Identification of Factors that Cause Heterophylly in *Ludwigia arcuata* Walt. (Onagraceae)

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Abstract: **The submerged leaves of *Ludwigia arcuata* are much narrower than the terrestrial leaves. Such heterophyllous changes in leaf shape have been observed in other aquatic angiosperms, such as *Callitriche heterophylla, Hippuris vulgaris* and *Ranunculus flabellaris*, but the cause of the formation of heterophyllous leaves in *L. arcuata* seems to be quite different. In contrast to other species, in which the shapes of epidermal cells have been changed, the change of leaf shape in *L. arcuata* was found to be due to changes in the numbers of epidermal cells aligned in transverse sections. The susceptibility of leaves to changes in shape above and below the water is dependent on the developmental stages: leaves younger than the fourth leaf responded to a change in environment, while leaves older than the fifth leaf did not. Treatment with ACC (1-aminocyclopropane-1-carboxylic acid), a precursor to ethylene, induced the formation of submerged-type leaves on terrestrial shoots, implying that ethylene might be the endogenous factor responsible for the change in leaf shape. The results are discussed with reference to the significance of the acclimation of plants to environmental changes.**

Key words: **Ethylene, heterophylly, leaf morphology, *Ludwigia arcuata*, submergence.**

Abbreviations:

ABA:	abscisic acid
ACC:	1-aminocyclopropane-1-carboxylic acid
BL:	brassinolide
DMSO:	dimethyl sulfoxide
GA_3:	gibberellic acid
LN:	leaf number
P:	primordium number
S-type:	submerged type
T-type:	terrestrial type

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Introduction

Plants that thrive near the water, and are occasionally submerged by flooding, can grow under water as well as under terrestrial conditions. Such plants often exhibit heterophylly, with the leaves on submerged shoots having quite a different shape from that of leaves on terrestrial shoots. Changes in leaf form are considered to represent acclimation to environmental changes. A number of unrelated species exhibit heterophylly upon submergence, and heterophylly in *Ranunculus flabellaris* (Ranunculaceae), *Callitriche heterophylla* (Callitrichaceae) and *Hippuris vulgaris* (Hippuridaceae) has been studied in detail. In *R. flabellaris,* the leaves on terrestrial shoots are short, with smaller surface area, a thick well-developed layer of mesophyll cells, and numerous stomata on leaf surfaces. These characteristics are supposed to be of benefit in the drier and brighter above-water environments. In contrast, submerged leaves are thin and elongated, with a larger leaf surface area and no stomata (Young et al., 1987[32]; Bruni et al., 1996[5]). Leaves with these attributes are thought to have less resistance to water flow, and are suited to absorb water and nutrients, and for harvesting the lower light levels under the submerged condition. This type of heterophylly has been reported in other species, such as *C. heterophylla* (Deschamp and Cooke, 1984[8], 1985[9]), *Callitriche intermedia* (Jones, 1955[15]), *H. vulgaris* (McCully and Dale, 1961[20]; Goliber and Feldman, 1990[13]), *Potamogeton nodosus* (Anderson, 1978[1], 1982[2]; Gee and Anderson, 1998[10]), *Proserpinaca palustris* (Wallenstein and Albert, 1963[30]), and *Ranunculus aquatilis* (Cooke, 1969[6]).

Attempts have been made to identify the factors responsible for such heterophyllous changes in aquatic angiosperms, focusing on the environmental factors that affect leaf shape. In addition to submergence, various environmental factors, such as temperature, relative humidity, the level of ambient CO_2, and the ratio of red to far-red light, appear to affect the formation of atypical leaves on such heterophyllous aquatic angiosperms. For example, submerged type (S-type) leaves are produced at high relative humidity on terrestrial shoots of *C. heterophylla* (McComb, 1965[19]), *H. vulgaris* (McCully and Dale, 1961[20]), and *R. flabellaris* (Deschamp and Cooke, 1984[8]). Low temperature or an abbreviated light period induces the formation of S-type leaves on terrestrial shoots of *R. flabellaris* (Johnson, 1967[14]), *Proserpinaca palustris* (Wallenstein and Albert, 1963[30]) and *Proserpinaca intermedia* (Kane and Albert, 1982[16]). In *H. vulgaris,* light at lower than normal intensity in-

