Frond Architecture of The Rootless Duckweed Wolffia Globosa

Jingjing Yang
Institute of Hydrobiology Chinese Academy of Sciences
Xuyao Zhao
Institute of Hydrobiology Chinese Academy of Sciences
Gaojie Li
Institute of Hydrobiology Chinese Academy of Sciences
Shiqi Hu
Institute of Hydrobiology Chinese Academy of Sciences
Hongwei Hou (houhw@ihb.ac.cn)
Institute of Hydrobiology Chinese Academy of Sciences https://orcid.org/0000-0003-4823-0270

Research article

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Abstract

Background: The plant body of duckweed species has undergone reduction and simplification from the ancient *Spirodela* species towards more-derived *Wolffia* species. Among the five duckweed genera, *Wolffia* members are rootless and represent the smallest and most-reduced species. However, we lack detailed knowledge about their structure.

Results: We conducted a comprehensive study of the morphology and anatomy of *Wolffia globosa,* the only *Wolffia* species in China. We first used X-ray microtomography imaging to reveal the three-dimensional and internal structure of the *W. globosa* frond. This showed that new fronds rapidly budded from the hollow reproductive pocket of the mother fronds and that several generations at various developmental stages could coexist in a single *W. globosa* frond. Using light microscopy, we observed that the meristem area of the *W. globosa* frond was located at the base of the reproductive pocket and composed of undifferentiated cells that continued to produce new buds. A single epidermal layer surrounded the *W. globosa* frond, and the mesophyll cells varied from small and dense palisade-like parenchyma cells to large, vacuolated cells from the ventral to the dorsal part. Furthermore, *W. globosa* fronds contained all the same organelles as other angiosperms; the most prominent organelles were chloroplasts with abundant starch grains.

Conclusions: Our study revealed that the reproductive strategy of *W. globosa* plants enables the rapid accumulation of biomass and the wide distribution of this species in various habitats. Despite their reduced body plan and size, the simplicity of the *W. globosa* frond might be overestimated. We propose that *W. globosa* plants are not only suitable for the study of structural reduction in higher plants, but also an ideal system to explore fundamental developmental processes of higher plants that cannot be addressed using other model plants.

Background

Duckweeds, aquatic monocotyledonous plants of the family *Lemnaceae*, include five genera (*Spirodela*, *Landoltia*, *Lemna*, *Wolffiella*, and *Wolffia*) with variable morphology and living habits, propagating mostly by vegetative reproduction (Les et al. 2002; Appenroth et al. 2013). Duckweeds have attracted attentions for their economic value and potential to ameliorate resource limitations and environmental problems (Appenroth et al. 2015). For example, duckweeds are widely used for standardized toxicity testing of various water contaminants including nitrogen, phosphorus, metals, and numerous organic compounds (Yang et al. 2018a; Ziegler et al. 2019). Duckweeds also possess good qualitative and quantitative nutritional profiles components without detectable anti-proliferative or cytotoxic effects and could serve as a new source of human food (Sree et al. 2019). Duckweed-based expression systems with strictly controlled formats have been developed to produce various recombinant proteins with relatively high yield (Pavel et al. 2018; Heenatigala et al. 2020). Duckweeds also may be valuable feedstock for biofuel production due to their high biomass and starch accumulation (Liu et al. 2018; Sun et al. 2020). Furthermore, their rapid growth rate, ease of cultivation and transformation, direct contact with water, and

ability to adapt to environmental changes make duckweeds suitable plant models and excellent materials for physiological studies (Appenroth et al. 2015).

Duckweeds have undergone reduction and simplification of the plant body from the ancient *Spirodela* species towards the more-derived *Wolffia* species (Landolt 1986). Among the five duckweed genera, *Wolffia* members are rootless and represent the smallest (0.5–1 mm) and most-reduced species; other species including *Spirodela*, *Landoltia*, and *Lemna* are rooted and produce additional adventitious roots. DNA content estimates also vary nearly thirteen-fold among duckweed species, ranging from *S. polyrhiza* (158 Mbp) to *W. arrhiza* (1881Mbp) (Wang et al. 2011), and negatively correlate with body size (Cao et al. 2015; Wang and Messing 2015). The striking variation in body plan and size among duckweeds is one of the most extreme examples of structural reduction in any families. However, we lack knowledge about the mechanisms driving its occurrence within the plant kingdom.

The morphology varies distinctively among 11 species of *Wolffia* (12 subspecies) (Fig. 1), but little else is known about their structure. Given its unique characteristics, we conducted a comprehensive study of the morphology and anatomy of *Wolffia* globosa, the only *Wolffia* species in China. The findings provide a foundation for future research on duckweed growth, development, physiology, and evolution. Biological research on duckweeds is growing as their genomes are being sequenced. We hope to attract more investigators and investors to join our efforts and realize the great potential of duckweed as a model system for basic and applied research in plants.

