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Visualization of green and red leaf structures in flowering pear *Pyrus calleryana* using integrated microscopy

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Abstract: Light and electron microscopy has become invaluable tools in plant science research and education. This project critically revisited both light and electron microscopy in the visualization of green and red leaves of flowering pear (*Pyrus calleryana*). Plant leaf color changes regularly occur in the fall, which is of both science and public interests. We used leaves of the flowering pear because this woody plant is commonly used in landscaping in many cities around the world. In order to visualize and compare the structures of green and red leaves, we prepared hand-sections from both green and red leaves. Sections were examined and imaged with a compound microscope and a laser scanning confocal microscope, respectively. Our data indicated that chlorophyll fluorescence intensity was significantly reduced in the red leaf compared to that of the green leaf. Histological data also showed that the pigment, which is called anthocyanin and makes the leaf red, was located in the leaf palisade and sponge parenchyma cells. Scanning electron microscopy provided detailed cellular pattern and stomatal distribution on leaf lower surfaces. And transmission electron microscopy data clearly revealed the chloroplast ultrastructure in the green leaf and its derivative – gerontoplast structure in the red leaf. This study provides us a new microscopic viewing of the outstanding natural phenomenon in the leaf color change in flowering pear and the microscopic methods described in this paper are useful for plant science research and education in general.

Keywords: Flowering Pear, Leaf Structure, Confocal Microscopy, Electron Microscopy

1. Introduction

Beautiful autumn leaf color has been attracting great attention from public and plant biologists. The development of most deciduous leaves, such as of maple and flowering pear, is closely associated with seasonal changes in nature. In spring, the leaves start to initiate and gradually mature to their final forms. Mature leaves take advantages of long-day sun energy and actively convert CO_2 and water into sugar, a process known as photosynthesis [1], all the way through summer. In the fall, the day gradually grows shorter and night grows longer. Leaves of deciduous plants begin to prepare for winter and fall off, a process known as senescence.

Leaf colors arise from three major chemical components: chlorophyll, carotenoid, and anthocyanin. Chlorophylls make leaves green; carotenoids orange to yellow; and anthocyanins red. Both chlorophylls and carotenoids are present in the chloroplasts in leaf cells throughout the growing season. The chloroplast is a photosynthetic organelle that has its own DNA and rich thylakoid membranes. Most anthocyanins are produced in the autumn, in response to bright light and excess plant sugars within leaf cells during senescence [2].

The chloroplasts, present in green leaves, differentiate into gerontoplasts during leaf senescence. The gerontoplast is a special form of the plastid, which loses stromal components and thylakoids; but increases the number and size of electron-dense lipophilic bodies, called plastoglobules, in the transition from chloroplast [3].

There has been a rebirth of microscopy applications in biological sciences since genome era. Significant advances in the microscopy fields, such as laser scanning confocal microscopy (LSCM) and digital technology, greatly enhance the capacity that biologists apply the microscopy in their research and teaching. Here, we report the visualization of green and red leaf structures in the flowering pear (*Pyrus calleryana*), by using light, LSCM, and electron microscopy. This woody plant is also called Bradford pear, a landscaping tree species that can be found in many cities around the world.

2. Materials and Methods

2.1. Leaf Collection

Leaves were collected in the middle of October, when approximately half of the leaves turned into red while the other half remained green on the same tree. Six red and six green leaves were collected from a flowering pear tree at the library parking lot on Appalachian State University campus. The leaves were sampled from the lower part of the tree and sun-shining side only. The leaves were immediately photographed with a Nikon Cool Pix 8800 VR digital camera (Tokyo, Japan).

2.2. Sample Preparation for Optical Microscopy

Chemical fixation is not suitable for studying anthocynins and chlorophylls because the former is water soluble and the later can be dissolved by ethanol. They will disappear during fixation and dehydration processes. Therefore, we prepared fresh leaf cross sections using freehand sectioning method for optical and laser scanning confocal microscopy.

