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Determination of the unequal fate of cotyledons of a one-leaf plant, *Monophyllaea*

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SUMMARY

One-leaf plants, belonging to the family Gesneriaceae, were described for the first time more than 150 years ago. One such unusual plant, *Monophyllaea*, has only one leaf at maturity. Only one of the two cotyledons grows continuously, without the formation of true leaves, and this feature, known as anisocotyledonous development, has been repeatedly mentioned in textbooks of plant morphology. However, the mechanism for the determination of the one-leaf phenotype remains to be ascertained.

In this study, meristematic regions were identified, by monitoring DNA synthesis, at the base of both cotyledons just after germination, while no such regions were found in the shoot apex. Surgical experiments with seedlings and analysis of the anisocotyledonous development revealed that the fate of the cotyledons is determined during their growth. Anisocotyledonous development seems to be the result of competition between the two cotyledons. The mechanism that governs the development of the shoot in the genus *Monophyllaea* is discussed in relation to apical dominance, which is the common mechanism that regulates shoot development in many plants.

Key words: anisocotyledonous development, competitive organogenesis, leaf, meristem, *Monophyllaea*, suppression

INTRODUCTION

Monophyllaea was first described in 1840 as an unusual genus of one-leaf plants (Brown, 1840). As the Latin name indicates, each plant has only one leaf at maturity (Fig. 1F). The single, large leaf (more than 30 cm in length in some species) is derived from one of the two cotyledons (Chifflot, 1909; Oehlkers, 1923; Hill, 1938; Jong and Burtt, 1975). One of the cotyledons continues to grow while the other does not. No new organs are formed in the shoot after germination until the appearance of the inflorescences (Weber, 1975, 1976a,b). Thus, this plant lacks true leaves. The unequal development of two cotyledons is known as anisocotyledonous development. This feature is common to all known one-leaf species in the family Gesneriaceae (Hill, 1938), which includes more than 30 species in the genus *Monophyllaea* (Burtt, 1978) and several species in the genus *Streptocarpus*. Thus, special terms have been created for the description of the unique organs and tissue. 'Phyllomorph' (Jong and Burtt, 1975) is the name given to a unit of mixed stem-leaf nature in one-leaf plants (including species in the genera *Streptocarpus* and *Monophyllaea*). A cotyledonary phyllomorph is composed of a large cotyledon and a 'petiolode', which is the petiole-like organ of the large, continuously growing cotyledon. It seems likely that the phyllomorph is maintained by the activity of three kinds of meristem (Jong and Burtt, 1975), namely, the 'basal meristem', which is involved in the continued growth of the lamina; the 'petiolode meristem', which produces a long 'petiolode'; and a 'groove meristem', which is essential for the differentiation of each inflorescence meristem. As the requirement for these individual terms suggests, the unusual development of *Monophyllaea* involves many issues of interest to plant morphologists (e.g., Wardlow, 1952; Bell, 1991). However, no details of the determination of the unique one-leaf phenotype of *Monophyllaea* are understood. In this study, re-examination of surgical experiments with seedlings, which were attempted previously by Oehlkers (1923), revealed for the first time that the fate of each cotyledon is determined, after germination, by competition.

MATERIALS AND METHODS

Seeds of *Monophyllaea horsfieldii* R. Br., collected in Malaysia, were sown on rockwool and plants were grown at 23°C under continuous white light as described by Tsukaya et al. (1994). Seeds were storaged at room temperature under dry conditions, without any significant loss of the capacity for germination (approx. >95%). For cultivation on plates, seeds were sterilized in a solution of NaClO (Tsukaya et al., 1991) and sown on the medium described by Okada and Shimura (1992). Microscopic observations were made as described previously, with an optic microscope (Leica DAS Mikroskop DMR; Leica, Wetzlar, FRG) and a scanning electron microscope (JMS-820S; JEOL Ltd., Tokyo, Japan; Tsukaya et al., 1993; Tsuge et al., 1996). For identification of cell nuclei in whole-mounted seedlings, fixed samples were stained with 1 μg/ml 4′,6′-diamino-3-phenylindole (DAPI) after washing with 50 mM sodium phosphate buffer (pH 7.0). Histology of seedlings was examined after fixation with FAA solution, as described previously by Tsukaya et al. (1993) and embedding in Technovit 7100 resin (Kulzer & Co. GmbH, Wehrheim, FRG).

