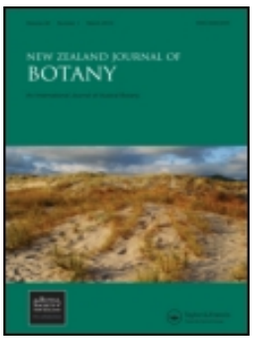

New Zealand Journal of Botany

ISSN: 0028-825X (Print) 1175-8643 (Online) Journal homepage: https://www.tandfonline.com/loi/tnzb20

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To cite this article: K Nishii , C-N Wang , A Spada , T Nagata & M Möller (2012) Gibberellin as a suppressor of lateral dominance and inducer of apical growth in the unifoliate *Streptocarpus wendlandii* (Gesneriaceae), New Zealand Journal of Botany, 50:3, 267-287, DOI: 10.1080/0028825X.2012.671775

To link to this article: https://doi.org/10.1080/0028825X.2012.671775

New Zealand Journal of Botany
Vol. 50, No. 3, September 2012, 267–287

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Gibberellin as a suppressor of lateral dominance and inducer of apical growth in the unifoliate *Streptocarpus wendlandii* (Gesneriaceae)

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(*Received 7 October 2011; final version received 28 January 2012*)

We report on the effects of exogenously applied hormones on the lateral and apical dominance that governs morphogenesis in the unifoliate *Streptocarpus wendlandii*. In this phenotype, lateral dominance is extreme as the plants only retain a macrocotyledon that develops into a leaf-like phyllomorph by means of a basal meristem and do not show apical growth. Gibberellin applications suppressed the basal meristem activity of the macrocotyledon resulting in an isocotylous seedling with two microcotyledons and caused the formation of a primary phyllomorph, which suggests that the groove meristem, a shoot apical meristem equivalent, is released from apical suppression by the basal meristem. Interestingly, uniconazol, a gibberellin biosynthesis inhibitor, also caused a reduction in basal meristem activity, but without primary phyllomorph formation, suggesting that some gibberellin is required for proper function of the basal meristem. Co-application of gibberellin and cytokinin resulted in two macrocotyledons also without phyllomorph formation, which is similar to previous results for cytokinin-only applications. Thus, cytokinin may act downstream in the regulatory pathway of the basal meristem. Our results suggest that the balance between gibberellin and cytokinin in the cotyledons appears thus as key factor in the regulation of lateral and apical dominance in *Streptocarpus*. Their interplay may well be the primary explanation for the great diversity in growth form exhibited in species of this genus. Our work shows that small imbalances of hormones in early stages of plant development can have major effects on the final phenotype.

Keywords: apical dominance; basal meristem; cytokinin; Gesneriaceae; gibberellin; lateral dominance; *Streptocarpus wendlandii*

Meristems are essential for plant morphogenesis and their interactions determine the shape of the plant body (Meyerowitz 1997). Plant hormones regulate these meristem functions (Davies 2004). Different meristems can interact and compete with each other: apical dominance, for example, is a classic example of the hormonal regulation of activities between the shoot apical meristem (SAM) and axillary meristems. In this case, the SAM suppresses the development of lateral (usually axillary) meristems. It is well known that apical dominance is primarily caused by auxins and its effect is counteracted by cytokinin (Phillips 1975; Ongaro & Leyser 2008).

In the family Gesneriaceae, another type of competition between meristems is observed, in which a lateral dominance is established at the seedling stage between the two cotyledons. Just after germination, both cotyledons are equal, but soon afterwards one continues to grow to become the macrocotyledon, suppressing the development of the other cotyledon (reviewed in Nishii et al. 2009). This phenomenon, termed anisocotyly, has been studied ever since plants of

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ISSN 0028-825X print/ISSN 1175-8643 online

http://dx.doi.org/10.1080/0028825X.2012.671775
http://www.tandfonline.com

the genus *Streptocarpus* Lindl. came into cultivation (Caspary 1858). In their extreme form, the enlarging cotyledon is the only foliar organ that the plant produces, and these unifoliate Gesneriaceae do not possess a shoot or SAM (Jong & Burtt 1975; Imaichi et al. 2000).

Jong (1970) introduced new terms to describe such unique development in *Streptocarpus*. In unifoliates, the macrocotyledon consists of a lamina and a 'petiolode', a stem-like petiole (Jong 1970; Tononi et al. 2010). Lamina and petiolode form a structure called 'phyllomorph'. Phyllomorphs retain a triad of meristems, the basal meristem for lamina growth, the petiolode meristem for petiolode growth and the groove meristem situated on the petiolode near the lamina forms inflorescences (Jong 1970). In rosulate *Streptocarpus*, further phyllomorphs are produced from the groove meristem, a SAM equivalent. Caulescent *Streptocarpus* species possess a shoot and SAM (Jong 1970; Jong & Burtt 1975; Imaichi et al. 2007). A SAM or groove meristem is not present in *Streptocarpus* embryos (Figs 1A, 1B; Imaichi et al. 2000, 2007, Mantegazza et al. 2007; Tononi et al. 2010), or for a while after germination (Figs 1C, 1D; Nishii et al. 2004, 2007). Its development is delayed by the activity of the basal meristem, for a short while in caulescents, longer in rosulates and completely suppressed in unifoliates (Jong 1970; Hilliard & Burtt 1971; Dubuc-Lebreux 1978). Likewise, anisocotyly is not predetermined during embryogenesis, because both embryonic cotyledons are equal in size in mature seeds (Figs 1A, 1B; Imaichi et al. 2000, 2007; Mantegazza et al. 2007; Tononi et al. 2010), and early on after germination (Figs 1C, 1D; Nishii et al. 2004, 2007).

In anisocotylous seedlings, the indumentum and venation of the lamina produced from the basal meristem of the cotyledonary phyllomorph (the macrocotyledon) in *Streptocarpus wendlandii* Sprenger differ from that of the lamina present at the early isocotylous seedling stage (Figs 1E, 1F; Fritsch 1904), and make this species an ideal model to study cotyledonary lamina extension (Nishii et al. 2004); isocotylous seedlings only have seed glandular trichomes (type 1 of Nishii et al. 2004) and short glandular trichomes (type 2 of Nishii et al. 2004; Figs 1E, 1G). In seedlings at the anisocotylous stage, the microcotyledon is unchanged but the macrocotyledon has additionally unbranched multicelled eglandular trichomes (type 3 of Nishii et al. 2004) at its base (Figs 1E, 1H). Lateral veins are also found in this added lamina tissue in the macrocotyledon, but not in the microcotyledon (Fig. 1F; Nishii et al. 2004). Thus, these two features can be used to define the activity of the basal meristem and can be utilised as criteria to identify the macrocotyledon in *S. wendlandii*.

An interplay of lateral and apical dominance can be hypothesised for the different growth forms in *Streptocarpus*. The SAM of the caulescents imposes the strongest apical dominance, and weakest lateral dominance, since the cotyledonary phyllomorphic phase is very short leading to a petioled macrocotyledon showing the absence of the basal meristem activity at that stage (e.g. Dickson 1883; Hill 1938). Conversely, acaulescents (unifoliate and rosulates) exhibit the least apical dominance and strongest lateral dominance (e.g. Hielscher 1883; Fritsch 1904; Rosenblum & Basile 1984). Among the acaulescents, the groove meristem is most extremely suppressed in unifoliates since it does not form new phyllomorphs, only inflorescences late in development (e.g. Jong 1970; Imaichi et al. 2000). In seedlings of the unifoliate *S. wendlandii* consequently, the groove meristem remains flat and undifferentiated (Figs 1I, 1K, 1L), unlike those of the rosulate *Streptocarpus rexii* Lindl., that form additional leaves during the vegetative phase (e.g. Nishii & Nagata 2007).

Several experiments have been carried out to suggest that plant hormones are involved in the establishment of apical and lateral dominance in *Streptocarpus* seedlings, these often involving exogenous applications of plant growth regulating substances; for example, cytokinin treatment of seedlings of acaulescent *Streptocarpus* induced the formation of two cotyledons with macrocotyledon features (Rosenblum & Basile 1984; Nishii et al. 2004; Mantegazza et al. 2009).