Methods

Plants cultivation and identification

W. globosa (5563) plants were collected from East Lake (N30°32', E114°21') at the city of Wuhan, Hubei Province, China (no permission was required to collect such plant samples). Plants were sterilized in 0.1% mercuric chloride for 2–3 min and then cultured in half-strength (1/2) SH medium at pH 5.5 containing 1% (w/v) sucrose and 0.8% (w/v) agar. Regenerated fronds of *W. globosa* were transferred to liquid 1/2 SH medium for longer preservation. Cultivation was conducted at 25 ± 1°C under white light of 85 μmol $m^{-2}s^{-1}$ and 16 h/8 h day-night photoperiod. *Wolffia* fronds in good condition were selected for experiments.

The identification of *W. globosa* (5563) was conducted by Jingjing Yang and P.P.M. Heenatigala using *atpF-atpH* barcode primers (Wang et al. 2010; Heenatigala et al. 2018). The identification results were submitted to the Rutgers Duckweed Stock Cooperative at the State University of New Jersey (http://www.ruduckweed.org/register.html). *W. globosa* (5563) plants were preserved at the National Aquatic Biological Resource Center.

3D structure observation of *W, globosa* frond by X-ray microtomography (MicroCT) imaging

We first used MicroCT to explore the morphology and internal structure of the *W. globosa* frond. The fronds were scanned at the MicroCT facility (Skyscan1267, Burker) and scans were obtained at a spatial resolution of 3 μm (4032 × 2688 pixel field of view), with an electron acceleration energy of 85 kV and a current of 100 μA. Detector exposure time was 750 ms, collecting 412 projections in “step and shoot” mode with no averaging, resulting in a scan duration of 9 min per sample. Radiograph reconstruction was carried out using NRecon reconstruction software (version 1.7.4.2, Bruker) with a beam hardening correction of 15. Finally, the scanned area beyond the plant sample was removed and reconstructed into 3D volumes using a filtered back-projection algorithm.

Light and electron microscope observation

For SEM, the fresh fronds were fixed in 2.5% glutaraldehyde in phosphate-buffered saline (PBS) buffer (1M, pH 7.4) overnight at 4°C followed by a stepwise ethanol and tert butanol dehydration. Then samples were dried using a freeze dryer (Hitachi ES-2030). The obtained specimens were examined with a Scanning Electron Microscope (Hitachi S4800) at 30 KV.

For light and transmission electron microscopy (TEM), the samples were washed in PBS buffer after fixing overnight at 4°C. Then samples were post-fixed with 1% OsO_4 in PBS for 2 h at 4°C following stepwise ethanol and acetone dehydration and infiltration with Spurr’s epoxy resin. The treated samples were embedded and polymerized in Spurr’s epoxy resin at 60°C for 48 h. Sections for light microscopy were cut using a LEICA EM UC 7 instrument with a glass knife and stained with 1% toluidine blue. The obtained specimens were photographed with an OLYMPUS BX53 camera. Ultra-thin sections (70 nm) for TEM were also cut using a LEICA EM UC 7 instrument and double-stained with 2% uranyl acetate and Sato’s lead citrate (28). The obtained specimens were examined with a transmission electron microscope (Hitachi-7700) at 120 kV.

Results

Morphology of the *W. globosa* frond

The three-dimensional (3D) volumes of the *W. globosa* frond are shown in Fig. 1 and Movies S1. The oval-shaped *W. globosa* frond could be divided into dorsal, ventral, and lateral parts (Fig. 2 A1). There was one big cavity in both the mother frond (MF) and daughter frond (DF1) named the reproductive pocket (RpM and RpD, respectively) (Fig. 2 A2–A5). The MF had two visible daughter fronds (DF1 and DF2), one (DF2) budding from the base of the RpM. The DF1 also had two new buds (GF1 and GF2) (Fig. 2 A3–A5). The empty RpM with the new bud (DF2) was exposed when the attached daughter frond (DF1) was separated (Fig. 2 B2–B5). It was located at one end of the MF and opened when DF1 protruded from the MF. Stomata were found only in the dorsal part of the frond; no stoma were found in the ventral and lateral parts (Fig. 2 A1–A2, B1–B2). We further observed the structure of the X–Y, X–Z, and Z–Y axes at two points on the dorsal part (Fig. 2 C1–C5). Stomata and substomatal cavities were clearly observed on

the dorsal side (Fig. 2 C1–C3). The RpM was one empty pouch where new generations budded (Fig. 2 C4–C5).

We also observed the morphology of the *W. globosa* frond by scanning electron microscopy (SEM). We found that the stomata were densely distributed on the dorsal surface with densities of 314.34 ± 46.99 /mm^2 (Fig. 3 A–B). The guard cells, accessory guard cells, and epidermal cells made the entire stoma form an unusual flower-ring structure while the cells on the ventral and lateral parts were pentagonal (Fig. 3 C–D). The daughter frond (DF) was released from the RpM and connected with their MF by a stalk structure (Fig. 3 E–F). The broken stalk connecting the MF and DF remained in the RpM when the DF was released. The structure of the stalk was similar to the vascular tissue of plants and filled with cavities. The other end of the stalk structure was located near the RpD and the detachment left a visible scar when the DF was released. The scar was similar in structure to the abscission layer (Fig. 3 H–I).