Incorporation of 5-bromodeoxyuridine (BrdU) into DNA was monitored after floating seedlings on a solution of 1 mM BrdU and 100 μM 5-fluoro-5′-deoxyuridine for 13 hours at 23°C. Fixation and embedding of samples in Technovit 7100 resin were performed as described by Fujie et al. (1994). The samples, embedded in resin, were sectioned at 1.2 μm thickness with glass knives. For detection of cells that had incorporated BrdU into their DNA, samples were treated with a BrdU-specific mouse monoclonal antibody (Becton Dickinson Immunochemistry Systems, San Jose, CA, USA) and sheep antibodies against mouse immunoglobulin, conjugated with fluorescein isothiocyanate (Amersham Japan Co. Ltd., Tokyo, Japan) as described elsewhere (Fujie et al., 1994).

For surgical experiments, one of the cotyledons was cut off with a razor blade at the base, at the stage indicated, and cultivation was continued. As a control to determine whether the death of seedlings could be due to damage resulting from the surgical treatment, whole seedlings were also grown on plates under sterile culture conditions.

For culture of segments of leaves in vitro, explants were cut into segments of approx. 1 cm in length and 1 cm in width with a razor blade. Explants were then put on MS101 medium (pH 6.3; Tsukaya et al., 1991) which was composed of Murashige-Skoog's inorganic salts supplemented with 1 mg/l benzyladenine as a cytokinin, 0.1 mg/l α-naphthyl acetic acid as an auxin, 2% (w/v) sucrose, 3 mg/l thiamine-HCl, 5 mg/l nicotinic acid, 0.5 mg/l pyridoxine HCl, and 0.2 % Gellan gum (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Cultivation was carried out at 23°C under continuous white light (at approx. 65 $\mu E/m^2/s$) as described by Tsukaya et al. (1994).

RESULTS

The germination of *Monophyllaea horsfieldii* R. Br. was analyzed first. Embryos of *Monophyllaea* in mature seeds contained two cotyledons of equal size without a shoot apical meristem (Fig. 1A). Germinated seedlings also had two cotyledons of equal size (Fig. 1B,C). Seedlings lacked a shoot apical meristem (Fig. 1C). However, a meristem-like cluster of small cells, including mitotic cells, was located at the base of the each half-blade of each cotyledon soon after germination (Fig. 1B). Since clusters of small cells are not necessarily indicative of mitotic tissues in leaf primordia (Dubuc-Lebreaux and Sattler, 1980), the meristematic activity in the basal regions of the cotyledons was examined by monitoring the incorporation of BrdU into DNA. Evidence of DNA synthesis was detected only at the base of each cotyledon (Fig. 1D). By contrast, no cells at the S phase of the cell cycle were detected at the shoot apex (Fig. 1E).

Next, the differential growth of the two cotyledons was analyzed during plant development. After germination, the meristematic regions disappeared from one of the cotyledons

