Doctoral Dissertation (Censored)

博士論文（要約）

Molecular Developmental Analysis of the Unique Development of a One-leaf Plant in the Genus *Monophyllaea*

（モノフィレア属における一葉植物の特異な発生様式に関する分子発生生物学的解析）

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ABSTRACT

Plants in the genus *Monophyllaea* in Gesneriaceae are called one-leaf plants. This is because one of the two cotyledons, whose size is identical just after germination, grow indeterminately and does not produce new organs in the vegetative phase leading to the appearance of harboring only one leaf. This development is contrasting to that of typical seed plants where organs are produced indeterminately whereas each lateral organ grows determinately. One-leaf plants are also found in the genus *Streptocarpus* in Gesneriaceae. Although these two genera are distantly related, how they develop is similar to each other. The uniqueness of the development of one-leaf plants has attracted botanists for more than 150 years, thus the developmental process and the tissue structure have been investigated in detail. Special terms to describe the unique body plan and tissues in one-leaf plants have also been established. Moreover, regarding *Streptocarpus*, the molecular background of the unique development has been investigated using species of one-leaf plants and related species. However, in terms of *Monophyllaea*, there are few previous studies investigating the molecular background in part due to the lack of applicable experimental methods. Therefore, I established bases for the molecular research on *M. glabra*, a species in the genus *Monophyllaea*, and by using the bases, I investigated the expression patterns of key genes known to be involved in the basic development of typical plants. Physiological experiments were also performed to investigate the involvement of phytohormones in the unique development. As a result, I revealed that the meristematic tissues of *M. glabra* are likely to have unique nature different from that of typical plants from molecular aspects. Moreover, from the results, the nature of the tissues is suggested to be considerably different from that of one-leaf plants and related species in *Streptocarpus*. A new model explaining underlying molecular mechanisms of the fate

determination of two cotyledons is also proposed based on the results obtained under a newly established experimental system and careful observation.

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LIST OF ABBREVIATIONS

Species name

A. majus: *Antirrhinum majus*

A. thaliana: *Arabidopsis thaliana*

M. glabra: *Monophyllaea glabra*

M. horsfieldii: *Monophyllaea horsfieldii*

M. lavandulacea: Microchirita

lavandulacea

O. sativa: *Oryza sativa*

P. hybrida: *Petunia hybrida*

S. dunni: *Streptocarpus dunni*

S. lycopersicum: *Solanum lycopersicum*

S. rexii: *Streptocarpus rexii*

S. wendlandii: *Streptocarpus wendlandii*

Z. mays: *Zea mays*

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CHAPTER 1

General introduction

The development of plants differs strikingly from that of animals. They can produce new organs from their meristems, proliferating tissues at their apices throughout their life, whereas morphogenesis in animals is primarily completed during their embryonic stages (Steeves and Sussex, 1989; Graham et al., 2000; Wolpert and Tickle, 2011). The aerial part of typical seed plants, the shoot, is composed of repeating phytomer units (Gray, 1879), each of which consists of a leaf, stem, and axillary bud (Figure 1A). All of these components are produced from the indeterminate meristem, the shoot apical meristem (SAM) at the tip of the shoot (Figure 1B). Because of the indeterminate nature of the SAM, the shoot system is indeterminate. Conversely, all the lateral organs, such as the leaves, produced from the SAM, have determinate growth, while having unique determinate intercalary meristems for organogenesis (Esau, 1977), the leaf meristem (LM) (Ichihashi and Tsukaya, 2015) in the basal part of leaf primordia (Figure 1B). For example, in a well-studied model plant, *Arabidopsis thaliana*, the activity of the leaf meristem is maintained for only ~1 week (Kazama et al., 2010; Andriankaja et al., 2012)

One-leaf plants, which belong to Gesneriaceae in Lamiales, have a developmental system unlike that of typical plants such as the model plant, *A. thaliana*. They lack the typical shoot system: They have indeterminately growing cotyledon and do not produce other new organs, such as stems or foliage leaves, until the reproductive phase (Jong, 1970; Jong and Burtt, 1975; Kinoshita and Tsukaya, 2018) (Figure 1C). Since they are eudicots, they develop two cotyledons of identical size immediately after germination in a stage, the isocotylous stage (Figure 1C, E). However, one of the cotyledons, called the microcotyledon, stops growing or wither away, whereas the other cotyledon, called the macrocotyledon, continues growing as the sole photosynthetic organ

in a stage, the anisocotylous stage (also called anisocotyledonous stage) (Figure 1F–G), leading to the appearance of the plant harboring a single leaf (Crocker, 1860; Ridley, 1906; Tsukaya, 1997; Nishii et al., 2017).

Studies of the one-leaf plants have focused on the genera *Monophyllaea* and *Streptocarpus* in Gesneriaceae, which, based on their phylogenetic position, evolved independently. In fact, these genera belong to two different tribes, Epithemateae and Trichosporeae (Jong and Burtt, 1975; Burtt, 1978; Smith, 1996; Smith et al., 1997; Möller et al., 2009; Weber et al., 2013) (Figure 2). Furthermore, their distributions differ: *Streptocarpus* is distributed mainly in Africa, whereas *Monophyllaea* is distributed in Southeast Asia (Hilliard and Burtt, 1971; Burtt, 1978). It has also been reported that there are some other one-leaf plants in other genera, but interestingly, all the one-leaf plants including the ones in *Monophyllaea* and *Streptocarpus* are included in the same subfamily (Burtt, 1963; Weber et al., 2013), Didymocarpoideae, whose important synapomorphy is anisocotyly, in which two cotyledon size becomes different after germination although they are in the same size just after germination (Fritsch, 1904, 1920; Weber et al., 2013). The extent of anisocotyly is known to be diverse (Huang et al., 2019), and the extreme case is the one-leaf plants. In the other two subfamilies in Gesneriaceae, two cotyledon size is identical, which is similar to most of the other eudicots.

The genus *Streptocarpus* contains over 150 species (Hilliard and Burtt, 1971; Nishii et al., 2015) and, interestingly, among them, at least three forms of shoot systems exist: a caulescent system that possesses a normal shoot structure; a unifoliate system, which is the one-leaf plant; and a rosulate system, which does not have a typical shoot but possesses multiple lamina structures each of which resembles the structure of the unifoliate species, phyllomorph, later mentioned (Hilliard and Burtt, 1971; Jong and Burtt, 1975). *Streptocarpus rexii* is one of the rosulate species. Species possessing the latter two

systems are referred to as acaulescent species. The *Streptocarpus* lineage is split into two lineages, the caulescent lineage and the primarily acaulescent lineage. In the acaulescent lineage, it is suggested that rosulate and unifoliate species evolved independently several times, and that the evolution back to caulescent species also seemed to have occurred (Möller and Cronk, 2007). Conversely, in the genus *Monophyllaea*, around 40 species are known (Burtt, 1978; Kiew, 2002; Kiew & Sang, 2013; Weber, 1998), all of which are basically unifoliate species.

Because the one-leaf plants have an indeterminate shoot-like character in their macrocotyledon and yet the macrocotyledon is a planar, photosynthetic organ, similar to the leaf of typical plants, the plant body system can be interpreted as "fuzzy morphology" (Rutishauser and Isler, 2001). The term "phyllomorph" has been proposed for this fuzzy morphological unit, which consists of a stem-/petiole-like structure, a petiolode, and an indeterminately growing lamina. The one-leaf plants are composed of this single unit (Jong, 1970; Jong and Burtt, 1975; Rutishauser and Sattler, 1985). And a rosulate species in *Streptocarpus*, such as *S. rexii* consists of multiple phyllomorphs arranged in an irregular rosette (Hilliard and Burtt, 1971). The first phyllomorph in the rosulate species is composed of a macrocotyledon and a petiolode same as one-leaf plants, then a subsequent phyllomorph is produced from the groove meristem (GM), later explained, of the first phyllomorph.

The growth of the phyllomorph is supported by three meristems: the groove meristem (GM), the basal meristem (BM) (Figure 1D), and the petiolode meristem (PM). The GM is located in the junction of the macrocotyledon and the petiolode (Jong, 1970; Jong and Burtt, 1975) and is thought to correspond to the SAM because of its position, its tunica-corpus structure (reminiscent of the SAM; Jong and Burtt, 1975; Imaichi et al., 2000; Ayano et al., 2005), and its ability to produce inflorescence (Imaichi et al., 2000;

Ayano et al., 2005) although the one-leaf plants do not produce new organs in the vegetative phase. The BM is positioned in the basal part of the lamina of the macrocotyledon, which is laterally adjacent to the GM and contributes to the growth of the lamina by active cell division. Moreover, the BM remains active indeterminately. Because of the indeterminate meristem, the cotyledon of most one-leaf plant species grows for several years; in some one-leaf plant species, the inflorescence-bearing mature cotyledon retains the BM (Hilliard and Burtt, 1971; Imaichi et al., 2001). The PM is positioned immediately below the GM (Imaichi et al., 2000, 2001) or below the two cotyledons (Ayano et al., 2005) according to species and contributes to the petiolode growth.

