Four-dimensional imaging with virtual reality to quantitatively explore jigsaw puzzle-like morphogenesis of Arabidopsis cotyledon pavement cells

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Abstract  In most dicotyledonous plants, leaf pavement cells exhibit complex jigsaw puzzle-like cell morphogenesis during leaf expansion. Although detailed molecular biological information and mathematical modeling of this jigsaw puzzle-like cell morphogenesis are now available, a full understanding of this process remains elusive. Recent reports have highlighted the importance of three-dimensional (3D) structures (i.e., anticlinal and periclinal cell wall) in understanding the mechanical models that describe this morphogenetic process. We believe that it is important to acquire 3D shapes of pavement cells over time, i.e., acquire and analyze four-dimensional (4D) information when studying the relationship between mechanical modeling and simulations and the actual cell shape. In this report, we have developed a framework to capture and analyze 4D morphological information of Arabidopsis thaliana cotyledon pavement cells by using both direct water immersion observations and computational image analyses, including segmentation, surface modeling, virtual reality and morphometry. The 4D cell models allowed us to perform time-lapse 3D morphometrical analysis, providing detailed quantitative information about changes in cell growth rate and shape, with cellular complexity observed to increase during cell growth. The framework should enable analysis of various phenotypes (e.g., mutants) in greater detail, especially in the 3D deformation of the cotyledon surface, and evaluation of theoretical models that describe pavement cell morphogenesis using computational simulations. Additionally, our accurate and high-throughput acquisition of growing cell structures should be suitable for use in generating in silico model cell structures.

Key words: 4D imaging, cell morphometry, image analysis, jigsaw puzzle-like cell morphogenesis, virtual reality.

Introduction

Plant leaf pavement cells show dynamic jigsaw puzzle-like cell morphogenesis during leaf expansion in most dicotyledonous plants, and research on these cells is attracting significant interest among plant cell biologists. A substantial body of research has revealed the molecular mechanisms and enhanced our understanding of the fundamental features of pavement cells. Although knowledge about key molecular players and hypothetical models to explain jigsaw puzzle-like cell morphogenesis are available, we remain far from a full understanding of this morphogenetic process.

Immunofluorescent labeling of microtubules and electron microscopic observations have shown that anticlinal cortical microtubule bundles and periclinal cortical microtubules splaying out from anticlinal microtubule bundles locally suppress pavement cell outgrowth via deposition of cellulose microfibrils which results in indentation of pavement cells in Vigna sinensis (Panteris et al. 1993). Important molecular regulators have been identified primarily by genetic approaches using Arabidopsis thaliana (A. thaliana), and it was hypothesized that a plant-specific Rho GTPase named Rho of plants 6 (ROP6) and a ROP-interactive CRIB motif-containing protein (RIC1) that interacts with ROP6 and microtubules contribute to the accumulation of cortical microtubules at the indent sites (Fu et al. 2002, 2005, 2009). However, a recent time-lapse imaging study showed that localization of cortical microtubules

Abbreviations: 2D, two-dimension; 3D, three-dimension; 4D, four-dimension; ABP1, auxin binding protein 1; DAS, days after sowing; GFP, green fluorescent protein; LED, light emitting diode; PIN1, PIN-FORMED 1; PIP2a, plasma membrane intrinsic protein 2a; ROP, Rho of plants; RIC, ROP-interactive CRIB motif-containing proteins; VR, virtual reality.