duces S-type leaves on terrestrial shoots. Terrestrial type (T-type) leaves appear at high osmotic pressure on submerged shoots of *C. heterophylla* (Deschamp and Cooke, 1984[8]), *C. intermedia* (Jones, 1955[15]), and *H. vulgaris* (McCully and Dale, 1961[20]). Similar changes are also induced by growing plants at elevated temperatures in the case of *C. heterophylla* (Deschamp and Cooke, 1984[8]), *Proserpinaca intermedia* (Kane and Albert, 1982[16]), *Proserpinaca palustris* (Kane and Albert, 1987 b[18]), and *R. flabellaris* (Johnson, 1967[14]). In *H. vulgaris,* strong light and a low ratio of red to far-red light induces such changes (Bodkin et al., 1980[4]; Goliber, 1989[11]). It is difficult to identify the mechanisms responsible for heterophylly by examining the effects of these environmental factors. An investigation of endogenous factors that cause heterophyllous changes seems critical to a full understanding of heterophylly.

The involvement of plant hormones as intermediate factors in the development of such heterophylly has been reported. Treatment with abscisic acid (ABA) leads to the formation of T-type leaves in various species, including *R. flabellaris* (Young and Horton, 1985[31]; Young et al., 1987[32], 1990[33], 1995[34]), *H. vulgaris* (Kane and Albert, 1987 a[17]; Goliber and Feldman, 1989[12], 1990[13]), *C. heterophylla* (Deschamp and Cooke, 1983[7], 1984[8], 1985[9]), *Limnophila indica* (Mohan Ram and Rao, 1982[22]), *Potamogeton nodosus* (Anderson, 1978[1], 1982[2]; Gee and Anderson, 1998[10]), and *Proserpinaca palustris* (Kane and Albert, 1987 b[18]). The levels of endogenous ABA in terrestrial shoots of *H. vulgaris* are higher than those in submerged shoots. However, upon submergence, endogenous levels of ABA decline, and S-type leaves develop (Goliber, 1989[11]; Goliber and Feldman, 1989[12]). Thus, it seems likely that ABA might be an endogenous regulator that induces the formation of T-type leaves in the natural environment. There are a few reports of endogenous factors that induce the development of S-type leaves on terrestrial shoots. For example, exogenously applied GA_3 induces S-type leaves on terrestrial shoots of *C. heterophylla* (Deschamp and Cooke, 1983[7], 1984[8]), and treatment with 1-aminocyclopropane-1-carboxylic acid (ACC, a precursor to ethylene) induces the formation of S-type leaves on terrestrial shoots of several species in the genus *Myriophyllum* (Haloragidaceae) (Aoki, 1985[3]). While the conversion of T-type leaves to S-type leaves upon exposure to plant hormones has been examined in some detail, anatomical and physiological analysis remains to be conducted. The leaves of *Ludwigia arcuata* Walt., which are easily induced to undergo heterophyllous changes, were used in the physiological analysis as part of an attempt to identify the causative factors of heterophylly. We found that ethylene appears to play an important role in the production of S-type leaves on terrestrial shoots of *L. arcuata.* The significance of this observation is discussed in relation to the acclimation of plants to different environments.