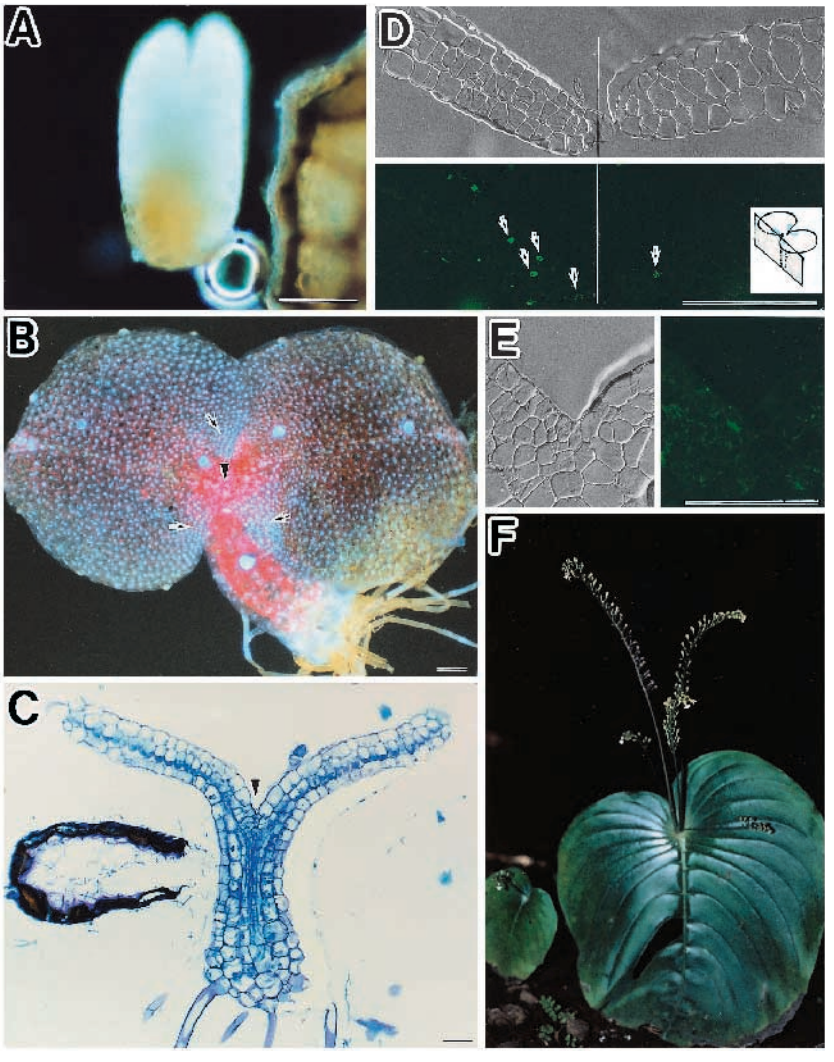

Fig. 1. Germination of seedlings of *Monophyllaea*. (A) A mature embryo of *Monophyllaea horsfieldii*. Bar, 100 μm. (B) A young seedling just after germination. Note the absence of meristematic cells in the apical region of the embryo (indicated by an arrowhead). Arrows indicate meristematic regions at the base of the two cotyledons. Nuclei were stained with DAPI. Bar, 100 μm. (C) Longitudinal section of a young seedling just after germination (see B). An arrowhead indicates the apical region, with no evidence of meristematic cells. Bar: 100 μm. (D,E) Detection of DNA-synthesizing cells that had incorporated BrdU, at the base of the two cotyledons of a seedling just after germination. Nuclei emitting green fluorescent signals indicate cells that had incorporated BrdU at the S phase of the cell cycle. Arrows indicate nuclei with positive signals. Bar, 100 μm. (D) Longitudinal section through the base of the two cotyledons. The upper photograph is a Nomarski image and the lower photograph was observed by a fluorescence microscopy. A vertical bar indicates the position of the shoot axis of the seedling (hypocotyl). The diagram shows the plane of the section in a seedling. A vertical dotted bar in the diagram indicates the position of the hypocotyl of the seedling. (E) Longitudinal section at the center of the seedling. The left photograph is a Nomarski image and right photograph is a fluorescence micrograph. (F) An adult specimen of *Monophyllaea horsfieldii*, with a single large leaf with inflorescences at its base. The photograph was taken in the plant's native habitat near Ipoh, Malaysia (Dec. 1995).