To fully understand the growth of one-leaf plants in *Monophyllaea*, it is necessary to investigate the molecular mechanisms in terms of anisocotyly and the unique nature of the meristems in the phyllomorph.

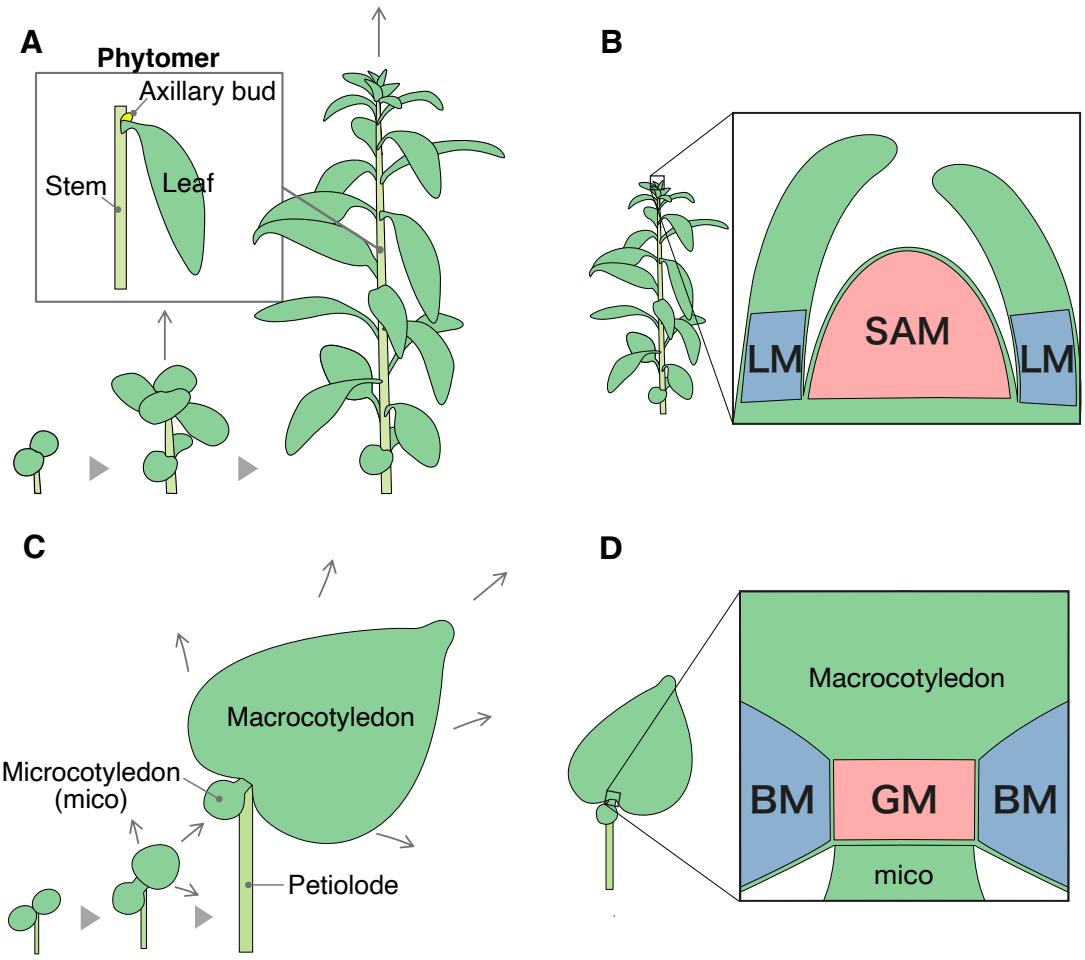

Figure 1. Development of typical seed plants and one-leaf plants.

(A) Development of a shoot of a typical seed plant composed of repetitive phytomers.

(B) Shoot apical meristem (SAM) and leaf meristem (LM) at the apex of the shoot.

(C) Development of a phyllomorph of a one-leaf plant, composed of a petiolode, a microcotyledon, and a macrocotyledon.

(D) Groove meristem (GM) and basal meristem (BM) in the basal part of the macrocotyledon.

（5 年以内に刊行のため非公開。）(G). Bar = 1 mm

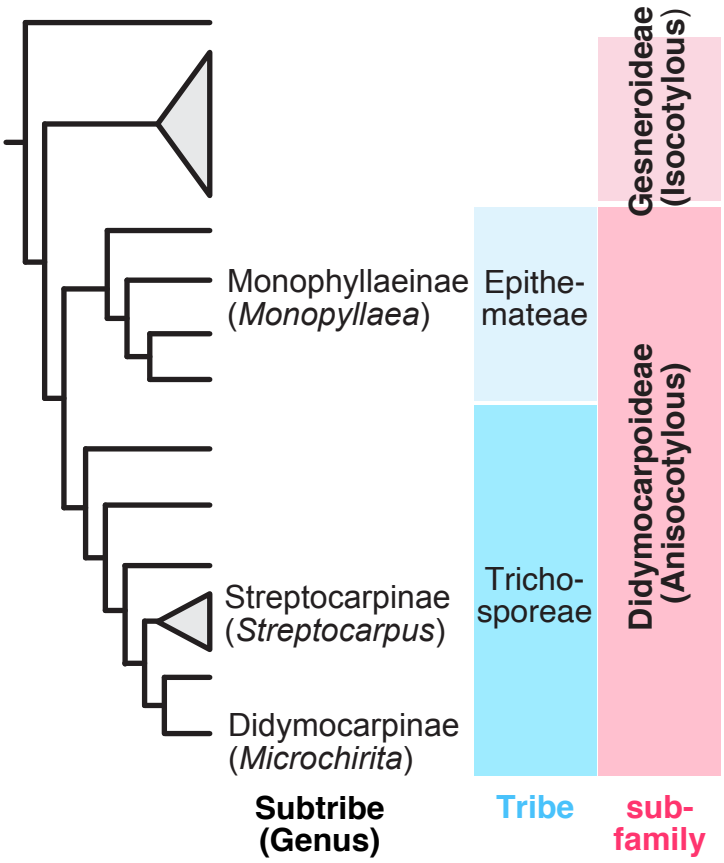

Figure 2. Phylogenetic relationship of subtribes in Gesneriaceae modified from Weber et al. (2013).

Triangles represent multiple subtribes. Under the subtribe names, a genus included in each subtribe is shown.

CHAPTER 2

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CHAPTER 3

Analysis of unique meristems in *M. glabra*

Introduction

The molecular aspects of the SAM and LM

As mentioned in Chapter 1, one of the unique natures of the plants is the indeterminacy in producing organs. All the aerial organs are produced from the SAM and the most important lateral organs, leaves are formed through the activity of the LM. Therefore, the molecular mechanisms underlying the formation and maintenance of these meristems have intensively been investigated in model plants to understand how the plants are shaped.

The SAM is gradually formed during embryogenesis. In *A. thaliana*, the embryo goes through the globular stage, where the embryo is spherical, the heart stage, where the embryo is heart-shaped due to the start of cotyledon outgrowth, the torpedo stage where cotyledons and hypocotyl enlarge, and the bent cotyledon stage where the elaborate structure of each organ is established. The three-layered structure of the mature SAM starts to be obvious in the torpedo stage and the domed structure typical of the SAM is established by the bent cotyledon stage (Capron et al., 2009).

The indeterminate nature of the SAM formed between two cotyledons is dependent on the maintenance of stem cells. Because of the indeterminate nature of the SAM, the shoot system is indeterminate. The established SAM is subdivided into three different zones based on cytoplasmic densities and cell division rates: central zone (CZ), peripheral zone (PZ), and rib zone (RZ) (Steeves and Sussex, 1989). At the CZ, stem cells replenish themselves and the cell division rate is relatively low. The PZ is at the peripheral of the CZ, and cells in the PZ divide actively and finally incorporated into new lateral

organ primordia. RZ is underneath the CZ contributing to the stem formation.

WUSCHEL (*WUS*) is an important homeobox transcription factor to form and maintain the stem cell niche in the SAM; a loss-of-function mutant has reduced stem cells and eventually the stem cells are used up (Laux et al., 1996; Mayer et al., 1998; Lenhard et al., 2002). In *A. thaliana*, it is initially expressed in the subepidermal apical part of the 16-cell stage (before the globular stage) in the embryo and gradually restricted to the inner part of the SAM. In post-embryonic stages, *WUS* is expressed just underneath the CZ (Mayer et al., 1998), which is defined as the organizing center (OC) because WUS non-cell autonomously controls the cell proliferation in the CZ by moving from the OC in the form of protein via plasmodesmata (Yadav et al., 2011; Daum et al., 2014) (Figure 10).