Light microscopy observation

The horizontal and vertical cross-sections of *W. globosa* are shown in Fig. 4. We found that the developing DFs were produced from the meristematic area in the base of the RpM (Fig. 4 A–C). This meristematic area was composed of some undifferentiated cells that continued to multiply, producing new DFs. The RpM became larger with the growth of DFs and opened when they were released (Fig. 4 G). Most chloroplasts were concentrated in the dorsal part (Fig. 4 D). The stomata were only found in the upper epidermis (dorsal side), and prominent substomatal cavities could be observed distinctly from the vertical cross-section. From the dorsal to ventral side, the mesophyll cells varied in size and changed from small and dense palisade-like parenchyma cells to large and empty vacuolated cells with many intercellular air spaces. Furthermore, the chloroplasts showed a developmental gradient from the youngest to the oldest fronds. Compared with the MF, DFs were at an earlier differentiation stage and mainly consisted of many dividing cells with larger nuclei (Fig. 4 E). The outermost layer of the MF was composed of a single layer of epidermal cells containing annular distributed chloroplasts (Fig. 4 F).

Ultrastructure of the *W. globosa* frond

Despite its reduction in body plan and size, the *W. globosa* frond contains the same organelles as other angiosperm plants (Fig. 5). The most prominent organelles were chloroplasts, which were mainly distributed in the mesophyll cells of the upper epidermis (Fig. 5 A). There were no significant differences in the size and elaboration of the thylakoid system among chloroplasts. The photosynthetic membrane system of these lens-shaped chloroplasts was well developed, and the individual grana were composed of three to eight thylakoids (Fig. 5 B–C). Starch grains occurred in the chloroplasts of almost all the palisade-like parenchyma cells but were more abundant in the chloroplasts of mature mesophyll cells than in the meristematic area or the developing DFs (Fig. 5 D–E). There were also more mitochondria in the meristematic area of the MF than the DFs, which had larger nuclei and smaller vacuoles (Fig. 5 F–G). Microbodies were often, but not always, found in close association with the chloroplasts. Other organelles such as Golgi, free ribosomes, and rough endoplasmic reticulum (RER) were not so prominent.

The outermost cells of the RpM were mostly vacuolated and organelles were almost invisible (Fig. 5 H). Furthermore, we found elaborate cell wall projections, which were classified as transfer cells, in most adjacent mature mesophyll cells. These transfer cells were ingrowths, increasing the area of the cell membrane (Fig. 5 I).

Discussion

Most plant species require all the vegetative (shoot, stem, and root) and reproductive (flower, fruit, and seed) organs to complete their life cycle. These plants produce numerous branches through the growth of the shoot apical meristem (SAM) and root apical meristem (RAM) (Weigel and Jurgens 2002). However, the morphology of *W. globosa* does not fit traditional botanical descriptions. Our study revealed that *W. globosa* normally budded new fronds from its unique meristematic area by vegetative propagation. The meristematic area of *W. globosa* was located at the base of the RpM and was a collection of poorly differentiated cells with the ability to divide; there were no morphologically strict divisions in the meristem area. The dividing cells may perform different functions than the SAM in *Arabidopsis thaliana*, including expression of some key genes involved in SAM activity and the distribution of auxin and cytokinin (Zadnikova et al. 2014; Maugarny-Calès et al. 2018).

The new generations produced by vegetative propagation were called DFs or new buds as in a budding yeast and were released horizontally from the RpM. Usually, several individuals at different developmental stages coexisted in a single *W. globosa* frond. A single *Wolffia* frond can produce 11 daughter fronds on average and live for about 17 days on average (Bernard and Bernard 1990). Each bud began to senescence on the 10th day of survival and the average life span was about 17 days (Bernard and Bernard 1990). Our study also confirmed the rapid propagation of *Wolffia* from the structural perspective. This reproductive strategy enabled rapid accumulation of biomass in *Wolffia*, which roughly doubled in 48 hours, and allows its wide distribution in various habitats around the world (Ziegler et al. 2013). *Wolffia* seldom flower under natural conditions, and no seed has been reported so far. However, *Wolffia* flowers have been reported in the laboratory; the causes of its reduction of sexual reproduction could be revealed in the future.

The reduction of the root in *Wolffia* is one of the most striking examples of structural reduction in the plant kingdom. Duckweeds include five genera; members of *Wolffia* and *Wolffiella* are rootless, while members of *Spirodela*, *Landoltia*, and *Lemna* produce either a single or few roots (Cao et al. 2016). There is a reduction of the number of roots from *Spirodela* to *Lemna* and they disappear entirely in *Wolffia*. Early studies suggested that duckweeds did not use their roots to acquire nutrients, and instead acquire nutrients through their fronds (Gorham 1941; Muhonen et al.1983; Ice and Couch 1987; Meijer and Sutton 1987).