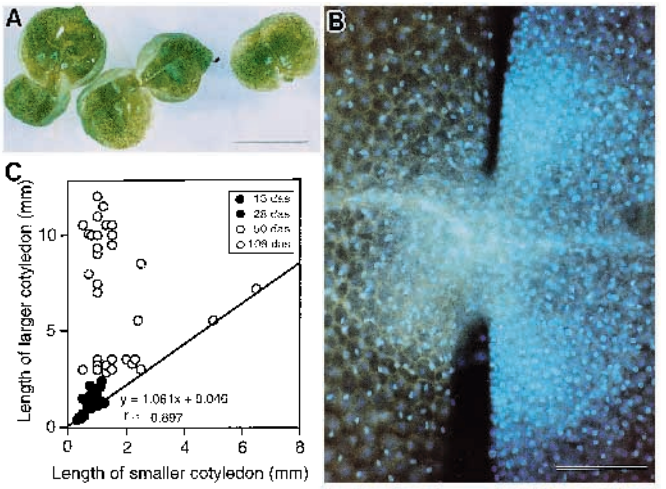

Fig. 2. Anisocotyledonous growth of *Monophyllaea* seedlings. (A) Variations in anisocotyledonous growth. Seedlings that had been cultivated on an agar plate were photographed 39 days after sowing. Bar, 5 mm. (B) Magnified view of the apical region of a seedling 15 days after sowing, showing the unequal distribution of meristematic cells in the two cotyledons. Note the disappearance of mitotic cells from the base of the left cotyledon. Bar, 100 μm. (C) Lengths of the cotyledons were measured, and the length of the smaller one was plotted against that of the larger one for each seedling. Note the equal sizes of the cotyledons soon after germination (13 days after sowing, closed circles). The relationship between the length of smaller cotyledon (x) and that of larger cotyledon (y), at this stage, is represented by the equation shown in the figure, where r is the correlation coefficient. Note also the varied anisocotyledonous growth. Ages of seedlings examined (das, days after sowing) are indicated by different symbols as shown in the Figure.

(Fig. 2B) and anisocotyledonous development subsequently became apparent (Fig. 2A,C). The pattern of anisocotyledonous development varied significantly among individual plants (Fig. 2A,C), as mentioned previously by Oehlkers (1923), suggesting that such development might not be strictly determined in an 'all or none' manner. A small number of plants even had cotyledons of equal size 109 days after sowing (Fig. 2A,C).

How is the unequal development of the two cotyledons determined? Ninety years ago, Chifflot (1909) noted that seedlings of *M. horsfieldii* occasionally developed a 'secondary cotyledon' after the larger cotyledon had been removed. In the present study, a re-examination of this phenomenon was made by surgical experiments at specific stages of development. When one of the two cotyledons was cut off soon after germination but before anisocotyledonous growth had become apparent, all the remaining cotyledons continued to develop (Table 1; Fig. 3B). This result implies that both cotyledons had the potential to grow continuously at a stage soon after germination. However, when one of the cotyledons was removed after a difference had become apparent in the sizes of the two cotyledons, different results were obtained. Seedlings from which smaller cotyledons had been removed continued to grow (Table 1; Fig. 3C). However, seedlings from which larger cotyledons had been removed retained a small cotyledon in many cases (Table 1; Fig. 3D). This result indicated that growth of smaller cotyledons had already been irreversibly interrupted. Oehlkers (1923) reported that some small cotyledons of seedlings, from which larger cotyledons had been surgically removed, continued to grow. However, in the present study, the percentage of determined cotyledons increased gradually with time after sowing (Table 1). Thus, the fate of the cotyledons, namely, the anisocotyledonous development of the seedling, seemed to be determined gradually after germination. It is noteworthy that, in many cases, a new leaf primordium differentiated irregularly at the base of the remaining cotyledon and/or on the hypocotyl (Table 2; Fig. 3D-G). This phenomenon was more frequently observed after the larger cotyledon had been removed than after the smaller one had been removed (Table 2). A similar phenomenon, with differentiation of secondary cotyledons, was also observed in a few seedlings without any surgical intervention (2 of 50 seedlings, Fig. 4).

To examine the role of the gravity in the determination of anisocotylly, some seeds were sown on vertically oriented agar plates (Okada and Shimura 1992). However, the direction of the force of gravity seemed to have only a weak influence on