Briefly, 8 mm in diameter carrot cylinders were obtained with a cork borer. The cylinders were split into two halves and cut small grooves (to fit the sample to be sectioned) on each half longitudinally. Roughly 5 mm^2 samples were cut through the midvein from fresh green and color leaves, respectively. The leaf sample was enclosed in the carrot cylinder, allowing the midvein to fit into the groove. Hand cross sections were immediately cut using a sharp razor blade. And sections were mounted onto glass slides with water and quickly imaged with Olympus IX81 (Olympus America Inc., Center Valley, PA) for bright field imaging or Zeiss 510 LSCM (Carl Zeiss Inc., Thornwood, NY) for fluorescence imaging.

2.3. Sample Preparation for Scanning Electron Microscopy

Several 10 x 10 mm^2 samples were cut from fresh green and red leaves collected above and were fixed, respectively in 2.5% glutaraldehyde diluted with 0.1 M sodium phosphate buffer. The samples were dehydrated in a graded ethanol series, and dried with a Polaron critical point drying apparatus (Polaron Instruments Inc., Doylestown, PA). All dried samples were mounted onto aluminum stubs, sputter coated with gold, and imaged with a Quanta 200 environmental scanning electron microscope (FEI Company, Hillsboro, OR).

2.4. Sample Preparation for Transmission Electron Microscopy

Several 2 x 5 mm^2 samples were cut from fresh green leaves collected above and fixed in 2.5% glutaraldehyde and postfixed in 1% OsO4 for about 2 h at room temperature. Red leaf samples were fixed in the same manner but in a separate vial. Samples were dehydrated through a graded ethanol series and embedded in Spurr's epoxy resin (Electron Microscopy Science, Hatfield, PA). All the samples were sectioned with an Ultracut E microtome (Reichert-Jung, Cambridge Instruments Inc., Buffalo, NY). Ultrathin sections (90–100 nm) were mounted onto 200-mesh copper grids and stained with 1% aqueous uranyl acetate and Reynolds lead citrate solution [4]. The sections were examined and imaged at 80 kV with a JEM-1400 transmission electron microscope (JEOL Ltd., Tokyo, Japan).

All digital images were processed and annotated for this paper with Adobe Photoshop (Adobe Systems Inc., San Jose, CA).

3. Results

Flowering pear (*Pyrus calleryana*) was one of the most beautiful and earliest spring blooming trees in the family Rosaceae. This species could tolerate most soil types, was pest- and pollution-resistant, and could be grown throughout the temperate regions. Its long-lasting beautiful white flowers appeared in April, and its glossy green leaves covered the tree all the way through summer and turned to scarlet red in October/November. The tree naturally grew into a tight and symmetrical shape and could reach to 40-50 feet in height (Fig. 1A). Therefore, flowering pear had been an ideal ornament for landscape in North America and Southeast Asia.

Leaves (Fig. 1B) were simple, broadly ovate to elliptical, shiny glossy on both green and red. Leaf margin was slightly dentate. Each leaf had one dominant midrib (midvein, MV) from which secondary vascular bundles extended. The secondary veins kept branching and produced fine tertiary veins, and so forth. Altogether they formed reticulate venation. Veins were important in distributing water and minerals from stem into leaf and simultaneously sending photosynthetic products – sugars from leaf to stem for use or storage elsewhere. Leaf blade

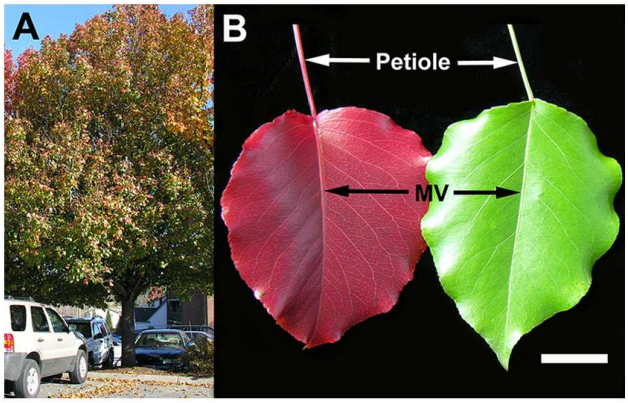

Figure 1. *Digital photographs showing a flowering pear tree (Pyrus calleryana) (A) and representative green and red leaves (B) sampled for this research. MV = midvein; Bar = 20 mm.*

(lamina) was connected to the stem/branch via a petiole. Mature leaves were 5 – 8 cm in length.

