

Green Globular Body (GGB) Induction and Differentiation in the Medicinal fern *Drynaria Roosii*

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Research article

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Abstract

Background: The green globular body (GGB) of ferns is a special propagule induced in plant *in vitro* culture systems. Owing to its high proliferation efficiency, GGB is widely used in the *in vitro* propagation of important ornamental and medicinal ferns. In addition, propagation using GGB shows great development prospects in the conservation of rare or endangered ferns and the breeding of new fern varieties. However, due to the lack of systematic studies on GGB ontogenesis, the morphogenetic aspects of GGB during induction and differentiation remain unclear.

Results: We characterized the response of five types of explants of *Drynaria roosii* to GGB inductive medium and further investigate morphological and anatomical changes of explants that developed GGBs. We found that the rhizome explants directly produced GGBs through cell proliferation of the shoot apical meristem and lateral meristem. The leaf and petiole explants produced GGBs indirectly through the proliferation of meristematic cells of somatic embryos derived from the epidermal cells of the explants. The root and gametophyte explants failed to produce GGB under our induction conditions. We further investigated the differentiation process of GGB. During GGB differentiation, shoot primordia and leaf primordia differentiate from meristematic cells on the epidermis, and the root primordia develop from an inner meristematic tissue with developing vascular tissue connecting all these primordia, which indicates the involvement of multiple organogenesis processes.

Conclusions: Our results suggested that preexisting or reestablished meristematic cells were the direct source of GGB in *D. roosii*. Somatic embryogenesis and organogenesis were involved in GGB induction and differentiation, respectively. The comparison with other common propagules revealed that GGB in *D. roosii* was largely different from somatic embryos, callus, and protocorm or protocorm-like bodies.

Background

Due to their high cellular plasticity, plants possess a high capacity to regenerate new individuals [1]. The regenerative capacity of plant cells can be enhanced *in vitro* by cultivating explants on media supplemented with plant growth regulators (PGRs) [2]. Previous studies have led to a better understanding of the two most common pathways during plant regeneration: somatic embryogenesis and *de novo* organogenesis, where regeneration may occur either directly from parental tissues or indirectly via the formation of a callus [2–4]. However, our current knowledge of *in vitro* plant regeneration is mainly based on studies of seed plants rather than non-seed plants, such as ferns. Despite the closest living relatives of seed plants, ferns exhibit considerably distinctive or even opposite characteristics on some key structures and regulatory mechanisms during growth and development, such as embryogenetic patterns [5], apical meristem organization [6], and regulatory roles of phytohormones in root meristem development [7]. Therefore, ferns are important subjects for research in development and morphology, comparative evolution, and functional genomics.

Ferns have a unique propagation system through the formation of a green globular body (GGB), a tissue produced by unlimited cell division of sporophytes in response to cytokinin during *in vitro* culture [8]. With many meristematic cells inside and/or near its surface, GGB displays a high proliferation rate [9–11]; therefore, it has excellent potential for micropropagation and conservation of fern species [8, 12, 13]. GGBs have been induced and applied to the micropropagation of several fern species. Liao and Wu [14] found that GGBs of *Platycerium bifurcatum* could differentiate into numerous new shoots following a process similar to organogenesis of callus. However, because the *SOMATIC EMBRYOGENESIS RECEPTOR KINASE* (*SERK*) gene, which is used as a marker to screen somatic cells that are capable of forming embryos, was expressed in the GGB of *Adiantum capillus-veneris*, Li et al. [15] suggested that GGB development followed the somatic embryogenesis pathway. In *Cibotium barometz*, GGBs were compared to protocorm-like bodies (PLBs) of orchids et al. [11]. These studies imply that although GGB induction is a common phenomenon during tissue culture in ferns, the regeneration pathway through which it is developed remains undefined. While previous studies have focused on establishing protocols for GGB induction, proliferation, and maintenance [9, 11, 16, 17]. However, the morphogenetic events that occur during the initiation and differentiation of GGB remain largely unidentified.

Here, we studied *in vitro* plant regeneration via GGB induction in the fern *Drynaria roosii*. The dried rhizome of *D. roosii* is the main source of the traditional Chinese medicine ‘Gusuibu’ for the treatment of bone-related diseases, such as bone fracture, osteoporosis and arthritis [18–20]. Qianggu capsule, whose main effective components are extracted from ‘Gusuibu’, has been developed into a postmarketing Chinese medicine for treating osteoporosis [21, 22]. Recently, the dried rhizome of *D. roosii* has also been proved to function in angiogenesis and treating Alzheimer’s disease [23, 24]. Hence, this species is being increasingly applied in clinical medicine, implying bright market prospect and high economic value [25]. However, owing to the lag of propagation and cultivation techniques of *D. roosii*, exploitation of wild populations is intensified by increasing market demands [26]. We focused on morphological and anatomical features that characterize GGBs during their initiation process in different explant sources. Our results indicated that meristematic cells played an important role in GGB formation, and both somatic embryogenesis and organogenesis were involved in the development of GGB in *D. roosii*. We further compared GGB with the other propagules: somatic embryo (SE), callus, and protocorm/PLB.

Results

GGB induction

Five types of explants were inoculated into GGB induction medium. After three months of culture, GGBs were generated on the surface of rhizome, leaf, and petiole explants (Fig. 1a–e). The GGBs of *D. roosii* were green and compact structures, covering many scales (Fig. 1c). Root explants and gametophyte explants failed to produce GGB even eight months later, when explants lost their vitality. Gametophyte explants proliferated continuously in the induction medium, and some of them developed large and substantially thick notch areas with numerous sexual organs (Fig. 1f).

GGBs induced in rhizome explants

We compared anatomical characteristics of the apical area of rhizome with that of rhizome explants bearing GGB. The tip of rhizome of *D. roosii* maintains the characteristics through the life cycle in PGR-free media. Numerous scales covered the rhizome, including the region of the shoot apical meristem (SAM), leaf primordia (LP), and root primordia (RP) (Fig. 2a). The apical meristematic area had numerous small meristematic cells, with a large inverted pyramidal apical cell (AC) at the tip (Fig. 2b). We observed that LP were close to AC (Fig. 2c). The meristematic cells located in the sub-apical region were differentiated into procambium and parenchyma cells (Fig. 2d). The vascular bundle of the rhizome was reticular (Fig. 3a) and composed of an endothelial layer, xylem, and phloem cells (Fig. 2e).

When rhizome explants were cultured for 20 days, the apical meristematic zone expanded due to an increase in cell number, rather than cell size (Fig. 3b). The meristematic region in the longitudinal section reached approximately 4–5 times the size of the SAM in explants cultured for 20 days, where the AC and LP could seldom be detected. The expansive apical region was composed of numerous meristematic cells, which showed similar cytological features to those in the rhizome SAM (Fig. 2f, g). Epidermal cells of GGB were rectangular and compact, exhibiting distinct anatomical characteristics when compared with the shoot apical cells (SAC) and leaf apical cells (LAC) (Fig. 2h). The morphological structure of the sub-apical region of the explant cultured for 20 days showed little difference from the uncultured explant. We observed basic parenchyma and procambial cells, as well as a differentiated root primordium in the sub-apical region (Fig. 2i, 3b). We also observed the formation of protoxylem and protophloem of vascular tissue in the sub-apical region of the rhizome explant (Fig. 2j). These results suggest that the morphological change of the rhizome explants in the inductive medium mainly occurred in the apical region, and the meristematic cell proliferation in this region resulted in the formation of GGB.

We also analyzed the development of GGBs born on the non-apical region of rhizome explants. We found that these GGBs could be traced back to 60 days after inoculation when we observed the formation of new SAM in the leaf axil of rhizome explant (Fig. 2k). The new SAM developed into leafy shoot later, and originated GGB after 3 months in the inductive medium (Fig. 3c). We used micro-CT to analyze the relationship of the non-apical GGB with the explant, and we found that there was a well-developed vascular tissue connecting GGB and the explant (Fig. 3c). These results suggest that the GGB formed in the non-apical region of rhizome explant was probably produced by the proliferation of meristematic cells of the leafy shoot of the rhizome explant.

GGBs induced in leaf and petiole explants

We observed morphological changes in leaf explants throughout the induction period. We found the epidermal cells of leaf explants increased in volume at the initial stage of induction (Fig. 4a). These cells then underwent several divisions to originate multicellular structures (Fig. 4b–d). These structures developed juvenile scales, and the larger structures presented SAM or LP morphology (Fig. 4e, f). Then, the multicellular structures developed into GGBs, which continuously increased in volume (Fig. 4g). We

detached the multicellular structures from the leaf explants and found that no vascular tissues connected them to the explants (Fig. 4h, i).

To verify the multicellular structures formed during GGB formation in leaf explants, we detached these structures before they presented a GGB-like appearance, transplanted them into $\frac{1}{2}$ MS medium with no PGRs, and observed their morphological changes. We found that the multicellular structures gradually developed LP (Fig. 5a–d). We also observed SAM and leaf apical meristem (LAM) developing in the multicellular structures (Fig. 5e). After the expansion of the first leaf, a rhizome tip-like structure developed at the basal part of the first leaf, with a second LP emerging (Fig. 5f–i). The SAM was progressively covered by developing scales (Fig. 5j). RP was not frequently detected in the multicellular structures during the early stage after transplanting, but several brown rhizoids were verified at the basal part of these structures (Fig. 5k, l). We observed no vascular tissue connecting the multicellular structures and the explant. Instead, cells that connected the multicellular structures to the explant seemed invasive into the explant tissue (Fig. 5m). These results suggested that these multicellular structures could be identified as SEs.