Echlin et al. (1981) found that most absorption of ions occurred in the root tip region of *Lemna minor*, and observed a Casparian band structure in the endodermis of the root tip. They suggested that the root of *L. minor* can not only absorb nutrients and water but also transport these to the frond. Kim (2007)

carried out a detailed study of root development of *S. polyrhiza* and found a large number of plasmodesmata between the cells of the root. They therefore concluded that the transport of metabolites between the root and frond may rely on the symplastic pathway. The root of duckweeds plays an important role in absorbing nutrients and maintaining their floating lifestyle. For example, the density of cells in the root tip of *S. polyrhiza* is high, such that it forms a pendulum-like structure to cope with water fluctuations and stabilize the plant on the water surface. However, for the rootless *Wolffia*, White and Wise (1998) suggested they stay afloat and upright not by buoyancy but by surface tension. In their opinion, if buoyancy kept *Wolffia* plants at the water's surface, then they would sink late in the day as their starch content reached a maximum. In our study, the dorsal part of *W. globosa* was always above the water, and it was difficult to submerge the plants or turn them over. In addition, most of the chloroplasts, which were filled with starch grains, were concentrated at the dorsal side. Previous studies have shown that dormant individuals of *Wolffia* were full of starch grains and sank in the water. We speculate that the content of starch grains affects the stable floating of *Wolffia*. Furthermore, the loss of the nutrient uptake and stabilization functions of the root in *Wolffia* may have allowed them to lose this organ. Phylogenetic analysis using different molecular markers has confirmed that duckweeds comprise a single monophyletic clade (Tippery et al. 2015), suggesting that rootlessness has a single evolutionary origin in Lemnoideae.

We also propose that *Wolffia* is a suitable model to study structural reduction in angiosperms and to explore the cause of rootlessness. First, *Wolffia* is easy to cultivate, completes its life cycle in the lab, and reproduces quickly. Second, *Wolffia* plants can be genetically transformed, as can the rooted *Spirodela* and *Lemna*, allowing us to conduct genetic studies (Cantó-Pastor et al. 2015; Heenatigala et al. 2018; Yang et al. 2018b). *Wolffia* species are the smallest flowering plants in the world, in both size and morphological structures, containing one leaf, one stamen and one gynoecium, which represent the core elements for angiosperms to complete their life cycle. Our study indicates that the simplicity of this species has been overestimated, because *Wolffia* has all the same organelles as other angiosperms at the ultrastructural level. Hillman (1961) pointed out that although the gross morphology and vegetative reproduction of *Lemnaceae* are somewhat unusual, their anatomy, particularly the prominent air spaces and reduced vascular structures, resembles that of many aquatic angiosperms. Anderson et al. (1973) also pointed out that although *Wolffia* lacks vascular tissue, the range of tissue and cell types appears as heterogeneous as in most leaves and varies considerably from meristematic to mature chlorenchymous tissue. Not only is it suitable to study structural reduction, but *Wolffia* would also be an ideal system to explore fundamental processes of angiosperm development that cannot be addressed using other model plants.

Conclusions

This is the first comprehensive study of the morphology and anatomy of *Wolffia globosa.* Our study revealed that the morphology of *W. globosa* did not fit the traditional botanical descriptions. The rootless *W. globosa* budded new fronds from the unique meristematic area by vegetative propagation, and usually several generations coexisted in a single frond. This reproductive strategy enabled their rapid

accumulation of biomass and wide distribution in various habitats around the world. Despite their reduction in body plan and size, *W. globosa* fronds contain the same organelles as other angiosperm plants, and their simplicity might be overestimated. Finally, we propose that *Wolffia* plants are not only suitable for the study of structural reduction in higher plants, but also an ideal system to explore the fundamental developmental processes of higher plants that cannot be addressed using other model plants.

Abbreviations

3D: Three-dimensional; RER: Rough endoplasmic reticulum; SAM: Shoot apical meristem; RAM: Root apical meristem; MicroCT: X-ray microtomography; PBS: Phosphate-buffered saline; TEM: Transmission electron microscopy; SEM: Scanning electron microscopy.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All data generated or analyzed during this study are included in this article (and its supplementary files) or available from the corresponding author on reasonable request. Plant materials are available from the corresponding author.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

JY and XZ performed experiments and wrote the initial draft. GL and SH designed the figures and contributed to the editing of this article. HH planned and designed the research. All authors have read and approved the manuscript.

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Authors' Information

[1]The State Key Laboratory of Freshwater Ecology and Biotechnology, The Key Laboratory of Aquatic Biodiversity and Conservation of Chinese Academy of Sciences, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan 430072, China. [2] University of Chinese Academy of Sciences, Beijing 100049, China.

