ABSTRACT

2D sections of rice (Oryza sativa L.) mesophyll cells are complex and the cell interior contains a large volume of chloroplasts. Furthermore, the structure of chloroplasts changes in response to salt stress. In this study, we used a 3D reconstruction method based on serial section light microscopy to analyze a wide range of structures in leaf tissues and compared the intracellular structure of mesophyll cells of rice under normal and salt-treated conditions. The 3D reconstructed models revealed that mesophyll cells appeared as ellipsoid discs with several lobes around the cell periphery, with the volumes showing no significant difference between control and salt-treated plants. The chloroplast structure in the whole mesophyll cell was altered under salt stress, showing a reduced coverage area, whereas their volumes did not differ between treatments. These findings suggest that 3D reconstruction based on serial light micrographs can reveal the morphology of cells and chloroplasts in plant tissue.
Introduction

The leaf is one of the most important organs in plants. Their structures are generally bifacial flat and thin and consist of three tissues; epidermal, mesophyll, and vascular tissues. Specific leaf tissues, cells, and organelles are involved in the process of photosynthesis. To understand the photosynthetic mechanism, numerous studies have been conducted to observe the structure of leaf tissue or cells by light or transmission electron microscopy after sectioning that shows only two-dimensional (2D) images. The volumes and surface areas of cells and chloroplasts have been estimated by using model-based formulas applied to 2D images (Burundukova et al., 2003; Chonan, 1967; 1970; Maksymowycz, 1963; Thain, 1983). However, accurately estimating the volumes and surface areas of cells with complex shapes, particularly the cell surfaces in spongy mesophyll, is difficult using these methods (Kubínová et al., 2017).

Stacking of serial 2D images can provide 3D volume data. Serial sectioning is one method useful for reconstructing 3D models (Théroux-Rancourt et al., 2017). Serial sectioning electron microscopy was introduced in the 1950s (Birch-Andersen, 1955; Sjöstrand, 1958), and several methods corresponding to different types of electron microscopy have been developed (Peddie & Collinson, 2014), such as serial section transmission electron microscopy (ssTEM; Birch-Andersen, 1955; Sjöstrand, 1958; Yamane et al., 2018), serial block face-scanning electron microscopy (SBF-SEM; Denk & Horstmann, 2004; Hughes, Hawes, Monteith, & Vaughan, 2014), and serial section scanning electron microscopy using a focused ion beam (FIB-SEM; Knott et al., 2008; Oi et al., 2017). Although ssTEM provides high-resolution data of the cellular ultrastructure, this method is laborious and requires high technical skills, such as sectioning, collection, staining, and washing of fragile ultrathin (~0.1 µm thickness) sections. Although FIB-SEM is rapid and accurate because serial sectioning and imaging are automated, the machine is quite costly (Kubínová et al., 2017). After serial sectioning by electron microscopy, manual segmentation of each compartment in the gray-scale images is necessary to reconstruct a 3D model of the target structure, which is time-consuming and labor-intensive (Oi et al., 2017). The use of X-ray microcomputed tomography (micro-CT) for 3D analysis of leaf tissue was recently reported; however, the intracellular structure cannot be accurately determined without light microscope support (Théroux-Rancourt et al., 2017).

Semithin (0.5–2 µm thickness) sectioning has been developed to fill the gap between light microscopy and electron microscopy (Grube & Kusumoto, 1986; Litwin, 1985). The semithin sections are cut from a block embedded in resin, as same as TEM, but are easier to handle compared to ultrathin sections. We can distinguish cells, organelles, and other substances in the semithin sections with a light microscope through histo-staining (Théroux-Rancourt et al., 2017). Blumer et al. (2002) reported semithin serial sectioning for light microscopy using a diamond knife with a large water trough. This method provided ribbons of serial sections aligned in the same orientation on a glass slide, which is highly advantageous for 3D reconstruction. Although serial sections of paraffin-embedded block cut with a rotary microtome are also used for 3D reconstruction (Gittenbeek et al., 2006), the paraffin sections are too thick (5–12 µm or more, thickness) to distinguish the cellular or subcellular structures clearly compared to resin sections (Ruzin, 1999). Furthermore, the paraffin is necessary to remove before observations (Yeung, 1999), and through the removing, some sections or parts of structures are lost or damaged frequently (Pichat et al., 2018).