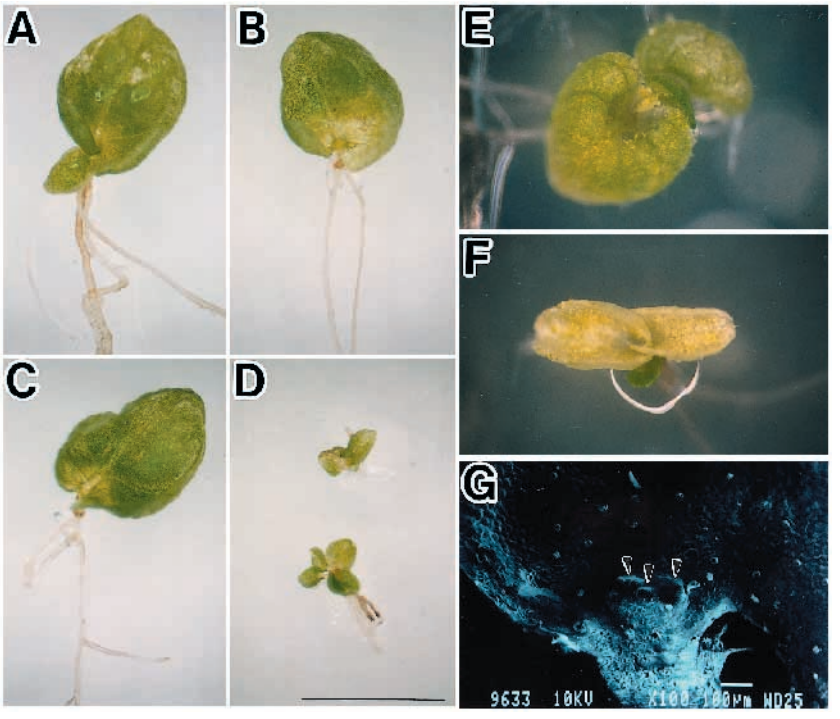

Fig. 3. Growth of cotyledons after surgical treatment. (A-D) Seedlings of *Monophyllaea* that had been cultivated on agar plates for 45 days. (A) A control plant. (B) A seedling from which one of the cotyledons had been removed 14 days after sowing, when both cotyledons were of equal size. (C) A seedling from which the smaller cotyledon had been removed 24 days after sowing. (D) A seedling from which the larger cotyledon had been removed 24 days after sowing. Bar, 5 mm. (E,F) Magnified views of seedlings that produced secondary cotyledons after removal of the larger cotyledon. (G) Scanning electron micrograph of primordia of secondary cotyledons that appeared at the base of the remaining cotyledon after surgical treatment. Arrowheads show primordia of secondary cotyledons. Bar, 100 μm.

Table 1A. The fate of the remaining cotyledons of seedlings that were grown on rockwool for 30 days after surgical treatment

	% of remaining cotyledons that continued to grow		
Day	Removal of 1 of 2*	Removal of larger	Removal of smaller
13	100.0 (24)	–	–
15	61.1 (18)	–	–
16	46.5 (43)	–	–
20	–	0.0 (13)	100 (13)
24	–	17.6 (17)	100 (8)
27	–	6.7 (15)	100 (10)

*Cotyledons were of equal size from day 13 to day 16.
Numbers in parentheses are the number of seedlings examined in each case.

Table 1B. The fate of the remaining cotyledons of seedlings that were grown on plates for 39 days after surgical treatment

	% of remaining cotyledons that continued to grow		
Day	Removal of 1 of 2*	Removal of larger	Removal of smaller
14	96.6 (29)	–	–
19	–	25.0 (20; 4 dead)	65.0 (20; 1 dead)
24	–	8.0 (25)	93.9 (33)

*Cotyledons were of equal size on day 14.
Numbers in parentheses are the numbers of seedlings examined in each case.

Table 2. The development of secondary cotyledons after surgical treatment of seedlings grown on plates for 39 days

	% plants with secondary cotyledons following surgery		
Day	Removal of 1 of 2*	Removal of larger	Removal of smaller
14	13.8 (29)	–	–
19	–	5.0 (20; 4 dead)	5.0 (20; 1 dead)
24	–	72.0 (25)	3.0 (33)

*Cotyledons were of equal size at 14 days.
Numbers in parentheses are the numbers of seedlings examined in each case.