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Figures

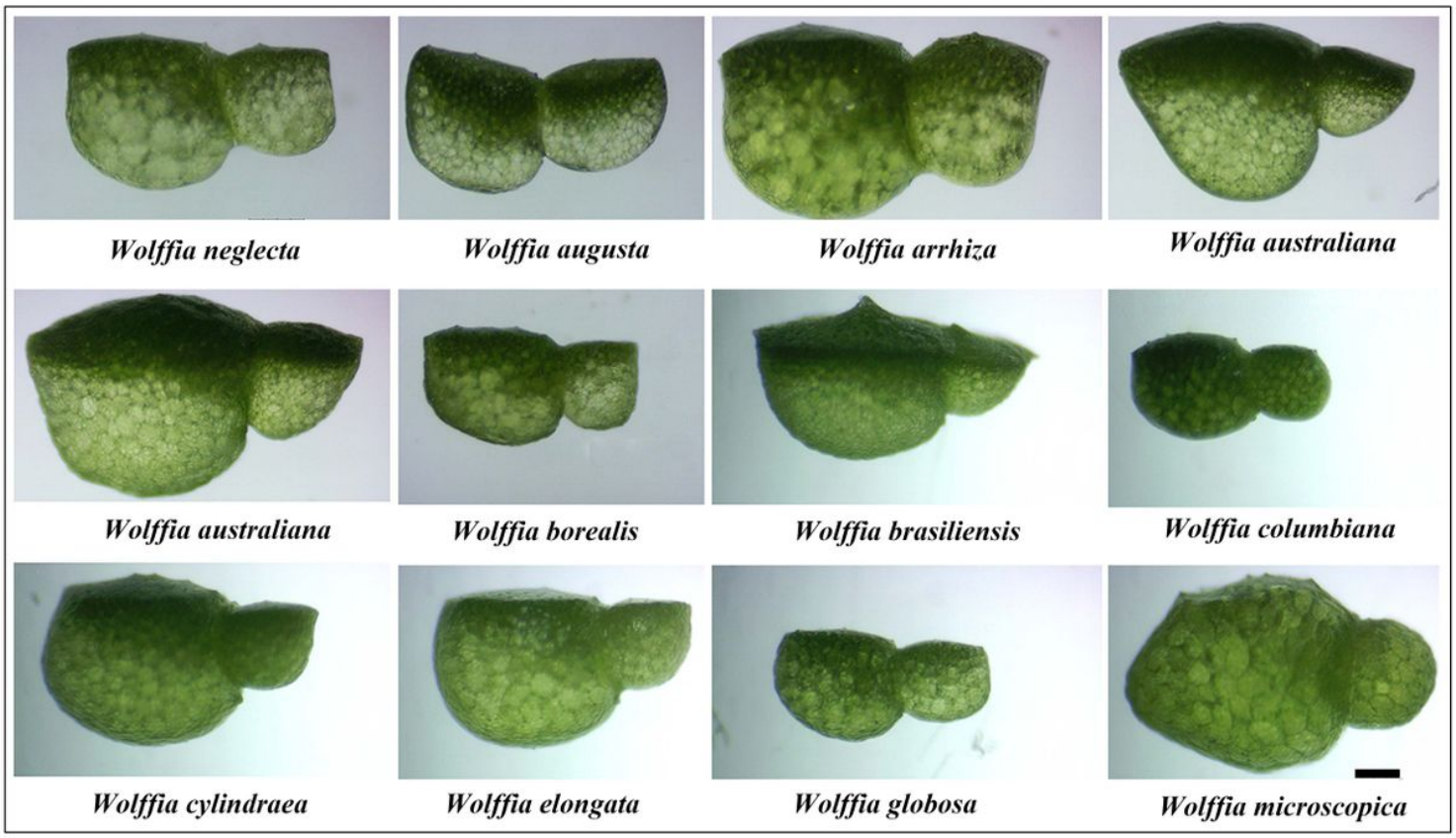

Figure 1

The morphology of 12 subspecies of Wolffia plants. Bar = 50 μm.

