Annals of Botany **94**: 457–467, 2004
doi:10.1093/aob/mch160, available online at www.aob.oupjournals.org

Characterization of Anisocotylous Leaf Formation in *Streptocarpus wendlandii* (Gesneriaceae): Significance of Plant Growth Regulators

KANAE NISHII*, ASUKA KUWABARA and TOSHIYUKI NAGATA

Department of Biological Sciences, Graduate School of Science, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

Received: 26 February 2004 Returned for revision: 27 April 2004 Accepted: 26 May 2004 Published electronically: 30 July 2004

• *Background and aims* Unifoliate species of Gesneriaceae are unique, as they bear only one leaf throughout their life history. The development of this leaf (termed a macrocotyledon) derived from one of two cotyledons is intriguing. The other cotyledon does not develop further and is termed a microcotyledon. This process of unequal cotyledon development is termed anisocotyly. In this study the process of macrocotyeldon formation was studied and the effects of plant hormones on the macrocotyledon development were investigated.
• *Methods Streptocarpus wendlandii* was chosen as the main subject material, as it was found to be suitable for experimental studies in laboratory conditions. Morphological analyses were carried out with light and scanning electron microscopy. Plant hormones were applied exogenously.
• *Key results* The macrocotyledon of *S. wendlandii* is produced through cell division activity in the basal meristem of the enlarging cotyledon. The newly developed region in the macrocotyledon displayed distinct morphological changes, including the formation of long, needle-shaped trichomes. The newly formed region was surrounded by lateral veins. No such change was observed in the microcotyledon. Furthermore, it was shown that development of anisocotyly is suppressed by the application of cytokinin, resulting in the formation of two nearly equal-sized cotyledons. Both cotyledons displayed macrocotyledon characteristics. This observation in *S. wendlandii* was confirmed using *Monophyllaea glabra*, another unifoliate species in the same family.
• *Conclusions* It is proposed that developmental changes of the macrocotyledon have characteristics of a developmental phase-change, and cytokinins may be involved in its formation. These results are discussed in the light of current knowledge of phase-change transitions in plant vegetative development.

Key words: Cotyledon, cytokinin, Gesneriaceae, *Monophyllaea glabra*, phase-change, *Streptocarpus wendlandii*, trichome, unifoliate.

INTRODUCTION

Leaves develop from the shoot apical meristem (SAM) and then differentiate into mature forms. As leaf formation is repeated along the shoot axis, this morphogenetic unit of formation of the plant body is frequently referred to as a phytomer (Sylvester *et al*., 1996). This type of leaf formation can be applied to monocotyledonous and dicotyledonous plants. However, there are several species among the Gesneriaceae (certain South African sub-genera of *Streptocarpus* and more than 30 species in *Monophyllaea*) to which this rule cannot be applied (Burtt, 1978). These species only produce one leaf throughout their life history and they do not retain the SAM. The single leaf, called the macrocotyledon, is derived from the enlarging cotyledon (Möller and Cronk, 2001). Cells produced from the basal meristem of the macrocotyledon form the vegetative organ, and at maturation develop an inflorescence. These inflorescences are developed from a specific part of the macrocotyledon, the groove meristem. Based predominantly on observations of the rosulate species of *Streptocarpus*, Jong and Burtt (1975) proposed a 'phyllomorph' concept, in which the unique developmental patterns of these species originate from three meristematic regions named the basal, petiolode and groove meristem. Some anatomical analyses have been reported in which the existence of a basal meristem was deduced from observations of small epidermal cells in the basal region of the macrocotyledons of *Monophyllaea* (Imaichi *et al*., 2000) and *Streptocarpus* (Jong and Burtt, 1975; Imaichi *et al*., 2001). Tsukaya (1997) described an early stage in the development of anisocotyly in *Monophyllaea*, in which active DNA synthesis was detected by the incorporation of 5-bromodeoxyuridine (BrdU) into the basal region of the enlarging cotyledon. It appears that the formation of the macrocotyledon occurs as a result of a competitive interaction between the two cotyledons.

We are interested in elucidating how this type of unifoliate plant differs from other dicotyledonous plants in terms of plant developmental biology. In order to understand the differences in development between anisocotylous plants and others, it is important to determine what causes the differences in development in the two unequal cotyledons. When we conducted initial studies on several different unifoliate as well as rosulate species of *Streptocarpus*, it was found that unifoliate *Streptocarpus wendlandii* was, for various reasons, a suitable species, particularly because it was convenient for cultivation under laboratory conditions. When the extent of meristematic activity was examined, the newly developed region displayed morphologically distinct

* For correspondence. E-mail: ss37201@mail.ecc.u-tokyo.ac.jp

features compared to the other parts of the leaf. This difference was confirmed by finding active DNA synthesis in this region, and by observing subsequent cell divisions in the basal region of the enlarging cotyledon. These unique features had been overlooked in previous studies. In addition, as cytokinin was suspected to be a likely internal factor involved in inducing the growth of the macrocotyledon, these novel findings are discussed in relation to the unique development of these unifoliate species.

MATERIALS AND METHODS

Plant materials

Seeds of *S. wendlandii* were kindly supplied by Mr M. Aizawa of Kyoto Prefectural Botanical Gardens (Kyoto, Japan), whilst those of *S. molweniensis*, *S. cooperi* and *S. grandis* were kindly supplied by Mr T. Okuto of Hyogo Flower Park (Hyogo, Japan). After preliminary inspection, *S. wendlandii* was chosen as the main study species, since this species could be easily cultured under our laboratory conditions. For *Monophyllaea*, seeds of two species of *M. glabra* and *M. horsfieldii* were kindly supplied by Mr K. Hirai in the Botanical Gardens of the University of Tokyo. However, most of the experiments were conducted with *M. glabra*, which was collected by Mr Hirai at Srakaew Cave, Thailand. After seeds were sterilized with a 0·2 % sodium hypochlorite solution that contained 0·02 % Nonidet P-40 (NP40; Sigma Chemicals, Missouri, USA) for 2 min and washed with distilled water, they were sown on a culture medium consisting of 30 % strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962), solidified with 0·8 % agarose in 9 cm plastic Petri dishes. Plants were cultured in a growth chamber at 23 °C under a cycle of 18 h light (80 μmol m^{-2} s^{-1}) and 6 h dark.

Plant hormone treatments

For treatment with plant hormones, seedlings were transferred just after germination to a medium that contained appropriate hormones, and were subsequently incubated on these plates for 30 or 45 d from imbibition. Germination was defined as unfolded cotyledons, which were examined from 10 d to 15 d after imbibition. Control plants, which were germinated at the same time, were incubated on the MS medium without hormones. The following plant hormones were used: the cytokinins 6-benzylaminopurine (BAP), *t*-zeatin and kinetin, the auxins indole-3-acetic acid (IAA) and 2,4-dichlorophenoxyacetic acid (2,4-D), the gibberellin GA_3, and abscisic acid (ABA), and the brassinosteroid, brassinolide. 1-aminocyclopropane-1-carboxylic acid (ACC) was used as a precursor of ethylene biosynthesis. ACC was purchased from TCI-EP Ltd. (Tokyo, Japan), while the other chemicals were purchased from Wako Pure Chem. Ind. Ltd. (Osaka, Japan).

For BAP, more detailed examinations were carried out. Germinated seedlings of *S. wendlandii* were cultured on medium containing 10^{-6} M BAP, and the areas of the cotyledons were measured at 15, 20, 30 and 40 d after imbibition. To examine how long responsiveness of a microcotyledon to cytokinin was preserved, seedlings were incubated on MS medium containing 10^{-6} M BAP for 3 d at selected growth stages between the 19th and 33rd day, and were then transferred to a medium without BAP. Sizes of cotyledons were then measured at 55 d after imbibition.