Materials and Methods

Plant material

Plants were purchased from a local market in Tokyo, Japan, and identified taxonomically as *L. arcuata* by Dr. Ching-I Peng, Herbarium, Institute of Botany, Academia Sinica, Taipei, Taiwan. Materials used in the present study were clones derived from a single plant. To maintain the vegetative growth of stock cultures, 3-cm long cuttings, taken from the apical parts of terrestrial shoots, were rooted and transplanted at weekly intervals. They were watered daily with 1000-fold diluted Hyponex (Hyponex Japan, Osaka, Japan) prepared with tap water. The stock solution of Hyponex contains 5% of total amounts of nitrogen (v/v), 10% of water-soluble phosphate (v/v), and 5% of potassium (v/v). Both stock cultures and physiological experiments were carried out under the same culture conditions, namely, in a growth chamber at 27 °C under continuous light at 80 μmol m^{-2} s^{-1}. Stock cultures were grown for four weeks and 3-cm long apical parts were used for physiological analysis.

Experimental conditions

The youngest primordium that was recognizable under a dissecting microscope (Olympus SZH; Olympus Optical Co., Ltd., Tokyo, Japan) at the start of each experiment, was designated LN 1 (leaf number one). The older leaves were numbered basipetally from LN 1, and leaves that emerged after LN 1 were designated LN-1, LN-2, and so forth (Fig. **2A**). After marking of leaves up to LN 6 or LN 8, plants were disinfected as described by Kane and Albert (1987 a[17]) with slight modifications. The plants were immersed for 14 min, in an aqueous solution of 14% (v/v) sodium hypochlorite that contained 0.01% (v/v) Tween-20. Then they were rinsed in sterile deionized water. The basal medium for cultures contained 65% strength Murashige & Skoog mineral salts (Murashige and Skoog, 1962[23]) and 1% (w/v) sucrose. It was adjusted to pH 5.7 with 1 N KOH before autoclaving and solidified with 0.9% (w/v) agar (Ina Agar, Funakoshi Co. Ltd., Tokyo, Japan). Four disinfected apical cuttings were cultivated in a 300 ml Erlenmeyer flask that contained 80 ml sterile basal medium. When shoots were grown under submerged conditions, 220 ml sterile deionized water was added above the solidified basal medium to generate submerged conditions. When shoots were grown under terrestrial conditions, 5 ml sterile deionized water was added above the solidified basal medium to maintain the relative humidity at 100%.

Treatment with plant hormones

We examined the effects of two plant hormones, namely, GA_3 (10^{-4} and 10^{-5} M), and BL (10^{-6} M). We also examined the effects of ACC (10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} M), a precursor to ethylene and ethylene gas released from 500 mg/l of 2-chloroethylphosphonic acid (Ethephon Standard) at neutral pH. The concentration of ethylene gas released from 500 mg/l of Ethephon Standard was 84.3 ± 18.9 ppm under this experimental condition, which was measured by gas chromatography (GC 390B, GL Sciences Inc., Tokyo, Japan). Solution of plant hormones, ACC, and Ethephon Standard were sterilized by passage through a 0.22 μm filter (Millipore Corp., Bedford, MA, USA). The basal medium and the sterile deionized water above the basal medium contained the desired concentration of each plant hormone. Plants were grown for three weeks on the basal medium with plant hormones or ACC. When treated with 10^{-3} M ACC, plants were grown for four weeks. BL was dissolved in 0.1% dimethyl sulfoxide (DMSO), but we confirmed in preliminary experiments that DMSO did not affect leaf form in any way (data not shown). BL was purchased from Fuji Chemical Industries, (Toyama, Japan), and other chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Fig. 1 Gross morphology of *L. arcuata* after cultivation under various experimental conditions for three weeks. (**A**) A terrestrial shoot with T-type leaves. (**B**) A submerged shoot with S-type leaves on the upper portion. (**C**) Terrestrial shoots after treatment with 10^{-4} M ACC for three weeks. Note the narrow S-type leaves on the secondary branches. (**D**) A terrestrial shoot after treatment with 10^{-6} M BL for three weeks. Note the petiole-like structures (arrowheads) that are not seen on any of the other shoots. Scale bar = 1 cm.

