed photosynthetic efficiency) peak, after analysis shown in the wild-type in Figure 2.2d (34,35). However, as our work progressed and data accumulated we found some wild-type data that had peaks at low *Pm*. These peaks now were impossible to ignore (for example, see Figure 2.3c).

Thus, we embarked on a wider examination of mutant and wild type spectra using the same statistical deconvolution (Figure 2.4). With these data we began to clearly see two populations of chloroplasts with different efficiencies not only in other mutants but also in wild-type *Arabidopsis* plants (Figure 2.4). This was quite disturbing, very puzzling and not especially tidy, for it disagreed with our initial hypothesis.

* Initial because this distribution was most common in wild type, we were happy to forget this little hint of something else. However later on, we realized that this observation represent something factual.

We could not attribute the difference in populations to an artifact of extraction because the technology used here was an in vivo method, and no extraction procedure is involved. What we see with this instrumentation represents the total population of functional or semi-functional chloroplasts of the area observed.

We asked ourselves many questions. For instance, were we really seeing two populations, or were we seeing the same kind of population at two different parts of its cycle of development? In this test model, separate new populations of chloroplasts would be increasing, maturing, and then decreasing in efficiency with age. This particular hypothesis was not very credible because one would expect that such a circumstance would result in not just two, but in many, populations.

However, the process of discovery was complex, with hypothesis after hypothesis failing to give reasonable explanations of the data observed. For instance, we considered the circumstance that some types of plants, known as C-4 plants, are long known to have different chloroplasts in the mesophyll and bundle sheath cells (49). The trouble with this explanation is that *Arabidopsis* are not C-4 plants; C-4 plants, it turned on, had even more complex distributions of chloroplast efficiencies (this will be discussed in the second article in this series).

Figure 2.1. Ranked and statistically processed data showing distinct populations chloroplast efficiencies in *Arabidopsis* spotty mutants (a) and (b) show data before statistical transformation; (c) and (d) show statistically transformed data; (a) and (c) show curve fitting to histograms in (b) and (d).

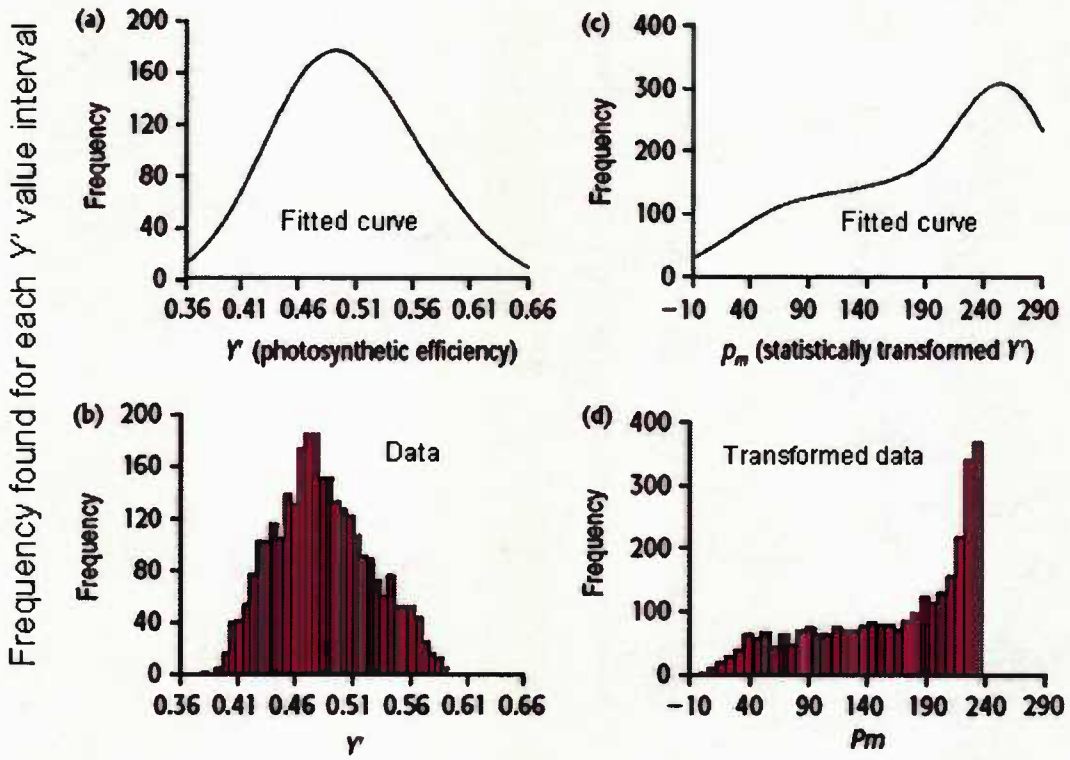
Frequency found for each Y' value interval
(a)
Frequency
Fitted curve
Y' (photosynthetic efficiency)
(c)
Frequency
Fitted curve
p_m (statistically transformed Y')
(b)
Frequency
Data
Y'
(d)
Frequency
Transformed data
Pm

FIGURE 2.1.

Figure 2.2. Ranked and statistically processed data showing distinct populations of chloroplast efficiencies in wild-type (non-mutant) *Arabidopsis* (a) and (b) show data before statistical transformation; (c) and (d) show statistically transformed data. Upper frames (a) and (c) show curve fitting to histograms in lower (b) and (d) frames.

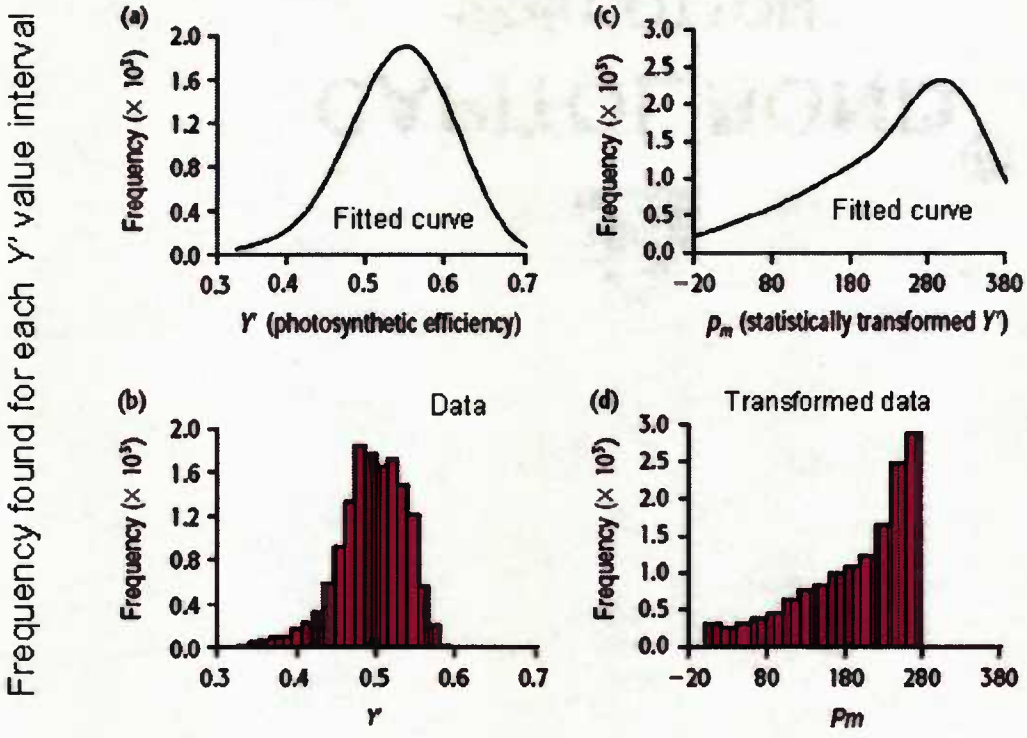
Frequency found for each Y' value interval
(a)
Frequency (× 10³)
2.0
1.6
1.2
0.8
0.4
0.0
Fitted curve
0.3
0.4
0.5
0.6
0.7
Y' (photosynthetic efficiency)
(c)
Frequency (× 10³)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Fitted curve
−20
80
180
280
380
p_m (statistically transformed Y')
(b)
Data
Frequency (× 10³)
2.0
1.6
1.2
0.8
0.4
0.0
0.3
0.4
0.5
0.6
0.7
Y'
(d)
Transformed data
Frequency (× 10³)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
−20
80
180
280
380
Pm

FIGURE 2.2.

Figure 2.3. Statistically processed curves showing dual populations of chloroplast efficiencies within mutant and wild-type leaves.

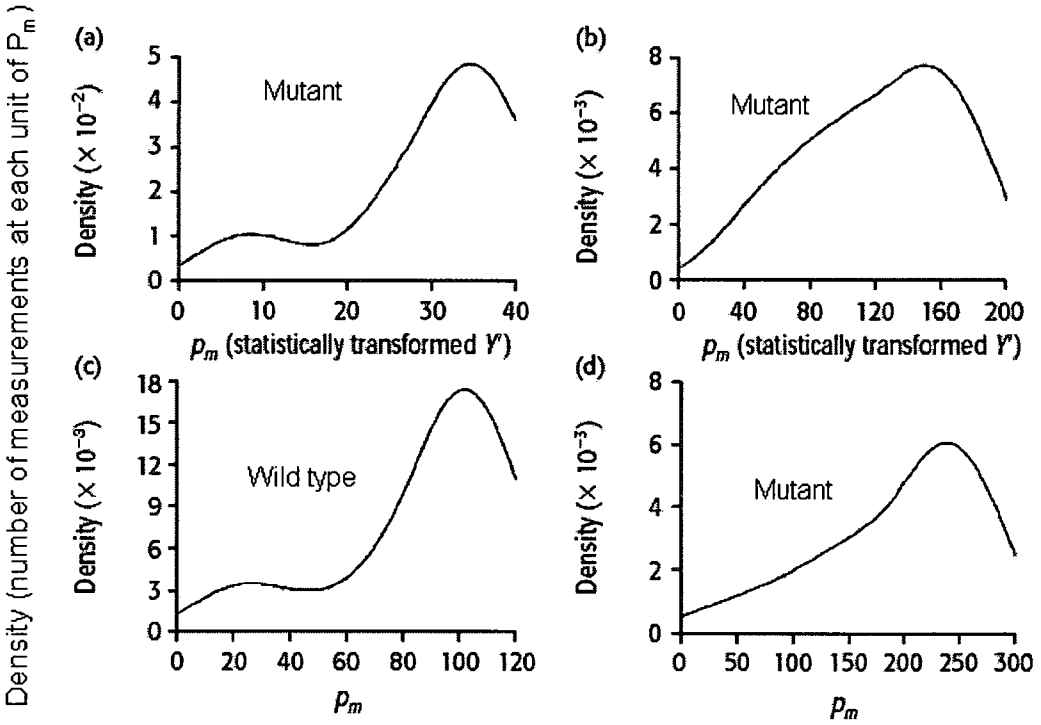
Density (number of measurements at each unit of P_m)
(a)
Density ($\times 10^{-2}$)
Mutant
0 1 2 3 4 5
0 10 20 30 40
p_m (statistically transformed Y')
(b)
Density ($\times 10^{-3}$)
Mutant
0 2 4 6 8
0 40 80 120 160 200
p_m (statistically transformed Y')
(c)
Density ($\times 10^{-3}$)
Wild type
0 3 6 9 12 15 18
0 20 40 60 80 100 120
p_m
(d)
Density ($\times 10^{-3}$)
Mutant
0 2 4 6 8
0 50 100 150 200 250 300
p_m

FIGURE 2.3.

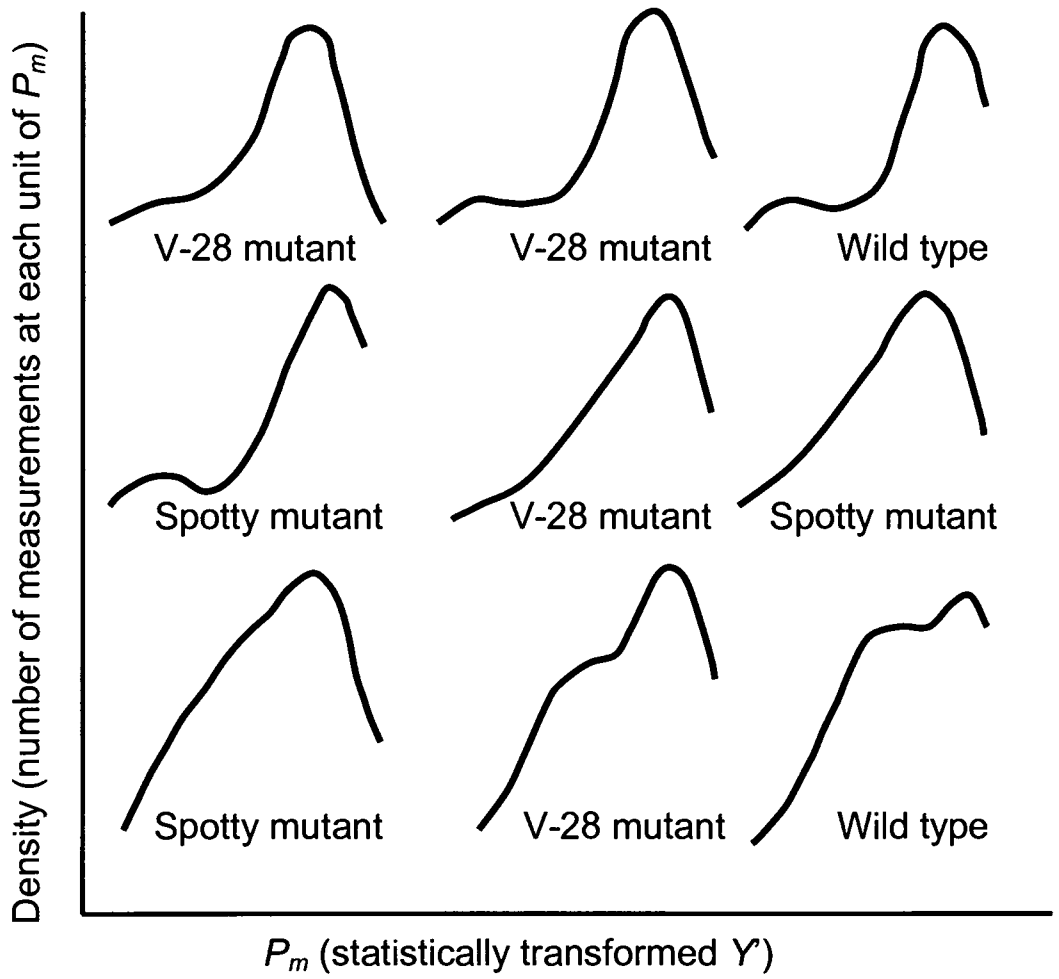

Figure 2.4. A larger set of data also showing statistical analysis from second variegated mutant (V-28).

We were getting nowhere looking for our explanation; thus we had to be sure that our observation was not merely a trait particular to our first experimental plant *Arabidopsis*. This unusual weed is known to have a reduced genome and to grow very quickly, and this might very well be a new peculiar of the weed (50). We explored other species; it turned out that the phenomenon occurred in other plants. An example of this is shown here for *Amaryllis* (Figure 2.5, Figure 2.6).

As Figure 2.6 illustrates, the phenomenon is indeed general, and is limited to the base of the *Amaryllis* plant where greening and, thus, chloroplast division takes place.

This information suggested that an explanation based on chloroplast division was possible. Research using an obscure alga, *Heterosigma carterae*, has demonstrated that when chloroplasts divide, photosynthetic efficiency decreases (51). This gave a theoretical explanation that fits our data as is shown in Figure 2.7.

Figure 2.5. The *Amaryllis* plants.

Figure 2.6. Plot of in vivo attenuance of photosystem I (estimated as 684-800 nm from [56]) viz. position on *Amaryllis* leaf, compared with pattern of photosynthetic efficiency in each part of leaf.

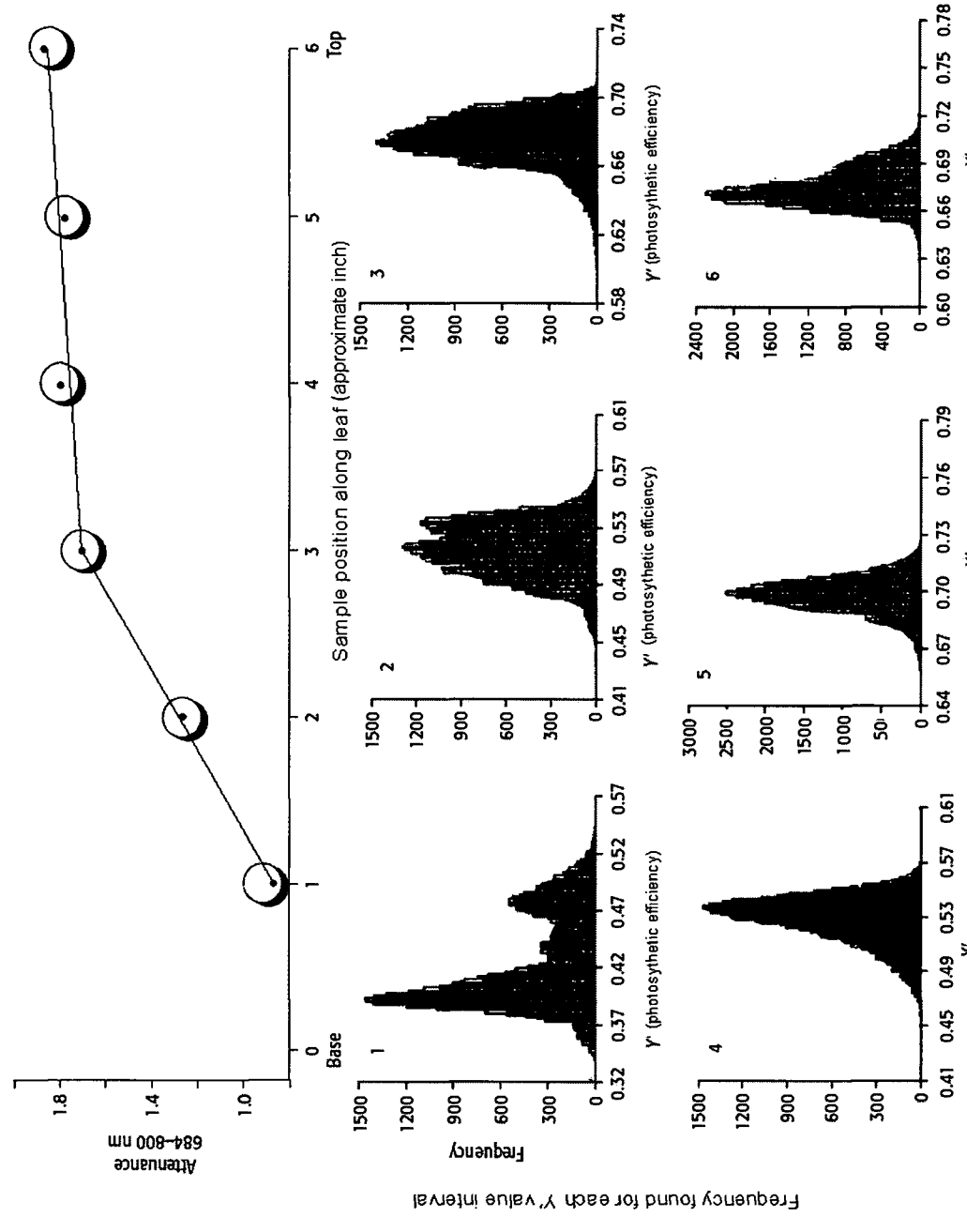
Attenuance 684–800 nm
1.0
1.4
1.8
Base
Top
Sample position along leaf (approximate inch)
Frequency
Y' (photosythetic efficiency)
Frequency found for each Y' value interval

FIGURE 2.6.