Morphological analyses

To measure leaf area, leaves were fixed with FAA solution (5 % acetic acid, 45 % ethanol, 5 % formaldehyde, 45 % distilled water; volume ratios) and spread on glass Petri dishes. Photographic images were taken using a dissecting microscope SZX9 (Olympus Optical Industries Co., Tokyo, Japan) and the images were transferred to a computer. Leaf areas were measured using a graphics program, NIH image (Scion Co., Maryland, USA). The ratio of the leaf area of a macrocotyledon and a microcotyledon was calculated by dividing the leaf area of a larger cotyledon with that of the smaller cotyledon in a given seedling.

For observation of the leaf surface and vascular pattern, samples were fixed in FAA, and decolourized overnight using a mixture of ethanol and acetic acid (4 : 1). The decolourized tissue was hydrated by passage through an ethanol series and then immersed overnight in distilled water. Hydrated tissue was further cleared by immersion in an aqueous solution consisting of 200 g chloral hydrate and 20 g glycerol in 50 mL of distilled water for 3 h. After samples were embedded in Technovit 7100 resin (Heraeus Kulzer GmbH & Co., KG, Wehrheim, Germany), sections were prepared as described in Kuwabara *et al*. (2001). After staining with Toluidine Blue, samples were observed under a microscope (BX51, Olympus). When trichome numbers were determined, the areas of cleared cotyledons were measured and therefore at this stage it was known which was the larger. After the total numbers of three types of trichomes were counted (see Results), numbers of each type per unit area were determined. As for the macrocotyledon, the number of trichomes in the distal and basal parts were measured; distal and basal parts were defined here as being partitioned at the position where lateral veins bifurcated from a midvein surrounded the basal parts.

For observation under a scanning electron microscope, the fixed samples were dehydrated using an ethanol series and immersed in isoamyl acetate. Following drying and ion-sputtering, samples were observed using a scanning electron microscope S-2250N (Hitachi), at the University Museum of the University of Tokyo.

Assessment of meristematic activity

Meristematic activity was assessed with the incorporation of BrdU into the nucleus, which was confirmed by staining with an antibody against BrdU. After 1 mM BrdU and 100 μM 5-fluoro-5′-deoxyuridine were added to the seedling 30 d after imbibition, seedlings were left for 16 h. Then fixation and embedding of samples in Technovit 7100 resin were performed as described in Fujie *et al*. (1994). The samples were sectioned to 3 μm and stained with a mouse monoclonal antibody against BrdU (Amersham Pharmacia Biotech Co.,

Tokyo, Japan). Subsequently, the secondary antibody from an anti-mouse IgG antibody from sheep (Sigma Chem. Co., Missouri, USA) was applied to identify the location of BrdU, as described in Tsukaya (1997). To further identity the exact location of DNA synthesis in the cotyledons, resin-embedded tissues were sectioned at 10 μm intervals along the long axis and stained with the antibody as above.

To identify the dividing cells of the macrocotyledons, decolourized and hydrated material was immersed in 0·15 M phosphate buffer (pH 8·6), followed by immersion in a solution of 0·005 % Aniline Blue in 0·15 M phosphate buffer (pH 8·6) for 2 h. Aniline Blue staining was conducted as described by Hayashi *et al*. (1986). Samples were observed under a fluorescence microscope (BX 51, Olympus) using U-excitation.

Culture of leaf explants

Eighty days after imbibition, a microcotyledon and a macrocoyledon were separated from seedlings. The latter was sectioned into the distal and basal regions using a razor blade. The resultant pieces of leaves were cultured on MS medium with or without 10^{-6} M BAP for 20 d. Morphological features of these segments were examined after culture under a microscope.

RESULTS

The emergence of anisocotyly in S. wendlandii

Anisocotyly could not be detected in *S. wendlandii* until 20 d after imbibition. A significant difference in cotyledon size was observed 30 d after imbibition (Fig. 1). When 30-d-old cotyledons were examined under a microscope, cell numbers along the long axis of leaves in the enlarging cotyledon (41·00 ± 6·19 cells per a leaf, mean ± s.d., $n = 10$) were twice those seen in the other cotyledon (20·43 ± 2·57). The increase in cell number in the enlarging cotyledon appeared to be due to newly added cells in the basal region of the enlarging cotyledon. To examine this possibility, incorporation of BrdU into the nuclei of cotyledons was measured. When the cotyledons were cut transversely along the long axis of the leaf, incorporation of BrdU was detected only in the nuclei of cells between the base of the enlarging cotyledon and the lateral veins (0–500 μm) (Fig. 2A). The distribution of cell division in the enlarging cotyledon was demonstrated by staining leaves with Aniline Blue, which stained the septum walls in divided cells (Fig. 2B, C). As shown in Fig. 2D, the newly formed cells were distributed in a broad area of the basal region of the enlarging cotyledon, while in the other cotyledon no such staining was detected. Thus, the emergence of the enlarging cotyledon was due to active cell division in the basal region, resulting in the enlargement of the macrocotyledon. In fact, cell size in the basal region was smaller than that in other regions. The size of epidermal cells in the basal region of the macrocotyledon (439·13 ± 40·28 μm^2, mean ± s.d., $n = 10$) was smaller than approx. one third of the size of those in the rest of the macrocotyledon (1575·91 ± 405·87 μm^2) ($P < 0·01$, one-way ANOVA), which was similar in the microcotyledon (1585·10 ± 290·20 μm^2) ($P > 0·05$) 30 d after imbibition.

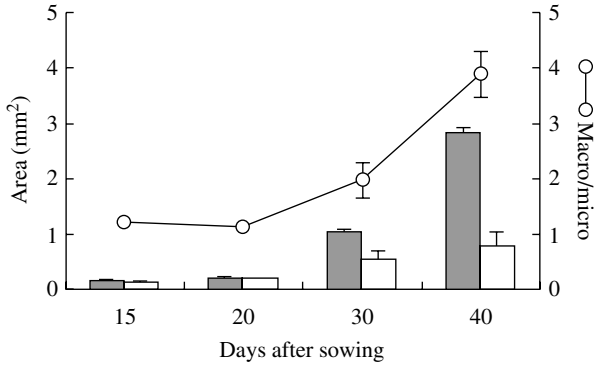

FIG. 1. Development of anisocotyly in *S. wendlandii*. Areas of the cotyledons were measured at 15, 20, 30 and 40 d after sowing, and the ratios of the area of a macrocotyledon to that of a microcotyledon were measured. Areas of both cotyledons were almost equal 15 d after sowing ($P > 0·05$, one-way ANOVA); at 20 and 30 d after sowing unequal growth had started ($0·05 > P > 0·01$), and the cotyledons were unequal 40 d after sowing ($P < 0·01$). Closed bars represent the surface area of the macrocotyledon, while open bars represent that of the microcotyledon. Open circles represent the ratio of the area of a microcotyledon to that of a macrocotyledon. Error bars represent s.e, $n = 13$ (15 d after sowing), 14 (20 d), 13 (30 d), 11(40 d).