SHOOT MERISTEMLESS (*STM*) is also indispensable for the formation and maintenance of the indeterminate SAM in model plants; in fact, loss-of-function mutants of this gene lack a SAM (Endrizzi et al., 1996; Long et al., 1996). *STM* encodes a class I KNOTTED-LIKE HOMEOBOX (KNOX I) transcription factor. In *A. thaliana* four class I KNOX genes, *STM*, *KNAT1*, *KNAT2*, and *KNAT6* redundantly support the maintenance of a meristematic state (Hay and Tsiantis, 2010).

The expression of *STM* is … （5 年以内に刊行のため非公開）

Leaf primordia are initiated from the flanking region, the PZ of the SAM. In the incipient leaf primordia, KNOX I genes are repressed and restricted to the SAM in the species of angiosperms possessing simple leaves and thus they are restricted to the SAM (Jackson et al., 1994; Long et al., 1996; Waites et al., 1998; Sentoku et al., 1999; Sakamoto et al., 2001). This repression is important for the proper organogenesis of simple leaves because when they are expressed ectopically in leaf primordia, leaves become lobed, likely due to abnormal cell proliferation activity (Sinha et al., 1993; Lincoln et al., 1994). Many genes are involved in the suppression of the class I KNOX genes such as *ASYMMETRIC LEAVES1*(*AS1*) and *ASYMMETRIC LEAVES2* (*AS2*) (Timmermans et al., 1999; Byrne et al., 2000; Ori et al., 2000; Byrne et al., 2002) and these are important for cells to differentiate. Similarly, the programs specific in SAM are shut-off in the leaf primordia by YABBY genes including *FILAMENTOUS FLOWER* (*FIL*), whose expression is confined to the lateral organ primordia (Kumaran et al., 2002; Sarojam et al., 2010). In *A. thaliana*, it was revealed that the repression of YABBY genes by miRNA leads to the reactivation of *WUS* expression at the tip of leaf primordia (Sarojam et al., 2010).

Although leaf primordia have a distinctly different nature from the SAM, they

have determinate meristem, known as the leaf meristem (LM) (Ichihashi and Tsukaya, 2015), that generates leaf lamina cells; therefore, leaves are determinate organs. Genes supporting the LM function have been identified in model plants. *ANGUSTIFOLIA3* (*AN3*)*/GRF-INTERACTING FACTOR1* (*GIF1*) regulates cell division in the leaf meristem by functioning as a transcriptional co-activator of transcription factors, such as *GROWTH-REGULATING FACTOR5* (*GRF5*), in *A. thaliana* (Horiguchi et al., 2005; Lee et al., 2009; Kim and Tsukaya, 2015). The leaf of the loss-of-function mutant *an3* has around 30% of the cells of the wild type; the cell number of cotyledons is also decreased (Horiguchi et al., 2005; Lee et al., 2009). In *A. thaliana* and *Oryza sativa*, *AN3* is expressed in the basal part of the leaf primordia but not in the SAM (Horiguchi et al., 2011; Shimano et al., 2018; Figure 10). In *Zea mays*, the *AN3* ortholog is expressed from the bottom to the center of the SAM but not at the tip (Zhang et al., 2018).

AN3 protein can move between cells (Kawade et al., 2013, 2017). Therefore, although the area of *AN3* expression is smaller than that of the active cell dividing area, it matches the meristematic area in leaf primordia, which suggests that it is a determinant thereof. This intercellular movement of AN3 protein is necessary for the proper regulation of leaf meristem activity; in one study, immobilized AN3 protein fused to three GFP molecules did not fully complement the reduced number of leaf cells in an *an3* mutant (Kawade et al., 2013).

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The molecular aspects of the GM and the BM

As mentioned in Chapter 1, the GM and the BM have been thought to be a corresponding tissue of the SAM and the LM, respectively but the GM and the BM have unique characters: that is, no-organ producing character in the vegetative phase in the GM, and indeterminate cell division in the BM unlike the SAM and the LM, respectively.

Unlike the typical SAM, the GM is formed after the germination. Therefore, in embryonic stages, or just after germination, there is no specific meristematic structure between two cotyledons (Imaichi et al., 2000, 2001; Ayano et al., 2005). The GM is gradually formed along with the macrocotyledon growth: in the basal part of the macrocotyledon of the future midrib, anticlinal cell division has been observed forming the tissue composed of small cells with tunica-corpus structure (Imaichi et al., 2000, 2001; Ayano et al., 2005). The GM has cell division activity (Ishikawa et al., 2017), and the area of the GM expands slowly, but it does not produce new organs until one-leaf plants enter the reproductive phase (Imaichi et al., 2000, 2001; Ayano et al., 2005). The SAM in a caulescent species in *Streptocarpus*, *S. pallidiflorus*, has a similar ontogeny as the abovementioned GM except that new foliage leaves are produced from the SAM formed after germination (Imaichi et al., 2007). As for the BM, smaller cells next to the

GM appear after germination in one of the cotyledons. The area of the BM also expands during the growth of macrocotyledon (Imaichi et al., 2000, 2001).

It has been hypothesized that no-organ producing phenotype in the GM in the vegetative phase is due to the impairment/loss of the SAM formation/maintenance mechanisms (Cronk and Möller, 1997; Tsukaya, 1997, 2000). On the other hand, it has been speculated that genes involved in the SAM formation/maintenance are expressed in the BM to maintain the indeterminate cell division activity (Cronk and Möller, 1997). In this aspect, the expression patterns of the key genes involved in the meristematic activity in the SAM and LM have been investigated to infer the nature of the unique meristems in one-leaf plants or related species.

First, the expression of KNOX I genes has been analyzed. KNOX I protein accumulation and *STM* ortholog expression patterns were investigated in a one-leaf plant, *S. dunni,* and a caulescent species *S. prolixus* (Harrison et al., 2005). In the one-leaf plant, neither KNOX I protein nor *STM* mRNA was detected in the GM whereas they were detected in the caulescent species in the SAM. An acaulescent species harboring rosulate body plan in *Strepotcarpus* is composed of the repetition of phyllomorphs. In this type, the SAM is unlike typical SAM; first, it is flat as the GM in one-leaf plants; however, the flat GM becomes domed shape and new foliage leaf primordia are produced; after finishing the formation of the foliage leaf primordia, the domed shape GM returns to the flat shape (Nishii and Nagata, 2007). This flat and domed state is repeated every time a new phyllomorph is produced. *S. rexii* is one of the species of this type and an *STM* ortholog, *SrSTM1* is expressed not in the flat type GM but expressed in the dome-shaped GM (Figure 11). Therefore, in *Streptocarpus*, the expression of *SrSTM1* in the SAM/GM coincides with the production of organs. In embryonic stages to the isocotylous stage, *SrSTM1* was not expressed in between two cotyledons (Mantegazza et al., 2007).

Contrarily, an ortholog of *KNAT1*, another KNOX I gene, *SrBP*, was expressed even in the flat type GM. As for *M. glabra*, the expression of an *STM* ortholog, *Mg-STM-B*, was investigated with RT-PCR combined with tissue collection with laser microdissection (LSM) and was suggested to be expressed in the GM (Ishikawa et al., 2017).

Interestingly, in *S. rexii*, *SrSTM1* and *SrBP* are also expressed in the BM consistent with the idea that the transfer of the SAM function to the BM caused the indeterminacy (Cronk and Möller, 1997; Mantegazza et al., 2007; Nishii et al., 2010) (Figure 11). In embryonic stages to isocotylous stages, *SrSTM1* was found to be expressed in both cotyledons consistent with the growth of both of the cotyledons (Figure 11).

Moreover, even in several species with the typical shoot system in Gesneriaceae family, it was suggested that the expression of *STM* orthologs coincides with the cell division activity in cotyledons and foliage leaves although they are simple leaves (Nishii et al., 2017). Therefore, the authors discussed that misexpression of KNOX I in the basal part of the leaves and gradual extension of KNOX I genes were in the background of the evolution of the indeterminate nature of leaves in acaulescent species, unifoliate and rosulate species in *Streptocarpus*. In terms of *Monophyllaea*, it was also discussed that an *STM* ortholog may be expressed in the BM (Ishikawa et al., 2017).

The expression pattern of a *WUS* ortholog in *S. rexii*, *SrWUS*, resembles that of *SrBP*: it is expressed in the domed GM and the BM but not expressed in between two cotyledons in the isocotylous stage (Mantegazza et al., 2009) (Figure 11). Interestingly, in embryonic stages, *SrWUS* was observed to be expressed in whole embryos.