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does not correlate with pavement cell shape in *A. thaliana* (Belteton et al. 2018). In addition, dynamic microtubule networks were observed instead of stable microtubule bundles, which were assumed to locally restrict cell outgrowth in the previous hypothetical models (Belteton et al. 2018). Although the observations do not repudiate the impact of cortical microtubules in pavement cell morphogenesis, we should rethink the conventional oversimplified model that cortical microtubule bundles are primarily responsible for jigsaw puzzle-like cell morphogenesis (Eng and Sampathkumar 2018; Sapala et al. 2019). Actin filaments have also been suggested to play a key role in jigsaw puzzle-like cell morphogenesis. The other ROP (ROP2) and its effector RIC4 were proposed to recruit actin filaments near plasma membranes of future protrusion sites (Djakovic et al. 2006; Fu et al. 2002, 2005), and accumulated actin filaments positively regulate polarized localization of auxin efflux carrier PIN-FORMED1 (PIN1) in plasma membranes (Nagawa et al. 2012; Xu et al. 2010). In the hypothetical model, extracellular auxin binds with the auxin binding protein 1 (ABP1), which interacts with plasma membrane-localized transmembrane kinase receptor-like kinases on the plasma membrane of neighboring cells, and they activate the ROP6-mediated pathway, which locally suppresses outgrowth in neighboring cells (Xu et al. 2014). These mutually exclusive ROP2 and ROP6 pathways have been proposed as a mechanism to cooperatively produce jigsaw puzzle-like pavement cell morphogenesis (Xu et al. 2010). However, recent reports showed no significant enrichment of actin filaments during pavement cell morphogenesis (Armour et al. 2015), and a lower impact of ABP1 and PIN1 in jigsaw puzzle-like morphogenesis (Belteton et al. 2018; Gao et al. 2015). Therefore, the roles of actin filaments and the auxin-signaling pathway in pavement cell morphogenesis remains unresolved. Even if the molecular regulators remain unidentified, we cannot exclude the possibility that cooperative jigsaw puzzle-like cell morphogenesis is controlled by intercellular signaling. Our previous mathematical model that incorporates both the cell wall remodeling activity of ROPs and the intercellular signaling molecules that act over a short range successfully explains jigsaw puzzle-like cell morphogenesis (Higaki et al. 2016). In this context, small membrane vesicles were observed in curved apoplastic regions between pavement cells, suggesting the possible involvement in jigsaw puzzle-like cell morphogenesis (Akita et al. 2017).

In contrast, recent computational simulations of mechanical models have proposed that mechanical stress can initiate jigsaw puzzle-like cell morphogenesis via cell wall buckling (Bidhendi et al. 2019; Higaki et al. 2017). In this scenario, cortical microtubules are proposed to subserviently accumulate in response to mechanical signals and align along the maximal tensile stress direction (Bidhendi et al. 2019; Hamant et al. 2008; Sampathkumar et al. 2014). Because microtubules and actin filaments are possibly involved in cell wall mechanics via cellulose synthesis and exocytic pathways, these mechanically-driven morphogenesis theories do not contradict previous experimental results that cytoskeletal mutants show defects in jigsaw puzzle-like cell morphogenesis. These computational approaches in combination with the assessment of cell wall mechanics should aid future research (Altartouri et al. 2019). In this context, recent reports revealed the importance of three-dimensional (3D) structures (i.e., anticlinal and periclinal cell wall) over two-dimensional (2D) approximations (i.e., only anticlinal cell wall) (Bidhendi and Geitmann 2019; Majda et al. 2017). In previous studies, it was standard practice to image and quantify pavement cell shape by 2D approximations (Möller et al. 2017; Sánchez-Corrales et al. 2018; Wu et al. 2016). The 2D approximation is suitable because the pavement cell constitutes a planar epidermal tissue, and the importance of quantitative analysis of the 2D cell shape will remain important in future research. Nonetheless, it is necessary to acquire 3D shapes of pavement cells over time, that is, acquire and analyze four-dimensional (4D) information, especially when studying the relationship between mechanical modeling and simulations and the actual cell shape.

Here, we report a framework to capture and analyze 4D morphological information of *A. thaliana* cotyledon pavement cells by combining direct water immersion observations and computational image analyses, including segmentation, surface modeling, virtual reality (VR) and morphometry. Our framework should facilitate analysis of mutant phenotypes in more detail, and the collection of real cell structural data for evaluation of theoretical models with computer simulations in the near future.

Materials and methods

**Plant materials and growth conditions**

Transgenic *A. thaliana* plants stably expressing GFP-tagged plasma membrane intrinsic protein 2a (PIP2a) (Cutler et al. 2000) were used. Sterilized seeds were sown on 0.5× Murashige–Skoog gellan gum dishes (φ40 mm) and incubated in a growth chamber [23.5°C, 86.2 µmol m⁻² s⁻¹ light emitting diode (LED) for plant growth (Plantflec)] (LH-241FPP-S, NK system, Tokyo, Japan).