Morphological characteristics of the basal region of the macrocotyledon

When morphological characteristics of the basal region of the macrocotyledon were examined, it was found that the most distinct feature of the newly added region of the enlarging cotyledon was the formation of needle-shaped trichomes. Three types of trichomes were observed on the epidermis of the adaxial surface of *S. wendlandii* (Fig. 3A). We named these type 1, type 2 and type 3 trichomes, respectively. These three types of trichomes were distinct in morphology and size (Fig. 3A); type 1 retained a round-shaped apical cell with a total length of 63 ± 9·9 μm ($n = 20$), type 2 had two flat-shaped apical cells, and a total length of 17·2 ± 3·4 μm, while type 3 had a pointed apical cell, and a total length of 338·7 ± 64·9 μm, being far larger than the others ($P < 0·01$, one-way ANOVA). Shortly after germination, type 1 and type 2 trichomes were observed throughout both cotyledons (Fig. 3C), while the needle-shaped type 3 trichomes were only observed in the newly formed region at the base of the enlarging cotyledon (Fig. 3B). Distribution of these trichomes was greater in the basal region of the macrocotyledon than elsewhere (Fig. 3E). On the abaxial side of the leaves, although emergence of type 3 trichomes was observed at much later stages (90 d after imbibition) than on the adaxial side, it was again observed only in the newly formed basal region of the macrocotyledon. Thus, the formation of type 3 trichomes in the basal region of the enlarging cotyledon was distinct to that area, suggesting that there is a close correlation between cell division at the base of the enlarging cotyledon and the formation of the needle-shaped trichomes there.

Another unique feature of the basal region of the enlarging cotyledon was recorded. When the development of

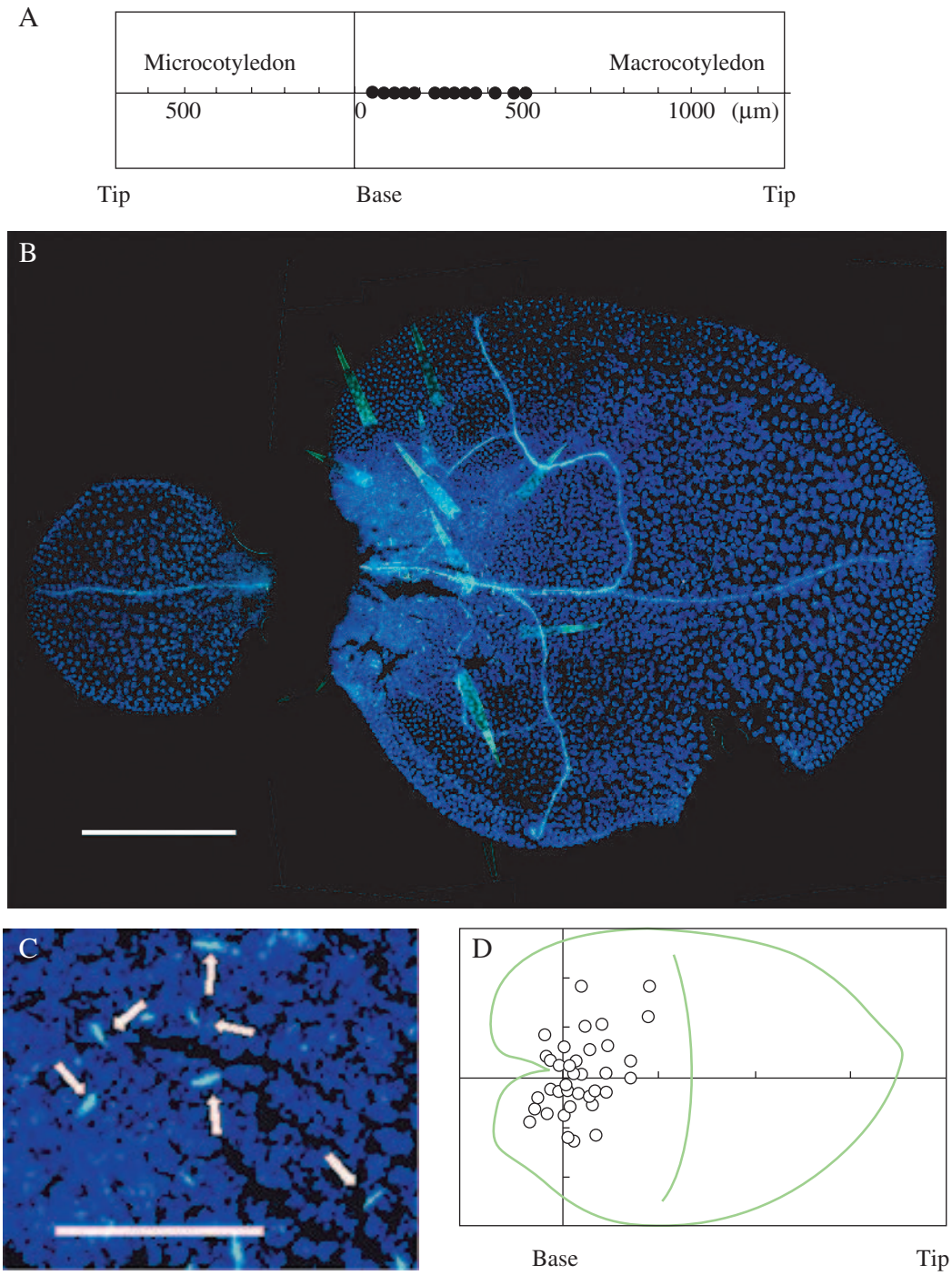

FIG. 2. Distribution of the meristematic region in cotyledons of *S. wendlandii*. (A) When the 30-d-old macrocotyledon was dissected into sections transversely along the long axis, incorporation of BrdU into certain cells of the sections was detected under a fluorescence microscope by staining with an antibody against BrdU. Closed circles indicate the regions where the incorporation of BrdU was observed. (B) Distribution of divided cells was demonstrated in the two 30-d-old cotyledons of *S. wendlandii* by staining with Aniline Blue. Scale bar = 500 μm. (C) Magnified views of the cells indicated by box in (B). Septum walls were visualized in the basal region of a macrocotyledon. Arrowheads indicate septum walls visualized with Anilin Blue staining. Scale bar = 100 μm. (D) Schematic illustration of the distribution of divided cells in the macrocotyledon. Circles indicate the distribution of cell division in the macrocotyledon as visualized by staining with Aniline Blue.

veins was observed, it became clear that lateral veins had formed, which surrounded the newly developed area in the basal region of the macrocotyledon (Fig. 3D). Moreover, the formation of procambial cells that became lateral veins was detected following recognition of the emergence of the needle-shaped trichomes 20 to approx. 25 d after sowing (data not shown). After 3 months, the numbers of lateral veins that were bifurcated from the midvein and were added