3.1. Leaf Structure and Color Compound Distribution

In a cross section view (Fig. 2A), the flowering pear leaf had two surface layers. The top layer was called the upper epidermis (or ventral surface). The bottom layer was the lower epidermis (or dorsal surface). The two layers wrapped around the entire leaf and protected the leaf. The shiny look from the upper surface (Fig. 1B) was due to a waxy cuticle layer on top of the upper epidermis (Fig. 2). The next layer to the upper epidermis was the palisade parenchyma (PP) (or palisade mesophyll), which was a layer of closely packed and elongated cells that performed photosynthesis. Between the PP and lower epidermis, there were loosely packed and oval-to-irregular shape photosynthetic cells. Those cells were collectively called spongy parenchyma (SP) (or spongy mesophyll). Vascular tissue that made up leaf veins included xylem (X), which brought water up from the roots, and phloem (P), which was responsible for transporting the products of photosynthesis. There were also supporting tissues – collenchyma (C) located both above and beneath the midvein (MV).

In green leaf, chlorophylls were concentrated in PP and SP (Fig. 2A). In red leaf, green chlorophylls diminished and red anthocyanin appeared in both PP and SP (Fig. 2B). It was also evident that anthocyanin synthesized first in PP because SP in some red leaves is yellowish (data not shown). The yellowish SP indicated chlorophyll degradation that unmasked the carotenoids but still lacked of anthocyanin synthesis.

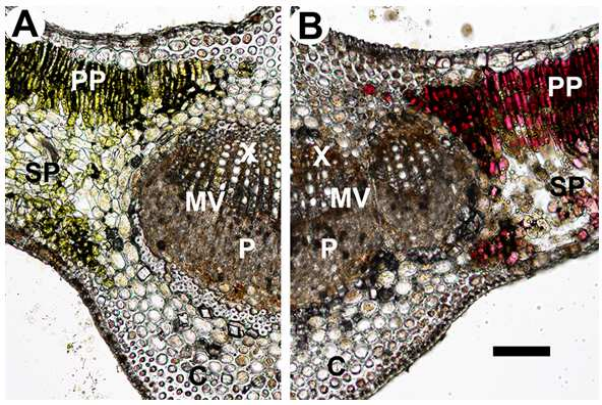

Figure 2. *Bright-filed imaging. Freehand sections were made from green (A) and red (B) leaves, respectively. In the green leaf, chlorophylls were dominant both in palisade parenchyma (PP) and spongy parenchyma (SP); while in the red leaf, anthocyanins were dominant in the PP and SP. The sections were imaged at the same magnification. C = collenchyma; MV = midvein; P = phloem; X = xylem; Bar = 100 µm.*

Scanning electron microscopy showed that the lower leaf surfaces had dense stomata (Fig. 3). The leaf margin and tertiary veins were also visualized in details (Fig. 3A and B). It was evident that color leaf lower surface had less wax and epidermal cells were smoother than those in the green leaf (Fig. 3C and D).

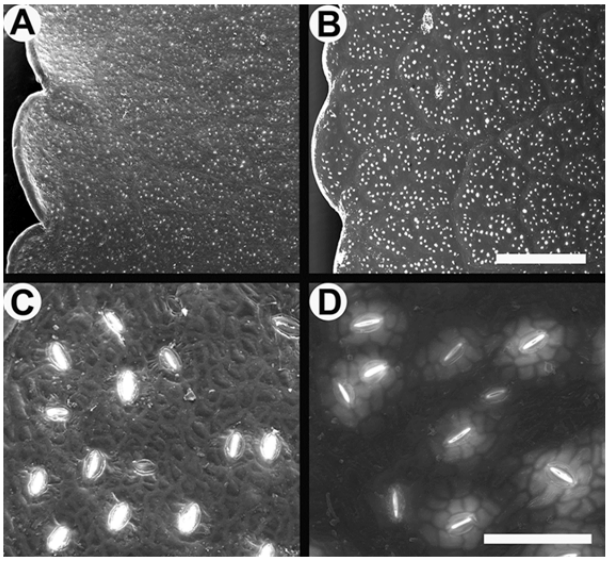

Figure 3. *Scanning electron micrographs showing the lower surfaces of green (A and C) and red (B and D) leaves of the flowering pear. Both leaves had dense stomata (A and B). The green leaf showed clear cellular patterns with wax on the surface (C); but the wax layer disappeared in the red leaf (D). Bar for A and B = 1 mm; for C and D = 100 µm.*