The expression pattern of *AS1* orthologs has also been investigated in phyllomorphs. Although their expression is confined to the lateral organs or their primordia in typical model plants harboring simple leaves, an ortholog in *S. rexii* is expressed both in the GM and the BM, overlapping with the expression area of a *KNAT1*

ortholog (Figure 11). In terms of *M. glabra*, the expression of an *AS1* ortholog was confirmed in the BM, and it was also discussed that may be expressed in the GM (Ishikawa et al., 2017). Contrarily, an ortholog of *FIL*, which is also confined to the lateral organ primordia in model plants, is found to be expressed only in the BM but not in the GM in *S. rexii* (Tononi et al., 2010).

Materials and methods

Plant materials and growth conditions

Refer to the section “Materials and methods” in Chapter 2.

Microscopy

Most of the WMISH samples were observed under a stereomicroscope, SZ61 (Olympus), and the images were taken by a digital camera, OM-D E-M10 (Olympus). Depending on the size of samples and magnification rate, an inverted microscope DM4500 (Olympus) was used for the observation and taking images through Leica Application Suite software (Leica).

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Isolation of genes in M. glabra

Total RNA was extracted from inflorescences of *M. glabra* with an RNeasy Plant Mini Kit (Qiagen) following the manufacturer's protocol. First-strand cDNA was synthesized from total RNA with the SuperScript III First-Strand Synthesis Kit (Invitrogen) according to the manufacturer's protocol. Primers for isolating genes were designed based on the *de novo* assembled sequences obtained from mRNA-seq of *M. glabra*. The primers for

cloning are listed in Table 4. The amplicons were ligated to *Eco*RV-digested pZErO-2 plasmids (Thermo Fisher Scientific) and were introduced to competent *Escherichia coli* (TOP10 or DH5a) to be amplified. The plasmids were used for sanger sequencing or direct colony sequencing was conducted to confirm the sequences of the amplicons. The nucleotide sequences deposited in DDBJ are listed also in Table 7.

Molecular phylogenetic analyses

The amino acid sequences other than from *M. glabra* were obtained from the databases in Table 5. GenBank accession number of sequences not from the databases were listed in Table 6. The amino acid sequences from *M. glabra* were inferred from the longest open reading frames in the cDNA sequences. Amino acid sequences were aligned by MAFFT v. 7.407 in auto mode (Katoh and Standley, 2013), and poorly aligned sequences were trimmed with trimAl v. 1.4. rev. 15 (Capella-Gutiérrez et al., 2009) in automated1 mode. RAxML v. 8.2.12 (Stamatakis, 2014) was used to analyze the phylogenetic relationship with the maximum likelihood method. Bootstrap analyses (Felsenstein, 1985) with 100 replicates was performed with the same software, and phylogenetic trees were generated with FigTree (http://tree.bio.ed.ac.uk/software/figtree/).

Whole-mount* in situ *hybridization

After confirming the plasmid sequences, we conducted PCR using M13 forward and M13 reverse primers (Table 4). The amplicons were used as the template for generating DIG-labelled antisense and sense probes by SP6 or T7 polymerase (Roche) using DIG RNA Labeling Mix (Roche). For WMISH, we slightly modified the protocol of Rozier et al. (2014) to facilitate the permeabilization of cells. First, we used 4% (w/v) paraformaldehyde (PFA) with 15% (v/v) dimethyl sulfoxide (DMSO) in phosphate-

buffered saline with 0.1% (v/v) Tween-20 (PBST) as the fixative unless stated otherwise. Second, the cell wall enzyme treatment was performed for 15 min for embryos and 30 or 60 min for the other samples with cell wall enzymatic solution (CWES), six times dilutes stock solution of 0.12% (w/v) Macerozyme R10 (Yakult), 0.5% (w/v) Cellulase Y-C (Kyowa Chemical), and 0. 25% (w/v) Pectolyase Y23 (Kyowa Chemical) in PBST. Hybridization was performed at 50°C or 55°C for 3–7 days unless noted. DIG was detected with a DIG Detection Kit (Roche) with a 1:2000 dilution of the anti-digoxigenin antibody conjugated with alkaline phosphatase (Roche). 1% (v/v) NBT/BCIP stock solution (Roche) diluted with a solution (50 mM $MgCl_2$, 100 mM NaCl, 100 mM Tris-HCl pH 9.5) was used for the chromogenic stain. The staining reaction was proceeded at 4°C for overnight–7 days. For some samples…（5 年以内に刊行のため非公開）

Results

Determining the precise position of the GM and the BM

I first defined the GM and the BM anatomically before analyses of spatio-temporal gene expression patterns. To determine precisely the position of the GM and the BM from the top of the phyllomorph, I stained the cell walls of anisocotylous-stage individuals with calcofluor white and took an image of a section including cells of 2nd and 3rd layers in the macrocotyledon with a confocal microscope (Figure 12A–C). Two positionally distinct meristems, the GM and the BM, were evident. One meristem resided in the most proximal part of the macrocotyledon around 100 μm from the mediolateral axis and adjacent to five rows of differentiated cells in the distal part of the meristem. This meristem is the GM—inflorescence was produced at this position, as evidenced by the presence of a bulge (Figure 12D–E). The other meristem was laterally adjacent to the GM, and its smaller cells were distributed more widely than the GM, both laterally and distally. In the basal part of macrocotyledon, changes in contour were observed at certain points (Figure 12A). Hereafter I regard the basal part of the tissue inside these points as the GM and the basal part of the tissue outside them as the BM.

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Isolation and characterization of orthologs of CYCB1

Although RNA-seq is a convenient method to analyze the spatio-temporal expression of genes, *in situ* hybridization can reveal spatial information more in detail. The conventional section *in situ* hybridization was conducted in *M. glabra* but no successful result has been obtained. Therefore, I decided to try WMISH, which was simpler and thus easier to examine various conditions than the conventional one.

CYCB1, a marker of the G2/M phase of the cell cycle is expressed in a scattered pattern in tissue with actively dividing cells, such as leaf primordia and floral leaf primordia in *A*. *thaliana* and other model plants, (Donnelly et al., 1999; Porceddu et al., 1999). I used this gene as the positive control for WMISH of *M*. *glabra*. Although a part of cDNA of a *CYCB1* ortholog in *M. glabra* was isolated (Kinoshita, master thesis), detailed analyses were not conducted.

I finally isolated cDNA of the *CYCB1* orthologs *Mg-CYCB1-1* and *Mg-CYCB1-2* using primers based on the sequence of a contig, Mgla2|TRINITY_DN5708_c0_g1 (Figure 15, Figure 16; Table 7), the nucleotide and amino acid sequences of which showed

98.3% and 98.8% similarity to each other, respectively.

Cyclin degradation at a particular cell cycle phase, which is important for progressing into the next cell cycle, depends on the destruction box motif, the key region for the regulation of the cyclin degradation (Glotzer et al., 1991). Therefore, Hemerly et al. (1992) was referred to determine the destruction box; in addition, Pfam was used (https://pfam.xfam.org/; *Cyclin_N*, PF00134; *Cyclin_C*, PF02984) to determine the other domains. Then, I found that both harbored a destruction box, a Cyclin_N domain, and a Cyclin_C domain (Figure 17).

WMISH in various stages of M. glabra

It has been revealed that fixing samples with 4% PFA/15 % DMSO in PBST and treatment with CWES for 30–60 minutes were critical factors for the WMIH of *M. glabra* in the anisocotylous stage (Kinoshita, master thesis). However, a negative control using a sense probe has not been checked, so I checked on this point in the present study. Moreover, to check the suitability of the WMISH condition I established, the *Mg-CYCB1* expression patterns were further examined in the isocotylous stage and reproductive stage.

Although anisocotylous samples treated with the antisense or sense probe exhibited a pale purple background, patchy dark purple signals were observed only when the antisense probe was used (Figure 18A–B). In addition, the *Mg-CYCB1* signal was denser in the BM than in the GM (Figure 18A). *Mg-CYCB1* was expressed in both cotyledons at the isocotylous stage (Figure 18D–I), consistent with the report of Tsukaya (1997) that BrdU is incorporated into both cotyledons immediately after germination. Moreover, the signal was detected in the inner tissue of the tip of the petiolode (Figure 18D) from which the first root newly emerges as reported in *Monphyllaea singularis* by Imaichi et al. (2001) and in *M. glabra* by Ayano et al. (2005). Therefore, the signals should

be from the proliferative cells which start to form the root primordia. WMISH in reproductive individuals, which had started to produce inflorescence meristems was also perfomed. The inflorescence meristem exhibited more signals than the vegetative-phase GM, confirming the suitability of the WMISH condition (Figure 18C).