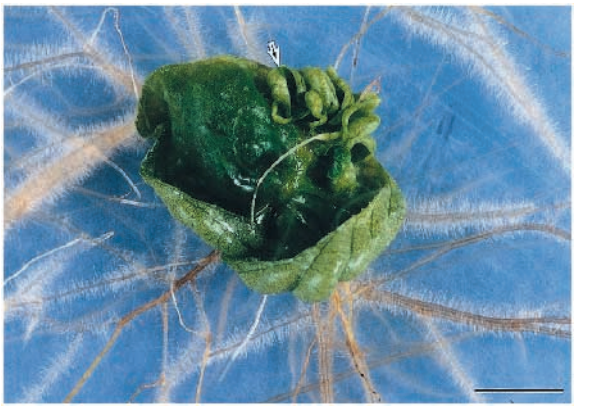

Fig. 4. Spontaneous occurrence of secondary cotyledons. At the base of the larger cotyledon, secondary cotyledons (one of them is indicated by an arrow) occurred spontaneously. Two of 50 seedlings exhibited this phenomenon 4 months after sowing, without any surgical treatment. Bar, 5 mm.

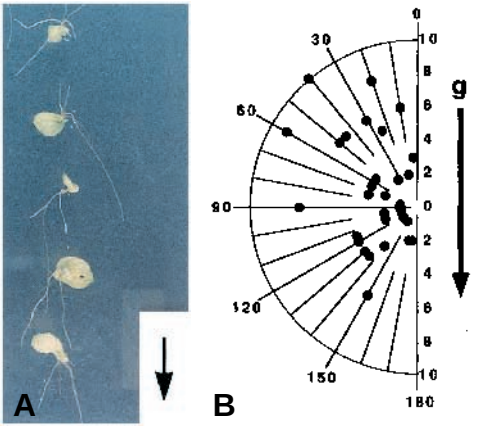

Fig. 5. Relationship between anisocotylly and the direction of the force of gravity. (A) Seedlings, 34 days after sowing that had been cultivated on a vertically oriented agar plate. The direction of the force of gravity is indicated by an arrow. (B) Summary of the directions in which the larger cotyledon elongated. The direction of the force of gravity is indicated by an arrow.

the gravitropism of hypocotyls, without any affect on the direction of the anisocotyledonous growth (Fig. 5).

As a first step towards and understanding of the roles of phytohormones in the regulation of leaf morphogenesis in *Monophyllaea*, segments of leaves were cultivated in vitro on medium supplemented with 1 mg/l benzyladenine as a cytokinin and 0.1 mg/l α-naphthyl acetic acid as an auxin. These conditions stimulate regeneration of shoots from explants of leaves of *Arabidopsis* (Tsukaya et al., 1991). As shown in Fig. 6A, explants differentiated many leaf-like structures, which resembled secondary cotyledons, on the surface of the explants. Scanning electron microscopy indicated that single secondary cotyledons seemed to occur independently of each other, without any shoot-like structures (Fig. 6B).

DISCUSSION

Apical dominance is the term used for the control exerted by the apex of the shoot over the outgrowth of lateral buds and it is observed in most higher plants (Cline, 1991). Apical dominance is maintained in a plant shoot only when the apical meristem of the shoot actively suppresses the growth of lateral meristem. When the apical meristem is damaged, competition occurs among the remaining lateral meristems, with the winner again establishing apical dominance. In such plants, competition and suppression control the development of shoots. From the present study, it appears that the phenomenon of anisocotyledonous development of the genus *Monophyllaea* is analogous to apical dominance, as will be discussed below.

In this study, several previously reported observations on one-leaf plants were confirmed in *Monophyllaea horsfieldii*, namely, the absence of a shoot apical meristem, the phenomenon of anisocotyledonous growth, and the presence of meristematic regions in the larger cotyledon. The locations of meristematic regions were demonstrated for the first time by direct monitoring of DNA synthesis. The following new findings were also made in this study. (1) Just after germination, the two cotyledons are completely identical morphologically. (2) Both cotyledons