Figure 2.7. Plots of photosynthetic efficiency versus time (left; figure modified from [51]) and frequency versus chloroplast area (right; figure modified from [7]).

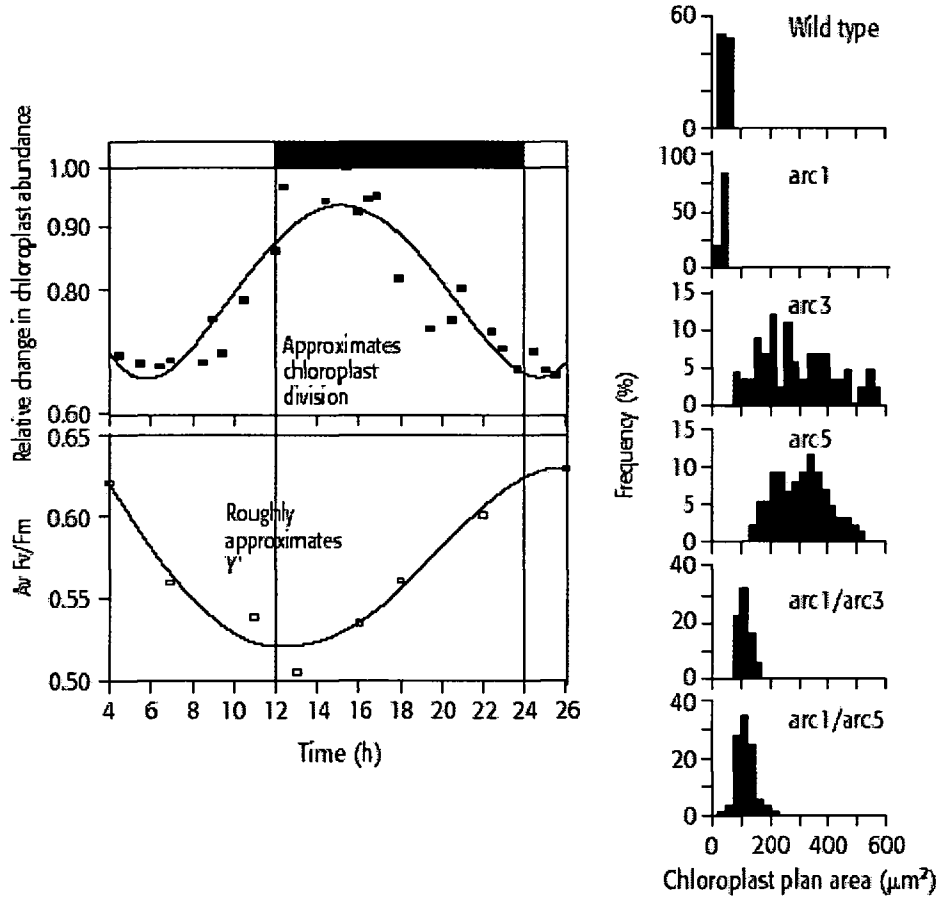
Relative change in chloroplast abundance
1.00
0.90
0.80
0.60
Approximates chloroplast division
Δv Fv/Fm
0.65
0.60
0.55
0.50
Roughly approximates γ'
4 6 8 10 12 14 16 18 20 22 24 26
Time (h)
Frequency (%)
Wild type
arc1
arc3
arc5
arc1/arc3
arc1/arc5
0 200 400 600
Chloroplast plan area (μm²)

FIGURE 2.7.

Now the explanation that what we observed was chloroplast division was quite novel and, if correct, quite important. Thus we applied for a grant; the reviewers did raise important criticisms.

One criticism of this chloroplast division explanation was that the number of chloroplasts per pixel of CCD covered by our detector was too large to explain the statistical discontinuity of our data.

This criticism would indeed be valid if we were observing all chloroplasts. We did a simple calculation of chloroplast size (see data in figure 2.7) using the data on chloroplast size of Pyke and Leach (7). This calculation showed that because the CCD pixels in our device are set up to cover 12, 626 μm^2 each pixel could gather signals from as many as 63, 200 chloroplasts. Such a group size of chloroplasts would be expected to generate an averaged efficiency signal rather than a biphasic one, damping out any differences. How then, the critics asked, could we see different populations with our device?

This question led us to reconsider basic concepts of plant function. One such assumption about photosynthetic function is that it is continuous. The fact that photosynthetic function is continuous on the macroscale of the whole leaf is easily provable (for example, see references 32-54). However, taking this assumption to the chloroplast level is not readily justified because measurements of the photosynthesis of a single chloroplast are difficult.

Although measuring a single chloroplast cycle is difficult, it is much easier to get a good number of chloroplasts synchronized, and this is almost as good for some purposes. What we (24), and many others before us (as cited in this article),

do when we measure chloroplast function is keep the leaf in the dark because then, after a few minutes of darkness, many of the chloroplasts have reset to the ready state.

When the instrument suddenly turns on the lights, these reset chloroplasts start from that point. The short wavelength excitation light impinges on the photosynthetic apparatus, which first responds by fluorescing strongly at longer wavelengths. Then, very rapidly, the fluorescence decreases as the chloroplast uses more of the incoming light to do chemical work (for example, see references 24, 41, 42, and 48). However, high background levels of fluorescence indicate that some chloroplasts continue to fluoresce and thus are inactive.

Another line of evidence (see APPENDIX "Factors that limit the number of functional chloroplasts") is that one can use this phenomenon to generate images; however, these images fade gradually in about 8-10 min (41). The gradual nature of the loss of these images informs us that not all chloroplasts recover immediately. Chloroplast function even under our conditions of prior dark adaptation and sudden influx of light are not synchronized; thus, for each chloroplast, light use is discontinuous.

An independent indication that chloroplasts do function intermittently is that, in vivo, sufficient chlorophyll to absorb 99.99% or more of the impinging light may be found in some leaves (see APPENDIX "Factors that limit the number of functional chloroplasts" in this article). This absorption level can be so complete that, were chloroplasts collecting light at all times, this excess synthesis of light-

collecting chlorophyll molecules would be an absurdly inefficient use of a plant's energies.

This makes it easy to see how, in such circumstance of low active chloroplast count per pixel, dividing chloroplasts with low efficiencies would be detected in a proportion of the pixel field.

So the number of active chloroplasts per pixel of the CCD may well be small enough to make the diversity among small populations of chloroplast that we observed, consistent with theoretical considerations.

CONCLUSIONS

Statistically we know that the existence of distinct populations of chloroplast efficiency is a real phenomenon, occurring both in wild-type and in variegated mutant *Arabidopsis*.

We infer from our data and logical analysis that what we are seeing is chloroplast division. This is consistent with data from another laboratory working a very phylogenetically distant species, *Heterosigma carterae* (51).

Preliminary experiments with other species using this method, along with confocal microscopy, support the idea that we are observing chloroplast division. This will be discussed in detail in the second part of this series.

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APPENDIX

The state of the art of chloroplast division observations is not yet Mature

DNA technology cannot be used directly to measure chloroplast division because there is not a linear correspondence of chloroplast genome to chloroplast number.

Scott et al. (15) described chloroplast DNA division in a young spinach leaf cell in rapid division as having ten or so immature chloroplasts with 70-100 copies of chloroplast DNA. As cell division ceases in most nuclei, the DNA remains diploid; however, there is a period of continued division of chloroplast DNA giving increased DNA per chloroplast. Then, as the leaf is still expanding, chloroplast DNA division ceases, and chloroplast, division and cell enlargement become the major processes. At the end of the process, the mature spinach leaf cell contains 25-50 chloroplasts with about 20-40 copies of chloroplast DNA per chloroplast.

From the above, it is clear that chloroplast genomes are multiple and the number of genomes not constant. Thus, measurement of chloroplast DNA is not an adequate measure of chloroplast division.

Another consideration that detracts from DNA measurement as a measure of chloroplast division is that chloroplast components can be directed by nuclear genes, such as is the small Rubisco subunit; therefore, probes used for this purpose must recognize bona fide chloroplast genes. Thus, it would seem that DNA technology is not, as yet, readily applicable to test our technology.

Microscopy has limitations. Until recently, the main methods used to observe chloroplast division were light and electron microscopy (10, 16). Using these methods and painstaking work, a number of characteristics of dividing chloroplasts were established. Yet, microscopic observation of chloroplast division is not one of the simpler tasks in biology. Physical observation of chloroplast division is enormously demanding. Chloroplasts exist as roughly ovoid to tubular objects moving in flexible undulating fashion in three dimensions. Thus, in most high plant species, it takes dedication, persistence, and a very large number of microscopy observations just to catch and verify chloroplasts in the act of division.

Some investigators have approached this problem using a different mutant of *Arabidopsis* having fewer, but larger, chloroplasts per cell (17). Using these *Arabidopsis* mutants, it was established that these plants compensate for reduced chloroplast number by allowing the few chloroplasts to grow much larger (20x) than in wild type (7, 17, 18). These mutants have allowed some very elegant studies of chloroplasts division (19); however, practical measurement of chloroplasts division by this approach is limited to the mutant in question.

Confocal microscopy, an increasingly used technology, (20, 21) allows one to observe and follow the shape of the chloroplast through the z direction ($z = \sim$ depth [22]); however, this technology requiring large fixed instrumentation is still very cumbersome for uses such as these that require repeated measurements.

Factors that limit the number of functional chloroplasts

CO_2 restrictions. Low CO_2 levels limit chloroplast function. Thus, when CO_2 limits photosynthesis, some chloroplasts do not have enough of this gas to function.

Shading. Chloroplasts can stack in leaf cells. For a chloroplast to function, it must be accessed by light for the machine to detect it. Thus, if there are too many chloroplasts to function at our light intensity, and they self-shade, we only work with the ones that have enough light to function.

Reaction center recovery. Photosynthetic systems in chloroplasts do not work all the time they need to for recovery. Use of light by living leaves is a slower process than many imagine. It is true that the first steps of the light reactions are extremely fast and these first steps occur in picoseconds. However, it is known that it takes about 12 s for the photosynthetic process to fully function, and it takes about 3 min for a leaf to reach steady state (24). However, this steady state still has considerable fluorescence indicating that, at any given time, nonfunctioning chloroplasts are present.

In experiments in which we measure the recovery time of leaf photosynthesis function by the disappearance of an imposed image, it takes about 8 min for the image to essentially disappear and before photosynthetic function is restored to all chloroplasts in sample (41). The observation also demonstrated that chloroplasts need to recycle.

Another observation is that leaves have a high optical density (A) at 684 nm, the peak absorption for photosystem II. This A is often, as in wheat (not shown) in

the order of 3-4 *A* (or more) at the 684 maximum (55). 1*A* absorbs 90% of the light, 2*A* 99%, 3*A* 99.9%, and 4*A* 99.99%, yet *A* is proportional to concentration. Because *A* is directly proportional to concentration, a 4*A* leaf has, at least for some structural factors, four times the chlorophyll of a 1*A* leaf.

Thus, for a 1*A* leaf to become a 2*A* leaf it has to double its chlorophyll for a return of 9% increase, and it has to double its chlorophyll for a return of 0.9% increase. This raises the question, why should leaves expend that kind of energy for such a small return? A more rational explanation is that the leaf chlorophyll is partitioned into photosynthetic units (here chloroplasts) that function at different times. And at any given time, most chloroplasts are recovering while only a few are in active light-gathering function.

AUTHORS

Ping Zheng is a graduate student in the Department of Horticulture, OSU (Corvallis, OR, 97331); she may be contacted by e-mail at zhengp@bcc.orst.edu.

Carolyn Wetzel is a recent graduate with a Ph.D. from in the Botany Department, Iowa State University (Ames). She may be contacted in care of Steven Rodermel, e-mail: rodermel@iastate.edu.

Karim Ammar is a breeder in the Hybrid Wheat Program at CIMMYT International, Apdo. Postal 6-641, 06600, Mexico D.F., Mexico; e-mail: k.ammar@cgiar.org.

Anne-Marie Michelle Girard is faculty research assistant at the Center for Gene Research and Biotechnology, OSU; e-mail: girarda@bcc.orst.edu.

Steven Rodermel is a professor in the Botany Department, Iowa State University (Ames); e-mail: rodermel@iastate.edu.

David R. Thomas is professor emeritus in the Department of Statistics, OSU; e-mail: thomas@stat.orst.edu.

Li Ning is a design engineer at Chrontel (San Jose, CA); (408) 544-2171, fax (408) 383-0338; e-mail: nli@chrontel.com.

James B. Callis is a professor in the Department of Chemistry, University of Washington (Seattle); (206) 543-1208, e-mail: callis@cpac.washington.edu.

Gerry E. Edwards is a professor in the Botany Department, Washington State University (Pullman); e-mail: edwardsg@ wsunix.wsu.edu.

Larry Daley is a professor in the Department of Horticulture, OSU; e-mail: daleyl@peak.org.

CHAPTER 3

TEST OF AN IN VIVO METHOD TO DETECT CHLOROPLAST DIVISION IN CROP PLANTS PART II: VERIFICATION OF THE PHENOMENON BY GERMPLASM METHODS AND CONFOCAL MICROSCOPY

Ping Zheng, Carolyn Wetzel, Karim Ammar, Anne-Marie Michelle Girard, Steve Rodermel, David R. Thomas, Li Ning, James B. Callis, Gerry E. Edwards, and Larry Daley

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The *Amaryllis* plants.

This article is the second installment in a series about a novel spectrofluorometric method that apparently allows for in vivo observation of division of chloroplast populations in leaves of *Arabidopsis thaliana.*

INTRODUCTION

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 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, monocotylednary 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

The *Amaryllis* plants.

Plant materials. Growth conditions were described in reference 1. We used Oregon State University (Corvallis, Oregon) soil mix [which is, by volume, two parts pumice, one of peat (Canadian medium horticultural grade), and one of sandy loam soil] (Ning et al., 1995) and Osmocote (Sierra Chemical, Milpitas, CA) slow-release fertilizer (14-14-14) in amounts and release times appropriate for each species; the greenhouse crew waters twice a day or as needs 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.

Data processing. Fluorometric analysis and data processing are described in reference 1.

Confocal microscopy. Confocal microscopy was done on Oregon State University's Leica (Hiedleberg, 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). 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 3.1) -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 3.1 and 3.2).

As expected, the most complex patterns were found in leaves of the C-4 plant sugarcane (Figure 3.1). 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 3.2). 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 3.2).

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.

Figure 3.1. Distribution plots of chloroplast efficiencies from the base to the top of sugarcane (*Saccharum officinarum L. or S. hybrid*) leaves.

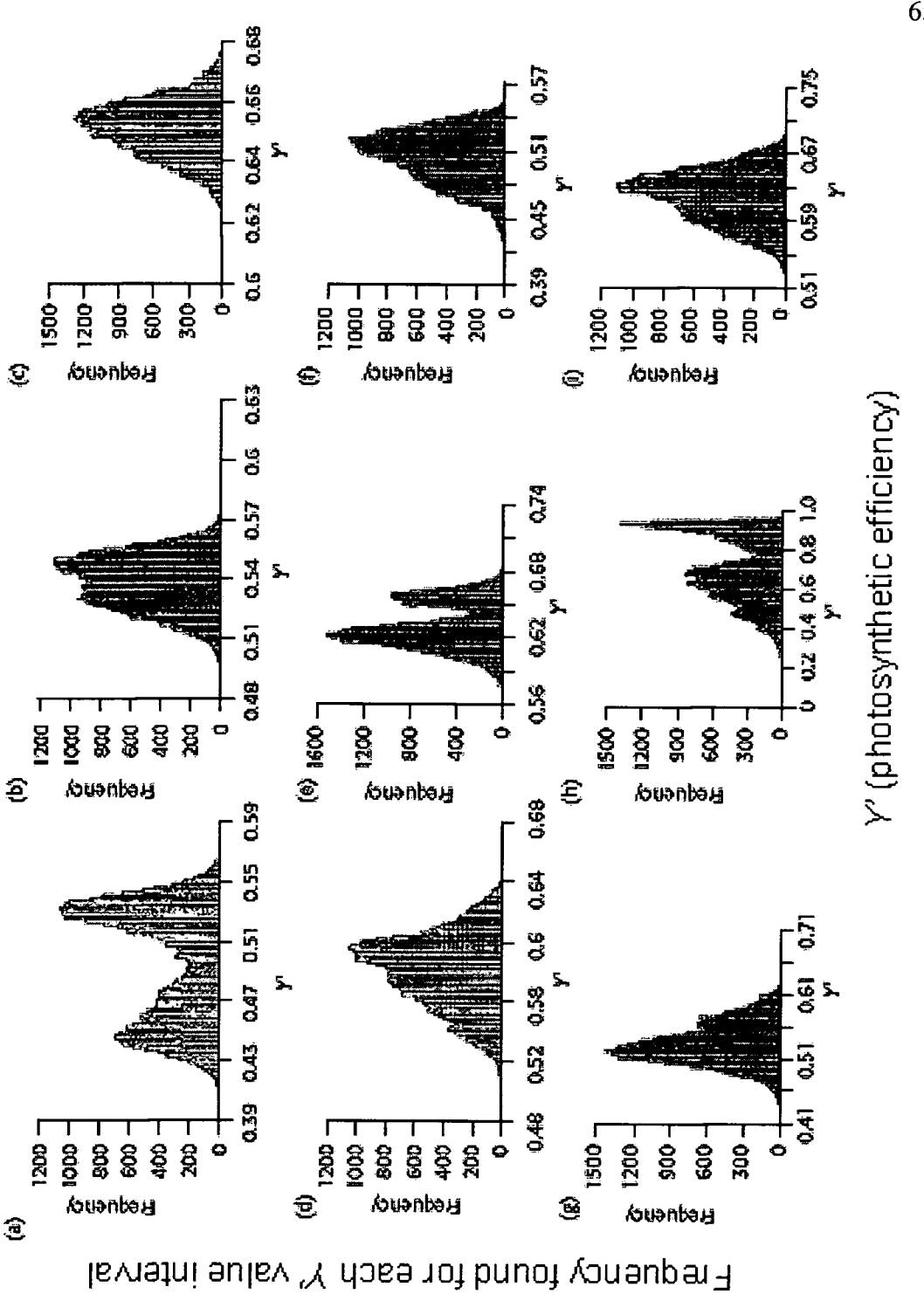

Frequency found for each Y' value interval
Y' (photosynthetic efficiency)
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
Frequency
Y'

FIGURE 3.1.

Figure 3.2. Populations of chloroplast rank by efficiency Y' along a leaf of an *Amaryllis* plant. In this plant, as in most monocots, cell growth occurs at the node where the leaf intersects the stem. Thus, the oldest, nondividing tissues are at the top of the leaf and the youngest are at the bottom.