Quantification of the heterophyllous changes

We used the ratio of leaf length to width for assessments of changes in leaf shape. Then, narrow S-type leaves had larger values of the ratio of leaf length to width. One leaf from a pair of leaves at a node was used for assessment of leaf shape, and the other leaf was used for anatomical analysis. After fixation of leaves in FAA [5% (v/v) formaldehyde, 5% (v/v) acetic acid, and 45% (v/v) ethanol], the maximum length and maximum width of each leaf were measured with a digital calibrator (Mitsutoyo Corp., Tokyo, Japan). Photographs of shoots with altered leaf shapes were taken after fixation for 1 h of leaves in a solution of 7.2% (v/v) formaldehyde, 10% (v/v) DMSO and 0.1% (v/v) Nonidet-P40 (Siguma, St. Louis, USA) in 50 mM sodium phosphate buffer (pH 7.0).

Anatomy

LN-2 leaves that had been newly generated under the appropriate experimental conditions were fixed in FAA. For observations of layers of epidermal cells, leaf tissues were decolourized by immersion in a mixture of ethanol and acetic acid (7:1) for 1 h. Then they were rinsed twice with 99% (v/v) ethanol. The decolourized tissue was hydrated by passage through an ethanol series (90%, 70%, 50%, 30%, and 10%, v/v), for 20 min each, and then immersed in distilled water for 20 min. Hydrated tissue was cleared by immersion for 10 min in an aqueous solution of chloral hydrate (200 g of chloral hydrate and 20 g of glycerol in 50 ml of distilled water) as described by Tsuge et al. (1996[28]). For observations of transverse sections, fixed leaves were embedded in Technovit 7100 resin (Kulzer & Co. GmbH, Wehrheim, Germany) and sectioned as described by Tsukaya et al. (1993[29]). Samples were cut from the centre of leaf blades with 5 μm or 7 μm sections. The sections of leaf tissues were stained with a 0.1% (w/v) solution of toluidine blue in 0.1 M sodium phosphate buffer (pH 7.0) for 1 min. For anatomical analysis, all samples were observed under a light microscope (IMT-2 or BH-2; Olympus Optical Co., Ltd., Tokyo, Japan).

Developmental analysis

Shoots that had been grown under terrestrial or submerged condition for four weeks, with stable expression of each phenotype, were used for developmental analysis. The youngest primordium on a submerged or terrestrial shoot was designated as P1 (leaf primordium no. 1). The other primordia and young leaves were designated, basipetally, P2, P3, and so forth (Fig. **5 A**). Samples were cut into 5 μm sections, and stained as described above. The number of adaxial epidermal cells, aligned in transverse sections, was counted under a light microscope (BH-2, Olympus Optical Co.) in the region where the number was maximal in each leaf.

Results

Characterization of heterophyllous changes in L. arcuata

Leaves produced under submerged conditions were narrower than those produced under terrestrial conditions (Figs. **1 A, B**). Compared to terrestrial leaves, submerged leaves had a higher ratio of leaf length to width with lower densities of stomata