In addition, some prothallus-like structures (Fig. 6a, b) showing only two-dimensional growth were occasionally observed on the leaf explants. Further analysis indicated that the initiating process of these structures had a similar cell division pattern to that of the prothallus. The prothallus-like structures underwent similar development stages of those in protonema and prothallus (Fig. 6c–f), but prothallus-like structures did not form a typical cordate prothallus with the notch meristem; instead, they maintained two-dimensional growth. Occasionally, the prothallus-like structures underwent three-dimensional growth and differentiated into a meristem similar to LM or SAM (Fig. 6g, h). It has been reported that diploid sporophytes of ferns can form haploid gametophyte bypass sporogenesis [27]. However, these prothallus-like structures were classified as diploid by flow cytometry (Fig. 6i). Therefore, we speculated that these prothallus-like structures might be another type of SE that underwent an unusual developmental program.

GGB formed on the petiole explants in inductive medium also underwent a multicellular structure phase. The epidermal cells on petiole explants were commonly elongated (Fig. 7a). At the initial stage of induction, some epidermal cells underwent two divisions perpendicular to the petiole axis, which originated eight cells in a linear arrangement (Fig. 7b). These cells were then divided tangentially to the petiole explant (Fig. 7c), forming a multicellular structure that protruded above the surface of the explant (Fig. 7d). The continuous division of the multicellular structure originated the juvenile GGB, with some scales and rhizoid developed from their surface (Fig. 7e, f). Due to their meristematic features manifested in their small size and dense large nuclei, cells of the multicellular structures could be easily distinguished from those of the explants (Fig. 7g). The cells touching the explant were slightly elongated, but they never connected with the vascular bundle of the explant. We inoculated these multicellular structures attached to the explant into PGR-free medium before they developed into GGBs, and we found that they gradually differentiated into SAM and LP, which were covered by several scales (Fig. 7h, i). Occasionally, the RP could be seen protruding from the inner side of the multicellular structures (Fig. 7j). Finally, these

multicellular structures developed into young sporophytes (Fig. 7k, l). Together, these results suggest that the multicellular structures formed on the petiole explant could develop into plantlets through the somatic embryogenesis pathway.

Additionally, we found that the SEs formed on the leaf and petiole explants could be induced to form GGB directly when they were detached from the explants and transplanted in the inductive medium (Fig. 8a). SEs present a meristematic tissue in the early stage (Fig. 8b), and the meristematic tissue increased rapidly in volume under inductive conditions (Fig. 8c–e). The expansion of meristematic tissue resulted from the proliferation of meristematic cells (Fig. 8f). We observed that SE commonly differentiated into LAC, which gave rise to the first LAM (Fig. 8f). Occasionally, LAM organization could not be established and the cells proliferated to form the second meristematic area (Fig. 8g). These results suggest that the GGB could be produced on leaf or petiole explants indirectly, with the involvement of the somatic embryogenesis pathway.

GGB differentiation

To observe the differentiation process of GGB, they were detached from the explants and transplanted into PGR-free medium, where they developed rapidly and differentiated into complexes with many leafy shoots (Fig. 9a, b). Several epidermal cells differentiated into inverted triangular cells that resemble SAC (Fig. 9c). Later, we observed a gradual organization of SAMs, which were covered in numerous scales (Fig. 9d, e). LACs, which were much larger than SACs, were also produced from the epidermal cells of GGB (Fig. 9f). After acquiring the LAC identity, the LAC underwent rapid cell division to produce LPs (Fig. 9g, h). We also observed that RP in differentiated GGBs originated from the meristematic cells (Fig. 9i). With continuous development, the growth of RPs eventually surpassed the limits of GGBs (Fig. 9j, k). Procambium differentiation occurred early at the inner face of GGB (Fig. 9l). Procambial development resulted in the formation of vascular tissue with xylem and phloem (Fig. 9m). Moreover, we analyzed the vascular tissue of differentiated GGB and found that the developing vascular tissue was spreading in the interior face of the GGB complex, connecting the SAMs, LPs, and RPs (Fig. 10).

Discussion

Preexisting meristematic tissue is the prerequisite for GGB formation in *D. roosii*

The maintenance of SAM organization in plants results from a balance between cell division and differentiation in this region. Cell fate in SAM is regulated spatially and temporally; cells in the central zone remain undifferentiated, while cells gradually acquire differentiation ability when recruited into organs in the peripheral zone [28]. SAM of ferns was once thought to be composed of a single apical cell, which would be the main source of the entire plant body [29–32]. However, cytohistochemical observations have shown that fern meristems are multicellular with specific zonation [6]. Recently, some regulatory factors have been identified for the maintenance of SAM in ferns [6, 30]. These results suggest that the organization of SAM in ferns may also be balanced by cell division and differentiation. Our

results suggest that the addition of 6-BA in the induction medium altered the organization of SAM in *D. roosii* by reverting the cell fate from differentiation to division, or by enhancing the division ability of those undifferentiated cells, which resulted in unscheduled cell proliferation. Then, an excessive cell division produced GGB with a large number of meristematic cells, suggesting that the meristematic cells originated GGB in *D. roosii*. Although GGB can be successfully induced on different explants in several fern species [9, 11, 14, 15], the precise cell origin of GGB has not been addressed. We believe this cognition was developed largely since the explants used for GGB induction already had meristematic cells.

In an *in vitro* culture system, plant regeneration largely depends on the ability to respecify cell fate [2]. For plant tissue culture, explants from juvenile parts usually exhibit more regenerative capacity than those from mature parts, because plant cells in immature or juvenile bodies tend to have a high potential to redirect the cellular fate [2]. The most frequently used explants for fern GGB induction are shoot tips and young leaves [8]. Under our inductive conditions, GGBs of *D. roosii* were generated on rhizomes, young leaves and petioles of young sporophytes. For rhizome explants, the GGB formed directly from cell proliferation of the preexisting SAM in the rhizome tip region, and indirectly from the newly developed shoot that transformed from LAM. In leaf and petiole explants, GGBs were formed by the meristematic cell proliferation of SEs, which were produced on the epidermis of both explants. These results suggest that the preexisting meristem is a prerequisite for the formation of GGB. In addition, under our induction conditions, the cells of both LAM and SAM can proliferate to form GGB, while the cells of root apical meristem failed to form GGB, which indicated that the meristematic cells from LAM resembled those from the SAM in that they were more easily proliferative. These results corroborate studies that reported that fern leaves are developmentally equivalent to shoots [33–35].

Plant regeneration from GGB underwent organogenesis in *D. roosii*

Fern regeneration from GGBs is highly efficient, as GGB differentiation gives rise to a large number of shoots, which then develop into juvenile sporophytes when inoculated on the proper medium. In an *in vitro* culture system, the dividing cells follow three developmental pathways: organogenesis, somatic embryogenesis, or disordered division [36]. However, the classification of the regeneration pathways of ferns via GGB induction and differentiation is still debated. This is probably because of overlapping of the two developmental states, proliferation and differentiation. Here, the induction and proliferation processes of GGB in *D. roosii* were analyzed. We found that the response of explants to 6-BA in the medium led to the formation of GGB, and the GGBs developed into shoots rapidly through the differentiation of epidermal cells into apical cells after they were inoculated into PGR-free medium, indicating the competency of GGB to recover the SAM organization. In addition, when GGB began to differentiate, the epidermal cells gave rise to the shoot primordia, leaf primordia, or even leafy shoots. We also verified the development of root primordia in the inner face of GGBs. Furthermore, the micro X-ray computed tomography analysis demonstrated that there were developing vascular tissues inside the GGB connecting all these primordia or organs. Indeed, the somatic embryos were observed to be involved in the process of GGB induction from the leaf and petiole explants, while in the differentiation process, no

intermediate state resembling the somatic embryogenesis was detected. Collectively, these results suggest that the regeneration of *D. roosii* initiated by GGB differentiation could be considered as organogenesis pathway.

Similarities and distinctions between GGB and other propagules

GGB vs. SE

Somatic embryogenesis is characterized by the establishment of polarity [37, 38] and the absence of vascular tissue connections from the maternal tissues [38]. Polarity is not necessarily established during the embryogenesis of ferns, that is, the shoot meristem and the root meristem do not develop as opposite to each other. In addition, the embryonic root only exists for a short time, and the young embryo produces foot cells that play an important role in fixing itself on the gametophyte before the root system is formed and of absorbing nutrients until the developing embryonic organs could be fully autotrophic [5, 7, 39]. The observation of somatic embryogenesis of ferns demonstrated that the developmental process of SE is similar to that of zygote embryogenesis, with no connection between SE and explant via vascular tissue [40]. Here, we found that although there was no obvious embryonic root formed in SE produced on leaf and petiole explants in *D. roosii*, the vascular tissue of SE was isolated from those of the explants. Moreover, the cells at the touching area between SE and explant exhibited similar characteristics to the foot cells of the zygotic embryo. However, none of these results were found during the process of GGB formation on rhizome explants, suggesting that the formation of GGB on rhizome explants in *D. roosii* under our induction condition did not involve the somatic embryogenesis pathway. Recently, the marker gene that is expressed during plant somatic embryogenesis, *SERK*, was identified in GGBs of the fern *Adiantum capillus-veneris* [15, 41], suggesting that the development of GGB shares similar characteristics of that of SE. However, the *SERK* gene is not unique to the somatic embryogenesis pathway, and it was also expressed during the organogenesis pathway in some plant species [42–45]. Considering that, in our study, all the SE derived from the leaf and petiole explants subsequently formed GGB in the induction medium, we speculate that the GGBs produced via SE formation on the leaf and petiole explants in *D. roosii* would probably be embryogenic. However, the somatic embryogenesis that occurred in this process might have occurred because the explants need to establish meristematic tissue for regeneration. Furthermore, even though somatic embryogenesis pathway was involved in the GGB formation process, we believe SE does not essentially characterize the generation of GGB.