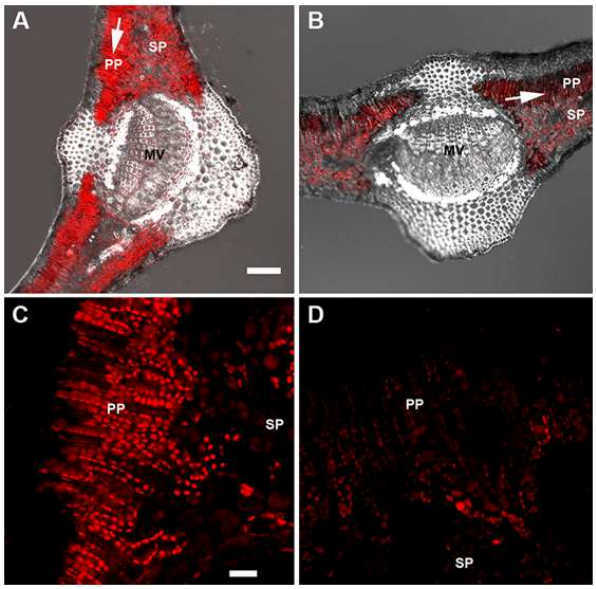

Figure 4. *Laser scanning confocal microscopy (LSCM). Freehand sections of green (A and C) and red (B and D) leaves were imaged with LSCM. Images A and B, each was recorded as a single optical section of LSCM with a 10x lens and merged with the corresponding differential interference contrast (DIC) image, indicating tissue locations of the chlorophylls in the leaves. Images C and D were fluorescence imaging only and captured with a 40x oil lens. Chlorophyll fluorescence in the green leaf was significantly stronger than that in the red leaf. Bars for A and B = 100 µm; for C and D = 20 µm.*

3.2. Leaf Fluorescence

Chlorophylls are the chemicals that absorb light energy: some of the energy is used for photosynthesis while another portion of the energy can be re-emitted as light. The latter is called chlorophyll fluorescence. When a fresh leaf was illuminated with HeNe 633 nm laser using a laser scanning

confocal microscope (LSCM), the chlorophyll fluorescence could be collected by using a LP650 nm filter. The fluorescence intensity was positively correlated to the amount of chlorophylls in the leaf, provided that the laser strength (excitation energy) was constant. Hand-sections of green and red leaves were imaged with the LSCM, respectively (Fig. 4).

Fluorescence image was merged with its correspondent differential interference contrast (DIC) image using a 10x objective lens and the same laser settings (Fig. 4A and B). Strong chlorophyll fluorescence was observed in the PP and SP of the green leaf (Fig. 4A), while the fluorescence intensity in the red leaf was much weaker (Fig. 4B). Using a 40x oil lens, the chloroplast morphology was clearer and chlorophyll fluorescence intensity was stronger in the green leaf (Fig. 4C) than those in the red leaf, respectively (Fig. 4D).

3.3. Leaf Ultrastructure

As indicated above, PP and SP are photosynthetic tissues where chlorophylls/chloroplasts are located. PP and SP in a mature green leaf all had large vacuoles in the cells and chloroplasts along with much of the cytoplasm were pushed closer to the cell walls (Fig. 5A). In contrast, vacuoles in the red leaf cells were even larger but the average size of chloroplasts was smaller (Fig. 5B).