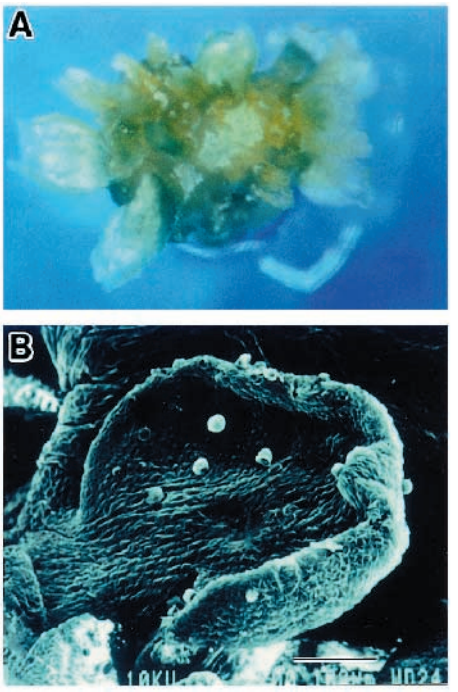

Fig. 6. Regeneration of secondary cotyledons on leaf segments in culture in vitro. (A) Occurrence of secondary cotyledons on the surface of a leaf segment. An explant after cultivation for two months on a plate of solidified medium is shown (see also Materials and Methods). (B) Scanning electron micrograph of a secondary cotyledon induced during culture in vitro. Note the single leaf-like organ without any structure, which resembles a shoot apical meristem at the base. Bar, 100 μm.

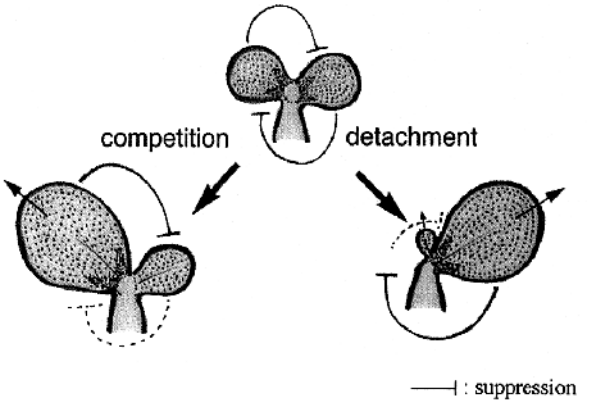

Fig. 7. Model of the mechanism responsible for the developmental control of anisocotylly in *Monophyllaea*. Areas with dense dots represent meristematic regions. Arrows on leaf blades indicate further growth. See text for details.

have meristematic regions, at the base of the blade, just after the germination. This conclusion strongly supports the hypothesis proposed by Oehlkers (1923) that the two cotyledons are physiologically equivalent. Oehlkers (1923) observed varied patterns of anisocotylly and performed some surgical experiments. Jong and Burtt (1975) suggested that, in *Streptocarpus fanniniae*, the meristematic region at the base of the larger cotyledon is the former embryonic meristem that has moved from its original position after the rapid growth of the larger cotyledon. However, this interpretation is not valid in the genus *Monophyllaea*. No embryonic meristem was recognized in mature embryos of *Monophyllaea*, and the meristematic regions at the base of the lamina were found only just after germination. Since anisocotyledonous growth is not necessarily accompanied by the absence of a shoot apical meristem in Gesneriaceae (as it is, for example, in species in the genera *Didymocarpus*, *Saintpaulia* and *Oreocharis*; Hill, 1938), absence of a shoot apical meristem can be considered to be independent of anisocotylly. (3) Anisocotyledonous growth is determined during growth of cotyledons and is irreversible. Prior to this study, the timing and mechanism of anisocotylly were unknown. Oehlkers (1923) suggested that the smaller cotyledons at later stages might still have ability to grow continuously. However, the present study strongly supports the irreversible determination of the fate of cotyledons at somewhat earlier stages. The difference between the conclusions might be due to the fact that Oehlkers succeeded in surgical experiments with only limited numbers of seedlings because of the high death rate of seedlings after surgical treatment. Oehlkers did not present quantitative results of his surgical experiments. In the present study, the combination of sterile culture with surgical treatment resulted a very low death ratio of treated seedlings. Thus, the surgical experiments in the present study showed for the first time that both cotyledons have equal potential for continuous growth just after germination. The present study proved for the first time that anisocotyledonous growth is irreversible. Moreover, the present study also showed for the first time that the timing of the choice of dominant cotyledon is not strictly programmed.