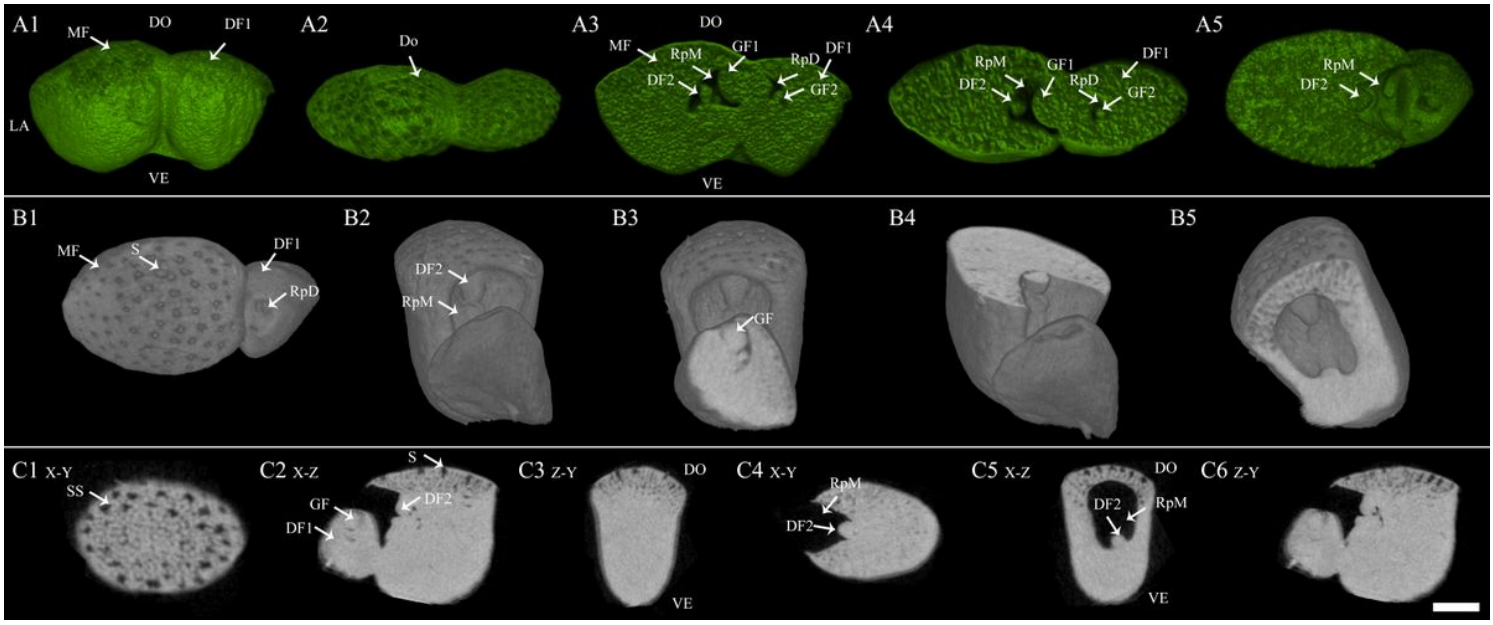

Figure 2

The 3D volumes of W. globosa fronds showing the mother frond with daughter fronds. DO- dorsal part, VE- ventral part, LA- lateral part, MF- mother frond, RpM- reproductive pocket of the mother frond (MF), RpD- reproductive pocket of the daughter frond, DF1- the first daughter frond of MF, DF2- the second

daughter frond of MF, GF1- the first daughter frond of DF1, GF2- the second daughter frond of DF1-, S- stoma, SS- substomatal cavity. Bar = 50 μm.