Rice (Oryza sativa L.) mesophyll tissues are among the most difficult structures to evaluate by conventional microscopy because their mesophyll cells are smaller (Chonan, 1978) and have a higher chloroplast density (Oi et al., 2017; Sage & Sage, 2009) compared to other crops. The unique shape of rice mesophyll cells, which is discoid with several lobes covered by chloroplasts inside of the cell periphery, is thought to enhance CO₂ diffusion from the intercellular space to the stroma (Oi et al., 2017; Sage & Sage, 2009). Therefore, accurately assessing mesophyll morphology is essential for estimating photosynthetic ability (Burundukova et al., 2003). Recently, the complicated structure of a whole rice mesophyll cell and the intracellular distribution of chloroplast have been revealed by 3D reconstruction using FIB-SEM (Oi et al., 2017). However, various forms of mesophyll cells were observed in the cross-sections of rice leaf tissues (Burundukova et al., 2003), and few studies have evaluated the 3D anatomy of rice mesophyll cells at the tissue level.

Salinity is an environmental stress that can reduce plant productivity. Excess salt accumulation decreases rice yields (Munns & Tester, 2008), and the leaves show higher salt sensitivity than the roots (Munns & Termaat, 1986). Chloroplast is known as an organelle that is sensitive to environmental stress (Omoto et al., 2009). Under salinity stress, the chloroplast structure in the leaf mesophyll cell is altered, which affects photosynthetic activity (Yamane et al., 2008). These structural changes are particularly prominent in the thylakoids (Omoto, Taniguchi, & Miyake, 2010), which become swollen under salinity stress (Mitsuya et al., 2003; Rahman et al., 2000; Yamane et al., 2008). Moreover, it has been reported that chloroplast protrusions (Bourett et al., 1999; Yamane, Mitsuya, Taniguchi, & Miyake, 2012) and pockets (Yamane et al., 2018) form in the presence of salt. Although previous studies based on TEM observations revealed changes in...
the chloroplast ultrastructure in response to salinity, the chloroplast volume in whole mesophyll cells has not been widely evaluated.

In this study, we established a method for 3D reconstruction of mesophyll cells and chloroplasts in rice leaf tissue using serial section light microscopy (ssLM). We also compared the shape and size of 3D models of rice leaves under normal growth and salt stress conditions.

Materials and methods

Plant materials and growth conditions

Caryopsis of rice (*Oryza sativa* L. ‘Nipponbare’) was soaked in distilled water and incubated in a growth chamber at 28°C/20°C (day/night) until the white tip of the coleoptile appeared. Then, 24 seedlings were transplanted onto a mesh above a plastic bucket containing tap water and grown in a growth chamber under a 14-h photoperiod (8:00 to 22:00 h) at 400–500 µmol m⁻² s⁻¹ and 28°C/20°C (day/night). After two days, the tap water was changed to nutrient solution (Mae & Ohira, 1981), and all solutions were changed once per week. At 21 days after transplantation, the plants were treated with 100 mM NaCl in the nutrition solution as salt stress or without NaCl as a control for four days.

Sample fixation and embedding

The middle parts of the fully expanded uppermost leaf (fifth leaf) blades of the control and salt-treated plants were cut into small segments (1 × 2 mm), and the segments were fixed with Karnovsky’s fixative (mixture of...
4% paraformaldehyde and 5% glutaraldehyde) in 50 mM sodium phosphate buffer (pH 7.2) for 5 h. The leaf segments were rinsed with the buffer for 2 h and post-fixed with 2% osmium tetroxide in the same buffer for 10 h. The segments were rinsed with the buffer and distilled water, and then dehydrated in a graded series of acetone (30%, 50%, 70%, 90%, 99%, and 100%), followed by treatment with propylene oxide. Finally, the segments were embedded in Spurr’s resin (Agar Low Viscosity Resin, Agar Scientific, UK) (Figure 1(a)).