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Molecular characterization and expression pattern of Mg-AN3

Next, I evaluated *AN3* expression in *M. glabra* to assess the leaf-meristem-like nature of the BM. I isolated the cDNA of two *AN3* orthologs, *Mg-AN3-1* and *Mg-AN3-2* by preparing primer sets based on the contigs, Mgla2|TRINITY_DN18644_c0_g1 and Mgla2|TRINITY_DN18644_c0_g1 (Figure 35A). These two contigs had highly similar sequences. The two cloned sequences had 97.4% and 99.5% sequence similarity at the nucleotide and amino acid levels, respectively. In the amino acid level, the only difference was residue 72, which was methionine or leucine. The putative AN3 protein of *M. glabra* possessed an SNH domain (Kim and Tsukaya, 2015) (Figure 35B), which is conserved among known AN3 orthologs and is necessary for interaction with GRF transcription factors (Kim and Kende, 2004; Horiguchi et al., 2005; Kim and Tsukaya, 2015).

RNA-seq results showed that *Mg-AN3* was expressed both in the GM and the BM (Figure 36A). WMISH revealed that *Mg-AN3* was expressed in the basal part of both cotyledons at the isocotylous stage (Figure 36B–C). Consistent with the RNA-seq result,

in the anisocotylous stage, WMISH showed that *AN3* was expressed in both the GM and the BM of the macrocotyledon (Figure 36D–F). The longitudinal sections revealed that *AN3* mRNA was not expressed in the epidermis (Figure 36F).

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Discussion

Applicability of WMISH to M. glabra

I established a WMISH technique for *M. glabra*, as confirmed by patchy *Mg-CYCB1* expression in mitotic regions in cotyledons, the petiolode, and the inflorescence meristem at the isocotylous and anisocotylous stages. The technique is rapid because it does not require laborious embedding or sectioning. Moreover, spatial patterns of expression can be easily evaluated because the three-dimensional structure is retained. I succeeded in identifying gene expression in the GM and/or the BM in a paradermal view, which is difficult with the traditional section *in situ* hybridization. Moreover, I successfully obtained a cross-section of the samples after WMISH, which enables to analyze gene expression patterns at a cellular level, showing that the WMISH technique can be a complete alternative to the traditional section *in situ* hybridization when samples are small enough. WMISH has been used less frequently in plant research compared to animal research (Tautz and Pfeifle, 1989; Hemmati-Brivanlou et al., 1990; Herrmann, 1991) and has rarely been used in studies of the photosynthetic organ of plants other than *A. thaliana* (Althoff et al., 2014). The established WMISH technique will definitely facilitate further studies of *Monophyllaea* and other non-model plants.

Expression of Mg-STM in the GM

STM is essential for the formation and maintenance of the SAM. The loss-of-function mutant *stm* lacks SAM; therefore, no new organ is formed after the cotyledons unfold. This phenotype is similar to that of one-leaf plants (Cronk and Möller, 1997; Tsukaya, 1997, 2000). *STM* expression and other class I KNOX protein accumulation have been investigated in some phyllomorphs. Harrison et al. (2005) reported that in the one-leaf plant *Streptocarpus dunni*, KNOX I protein was detected in the GM during the

reproductive phase but not the vegetative phase, whose result seems to be consistent with the hypothesis that SAM formation/maintenance system is lost/suppressed in the vegetative GM (Cronk and Möller, 1997; Tsukaya, 1997; Tsukaya, 2000). *SdSTM1*, an ortholog of *STM*, is not expressed in aboveground parts of *S. dunni* during the vegetative phase. A rosulate *Streptocarpus* species has repeating phyllomorphs (Jong,1970; Jong and Burtt, 1975) because the GM can produce new phyllomorphs even in the vegetative phase (Nishii and Nagata, 2007). In a rosulate species *S. rexii*, *SrSTM1* expression varies according to the stage of the GM and correlates with the production of new phyllomorphs (Mantegazza et al., 2007). Therefore, *SrSTM1* expression in the GM is correlated with additional organ formation in *Streptocarpus*. In this study, *Mg-STM* expression was detected in the proximal part of the future midrib in *M. glabra*, consistent with Ishikawa et al. (2017), which suggests that no organ is formed in the GM irrespective of *Mg-STM* expression. Therefore, the molecular mechanism underlying GM formation and maintenance may differ between *Streptocarpus* and *Monophyllaea*. Alternatively, because an ortholog of *KNAT1* in class I KNOX gene, *SrBP*, is expressed in the GM at the no-organ-producing stage in *S. rexii* (Nishii et al., 2010), and *KNAT1* is functionally similar to *STM* (Kim et al., 2003), *SrBP* may replace *SrSTM1* in the flat-stage GM of *Streptocarpus*.

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Prolonged cell division activity in cotyledons coincides with Mg-AN3 expression

AN3, a transcription co-activator expressed in the basal part of leaf primordia, promotes the division of leaf meristem cells in *A. thaliana*, *O. sativa*, and *Z. mays* (Kim and Kende, 2004; Horiguchi et al., 2005; Shimano et al., 2018; Zhang et al., 2018). In isocotylous-stage *M. glabra*, cell division occurs in both cotyledons, as evidenced by the *Mg-CYCB1* expression pattern. At this stage, *AN3* was expressed in the basal part of each cotyledon. At the anisocotylous stage, *Mg-CYCB1* was expressed in the basal part of the macrocotyledon but not in the microcotyledon, which indicates that cell division is

confined to this area. *Mg-AN3* expression is also confined to the basal part of the area of cell division in the BM, as in leaf primordia of *A. thaliana* (Kawade et al., 2017). This suggests that the cell division activity of the BM is, at least in part, supported by *Mg-AN3*. The greater area of *Mg-CYCB1* than *Mg-AN3* expression could be caused by intercellular diffusion of Mg-AN3 proteins, as in *A. thaliana* (Kawade et al., 2017). Therefore, the BM is equivalent to the leaf meristem in terms of the *AN3* expression patterns

Expression of Mg-AN3 in the GM

In *A. thaliana* and *O. sativa*, *AN3* is not expressed in the SAM (Horiguchi et al., 2011; Shimano et al., 2018). In maize, *AN3* is expressed from the bottom to the center of the SAM but not in the tip (Zhang et al., 2018). In this study, an *AN3* ortholog in *M. glabra* was expressed not only in the BM but also in the GM, together with an *STM* ortholog. I confirmed that the expression area of these genes was almost completely overlapped. It may suggest that the GM has a leaf-meristem-like as well as a SAM-like nature, which may explain the fuzzy plant-body system of one-leaf plants. Moreover, Ishikawa et al. (2017) reported that *Mg-AS1* and *Mg-STM* expression is not mutually exclusive, which suggests that *Mg-STM* and *Mg-AS1* are co-expressed in the BM or GM of *M. glabra*. In the present study, I found that *Mg-STM* is not expressed in the BM in WMISH, but Ishikawa et al. (2017) reported that *Mg-STM* was expressed in tissue defined as the BM in their study. This suggests that tissue defined as the BM included a portion of the GM in their study. Nevertheless, *Mg-AS1* expression was detected in the tissue collected as the GM, suggesting that both *Mg-AS1* and *Mg-STM* are expressed in the GM. The genes that maintain SAM function are believed to repress genes that promote differentiation. For example, in model plants with simple leaves, the SAM-maintaining *STM* suppresses *AS1* (Byrne et al., 2000, 2002) to maintain an undifferentiated SAM. In addition, the SAM

stem cell niche gene *WUS* represses genes that promote differentiation, such as KANADI1 (Yadav et al., 2013). Thus, the suppression of genes that promote differentiation by genes that maintain the SAM might be impaired in vegetative-stage *Monophyllaea*. This suppression might explain the expression of *Mg-AN3* in the GM. In summary, *Mg-AN3* expression in the GM suggests that it has a leaf-like, as well as a SAM-like, nature.