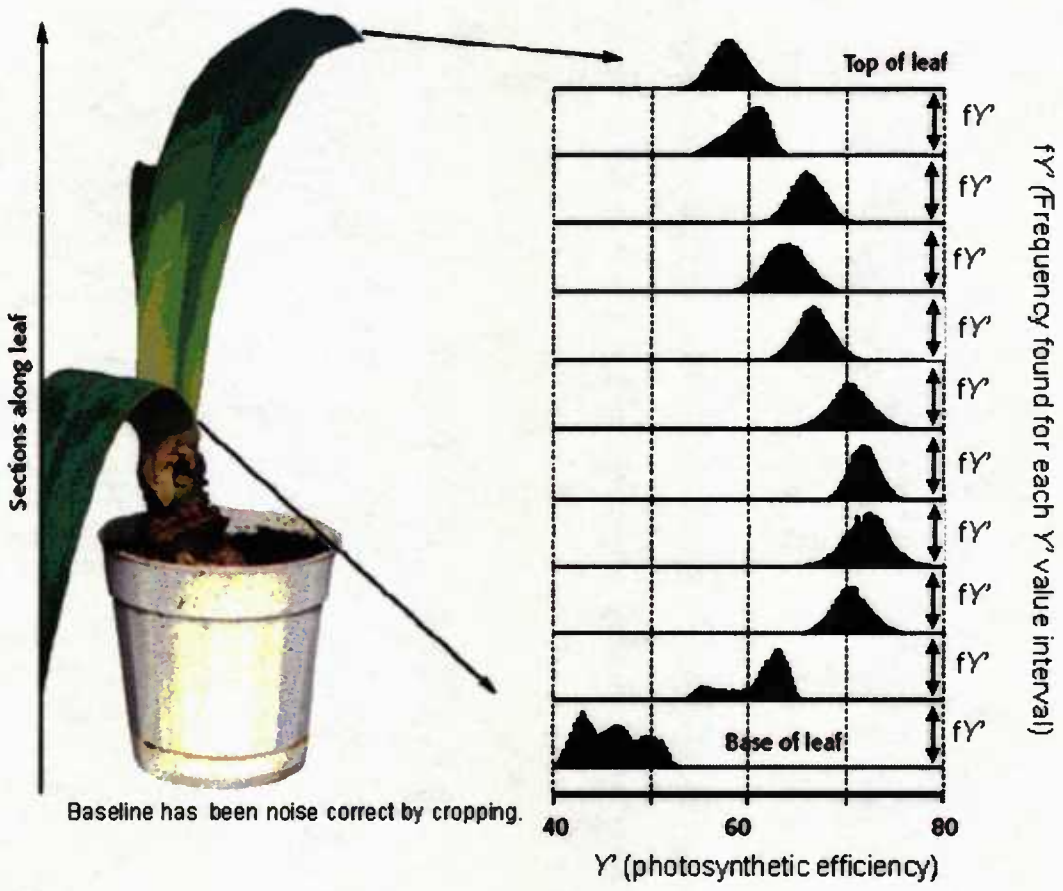

Sections along leaf
Top of leaf
Base of leaf
fY'
fY' (Frequency found for each Y' value interval)
Baseline has been noise correct by cropping.
40
60
80
Y' (photosynthetic efficiency)

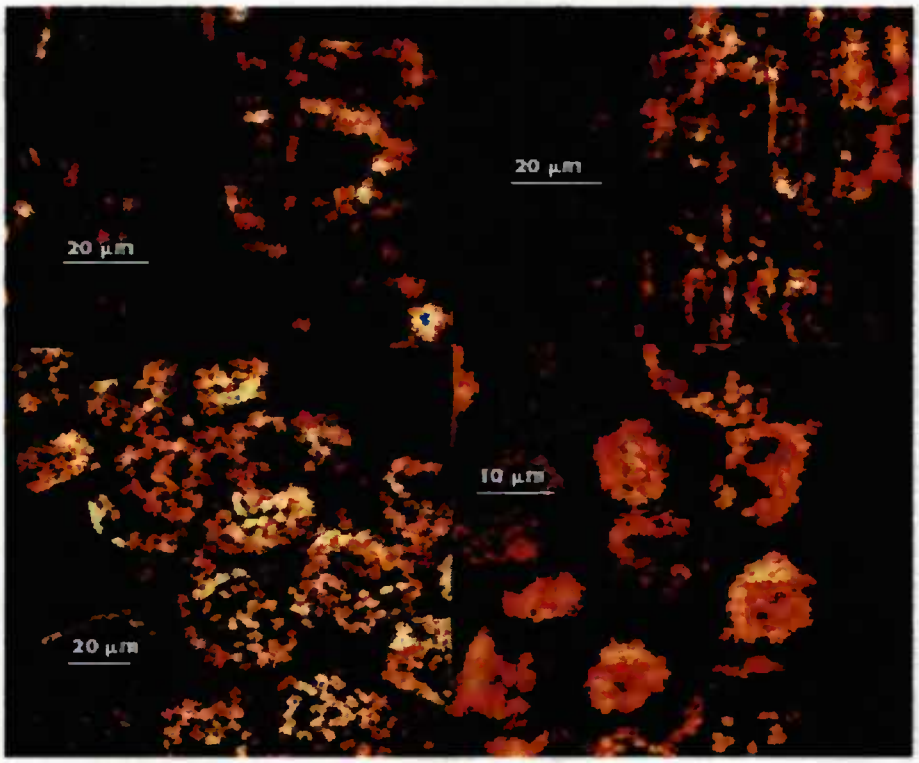
FIGURE 3.2

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 3.3 and 3.4).

Figure 3.3 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 3.3, 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 resolution (Figure 3.4), and more dumbbell-shaped chloroplasts were apparent.

Figure 3.3. 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).


20 µm
20 µm
10 µm
20 µm

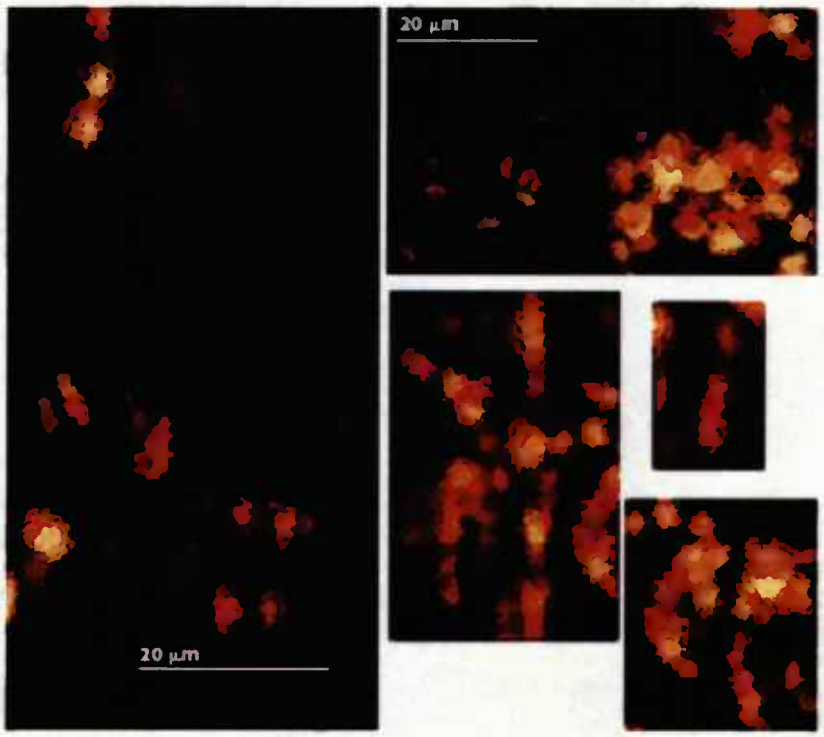


Figure 3.4. Confocal images of young tissues

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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The *Amaryllis* plants.

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AUTHORS

Ping Zheng is a graduate student in the Department of Horticulture, OSU (Corvallis, OR, 97331); she may be contacted by e-mail at zhengp@bcc.orst.edu.

Carolyn Wetzel is assistant professor at Smith College (Northampton, MA). She may be contacted by e-mail at cwetzel@smith.edu.

Karim Ammar is a breeder in the Hybrid Wheat Program at CIMMYT International, Apdo. Postal 6-641, 06600, Mexico D.F., Mexico; e-mail: k.ammar@cgiar.org.

Anne-Marie Michelle Girard is faculty research assistant at the Center for Gene Research and Biotechnology, OSU; e-mail: girarda@bcc.orst.edu.

Steven Rodermel is a professor in the Botany Department, Iowa State University (Ames); e-mail: rodermel@iastate.edu.

David R. Thomas is professor emeritus in the Department of Statistics, OSU; e-mail: thomas@stat.orst.edu.

Li Ning is a design engineer at Chrontel (San Jose, CA); (408) 544-2171, fax (408) 383-0338; e-mail: nli@chrontel.com.

James B. Callis is a professor in the Department of Chemistry, University of Washington (Seattle); (206) 543-1208, e-mail: callis@cpac.washington.edu.

Gerry E. Edwards is a professor in the Botany Department, Washington State University (Pullman); e-mail: edwardsg@ wsunix.wsu.edu.

Larry Daley is a professor in the Department of Horticulture, OSU; e-mail: daleyl@peak.org.

CHAPTER 4

TEST OF AN IN VIVO METHOD TO DETECT CHLOROPLAST DIVISION IN CROP PLANTS PART III: STATISTICAL PROOFS OF OBSERVATION AND GENERAL UTILITY OF THE METHOD

Ping Zheng, Karim Ammar, Anne-Marie Michelle Girard,
Steve Rodermel, David R. Thomas, Li Ning, James B. Callis, Gerry E. Edwards,
and Larry Daley

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Ping Zheng, Karim Ammar, Anne-Marie Michelle Girard, Steve Rodermel, David R. Thomas, Li Ning, James B. Callis, Gerry E. Edwards, and Larry Daley

This article is the third installment in a series about a novel spectrofluorometric method that allows for in vivo observation of the division of chloroplast populations in leaves of *Arabidopsis thaliana*.

INTRODUCTION

This article provides a statistical approach allowing quantification of probability in two- dimensional in vivo fluorescence spectroscopy of biological samples.

When using spectroscopy for chemicals, the number of molecules, atoms, or other moieties, even when observed in complex matrices, is commonly very large, and their state relatively homogeneous. During biological in vivo experiments, the moiety units explored are usually far larger in size, less concentrated, more variable, and found in far fewer numbers than common in chemicals or even biochemical work. Thus, in vivo fluorescent spectroscopy of the relatively small numbers, large variability and great complexity of these biological moieties offers exceptional statistical challenges.

Chloroplast division is critical in plant development. A novel spectrofluorometric method which allows for in vivo observation of division of chloroplast populations in leaves of *Arabidopsis thaliana* mutants was described in part one of this series (1). A second paper followed that described preliminary confocal microscopic work and confirming investigations using amaryllis, wheat, sugarcane, narcissus and other plants (2).

The technology of this series of articles holds promise in applied, as well as theoretical, areas of plant science. This article describes the novel statistical methods used for the initial discovery. We expect this statistical approach to have more general applications.

Chloroplasts, the green organelles found in plant cells, are central to life on Earth because of their photosynthetic functions and the leaves of higher plants are packed with them; however, the seeds from which these plants grow are small compared the mature plant, and seeds commonly have few or no functional chloroplasts. Chloroplasts have a separate genome, which codes much of the chloroplast's function. Thus chloroplasts in plants must arise through repeated chloroplast division.

Chloroplast division occurs primarily in developing leaf mesophyll cells, where chloroplast division is essential to mesophyll development. The first article in this series identifies the environmental factors that influence chloroplast division including cell size and nuclear ploidy and the environment of the leaf, especially light quality (1).

Chloroplast accumulation in developing leaves enormously affects photosynthetic production, yet control of chloroplast division process itself is one of the least understood areas of chloroplast biology (3). This situation calls for improved technology, such as that which we have contributed (1, 2).

When we were trying to distinguish whether the observations, we made comparing *Arabidopsis* mutants and wild type (natural or unmutated) plants, we found that in both there were multiple populations of chloroplast efficiencies. Puzzled, we were forced to develop novel statistical tools to distinguish between really different and spurious segregated populations.

EXPERIMENTAL

Fluorometric analysis. Fluorometric analysis followed the procedure described by Ning et al. (4). Our laboratory developed an imaging fluorometer/spectrophotometer (4-10) that can quantify the fluorescence characteristics of leaves in two-dimensional space. This instrument detects changes related to the photosynthetic function and pathology of plants. Our imaging fluorometer uses a charge coupled device (CCD) as detector element, acquiring spectra for 31,680 positions per sample (12-bit, Lynxx, PC CCD Imaging System, SpectraSource Instruments, Westlake Village, CA).

Plant materials. The material was grown in the greenhouses adjacent to the laboratory, sampled with permission from some of the material grown by other researchers at Oregon State University (OSU, Corvallis), or sent by express mail from other researchers across the country.

The *Arabidopsis thaliana* used for these experiments were prepared and grown (11) by Steve Rodermel at Iowa State University of Science and Technology (Ames). These materials consisted of grown Arabidopsis plants. The *Arabidopsis thaliana* germplasm used was: V28, spotty and wild type. Plants were shipped, carefully protected and by express mail service, from the Iowa laboratory site to the OSU laboratory site. Once the plants arrived at OSU, they were carefully unpacked and allowed to grow overnight to permit recovery of photosynthetic function from shipping stresses.

Leaves were sampled and then analyzed by the imaging fluorometer (1, 2). These *Arabidopsis thaliana* leaves were taken at zero, four, and eight days after arrival from Iowa. Mutants and their growing conditions have been described previously (1).

When we grew our own plants we used OSU soil mix [which is, by volume, two parts pumice, one of peat (Canadian medium horticultural grade), and one of sandy loam soil] (Ning et al., 1995) and Osmocote (Sierra Chemical, Milpitas, CA) slow-release fertilizer (14-14-14) in amounts and release times appropriate for each species. The greenhouse crew watered each plant twice daily or as needs and controlled any pathogens and pests that could cause problems.

Program for data analysis. One of the most computation-intensive problems is to calculate maximum-likelihood estimate for a mixture of normal distributions. This process can be thought of as parametric estimations of likelihood functions. Maximizing these likelihood functions is complicated by singularities and numerous spurious maximizers. Here we used the Expectation Maximization (EM) algorithm, which is a technique for finding maximizers of likelihood functions (12). The program running the EM was written using MATLAB (The Math Work, Natick, MA) numerical software package.

This iterative EM algorithm is extremely reliable and usually finds the most appropriate maximizer from most reasonable initial guesses. However, it is very slow in cases where there is considerable overlap between component normal distributions.

We set up a model where the resultant distribution is considered as a mixture of normal distributions. To do this we wrote a novel program in numerical software package called MATLAB using the established formulae and mathematical conventions. In these formulas we consider the unknown distribution as mixture of a number of subdistributions from *1...m*; where *m* is the number of normal components in the mixture.

A *t*- variable normal density $p(x \mid \mu, \Sigma)$ with *t*- variable mean vector μ and *t* x *t* symmetric, positive definite covariance matrix Σ, is defined for *t*- variable real *x* by

$$p(x \mid \mu, \Sigma) = (\exp(-(x-\mu)^T \Sigma^{-1}(x-\mu)/2))/((2\pi)^{1/2} \mid \Sigma \mid^{1/2}))$$

Here the mixture has a *t*-variable normal distribution $p_m(x \mid \Theta)$, and is defined by the following equation:

$$p_m(x \mid \Theta) = \alpha_1 p(x \mid \mu_1, \Sigma_1) + ... + \alpha_m p(x \mid \mu_m, \Sigma_m) \qquad (1)$$

In the equation form $\alpha_1 p(x \mid \mu_1, \Sigma_1)$, the vertical line means "given." The notation $\{\alpha_1 p(x \mid \mu_1, \Sigma_1)\}$ means: given the values for μ_1 and Σ_1, the equation defines the probability that the *x* occurs. Here *x* is observed random samples; Θ collectively refers to all the individual component mean and covariance parameters along with the mixing proportions $\alpha_1, \alpha_2, ..., \alpha_m$, which must sum unity (that is 1) and have values between 0 and 1.

The theory of maximum-likelihood states that good estimates of the unknown parameter Θ_0 can be obtained from the log-likelihood function $L(\Theta)$. The function ($L(\Theta)$) is defined for a given t - variable sample $\{x_1,...,x_n\}$ and density of the form in (1), by

$$L(\Theta) = \log(p_m(x_1 \mid \Theta)) + \log(p_m(x_2 \mid \Theta)) + \log(p_m(x_n \mid \Theta)).$$

The chosen maximizer is denoted here by Θ^*. The approach considered here results from applying different optimization techniques to find Θ^* by maximizing $L(\Theta)$.

Optimization algorithms are iterative in nature. These algorithms will, in theory, generate an infinite sequence $\{\Theta_{(r)}\}$ of approximations to Θ^*. In this sequence each successive $\Theta_{(r)}$, tends to more closely approximate Θ^*.

The procedure starts with supplying an initial, and usually rough, estimate $\Theta_{(1)}$ of Θ^*. The main check used in these tests compares the new iterate $\Theta_{(r+1)}$ with the old iterate $\Theta_{(r)}$ at each step. When there is very little difference between successive iterates the process is near completion. This is because this circumstance usually means that the accuracy of $\Theta_{(r+1)}$ as an approximation to Θ^* will not be much improved by further iteration.

For above reason the implemented iteration is terminated as soon as two successive iterates are very similar. For $i = 1, ...m$, where m is the number of normal components in the mixture, the following calculations are done for $\alpha_{i(r+1)}, \mu_{i(r+1)}$ and $\Sigma_{i(r+1)}$:

$$p(x_k \mid \mu_{i(r)}, \Sigma_{i(r)})$$

Where r is the iteration, $i = 1,\ldots,m$, $k = 1,\ldots,n$

$$p_m(x_k \mid \Theta_{(r)}) = \sum \alpha_{i(r)} p(x_k \mid \mu_{i(r)}, \Sigma_{i(r)})$$

where $i=1$

$$W_{ik(r)} = \alpha_{i(r)} p(x_k \mid \mu_{i(r)}, \Sigma_{i(r)}) / p_m(x_k \mid \Theta_{(r)})$$

$$A_{i(r)} = (\sum_{k=1}^{n} W_{ik(r)})$$

$$\alpha_{i(r+i)} = A_{i(r)} / n$$

$$\mu_{i(r+1)} = (\sum_{k=1}^{n} x_k W_{ik(r)}) / A_{i(r)}$$

$$\Sigma_{i(r+1)} = (\sum_{k=1}^{n} (x_k - \mu_{i(r+1)})(x_k - \mu_{i(r+1)})^T W_{ik(r)}) / A_{i(r)}$$

The property of EM is such that $L(\Theta_{(r+1)}) \geq L(\Theta_{(r)})$ for each iteration, so that progress towards finding a maximizer is always being made.

RESULTS AND DISCUSSION

For this particular series of experiments seeking differences in photosynthetic efficiency between the mutant and the normal parts of these *Arabidopsis*, we reprocessed the photosynthetic efficiency data (1, 2). We ranked the data, with respect to efficiency on the x-axis with the y-axis as the number of pixels at that efficiency range.

This generated normal curves of the efficiency of the chloroplasts within each image. Our original intent was, in this way, to measure the relative proportions of mutated and normal tissue.