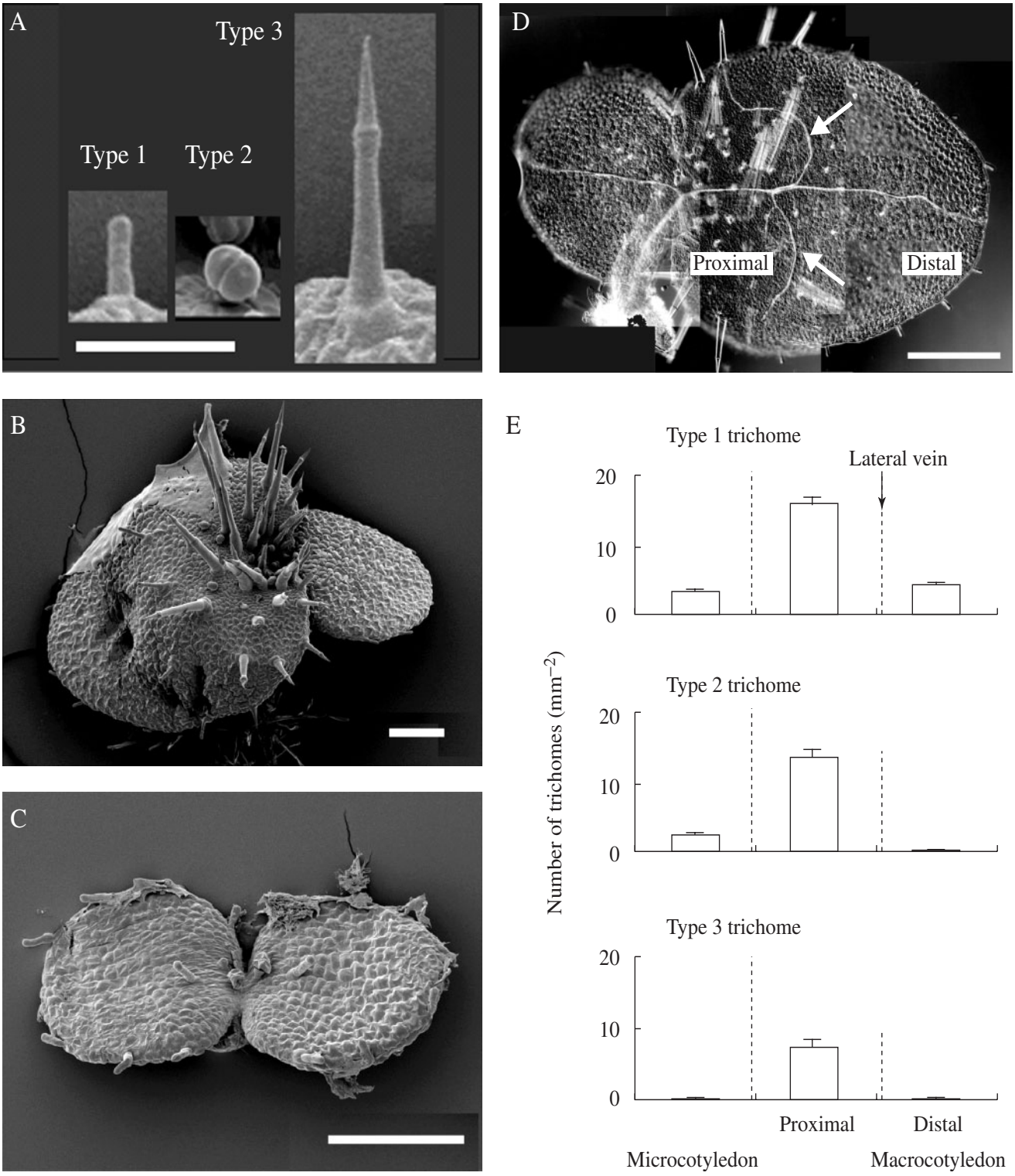

FIG. 3. (A–C) Scanning electron micrographs of cotyledons of *S. wendlandii*. (A) Three types of trichomes were observed on the surface of cotyledons of *S. wendlandii*. Scale bar = 100 μm. (B) Needle-shaped type 3 trichomes were only observed in the newly developed region at the base of the macrocotyledon. (C) Shortly after germination, when the cotyledons were equal, type 1 and type 2 trichomes were observed in both cotyledons. (D) Vascular patterns and the distribution of trichomes in 30-d-old seedlings. Lateral veins were observed in the macrocotyledon (arrow), but not in the microcotyledon. The newly developed region surrounded by lateral veins retains type 3 trichomes. Scale bars = 200 μm (B–D). (E) Distribution of the three types of trichomes in the proximal and distal regions of the macrocotyledon across the lateral veins and in the microcotyledon. Type 3 trichomes were only observed in the proximal region of the macrocotyledon. Distributions of trichomes on the proximal region of macrocotyledon were different from that of the distal region of the macrocotyledon ($P < 0{\cdot}01$, ANOVA), and distributions of trichomes on the microcotyledon were fairly similar to that of the distal region of the macrocotyledon ($P > 0{\cdot}05$). Error bars represent s.e. ($n = 10$).

basipetally were determined to be 11·91 ± 2·62 ($n = 11$), and the newly formed regions were always characterized with the type 3 trichomes. There appeared to be a close link between formation of lateral veins and the newly developed area caused by cell division. It is suggested that after the newly developed area of dividing cells at the base of the enlarging cotyledon had been established, the area was surrounded by lateral veins. The process of formation of a new area and surrounding it with lateral veins was repeated continuously, resulting in the continual growth of macrocotyledon.

Cytokinin as a causative factor upon the formation of the macrocotyledon

The effects of plant hormones (or a precursor) upon the anisocotylous leaf formation of *S. wendlandii* were examined. As shown in Table 1, different plant hormones affected the growth of cotyledons in various ways. However, only cytokinin treatments resulted in the suppression of anisocotyly when strictly defined as the suppression of the growth of macrocotyledon without growth of microcotyledon also being suppressed. In this case, the ratio of macrocotyledon to microcotyledon was calculated to be lower than 1·4. Even the treatment of cytokinins BAP at 10^{-8} M and kinetin at 10^{-7} M showed distinct anisocotyly, implying that these concentrations were not effective for suppressing anisocotyly. On the other hand, *t*-zeatin at 10^{-5} M suppressed the growth of the microcotyledon, suggesting an inhibitory effect at this higher concentration. Other cases in which the ratio of two cotyledons was close to 1·4 were observed in the treatment of ABA at 10^{-7} M and GA_3 at 10^{-5} M. Regarding the former, although the suppression of the anisocotyly at this concentration of ABA was not distinct as the ratio of macrocotyledon to microcotyledon was 1·5, this may be a transition level

TABLE 1. *Effects of plant hormones and their precursors on the development of cotyledons of S. wendlandii. Seedlings were placed on medium containing plant hormones after germination and compare with control plants that were cultured without plant hormones*

Plant hormone or precursor	Days after sowing	Number of seedlings examined	Area of macrocotyledon (mm^2) (A)	Area of microcotyledon (mm^2) (B)	Ratio of A : B
Experiment 1					
IAA 10^{-5} M	30	20	0·51 ± 0·04	0·22 ± 0·01	2·26 ± 0·13
IAA 10^{-6} M		14	0·92 ± 0·06	0·32 ± 0·02	2·95 ± 0·19
IAA 10^{-7} M		20	0·91 ± 0·04	0·33 ± 0·02	2·96 ± 0·21
Control 1		10	1·10 ± 0·12	0·36 ± 0·03	3·05 ± 0·30
Experiment 2					
2,4-D 10^{-5} M	45	15	0·36 ± 0·03	0·30 ± 0·03	1·2 ± 0·06
2,4-D 10^{-6} M		13	0·20 ± 0·01	0·17 ± 0·01	1·26 ± 0·08
Control 2		16	3·60 ± 0·39	0·86 ± 0·20	6·15 ± 0·86
Experiment 3					
BAP 10^{-5} M	30	10	0·85 ± 0·08	0·81 ± 0·08	1·13 ± 0·11
BAP 10^{-6} M		13	1·98 ± 0·10	1·38 ± 0·12	1·28 ± 0·08
BAP 10^{-7} M		11	1·66 ± 0·11	1·46 ± 0·12	1·15 ± 0·04
BAP 10^{-8} M		13	1·77 ± 0·16	0·64 ± 0·04	2·82 ± 0·25
Control 3		10	1·24 ± 0·14	0·62 ± 0·05	2·22 ± 0·44
Experiment 4					
Kinetin 10^{-5} M	30	14	0·78 ± 0·04	0·68 ± 0·05	1·21 ± 0·08
Kinetin 10^{-6} M		11	0·67 ± 0·03	0·59 ± 0·03	1·14 ± 0·04
Kinetin 10^{-7} M		16	0·89 ± 0·44	0·35 ± 0·24	2·57 ± 0·76
Control 4		11	1·20 ± 0·12	0·45 ± 0·06	2·91 ± 0·28
Experiment 5					
t-zeatin 10^{-5} M	30	19	0·38 ± 0·02	0·33 ± 0·02	1·19 ± 0·04
t-zeatin 10^{-6} M		13	0·74 ± 0·04	0·65 ± 0·03	1·15 ± 0·03
t-zeatin 10^{-7} M		9	0·70 ± 0·06	0·55 ± 0·04	1·31 ± 0·15
Control 5		14	0·95 ± 0·07	0·36 ± 0·02	2·68 ± 0·18
Experiment 6					
ABA 10^{-5} M	30	9	0·17 ± 0·01	0·14 ± 0·01	1·18 ± 0·04
ABA 10^{-6} M		11	0·25 ± 0·02	0·21 ± 0·02	1·17 ± 0·03
ABA 10^{-7} M		5	0·60 ± 0·11	0·40 ± 0·06	1·50 ± 0·14
Control 6		8	0·85 ± 0·12	0·30 ± 0·04	3·00 ± 0·33
Experiment 7					
GA_3 10^{-5} M	30	10	0·62 ± 0·03	0·40 ± 0·02	1·58 ± 0·13
GA_3 10^{-6} M		10	0·56 ± 0·08	0·35 ± 0·03	1·53 ± 0·13
GA_3 10^{-7} M		10	0·54 ± 0·04	0·37 ± 0·03	1·54 ± 0·20
ACC 10^{-4} M	30		Plants were dead		
ACC 10^{-5} M		7	0·68 ± 0·03	0·44 ± 0·04	1·59 ± 0·09
Control 7		12	1·12 ± 0·14	0·38 ± 0·04	3·31 ± 0·67