Figures and tables

Table 2. （5 年以内に刊行のため非公開）

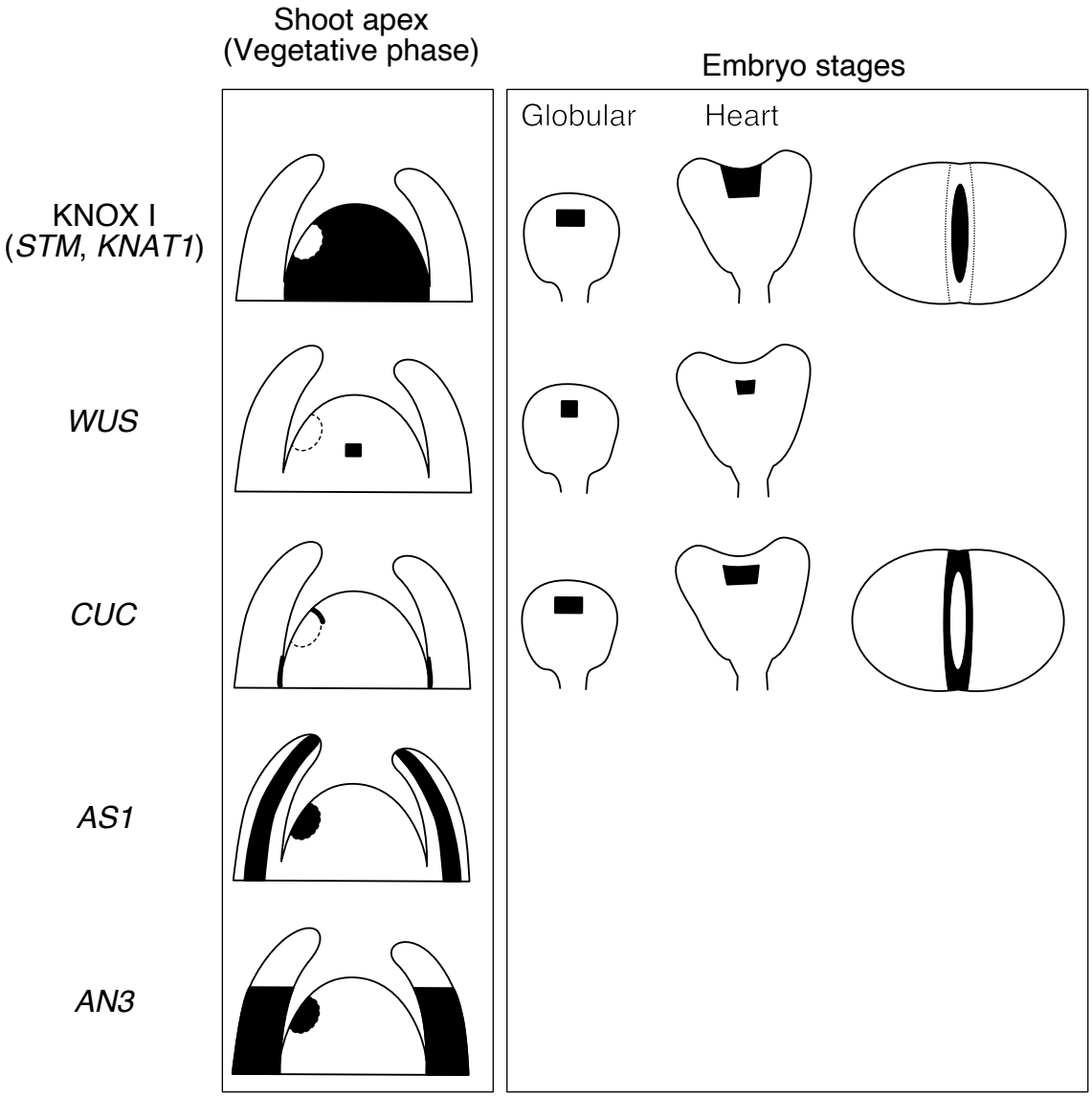

Figure 10. Expression patterns of key genes for meristem activity in *A. thaliana.*
Expression patterns of genes important for meristem formation and functions in *A. thaliana* at a shoot apex in a postembryonic stage and embryonic stages are colored with black color. The broken line in the shoot apex shows an emerging leaf primordium. The figures of the shoot apex and embryonic stages are modified from Tsukaya (2013) and Capron et al. (2009), respectively.

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Table 3. （5 年以内に刊行のため非公開）

Table 4. （5 年以内に刊行のため非公開）

Table 5. Database used for phylogenetic analysis

Species	Database	Version	URL
Amborella trichopoda	Phytozome	1.0	https:// phytozome.jgi.doe.gov/pz/portal.htm
Oryza sativa	Phytozome	7.0	https:// phytozome.jgi.doe.gov/pz/portal.htm
Solanum lycopersicum	Phytozome	iTAG 2.4	https:// phytozome.jgi.doe.gov/pz/portal.htm
Zea mays	Phytozome	5.0	https:// phytozome.jgi.doe.gov/pz/portal.htm
Antirrhinum majus	Snapdragon Genome Database	3.0	http://bioinfo.sibs.ac.cn/Am
Arabidopsis thaliana	TAIR	10.0	https://www.arabidopsis.org/

Table 6. （5 年以内に刊行のため非公開）

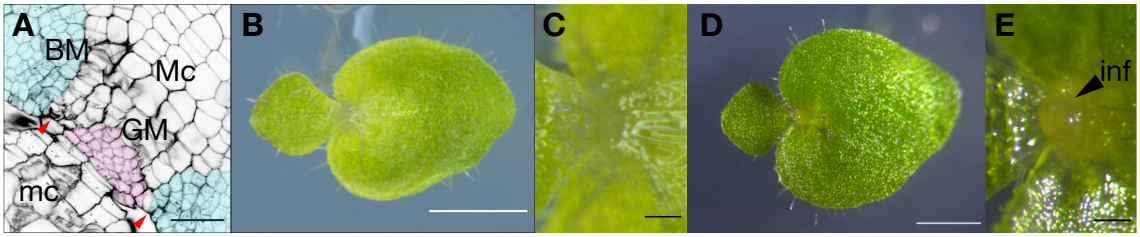

Figure 12. Position of meristems in anisocotylous-stage *M. glabra*.

(A) Paradermal view confocal micrograph of the tissue structure in an anisocotyledonous individual stained with calcofluor white. The upper right leaf is the macrocotyledon, and the lower-left leaf is the microcotyledon. The positions of the GM and the BM are colored pink and pale blue, respectively with an image processing software. The red arrowheads show the position where changes in contour in the macrocotyledon were observed.

(B) An anisocotyledonous-stage (17 DAS) individual in the vegetative phase grown under continuous light.

(C) The basal part of the macrocotyledon of the individual in (B).

(D) An anisocotyledonous-stage (32 DAS) individual in the reproductive phase grown under short-day conditions.

(E) The basal part of the macrocotyledon of the individual in (D). Black arrowhead, bulging inflorescence produced from the basal part of the macrocotyledon.

BM, basal meristem; GM, groove meristem; inf, inflorescence; mc, microcotyledon; Mc, macrocotyledon. Bar = 50 μm in (A), 1 mm in (B, D), 100 μm in (C, E).

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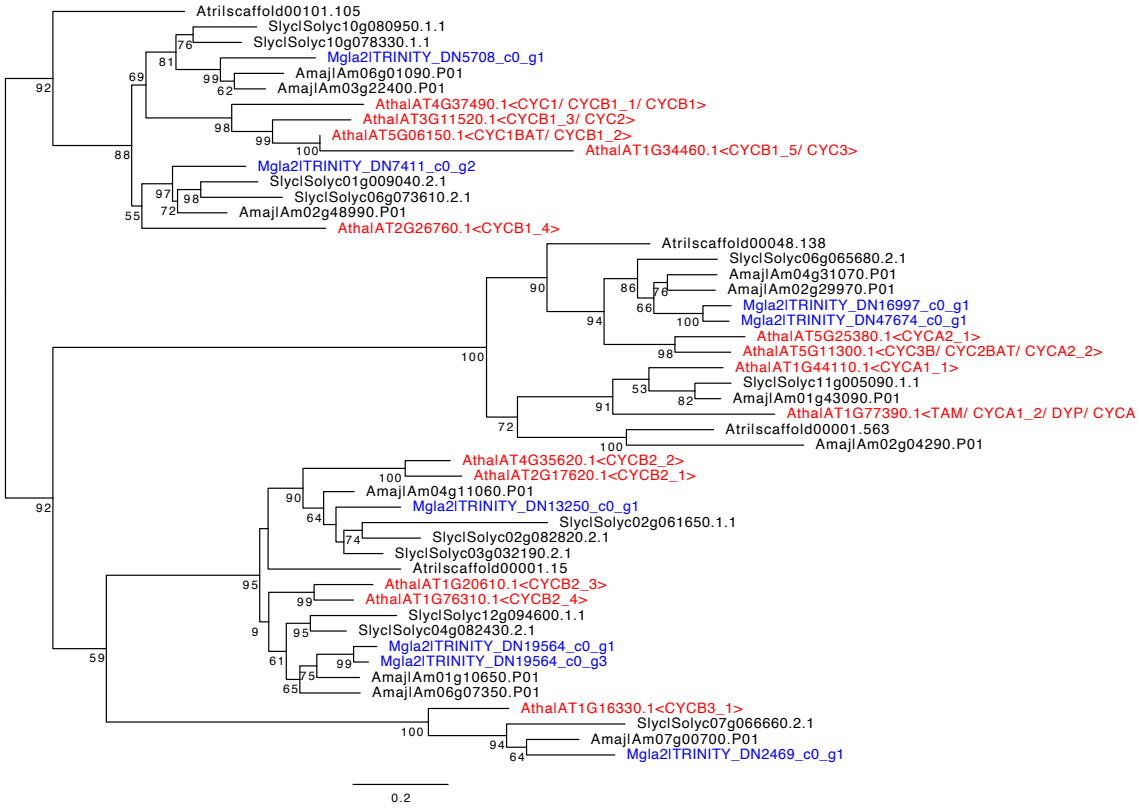

Figure 16. Phylogenetic analysis to find a *CYCB1* ortholog in *M. glabra*.
Maxim likelihood tree of amino acid sequences. Bootstrap values > 50 are shown at the branches. Amaj, *A. majus*; Atha, *A. thaliana*; *Atri, Amborella trichopoda*; Slyc, *S. lycopersicum*. Genes from *A. thaliana* and *M. glabra* are highlighted by red and blue, respectively.