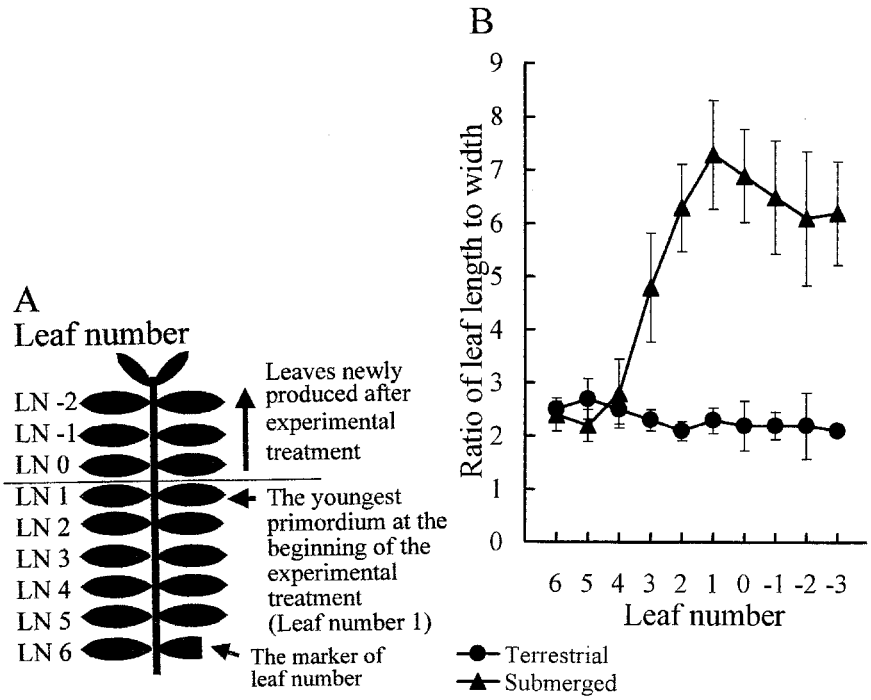

Fig. 2 Quantification of heterophyllous changes. (**A**) A diagram showing the designation of leaf numbers. The youngest leaf primordium is designated LN 1. (**B**) Comparison of leaf shape between terrestrial and submerged leaves: The ratio of leaf length to width was determined for the specified leaves. Symbols and bars represent means ± SD. For each measurement 20 leaves were collected from different plants of the clone.

Table 1 Comparison of numbers of adaxial epidermal cells and stomatal densities on terrestrial and submerged leaves and of leaves on terrestrial shoots after treated with 10^{-4} M ACC for three weeks

Conditions	Number of epidermal cells aligned in transverse sections (adaxial side)	Stomatal density on adaxial side (/mm²)	Stomatal density on abaxial side (/mm²)
Terrestrial	177.3 ± 22.8	329.1 ± 48.1	158.6 ± 35.7
Submerged	90.1 ± 16.2	133.0 ± 26.9	47.5 ± 11.4
Terrestrial, 10^{-4} M ACC	73.4 ± 9.9	144.0 ± 27.6	28.6 ± 22.4

The data for terrestrial and submerged leaves were obtained from LN-2 leaves. The data for leaves treated with 10^{-4} M ACC were obtained from LN-4 leaves. Data represent means ± SD. For each measurement 20 leaves were collected from different plants of the clone.

and smaller numbers of epidermal cells aligned in transverse sections (Table **1**). Leaves that had been newly produced under submerged conditions (LN 0 to LN-3) had a higher ratio of leaf length to width, ranging from 6 to 7, whereas those of terrestrial leaves were close to 2 (Fig. **2B**). Leaves older than LN 5 or LN 6 did not respond to the treatments. However, the primodia and young expanding leaves (LN 1 to LN 4) present before the start of submergence did respond by changes in shape (Fig. **2B**). In contrast to the dramatic change in leaf shape, there were no distinct differences in terms of the shape of epidermal cells between terrestrial and submerged leaves (Figs. **3A,B**). Densities of stomata on submerged leaves were significantly lower than those on terrestrial leaves, on both sides of leaves (Table **1**).