**Confocal microscopy**

Five-days-old *A. thaliana* seedlings on the 0.5× Murashige–Skoog gellan gum medium dish were put on a customized microscope stage of an upright confocal microscope (Mizuno et al. 2014, 2018a, b), and then moved to an appropriate position...
to take time-lapse 3D images of pavement cells by observations with reflection LED illumination and a low-magnified air lens (HC PL FLUOTAR 10×/0.32, Leica, Wetzlar, Germany), as shown in Figure 1C. After sample positioning, 50 µl tap water was mounted on the water lens (HCX IRAPO L 25×/0.95 W), and the lens was carefully lowered so that the water touched the adaxial cotyledon surface of the seedling on the medium dish (Figure 1A, B). The serial optical sectional images of GFP-PIP2a-labeled plasma membranes of pavement cells were then acquired at 1.7-µm intervals, using an upright confocal microscope (TCS SP8, Leica). After image acquisition, the seedlings on the dish were placed back in the growth chamber. The same confocal image acquisitions were repeated three-times, at 5-, 6- and 7-DAS, and every 24 h.

Image processing, visualization and quantification
The acquired time-lapse 3D (i.e., 4D) images were blurred with a Gaussian filter (sigma = two pixels) and then the cell regions in serial confocal sections were segmented with the ImageJ plug-in, Morphological Segmentation (Legland et al. 2016). The segmented 4D images were used to generate and visualize surface models with the bioimage analysis software Imaris (Bitplane, Belfast, UK) or AIVIA (DRVision, Bellevue, WA, USA). For VR observation of the 4D cell models, we used the VR add-on in the AIVIA software with the head mounted display (Vive Pro, HTC, New Taipei, Taiwan). The Imaris Measurement Pro tool was used for quantification of cell volume, surface area and sphericity. The sphericity is defined by

\[
\text{Sphericity} = \frac{\pi^{3/2} (6V)^{2/3}}{A}
\]

where \(V\) and \(A\) are the cell volume and surface area. A maximum sphericity value of 1 indicates a perfect sphere, and this value decreases and approaches 0 as the complexity of the shape increases.

Results and discussion
In a previous study, we monitored pavement cell morphogenesis in cotyledons, which were mounted on a chamber slide and covered with medium agar gel (Higaki et al. 2016), mainly according to Peterson and Torii (2012). This chamber slide system worked well and we succeeded in acquiring time-lapse images of pavement cell growth with jigsaw puzzle-like morphogenesis (Higaki et al. 2016). However, this system may cause mechanical stress to the cotyledons sandwiched between the glass slide and medium gel. In this study, to monitor 3D cell growth and morphogenesis of pavement cells in a more natural environment, the adaxial surface of cotyledons from seedlings standing on medium gel were in direct contact with immersion water after sample positioning and observed repeatedly at 5-, 6- and 7-DAS with an upright confocal microscope equipped with a water immersion lens (Figure 1). For visualization of plasma membranes, transgenic lines stably expressing GFP-tagged GFP-PIP2a (Cutler et al. 2000) were used. In this study, a 25× objective lens was chosen for accurate capturing of cell morphology and analysis of many cells over a wide range, i.e., 590.8 µm square regions by 1,024×1,024 pixels (Figure 2A). Because cotyledon surfaces are not completely flat and not parallel to the focal plane, the single optical section shows a ‘contour-
Like' pattern (Figure 2A). Therefore, obtaining serial optical sections was required to cover wide-range regions of the cotyledon epidermis, as imaged by a projection image generated by serial optical sections (Figure 2B). We obtained serial optical sections at 5-, 6- and 7-DAS without significant photobleaching and suppression of cotyledon expansion. In our dataset, in which three independent cotyledons were captured at three time points, the average sectional frame number was 174.2, which corresponds to 294.4 \( \mu \text{m} \) of the Z-axis height with a standard deviation of 67.46 frames (114.7 \( \mu \text{m} \)).