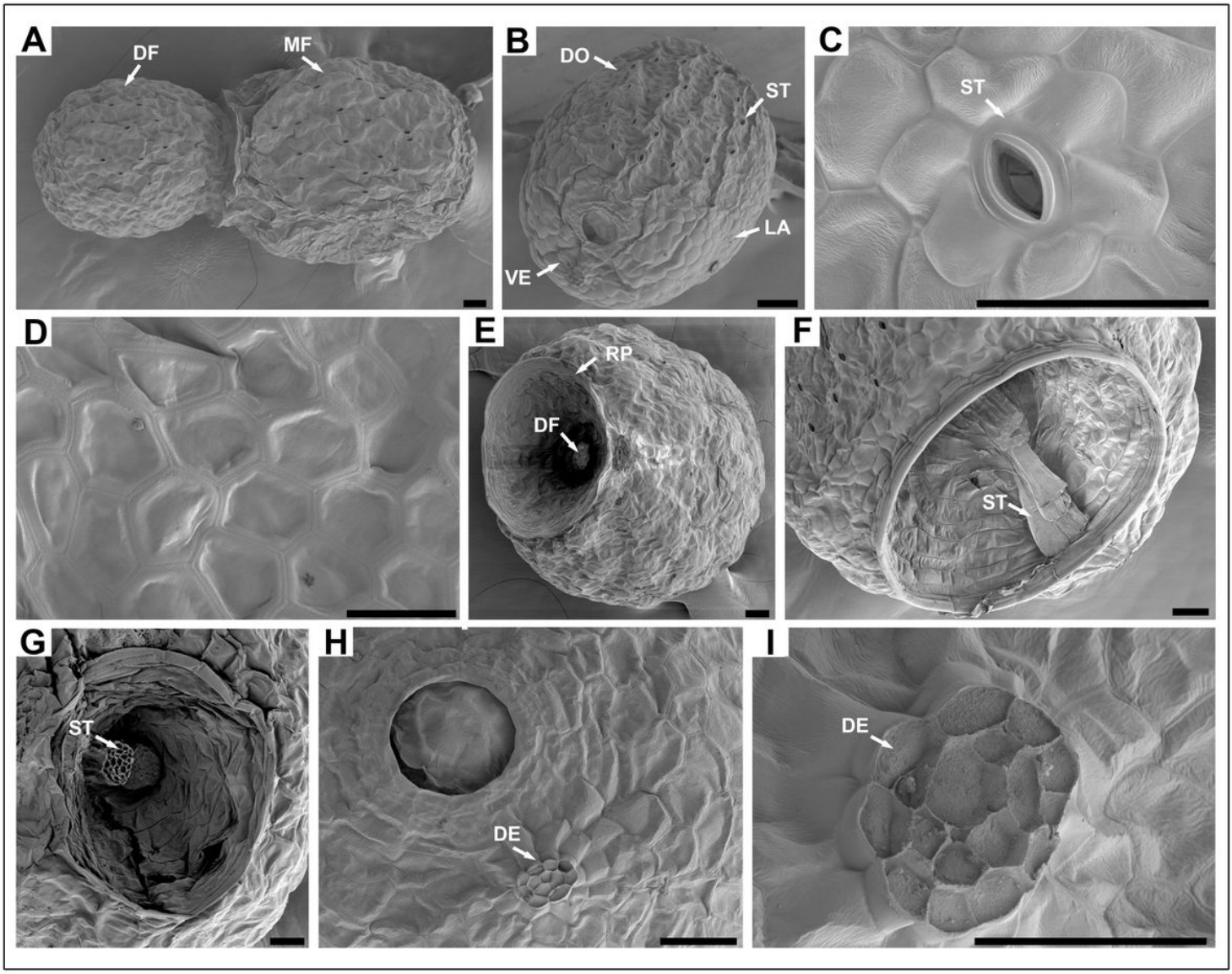

Figure 3

Scanning electron micrographs of W. globosa fronds. A, A single frond composed of mother frond (MF) and daughter frond (DF). B, The frond was divided into dorsal (DO), ventral (VE) and lateral parts (LA). C–D, Stomata (S) and epidermal cells on the dorsal part. E-G, The daughter frond produced from reproductive pocket (RP) and connected with the mother frond by the stalk structure (ST). H–I, The detachment of stalk (DE) and its magnification. Bars = 50 μm.

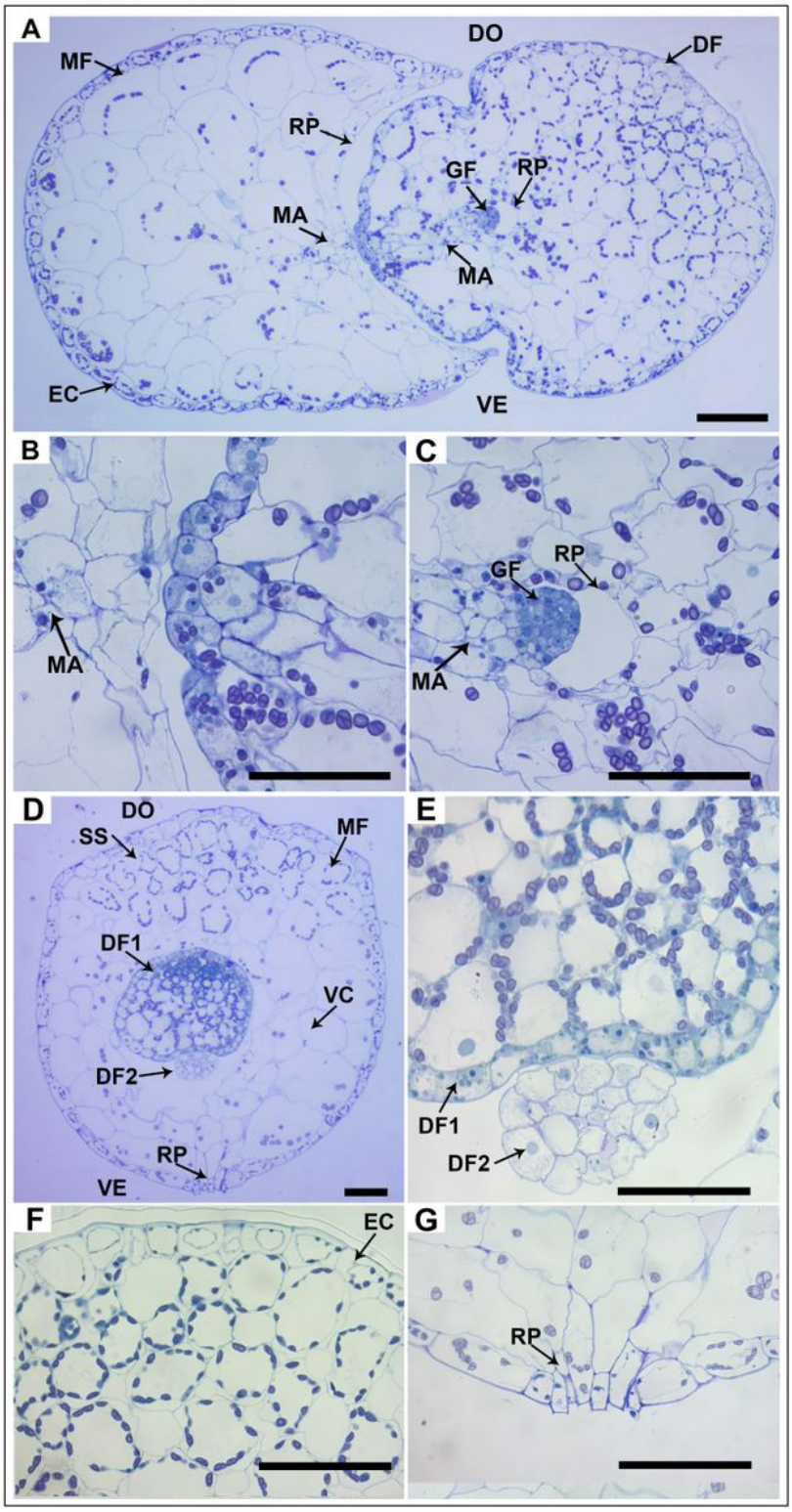

Figure 4

Microscopic observations of W. globosa fronds. A, Light micrograph of vertical cross-sections of a W. globosa frond showing mother frond (MF) and daughter fronds (DF, GF). B-C, Magnification of the meristematic area (MA) showing the growing daughter frond in the reproductive pocket (RP). D, Light micrograph of horizontal cross-section of a W. globosa frond showing mother frond and daughter fronds

(DF1, DF2), vacuolated cells (VC) and substomatal cavity (SS). E-F, Magnification of daughter fronds (DF1, DF2), epidermal cells (EC) and reproductive pocket (RP). Bars = 200 μm.