GGB vs. callus

Here, shoots and roots were regenerated by GGB in a way that resembles the regeneration of callus via *de novo* organogenesis. However, recent studies have revealed that callus formation on callus-induced medium followed a lateral root development pathway, and the callus was initiated from xylem-pole pericycle cells of root explants and pericycle-like cells of aerial organs [46–48]. In contrast to callus, GGB was initiated from meristematic cells in SAM and/or LAM, as discussed above. Callus formation and

shoot regeneration are two different stages in plant tissue culture, and callus formation alone is not sufficient for shoot regeneration [49, 50]. However, the regeneration in GGB differs from that in callus, because shoot regeneration can be easily obtained when GGBs are transplanted into PGR-free media. Nevertheless, the formation of both callus and GGB requires an important process to establish meristematic tissues. A callus is a disorganized cell mass formed by the cell proliferation process after meristematic cells are generated by the explants [51], rather than a process of reprogramming to an embryonic state [48, 52]. As to GGB, they are formed directly by meristematic cells in the SAM of rhizome explants. The formation of GGB on leaf and petiole explants was indirect and needed a process that established the meristematic tissue via somatic embryogenesis. Therefore, we speculate that during the induction period, the dividing state of cells might be essential for promoting the proliferation capability to respond to the induction factors, both in callus and GGB.

GGB vs. protocorm/PLBs

It has been reported that GGB of ferns is a structure reminiscent of the PLB of orchids [11, 14]. In orchids, protocorm typically describes an indispensable stage after seed germination and before the formation of the first shoot or root, while PLBs appear in plant *in vitro* culture systems [53]. In these plants, seeds lack embryos or meristem and the protocorm is responsible for the formation of the meristem [54]. In contrast, our analysis indicated that GGB in *D. roosii* was succeeded by preexisting or reestablished meristematic tissues. Furthermore, protocorms grow spontaneously, whereas GGBs in *D. roosii* could only be generated under proper inductive conditions, as corroborated by Fernández [8]. Nevertheless, GGBs in *D. roosii* and PLBs share similarities as both produce multiple shoot meristems when transferred to differentiation media, showing high plant regeneration efficiency.

Conclusions

Here, we induced GGB and SE in explants from *D. roosii*, and identified regeneration pathways and important anatomical events occurring during initiation and differentiation of GGB in *D. roosii*. This practice could contribute to establishing an *in vitro* propagation system for *D. roosi*. We proposed a scheme of the process of *in vitro* propagation of *D. roosii* indicated in Fig. 11. It initiates with sterile sporophytes, passes through GGB induction, GGB proliferation, GGB differentiation, shoot segmentation, and ends with new generations of sterile sporophytes. Furthermore, SEs generated on leaf and petiole explants during the early stages of GGB induction could directly develop into young sporophytes (Fig. 11). This *in vitro* propagation system could be beneficial to the large-scale production of plantlets for artificial cultivation of *D. roosii*, thereby protecting the wild resources of this species. Our findings will contribute to filling gaps in knowledge regarding *in vitro* plant regeneration of non-seed plants.

Methods

Plant material

We selected five explant sources for GGB induction in *D. roosii*: young rhizomes, leaves, petioles, roots of sterile sporophytes, and sterile gametophytes. *D. roosii* plants were cultivated by Mr. Dong Li in a greenhouse at the Institute of Botany of the Chinese Academy of Sciences. Fertile leaves with mature sporangia were harvested and dried at room temperature (24 ± 2 °C). After sporangial dehiscence, spores were collected and stored at 4 °C until use. These spores were later cultivated *in vitro* to generate sporophytes and gametophytes used as sources of explants as described below.

Disinfection of spores and preparation of explants

Spores were placed in 1.5 mL centrifuge tubes and washed twice with deionized water. Spores were then surface-sterilized with 5% NaClO for 7 min and washed five times with sterile deionized water. Next, spores were sown in plastic Petri dishes containing 1/2 MS media with 0.65% plant agar and incubated at room temperature (24 ± 2 °C) at a 16 h photoperiod. After spores germinated and the prothalli developed into gametophytes with archegonia and antheridia, deionized water was added daily into the Petri dishes to promote sporophyte formation. After the juvenile sporophytes could be observed by the naked eye, they were detached from the gametophytes and sub-cultured in MS media with 0.65% plant agar and 3% sucrose to accelerate rhizome development.

As explants, gametophytes were sub-cultured into GGB inductive medium before fertilization. The leaves from juvenile sporophytes were separated into blades and petioles, which were used as leaf and petiole explants, respectively. The rhizomes were sub-cultured into the inductive medium, when they grew to a length of approximately 5–8 mm. The roots were cut at approximately 5–6 mm from the root tip and used as root explants.

GGB induction and differentiation

For GGB induction, the explants were cultured on MS media supplemented with 2.0 mg/L 6-BA, 3% sucrose, and 0.65% plant agar. We observed that both leaf and petiole explants produced GGBs through multicellular structures. To evaluate these multicellular structures, the leaf and petiole explants were pre-cultured in inductive medium for four weeks and then transplanted into PGR-free medium, where the induced multicellular structures developed into shoots in the following eight weeks. In addition, the juvenile plantlets formed on the leaf and petiole explants in the PGR-free medium were detached from the donor explants and inoculated into the inductive medium to assess their responses. Induced GGBs were detached from the rhizome, leaf and petiole explants, and transplanted into PGR-free medium to stimulate plantlet regeneration through GGB differentiation. All cultures were incubated under the aforementioned conditions.

Anatomical analysis

To observe the morphological and anatomical changes during GGB initiation and differentiation, the samples from each treatment were investigated every three days under a Nikon SMZ800 stereoscope (Nikon, Tokyo, Japan) and photographed with an INFINITY2-5C camera (Teledyne Lumenera Company, Ottawa, Canada). The plant materials (explants, GGBs and SEs) were dissected and fixed in FAA (70%

ethanol: glacial acetic acid: formaldehyde, 89:6:5) and glutaraldehyde (2.5%) in phosphate buffer (0.1 M, pH 7.2), respectively.

The samples fixed in FAA were dehydrated through an ethanol-acetone and acetone-isoamyl acetate series, and then critical-point dried with CO₂. For morphological observation, the samples were mounted on aluminum stubs and sputter-coated with gold and then examined using an S-4800 FESEM scanning electron microscope (Hitachi, Tokyo, Japan). To directly observe the histological characteristics of rhizome explants and GGBs, the samples were mounted into paraffin wax and examined using a Bruker SkyScan1172 micro-computed tomography (CT) (Bruker Company, Billerica, Massachusetts). Stacks of images were obtained by scanning samples and reconstructing them using CTvox software. In addition, the vascular tissues of GGBs were examined using a micro X-ray computed tomography from ZEISS Xradia 510 Versa (Carl Zeiss Meditec AG, Jena, Germany).

The samples fixed in glutaraldehyde were dehydrated with an ethanol–acetone series and embedded in spurr resin. The sections were cut to a thickness of 1–2 µm using a Leica EM UC7 ultramicrotome (Menzel-Gläser, Braunschweig, Germany) with a diamond knife. After staining with toluidine blue (0.1%), the samples were examined under a Nikon ECLIPSE E600 light microscope (Nikon) equipped with an INFINITY2-5C digital camera (Teledyne Lumenera Company).

Flow cytometry analysis

We observed that leaf explants produced prothallus-like bodies when cultured in inductive medium. To compare the ploidy level of prothallus-like bodies with gametophytes and sporophytes, these parts were collected and chopped in the nuclei extraction buffer using straight-edge razor blades. The crude nuclear suspension was filtered through a 50 µm nylon mesh. After staining with propidium iodide, the ploidy levels of each sample were detected from the fluorescence intensities of stained nuclei using a BD LSRFortessa flow cytometer (Becton, Dickinson and Company, San Jose, California).

Abbreviations

AC: Apical cell; GGB: Green globular body; LAC: Leaf apical cells; LAM: Leaf apical meristem; LP: Leaf primordia; MS: Murashige & Skoog; PGR: Plant growth regulator; PLB: Protocorm-like body; RP: Root primordia; SAC: Shoot apical cells; SAM: Shoot apical meristem; SE: Somatic embryo.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

Competing interests

The authors declare that they have no conflict of interest.

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Authors' contributions

LS supervised the research work. DW and YL conceived the idea and performed the experiments. DW wrote the manuscript. DL collected the samples. All authors read and approved the manuscript.

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Authors' Information

Not applicable.