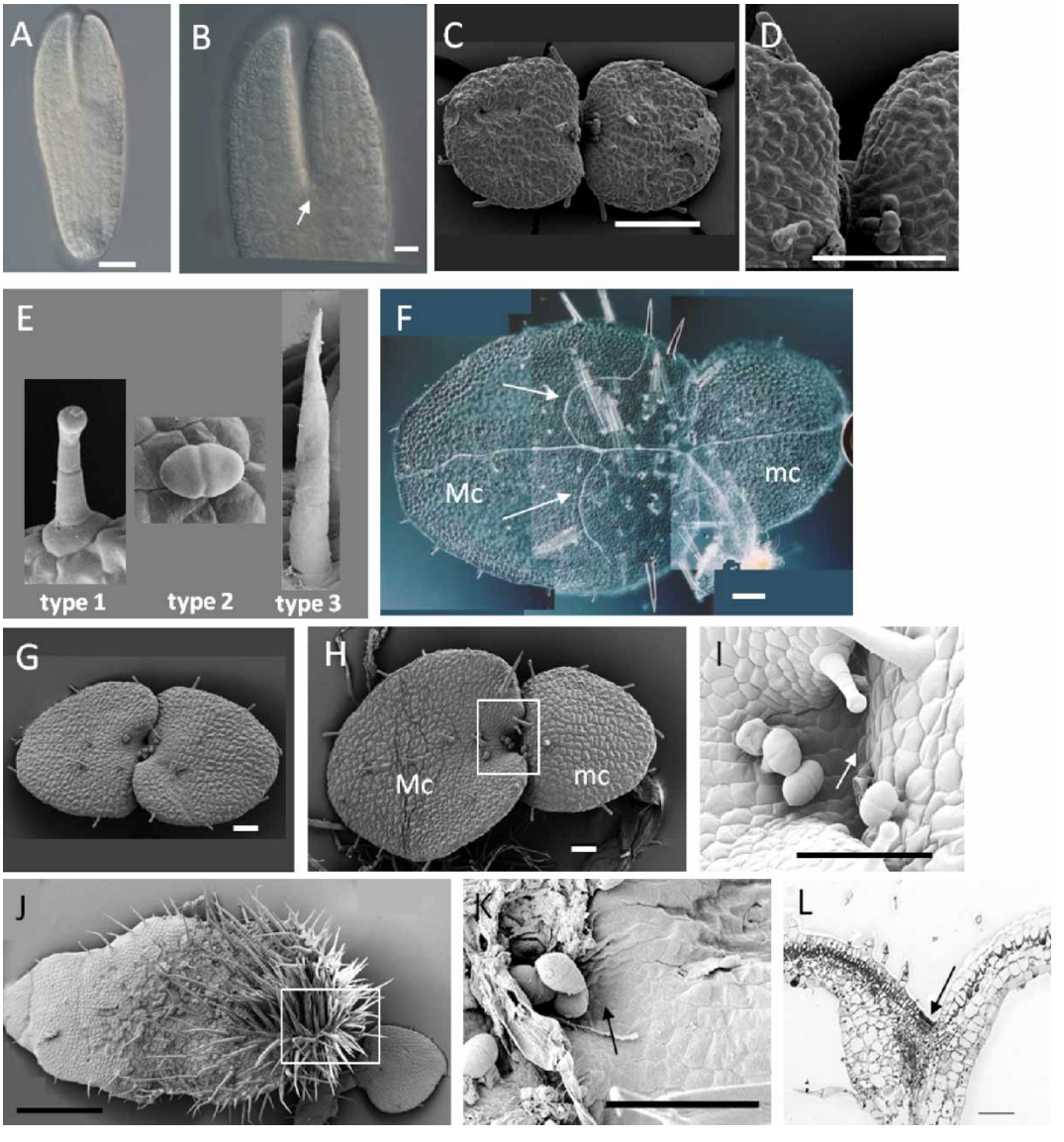

Figure 1 The morphology of mature embryo and early seedling development in *S. wendlandii*. Cleared mature embryo (**A, B**). Scanning electron micrographs (**C–E, G–K**), cleared (**F**) and longitudinal section (**L**) of *S. wendlandii* seedlings at different growth stages. **A,** Mature embryo. **B,** Magnified inset of **A**. No embryonic SAM is observed (arrow). **C,** Isocotylous seedling just after germination, 10 DAI. Both cotyledons are still indistinguishable in size. **D,** Magnified inset of **C**. **E,** Types of trichomes observed on the cotyledons of *S. wendlandii*, following the categories of Nishii et al. (2004). **F,** Cleared anisocotylous seedling 30 DAI. Arrows indicate the lateral veins in the macrocotyledon. **G,** Isocotylous seedling 15 DAI. **H,** Anisocotylous seedling 20 DAI. **I,** Magnified inset of **H**. At this point the rudimentary groove meristem is visible only as a few small cells between the macrocotyledon and microcotyledon (arrow)**. J,** Seedling with 4-mm-long macrocotyledon and a dense indumentum of eglandular hairs defining the added area by the basal meristem activity. **K,** Magnified inset of **J** showing the groove meristem (arrow in **K**). Lamina of proximal region of the macrocotyledon to the right removed. **L,** Longitudinal section of anisocotylous seedling 30 DAI. A flat groove meristem is visible near the base of the macrocotyledon (arrow). Mc, macrocotyledon; mc, microcotyledon. Scale bars: 50 μm (**A**), 20 μm (**B**), 200 μm (**C**), 100 μm (**D, F–I, K, L**), 1 mm (**J**).

It was suggested that cytokinin removes the developmental block from the microcotyledon, imposed by the macrocotyledon. On the other hand, gibberellin treatments prevented the formation of a macrocotyledon in acaulescent species (Rosenblum & Basile 1984; Mantegazza et al. 2009).

Both gibberellin and cytokinin treatments in fact cause the formation of isocotylous seedlings (two microcotyledons with gibberellin, two macrocotyledons with cytokinin) with a groove meristem displaced to a central position between the cotyledons (Rosenblum & Basile 1984; Mantegazza et al. 2009). Seedlings of the plurifoliate *Streptocarpus prolixus* C.B.Clarke, that show a phenotype between unifoliate and rosulate and form a few phyllomorphs from the groove meristem, treated continuously with gibberellin loosely reminisce caulescent plants with several leaves and elongated petioles (Rosenblum & Basile 1984). Whether this elongation is due to increased cell division or cell elongation was not investigated.

In this study, we focus on the anatomical effects of the applications of gibberellin (GA_3) and uniconazol, a gibberellin biosynthesis inhibitor (Nambara et al. 1991), on the anisocotylous seedling development in *S. wendlandii*, because this unifoliate shows the most pronounced features of apical suppression and lateral dominance, and to determine the role of gibberellin in this process. To understand the interactions between gibberellin and cytokinin in this respect, we also assessed the effects of GA_3/BAP co-treatments. We specifically used *S. wendlandii* because the cotyledons of its seedlings show morphological features to easily assess basal meristem activity and macrocotyledon determination, such as the occurrence of trichomes and a branched vasculature only in the macrocotyledon.

Materials and methods

Plant materials

Seeds of *S. wendlandii* were kindly provided by the Kyoto Prefectural Botanical Gardens (Kyoto, Japan) and the Royal Botanic Garden Edinburgh (RBGE; Edinburgh, UK). Mature plants of *S. wendlandii*, *Streptocarpus goetzei* Engl. and *Streptocarpus grandis* N.E.Br. were cultivated at RBGE. After seeds were sterilised with a 0.2% sodium hypochlorite solution that contained 0.02% Nonidet P-40 (Sigma-Aldrich, MO, USA) and washed with distilled water, they were sown in 9-cm plastic Petri dishes on a culture medium consisting of 30% strength Murashige & Skoog (MS) medium (Murashige & Skoog 1962), solidified with 0.8% agarose. Seedlings 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

Treatment with plant hormones was conducted as described in Nishii et al. (2004). Seedlings were transferred just after germination to a medium that contained the respective hormones (see below), and grown on as described above. Germination was here defined as cotyledon unfolding, which occurred about 10–15 days after imbibition (DAI). Control plants, which were germinated at the same time, were incubated on MS medium without hormones. For gibberellin treatments, GA_3, and for cytokinin treatments, 6-benzylaminopurine (BAP) was used. The gibberellin biosynthesis inhibitor uniconazol was also used (all hormones from Wako Pure Chem. Ind. Ltd., Osaka, Japan). Uniconazol inhibits the *ent*-kaurene oxidase that catalyses *ent*-kaurene to *ent*-kaurenoic acid, a precursor of gibberellin (Nambara et al. 1991; Todoroki et al. 2008). The stock solutions of plant hormones were prepared in dimethyl sulfoxide (DMSO, Sigma-Aldrich), which was also added to the medium of the control seedlings.

Seedlings cultured on MS medium containing 10^{-5}, 10^{-6} or 10^{-7} M GA_3 were observed 30 DAI. Seedlings cultured on 10^{-5}M GA_3 were also observed 20 DAI to examine early effects at the isocotylous stage. Seedlings grown on MS medium with 10^{-5} to 10^{-8} M

uniconazol were evaluated 30 DAI. GA_3/BAP co-treated seedlings were cultured on MS medium containing 3×10^{-5}M GA_3 with a combination of 10^{-5}, 10^{-6} or 10^{-7} M BAP, and their morphology observed 50 DAI. The number of seedlings examined for each treatment was as follows; 11 (20 DAI 10^{-5} M GA_3), 10 (20 DAI control, 30 DAI 10^{-5}, 10^{-6}, 10^{-7} M GA_3), 12 (30 DAI control). For assessing the effect of uniconazol, 12 (10^{-5} M), 10 (10^{-6} M), 8 (10^{-7} M), 11 (10^{-8} M), and 8 (control) seedlings were examined. For the GA_3/ BAP co-treatment, 12 (GA_3/10^{-5} M BAP), 8 (GA_3/10^{-6} M BAP) and 10 (GA_3/10^{-7} M BAP) seedlings were used.

Anatomical analyses

Anatomical analyses were carried out as described in Nishii et al. (2004). Briefly, to measure the cotyledon area, images of cotyledons were captured under an Olympus SZX9 dissecting microscope (Olympus Optical Industries Co., Tokyo, Japan). The images were analysed using a graphics program, NIH image (Scion Co., Maryland, USA), and the macro- and microcotyledon area and their ratio calculated.