In these mutants we often, but not always, saw two populations with different photosynthetic efficiencies. This observation seemed to explain the differences between dark green and much lighter white or yellow sectors of the mutation plant's leaves (for example, unprocessed spotty mutant data presented in Figure 4.1). Preliminary examination of data made us realize that these divided efficiency populations of chloroplast suggested that this did not existed in wild type plants (unprocessed data in Figure 4.2)

To obtain statistical proof of this difference we tested the data statistically by a de novo rigorous statistical analysis for sub-populations (see Experimental). This statistical approach surprisingly showed that the presence of these sub-populations, in both wild and mutant plants (processed data in figures 4.3 and 4.4). This effect is attributed to the energy costs of chloroplast division (1, 3, 13).

Figure 4.1. Ranked and statistically processed data showing distinct populations of chloroplast efficiencies in *Arabidopsis* spotty mutants. Right frames (A and B) show data before statistical transformation. Left frames (C and D) show statistically transformed data. Upper frames (A and C) show curve fitting to histograms in lower (B and D) frames.

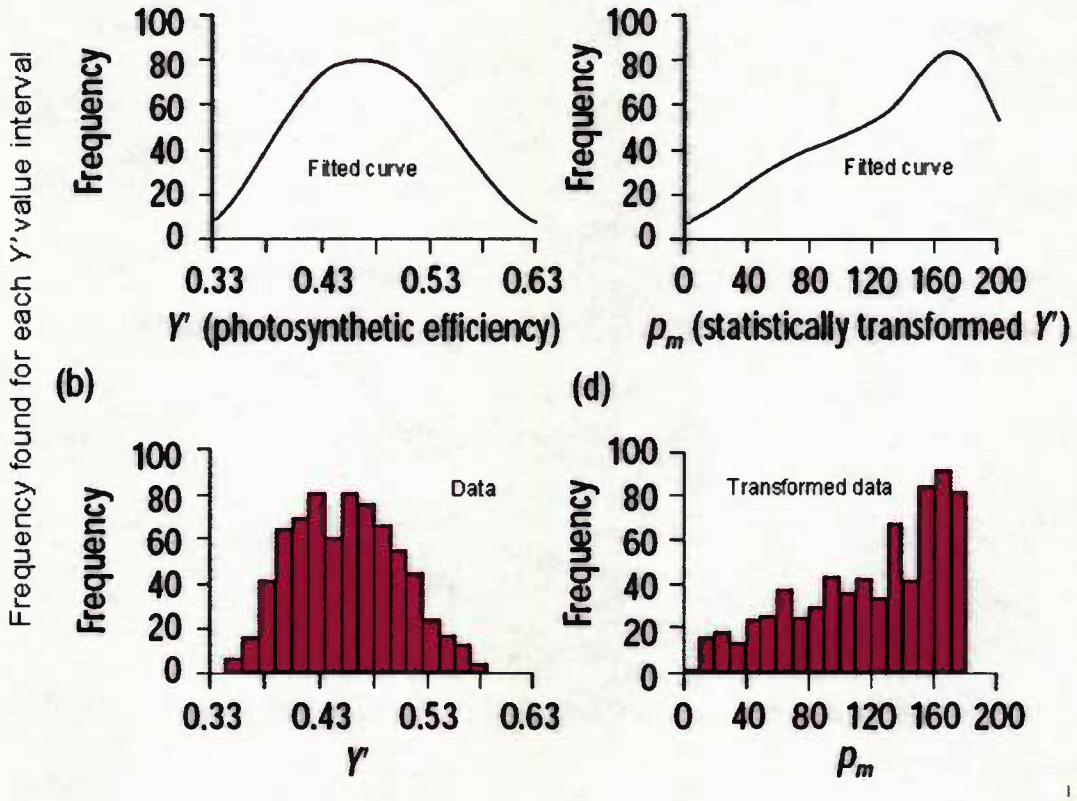
Frequency found for each Y' value interval
Frequency
100
80
60
40
20
0
Fitted curve
0.33
0.43
0.53
0.63
Y' (photosynthetic efficiency)
Fitted curve
0
40
80
120
160
200
p_m (statistically transformed Y')
(b)
Data
Y'
(d)
Transformed data
p_m

FIGURE 4.1.

Figure 4.2. Ranked and statistically processed data showing distinct populations of chloroplast efficiencies in wild type (non- mutant) *Arabidopsis*. Right frames (A and B) show data before statistical transformation. Left frames (C and D) show statistically transformed data. Upper frames (A and C) show curve fitting to histograms in lower (B and D) frames.

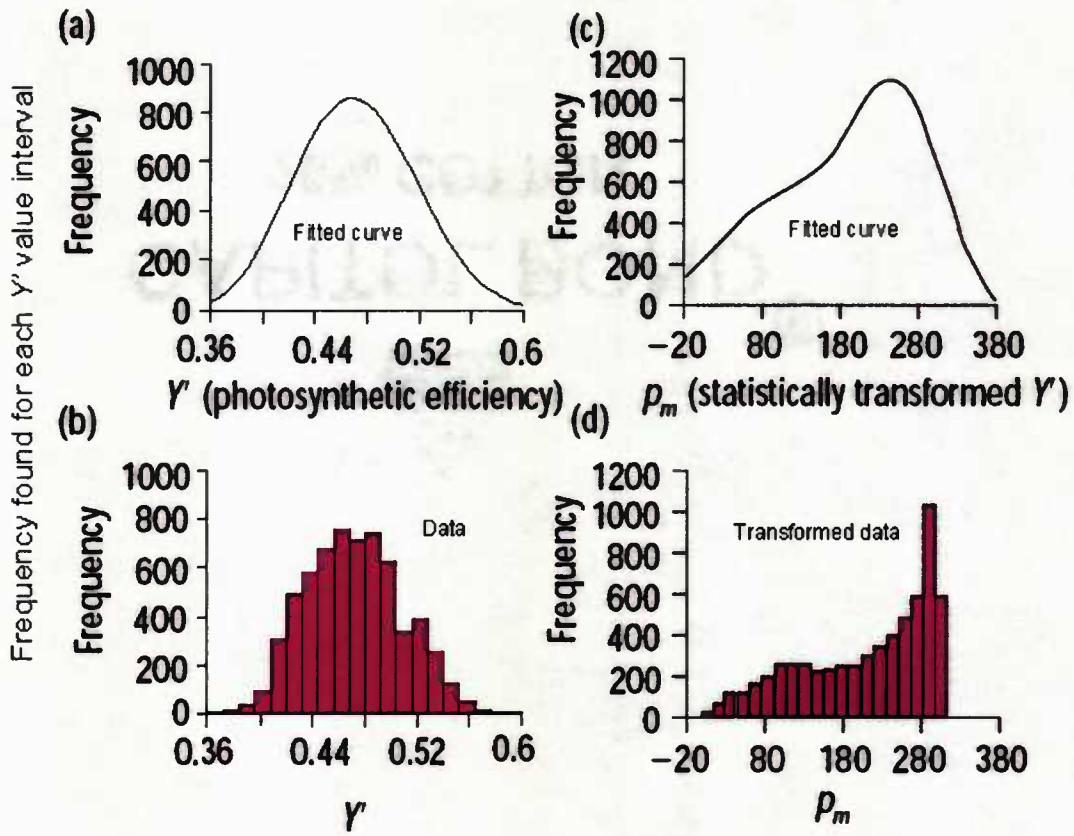
(a)
Frequency
1000
800
600
400
200
0
Fitted curve
0.36
0.44
0.52
0.6
Y' (photosynthetic efficiency)
(c)
Frequency
1200
1000
800
600
400
200
0
Fitted curve
−20
80
180
280
380
p_m (statistically transformed Y')
Frequency found for each Y' value interval
(b)
Frequency
1000
800
600
400
200
0
Data
0.36
0.44
0.52
0.6
Y'
(d)
Frequency
1200
1000
800
600
400
200
0
Transformed data
−20
80
180
280
380
p_m

FIGURE 4.2.

Figure 4.3. Statistically processed curves showing dual populations of chloroplast efficiencies within mutant and wild type leaves.

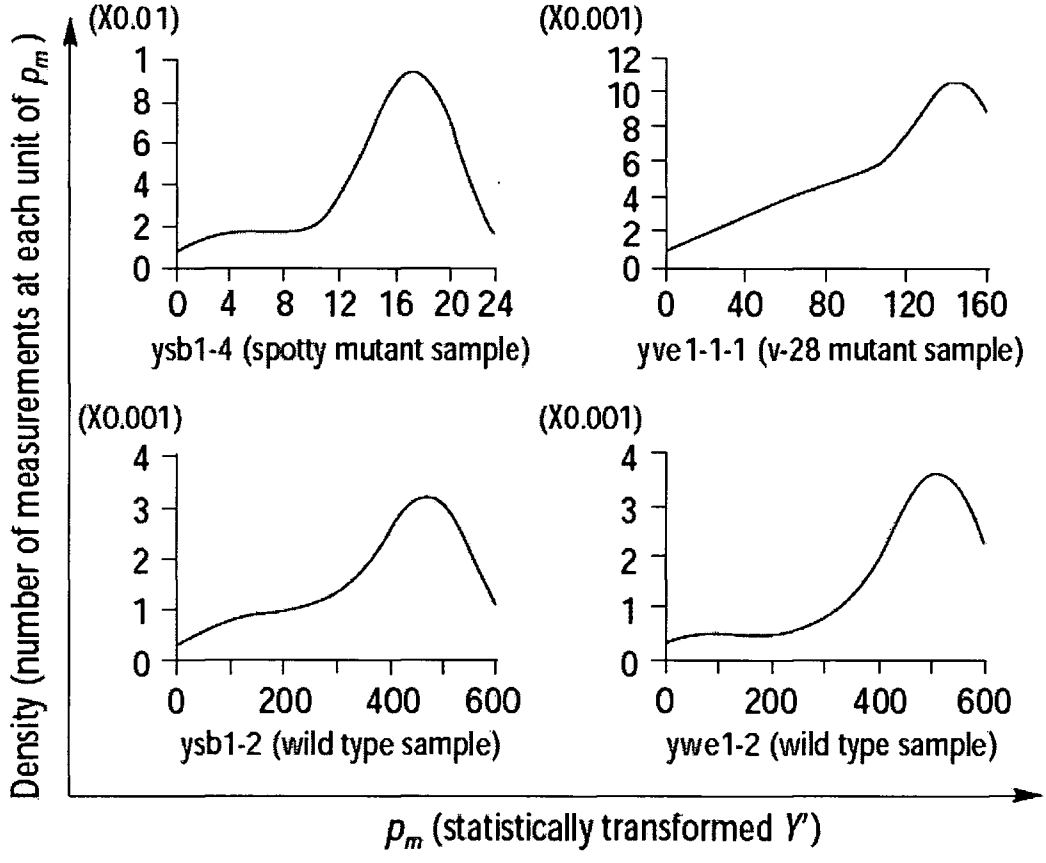
(X0.01)
1
8
6
4
2
0
0 4 8 12 16 20 24
ysb1-4 (spotty mutant sample)
(X0.001)
12
10
8
6
4
2
0
0 40 80 120 160
yve1-1-1 (v-28 mutant sample)
(X0.001)
4
3
2
1
0
0 200 400 600
ysb1-2 (wild type sample)
(X0.001)
4
3
2
1
0
0 200 400 600
ywe1-2 (wild type sample)
Density (number of measurements at each unit of p_m)
p_m (statistically transformed Y')

FIGURE 4.3.

Thus we embarked on a wider examination of mutant and wild type spectra using the same statistical deconvolution (Figure.4.4). With this data we began to clearly see two populations of chloroplasts with different efficiencies not only in other mutants but also in wild type *Arabidopsis* plants (Figure 4.4). This binary distribution did not occur in all samples and thus clearly was not some general property of statistical approach (for example, Figure 4.4). This frequent appearance of binary distribution of photosynthetic efficiency in both wild type and mutant plants was surprising, quite disturbing, very puzzling, and not especially tidy, for it blew our initial hypothesis clear out of the water.

Thus we came to the surprising conclusion that this phenomena, of which we were not sure of at the time but now know was related to chloroplast division, occurred in both mutant and wild type plants.

This showed that we had unwittingly developed a method to statistically show chloroplast division in plants (1, 3).

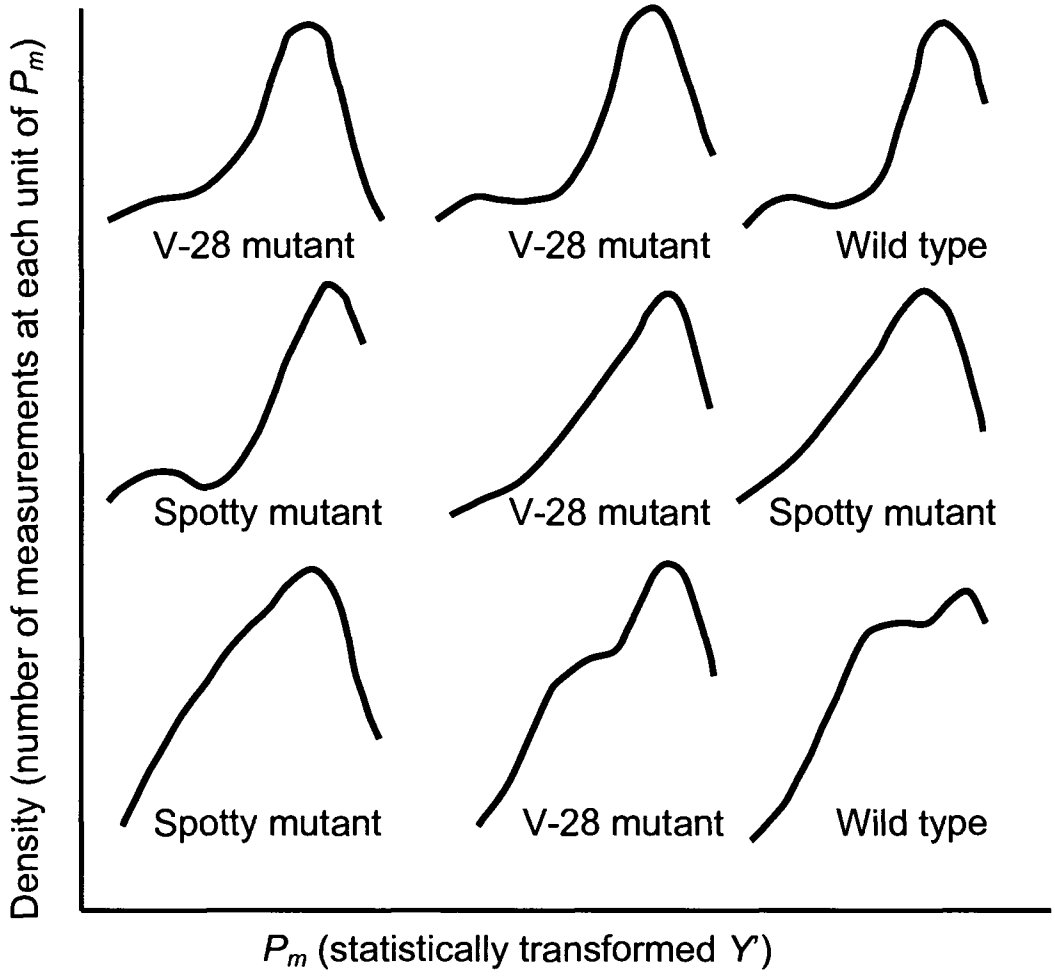

Figure 4.4. A larger set of data also showing statistical analysis from second variegated mutant (V-28) (adapted from reference 3)

CONCLUSIONS

Statistically we demonstrated that distinct populations of chloroplast efficiency are real phenomena, occurring both in wild type and in variegated mutant *Arabidopsis*. That these populations were resulted from the measurement of chloroplast division also became clear. Thus, a statistical method that we thought only applied to a particular use has far more general applications in deconvoluting biological data.

Once this method was applied and the phenomena found real, it was possible to consider that the large number of pixels sampled was sufficient to fulfill statistical requirements without further statistical analysis.

General use of this method allows statistical verification of bimodal and poly-modal distribution phenomena of data from obtained CCD cameras and other devices.

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AUTHORS

Ping Zheng is a graduate student in the Department of Horticulture at Oregon State University (OSU, Corvallis, OR). She may be contacted by e-mail at zhengp@bcc.orst.edu.

Karim Ammar is a breeder in the Hybrid Wheat Program at international Maize Wheat Improvement Center, CIMMYT (Texcoco, Mexico); e-mail: k.ammar@cgiar.org.

Anne-Marie Michelle Girard is a faculty research assistant at the Center for Gene Research and Biotechnology at OSU; e-mail: girarda@bcc.orst.edu.

Steven Rodermel is a professor in the Botany Department at Iowa State University (Ames); e-mail: rodermel@iastate.edu.

David R. Thomas is professor emeritus in the Department of Statistics at OSU; e-mail: thomas@stat.orst.edu or thomasd541@msn.com.

Li Ning is a design engineer for Broadcom Corporation (Fremont, CA); (408)922-7523, e-mail: nli@broadcom.com.

James B. Callis is a professor in the Department of Chemistry, University of Washington (Seattle); (206) 543-1208, e-mail: callis@cpac.washington.edu.

Gerald E. Edwards is a professor in the Botany Department, Washington State University (Pullman); e-mail: edwardsg@wsunix.wsu.edu.

Larry Daley is a professor in the Department of Horticulture at OSU; e-mail: daleyl@peak.org.

CHAPTER 5

A DIGITAL IMAGE PROCESSING TOOL

INTRODUCTION

We have developed an image processing tool to analyze an image/picture taken by confocal microscopy. This tool helps us answer our experimental questions. These questions are:

1. *Does the distribution of intensity of pixel within the large clusters of chloroplasts (where one presumes most chloroplast division occurs) follow a biphasic curve in each cluster?*
2. *Does cluster size influence this distribution?*

In order to achieve these goals, we: (1). Group pixels within the edges of chloroplasts. Each identified group is represented as a chloroplast. (2). Provide an interactive interface that allows the user to correct misdiagnoses made by computer. By utilizing an innovative configuration of image processing techniques, the tool is able to detect chloroplast division and test the hypothesis.

OVERVIEW OF PROGRAM

In this section, we describe each phase of the program by following the flow of the program. The screenshots taken during the execution of the program are presented. The program flow diagram can be seen in Appendix A

Program Phases:

1. Load confocal microscopy image.

When executing the program the first time, a user sees the main window with default image. To load a picture taken by confocal microscopy, a user clicks File->Open to select a desired image. Figure 5.1 shows the main widow displaying a loaded confocal microscopy image.