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Figures

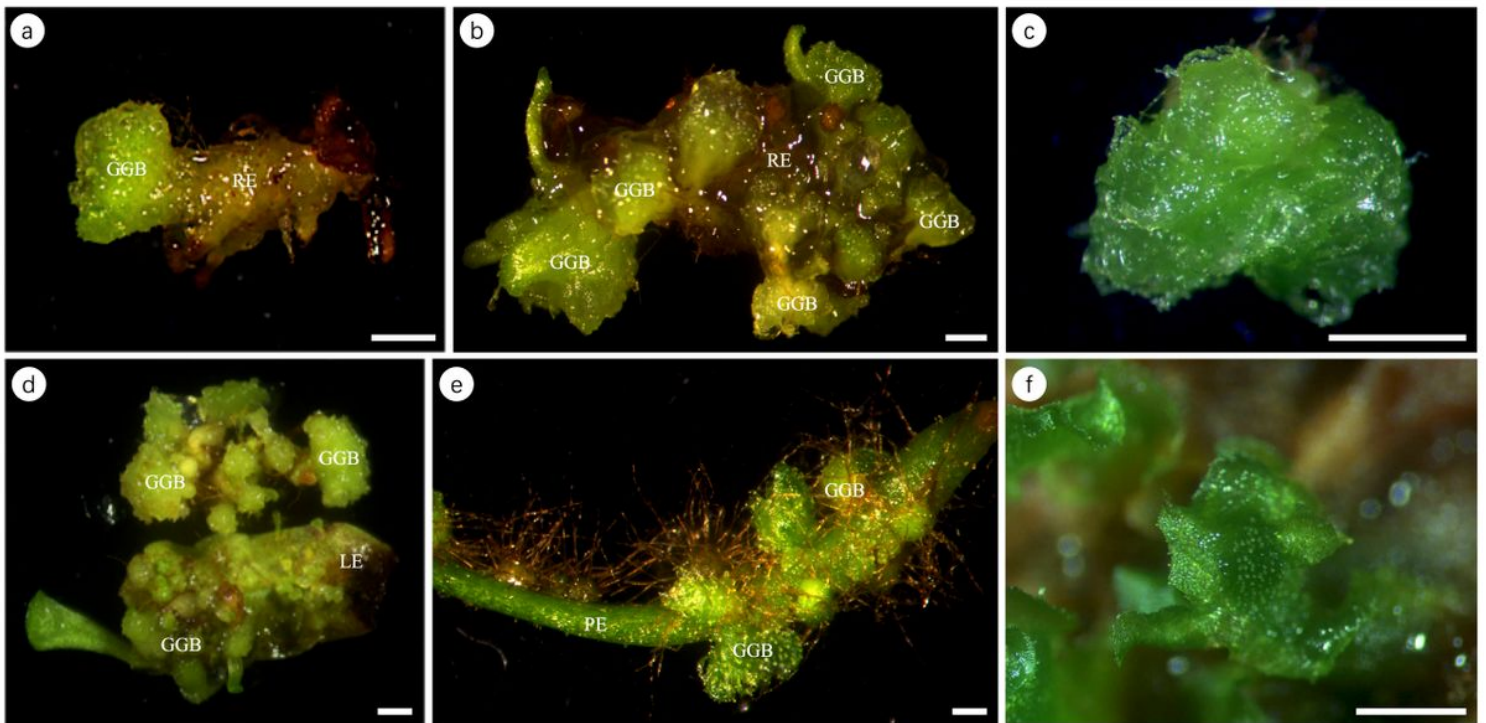


Figure 1

In vitro formation of green globular bodies (GGBs) on *Drynaria roosi* explants in induction medium after three months of inoculation. a GGB formation on the tip of rhizome explant. b GGB formation on the non-tip region of rhizome explant. c morphological characteristics of GGB. d GGB formation on leaf explants. e GGB formation on the petiole explant. f gametophyte explant lacking GGB. GGB: green globular body, LE: leaf explant, PE: petiole explant, RE: rhizome explant. Scale bars: 2 mm (a, b), 1 mm (c–f)

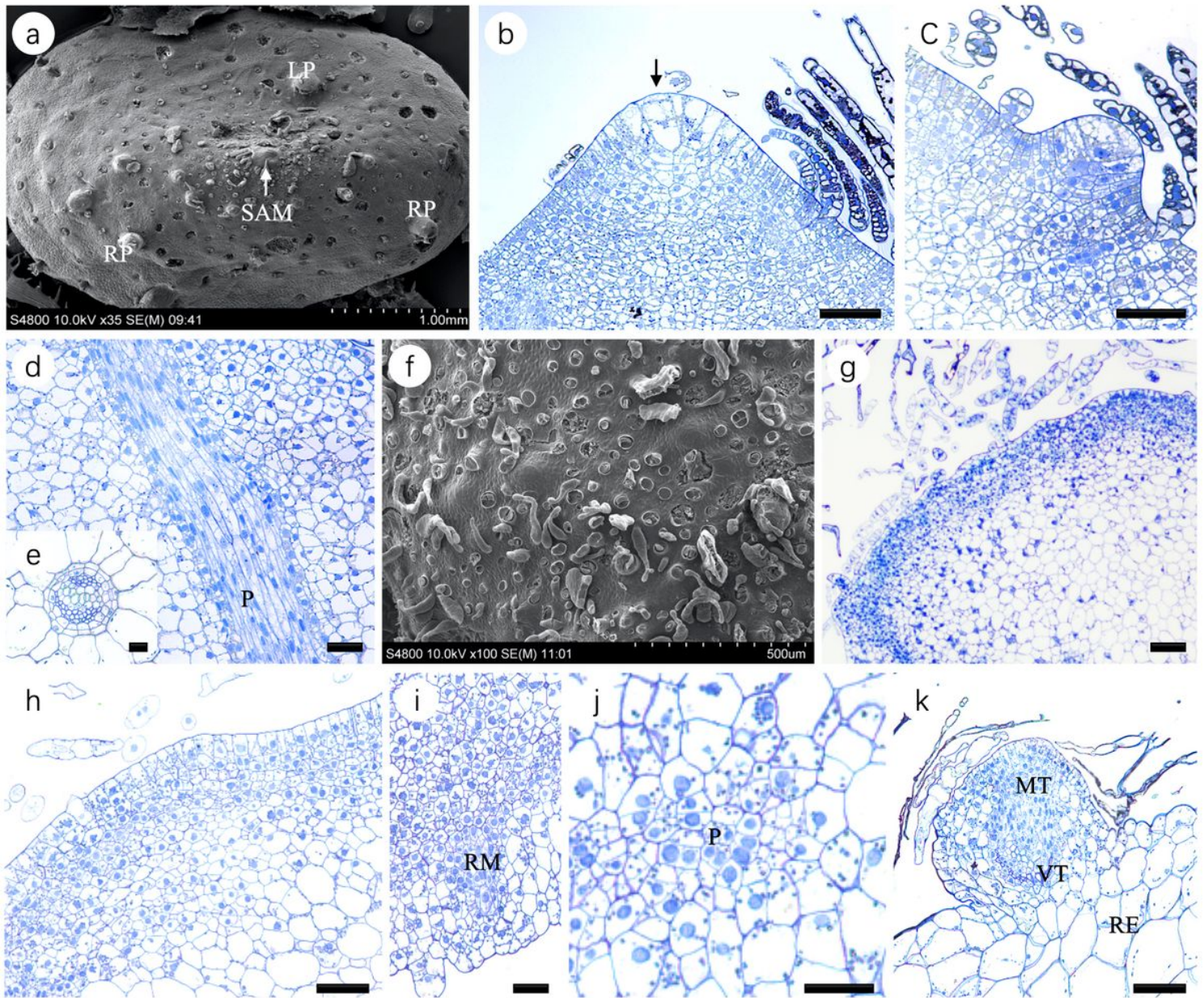


Figure 2

Anatomical characteristics of rhizome tip and GGB formed on rhizome explant. a morphological characteristics of the rhizome tip. b anatomical characteristics of the rhizome tip. c leaf primordium of rhizome. d procambium of the rhizome. e vascular tissue of rhizome. f morphological characteristics of GGB. g anatomical characteristics of the GGB. h anatomical characteristics of the meristematic cells of GGB. i root primordium of GGB. j procambium of GGB. k SAM formed in the leaf axil. LP: leaf primordium, MT: meristematic tissue, P: procambium, PE: petiole explant, RE: rhizome explant, RM: root meristem, RP: root primordium, SAM: shoot apical meristem, VT: vascular tissue. Scale bars: 25 μm (b–e, i, j), 100 μm (g, h, k)