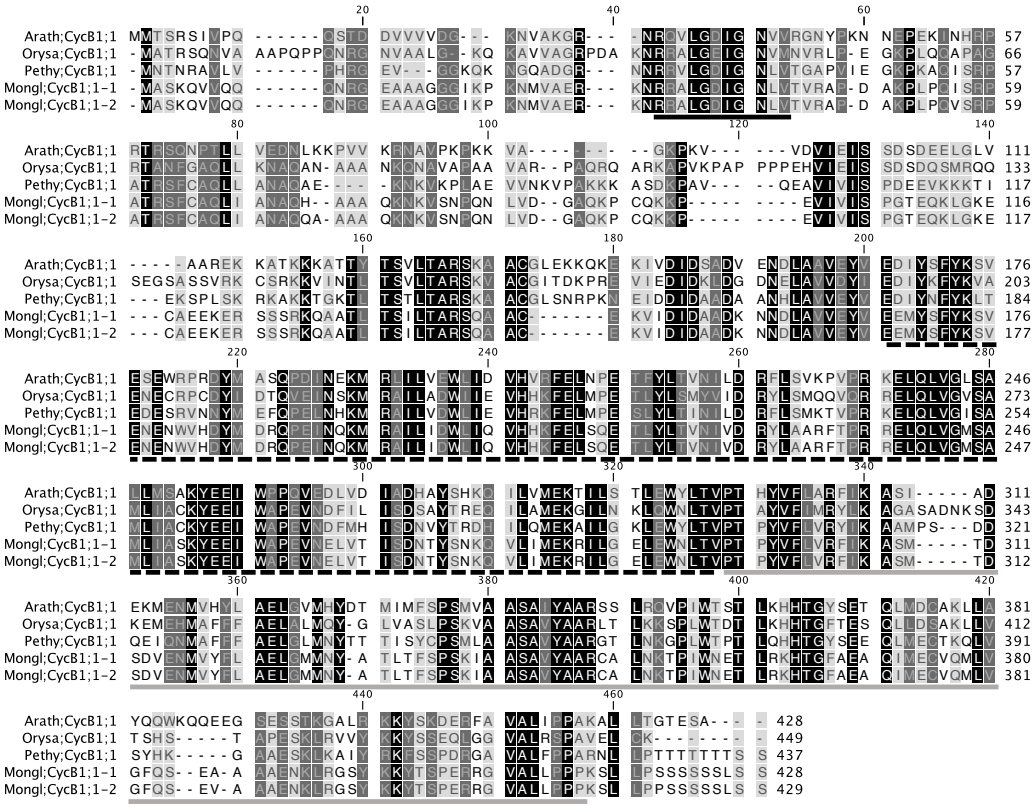

Figure 17. Alignment of amino acid sequences among *CYCB1* orthologs.

Alignment of amino acid sequence of CYCB1 orthologs; dashes indicate gaps. The destruction box, cyclin C-terminal domain, and cyclin N-terminal domain are indicated by black, black hatched, and gray underlining, respectively. The darker background color indicates an amino acid conserved among the five sequences. Arath, *A. thaliana*; Orysa, *O, sativa*; Pethy, *P. hybrida*; Mongl, *M. glabra*.

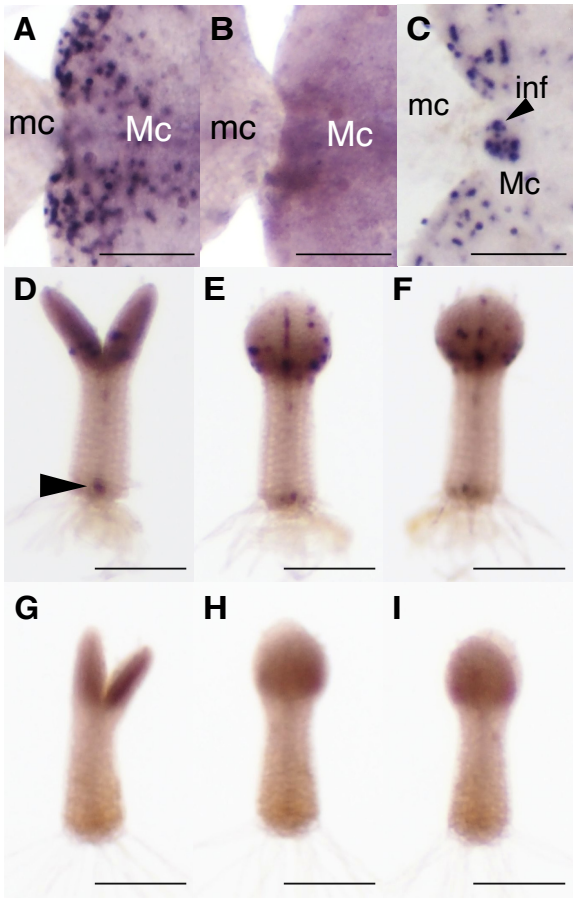

Figure 18. Applicability of the established protocol of the WMISH for *M. glabra* in various stages confirmed by *Mg-CYCB1* probe.

(A–B) The proximal part of the macro- and microcotyledon in an anisocotylous individual (17 DAS). Antisense probe (A). Sense probe (B).

(C) The proximal part of the macro- and microcotyledon at the reproductive stage (38 DAS).

(D–I) Whole plants at 7 DAS in isocotylous stage. Antisense probe (D–F). The black arrowhead indicates signals in the distal part of the petiolode. Sense probe (G–I). Frontal view (D, G). Side view (E–F, H–I).

inf, inflorescence; mc, microcotyledon; Mc, macrocotyledon. Bar = 200 μm.

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Figure 21. Alignment of nucleotide sequences of *Mg-STM-B* obtained from RNA-seq data and a previous study.

TRINITY_DN6864_c0_g1 and TRINITY_DN6864_c0_g3_complementary are obtained from RNA-seq data. *Mg-STM-B-1* and *Mg-STM-B-2* are obtained from gene cloning.

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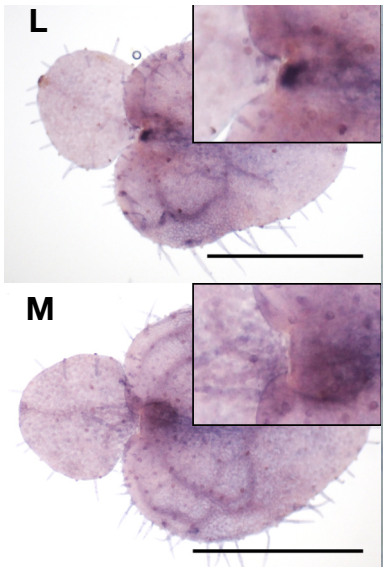

Figure 27. Expression patterns of *Mg-STM-B* investigated with WMISH.
The same individuals are in the panels with the same alphabet.
（5 年以内に刊行のため非公開）(L–O) Individuals in anisocotylous stage in the vegetative phase. Insets in (L) and (M) are the magnified view of the basal part of the macrocotyledons. The longitudinal section along the midrib （5 年以内に刊行のため非公開）
1 mm in (L–M)