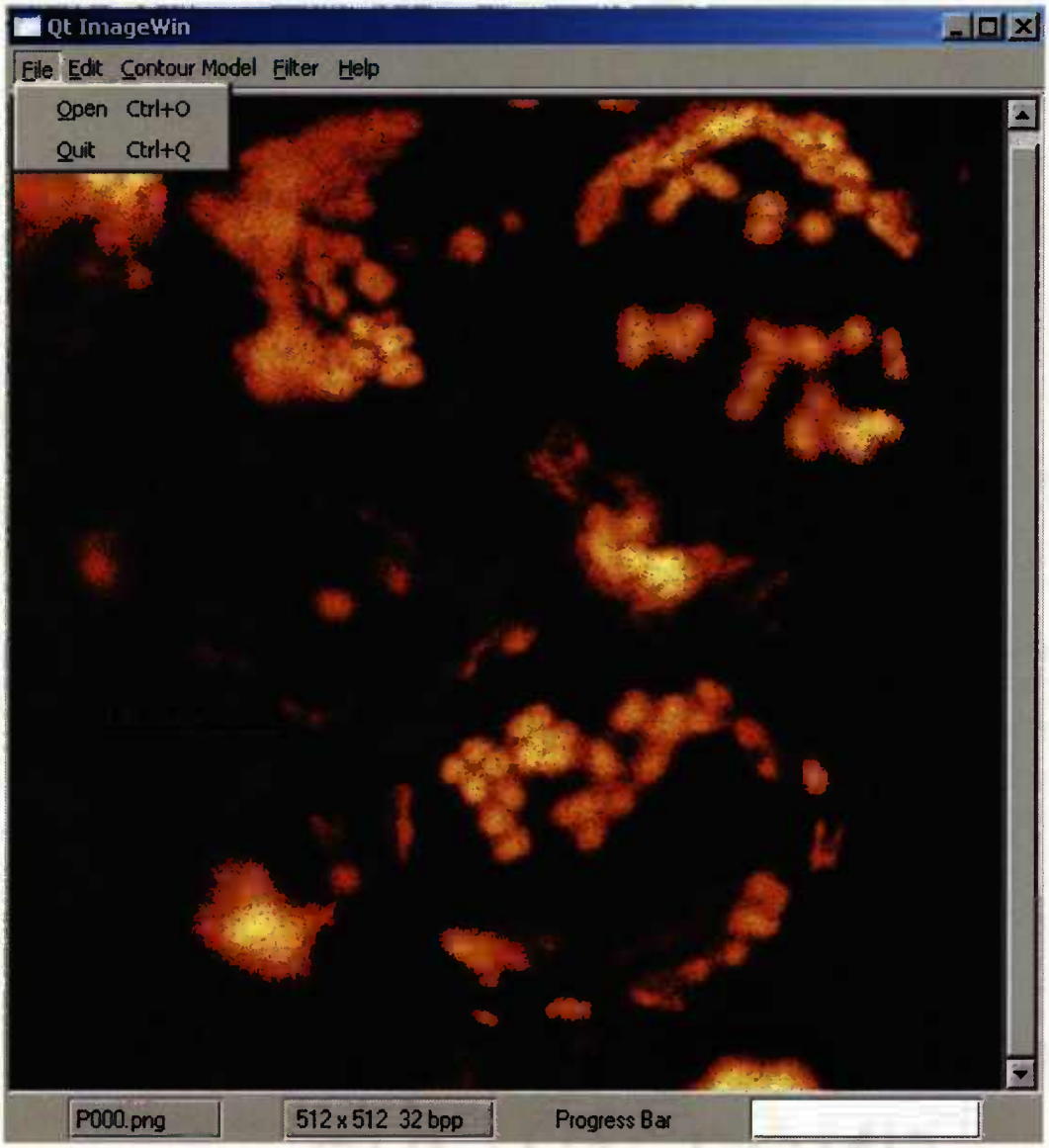

Figure 5.1. Shows main widow displaying a loaded confocal microscopy image.

2. Filter a loaded image.

The program provides Gaussian, Median and Convolution filters to remove noises of the loaded image (Figure 5.2). These filters are used to sharpen or blur an image. A user needs to select and apply one of these filters on the image at a time, until major noises of the image are removed (3). The selection of a filter depends on the conditions of the loaded image. A user may need to apply a different filter to the image several times.

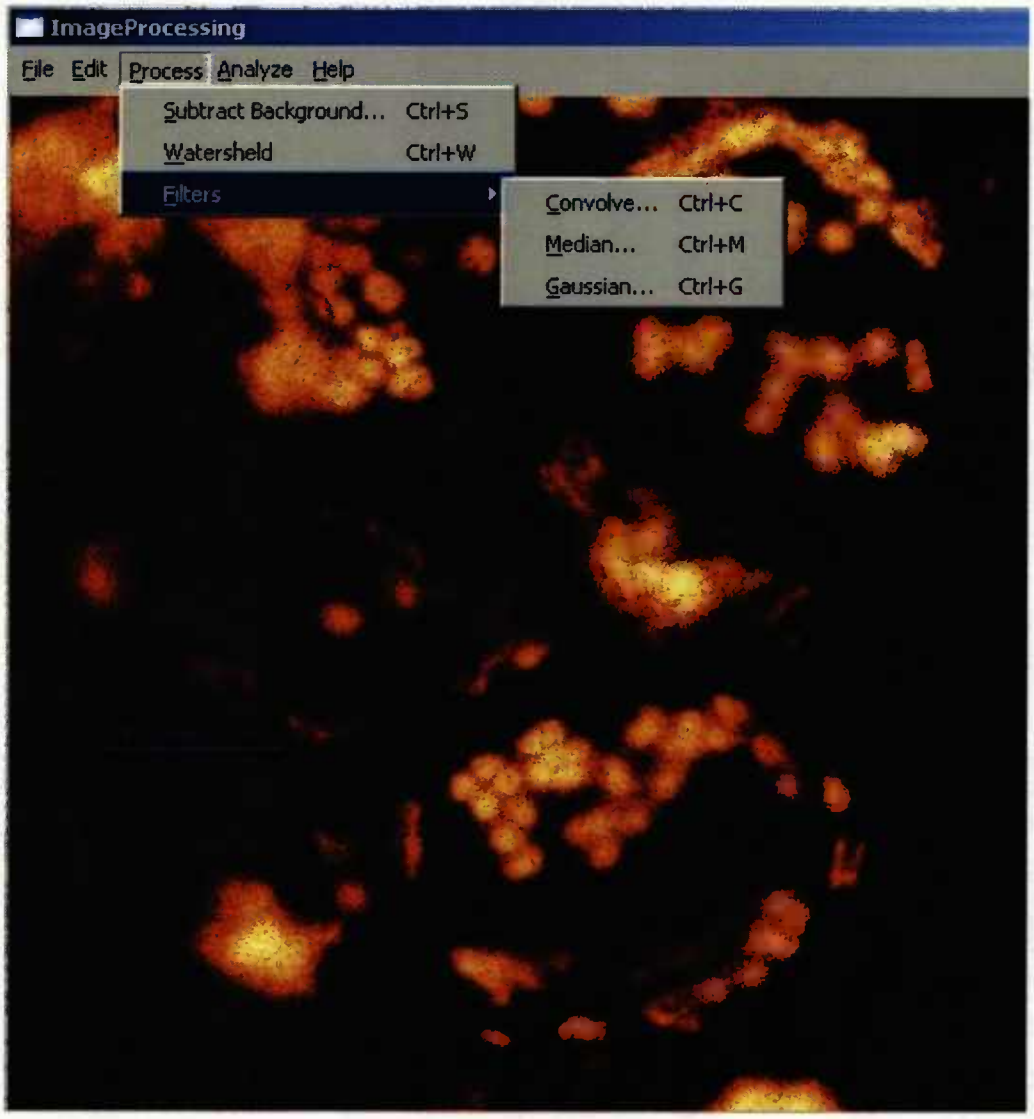

Figure 5.2. Filter menu: selection of filters

3. Identify Chloroplasts

3.1 Edge Detection

After the image is filtered and major noises are removed, the IPtool program performs an edge-detection algorithm to identify the edges of the objects in the filtered image.

3.2 Grouping pixels

Once all the edges of the objects in the image are detected, pixels are "grouped" within the edges. To group pixels, the program performs a breadth search algorithm on the labeled matrix computed in the edge detection phase. Each identified group represents a chloroplast.

4. Open Analysis window

When the Analysis window pops up, all computation has been done. The necessary information is stored in the internal data structure of the program. Figure 5.3 (a) highlights all chloroplasts identified by the previous process in the image.

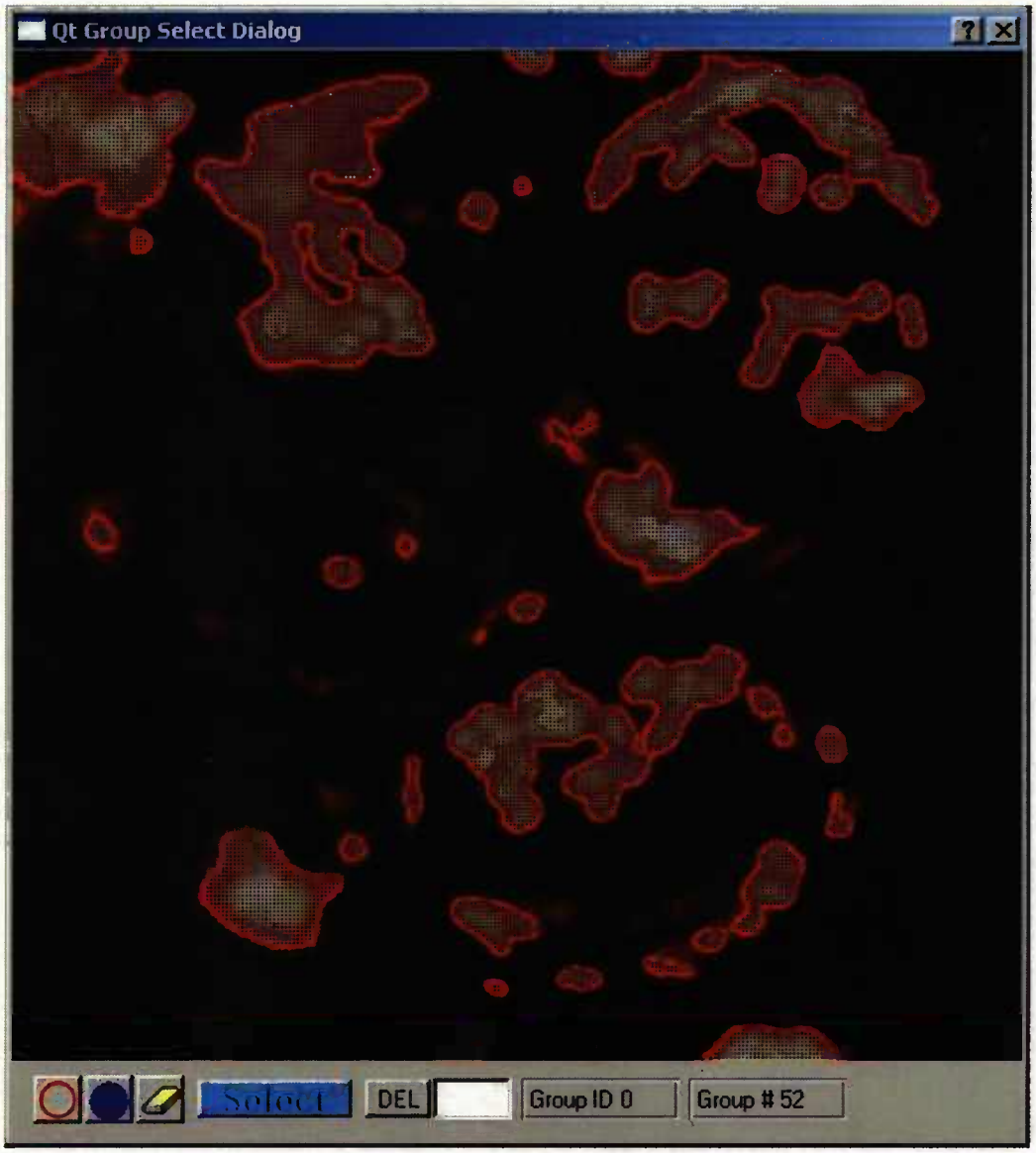

Figure 5.3(a) Highlights all chloroplasts in the image.

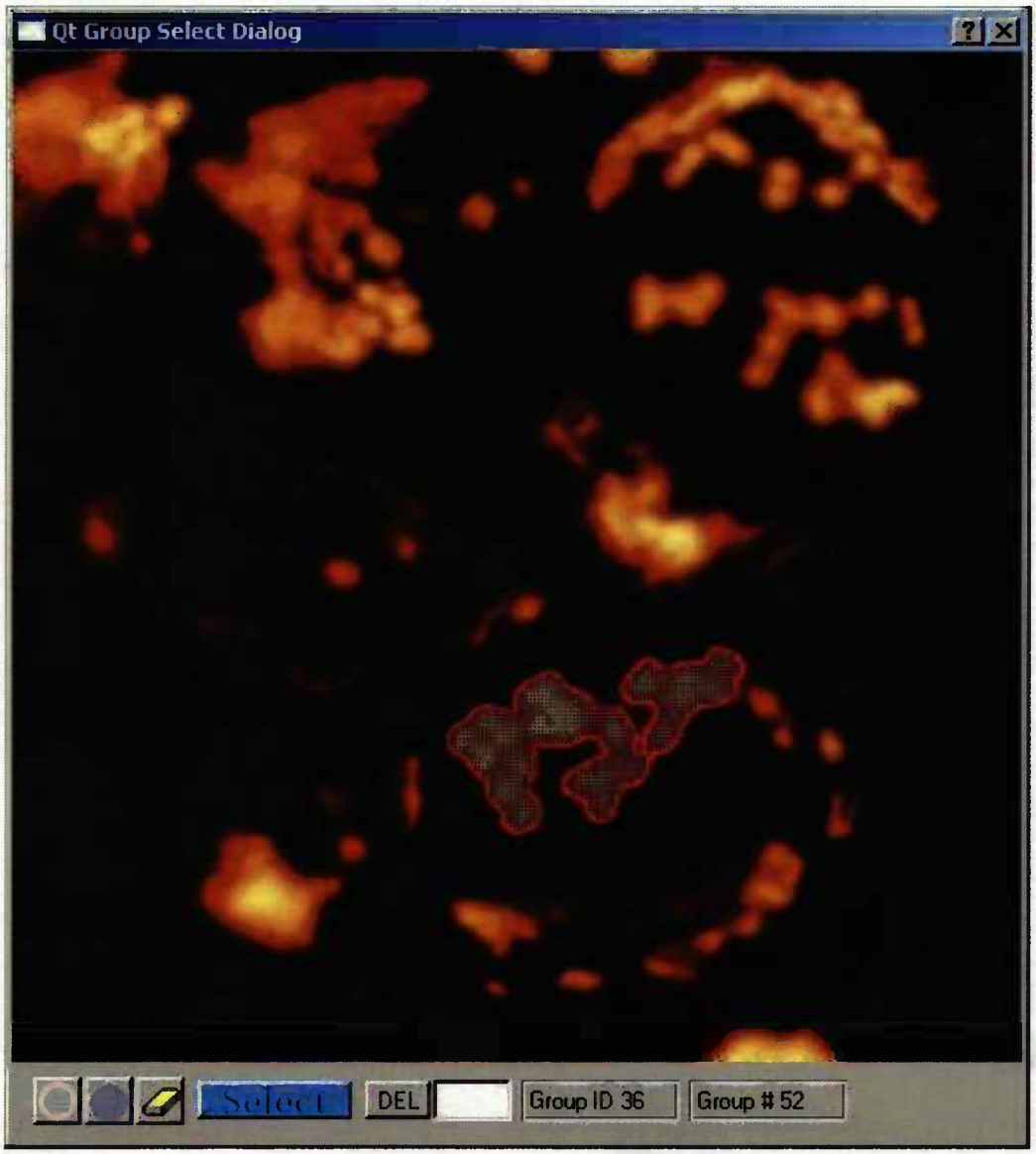

Figure 5.3(b) Select Group ID 36.

5. Correction

Given the nature of this image processing method, the program can not always give a perfect solution for a user. It is most likely not to have identified all chloroplasts in the image. Therefore, the IPtool provides add/delete functions to give a user an opportunity to visually correct the output from the preprocessing phase. Figure 5.4 (a) and Figure 5.4(b) show screenshots of performing the add/delete chloroplasts function. To delete one or more chloroplasts, a user first selects the chloroplasts to be removed. The selected chloroplasts are highlighted, then the user selects "Delete Group" from the popup menu by placing the cursor on the chloroplast(s) and clicking the right mouse button. To add a new chloroplast, a user draws an edge of a chloroplast. The QPen tool enables a user to draw an edge on the screen.

6. Retrieve Information

A user can retrieve the statistical information of any identified chloroplast in the image. The dialog box (Figure 5.5) shows the statistical information of the selected chloroplast. The top portion of the dialog displays the histogram of the selected chloroplast. The bottom of the dialog shows the group ID and size of a chloroplast.