**Serial sectioning and staining**

Semithin serial sectioning was performed as described by Blumer et al. (2002) using a diamond knife with a large water trough (Histo-Jumbo, Diatome, Switzerland) mounted on an ultramicrotome (EM UC6, Leica, Germany). The segment block was trimmed into the usual shape of a truncated pyramid. The leading face of the trimmed block was coated with a thin layer of the glue (SU multi-purpose glue, Konishi, Japan) diluted in xylem (3:1) to improve adhesion between the sections and obtain stable ribbons. A glass slide was placed in distilled water one day before sectioning to ensure that the whole surface was hydrophilic, providing a stable condition for mounting the ribbons. The wet glass slide was dipped in the large water trough of the diamond knife before sectioning. Then the block was cut into serial longitudinal sections 0.5 µm thick (Figure 1(b)). The ribbons of semithin sections were placed on a glass slide, which then was placed on a hot plate at 60°C for 10 min to stretch the ribbons.

Serial sections were stained with thionin and acridine orange solution (Paul, 1980) (Figure 1(c)). The sections were stained with thionin for 10 min and then counterstained with acridine orange for 2 min on a hot plate at 42–45°C.

**Three-dimensional reconstruction**

The stained sections were observed under a light microscope (BX 51, Olympus, Japan) with a CMOS camera (DP74, Olympus) and then images were acquired successfully. Brightness and contrast of images were adjusted, and then the images were aligned and cropped (Figure 1(d)), using ImageJ software (http://fiji.sc/Fiji, National Institutes of Health, USA). The processed image stacks were shown as orthogonal slide images based on volume rendering using Image-Pro Premier 3D software (Ver.9.3, Media Cybernetics, USA).

To reconstruct the 3D models, the cell walls of each mesophyll cell were traced manually (Figure 1(e)), painted with different colors on a black background (Figure 1(f)), and saved in bitmap (BMP) format using PaintTool SAI software (Ver.1, Systemax, Japan). The color-segmented image stacks were then reconstructed into 3D surface rendering models of mesophyll cells using Image-Pro Premier 3D software (Figure 1(f')). In this study, the mesophyll cells without close contact with each other were selected from the middle portion of leaf tissue.

The traced mesophyll cells (Figure 1(e)) were processed into painted mesophyll cells (Figure 1(f)), mesophyll cell wall lines (Figure 1(g)), and binarized chloroplast regions (Figure 1(h)). The painted image stacks of mesophyll cells (Figure 1(f)) were masked using the ‘Threshold’ function in Image-Pro and then added to the original images to extract chloroplast regions in the segmented mesophyll cells. The chloroplast regions in segmented mesophyll cells were extracted using the ‘Threshold’ function in Image-Pro and reconstructed into 3D surface rendering models (Figure 1(h')).

To estimate the cytoplasm periphery covered by chloroplasts, the chloroplast regions (Figure 1(h)) were dilated using the function ‘3D filters’ (width x height x depth = 7 x 7 x 1) in Image-Pro (Figure 1(h')) and the obtained images were overlapped with the cell wall lines (Figure 1(g)). In the overlapped images (Figure 1(i)), the region where chloroplasts contacted the cell wall was detected (Figure 1(j)). The detected region was reconstructed as a 3D surface rendering model using Image-Pro as cytoplasm periphery coverage by chloroplasts (Figure 1(k)). The cytoplasm periphery coverage was shown with mesophyll cells, and the combined model showed gap regions (blue) among the coverage (yellow) (Figure 1(l)).

All 3D surface rendering models were smoothed with ‘Low-pass filter’ (3:3:3), and their volume and surface area were calculated using the ‘3D Measure’ function in Image-Pro.

**Statistical analysis**

The data were statistically analyzed with Student’s t-test using add-in software for Microsoft Excel (Statcel 3, OMS Publishing Inc., Japan) for Windows. Differences were considered as significant when P < 0.05.