For observation of cotyledonary leaf surface and vascular pattern, seedlings were fixed in ethanol and acetic acid (4:1). For embryo observations, the seed coat was removed before fixation. The fixed samples were hydrated in an ethanol series then cleared in chloral hydrate. For sectioning, samples were fixed in 5% acetic acid, 45% ethanol, 5% formaldehyde, 45% distilled water (FAA), dehydrated in an ethanol series and embedded in Technovit 7100 resin (Heraeus Kulzer GmbH & Co., KG, Wehrheim, Germany). Sections were prepared as described in Nishii et al. (2004) and stained with Toluidine Blue (Wako Pure Chem. Ind. Ltd., Osaka, Japan). Images were taken with an optical microscope BX51 (Olympus). Nomarski optics was used for cleared samples.

For scanning electron microscopy observations, samples were fixed in FAA as above, dehydrated in an ethanol series and immersed in isoamyl acetate. Following drying and ion-sputter coating, samples were observed with a Hitachi S-2250N SEM (Hitachi, Tokyo, Japan) at the university museum of the University of Tokyo (Japan) or with a LEO Supra 55VP SEM (Zeiss, Cambridge, UK) at RBGE.

Statistical analyses

Data were analysed in Microsoft Office Excel (Microsoft Corporation, WA, USA); cotyledon area, length and width, hypocotyl length, petiole length. A one-way analysis of variance (ANOVA) was performed and *P*-values calculated between the treatments. Values given are treatment means and their standard error of means (SEM).

Results

Embryo morphology

In the mature embryo of *S. wendlandii*, both cotyledons are virtually indistinguishable in morphology and size (length of larger cotyledon: 130.9 ± 3.8 μm, smaller cotyledon: 132.2 ± 3.3 μm, $n = 4$, $P = 0.840$; Figs 1A, 1B).

Effects of GA_3 on the morphology of S. wendlandii *seedlings*

Just after germination (10 DAI) the cotyledons were still equal in size varying byless than 5% (length of larger cotyledon: 310.54 ± 31.24 μm, smaller cotyledon: 296.64 ± 34.49 μm, $n = 4$, $P = 0.775$; Figs 1C, 1D; see also Nishii et al. 2004). At 20 DAI, the control seedlings were still isocotylous and the cotyledon area not different from seedlings treated with GA_3 (10^{-5} M) ($P = 0.692$; Fig. 2A). At 30 DAI, the control seedlings were clearly anisocotylous and the area of the macrocotyledons about three times larger than that of their microcotyledons ($P < 0.001$), whereas in seedlings treated with GA_3 (10^{-7} to 10^{-5} M) the ratio of macro-/microcotyledon area was about 1.5, significantly lower than in control seedlings

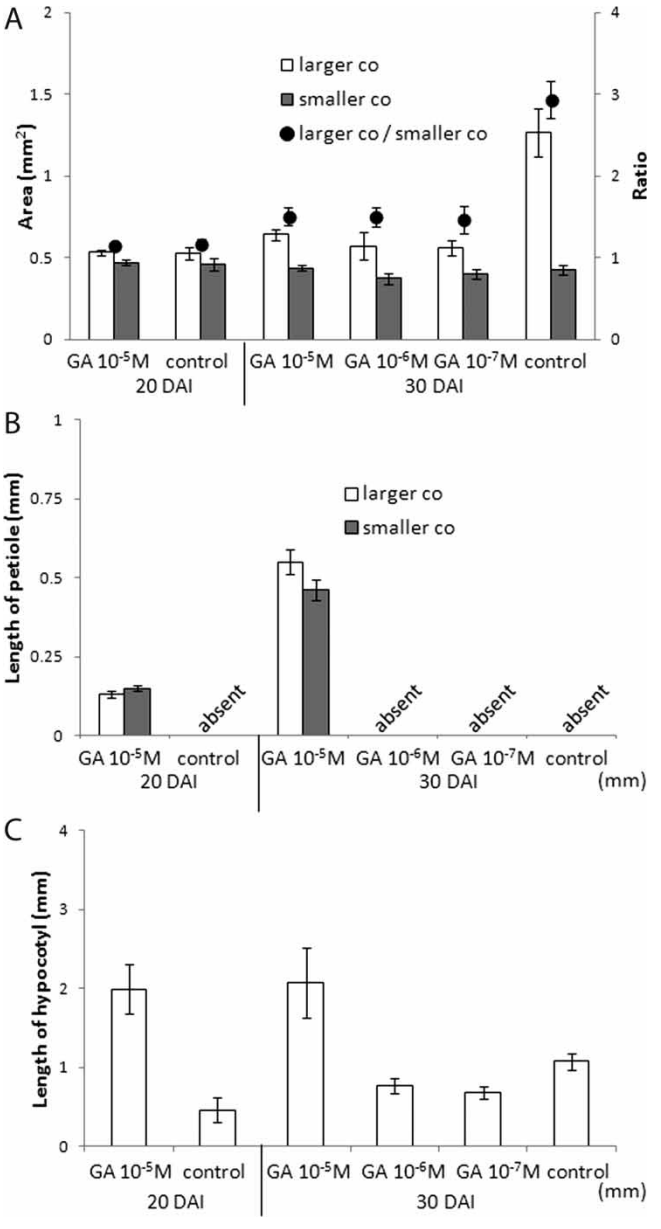

Figure 2 Effect of GA_3 on the macrocotyledon of *S. wendlandii* seedlings 20 and 30 DAI. **A,** Cotyledon area and ratio between larger and smaller cotyledons. □, area of larger cotyledon (co); ■, area of smaller cotyledon (co); ●, ratio between the area of the larger over the smaller cotyledon (co). **B,** Petiole length. Petiole formation was only observed in the seedling treated with 10^{-5} M GA_3. □, length of petiole of larger cotyledon (co); ■, length of petiole of smaller cotyledon (co); absent, no petiole formation was observed. **C,** Hypocotyl length. Hypocotyl elongation is induced by GA_3. Error bars: SEM, $n = 11$ (10^{-5} M GA_3 20 DAI), $n = 10$ (control 20 DAI, 10^{-5} M to 10^{-7} M GA_3 30 DAI), $n = 12$ (control 30 DAI).

($P < 0.001$; Fig. 2A). The lower ratio in GA_3-treated seedlings 30 DAI resulted from a reduced area of the larger cotyledons, the putative macrocotyledons, while the area of the smaller cotyledons was virtually identical in the control and GA_3-treated seedlings ($P > 0.5$; Fig. 2A).

While control seedlings (Figs 2B, 3A, 3B) and seedlings treated with 10^{-6} M or 10^{-7} M GA_3 developed no petioles (Figs 2B, 3B, 3E, 3F), seedlings treated with 10^{-5} M GA_3 possessed distinct petioles (Figs 2B, 3C, 3D, 4C–4E). Seedlings treated with 10^{-5} M GA_3 had greatly elongated cells in the petiole (cell length: 170.60 ± 21.01 μm, $n = 5$), very different from that of cells on the lamina (57.47 ± 2.38 μm, $P < 0.001$; Figs 4D, 4E), and that of cells between the lamina and hypocotyl on the macrocotyledons of control seedlings (14.51 ± 1.69 μm, $P < 0.001$; Figs 1H).

Hypocotyl elongation also showed no significant difference between control and 10^{-6} M or 10^{-7} M GA_3 treatments at 30 DAI ($P > 0.05$; Figs 2C, 3B, 3E, 3F). Seedlings treated with 10^{-5} M GA_3 had hypocotyls that were nearly four times longer at 20 DAI ($P < 0.001$; Fig. 2C), which had not further elongated at 30 DAI (Figs 2C, 3D, 4C). The hypocotyl cell size in control seedlings was 17×23 μm (length × width), whereas that of 10^{-5} M GA_3-treated seedlings 30 DAI was almost nine times longer, 157×20 μm ($P < 0.001$).

The most conspicuous effect of the GA_3 treatment was observed in 10^{-5} M-treated seedlings where 6 out of 10 seedlings formed leaf primordia 30 DAI (Fig. 4F; Table 1), which developed into leaf-like structures 45 DAI (Figs 3G, 3H).

The macrocotyledons of control seedlings possessed three trichome types (Fig. 1E; Nishii et al. 2004), while their microcotyledons had only type 1 and 2 trichomes (Figs 1F, 4A, 4B). With increasing GA_3 concentrations fewer seedlings showed type 3 trichomes on the larger cotyledons 30 DAI, and both cotyledons of 10^{-5} M GA_3-treated seedlings had only type 1 and type 2 trichomes (Fig. 4E; Table 1). In control seedlings only the macrocotyledons formed lateral veins. Increased GA_3 concentrations reduced the formation of lateral veins, and in 10^{-5} M GA_3-treated seedlings none had formed (Table 1).

Effects of uniconazol on the morphology of S. wendlandii *seedlings*

Uniconazol reduced the cotyledon size ratios. In seedlings treated with uniconazol (10^{-5} M to 10^{-8} M) these were between 1.24 ± 0.18 and 1.48 ± 0.31, significantly lower than in control seedlings (3.65 ± 0.75, $P < 0.001$; Fig. 5A). There was no significant difference in the length of epidermal cells on the lamina between control and uniconazol-treated seedlings ($P = 0.854$).

Within the applied range of uniconazol concentrations the formation of petioles was not observed (Figs 6B, 6C). On the other hand, hypocotyl elongation was reduced by uniconazol 50% (10^{-8} M) to 36% (10^{-5} M) compared with the control ($P < 0.001$). The hypocotyl length in seedlings treated with 10^{-5} M uniconazol was slightly but significantly shorter than in 10^{-8} M uniconazol ($P < 0.001$; Fig. 5B).