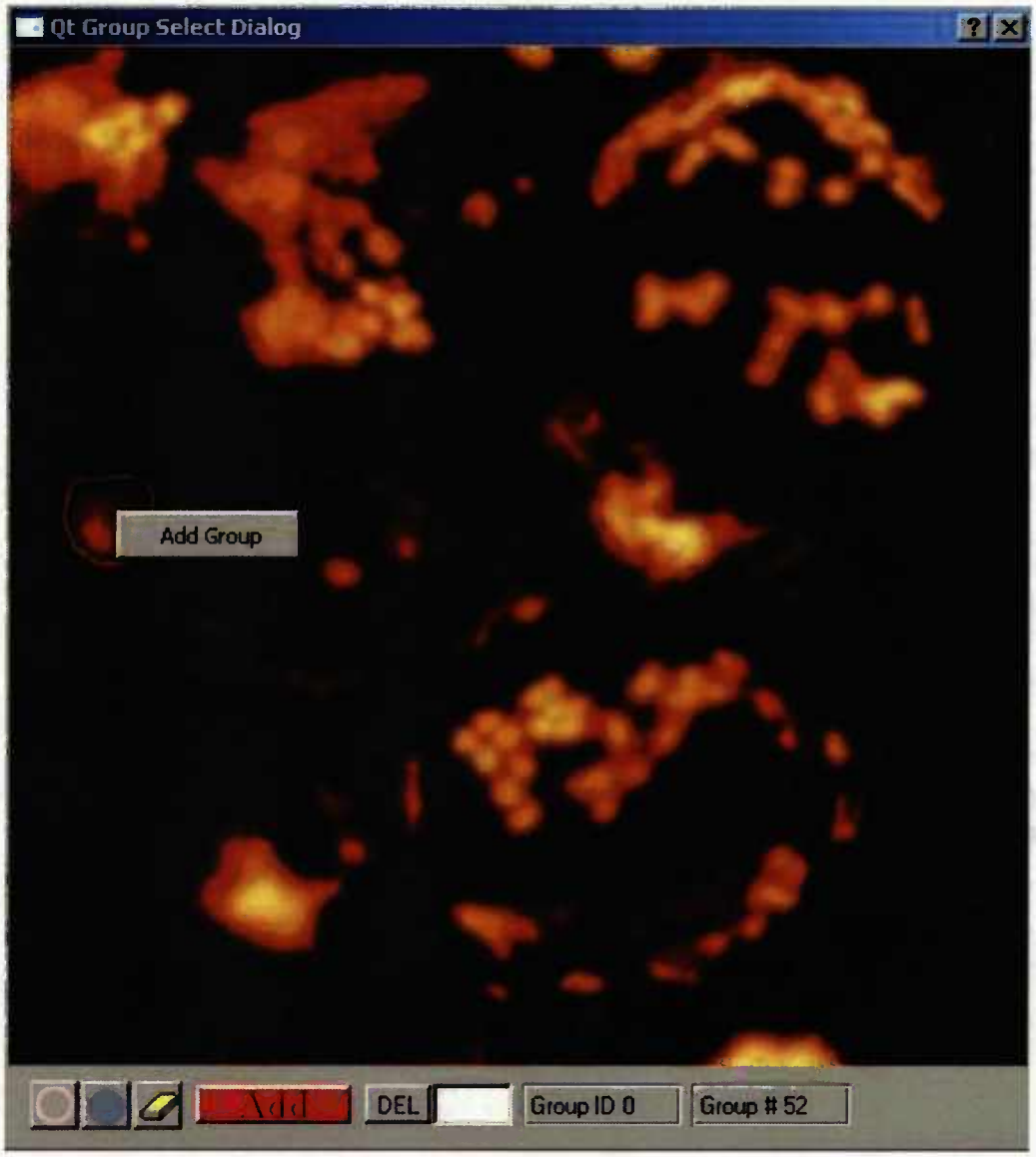

Figure 5.4 (a) Adding chloroplast

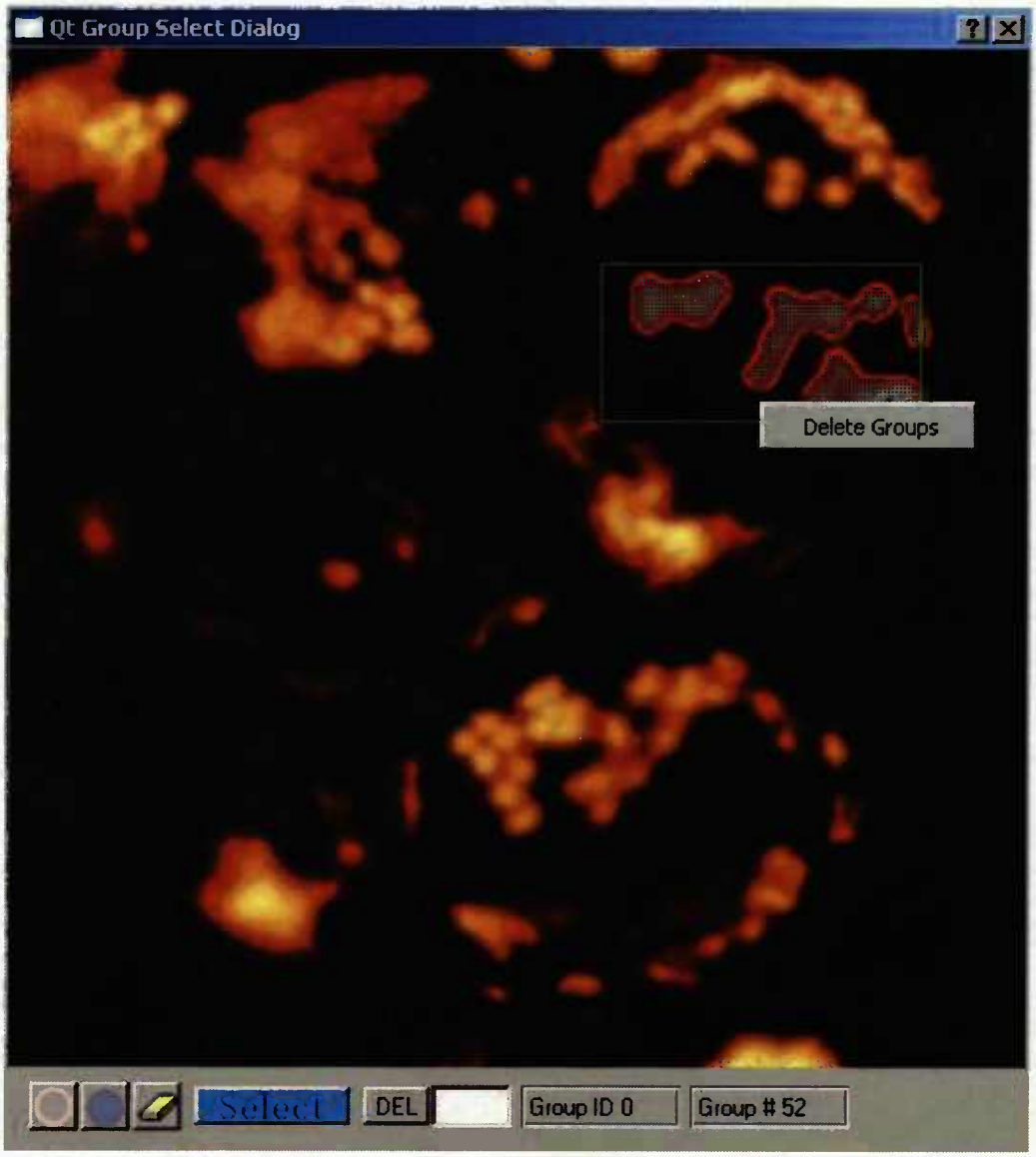

Figure 5.4 (b) Delete chloroplast(s)

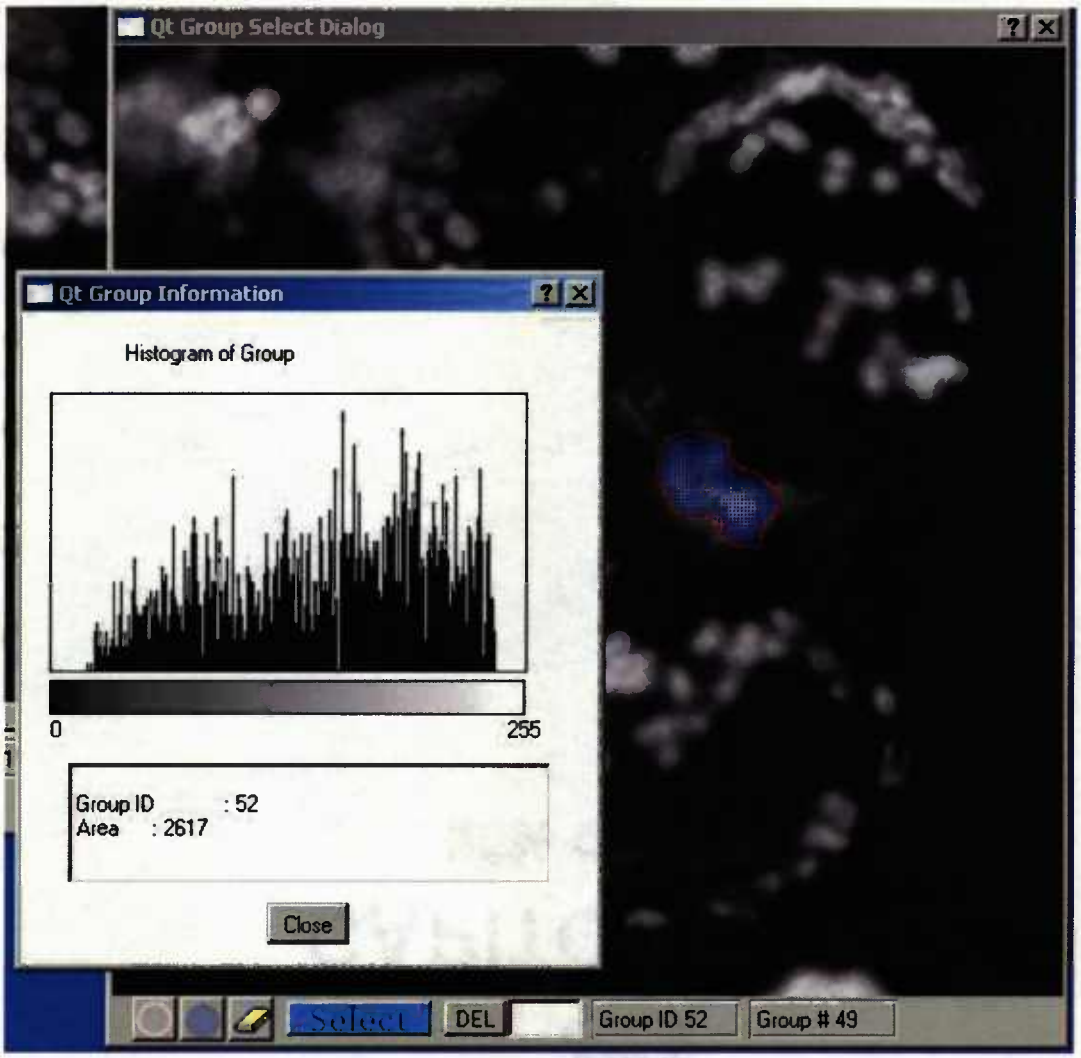

Figure 5.5. Information Dialog Box

IMPLEMENTATION

1. Program Languages and environments

We used C++ with Qt library (1) to implement this image processing tool (IPtool). With Qt library, we can rapidly create a window-based application that can be compiled and executed in both Windows and Unix/Linux environment.

2. Filter Image

Filters are used to blur or sharpen an image. Generally, intensities of pixels are stored in the matrix the same size as the given image. Then the matrix is convolved with a kernel (a square matrix with odd size).

The filters are:

- Gaussian filter (6)
- Median filter (5,9)
- Convolution filter

3. Edge Detection

Edge detection or object segmentation has been studied for long time in Computer Vision. There are millions of methods to identify edges of the objects in the image.

For this version IP tool, we use a simple threshold method to detect objects. We set a threshold on certain intensity. While scanning the image, we ignore pixels that fall below the threshold. Each time a pixel above the threshold is found,

we assign a new label to it. Then we use a breadth-first search to assign the same label to neighboring pixels. This method is not ideal since it is weak on noises inside of an object. In addition, it can not detect an object that appears dark (lower intensity).

We can improve object detection using some other methods, such as watershed algorithm (2, 4, and 11), toboggan (7) or rainfalling simulation (10).

4. Identify Chloroplasts

The edge detection method creates a labeled matrix. The matrix is scanned from top left. Each time a new label is encountered, a new instance of Group class is created and all pixels with the same label are added to the pixel list in Group class by performing a breath-first search. The created Group class is added to the group list in Groups class. The diagram of Groups and Group class can be seen in Appendix B).

5. Computing Histogram

A histogram shows the distribution of intensity of pixel of a specified region within the image. To compute a histogram of a certain group, we count intensities of pixels belonging to the group. Since each group class keeps coordination of the pixels in the list, we refer to intensity matrix and compute histogram of a group.

CONCLUSIONS

Human eyes have limitations when interpreting an image. To visually compute an area or perimeter of one chloroplast takes a lot of time. And since details of the image may escape undetected by eye, we may be less accurate. Furthermore, it is almost impossible to compute distribution of intensity of a certain chloroplast in the image by eye.

To overcome these limitations and reduce time, we analyze images with our Image Processing Tool (IPTool). This way, we tremendously reduce the time taken to analyze an image. However, our first concern is the accuracy of the computation.

Accuracy is always the issue in Computer Vision. Accuracy is, of course, dependent on the quality of an image, but also is heavily dependent on which algorithms or techniques are used.

The quality of the image was improved with IPTool, Median, Gaussian, and Convolution filters. Some algorithms from graph theory were also used. These simple and accurate algorithms considerably facilitated our end result, the identification of chloroplast division. This tool allows semi-automation of the identification and localization of chloroplast division, and is potentially very useful for monitoring crop development.

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CHAPTER 6

GENERAL CONCLUSION

In our laboratory a novel imaging spectrofluorometric method was developed that allows in vivo observation of chloroplast structures and functions in leaves. With this method, we started by examining leaves of *Arabidopsis thaliana,* looking for differences between mutant and wild type plants. In the process of analyzing the results of these experiments, we realized that it was possible non-invasively to observe division of chloroplast in plants.

Discovery of the Phenomenon

With the objectives and intent previously described, we estimated photosynthetic efficiency by measuring various points of the transient spectra of fluorescence released on the onset of photosynthesis. These data were collected with a CCD for 31,680 data collecting units (pixels) per image. With these data we generated images that showed estimated photosynthetic efficiency across sections of leaves

In this way, we explored differences in photosynthetic efficiency between the mutant and the normal part of these *Arabidopsis* mutants. Then we processed the resulting photosynthetic efficiency data in these images. To do this we ranked

the data with respect to efficiency on the x axis, plotting as y axis the number of pixels at that efficiency range.

We processed the data by rigorous statistical analysis for subpopulations. This statistical approach verified the presence of these subpopulations. In these mutants we often, but not always, saw two populations chloroplasts with different photosynthetic efficiencies. It seemed to explain the differences between the dark green and the much lighter white or yellow sectors of the mutation plant's leaves.

Application of the deconvolution program to the collected data shows that, except for a hint of a low peak at low density, the data support a single normally distributed population of Y' (estimated photosynthetic efficiency) data. This distribution was found most commonly in wild type leaves. It suggests that one major population of chloroplast has a common photosynthetic efficiency.

As our work progressed and data accumulated we found some wild-type data that had peaks at low Pm (statistically transformed photosynthetic efficiency). Thus we embarked on a wider examination of mutant and wild type spectra using the same statistical deconvolution. With these data we began to clearly see two populations of chloroplasts with different efficiencies, not only in other mutants but also in wild type *Arabidopsis* plants.

Statistically we determinate that the existence of distinct populations of chloroplast efficiency is a real phenomenon, occurring both in wild-type and in variegated mutant *Arabidopsis*.

However, the process of discovery was complex, with hypothesis after hypothesis failing to give reasonable explanations of the data observed. For instance, C-4 plants are long known to have different chloroplasts in the mesophyll and bundle sheet cells (Laetsch and Price 1969). The trouble with this explanation is that *Arabidopsis* are not C-4 plants. Testing the phenomenon in other species, we found that the phenomenon is general, and is limited to the base of the *Amaryllis* plant where greening and thus, chloroplast division takes place.

We infer from our data and logical analysis that what we are seeing is chloroplast division. This is consistent with data from another laboratory working a very phylogenetically distant species, *Heterosigma carterae.*

We could not attribute the difference in populations to an artifact of extraction because the technology used here was an in vivo method, and no extraction procedure is involved. What we see with this instrumentation represents the total population of functional or semi-functional chloroplasts of the area observed.

We did a simple calculation of chloroplast size using the data on chloroplast size of Pyke and Leach (Pyke and Leach, 1994). This calculation showed that because the CCD pixels in our device are set up to cover 12,626 μm^2, each pixel could gather signals from as many as 63, 200 chloroplasts. Such a group size of chloroplasts would be expected to generate an averaged efficiency signal rather than a biphasic one, damping out any differences.

Although measuring a single chloroplast cycle is difficult, it is easier to get a good number of chloroplasts synchronized. Our device and practice is designed

to produce this effect using pre-treatment of tissue in the dark (Ning et al., 1995). We could observe different populations with our device, because the number of active chloroplasts per pixel of the CCD may well be small enough to make manifest the diversity populations among small populations of chloroplasts observed.

Verification of the phenomenon by germplasm methods and confocal microscopy

After we worked on *Arabidopsis,* we further tested the phenomenon by examining the leaves of monocots (sugarcane, wheat, *Narcissus*, and especially *Amaryllis*). Of all the germplasm examined, *Amaryllis* proved to be the most favorable for our purposes. In the rapidly dividing tissues of the *Amaryllis* the dual nature of the subpopulations of chloroplasts was clearly manifested. We also found multiple populations of chloroplast efficiencies in other leaves. The phenomenon is general, and is limited to the base of the *Amaryllis* plant where greening and thus, chloroplast division takes place.

To accumulate further evidence for existence of the phenomenon, we were given some time on a confocal microscope to study the phenomenon in *Amaryllis*. 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.

At first we found resolution difficulties common with this type of in vivo measurements. But with a little more resolution, we are able to see more dumbbell shaped chloroplasts that are dividing at the section of the leaf where the chloroplast division takes place as judged by the incipient greening of the tissue.

Statistical proofs of observation and general utility of the method

Biophysical considerations, statistical theory, and preliminary experimental evidence support the idea that we are observing chloroplast division.

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

To obtain statistical proof of two populations with different photosynthetic efficiencies, we tested the data statistically by rigorous statistical analysis for subpopulations. This statistical approach surprisingly showed the presence of these subpopulations, in both wild and mutant plants. This biphasic distribution of photosynthetic efficiencies is attributed to demand for extra energy (and thus lower photosynthetic efficiencies) during chloroplast division.

Statistically we demonstrated that distinct populations of chloroplast efficiency are real phenomena, occurring both in wild type and in variegated

mutant *Arabidopsis*. That these populations resulted from the measurement of chloroplast division also became clear.

Once this method was applied and the phenomena found real and repeatedly verified, we realized that the large number of pixels sampled was sufficient to fulfill statistical requirements without further statistical analysis.

General use of this method allows statistical verification of bimodal and poly-modal distribution phenomena of data obtained from CCD cameras and other devices. Thus, we began to realize that a statistical method that we thought only applied to a particular use has far more general applications in deconvoluting other biological data.

A digital image processing tool

Human eyes have limitations when interpreting an image. To visually compute an area or perimeter of one chloroplast takes a lot of time. Since details of the image may escape undetected by eye, we may be less accurate. Furthermore, it is almost impossible to compute distribution of intensity of a certain chloroplast in the image by direct visual observation.

To overcome these limitations and reduce time, we analyzed images with our Image Processing Tool (IPTool). IPTool is used to detect chloroplast division from photographs of confocal microscopy images. To achieve this goal, we grouped pixels within the edges. Each identified group is represented as a

chloroplast. This provides an interactive interface that allows the user to manually correct misdiagnoses made by computer.

In this way, we reduce the time to analyze an image by using IPTool. Accuracy is always the issue in Computer Vision, it depends on the quality of an image, but is also heavily dependent on which algorithms or techniques are used.

The quality of the image was improved with IPTool, and by using median, Gaussian, and Convolution filters. Some algorithms from graph theory were also used. These simple and accurate algorithms considerably facilitated our end result, the identification of chloroplast division.