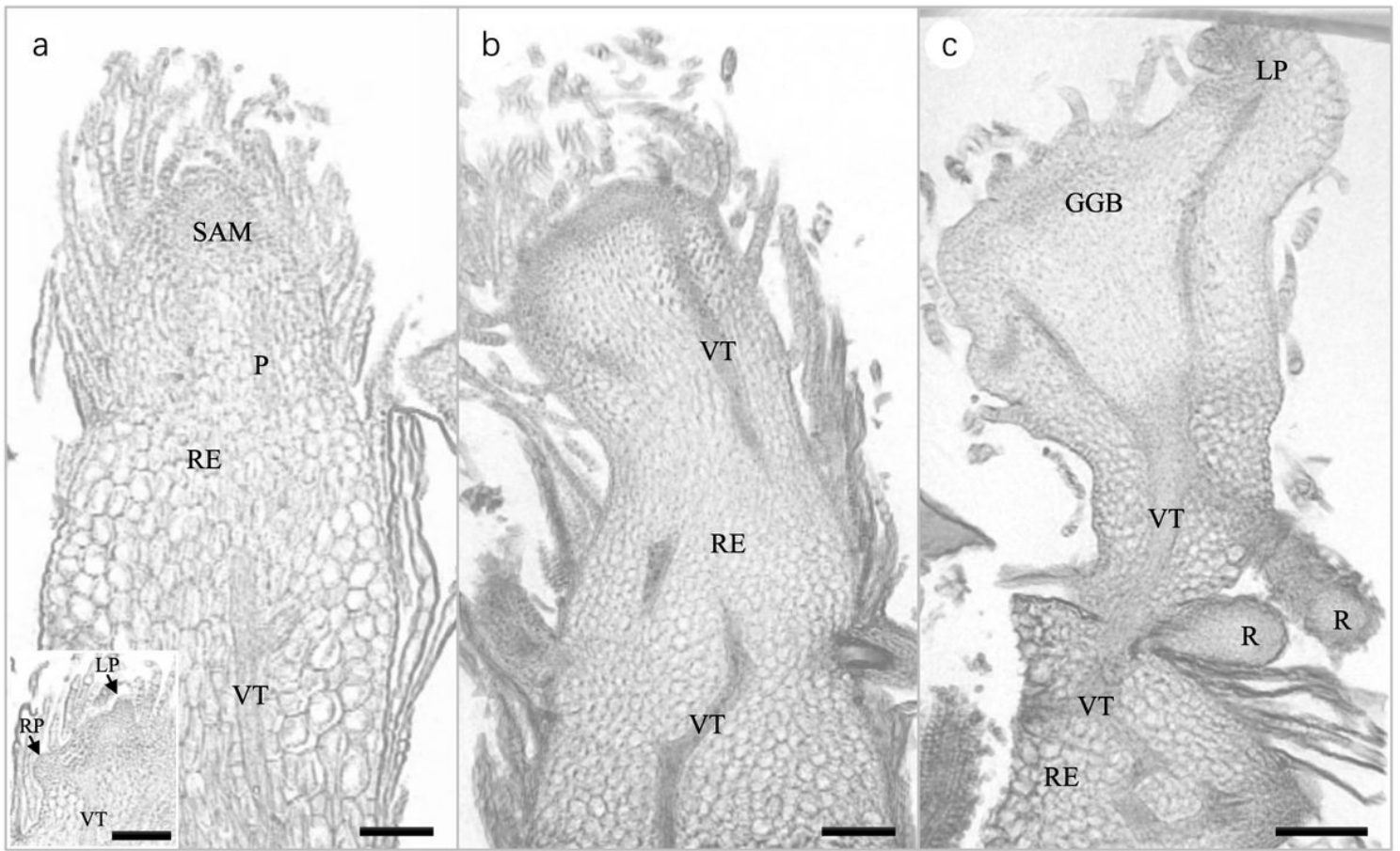


Figure 3

Rhizome tip and green globular body (GGB) formed on rhizome explant. a morphological characteristics of rhizome explant, small box indicating the leaf primordium and the root primordium. b morphological characteristics of GGB derived from the rhizome tip region. c morphological characteristics of GGB derived from the non-tip region of rhizome explant. GGB: green globular body, LP: leaf primordium, P: procambium, RE: rhizome explant, R: root, RP: root primordium, SAM: shoot apical meristem, VT: vascular tissue. Scale bars: 200 μm

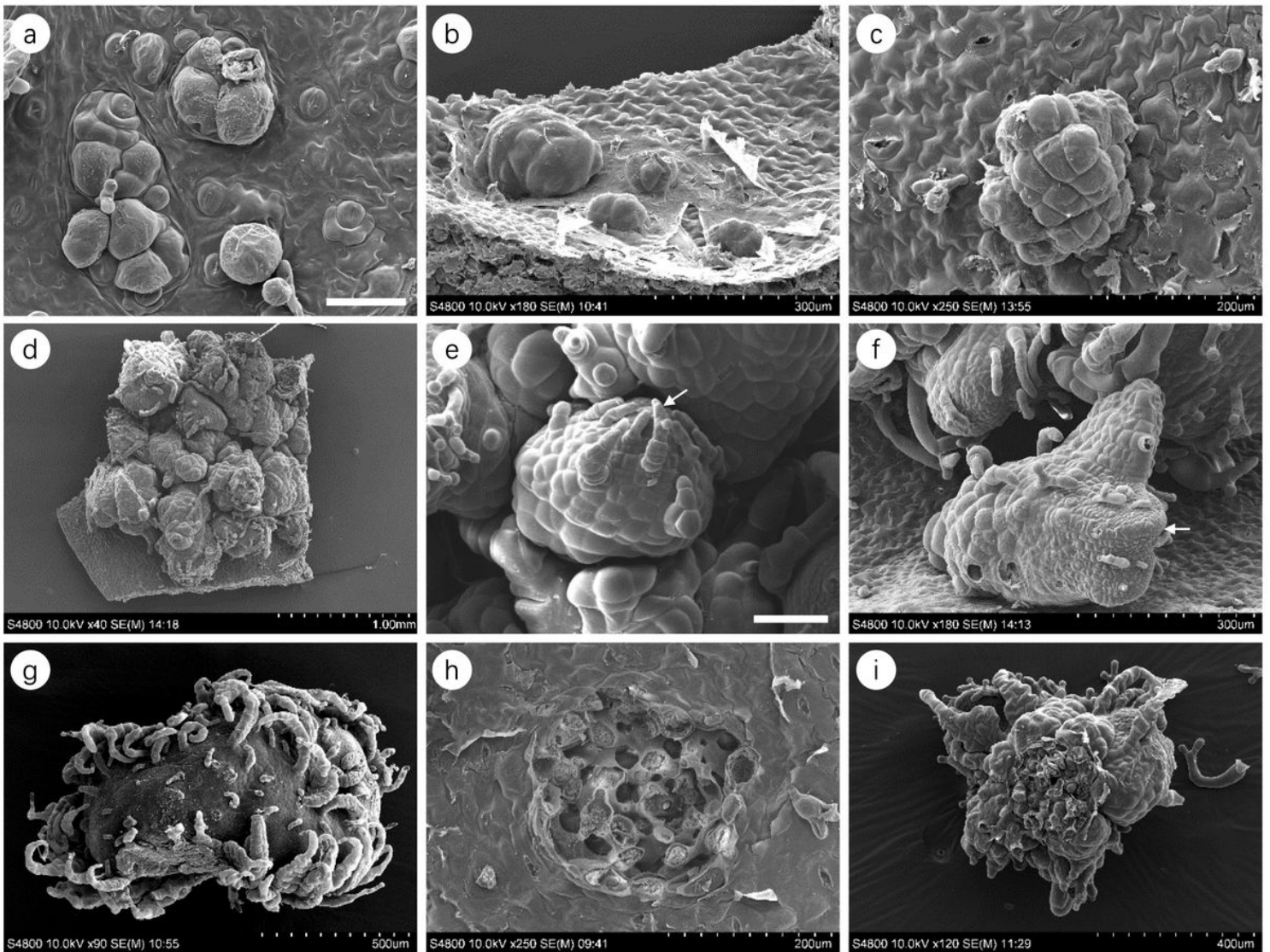


Figure 4

Green globular body (GGB) developmental process on leaf explants. a morphological change of epidermal cells in the leaf explant. b–d multicellular structures formed on leaf explants. e multicellular structure showing SAM morphology (arrow). f multicellular structure showing LP morphology (arrow). g multicellular structure increased in size to form GGB. h–i leaf explant (h) and multicellular structure (i) with no connective vascular tissue. Scale bars: 100 μm