Macrocotyledon features, such as lateral veins or type 3 trichomes, were observed only in the larger cotyledons, with a tendency of a reduced occurrence with increasing uniconazol concentrations (Table 2). Only two seedlings, at 10^{-6} M and 10^{-7} M uniconazol, formed a few type 3 trichomes also on the microcotyledons. Unlike the result of GA_3 treatments, the formation of new leaf primordia was not observed in the seedlings treated with uniconazol.

Effects of GA_3/BAP co-treatment on S. wendlandii *seedlings*

The morphology of GA_3/BAP co-treated seedlings was analysed 50 DAI to ensure that the leaf formation was fully captured (see 10^{-5} M GA_3 treatment after 45 DAI; Figs 3G, 3H). We used 3×10^{-5} M GA_3 with a combination of

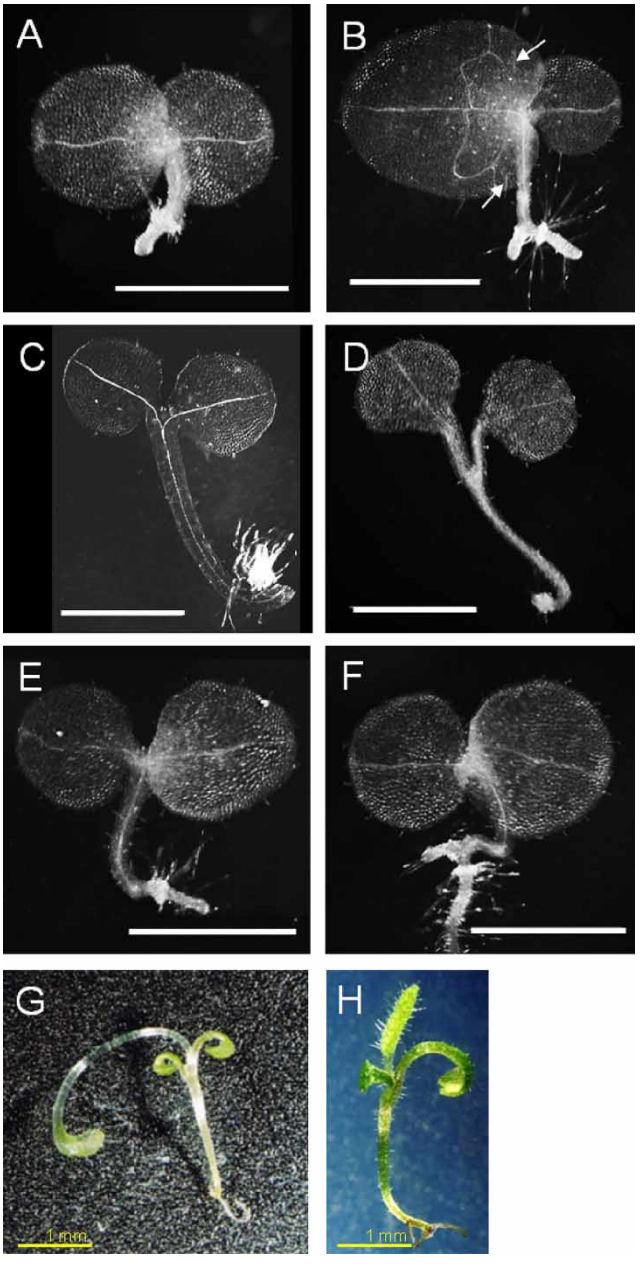

Figure 3 Morphology of untreated and GA_3 treated *S. wendlandii* seedlings. 20 DAI (**A**, **C**), 30 DAI (**B**, **D**–**F**). **A–F**, Cleared seedlings. **A, B**, Control seedlings. **C, D,** Seedlings treated with 10^{-5} M GA_3. **E**, Seedling treated with 10^{-6} M GA_3. **F,** Seedling treated with 10^{-7} M GA_3. Seedlings treated with 10^{-5} M GA_3 (**C**, **D**) showing isocotylous growth without macrocotyledon features, while control seedlings 30 DAI (**B**) show anisocotylous growth with lateral veins and type 3 trichomes (arrows) on the macrocotyledon. **G, H,** Seedlings treated with 10^{-5} M GA_3 at 45 DAI showing leaf formation between cotyledons. Scale bars, 1 mm.

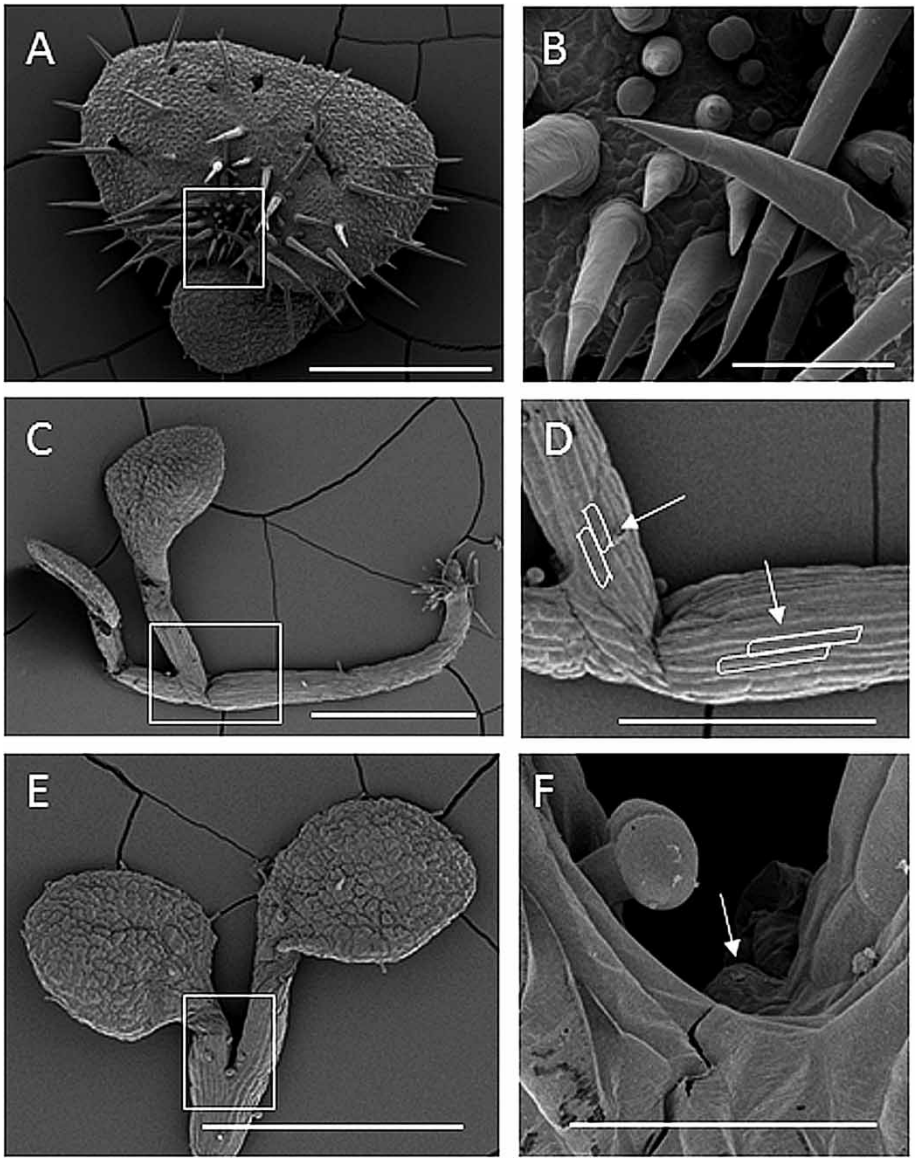

Figure 4 Scanning electron micrographs of untreated and 10^{-5} M GA_3-treated *S. wendlandii* seedlings 30 DAI. **A, B**, Control seedlings showing basal meristem with small cells at the proximal region of macrocotyledon and type 3 trichomes. **B**, magnified area indicated in **A**. **C–F,** Seedlings treated with 10^{-5} M GA_3, showing induced cell elongation on the hypocotyl and petiole (**C**, magnified in **D**). Elongated cell shapes are outlined in **D** (arrows). A leaf primordium is formed between cotyledons (**E**, magnified in **F**, arrow indicates leaf primordium). None of the cotyledons show type 3 trichomes and no basal meristem with small cells (**E**). Scale bars, 1 mm (**A–C, E, F**), 0.5 mm (**D**), 0.2 mm (**B**), 0.1 mm (**F**).

different BAP concentrations, because 3×10^{-5} M GA_3 treated seedlings showed a similar but more stable phenotype compared with seedlings treated with 10^{-5} M GA_3 (K Nishii, pers. obs.). The co-treatment of GA_3/BAP resulted in isocotylous seedlings for all

Table 1 Effect of gibberellin (GA_3) treatment on the seedling development in *S. wendlandii*.

Days after imbibition (DAI)	20 DAI		30 DAI			
Treatments: Effects	GA_3 10^{-5} M	Control	GA_3 10^{-5} M	GA_3 10^{-6} M	GA_3 10^{-7} M	Control
Percentage of seedlings with lateral veins on larger cotyledon	0	30	0	30	50	83
Percentage of seedlings with lateral veins on smaller cotyledon	0	0	0	0	0	0
Number of lateral veins per larger cotyledon (mean ± SEM)	0.0 ± 0.0	0.2 ± 0.1	0.0 ± 0.0	0.4 ± 0.2	0.5 ± 0.2	1.2 ± 0.3
Number of lateral veins per smaller cotyledon (mean ± SEM)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Percentage of seedlings with type 3 trichomes on larger cotyledon	9	30	0	30	60	92
Percentage of seedlings with type 3 trichomes on smaller cotyledon	0	0	0	0	0	0
Percentage of seedlings with leaf primordium	0	0	60	0	0	0

Note: SEM, standard error of the mean.

hormone combinations (ratio from 1.29 at $GA_3/10^{-5}$ M BAP to 1.18 at $GA_3/10^{-7}$ M BAP; Figs 7, 8). The cotyledon size of hormone-treated seedlings was intermediate between that of the micro- and macrocotyledons of control seedlings (Fig. 7A).