Data represent mean ± s.e.

for the suppression of anisocotyly, something which remains to be examined in further studies. As regards GA_3, the suppression of anisocotyly at this concentration was also not distinct, as the ratio of macrocotyledon to microcotyledon was 1·58. In this case, just as Rosenblum and Basile (1984) noted caulescence formation in *S. prolixis*, a certain level of petiole-like formation was observed in *S. wendlandii*, although this effect was not so distinct in comparison with cytokinins. Furthermore, when the effect of brassionostelide upon the anisocotyledonous growth of cotyledon was examined, no distinct effects were observed (data not shown).

Thus, the effect of cytokinins upon anisocotylous leaf formation was distinctive between the plant hormones that were examined. In the presence of 10^{-6} M BAP, both cotyledons grew almost equally, both ending up the size of the macrocotyledon in the untreated plants, implying that a microcotyledon was converted to a macrocotyledon by the addition of cytokinin. As both cotyledons grew continuously, the ratio of cotyledons was lower than plants without BAP treatment, and formed nearly equal-sized cotyledons (Fig. 4A, see also Fig. 1). In addition, an examination was made of how long the responsiveness of a microcotyledon to cytokinin was preserved. When sizes of cotyledons were measured 55 d after imbibition, the microcotyledon of seedlings that were incubated with BAP from the 33rd day to the 35th day became larger than the microcotyledon of the untreated control (Fig. 4B). This suggests that the microcotyledon was responsive to the treatment of BAP until this time. However, the microcotyledon of seedlings approx. 2–3 months after imbibition was no longer responsive to cytokinin (data not shown).

As the macrocotyledon exhibited unique morphological features (as illustrated above), we examined whether the observed characteristics would be seen in both the cotyledons produced by cytokinin-treated plants. Cytokinin-treated seedlings showed active DNA synthesis in the basal region of both cotyledons (Fig. 5). Type 3 trichomes were observed in the basal regions of both cotyledons (Fig. 6B, C), and the development of lateral veins was observed surrounding the basal area (Fig. 6A). Thus, both cotyledons displayed the basic characteristics of the macrocotyledon. As the change observed in the formation of the two macrocotyledons can be interpreted as conversion of two cotyledons to two macrocotyledons, cytokinin could be an endogenous factor involved in triggering the formation of the macrocotyledon.

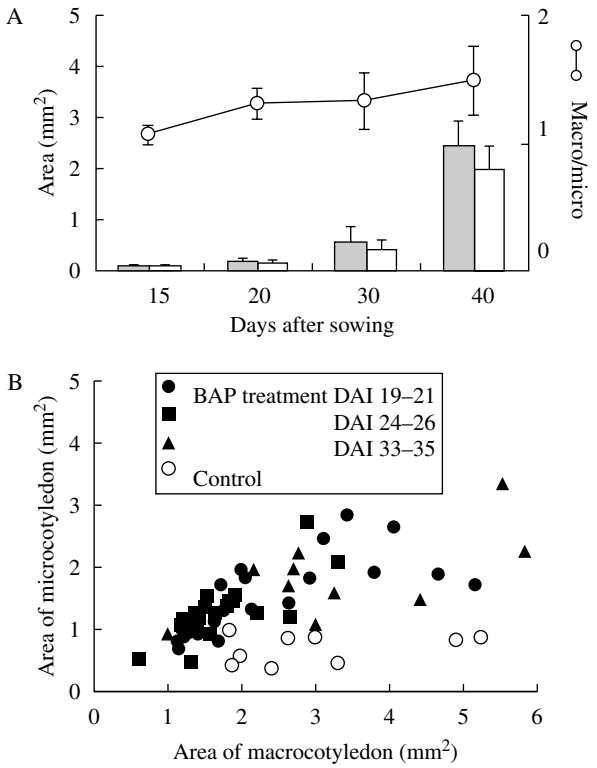

FIG. 4. Effects of cytokinin on the early development of *S. wendlandii*. (A) Germinated seedlings were cultured in medium containing 10^{-6} M BAP. Leaf areas were measured at 15, 20, 30 and 40 d after sowing (DAI) on the culture media. The area of the macrocotyledon and the area of the microcotyledon were almost equal during the period of observation ($P >$ 0·05, one-way ANOVA). Closed bars = areas of macrocotyledon; open bars = areas of microcotyledon; open circles = the ratios of the area between the microcotyledon and the macrocotyledon; error bars = s.e., n = 15, (15, 20, 30 d after sowing), 12 (40). (B) Additional growth of the microcotyledon was induced by treatment with BAP. The time when growth of cotyledons was affected by treatment of BAP was examined. Seedlings were placed on the medium with 10^{-6} M BAP for 3 d at different times, and areas of cotyledons were measured 55 d after sowing. Microcotyledons of plants treated BAP were larger than those of control plants.

Responsiveness to cytokinin in different parts of cotyledons

As described above, exogenous application of cytokinins to seedlings of *S. wendlandii* produced distinct responses between the macrocotyledon and microcotyledon. There might be differences of responsiveness to cytokinins between the microcotyledon and the distal and basal portion of the macrocotyledon. To examine this, we tried to culture separated portions of the cotyledons. As shown in Fig. 7B and E, in the basal portion of the macrocotyledon, larger numbers of type 3 trichomes were observed in comparison with other portions (Fig. 7 A, C, D, F). Thus, the responsiveness to cytokinins was confirmed to be contained within the basal portion of the macrocotyledon. It remains to be examined where cytokinins might be produced and transported to the target tissues.