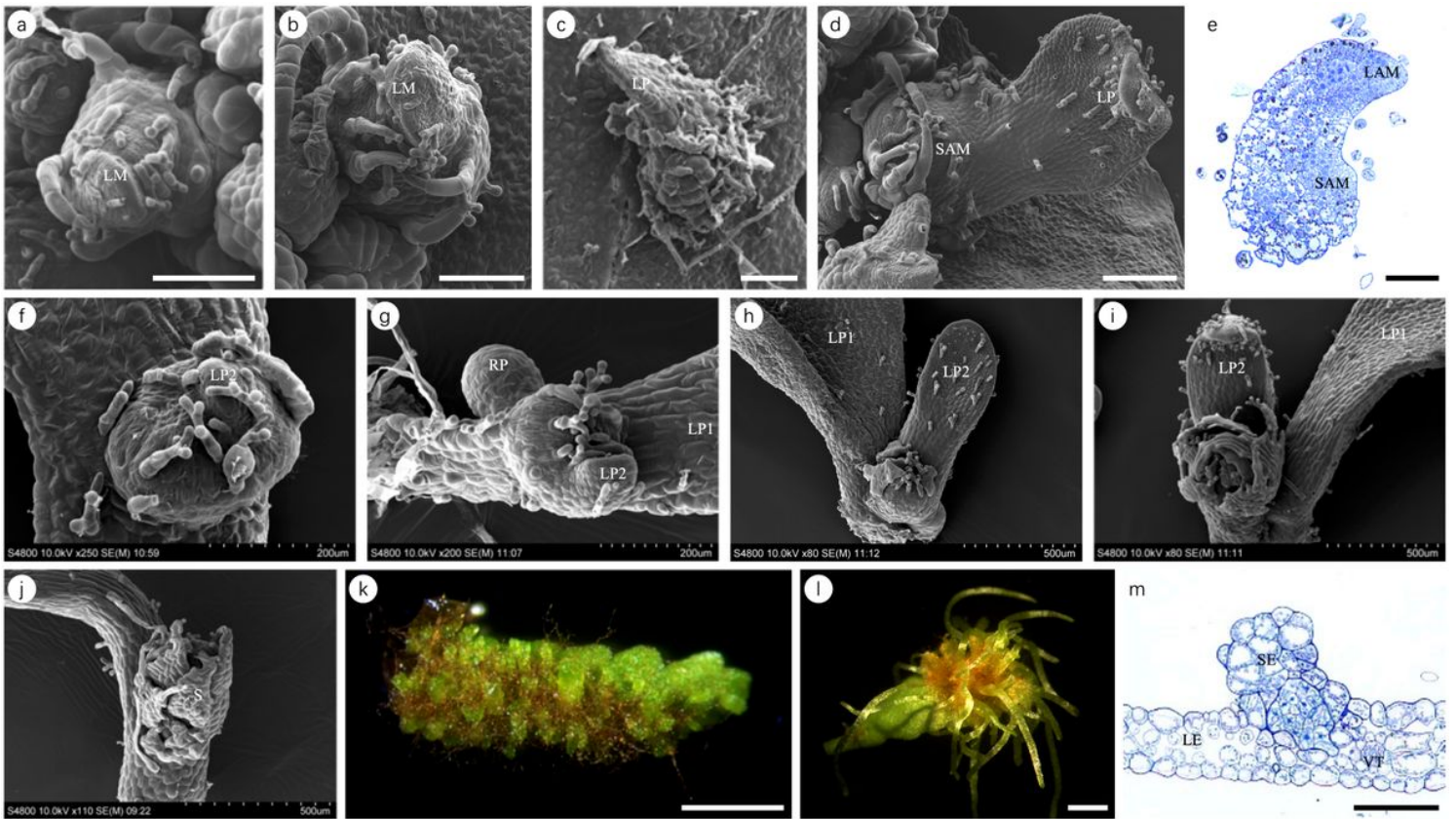


Figure 5

Development of multicellular structures derived from leaf explant into young sporophyte. a–d leaf primordium differentiated on the multicellular structures. e development of SAM and LAM from multicellular structures. f–i development of second leaf primordium from multicellular structures. j development of SAM (covered in scales) from multicellular structures. k–l development of rhizoids from multicellular structures on leaf explants. m no vascular tissue connecting the SE and the leaf explant. LAM: leaf apical meristem, LM: leaf meristem, LP: leaf primordium, LP1: first leaf primordium, LP2: second leaf primordium, RP: root primordium, S: scale, SAM: shoot apical meristem, SE: somatic embryo, VT: vascular tissue. Scale bars: 200 μ m (a–e, m), 2 mm (k, l)

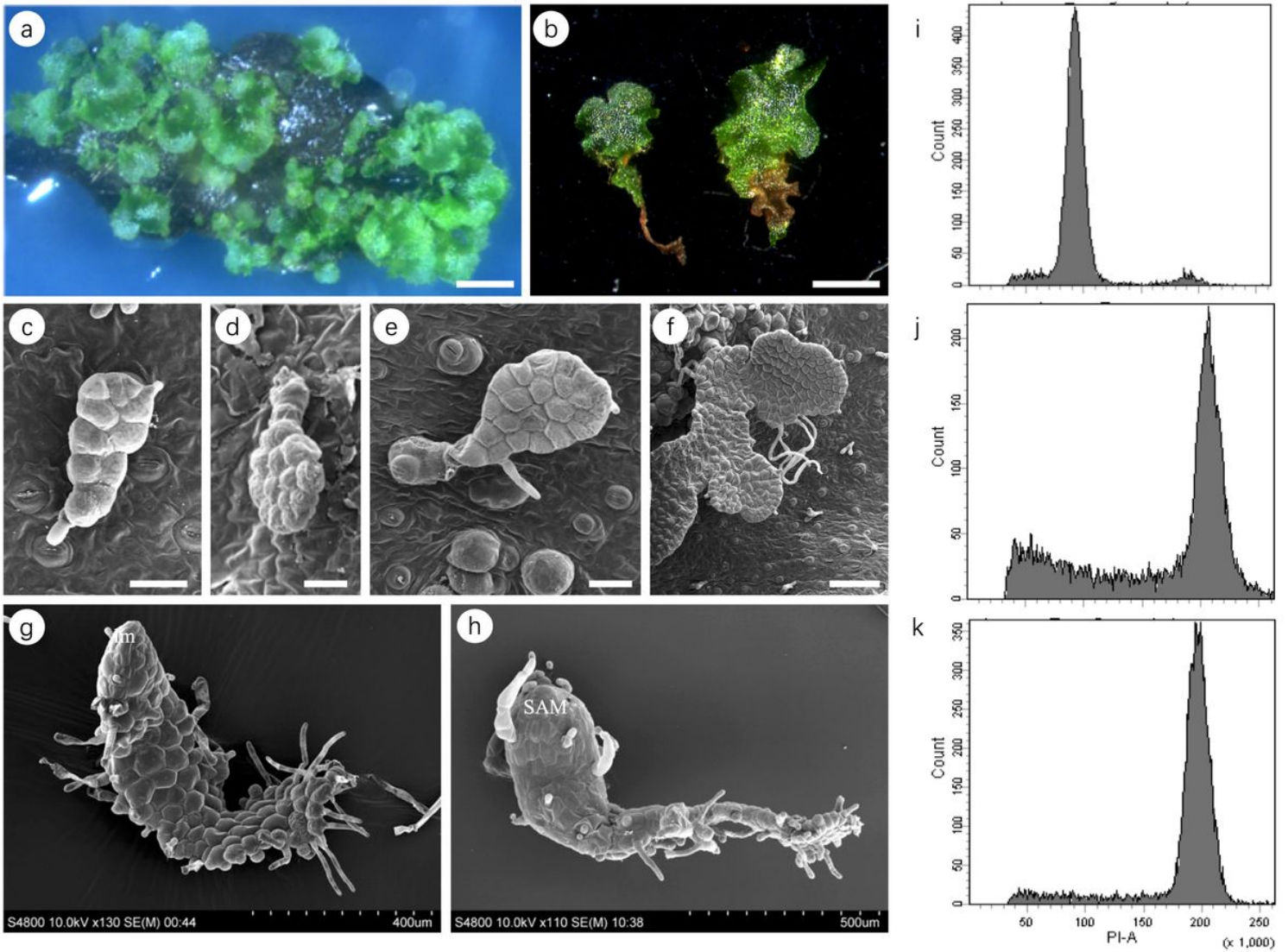


Figure 6

Prothallus-like bodies formed on leaf explants. a prothallus-like bodies formed on leaf explants. b prothallus-like bodies detached from the leaf explant. c–f prothallus-like bodies maintain two-dimensional growth. g–h three-dimensional growth of prothallus-like bodies, giving rise to the meristematic tissue. i–k ploidy level of gametophytes (i), leaves (j), and prothallus-like bodies (k) detected by flow cytometry. LM: leaf meristem, SAM: shoot apical meristem. Scale bars: 1 mm (a, b), 50 μ m (c–e), 200 μ m (f)