The surgical experiments suggested that the two cotyledons compete with one another. The determination of anisocotyledonous development seemed to be the result of competition between the two cotyledons for the following reasons: (1) Surgical detachment of one cotyledon at an early stage resulted in further growth of all of the remaining cotyledons. (2) Determination of anisocotyledonous growth is not strictly programmed (in some plants both cotyledons developed equally). Fig. 7 shows a proposed model of the developmental control of anisocotylly in *Monophyllaea*, as deduced from this study. Just after germination, both cotyledons are identical and each has the ability to grow continuously. Cotyledons compete with one another and one finally inhibits the further growth of the other, perhaps winning the competition as a result of the influence of some environmental factor. The model shown in Fig. 7, which suggests that one cotyledon inhibits the growth of the other cotyledon, is supported by the fact that seedlings with equal-sized cotyledons tended to grow more slowly than seedlings with typical anisocotyledonous development (data not shown). There remains the possibility that 'self-activation' of cotyledons might play a role in the regulation of anisocotyledonous growth. With respect to the roles of phytohormones in anisocotylly, Rosenblum and Basile (1984) studied the effects of phytohormones on anisocotyledonous growth of some species in the genus *Streptocarpus*. They showed that some hormones, when supplied exogenously, influenced the fate of the cotyledons and the apical meristems in *Streptocarpus*. Since the combination of a cytokinin and an auxin resulted in differentiation of secondary cotyledons on the surface of leaf segments (Fig. 6), phytohormones might be involved in the regulation of anisocotylly. However, the roles of endogenous

phytohormones in anisocotylly cannot be discussed on the basis of experiments with exogenously supplied phytohormones.

The present study showed that the direction of the force of gravity had no effect on the determination of anisocotyledonous development. Oehlkers (1923) suggested the possibility that growth of cotyledons above the horizontal line might be promoted. However, in the present study, no results clearly supported this hypothesis. Further analysis is needed to define the physiological nature of the competition between the two cotyledons in *Monophyllaea*.

'Competitive organogenesis' has not been reported in other organisms, but this phenomenon might be analogous to the apical dominance that is commonly observed during the regulation of development of plant shoot systems. When one of the cotyledons was cut off, a secondary cotyledon frequently appeared at the base of the remaining cotyledon and/or on the hypocotyl, as noted by Chifflot (1909). Although this phenomenon might be a secondary effect induced by wounding stress, the appearance of a secondary cotyledon from the remaining cotyledon and/or hypocotyl can also be explained by the model shown in Fig. 7 (right). When the two cotyledons inhibit the meristematic activity of the other part of shoot and then the inhibitory effect of one of the cotyledons is abruptly terminated, the meristematic activity of the remaining cotyledon and the potential meristems in the hypocotyl might be enhanced. The appearance of secondary cotyledons might be the result of such sudden release from inhibition. The model is supported by the tendency of secondary cotyledons to appear more frequently when the larger cotyledon had been detached than when the smaller cotyledon had been detached (Table 2; Fig. 3D). This explanation is also applicable to the spontaneous differentiation of secondary cotyledons at the base of larger cotyledons (Fig. 4).

The present results suggests that, although the one-leaf phenotype of *Monophyllaea* is unusual, the mechanism that governs anisocotylly might be based on a principal that is common to and typical of all plant shoots, namely, competition and suppression. Apical dominance, which governs shoot morphogenesis in plants, is a more common example of competition and suppression. The plant-specific developmental principal that is based on competition and suppression can be considered to represent 'competitive organogenesis'. Thus, the anisocotyledonous phenotype is an extreme example of competitive organogenesis. Future studies of *Monophyllaea* should provide new insights into plant morphogenesis. The apparent similarity between seedlings of *Monophyllaea* and the seedlings of mutants of *Arabidopsis thaliana* that lack a shoot apical meristem (Barton and Poethig, 1993; Long et al., 1996; Laux et al., 1996) is also of interest in terms of the molecular mechanisms required for the establishment and maintenance of meristematic regions in plants.

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