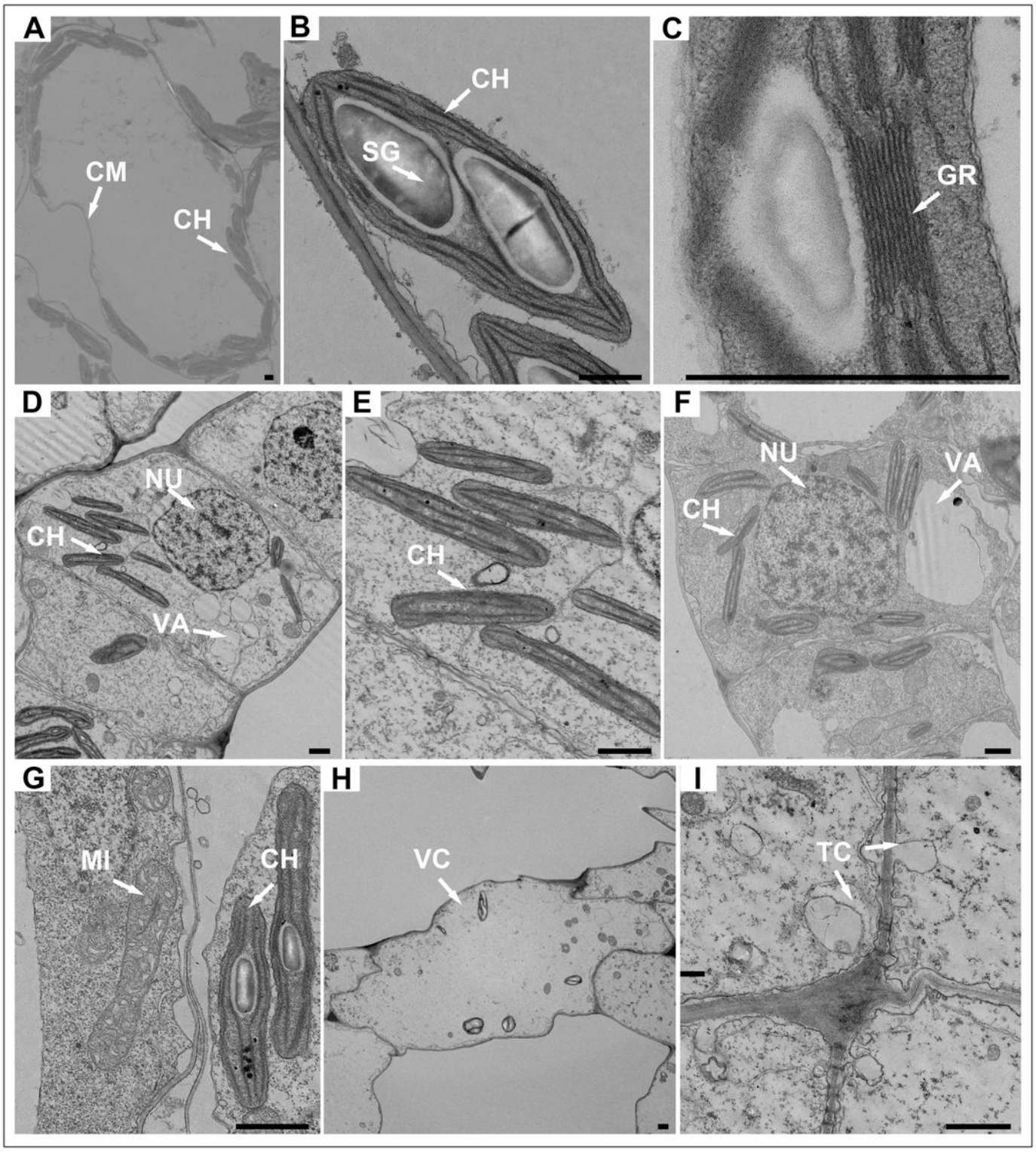

Figure 5

Transmission electron micrographs of W. globosa fronds. A-B, Lens-shaped chloroplasts were distributed in mature mesophyll cells and its magnification. C, The photosynthetic membrane system of chloroplasts containing a granum composed of thylakoids. D–E, Cell of meristematic area and its chloroplasts. F–G,

Cell of daughter frond and its chloroplasts. H, Cell of reproductive pocket was highly vacuolated. I, Transfer cells in most adjacent mature mesophyll cell. Bars = 1 μm.

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