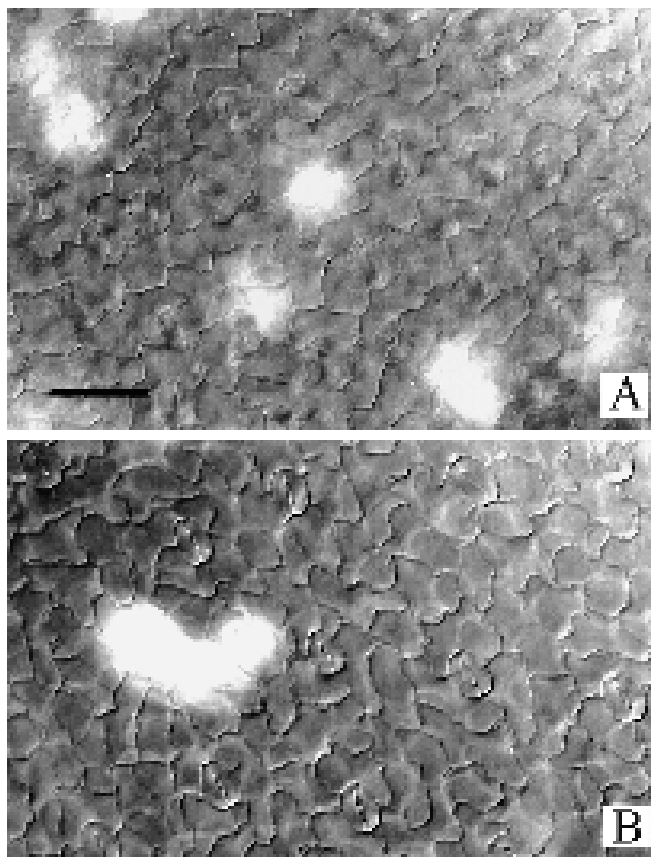

Fig. 3 Adaxial epidermal cells viewed with Nomarski optics. Details of a typical T-type leaf on a terrestrial shoot (**A**) and a typical S-type leaf on a submerged shoot (**B**) are shown. Scale bar = 20 µm.