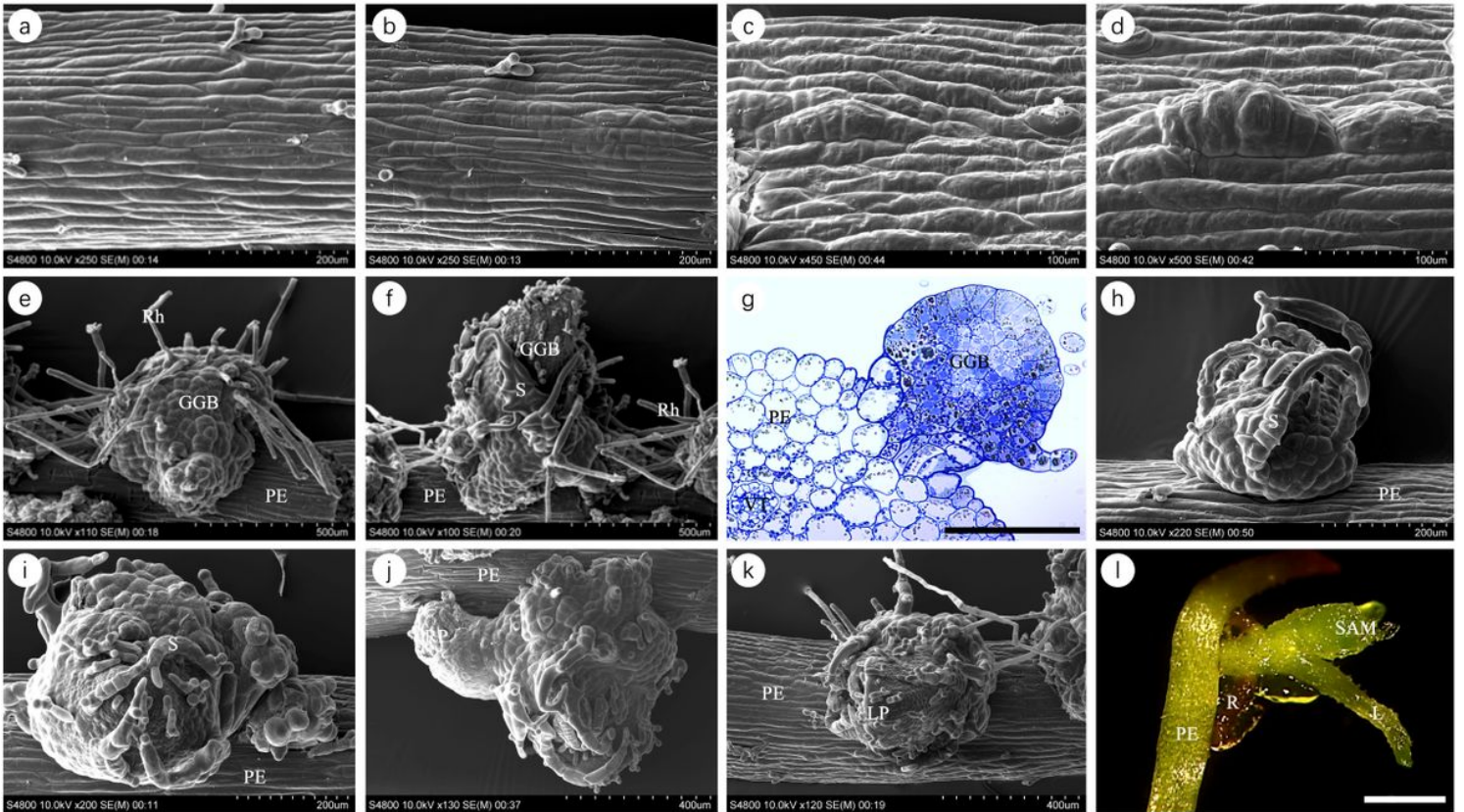


Figure 7

Green globular body (GGB) developmental process on petiole explants. a petiole explant before inoculation in inductive medium. b epidermal cells divided perpendicular to the axis of the petiole explant. c–d multicellular structures produced by several cell divisions of epidermal cells in petiole explants. e–f multicellular structures developed into GGBs. g anatomical characteristics of the leaf explant bearing GGB. h–k multicellular structures inoculated in PGR-free media developed the LP, RP, and SAM covered by several scales. l multicellular structure developed into young sporophyte in the PGR-free medium. GGB: green globular body, L: leaf, LP: leaf primordium, PE: petiole explant, R: root, Rh: rhizoid, RP: root primordium, S: scale, SAM: shoot apical meristem, VT: vascular tissue. Scale bars: 200 μ m (g), 2 mm (l)

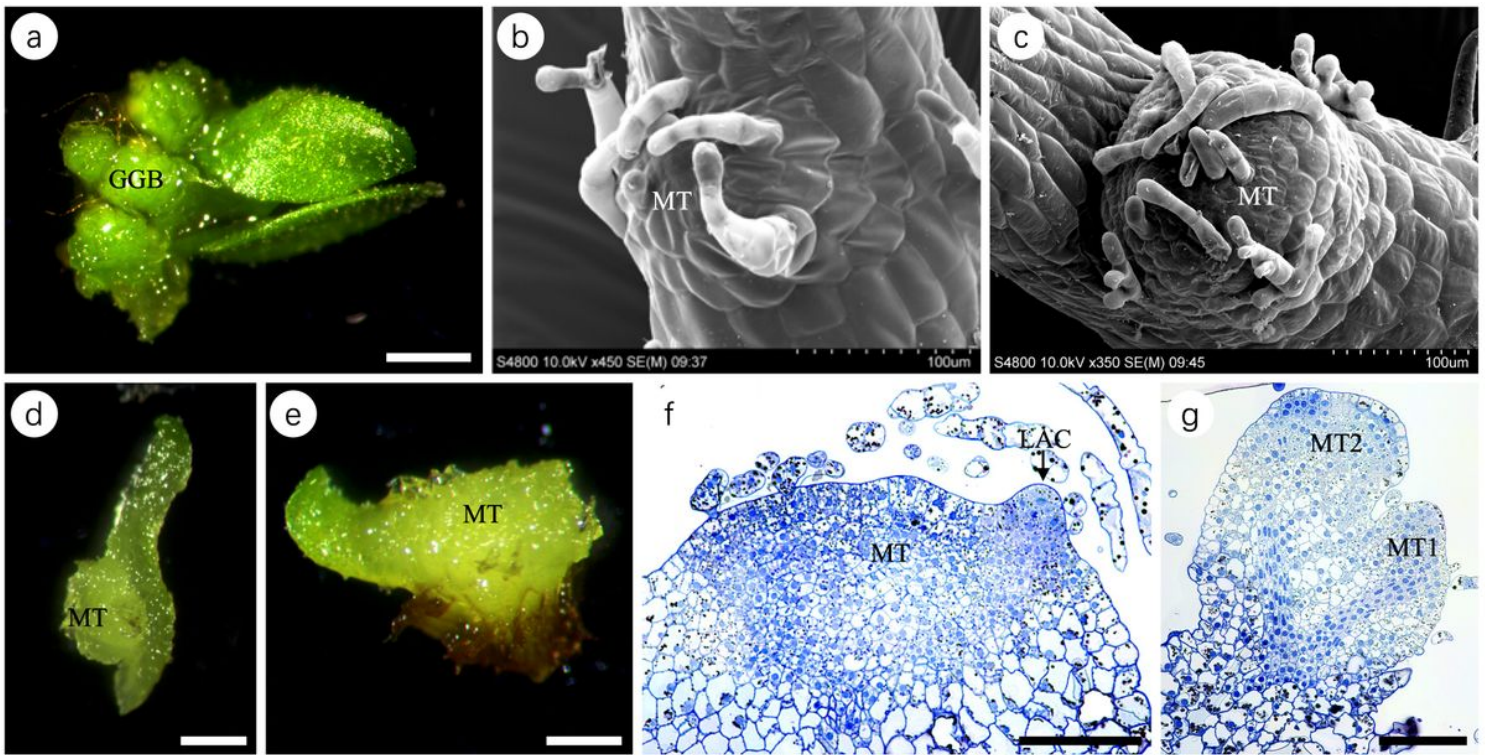


Figure 8

Green globular body (GGB) derived from the multiplication of meristematic cells of somatic embryos (SE). a GGB derived from SE when inoculated in inductive medium. b meristematic tissue in SE. c–e meristematic tissue of SE enlarged when inoculated in inductive medium. f semi-thin sectioning of SE indicated an enlarged meristematic tissue with an obvious LAC. g 2 meristematic regions on a single SE. GGB, green globular body; LAC, leaf apical cell; MT, meristematic tissue; MT1, meristematic tissue formed by SAM; MT2, meristematic tissue formed by LAM. Scale bars: 1 mm (a), 200 μ m (d, e), 100 μ m (f, g)