None of the GA_3/BAP treatments caused the formation of petioles (Fig. 8). Instead, in seedlings treated with 3×10^{-5} M $GA_3/10^{-7}$ M BAP the lamina of the cotyledons was elongated with a gradual tapering at the proximal end (Figs 7B, 8B).

Seedlings treated with 3×10^{-5} M $GA_3/10^{-7}$ M BAP showed hypocotyls three times longer than the control ($P < 0.001$) and two times longer in 3×10^{-5} M $GA_3/10^{-5}$ M BAP ($P < 0.001$; Fig. 7C). At higher concentrations of BAP (3×10^{-5} M $GA_3/10^{-5}$ M BAP), the cytokinin appeared to counteract the GA_3 effect on hypocotyl elongation. Macrocotyledon features, type 3 trichomes and lateral veins, were observed in both cotyledons of all hormone-treated seedlings (Table 3). Interestingly, none of the seedlings treated with GA_3/BAP formed new leaf primordia between the cotyledons (Fig. 8, Table 3).

Discussion

Gibberellin as a factor for regulating the macrocotyledon formation in S. wendlandii

In the present study, we have shown the effects of hormones on anisocotyly, a phenomenon that is not predetermined during embryogenesis, but established post-germination in seedlings of the unifoliate *S. wendlandii*. In this process, gibberellin can suppress the formation of the macrocotyledon. This is in line with previous findings (Nishii et al. 2004) and not restricted to this species or growth form and has been observed in the plurifoliate *S. prolixus* (Rosenblum & Basile 1984) and rosulate *S. rexii* (Mantegazza et al. 2009). Our studies here revealed the anatomical effects of gibberellin on seedling morphology; since lateral veins and type 3 trichomes in both cotyledons of *S. wendlandii* were absent, GA_3 appeared to have prevented the proliferation of cell division and the establishment and development of a basal meristem. This is supported by the lack of a region of small meristematic cells at the base of the lamina (Figs 4C, 4E), and the presence of a petiole-like structure containing significantly

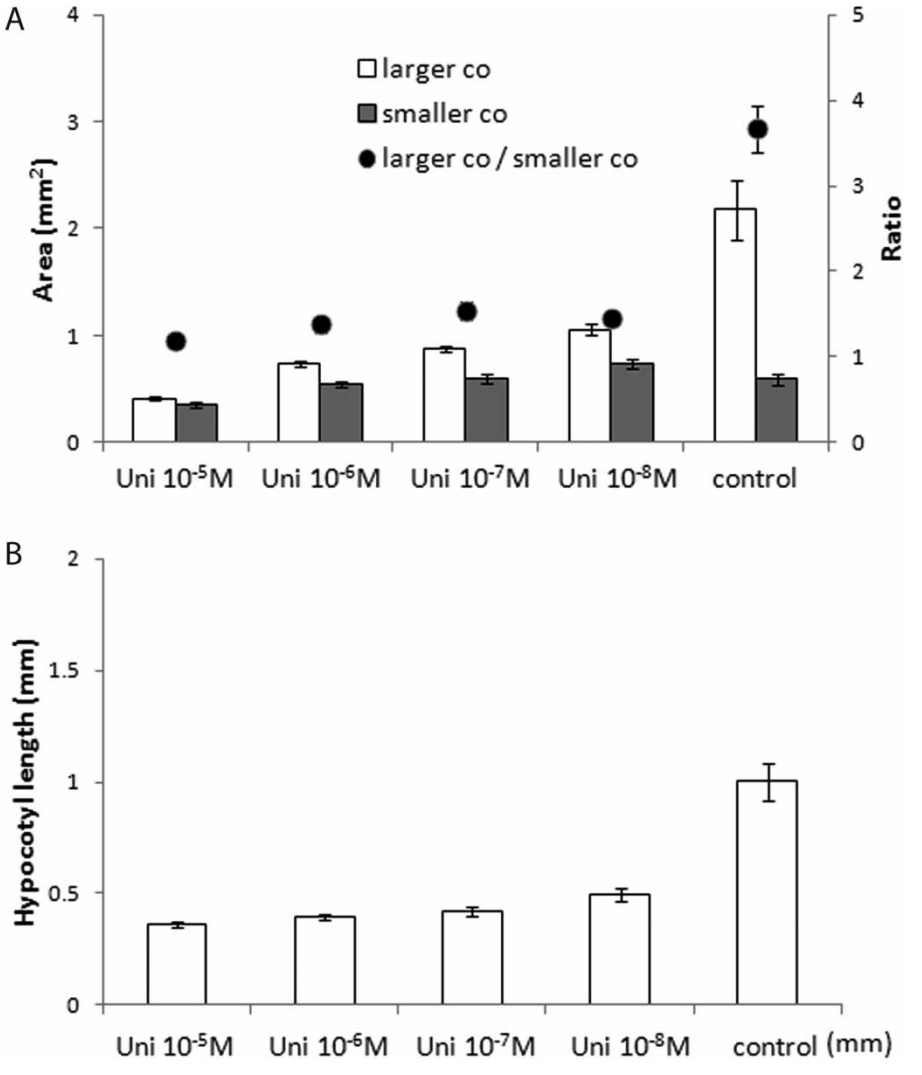

Figure 5 Effect of the gibberellin biosynthetic inhibitor, uniconazol, on *S. wendlandii* seedlings 30 DAI. **A,** Cotyledon area and ratio between larger and smaller cotyledons. □, area of larger cotyledon (co); ■, area of smaller cotyledon (co); ●, ratio between the area of the larger over the smaller cotyledon (co). **B,** Hypocotyl length. Hypocotyl elongation was suppressed by uniconazol. Error bars: SEM, $n = 12$ (10^{-5} M Uni), $n = 10$ (10^{-6} M Uni), $n = 18$ (10^{-7} M Uni), $n = 11$ (10^{-8} M Uni), $n = 8$ (control). Uni, uniconazol at the concentrations indicated.

elongated cells. Similarly, the longer hypocotyls of GA_3 treated seedlings were due to cell elongation. This is a commonly observed effect of gibberellins (Cowling & Harberd 1999). In *S. wendlandii*, GA_3 had an additional effect, combining cell elongation with inhibition of the basal meristem activity, which resulted in the formation of etiolated isocotylous seedlings with petioled cotyledons.

Intriguingly, uniconazol did not cause the formation of typical anisocotylous seedlings (Fig. 6, Table 2). The counteraction of classical gibberellin effects, such as hypocotyl elongation (Fig. 5B) suggests that this compound indeed

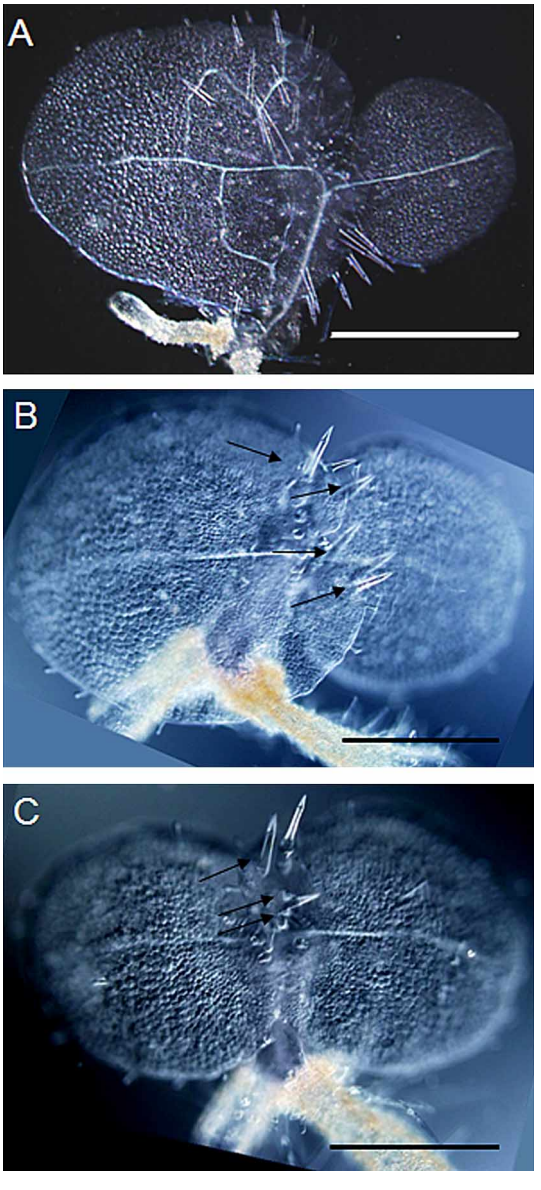

Figure 6 Morphology of untreated and uniconazol-treated *S. wendlandii* seedlings 30 DAI. Cleared seedlings. **A,** Control. **B,** 10^{-6} M uniconazol. **C,** 10^{-5} M uniconazol. Uniconazol suppressed growth of the macrocotyledons, which are characterized by type 3 trichomes (arrows in **B, C**). Scale bars, 0.5 mm.

interfered with gibberellin synthesis in *S. wendlandii*, similar to *Arabidopsis thaliana* (L.) Heynh. (Cowling & Harberd 1999). In *S. wendlandii*, uniconazol caused a reduction in cotyledon size seemingly similar to GA_3. However, while gibberellin caused the formation of two microcotyledons (absence of lateral veins, no type 3 trichomes), uniconazol-treated seedlings still showed one cotyledon with macrocotyledon features, although of much

Table 2 Effects of the gibberellin biosynthetic inhibitor, uniconazol, on the seedling development in *S. wendlandii* (30 DAI).