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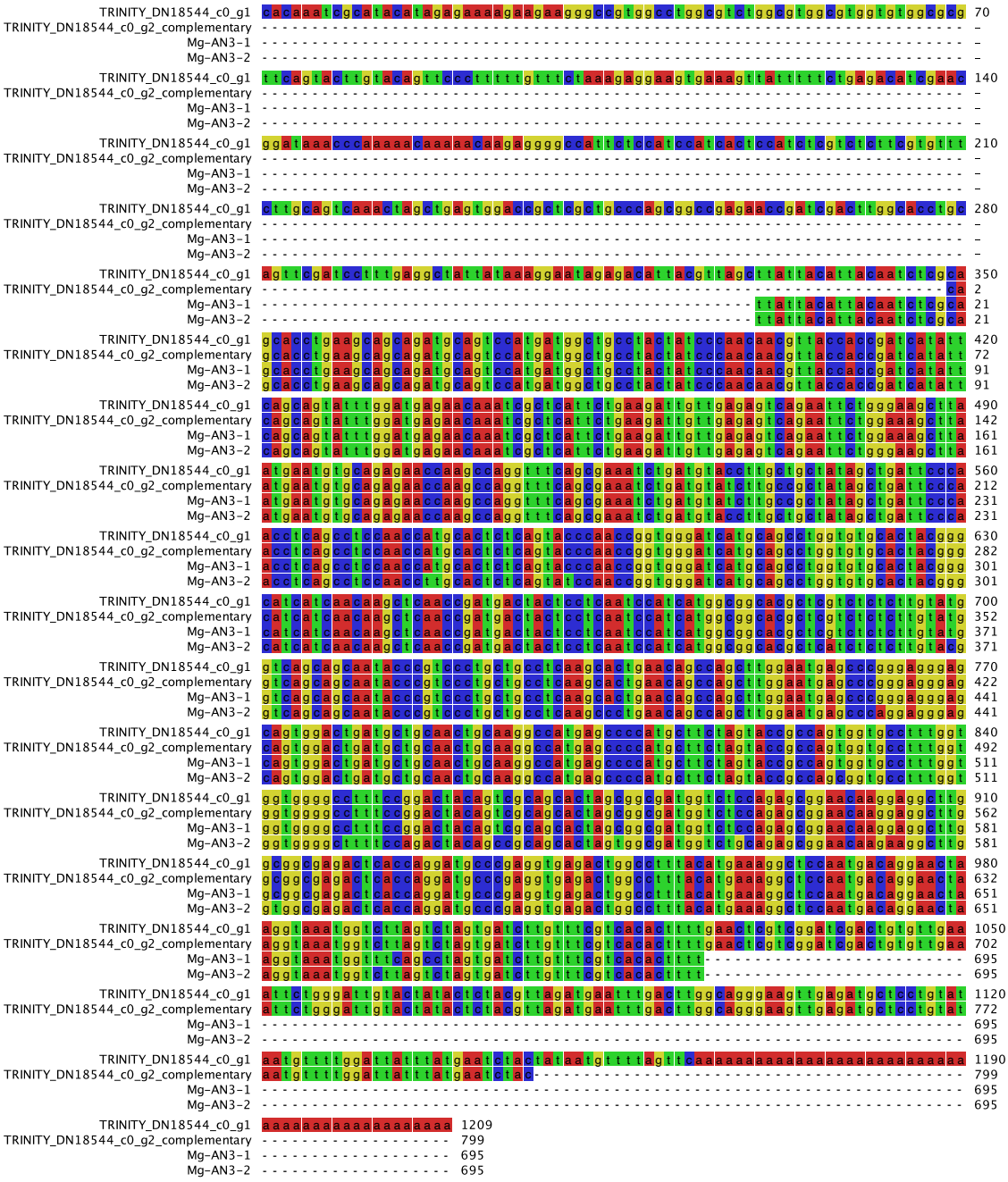
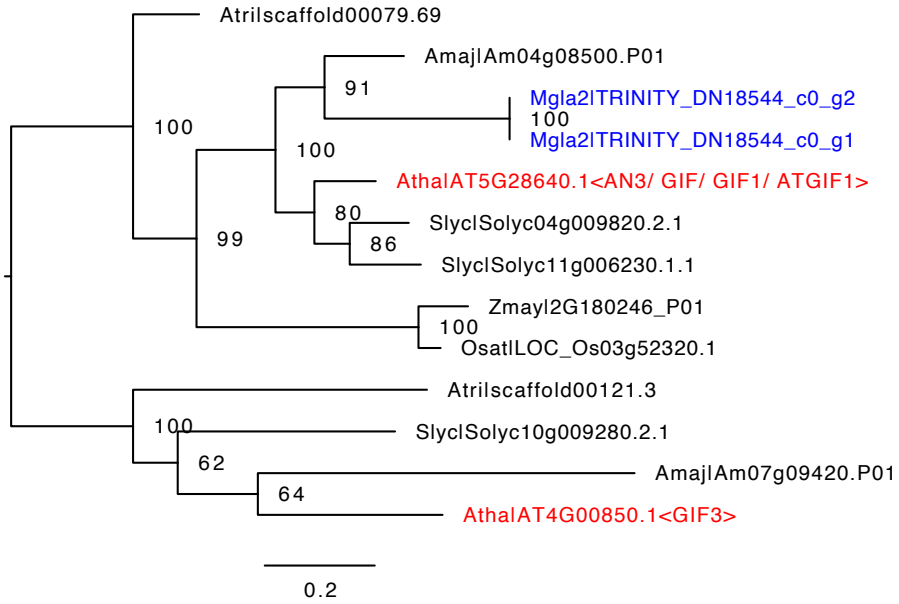


Figure 34. Alignment of nucleotide sequences of *Mg-AN3* obtained from RNA-seq data and cDNA cloning.

TRINITY_DN18544_c0_g1 and TRINITY_DN18544_c0_g2_complementary are sequences obtained from the RNA-seq data. *Mg-AN3-1* and *Mg-AN3-2* are sequences obtained from the gene cloning.

A



B

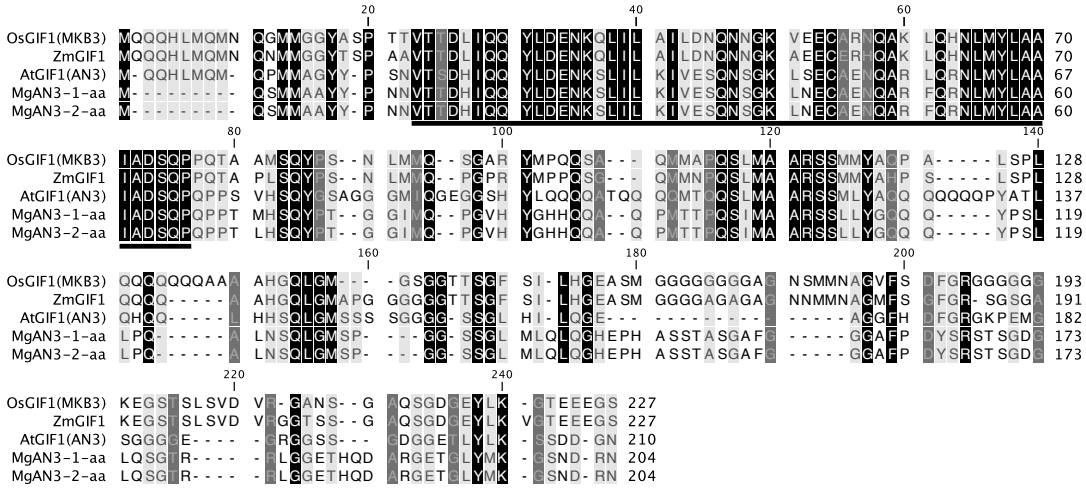


Figure 35. Phylogenetic analysis to find AN3 orthologs in *M. glabra* and the alignment of amino acid sequences among AN3 orthologs.

(A) A maximum likelihood tree of amino acid sequences of GIF genes. Bootstrap values > 50 are shown at the branches. Amaj, *A. majus*; Atri, *A. trichopoda*; Atha, *A. thaliana*; Slyc, *S. lycopersicum*, Mgla, *M. glabra*, Osat, *O. sativa,* and Zmay, *Z. mays*. Genes from *A. thaliana* and *M. glabra* are highlighted by red and blue, respectively.

(B) Amino acid sequence alignment of the AN3 homologs from *O. sativa* (MKB3), Z. mays (ZmGIF1), *A. thaliana* (AN3), and *M. glabra* (Mg-AN3-1, Mg-AN3-2). SNH domain is underlined with a black bold line.

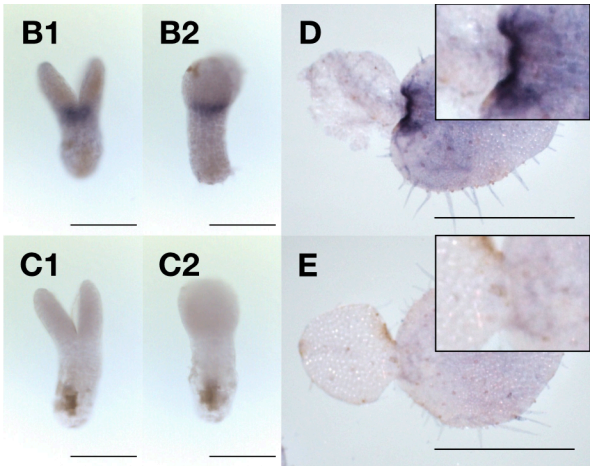


Figure 36. Expression pattern of *Mg-AN3* investigated with RNA-seq and WMISH.
The same individuals are in the panel with the same alphabet.
（5 年以内に刊行のため非公開）(B–F) Expression patterns of *Mg-AN3* investigated with WMISH. Samples hybridized with antisense probe (B, D, F) and sense probe (C, E).
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Bar = 100 μm in (B–C, F), 1 mm in (D, E)

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CHAPTER 4

General discussion and perspectives

The molecular background behind the unique development of one-leaf plants in *Monophyllaea* had not been revealed until the present study nearly at all in part because of limited applicable experimental systems. In the present study, I established some bases for the molecular studies on *M. glabra*: I obtained *de novo* assembled transcriptome data and RNA-seq data from each tissue; （5 年以内に刊行のため非公開）

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