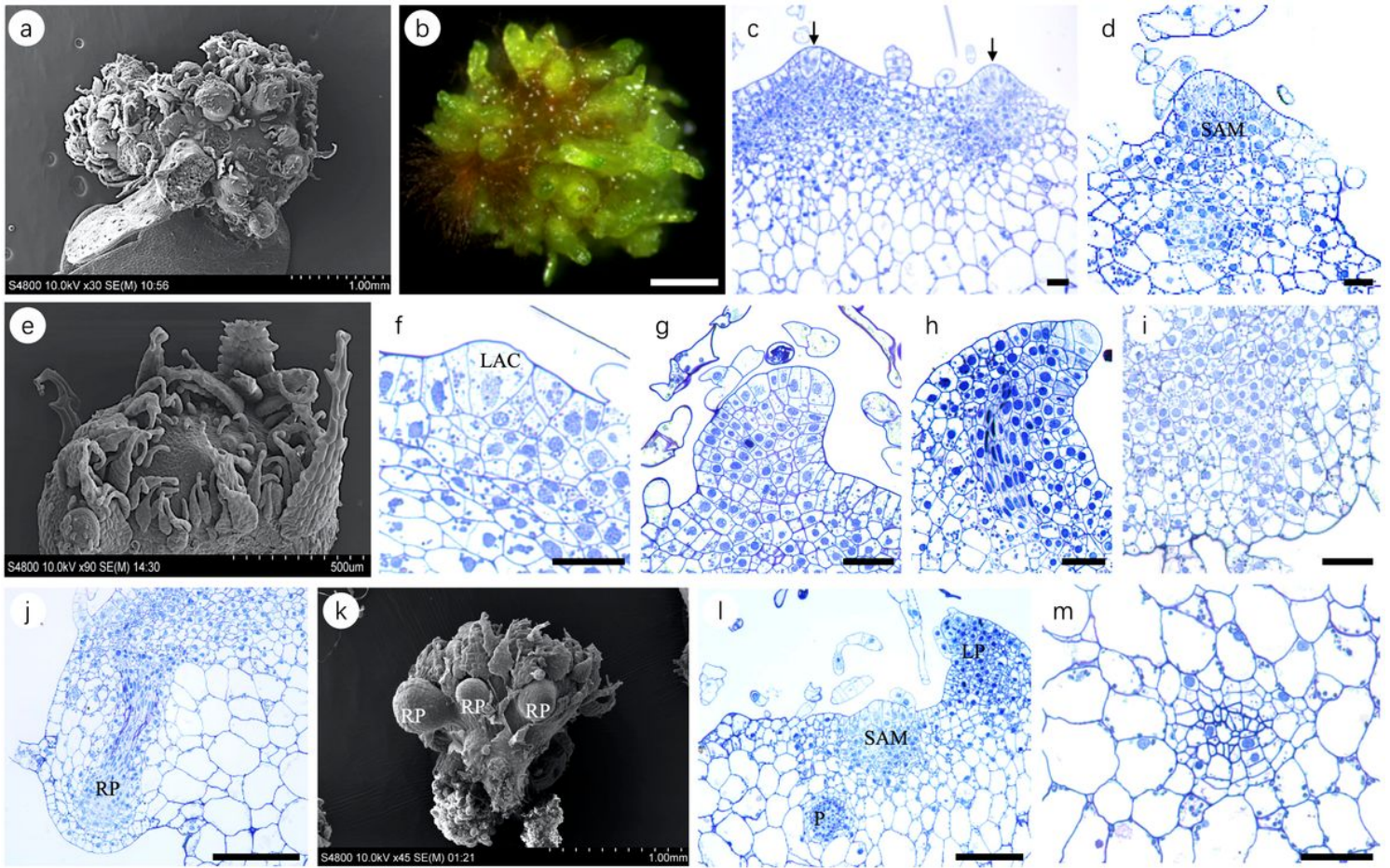


Figure 9

Green globular body (GGB) differentiation. a–b leafy shoots produced on GGB. c SAC acquired the identity. d–e SAM organized on GGB. f–h LAC acquired the identity and gave rise to the LP. i–k RM developed inside the GGB and gave rise to the RP. l a newly formed shoot with SAM, LP, and procambium. m the developing vascular tissue inside the GGB. LAC: leaf apical cell, LP: leaf primordium, P: procambium, RP: root primordium, SAM: shoot apical meristem. Scale bars: 500 μm (b), 20 μm (c, d, f–i), 100 μm (j, l, m)

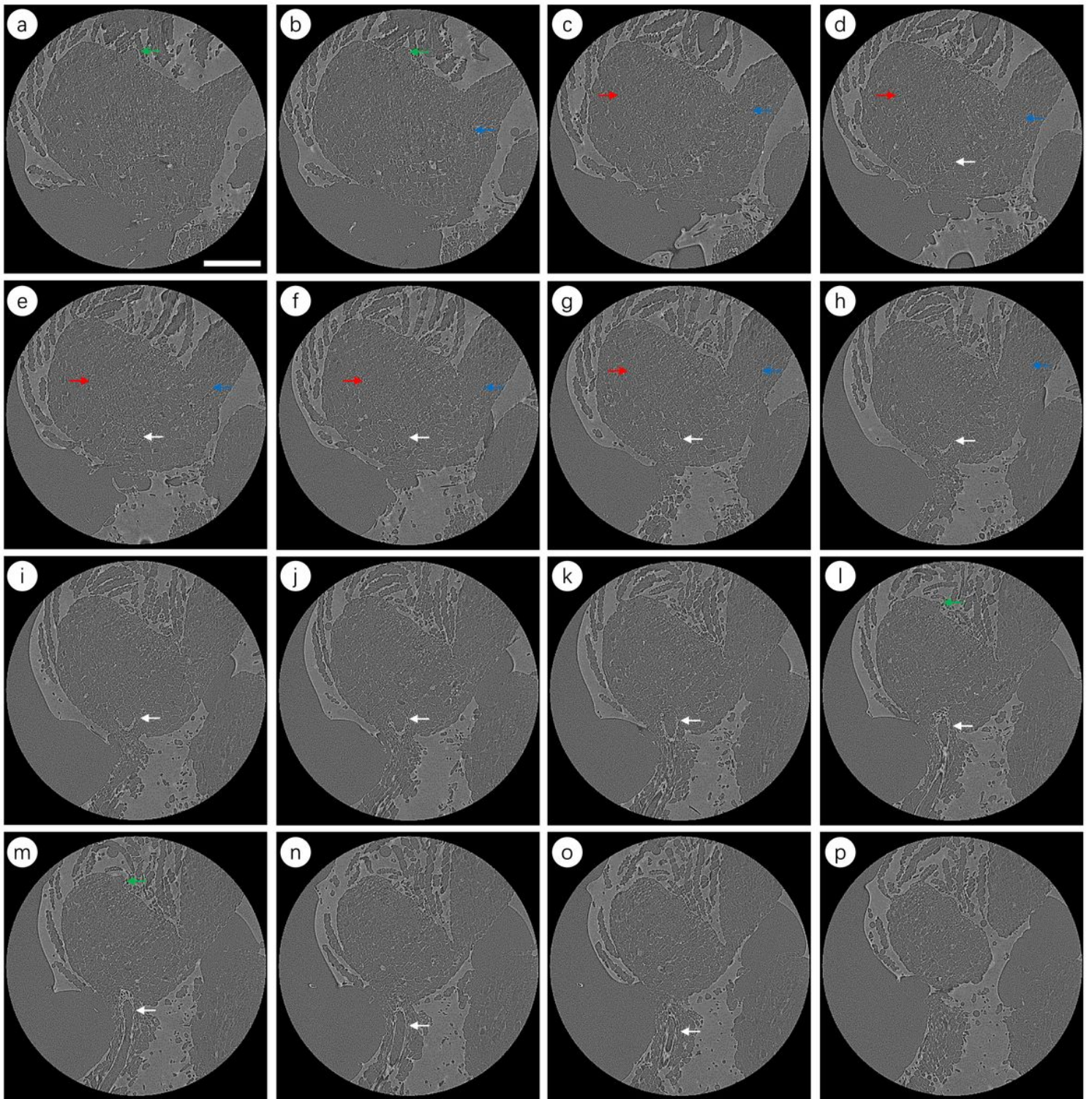


Figure 10

Different sections of one differentiating green globular body (GGB), representing the vascular tissue connecting the leaf primordium (LP), root primordium (RP), and shoot apical meristem (SAM). GGBs showed several LP, SAM and RP. Red arrows indicate the vascular tissue connecting the SAM, blue arrows indicate the vascular tissue of LP, white arrows indicate the vascular tissue of RP, and green arrows indicate the SAMs. Scale bar: 300 μ m

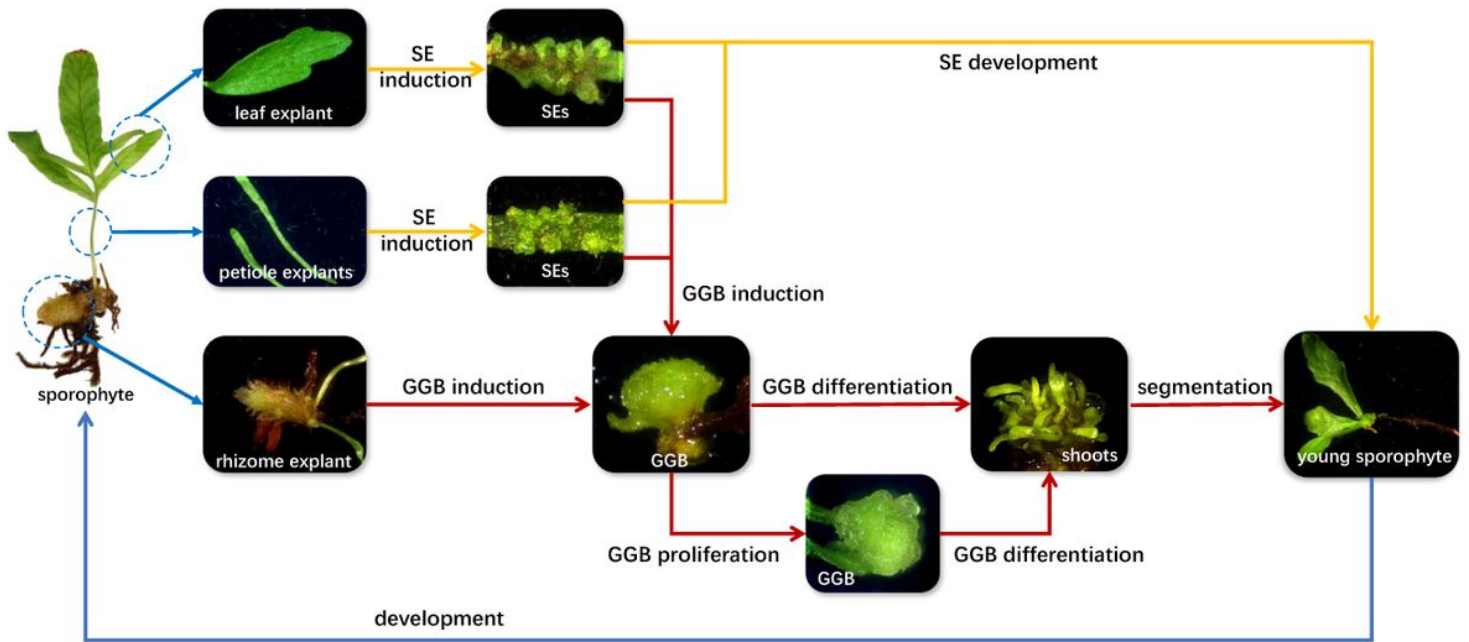


Figure 11

Schematic diagram representing the in vitro propagation process of *D. roosii*. The green globular body (GGB) pathway is indicated by red lines, and the somatic embryogenesis pathway is indicated by yellow lines.