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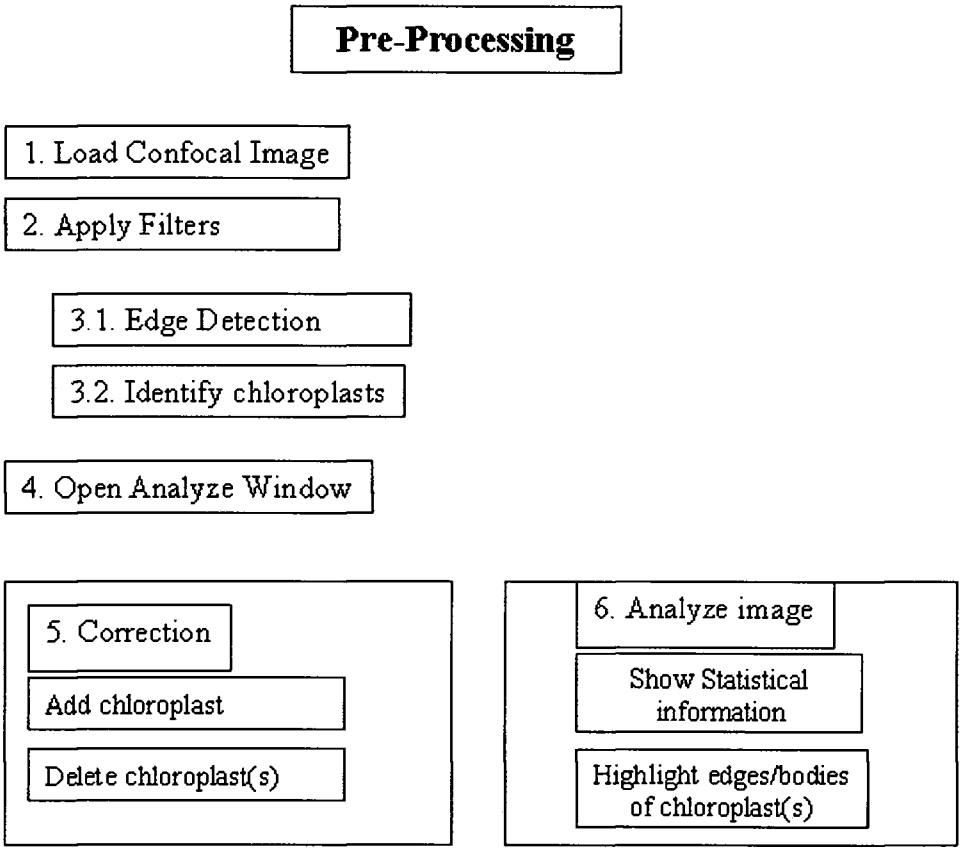
APPENDICES