Comparative analyses of the morphogenesis of cotyledons between Streptocarpus *and* Monophyllaea

Similar observations have been made using another unifoliate species, *Monophyllaea glabra*, in the same experiments as those performed using *S. wendlandii*. When the early morphogenesis of *M. glabra* was followed, the development of lateral veins (Fig. 8A) and the formation of needle-shaped type 3 trichomes (Fig. 8B, C) were observed in the newly developed region of the enlarging cotyledon. In the case of *M. glabra*, however, type 3 trichomes were first observed on the edges of the basal region newly formed in the enlarging cotyledon, and then were found right across the surface. This area was subsequently surrounded by lateral veins (Fig. 8). Furthermore, when cytokinins were

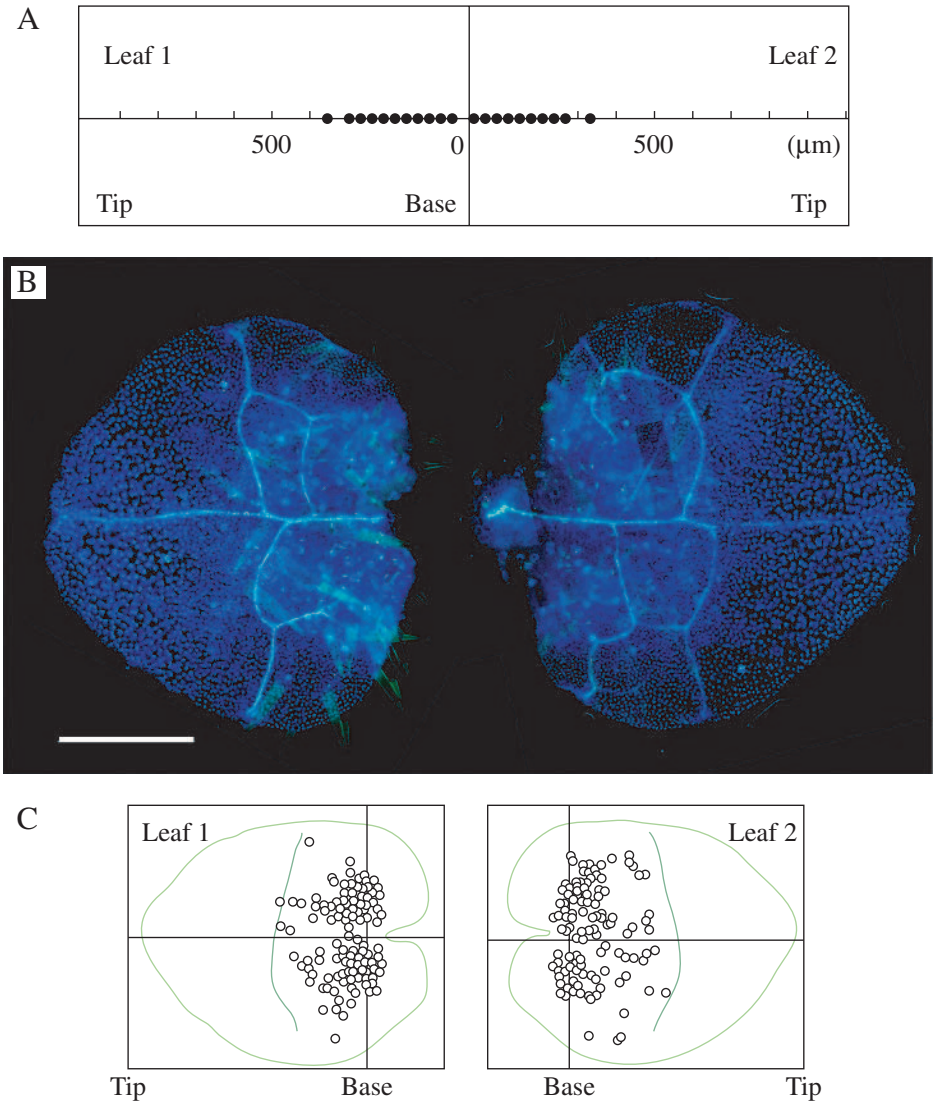

FIG. 5. Distribution of meristematic regions in the cotyledons cultured in the presence of 10^{-6} M BAP. (A) When the 30-d-old seedling was dissected into sections transversely along the long axis, incorporation of BrdU into cells of the sections was detected by staining with an antibody against BrdU. Closed circles indicate the regions where the incorporation of BrdU was observed. (B) Distribution of divided cells was demonstrated in the two 30-d-old cotyledons by staining with Aniline Blue. Scale bar = 500 μm. (C) Schematic illustrations of distribution of septum walls of divided cells in both cotyledons that were treated with 10^{-6} M BAP. Circles indicate the distribution of septum walls stained with Aniline Blue.

applied to *M. glabra*, the two cotyledons grew equally, resulting in the formation of two equal-sized macrocotyledons (data not shown). Thus, the principle of the development of the cotyledons of *M. glabra* was identical to that of *S. wendlandii*.

DISCUSSION

It has been demonstrated in this study that in normal development the formation of the macrocotyledon of *S. wendlandii* was achieved by the addition of new cells supplied by meristematic activity in the basal region of the macrocotyledon. This newly added region displayed distinct morphological changes, which have been overlooked in previous studies. The needle-shaped type 3 trichomes were formed only in the newly developed region, and not in the distal region of the macrocotyledon, or in the microcotyledon. This observation is consistent with the notion that needle-shaped type 3 trichomes are ordinary trichomes of foliage leaves of Gesneriaceous plants (Michael Möller, Edinburgh, pers. comm.). The new region might have been separated from existing cotyledon tissues by the newly developed lateral veins. These changes were also confirmed in another remotely related species, *M. glabra*. Kerstetter and Poethig (1998) have discussed the relationship of trichome formation to phase-change in the context of plant developmental biology. In fact, the formation of specific types of trichomes is considered to be a sign of 'phase-change' in *Arabidopsis thaliana* (Telfer and Poethig, 1997). Thus the formation of a distinct type of trichome in the newly developed region of the enlarging

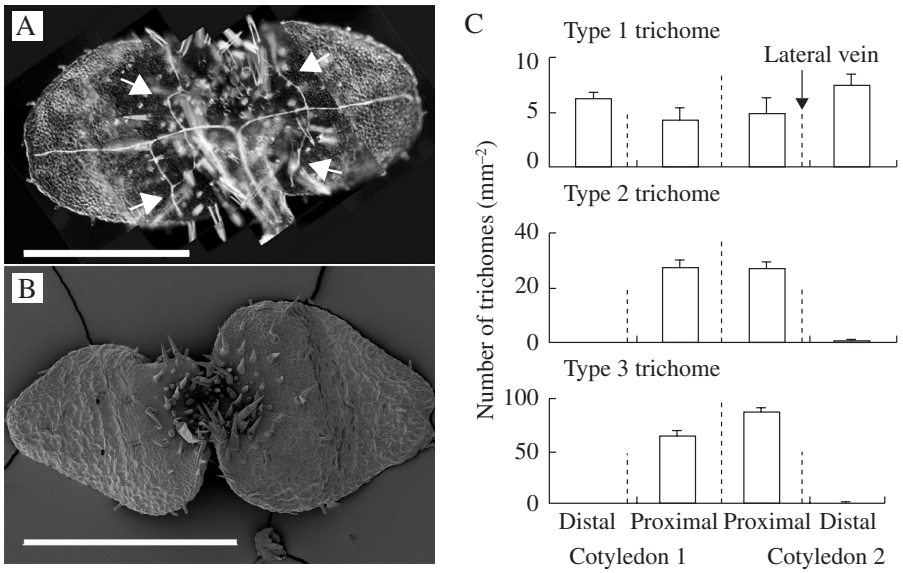

FIG. 6. Morphological characteristics of the cotyledons of *S. wendlandii* cultured in the presence of 10^{-6} M BAP. Analyses were carried out on 30-d-old seedlings incubated on a medium containing 10^{-6} M BAP. (A) Cleared cotyledons indicated that both cotyledons showed features characteristic of the macrocotyledon. Note that lateral veins were formed in both cotyledons (arrows). (B) Under scanning electron microscopy, type 3 trichomes were observed to be distributed in the proximal regions of both cotyledons. Scale bars = 1 mm (A, B). (C) Distribution of the three types of trichomes was examined in the distal and proximal regions of both cotyledons across the lateral veins. The number of trichomes was different between cotyledons ($0{\cdot}05 > P > 0{\cdot}01$, ANOVA), but both cotyledons displayed type 3 trichomes on the proximal region, and the distribution of trichomes was significantly different between distal and proximal regions ($P < 0{\cdot}01$). Error bars represent s.e. ($n = 10$).