	Treatments				
Effects	Uniconazol (10^{-5} M)	Uniconazol (10^{-6} M)	Uniconazol (10^{-7} M)	Uniconazol (10^{-8} M)	Control
Percentage of seedling with lateral veins on larger cotyledon	67	70	67	100	100
Percentage of seedling with lateral veins on smaller cotyledon	0	0	0	0	0
Number of lateral veins per larger cotyledon (mean ± SEM)	0.6 ± 0.2	0.5 ± 0.2	0.9 ± 0.2	1.2 ± 0.2	2.6 ± 0.4
Number of lateral veins per smaller cotyledon (mean ± SEM)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Percentage of seedlings forming type 3 trichomes on larger cotyledon	75	80	83	82	100
Percentage of seedlings forming type 3 trichomes on smaller cotyledon	0	10	5	0	0
Percentage of seedlings with leaf primordium	0	0	0	0	0

Note: SEM, standard error of the mean.

reduced size (Table 2). This suggests that uniconazol acted by reducing the meristematic activity of the basal meristem. A comparable result was observed for *S. rexii* with another gibberellin biosynthesis inhibitor, paclobutrazol (Mantegazza et al. 2009), that interferes with *ent*-kaurene oxidase similar to uniconazol (Wang et al. 1986). These results suggest that certain levels of gibberellin are required for a proper development of the basal meristem. It has indeed been shown that the gibberellin biosynthesis genes, *GA20ox-2* in rice or *AtGA20ox* in *A. thaliana* expressed in leaf primordia, are required to control normal leaf growth in *A. thaliana* and rice, respectively (Ashikari et al. 2002; Hay et al. 2002). Excess amounts of gibberellin, by contrast, inhibited the activity of the basal meristem and thus prevent the proliferation of the macrocotyledon.

Cytokinin may act epistatically to control the basal meristem activity in Streptocarpus

In contrast to exogenous gibberellin treatments, cytokinin induced two macrocotyledons in *Streptocarpus* (Rosenblum & Basile 1984; Nishii et al. 2004; Mantegazza et al. 2009; Fig. 9A). Thus, it has been suggested that cytokinin and gibberellins have mutually opposing roles in the macrocotyledon formation in *Streptocarpus* (Fig. 9). To better understand the relationship between these two hormones, we co-applied GA_3 and BAP. The morphology of co-treated *S. wendlandii* seedlings also resulted in two macrocotyledons similar to cytokinin-alone treatments (Figs 7–9; Nishii et al. 2004). This indicated that cytokinin can indeed partly reverse the inhibitory effects of gibberellin, because two macrocotyledons were formed. This was the case even at a low concentration

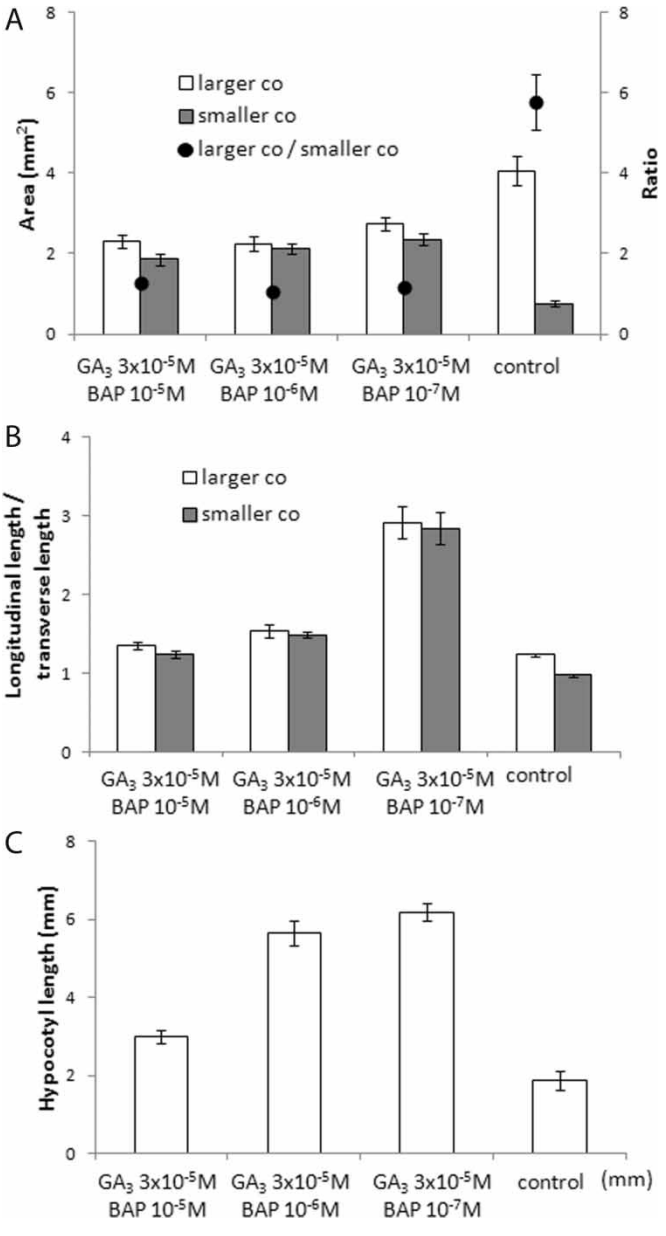

Figure 7 Effect of co-treatment of GA_3/BAP on *S. wendlandii* seedlings 50 DAI. **A,** Cotyledon area and ratio between larger and smaller cotyledons. □, area of larger cotyledon (co); ■, area of smaller cotyledon (co); ●, ratio between the area of the larger over the smaller cotyledon (co). **B,** Cotyledon length/width ratio. Seedlings treated with 3×10^{-5} M $GA_3/10^{-7}$ M BAP show elongated cotyledons compared to the control. □, larger cotyledon (co); ■, smaller cotyledon (co). **C,** Hypocotyl length. At 3×10^{-5} M $GA_3/10^{-5}$ M BAP seedlings had a reduced hypocotyl elongation induced by GA_3. Error bars: SEM, $n = 12$ (3×10^{-5} M GA_3/ 10^{-5} M BAP), $n = 8$ ($-/10^{-6}$ M BAP), $n = 10$ ($-/10^{-7}$ M BAP, control).

Table 3 Effects of co-treatment of gibberellin (GA_3) and cytokinin (BAP) on the seedling development in *S. wendlandii* (50 DAI).

	Treatments			
	GA_3 3×10^{-5} M BAP 10^{-5} M	GA_3 3×10^{-5} M BAP 10^{-6} M	GA_3 3×10^{-5} M BAP 10^{-7} M	Control
Effects				
Percentage of seedlings with lateral veins on larger cotyledon	100	100	100	100
Percentage of seedlings with lateral veins on smaller cotyledon	100	100	100	30
Number of lateral veins per larger cotyledon (mean ± SEM)	5.9 ± 0.3	6.3 ± 0.5	4.4 ± 0.6	4.6 ± 0.6
Number of lateral veins per smaller cotyledon (mean ± SEM)	5.3 ± 0.5	6.0 ± 0.5	3.9 ± 0.7	0.3 ± 0.2
Percentage of seedling with type 3 trichomes on larger cotyledon	100	100	100	100
Percentage of seedling with type 3 trichome on smaller cotyledon	100	100	100	0
Percentage of seedling with leaf primordium	0	0	0	0

Note: SEM, standard error of the mean.

of 10^{-7} M BAP (Fig. 7A, Table 3), which was also the lowest concentration where BAP alone had the same effect in *S. wendlandii* (see Nishii et al. 2004). However, it seemed this BAP concentration was too low to sustain a continued basal meristem activity when applied together with GA_3, as indicated by a tapered proximal part of the cotyledons (Figs 7B, 8B). Notwithstanding the potential pitfalls of exogenous hormone applications and effective concentrations, it is interesting to note that, when GA_3 and BAP were applied together, the effect of the cytokinin on cotyledons of *S. wendlandii* appeared to be epistatic. This suggests that cytokinin may act downstream from gibberellin.

Lateral and apical dominance co-exist in unifoliate Streptocarpus

Unifoliates in Gesneriaceae, such as *S. wendlandii*, display a complex interaction between lateral and apical dominance, or more precisely apical suppression (e.g. Dubuc-Lebreux 1978; Rosenblum & Basile 1984; Tsukaya 1997). They exhibit extreme lateral dominance, with macrocotyledons growing up to 45 cm long (*Streptocarpus dunnii* J.D.Hooker in cultivation up to 1 m; M Möller, pers. obs.) and basal meristems remaining active over more than 6 years (e.g. *Streptocarpus trabeculatus* Hilliard; Hilliard & Burtt 1971). They do not produce additional phyllomorphs and die after flowering and seed set (Hilliard & Burtt 1971). One plausible interpretation of the fact that the groove meristem in monocarpic unifoliates (Figs 1G, 1J) does not produce phyllomorphs (Jong 1970; Imaichi et al. 2000) could be that this meristem is apically suppressed by the basal meristem of the growing macrocotyledon.