**Results**

**Mesophyll cells in rice leaf tissue**

Leaf segments were continuously cut, and several ribbons (30–50 serial sections) were aligned in the same orientation on a glass slide (total of 100–200 sections) (Figure 1(c)). The longitudinal (xy) serial sections were stacked, and the virtual sections in the transversal (yz) and paradermal (xz) views were obtained from the reconstructed images (Figure 2). Although the sharpness of the virtual sections (Figure 2(b,c)) was lower
compared to that of the actual sections (Figure 2(a)), the shape of mesophyll cells and their intracellular structure were identifiable. In the stacked images, we acquired the mesophyll cell layers located between two small vascular bundles. There were four to five layers of mesophyll cells sandwiched between the upper and lower epidermis (Figure 2(a,b)), and mesophyll cells in the middle layer were selected (Figure 2, Video S1). In the longitudinal and paradermal sections, the mesophyll cells appeared to be oblong extended in a direction perpendicular to the vascular bundle (Figure 2(a,c)). In contrast, in the transversal sections, the mesophyll cells appeared as profusely lobed-shaped (Figure 2(b)). Similar images of the salt-treated leaf were reconstructed and virtual sections were obtained (Figure 3, Video S2). There were five to six layers of mesophyll cells between the upper and lower epidermis (Figure 3(a,b)), and mesophyll cells were selected from the middle layer. In both the control and salt-treated plants, eight mesophyll cells were segmented in the stacked images and reconstructed into 3D models (Figure 4; Video S3, S4). In longitudinal view of control and salt-treated plants, the mesophyll cells appeared to be oblong with a few projections (Figure 4(a,d)). In the transversal views, the mesophyll cells appeared as ellipsoid discs with several lobes in control and salt-treated plants (Figure 4(b,e)). In the paradermal views, the mesophyll cells appeared similar to in the longitudinal view but with a greater number of protuberances in both plants (Figure 4(c,f)).

Based on the reconstructed models, the volume and surface area of mesophyll cells were calculated (Figure 5). In the control plant, the average volume of the eight mesophyll cells was 2829 ± 609 µm$^3$ (mean ± SD) and the average of surface area was 1397 ± 208 µm$^2$ (Figure 5(a,b); Table S1). The smallest mesophyll cell in the control plant had a volume and surface area of 2086 µm$^3$ and 1131 µm$^2$, respectively (#5). The largest mesophyll cell had a volume and surface area of 3757 µm$^3$ and 1685 µm$^2$, respectively (#2). The ratio of surface area to volume was 0.50 ± 0.04 (Table S1). In salt-treated plant, the average volume and

Figure 2. Orthogonal slice images of leaf tissue in control rice plant. (a) One longitudinal image (xy) in a sequence of 137 images of rice leaf observed under a light microscope. (b, c) Orthogonal slice images of virtually imaged by volume rendering. (B) Transversal view (yz) of the image stacks, (C) paradermal view (xz) of the image stacks. Each slice plane (a, b, c) crossed the lines marked as A, B, C showed in other orientation images. BS; bundle sheath cell, IC; intercellular space, LE; lower (abaxial) epidermis, M; mesophyll cell, UE; upper (adaxial) epidermis. Different colors were used to distinguish mesophyll cells. Cutting interval (z-steps) = 0.5 µm. Total number of cuttings = 137.
surface area in mesophyll cells were 3069 ± 528 µm$^3$ and 1386 ± 180 µm$^2$, respectively (Table S1). The ratio of surface area to volume was 0.45 ± 0.02 (Table S1). Among the eight mesophyll cells in the salt-treated plant, the smallest mesophyll cells had a volume and surface area of 2313 µm$^3$ and 1132 µm$^2$, respectively (#8). The largest volume and surface area of mesophyll cells in salt-treated plant were 3919 µm$^3$ and 1716 µm$^2$, respectively (#6).

There were no significant differences in the sizes of mesophyll cells between control and salt-treated plants (Figure 5(a,b)). The volume and surface area of mesophyll cells were strongly correlated in both control and salt-treated plants ($r^2 = 0.95$ and 0.90, respectively) (Figure 5(c)).

**Chloroplasts in mesophyll cells**

Orthogonal sections showed that mesophyll chloroplasts in the control leaf appeared to be narrow and spread along the cell wall in three-angle views (Figure 2), while those in the salt-treated leaf appeared to be widespread in mesophyll cells (Figure 3). The area occupied by chloroplasts in the cell (chloroplast region) was extracted from each of the segmented cell in the control or salt-treated plant and was reconstructed into 3D model (Figure 6(a–f)). The longitudinal views showed chloroplast regions covered nearly the entire cell periphery with a few gaps in the control and salt-treated plants (Figure 6(a,d)). In the transversal view, the chloroplast regions filled nearly the entire periphery; however, there were wider gaps in the center part of the cell in the salt-treated plant than in the control plant (Figure 6(b,e)). In the paradermal view, salt-treated plant appeared to have fewer gaps compared to control plant (Figure 6(c,f)). Furthermore, 3D models of the cytoplasm periphery regions covered by chloroplasts (cytoplasm periphery coverage) were reconstructed (Figure 6(a–f), yellow) and combined with the 3D models of mesophyll cells to show the gap areas among the chloroplasts (Figure 6(a–f), blue) more prominently. In the longitudinal views, the chloroplasts showed less coverage at the cytoplasm periphery in both control and salt-treated plants (Figure 6(a,d')). In the transversal view, the