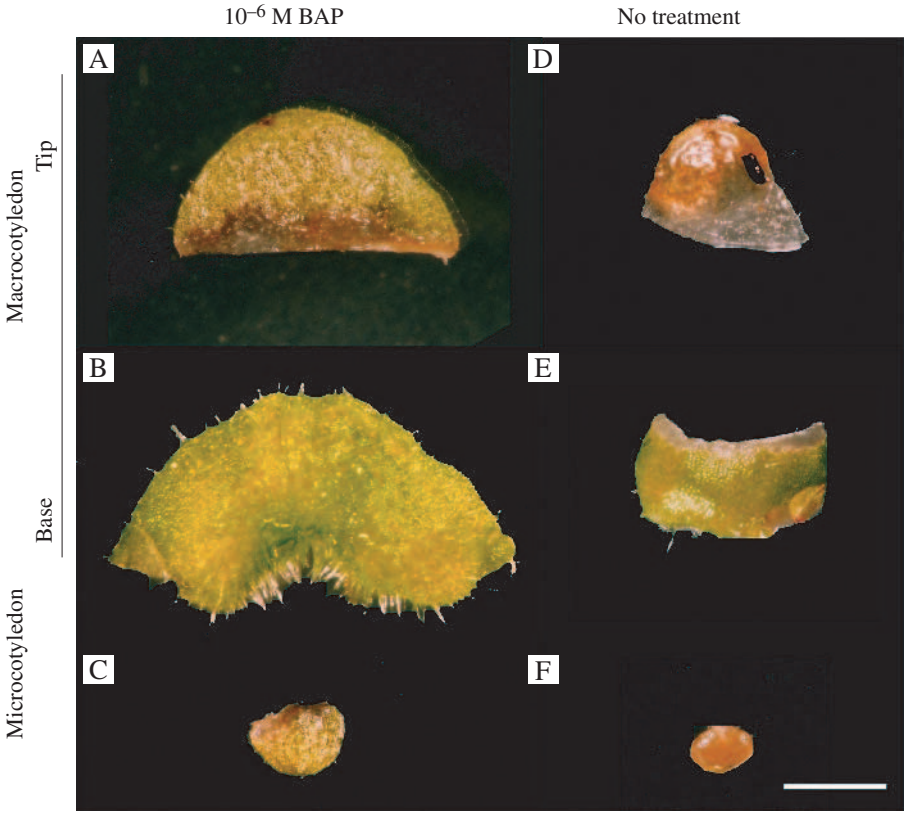

FIG. 7. *In vitro* culture of leaf explants. 80-d-old *S. wendlandii* plants were dissected into a distal half and a basal half of the macrocotyledon and the microcotyledon. These explants were cultured for 20 d on the medium with or without 10^{-6} M BAP. (A–C) Explants cultured with 10^{-6} M BAP. (D–F) explants cultured without BAP. (A, D) Explants of the distal region of the macrocotyledon. (B, E) Explants of the basal region of the macrocotyledon. (C, F) Detached microcotyledon. Scale bar = 2 mm.

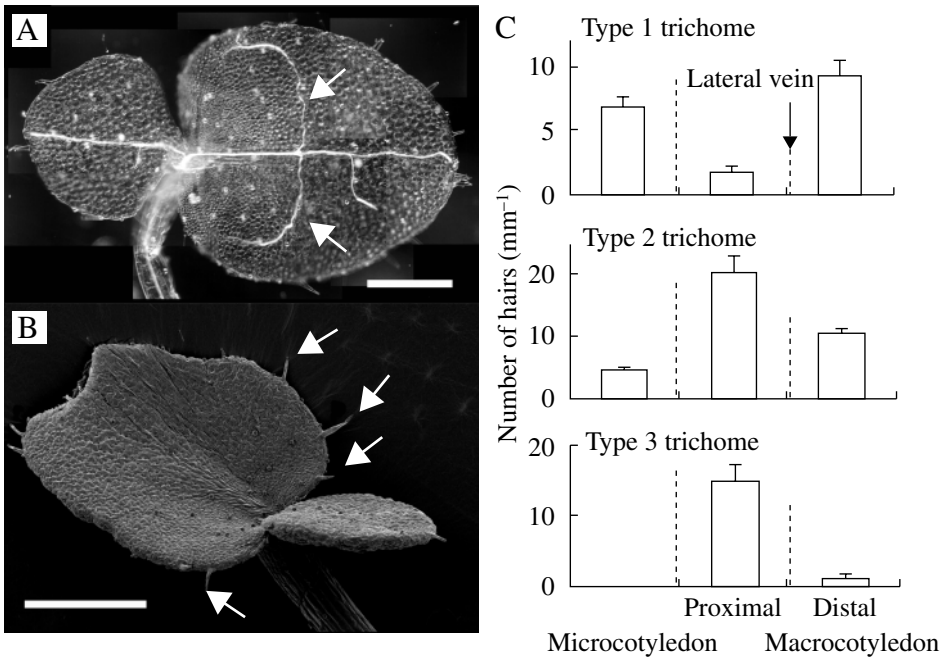

FIG. 8. Morphological characteristics of the cotyledons of *Monophyllaea glabra*. (A, B) Seedlings 20 d after imbibition were examined. Areas of the cotyledons differed at that time. (A) Cleared seedlings showing a newly formed region of the macrocotyledon surrounded by lateral veins (arrow). (B) Scanning electron microscopy image showing three types of trichomes were formed on the macrocotyledon (as in *S. wendlandii*). Needle-shaped type 3 trichomes were distributed only on the edge of the proximal region of the macrocotyledon (arrows). Scale bars = 500 μm (A, B). (C) Distribution of the trichomes on the proximal and the distal region of the macrocotyledon across the lateral veins and on the microcotyledon. Seedlings 36 d after imbibition were cleared and trichomes on the edges of cotyledons were counted. The number of type 3 trichomes was different between the basal region of the macrocotyledon and the distal regions of the macrocotyledon and microcotyledon ($P < 0{\cdot}01$, one-way ANOVA). Error bars represent s.e. ($n = 10$).

cotyledon in *S. wendlandii* as well as *M. glabra* can also be considered as a phase-change.

Mutational changes of cotyledons to foliage leaves or of foliage leaves to cotyledons in *A. thaliana* have been reported in several cases. *Leafy cotyledon* causes the conversion of cotyledons to foliage leaves, whereupon the formation of trichomes is observed, while in the wild type no trichomes are formed on the cotyledons (Meinke and Yeung, 1994). On the other hand, in the *extra cotyledon* mutants, the first pair of foliage leaves display the shape of cotyledons (Conway and Poethig, 1997). When *Brassica napus* seeds are precociously germinated, the foliage organ that is formed at the tip of the shoot has the appearance of a cotyledon, while its base shows leaf-like characteristics (Fernandez, 1997). In all of these cases, the conversion of cotyledons to leaves and *vice versa* is accompanied by the formation of distinct types of trichomes. In addition, recent reports have revealed that several genes relating to the cytoskeleton and cell-division-related genes are involved in the morphogenesis of trichomes in *A. thaliana* (Schnittger and Hülskamp, 2002). Thus, it is likely that the observed morphological changes in the macrocotyledon of *S. wendlandii* or *M. glabra* would reflect a phase-change.