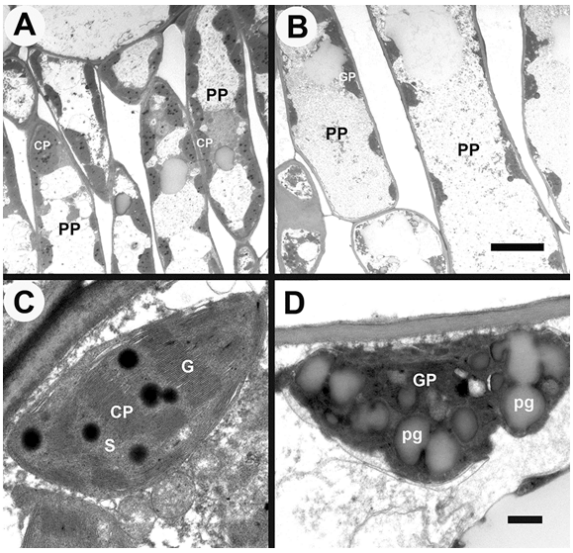

Figure 5. *Transmission electron microscopic (TEM) images of palisade parenchyma (PP) and chloroplasts (CP) in the PP from green (A and C) and red (B and D) leaves in the flowering pear. CP in the green leaf (C) exhibited thylakoid membrane stacks called grana (G) and amorphous stroma (S). Thylakoid membrane of CP in the red leaf disappeared while the number and size of lipid globules (plastoglobuli) (pg) increased; but more electron translucent (D). The CP now turned into a gerontoplast (GP). Bars for A and B = 10 μm; for C and D = 0.5 μm.*

An individual chloroplast in the green leaf had outer and inner membranes (Fig. 5C). The inner membrane was extensive and highly folded to increase the area for photosynthetic pigments – chlorophyll. Single membranes were thylakoids (T) and multi-membrane stacks were grana (G). The rest fluid in the chloroplast was stroma (S). A few electron-dense oil bodies already appeared in the green leaf (Fig. 5C). In contrast, the thylakoids and grana all disappeared in the red leaf "chloroplast"; but more lipophilic droplets – plastoglobuli (pg) were visualized in the organelle (Fig. 5D). The chloroplast from a green leaf now turned into a gerontoplast (GP) in the red leaf.

4. Discussion

This project has further demonstrated that different microscopy methodologies complement each other, providing scientific data from different perspectives in the study of the same object. Our digital photography documents the fantastic colors and morphology of the flowering pear tree. Optical compound microscopy reveals color distributions at tissue level and it is a critical technology for histology. Confocal microscopy is powerful in chlorophyll auto-fluorescence imaging; certainly LSCM is even more useful in all kinds of live cell imaging [5]. Electron microscopy has been broadly used in biology, medicine, and material sciences [6]. Here scanning electron microscopy details leaf lower surface morphology. And transmission electron microscopy visualizes leaf senescence process at sub-cellular level and clearly shows the ultrastructural differences between chloroplast and gerontoplast.

Even though freehand sectioning is an "old" microscopy sample preparation technique, this method is easy, fast, cost-efficient, and very suitable for visualization of live structures/chemicals that can not be processed with chemical fixation and dehydration. Certainly, a vibratome and a cryostat will have a control in section thickness of live tissues, if the resource is available.

Chlorophyll/chloroplast color has long been a confusing subject for many biology students. The reason that plant leaves look green to the naked eye is that chlorophyll/chloroplasts absorb mainly the red (around 670 nm) and infrared (750 nm – 1 mm) wavelengths of light; but reflect green wavelength (around 530 nm). The color we see is the color that is reflected from the object. In confocal microscopy, a beam splitter and filters are used to separate the original excitation light/laser from the fluorescent signals. The fluorescent information we see/collect is re-emitted light from the object. Chloroplasts look red under the confocal microscope because chlorophyll absorbs 633 nm laser energy and gives off fluorescence between 660 and 700 nm.

The beautiful autumn colors come from leaf morphological and physiological changes of the deciduous plants. Flowering pear has become a promising landscaping deciduous species and it will decorate more cities around the world. Integrated microscopy helps us understand this natural phenomenon at morphological, histological, cellular and sub-cellular levels. Importantly, integrated microscopy has been and will continue to be an indispensable technology for biological research and science education.

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