In this respect it is noteworthy that gibberellin treatments initiated a phyllomorph from the groove meristem in the unifoliates *S. wendlandii* (this study) or *S. grandis* (Rosenblum 1981). This phenomenon was observed when the basal meristem was strongly suppressed by GA_3 concentrations of 10^{-5} M (Table 1) or higher (K Nishii, pers. obs.).

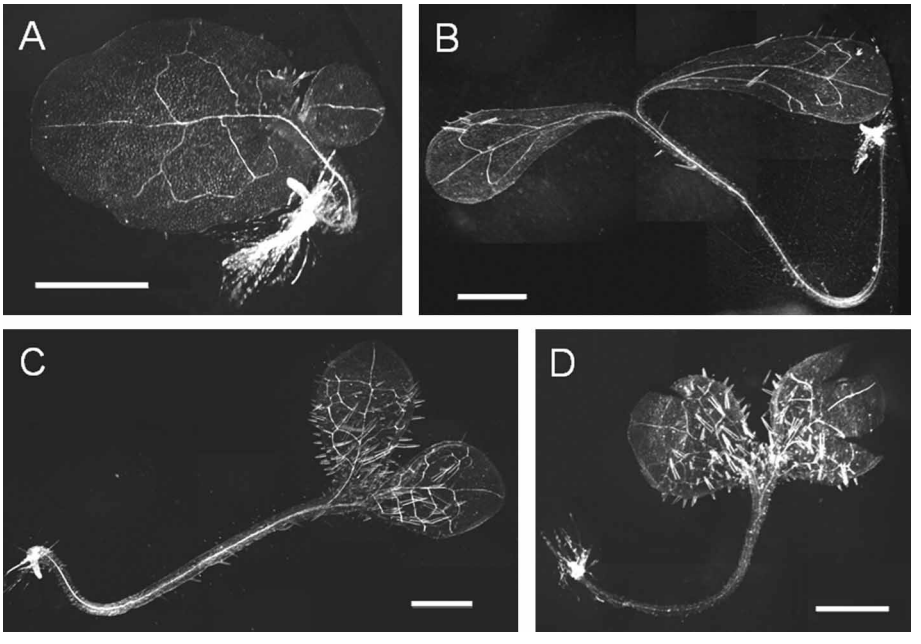

Figure 8 Morphology of *S. wendlandii* seedlings co-treated with GA_3 and BAP, 50 DAI. **A,** Control seedling. **B,** Seedling treated with 3×10^{-5} M GA_3/BAP 10^{-7} M showing tapering lamina. **C, D,** Seedlings treated with 3×10^{-5} M GA_3/BAP 10^{-6} M (**C**) and 3×10^{-5} M GA_3/BAP 10^{-5} M (**D**) showing two cotyledons with macrocotyledon features, such as type 3 trichomes and lateral veins. All seedlings are cleared. Scale bars, 1 mm.

None of the treatments that resulted in even a slight macrocotyledon growth developed a new leaf primordium. This supports the idea that the basal meristem activity suppresses the groove meristem, at least in the unifoliates *S. grandis* and *S. wendlandii* (Fig. 9).

In terms of hormone involvement in the competition between the meristems, i.e. dominances in *Streptocarpus*, the balance between gibberellin and cytokinin appears to be critical; a low ratio (i.e. low gibberellin/high cytokinin) sustains basal meristem activity, and suppresses the groove meristem. A high ratio releases the groove meristem from suppression in this apical dominance scenario (Fig. 9). The phenotypic expression of lateral dominance (anisocotyly), may also be based on differential levels of gibberellin and cytokinin, with a high ratio being present in the microcotyledon and low ratio in the macrocotyledon. How such gradient is established remains at present unknown. The environmental factor such as light (Sauer-egger & Weber 2004) may regulate this physiological pathway, although more detailed experiments are needed here.

In model plants, cytokinin and gibberellin also affect growth dominance (Jacobs & Case 1965; Phillips 1975; Cline 1991). In tobacco, for example, overexpression of the cytokinin biosynthesis gene *ipt* induced lateral shoot growth and reduced apical shoot growth (Faiss et al. 1997; Fig. 9A). On the other hand, overexpression of the gibberellin synthetic genes, *GA3* and/or *GA20 oxidase*, in tobacco plants caused elongated stems due to an increased number of leaves produced (Gallego-Giraldo et al. 2008; Fig. 9A). Thus, gibberellin appears to increase apical dominance, whereas cytokinin increases lateral dominance, similarly in tobacco and *S. wendlandii* (Fig. 9), which might suggest a common mechanism for regulating dominance in the model plant and *Streptocarpus*.

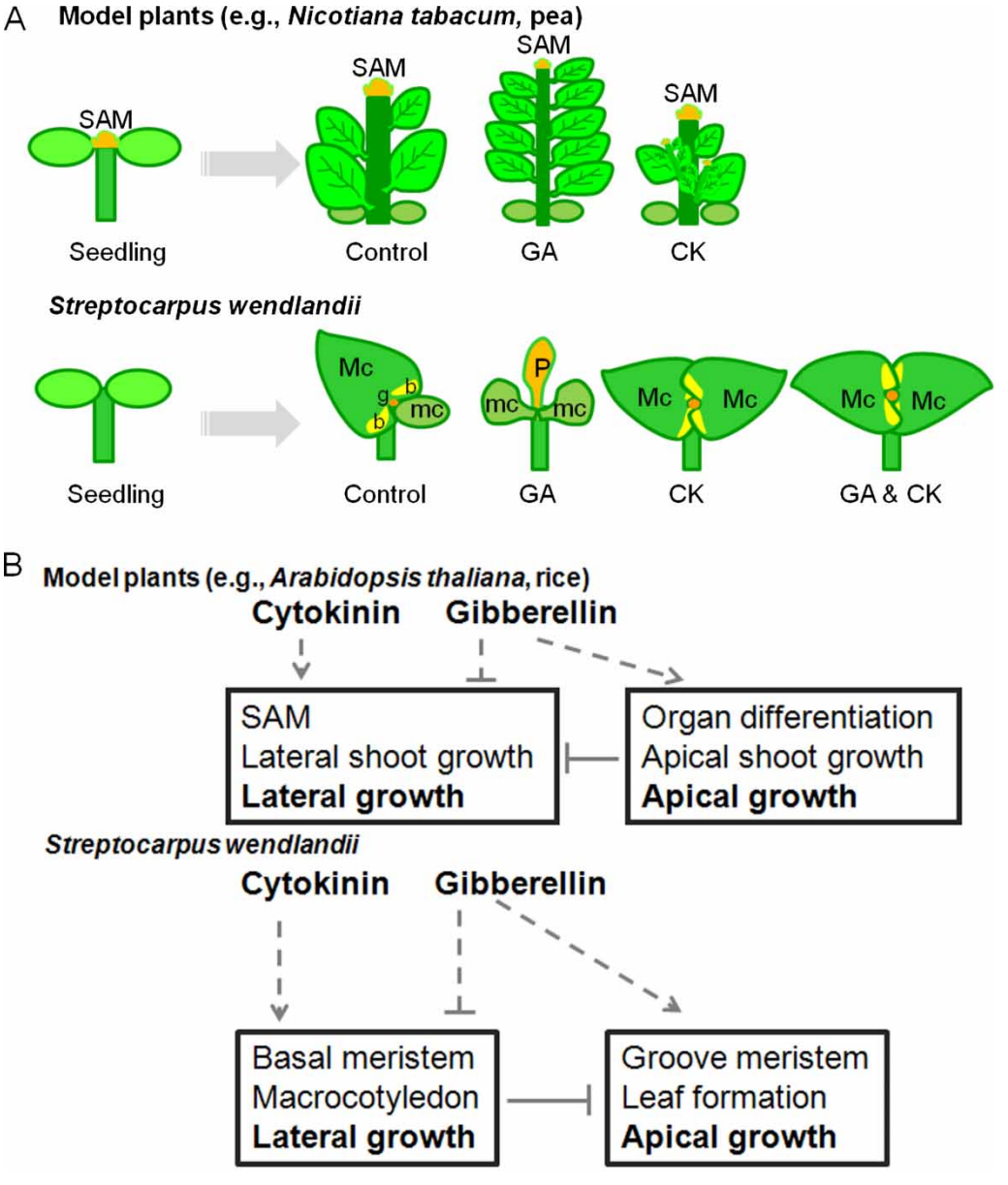

Figure 9 Schematic representations and interpretations of hormone treatment effects in *S. wendlandii* seedlings and ordinary plants. **A,** Schematic illustrations of hormonal effects. Upper: *Nicotiana tabacum* seedling show SAM, and form shoot (control). Plants overexpressing cytokinin and gibberellin synthesis genes (modified from Faiss et al. 1997; Gallego-Giraldo et al. 2008). Lower: *S. wendlandii* do not retain SAM in seedling, but form the macrocotyledon by means of the basal meristem, and retain vegetatively suppressed groove meristem (control). Gibberellin, cytokinin-treated plants, and gibberellin/cytokinin co-treated plant (modified from Nishii et al. 2004; this study). GA, gibberellin; CK, cytokinin; b, basal meristem; g, groove meristem; Mc, macrocotyledon; mc, microcotyledon; P, phyllomorph. **B,** Hypotheses of hormonal regulation of the morphogenesis in unifoliate *Streptocarpus*, in comparison with the suggesting pathway of hormonal regulation of shoot formation in model plants.