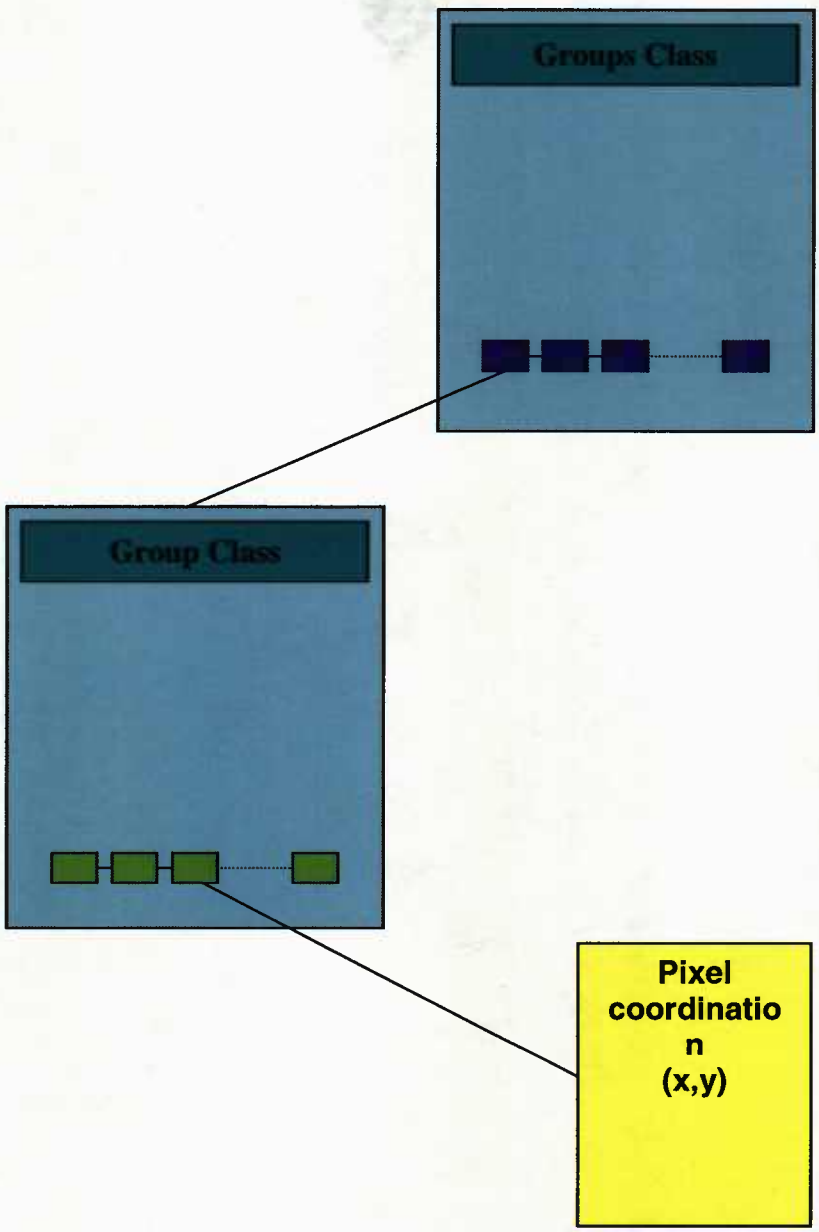
APPENDIX A

PROGRAM FLOW DIAGRAM



APPENDIX B

THE DIAGRAM OF GROUPS AND GROUP CLASS



APPENDIX C

PARTIAL DEMONSTRATION OF SOURCE CODE (One of six functions)

```
#ifndef GROUPSELECTDLG_H
#define GROUPSELECTDLG_H
#include "main.h"

#define MENU_DEL              100
#define MENU_ADD              101
#define MENU_INFO             102
#define MODE_SELECT           201
#define MODE_ADD_GROUP        202
#define MODE_MULT_SELECT      203

class GroupSelectDlg;

/*****************************************************/
/*    Group Class Declaration                        */
/*****************************************************/
class Group
{
      public:
            Group(int,int,int);
            ~Group();
            int getID() { return _gID; }
            void deleteGroup(int**);
            void addGroup(list<QPoint*>*,int**);
            void addInnerPixels(int**);
            void addOuterPixels(list<QPoint*>*,int**);
            void highlightGroup(QPainter*,bool,bool);
            void addPixel(QPoint* p,bool);
            long* getHistogram(int*);
           int getArea() { return (_inner.size() +_outer.size()); }
            void interpolate(QPoint*,QPoint*,int**);

      private:
            int _gID;
            int _height;
            int _width;
            list<QPoint*> _inner;
            list<QPoint*> _outer;
            list<QPoint*>::iterator _itr;
};

/*****************************************************/
/*    Groups Class Declaration                       */
/*****************************************************/
class Groups
{
      public:
            Groups(QImage*);
            ~Groups();
            void highlightAllGroups(QPainter*,bool,bool);
```

```
            void
highlightSelectedGroups(list<int>*,QPainter*,bool,bool);
            void highlightGroup(int,QPainter*,bool,bool);
            void findGroups(list<int>*,QRect*);
            Group* getGroup(int id);
            bool deleteGroup(int);
            void deleteMinGroups(int);
            int addGroup(list<QPoint*>*);
            int getGroupID(int x, int y);
            int getNumGroups(){ return _groups.size(); }

      private:
            Group* createNewGroup(int,int,int);
            void groupPixels();
            void printLabelMap(int);

      private:
            int _width;
            int _height;
            int _maxID;
            map<int,Group*> _groups;
            map<int,Group*>::iterator _itr;
            int** _labelMap;
};

/*************************************************/
/* GroupSelectCtlBar Class Declaration           */
/*************************************************/
class GroupSelectCtlBar : public QWidget
{
      Q_OBJECT
      public:
            GroupSelectCtlBar(int, QWidget *parent=0, const char
*name=0);
            ~GroupSelectCtlBar();
            void setNumGroups(int n)      { _numGroupLabel-
>setText("Group # "+QString::number(n)); }
            void setGroupID(int n)        { _groupIDLabel-
>setText("Group ID "+QString::number(n)); }
            void setButtons(bool,bool);
            void setMode(int);
            int getMinArea();

      private:
            QPushButton* _modeBtn;
            QPushButton* _showAllEdgeBtn;
            QPushButton* _showAllBodyBtn;
            QPushButton* _clearBtn;
            QPushButton* _delMinGBtn;
            QLineEdit* _delMinGLine;
            QLabel* _numGroupLabel;
            QLabel* _groupIDLabel;
            QPixmap *_epmapON, *_epmapOFF;
            QPixmap *_bpmapON, *_bpmapOFF;
            QPixmap *_cpmap;
```

```
            QPixmap *_selectMode;
            QPixmap *_addMode;
};

/*************************************************/
/*    GroupSelectInfoDlg Class Declaration       */
/*************************************************/
class GroupSelectInfoDlg : public QDialog
{
      Q_OBJECT
      public:
            GroupSelectInfoDlg(QWidget* parent= 0, const char*
name= 0, bool modal= false, WFlags f= 0);
            ~GroupSelectInfoDlg();
            void display(Group*,QImage*);

      private:
            QMultiLineEdit* _multiLine;
            QPushButton* _closeBtn;
            QLabel* _histLabel;
};

/*************************************************/
/*    GroupSelectDlg Class Declaration           */
/*************************************************/
class GroupSelectDlg : public QDialog
{
      Q_OBJECT
      public:
            GroupSelectDlg(QImage*, QWidget* parent= 0, const char*
name= 0, bool modal= false, WFlags f= 0);
            ~GroupSelectDlg();
            void highlightGroups(bool,bool);
            void highlightGroup(bool,bool);

      public slots:
            void deleteGroups();
            void deleteMinGroups();
            void addGroup();
            void changeMode();
            void showAllEdge();
            void showAllBody();
            void clear();

      private:
            void refresh();
            void drawFreeLine();
            void drawRectangle();
            void updateRect(int,int);
            void setMenuItems();
            void setSelectedGIDs(int);
            void showInfoDlg();
            void showPopupMenu(QPoint);

      protected:
```

```
            void mouseMoveEvent(QMouseEvent*);
            void mousePressEvent(QMouseEvent*);
            void mouseReleaseEvent(QMouseEvent*);
            void mouseDoubleClickEvent(QMouseEvent*);
            void paintEvent(QPaintEvent*);

      private:
            QImage* _img;
            int _width;
            int _height;
            int _curMode;
            bool _showAllEdge, _showAllBody;
            list<QPoint*> _contour;
            list<int> _selectedGIDs;
            QRect _curRect;
            QPoint _originRect;
            QPopupMenu* _popup;
            QPainter* _painter;
            GroupSelectCtlBar* _ctlBar;
            Groups* _groups;
};
#endif

#include "GroupSelectDlg.h"

/*****************************************************/
/* Group Class Definition                            */
/*****************************************************/
Group::Group(int id, int width, int height)
{
      //Initialization
      _gID= id;
      _width= width;
      _height= height;
}

Group::~Group()
{}

/*---------------------------------------------------*/
/* Add pixel                                         */
/*---------------------------------------------------*/
void Group::addPixel(QPoint* p, bool inner)
{
      if (inner)
        _inner.push_back(p);
      else
        _outer.push_back(p);
}

/*---------------------------------------------------*/
/* Interpolate pixels between two points             */
/*---------------------------------------------------*/
void Group::interpolate(QPoint* p0, QPoint* p1, int** _labelMap)
{
```

```
	int i,j;
	float x0= (float)p0->x(), x1= (float)p1->x();
	float y0= (float)p0->y(), y1= (float)p1->y();

	//Check if p0 is adjacent with p1
	for (i= -1; i < 2; i++)
		for (j= -1; j < 2; j++)
		{
			int qx= x0+j;
			int qy= y0+i;
			if ((qx == x1) && (qy == y1))
			{
				_labelMap[p0->y()][p0->x()]= -9999;
				_outer.push_back(p0);
				return;
			}
		}

	//Compute an equation of p0 and p1
	float dx= x0 - x1;
	float dy= y1 - y0;
	float slope;
	if ((dx == 0.0) || (dy == 0.0))
		slope= 0.0;
	else
		slope= dy/dx;
	float intersect= -y0 - slope*x0;

	//Interpolate p0 and p1
	int start, end, increment, newPoint;
	if (abs(dx) > abs(dy))
	{
		start= p0->x();
		end    = p1->x();
	}
	else
	{
		start= -p0->y();
		end    = -p1->y();
	}

	if (start < end)
		increment= 1;
	else
		increment= -1;

	while (start != end)
	{
		if (abs(dx) > abs(dy))
		{
			if (slope == 0.0)
				newPoint= p0->y();
			else
				newPoint= (int)(start*slope + intersect)*(-
1);
```

```
                _labelMap[newPoint][start]= -9999;
                _outer.push_back(new QPoint(start,newPoint));
            }
            else
            {
                if (slope == 0.0)
                    newPoint= p0->x();
                else
                    newPoint= (int)((start - intersect)/slope);
                _labelMap[-start][newPoint]= -9999;
                _outer.push_back(new QPoint(newPoint,-start));
            }
            start+= increment;
        }
}

/*-------------------------------------------------*/
/* Add outer pixels                                */
/*-------------------------------------------------*/
void Group::addOuterPixels(list<QPoint*>* contour, int** _labelMap)
{
    list<QPoint*>::iterator current,next;
    current= next= contour->begin();
    next++;
    while (next != contour->end())
    {
        interpolate(*current,*next,_labelMap);
        next++;
        current++;
    }
}

/*-------------------------------------------------*/
/* Add inner pixels                                */
/*-------------------------------------------------*/
void Group::addInnerPixels(int** _labelMap)
{
    //Fill outside of contour
    int i,j,qx,qy;
    list<QPoint*> queue;
    queue.push_back(new QPoint(0,0));
    _labelMap[0][0]= 9000;
    while(!queue.empty())
    {
        QPoint* p= queue.front();

        //Check Neighbor pixels
        bool border= false;
        for (i= -1; i < 2; i++)
            for (j= -1; j < 2; j++)
            {
                qx= p->x()+j;
                qy= p->y()+i;
                if ((qx > -1 && qx < _width) && (qy > -1 &&
qy < _height))
```

```
                        {
                              if (_labelMap[qy][qx] == -9999)
                                    border= true;
                        }
                  }

            if (!border)
            {
                  for (int i= -1; i < 2; i++)
                        for (int j= -1; j < 2; j++)
                        {
                              qx= p->x()+j;
                              qy= p->y()+i;
            if ((qx > -1 && qx < _width) && (qy > -1 && qy <
_height))
                         {
                              if (_labelMap[qy][qx] >= 0)
                                    {
                                    if (_labelMap[qy][qx] == 0)
                                        _labelMap[qy][qx]= -9000;
                                          else
                                        _labelMap[qy][qx]*= -1
                                    queue.push_back(new
QPoint(qx,qy));
                                    }
                              }
                        }
            }
            queue.pop_front();
      }

      //Add inner pixel
      for (int y= 0; y < _height; y++)
            for (int x= 0; x < _width; x++)
            {
                  if (_labelMap[y][x] > -1)
                        _inner.push_back(new QPoint(x,y));

                  if (_labelMap[y][x] == -9000)
                        _labelMap[y][x]= 0;
                  else if (_labelMap[y][x] > -9999)
                        _labelMap[y][x]*= -1;
            }
}

/*-------------------------------------------------*/
/* Create new group                                */
/*-------------------------------------------------*/
void Group::addGroup(list<QPoint*>* contour, int** _labelMap)
{
      //Add outer pixels
      addOuterPixels(contour,_labelMap);

      //Add inner pixels
      addInnerPixels(_labelMap);
```

```
      //Update LabelMap
      for (_itr= _outer.begin(); _itr != _outer.end(); _itr++)
            _labelMap[(*_itr)->y()][(*_itr)->x()]= _gID;
      for (_itr= _inner.begin(); _itr != _inner.end(); _itr++)
            _labelMap[(*_itr)->y()][(*_itr)->x()]= _gID;

      //Clear contour points
      contour->clear();
}

/*-----------------------------------------------------*/
/* Delete group                                        */
/*-----------------------------------------------------*/
void Group::deleteGroup(int** _labelMap)
{
      //Update LabelMap
      for (_itr= _outer.begin(); _itr != _outer.end(); _itr++)
            _labelMap[(*_itr)->y()][(*_itr)->x()]= 0;
      for (_itr= _inner.begin(); _itr != _inner.end(); _itr++)
            _labelMap[(*_itr)->y()][(*_itr)->x()]= 0;
}

/*-----------------------------------------------------*/
/* Highlight group                                     */
/*-----------------------------------------------------*/
void Group::highlightGroup(QPainter* painter, bool body, bool edge)
{
      //Highlight Body of Group
      if (body)
      {
            painter->setPen(Qt::blue);
           for (_itr= _inner.begin(); _itr != _inner.end(); _itr++)
            {
                  int x= (*_itr)->x();
                  int y= (*_itr)->y();
                  if ((x%2==0) && (y%2==0))
                 painter->drawPoint(x,y);
            }
      }

      //Highlight edge of Group
      if (edge)
      {
            painter->setPen(Qt::red);
           for (_itr= _outer.begin(); _itr != _outer.end(); _itr++)
                  painter->drawPoint((*_itr)->x(),(*_itr)->y());
      }
}
/*****************************************************/
/* Groups Class Definition                           */
/*****************************************************/
Groups::Groups(QImage* img)
{
      //Initialization
```

```
	_width=  img->width();
	_height= img->height();
	_maxID= 0;

	//Create label map by watershed algorithm
	Watershed* wshed= new Watershed(img);
	_labelMap= wshed->createLabelMap(true);

	//Group pixels of the image
	groupPixels();
}

Groups::~Groups()
{
	//Release memory
	for (int i= 0; i < _height; i++)
		delete[] _labelMap[i];
	delete[] _labelMap;
}

/*-----------------------------------------------------*/
/* Create Group                                        */
/*-----------------------------------------------------*/
Group* Groups::createNewGroup(int id, int x, int y)
{
	list<QPoint*> queue;
	queue.push_back(new QPoint(x,y));

	//Create a new Group
	Group* g= new Group(id,_width,_height);

	//Add pixels to the group
	_labelMap[y][x]= -id;
	while(!queue.empty())
	{
		QPoint* p= queue.front();

		//Check Neighbor of p
		bool inner= true;
		for (int i= -1; i < 2; i++)
			for (int j= -1; j < 2; j++)
			{
				int qx= p->x()+j;
				int qy= p->y()+i;
				if ((qx > -1 && qx < _width) && (qy > -1 &&
qy < _height))
				{
					if (_labelMap[qy][qx] == id)
					{
					_labelMap[qy][qx]= -id;
					queue.push_back(new QPoint(qx,qy));
					}
					else if (_labelMap[qy][qx] != -id)
						inner= false;
				}
```

```
                }

                g->addPixel(p,inner);
                queue.pop_front();
        }
        return g;
}

/*-----------------------------------------------------*/
/* Group pixels from label map                         */
/*-----------------------------------------------------*/
void Groups::groupPixels()
{
        //Clear groups
        _groups.clear();

        //Group Pixels
        int x,y;
        for (y= 0; y < _height; y++)
                for (x= 0; x < _width; x++)
                {
                        if (_labelMap[y][x] > 0)
                        {
                                int id= _labelMap[y][x];
                                if (id > _maxID)
                                        _maxID= id;

      _groups.insert(pair<int,Group*>(id,(createNewGroup(id,x,y))));
                        }
                }

        for (y= 0; y < _height; y++)
                for (x= 0; x < _width; x++)
                        _labelMap[y][x]= -_labelMap[y][x];
}

Group* Groups::getGroup(int id)
{
        //Find and delete group
        _itr= _groups.find(id);
        if (_itr != _groups.end())
                return _itr->second;
        else
                return NULL;
}

int Groups::getGroupID(int x, int y)
{
        //Return Group ID
        if ((x > -1 && x < _width) && (y > -1 && y < _height))
        {
                int id= _labelMap[y][x];
                _itr= _groups.find(id);
                if (_itr != _groups.end())
                        return id;
```

```
        }
        return 0;
}

int Groups::addGroup(list<QPoint*>* contour)
{
        if (contour->size() > 3)
        {
                //Create a group
                _maxID++;
                Group* g= new Group(_maxID,_width,_height);

                //Add pixels to the group and update _labelMap
                g->addGroup(contour,_labelMap);

                //Re-group from _labelMap
                groupPixels();

                return _maxID;
        }
        return 0;
}

bool Groups::deleteGroup(int gid)
{
        //Find and delete group
        _itr= _groups.find(gid);
        if (_itr != _groups.end())
        {
                //Update _labelMap
                (_itr->second)->deleteGroup(_labelMap);

                //Re-group from _labelMap
                groupPixels();
                return true;
        }
        return false;
}

/*-------------------------------------------------*/
/* Delete groups with area less than min           */
/*-------------------------------------------------*/
void Groups::deleteMinGroups(int minArea)
{
        //Delete groups with area less than minArea
      _itr= _groups.begin();
        while (_itr != _groups.end())
        {
                Group* g= (*_itr).second;
          if (g->getArea() < minArea)
                {
                        g->deleteGroup(_labelMap);
                        _itr= _groups.erase(_itr);
                }
                else
```

```
                _itr++;
        }
}

void Groups::highlightAllGroups(QPainter* painter, bool body, bool
edge)
{
        //Highligh all object
        for (_itr= _groups.begin(); _itr != _groups.end(); _itr++)
                (_itr->second)->highlightGroup(painter,body,edge);
}

void Groups::highlightSelectedGroups(list<int>* glist, QPainter*
painter, bool body, bool edge)
{
        //Highlight selected groups
        if (glist->size() > 0)
        {
                list<int>::iterator it;
                for (it= glist->begin(); it != glist->end(); it++)
                {
                        _itr= _groups.find(*it);
                if (_itr != _groups.end())
                                (_itr->second)-
>highlightGroup(painter,body,edge);
                }
        }
}

void Groups::findGroups(list<int>* glist,QRect* rect)
{
        //Find Groups
        glist->clear();
        int* label= new int[_maxID+1];
        for (int i= 0; i < _maxID+1; i++)
                label[i]= 0;

        for (int y= rect->top(); y < rect->bottom(); y++)
                for (int x= rect->left(); x < rect->right(); x++)
                {
                        if (_labelMap[y][x] > 0)
                                label[_labelMap[y][x]]++;
                }

        //Insert selected IDs
        for (int k= 0; k < _maxID+1; k++)
        {
                if (label[k] > 0)
                        glist->push_back(k);
        }
        delete[] label;
}

void Groups::printLabelMap(int n)
{
```

```
      ofstream out;
      QString
fn("Data//logs//log_GroupSelect_"+QString::number(n)+".txt");
      out.open(fn);

      for (int y= 0; y < _height; y++)
      {
            for (int x= 0; x < _width; x++)
            {
                  int value= _labelMap[y][x];
                  if (value > 0)
                        out<<value;
                  else if (value > -1)
                        out<<" ";
                  else if (value > -2)
                        out<<"A";
                  else if (value > -3)
                        out<<"B";
                  else
                        out<<"C";
            }
            out<<endl;
      }
      out.close();
}

/*************************************************/
/*    GroupSelectCtlBar Class Definition         */
/*************************************************/
GroupSelectCtlBar::GroupSelectCtlBar(int width, QWidget *parent,
const char *name)
                  : QWidget(parent,name)
{
      //Create and add components
      _showAllEdgeBtn   = new QPushButton(this);
      _showAllBodyBtn   = new QPushButton(this);
      _modeBtn          = new QPushButton(this);
      _clearBtn         = new QPushButton(this);
      _delMinGBtn       = new QPushButton("DEL",this);
      _delMinGLine      = new QLineEdit("",this);
      _groupIDLabel     = new QLabel("Group ID: 0",this);
      _numGroupLabel    = new QLabel("Group #: 0",this);

      //Tuneup components
      _epmapON    = new QPixmap("Images//edgeONBtn.png");
      _bpmapON    = new QPixmap("Images//bodyONBtn.png");
      _epmapOFF   = new QPixmap("Images//edgeOFFBtn.png");
      _bpmapOFF   = new QPixmap("Images//bodyOFFBtn.png");
      _cpmap            = new QPixmap("Images//clearBtn.png");
      _selectMode = new QPixmap("Images//selectMode.png");
      _addMode    = new QPixmap("Images//addMode.png");
      _showAllEdgeBtn->setPixmap(*_epmapOFF);
      _showAllBodyBtn->setPixmap(*_bpmapOFF);
      _clearBtn->setPixmap(*_cpmap);
      _groupIDLabel->setFrameShape(QFrame::Box);
```

```
        _groupIDLabel->setFrameShadow(QFrame::Sunken);
        _numGroupLabel->setFrameShape(QFrame::Box);
        _numGroupLabel->setFrameShadow(QFrame::Sunken);
        Prims::setWidgetSize(this,width,40);
        Prims::setWidgetSize(_showAllEdgeBtn,26,26);
        Prims::setWidgetSize(_showAllBodyBtn,26,26);
        Prims::setWidgetSize(_clearBtn,26,26);
        Prims::setWidgetSize(_modeBtn,80,21);
        Prims::setWidgetSize(_delMinGBtn,35,21);
        Prims::setWidgetSize(_delMinGLine,40,21);
        Prims::setWidgetSize(_clearBtn,26,26);
        Prims::setWidgetSize(_groupIDLabel,80,21);
        Prims::setWidgetSize(_numGroupLabel,80,21);

        QHBoxLayout* layout= new QHBoxLayout(this);
              layout->addSpacing(10);
              layout->addWidget(_showAllEdgeBtn);
              layout->addWidget(_showAllBodyBtn);
              layout->addWidget(_clearBtn);
              layout->addSpacing(5);
              layout->addWidget(_modeBtn);
              layout->addSpacing(5);
              layout->addWidget(_delMinGBtn);
              layout->addWidget(_delMinGLine);
              layout->addSpacing(5);
              layout->addWidget(_groupIDLabel);
              layout->addSpacing(5);
              layout->addWidget(_numGroupLabel);
              layout->addStretch(100);

        //Connections
        connect(_showAllEdgeBtn,SIGNAL(pressed()),parent,SLOT(showAll
Edge()));
        connect(_showAllBodyBtn,SIGNAL(pressed()),parent,SLOT(showAll
Body()));
        connect(_delMinGBtn,SIGNAL(pressed()),parent,SLOT(deleteMinGr
oups()));
       connect(_modeBtn,SIGNAL(pressed()),parent,SLOT(changeMode()));
        connect(_clearBtn,SIGNAL(pressed()),parent,SLOT(clear()));
}

GroupSelectCtlBar::~GroupSelectCtlBar()
{}

int GroupSelectCtlBar::getMinArea()
{
        //Return minimum area
        bool ok;
        int min= _delMinGLine->text().toInt(&ok,10);
        if (ok)
              return min;
        else
          return -1;
}
```

```
void GroupSelectCtlBar::setButtons(bool edge, bool body)
{
      //Set edge and body buttons
      if (edge)
            _showAllEdgeBtn->setPixmap(*_epmapON);
      else
            _showAllEdgeBtn->setPixmap(*_epmapOFF);
      if (body)
            _showAllBodyBtn->setPixmap(*_bpmapON);
      else
            _showAllBodyBtn->setPixmap(*_bpmapOFF);
}

/*-------------------------------------------------*/
/* Change current mode                             */
/*-------------------------------------------------*/
void GroupSelectCtlBar::setMode(int mode)
{
      if (mode == MODE_ADD_GROUP)
            _modeBtn->setPixmap(*_addMode);
      else if (mode == MODE_SELECT || mode == MODE_MULT_SELECT)
            _modeBtn->setPixmap(*_selectMode);
}

/*************************************************/
/*    GroupSelectInfoDlg Class Definition        */
/*************************************************/
GroupSelectInfoDlg::GroupSelectInfoDlg(QWidget * parent, const char
* name, bool modal, WFlags f)
                  :QDialog(parent,name,modal)
{
      //Create and add components
      _multiLine  = new QMultiLineEdit(this);
      _closeBtn   = new QPushButton("Close",this);
      _histLabel  = new QLabel("Histogram of Group",this);

      //Tuneup components
      setCaption("Qt Group Information");
      setBackgroundColor(white);
      _histLabel->setBackgroundColor(white);
      Prims::setWidgetSize(this,310,300);
      Prims::setWidgetSize(_multiLine,260,65);
      Prims::setWidgetSize(_histLabel,200,23);
      Prims::setWidgetSize(_closeBtn,45,23);

      //Layout components
      QVBoxLayout* layout= new QVBoxLayout(this);
      layout->addSpacing(10);
      layout->addWidget(_histLabel);
      layout->addSpacing(10);
      layout->addWidget(_multiLine);
      layout->addSpacing(10);
      layout->addWidget(_closeBtn);
      layout->addSpacing(10);
```

```
      //Connections
      connect(_closeBtn,SIGNAL(pressed()),SLOT(close()));
}

GroupSelectInfoDlg::~GroupSelectInfoDlg()
{}

/*-------------------------------------------------*/
/* Display selected group information              */
/*-------------------------------------------------*/
void GroupSelectInfoDlg::display(Group* g, QImage* img)
{
      //Display histogram of image
      int* gray= Prims::getGrayScale(img);

      //Display group information
      _multiLine->insertLine(" ",1);
      _multiLine->insertLine("Group ID\t: "+QString::number(g-
>getID()),2);
      _multiLine->insertLine("Area    \t: "+QString::number(g-
>getArea()),3);

      delete[] gray;

      show();
}

/*************************************************/
/*    GroupSelectDlg Class Definition            */
/*************************************************/
GroupSelectDlg::GroupSelectDlg(QImage* img, QWidget * parent, const
char * name, bool modal, WFlags f)
                  :QDialog(parent,name,modal)
{
      //Initialization
      _img= img;
      _height= img->height();
      _width=  img->width();
      _painter= new QPainter(this);
      _groups= new Groups(img);
      _curMode= MODE_SELECT;

      //Create and add components
      _ctlBar= new GroupSelectCtlBar(_width,this);
      _popup= new QPopupMenu(this);

      //Layout components
      QVBoxLayout* layout= new QVBoxLayout(this);
      layout->addStretch();
      layout->addWidget(_ctlBar);

      //Tuneup components
      Prims::setWidgetSize(this,_width,_height+40);
      setCaption("Qt Group Select Dialog");
```

```
	//Set Image
	QPixmap pmap;
	pmap.convertFromImage(*_img);
	setBackgroundPixmap(pmap);

	//Clear drawing area
	clear();
}

GroupSelectDlg::~GroupSelectDlg()
{}

/*-----------------------------------------------------*/
/* clear                                               */
/*-----------------------------------------------------*/
void GroupSelectDlg::clear()
{
	_showAllEdge= false;
	_showAllBody= false;
	refresh();
}

/*-----------------------------------------------------*/
/* refresh                                             */
/*-----------------------------------------------------*/
void GroupSelectDlg::refresh()
{
	_ctlBar->setGroupID(0);
	_ctlBar->setMode(_curMode);
	_ctlBar->setNumGroups(_groups->getNumGroups());
	_ctlBar->setButtons(_showAllEdge,_showAllBody);
	_selectedGIDs.clear();
	_contour.clear();
	update();
}
/*-----------------------------------------------------*/
/* Set selected Group ID                               */
/*-----------------------------------------------------*/
void GroupSelectDlg::setSelectedGIDs(int gid)
{
	//Set Group ID
	_selectedGIDs.clear();
	_contour.clear();
	if (gid > 0)
		_selectedGIDs.push_back(gid);

	//Update control bar
	_ctlBar->setGroupID(gid);
	_ctlBar->setNumGroups(_groups->getNumGroups());
}

/*-----------------------------------------------------*/
/* Show edges of groups                                */
/*-----------------------------------------------------*/
void GroupSelectDlg::showAllEdge()
```

```
{
      _showAllEdge= !_showAllEdge;
      _ctlBar->setButtons(_showAllEdge,_showAllBody);
      update();
}

/*-------------------------------------------------*/
/* Show bodies of groups                           */
/*-------------------------------------------------*/
void GroupSelectDlg::showAllBody()
{
      _showAllBody= !_showAllBody;
      _ctlBar->setButtons(_showAllEdge,_showAllBody);
      update();
}

/*-------------------------------------------------*/
/* Change current mode                             */
/*-------------------------------------------------*/
void GroupSelectDlg::changeMode()
{
      if (_curMode == MODE_ADD_GROUP)
            _curMode= MODE_SELECT;
      else if (_curMode == MODE_SELECT || _curMode ==
MODE_MULT_SELECT)
            _curMode= MODE_ADD_GROUP;
      refresh();
}

/*-------------------------------------------------*/
/* Set menu items in popup menu                    */
/*-------------------------------------------------*/
void GroupSelectDlg::setMenuItems()
{
      //clear all menu items
      _popup->clear();

      //Add menu items
      if (_curMode == MODE_SELECT)
      {
            _popup->insertItem("Delete
Group",this,SLOT(deleteGroups()));
      }
      else if (_curMode == MODE_MULT_SELECT)
      {
            _popup->insertItem("Delete
Groups",this,SLOT(deleteGroups()));
      }
      else if (_curMode == MODE_ADD_GROUP)
      {
            _popup->insertItem("Add Group",this,SLOT(addGroup()));
      }
}
```

```
/*-------------------------------------------------*/
/* Draw rectangle                                  */
/*-------------------------------------------------*/
void GroupSelectDlg::drawRectangle()
{
      //Draw rectangle
      _painter->setPen(Qt::green);
      _painter->drawRect(_curRect);
}

/*-------------------------------------------------*/
/* Draw free line                                  */
/*-------------------------------------------------*/
void GroupSelectDlg::drawFreeLine()
{
      //Draw free line
      ptsList::iterator current,next;
      current= next= _contour.begin();
      next++;
      _painter->setPen(Qt::red);
      while(next != _contour.end())
      {
            _painter->drawLine(*(*current),*(*next));
            next++;
            current++;
      }
}

/*-------------------------------------------------*/
/* Update current rectangle                        */
/*-------------------------------------------------*/
void GroupSelectDlg::updateRect(int x, int y)
{
      //Update current rectangle
      if (x < _originRect.x())
      {
            _curRect.setLeft(x);
            _curRect.setRight(_originRect.x());
      }
      else
      {
            _curRect.setLeft(_originRect.x());
            _curRect.setRight(x);
      }

      if (y < _originRect.y())
      {
            _curRect.setTop(y);
            _curRect.setBottom(_originRect.y());
      }
      else
      {
            _curRect.setTop(_originRect.y());
            _curRect.setBottom(y);
      }
```

```
}

/*-------------------------------------------------*/
/* Delete group(s) in Group ID list                */
/*-------------------------------------------------*/
void GroupSelectDlg::deleteGroups()
{
      //Delete group(s)
      while (!_selectedGIDs.empty())
      {
            int gid= _selectedGIDs.front();
            _groups->deleteGroup(gid);
            _selectedGIDs.pop_front();
      }
      _curMode= MODE_SELECT;
      setSelectedGIDs(0);
      update();
}

/*-------------------------------------------------*/
/* Delete group(s) with gid                        */
/*-------------------------------------------------*/
void GroupSelectDlg::deleteMinGroups()
{
      //Delete group(s) with area less than min value
      int min= _ctlBar->getMinArea();
      if (min > 0)
      {
            _groups->deleteMinGroups(min);
            refresh();
            update();
      }
}

/*-------------------------------------------------*/
/* Add a new group                                 */
/*-------------------------------------------------*/
void GroupSelectDlg::addGroup()
{
      //Add a new group
      setSelectedGIDs(_groups->addGroup(&_contour));
      update();
}

/*-------------------------------------------------*/
/* Show popup menu                                 */
/*-------------------------------------------------*/
void GroupSelectDlg::showPopupMenu(QPoint pos)
{
      //Show popup menu
    QPoint p(mapToGlobal(pos));
    _popup->move(p.x(),p.y());
    setMenuItems();
      _popup->show();
}
```

```
void GroupSelectDlg::showInfoDlg()
{
	//Popup group information dialog
	if (!_selectedGIDs.empty())
	{
		Group* g= _groups->getGroup(_selectedGIDs.front());
		GroupSelectInfoDlg* infoDlg= new
GroupSelectInfoDlg(this);
		infoDlg->display(g,_img);
	}
}

/*-------------------------------------------------*/
/* MouseEvent handlers                             */
/*-------------------------------------------------*/
void GroupSelectDlg::mousePressEvent(QMouseEvent* e)
{
	if (_curMode == MODE_ADD_GROUP)
	{
		if (e->button() == LeftButton)
		{
			//Clear contour points and add new contour point
			_contour.clear();
			_contour.push_back(new QPoint(e->x(),e->y()));
		}
		else if (e->button() == RightButton)
		{
			//Show popup menu
			showPopupMenu(e->pos());
		}
	}
	else if (_curMode == MODE_SELECT || _curMode ==
MODE_MULT_SELECT)
	{
		if (e->button() == Qt::LeftButton)
		{
			//Change current mode
			if (e->state() == Qt::ControlButton)
				_curMode= MODE_MULT_SELECT;
			else
				_curMode= MODE_SELECT;

			//Select group(s)
			if (_curMode == MODE_SELECT)
				setSelectedGIDs(_groups->getGroupID(e-
>x(),e->y()));
			else if (_curMode == MODE_MULT_SELECT)
			{
				_selectedGIDs.clear();
				//Set a new rectangle
				_originRect.setX(e->x());
				_originRect.setY(e->y());
				_curRect.setRect(e->x(),e->y(),0,0);
			}
```

```
            }
            else if (e->button() == Qt::RightButton)
            {
                  //Show popup menu
                  if (_curMode == MODE_SELECT)
                  {
                        setSelectedGIDs(_groups->getGroupID(e-
>x(),e->y()));
                        if (!_selectedGIDs.empty())
                              showPopupMenu(e->pos());
                  }
                  else if (_curMode == MODE_MULT_SELECT)
                  {
                        if (_curRect.contains(e->x(),e->y()))
                              showPopupMenu(e->pos());
                  }
            }
      }
      update();
}

void GroupSelectDlg::mouseMoveEvent(QMouseEvent* e)
{
      if (_curMode == MODE_MULT_SELECT)
      {
            //Update current rectangle
            updateRect(e->x(),e->y());
      }
      else if (_curMode == MODE_ADD_GROUP)
      {
            //Add a new contour point
            if (e->state() == Qt::LeftButton)
                  _contour.push_back(new QPoint(e->x(),e->y()));
      }
      update();
}

void GroupSelectDlg::mouseReleaseEvent(QMouseEvent* e)
{
      if (_curMode == MODE_MULT_SELECT)
      {
            if (e->button() == Qt::LeftButton)
            {
                  updateRect(e->x(),e->y());
                  _groups->findGroups(&_selectedGIDs,&_curRect);
            }
      }
      else if (_curMode == MODE_ADD_GROUP)
      {
            if (e->state() == LeftButton)
            {
                  //Enclose contour line
                  _contour.push_back(new QPoint(e->x(),e->y()));
                  _contour.push_back(new QPoint(_contour.front()-
>x(),_contour.front()->y()));
```

```
            }
        }
        update();
}

void GroupSelectDlg::mouseDoubleClickEvent(QMouseEvent* e)
{
        if (_curMode == MODE_SELECT)
        {
                //Get and set Group ID
                setSelectedGIDs(_groups->getGroupID(e->x(),e->y()));

                //Show Information Dialog
                showInfoDlg();
        }
}

/*-------------------------------------------------*/
/* PaintEvent handler                              */
/*-------------------------------------------------*/
void GroupSelectDlg::paintEvent(QPaintEvent*)
{
        //Highlight selected groups
        _groups-
>highlightAllGroups(_painter,_showAllBody,_showAllEdge);
        _groups-
>highlightSelectedGroups(&_selectedGIDs,_painter,true,true);

        if (_curMode == MODE_MULT_SELECT)
        {
                //Draw rectangle
                drawRectangle();
        }
        else if (_curMode == MODE_ADD_GROUP)
        {
                drawFreeLine();
        }
}
```