In this context, the uniqueness of the phase-change in the unifoliate species of Gesneriaceae lies in its occurrence in different parts of the same leaf. The newly developed leaf area was supplied by the basal meristem, but not by the SAM, as in most higher plants. Although the newly developed region displayed morphologically distinct features, this region may fulfill certain distinct functions such as photosynthetic activity (Burtt, 1970), although this remains to be examined. As this phase-change is distinct in this group of plants, it is important to determine its trigger.

Experimental results revealed that cytokinin is a likely factor in expression of such phase-changes, but it remains to be determined where this cytokinin is synthesized and how it is transported. It can be inferred from our results (Fig. 1) that under natural conditions at about 20 d post-imbibition, cytokinins may be supplied to the enlarging cotyledon, and that this triggers the conversion of a microcotyledon into a macrocotyledon, resulting in the two unequal cotyledons. The susceptibility of tissues to cytokinin evidently existed in the newly developed region, as was confirmed by *in vitro* culture of different portions of cotyledons. This may have some relevance to the irreversible growth of the microcotyledon of *M. horsfieldii* after the cotyledon fates have been determined, as described by Tsukaya (1997). There have been a few reports in which plant hormones have been shown to be involved in the induction of phase-changes. Trichome formation in *leafy cotyledon* of *A. thaliana* has been proposed to be caused by plant hormones (Meinke, 1992). The conversion of cotyledons in *Eranthis himalis* to foliage leaves by cytokinin (Haccius, 1972) may have some relevance to the observations made in this study. Although

conversion of the cotyledons to foliage leaves in a cytokinin-overproducing *amp1* of *A. thaliana* is accompanied by accumulation of cytokinin (Chaudhury *et al*., 1993), the effects of cytokinin are not adequately understood. Another plant hormone, GA, seems to be involved in this phase-change in certain species of *Streptocarpus* (Rosenblum and Basile, 1984). However, as shown in Table 1, effects of cytokinin were more distinct than GA, at least in the early stages of development of *S. wendlandii*. Although lower concentrations of ABA (10^{-7} M) and ACC seem to have some effects upon anisocotyly in *S. wendlandii*, the effect of cytokinin was more distinct than for either of these. Nonetheless, it is important to examine the interactions of these plant hormones upon this developmental process.

Finally, it may be argued that the phase-change observed in the unifoliate species of Gesneriaceae has happened within one leaf, which implies that the presence of a SAM is not a prerequisite for causing the phase-change. This also suggests that different developmental controls can occur within a single organ, as shown here for the formation of macrocotyledons. This is an intriguing question to be considered in future analyses of the leaf identity of plants.

ACKNOWLEDGEMENTS

We thank Dr Miltos Tsiantis (University of Oxford, UK) for his critical reading of the manuscript. Thanks are also due to Dr H. Ohba of the University of Tokyo for use of the scanning electron microscopic facility. We are also indebted to Mr T. Okuto (Hyogo Flower Park, Japan), Messrs M. Yasukawa, K. Hiratsuka, J. Nagasawa and Y. Nakamura (Kyoto, Prefectural Botanical Gardens, Japan), Dr J. Van Staden (University of Natal, South Africa) for providing *Streptocarpus* seeds, and to Mr K. Hirai of the Botanical Gardens of the University of Tokyo for providing *Monophyllaea* seeds.

LITERATURE CITED

Burtt BL. 1970. Studies in the Gesneriaceae of the old world. XXXI. Some aspects of functional evolution. *Notes from the Royal Botanic Garden, Edinburgh* **30**: 1–10.

Burtt BL. 1978. Studies in the Gesneriaceae of the old world. XLV. A preliminary revision of *Monophyllaea*. *Notes from the Royal Botanic Garden, Edinburgh* **37**: 1–59.

Chaudhury AM, Letham S, Craig S, Dennis ES. 1993. *amp1*—a mutant with high cytokinin levels and altered embryonic pattern, faster vegetative growth, constitutive photomorphogenesis and precocious flowering. *The Plant Journal* **4**: 907–916.

Conway LJ, Poethig RS. 1997. Mutations of *Arabidopsis thaliana* that transform leaves into cotyledons. *Proceedings of the National Academy of Sciences of the USA* **94**: 10209–10214.

Fernandez DE. 1997. Developmental basis of homeosis in precociously germinating *Brassica napus* embryos: phase change at the shoot apex. *Development* **124**: 1149–1157.

Fujie M, Kuriowa H, Kawano S, Mutoh S, Kuroiwa K. 1994. Behavior of organelles and their nucleotides in the shoot apical meristem during leaf development in *Arabidopsis thaliana* L. *Planta* **194**: 395–405.

Haccius VB. 1972. Primarblatt-ähnliche Fiederkotyledonen nach Cytokinin-Behandlung junger Embryonen von *Eranthis hiemalis*. *Beiträge zur Biologie der Pflanzen* **48**: 301–311.

Hayashi T, Polonenko DR, Camirand A. 1986. Pea xyloglucan and cellulose IV. Assembly of β-glucans by pea protoplasts. *Plant Physiology* **82**: 301–306.

Imaichi R, Nagumo S, Kato M. 2000. Ontogenetic anatomy of *Streptocarpus grandis* (Gesneriaceae) with implication for evolution of monophylly. *Annals of Botany* **86**: 37–46.

Imaichi R, Inokuchi S, Kato M. 2001. Developmental morphology of one-leaf plant *Monophyllaea singularis* (Gesneriaceae). *Plant Systematics and Evolusion* **229**: 171–185.

Jong K, Burtt BL. 1975. The evolution of morphological novelty exemplified in the growth patterns of some Gesneriaceae. *New Phytologist* **75**: 297–311.

Kerstetter RA, Poethig RS. 1998. The specification of leaf identity during shoot development. *Annual Review of Cell and Developmental Biology* **14**: 373–398.

Kuwabara A, Tsukaya H, Nagata T. 2001. Identification of factors that cause heterophylly in *Ludwigia arcuata* Walt. (Onagraceae). *Plant Biology* **50**: 98–105.

Meinke DW. 1992. A homeotic mutant of *Arabidopsis thaliana* with leafy cotyledons. *Science* **258**: 1647–1650.

Meinke DW, Yeung EC. 1994. *Leafy cotyledon* mutants of *Arabidopsis*. *The Plant Cell* **6**: 1049–1064.

Möller M, Cronk QCB. 2001. Evolution of morphological novelty: a phylogenetic analysis of growth patterns in *Streptocarpus* (Gesneriaceae). *Evolution* **55**: 918–929.

Murashige T, Skoog F. 1962. A revised medium for rapid growth and bioassay with tobacco cultures. *Physiologia Plantarum* **15**: 473–497.

Rosenblum IM, Basile DV. 1984. Hormonal regulation of morphogenesis in *Streptocarpus* and its relevance to evolutionary history of the Gesneriaceae. *American Journal of Botany* **71**: 52–64.

Schnittger A, Hülskamp M. 2002. Trichome morphogenesis: a cell-cycle perspective. *Philosophical Transactions of the Royal Society of London* **357**: 823–826.

Sylvester AW, Smith L, Freeling M. 1996. Acquisition of identity in the developing leaf. *Annual Review of Cell and Developmental Biology* **12**: 257–304.

Telfer A, Poethig RS. 1997. Phase change and the regulation of trichome distribution in *Arabidopsis thaliana*. *Development* **124**: 645–654.

Tsukaya H. 1997. Determination of the unequal fate of cotyledons of a one-leaf plant, *Monophyllaea*. *Development* **124**: 1275–1280.