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**Figure 3.** Orthogonal slice images of leaf tissue in salt-treated rice plant.

(a) One longitudinal image (xy) in a sequence of 178 images of rice leaf observed under a light microscope. (b,c) Orthogonal slice images of virtually imaged by volume rendering. (B) Transversal view (yz) of the image stacks, (c) paradermal view (xz) of the image stacks. Each slice plane (a, b, c) crossed the lines marked as 'A, B, C' in other orientation images. BS; bundle sheath cell, IC; intercellular space, LE; lower (abaxial) epidermis, M; mesophyll cell, UE; upper (adaxial) epidermis. Different colors were used to distinguish mesophyll cells. Cutting interval (z-steps) = 0.5 µm. Total number of cuttings = 178.
chloroplasts showed less coverage on the cytoplasm periphery in salt-treated than in control plants (Figure 6(b')). In the paradermal view, the chloroplasts showed higher coverage on the cytoplasm periphery in the salt-treated plant compared to the control plant, and appeared to cover the interior of whole mesophyll cell lobes (Figure 6(c',f')).

The volume and surface area of chloroplasts were calculated based on the 3D models and showed no significant difference between control and salt-treated plants (Figure 7(a,b)). However, the ratio of surface area to volume of the chloroplast regions in salt-treated plant was significantly lower than that in control plant (Figure 7(c)). The volume ratio of chloroplasts to mesophyll cells did not differ between control (41.4%) and salt-treated (40.6%) plants (Figure 7(d)). The surface area ratio of chloroplasts to mesophyll cells was significantly lower in salt-treated (128.5%) than in control plants (139.4%) (Figure 7(e)). In addition, the

Figure 4. Reconstructed 3D models of mesophyll cells in rice leaf tissues.
(a–c) Control plant. (d–f) Salt-treated plant. (a, d) Longitudinal views. (b, e) Transversal views. (c, f) Paradermal views. The numbers indicate the order of mesophyll cells on a leaf tissue.

Figure 5. Quantitative comparison of mesophyll cells in control and salt-treated plants.
(a) Volume. (b) Surface area. (c) Relationship between volume and surface area. Circle: control, square: salt-treated. Mean ± standard deviation (n = 8). Mean between control and salt-treated was compared by Student’s t-test (*P < 0.05).
ratio of cytoplasm periphery coverage in mesophyll cells was significantly lower in salt-treated plant than in control plant (Figure 7(f)). This suggests that chloroplasts covered a smaller surface area of mesophyll cells in the salt-treated plant compared to in the control plant.

**Discussion**

In this study, ssLM was used to reconstruct 3D structures of mesophyll cells and chloroplast regions in leaf tissues based on consecutive sections. This method does not
require a specific apparatus and is less time-consuming compared to ssTEM, SBF-SEM or FIB-SEM. Although the spatial resolution of the light microscope is low (0.2 µm) compared to the electron microscope (0.1 nm) (Bozzola & Russell, 1992), this resolution is enough to distinguish the cellular and subcellular structures in tissue level. The serial sections are manually prepared using an ultramicrotome at 0.5-µm-thick intervals. Using a diamond knife with a large water trough assists to acquire hundreds of stable semithin serial sections and to examine the wide scale of leaf tissue (Figure 1(b,c)). Moreover, ssLM followed by 3D reconstruction using image processing software provides virtual sectional images at various angles (Figures 2, 3); unlike traditional microscopy techniques which many sections at different angles are needed to understand the leaf structure (Chonan, 1967; Turrell, 1936).