To make a 3D structure model of pavement cells, the cell regions were segmented with an ImageJ plug-in, Morphological Segmentation (Legland et al. 2016). After tuning parameters in Gaussian filtering as preprocess and morphological segmentation, the 3D images could be automatically binarized (Figure 2C, D). Based on the binary images, 3D surface models were also automatically produced after the tuning of the parameters using functions of the bioimage software, Imaris or Aivia (Figures 2E, F, 3). As a post processing approach, over-segmented pavement cells, guard cells and mesophyll cells were eliminated by thresholding based on cell size and visual inspection using the software. The final 3D models showed a slightly bulging shape of the adaxial side of the cotyledon surface composed of jigsaw puzzle-like shaped pavement cells (Figure 2F). Additionally, various morphometrical results could be visualized in 4D models by the software. For example, an increase in cell volume was confirmed by pseudo-color visualization (Figure 3A), suggesting the usefulness of our imaging method in pavement cell growth analysis.

In addition to the visual inspections with a 2D monitor (Figures 2E, F, 3A), VR with a head mounted display was applied to our 4D models (Figure 3B, C). VR system not only allowed us to observe the pavement cells as if an observer was walking on a cotyledon, but also greatly helped to carefully check the cell structural models (Figure 3B, C). In VR observations, it was possible to change the field of view more intuitively by physical movement when compared with using a 2D display, and time frames are easily changed by simple operation of the controller. These features of VR increase user’s engagement for exploring cells and facilitated rapid identification of under-segmented pavement cells (Figure 3A, arrow), guard cells or mesophyll cells to eliminate before morphometry. VR observations not only find inappropriate cells but also afford the frequency and tendency of inappropriate cells and provide rapid feedback for tuning the 3D modeling parameters. In the future, we will be able to perform mechanical simulations of the ‘actual cell shape,’ which will be modeled based on microscopic data and by using the physics engine in VR. In addition, we notice that the 3D models of the cells can be applied to mechanical modeling and simulation as previously we recognized the potential of VR to promote interdisciplinary research that uses microscopic living...
The 4D cell models allowed us to perform time-course 3D morphometrical analysis (Figure 4). In our dataset, 251 3D cell structures from three independent cotyledons were successfully traced from 5- to 7-DAS. Quantification of the cell volumes showed that all cells increased for 2 days (48 h; from 5- to 7-DAS) with an average increase of 16,380 µm³ (Figure 4A). We also detected a strong positive correlation between cell volume at 5-DAS and the volume increase between 5- and 7-DAS (Figure 4B), suggesting that larger cells generally have larger volume changes under these conditions. Calculation of the relative growth rate, which is defined as the cell volume per cell volume at 5-DAS (starting point of observations), indicated that its average and maximum value at 7-DAS was 1.899 (standard deviation=0.2741) and 2.630-fold (Figure 4C). The cell, which had the maximum growth rate in our dataset, was easily identified and its growth confirmed in raw confocal images and 3D models (Figure 4D). The time-course analysis of cell surface area showed similar results with cell volume (Figure 4E). Correlation analysis between cell volume and surface area revealed a very strong positive correlation (Figure 4E, F), suggesting that the cells have almost constant thickness during cell growth. Finally, sphericity, which is an indicator of cell surface area per volume (see Materials and methods for the definition), was measured (Figure 4G). Sphericity changes were not drastic from 5- to 7-DAS, but had a
negative correlation with cell volume (Figure 4H). The decrease in sphericity for the larger cells might be due to not only lateral cell wall waving but also flat growth with constant thickness during cell growth.

In conclusion, we developed a framework to capture and analyze 4D information of cotyledon pavement cell growth and morphogenesis. We believe that this framework should aid analysis of mutant phenotypes, especially in the 3D deformation of the cotyledon surface, as recently reported by Gunji et al. 2020. Additionally, our accurate and high-throughput acquisition of growing cell structures might be suitable for use in making an *in silico* model cell structure, which is generated by the observed data. The framework should be useful for computational simulations evaluating theoretical models that describe pavement cell morphogenesis (e.g., mechanical model) in future studies.

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