In the model plants, such as *A. thaliana* or rice, gibberellin and cytokinin were found to regulate the expression of class 1 *KNOX* homeobox genes (*KNOX1*) that maintain the SAM (Jasinski et al. 2005; Sakamoto et al. 2006). In the rosulate *S. rexii*, *KNOX1* genes are also expressed in the groove meristem and the basal meristem, and external application of gibberellin and cytokinin altered their expression patterns (Harrison et al. 2005; Mantegazza et al. 2007, 2009). Thus, *KNOX1* genes may include candidate genes working downstream of gibberellin or cytokinin for maintaining the meristems of *Streptocarpus*. More molecular genetic studies are required here.

Interplay of dominances in the genus Streptocarpus

Dubuc-Lebreux (1978), Rosenblum (1981) and Rosenblum & Basile (1984) proposed a sequence of events in the expression of growth dominances in *Streptocarpus*, with the unifoliates having the longest sequential intervals, while in caulescents the meristems develop most simultaneously.

In unifoliates the cotyledonary basal meristem is active until flowering, and suppresses the groove meristem throughout its development. In rosulates, the cotyledonary basal meristem is equally active until flowering, but allows the groove meristem to produce phyllomorphs throughout its growth. In caulescent species the phase of cotyledonary basal meristem is very short, then forming a petioled macrocotyledon, and allowing the SAM and shoots to form quickly (Jong 1970).

The gibberellin application to seedlings induced the formation of a phyllomorph in *S. wendlandii*, rather than inflorescences. This developed into a typical phyllomorph when transferred to soil, that flowered later on, and the plants behaved like typical unifoliates (K Nishii, pers. obs.). One explanation could be that at the seedling stage the groove meristem is too underdeveloped and programmed for a vegetative pathway (see Jong 1970). Observations on *Streptocarpus fanniniae* Harv. ex C.B. Clarke suggested that the inflorescence meristem splits off the groove meristem when the plants reached reproductive maturity (Plate 5, No. 2 in Jong & Burtt 1975). Meanwhile, Dubuc-Lebreux (1978) observed that the unifoliate *S. wendlandii* induced additional phyllomorphs towards the end of their flowering. We widely observed this phenomenon in unifoliate *Streptocarpus* in cultivation (e.g. *S. wendlandii*, *S. goetzei*, *S. grandis*; Fig. 10). The accessory phyllomorph formed at the base of the acropetally extending inflorescence series on the petiolode near the lamina, i.e. at the proximal, developmentally older end of the inflorescence series. Inflorescences were also formed in the proximal region of the accessory phyllomorph (Fig. 10). With the elongated petiolode, and when more than one flowering accessory phyllomorph is formed, the plants then appeared similar in phenotype to some basal species of acaulescent rosulate *Streptocarpus* (Möller & Cronk 2001), such as *S. bullatus* Mansf. or *S. davyi* S.Moore (Hilliard & Burtt 1971).

It seems, therefore, that in unifoliates the groove meristem remains intact but is released towards the end of the flowering season. It is interesting at this point that when applied to 9–14-month-old *S. wendlandii* plants, gibberellin was found to increase the number of accessory phyllomorphs (Dubuc-Lebreux 1978), further supporting the role of gibberellin as an inducer and modifier of apical growth in *Streptocarpus*.

Conclusions

In conclusion, we demonstrated that the application of exogenous gibberellin to seedlings of the unifoliate *S. wendlandii* inhibited the macrocotyledon formation through the suppression of its basal meristem activity and induced the initiation of a leaf primordium. On the other hand, cytokinin alone or co-applied with gibberellin induced the basal meristem activity in both cotyledons, and no leaf primordia developed. Thus, gibberellin is a suppressor of lateral growth and an inducer of apical growth in the

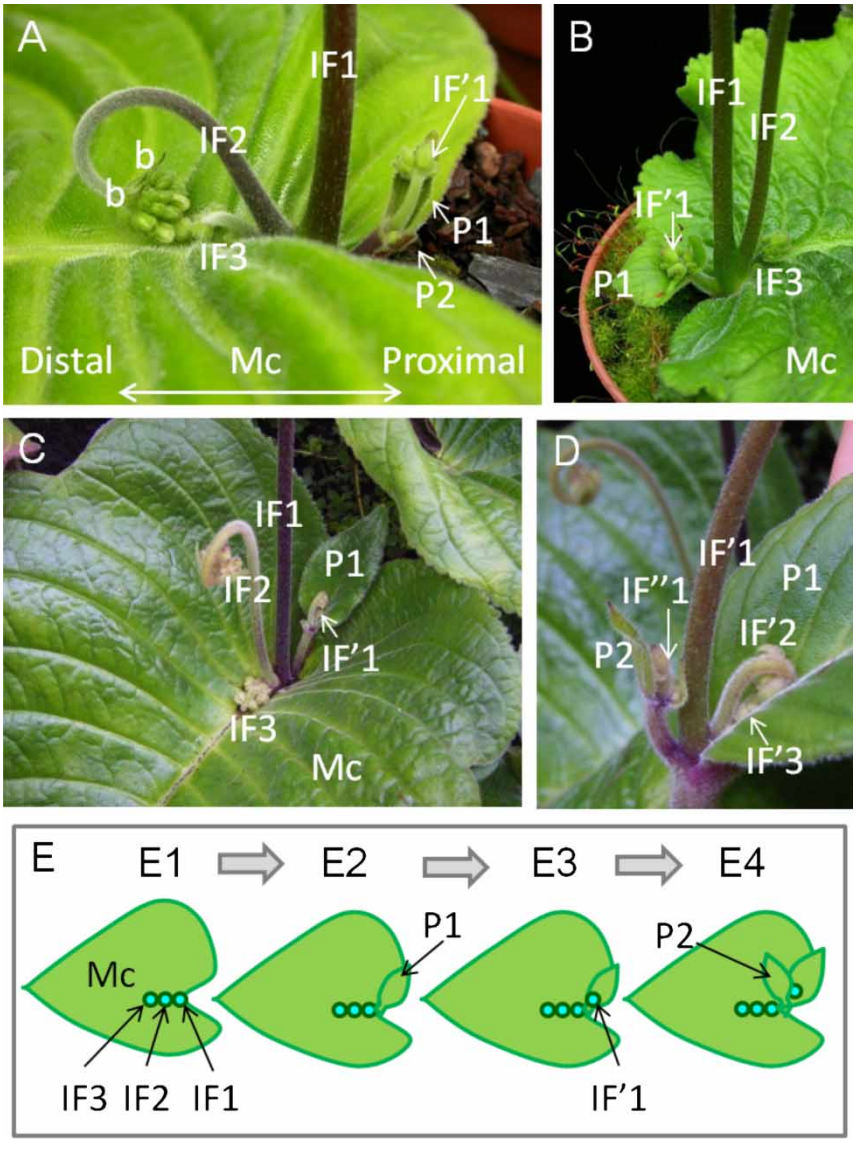

Figure 10 Developmental succession of inflorescences and accessory phyllomorphs in unifoliate *Streptocarpus*, showing the order of first to third inflorescence initiation (IF1 to IF3) on the midrib in acropetal succession towards the distal end of the lamina. An additional inflorescence develops out of developmental sync in front of the oldest inflorescence, subtended by 'the accessory phyllomorph' (*sensu* Dubuc-Lebreux 1978). **A,** *S. wendlandii*. **B,** *S. goetzei*. **C, D,** *S. grandis*. **E,** Schematic illustration of the inflorescence and accessory phyllomorph formation in unifoliate *Streptocarpus*. **E1,** Inflorescences (closed circles) form at the base of the macrocotyledon in acropetal succession. **E2,** First accessory phyllomorph forms at the base of the macrocotyledon. **E3,** Additional inflorescences are formed at the base of first accessory phyllomorph. **E4,** Second accessory phyllomorph forms at the base of first accessory phyllomorph. Mc, macrocotyledon; IF, inflorescences; b, bracts; P, accessory phyllomorph; IF′, additional inflorescences subtended by the first accessory phyllomorph; IF″, additional inflorescences subtended by the second accessory phyllomorph.

unifoliate *S. wendlandii*. Regulatory differences, i.e. hormone levels, and sensitivity to these hormones during plant development may explain the existence of the different growth forms found in the genus *Streptocarpus*. Further evolutionary developmental studies may reveal the significance of genes involved in the hormone cascade linked to the establishment of plant diversity.

Acknowledgements

We thank Hideaki Ohba (University of Tokyo, Japan) and Frieda Christie (RBGE, UK) for supporting the microscopy work. We are grateful to Sadie Barber and Steve Scott (horticulture division, RBGE) for growing plant material. We thank Asuka Kuwabara (ETH Zürich, Switzerland) for her helpful advice. *S. wendlandii* seeds were kindly gifted by the Kyoto Prefectural Botanical Gardens (Japan). This study was initially supported by the Japan Society for the Promotion of Science, also by the National Science Council of Taiwan [grant number NSC97-2811-B-002-027, 99-2923-B-002-007-MY2], the top 100 research program of the National Taiwan University [96R0044, 96R8044], and the RBGE Sibbald Trust. RBGE is supported by the Rural and Environment Science and Analytical Services division (RESAS) in the Scottish Government.

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