In this study, eight whole mesophyll cells in the control or salt-treated leaves were reconstructed based on stacks of serial sections and observed at three-angle views (Figures 2, 3). The eight mesophyll cells were packed in leaf tissues that were at least 70–90 µm thick (Figures 2(b), 3(b)). Meanwhile, FIB-SEM methods can obtain the volume at a maximum thickness of 50 µm (Zankel et al., 2014). In this study, we observed mesophyll cells over a wider range compared to previous studies using FIB-SEM (Oi et al., 2017, in press); Oi et al. (in press) observed only three cells for each treatment, without information of tissue structures. The typical rice mesophyll cell appeared as an armed discoid in the transversal view, while mesophyll cells in the longitudinal and paradermal views appeared as oblong with several projections on the surface (Figure 4) (Chonan, 1967; Oi et al., 2017; Sage & Sage, 2009). The larger mesophyll cells exhibited armed ellipsoid structures that were concavo-convex on the cell periphery (Figure 4(b), control #7; 4E, salt-treated #4, #6), located in the middle of mesophyll tissue and touching the bundle sheath cells (Sage & Sage, 2009). Comparison of the reconstructed 3D models revealed that salt-stress did not affect the shape and size of mesophyll cells. The volume and surface area in both treatments were significantly correlated (Figure 5(c)), and the ratio of surface area to volume of mesophyll cells (Table S1) was similar to that found in a previous study of rice mesophyll cells (Chonan, 1967). Therefore, exposure to salt stress in this study did not affect the morphogenesis of mesophyll cells.

The chloroplast regions in each mesophyll cell were also reconstructed based on serial images of mesophyll cells. The chloroplast regions almost covered the cytoplasm periphery and fit to the shape of a mesophyll cell (Figure 6) (Oi et al., 2017; Sage & Sage, 2009). The volume, surface area, and ratio of chloroplasts in mesophyll cells
were calculated based on the voxels of the 3D models (Figure 7), which is more accurate compared to model-based and formula approaches in which equations were required to estimate ideal cell shapes assumed from 2D images (Burundukova et al., 2003; Thain, 1983). In addition, ssLM for leaf tissue showed intercellular space and stomata, which are important factors affecting gas exchange and photosynthetic capacity (Théraux-Rancourt et al., 2017).

The morphology of chloroplasts in rice mesophyll cells under normal and salt stress conditions was compared in this study. Previous studies reported that salt stress affects the chloroplast ultrastructure; the swelling of thylakoid (Lee et al., 2013; Mitsuya et al., 2003; Rahman et al., 2000; Yamane et al., 2003), stromule or protrusion formation (Buchner et al., 2007; Yamane et al., 2012), and formation of pockets mainly associated within the thin layer of the chloroplast stroma (Yamane et al., 2018). Although such as ultrastructures in chloroplasts were not observed in this study because of the lower resolution of light microscope than TEM, ssLM followed by 3D reconstruction revealed structural changes caused by salt stress (Figure 6). Chloroplasts in the control plant covered nearly the whole periphery of mesophyll cells (Figure 6(a–c)) (Oi et al., 2017; Sage & Sage, 2009), whereas in the salt stress plant, the chloroplasts covered a part of the cell periphery (Figure 6(d–f)). However, the volume of chloroplasts in mesophyll cells did not differ between control and salt-treated plants (Figure 7(a,d)). A recent study using FIB-SEM reported that chloroplasts in rice mesophyll cells changed to a spherical shape under salinity stress (Oi et al., in press). In the present study, the coverage area of chloroplasts on cytoplasm periphery was lower in salt-treated mesophyll cells (Figure 7(f)). Therefore, chloroplasts may respond to salt stress by altering their shape from a flat form, which was typically observed in the control plant, to a round form with lower cytoplasm periphery coverage.

In conclusion, the ssLM method was used to reconstruct 3D models of mesophyll cells in rice leaf tissue. Widely varying rice tissue structures were observed, and 3D models of eight mesophyll cells and chloroplast regions were reconstructed from light micrographs. Furthermore, the 3D models indicated that the chloroplasts decreased their coverage of the cytoplasm periphery by changing their shape without decreasing their volume under salt stress, which did not change the shape and size of mesophyll cells. Although the resolution of light microscope is lower than that of the electron microscope, ssLM is accurate enough to observe a wide scale of leaf tissues and provide information on the intracellular structure.

Disclosure statement

No potential conflict of interest was reported by the authors.

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