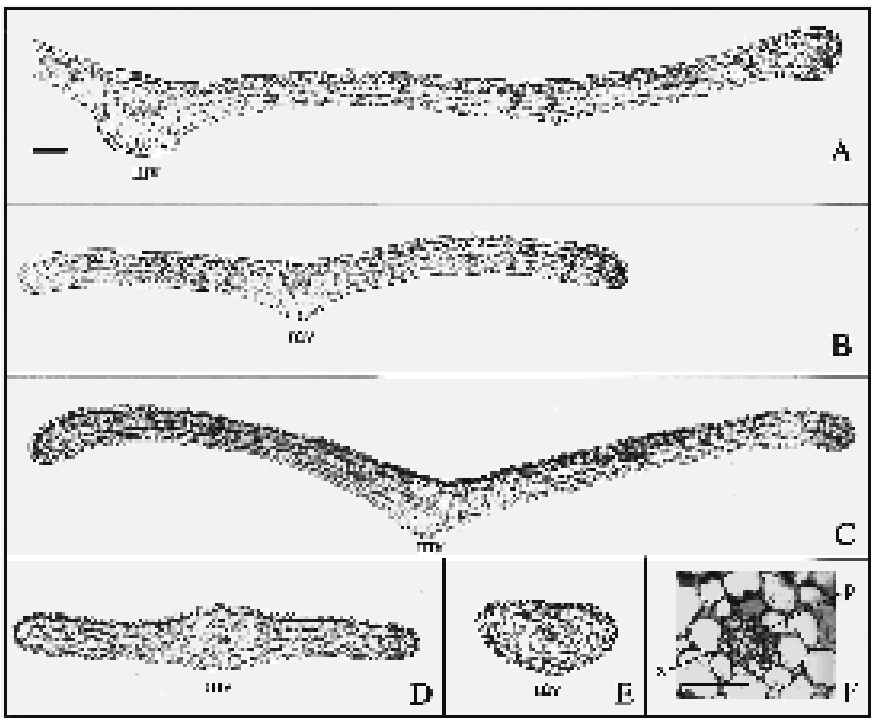

Fig. 4 Transverse sections of leaves. (**A**) A typical T-type LN-2 leaf on a terrestrial shoot. (**B**) A typical S-type LN -2 leaf on a submerged shoot. (**C**) A LN-2 leaf on a terrestrial shoot after treatment with 10^{-5} M ACC for three weeks. (**D**) A LN-5 leaf on a terrestrial shoot after treatment with 10^{-4} M ACC for three weeks. (**E**) A leaf on a terrestrial shoot after treatment with 10^{-3} M ACC for four weeks. Note the lacking of the leaf blade. (**F**) Vascular bundle of the leaf on a terrestrial shoot after treatment with 10^{-3} M ACC for four weeks (enlarged view of part of the sample in **E**). The adaxial surface of the leaf is uppermost. Mv, Mid vein; p, phloem; and x, xylem. Scale bars = 100 µm.

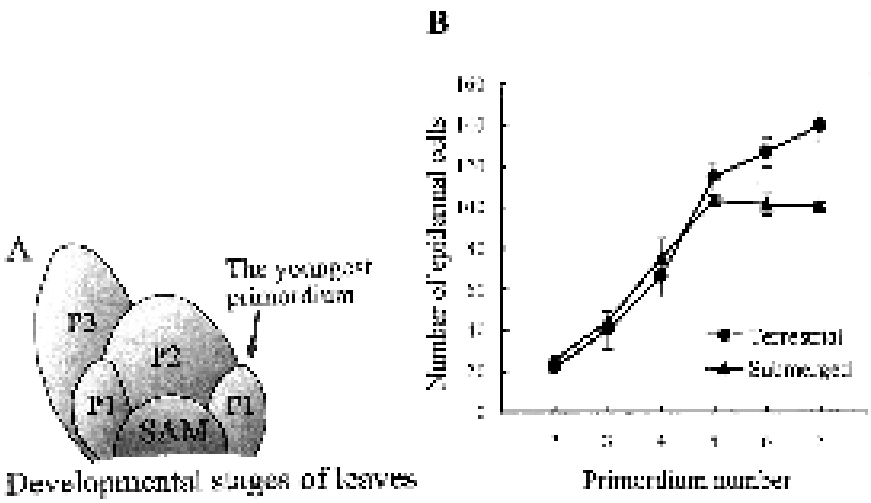

Fig. 5 Analysis of developmental stages. (**A**) A diagram showing developmental stages of leaves with primordium numbers (P1, P2, and P3). SAM, Shoot apical meristem. (**B**) The number of epidermal cells aligned in transverse sections of young leaf primordia at various stages of development. The adaxial epidermal cells aligned in transverse sections of each primordium were counted. Symbols and bars represent means ± SD. For each measurement 20 leaves were collected from different plants of the clone.

A detailed examination of transverse sections revealed that the numbers of adaxial epidermal cells in transverse sections of submerged leaves were smaller than in those of terrestrial leaves (Fig. **4**). Thus, it appeared that the cause of the change in leaf form might mainly have been a change in number of epidermal cells produced. We next tried to identify the stage of leaf development at which such changes occurred. As shown in Fig. **5 B**, there was a continuous increase in numbers of leaf cells aligned in transverse sections until developmental stage P4, irrespective of submerged or terrestrial conditions. However, after developmental stage P5, we observed distinct differences between the two conditions: terrestrial leaves continuously increased the leaf width, with increases in cell number, whereas in submerged leaves, no further increase occurred in the number of epidermal cells aligned in transverse sections. Thus, the cessation of the increase in cell number under submerged conditions at a particular developmental stage was responsible for the narrower leaves that formed under these conditions.

Hormonal treatments

In order to identify endogenous factors that might mediate the change in leaf form from the T-type to the S-type, we treated terrestrial shoots with GA_3, BL, ACC (a precursor of ethylene biosynthesis). Three weeks of treatment with GA_3 did not induce any substantial changes in leaf form, and the ratio of leaf length to width was similar to those of controls (Fig. **6**). Treatments with BL and ACC affected the leaf shape of *L. arcuata*. Leaves had a higher ratio of leaf length to width, which ranged

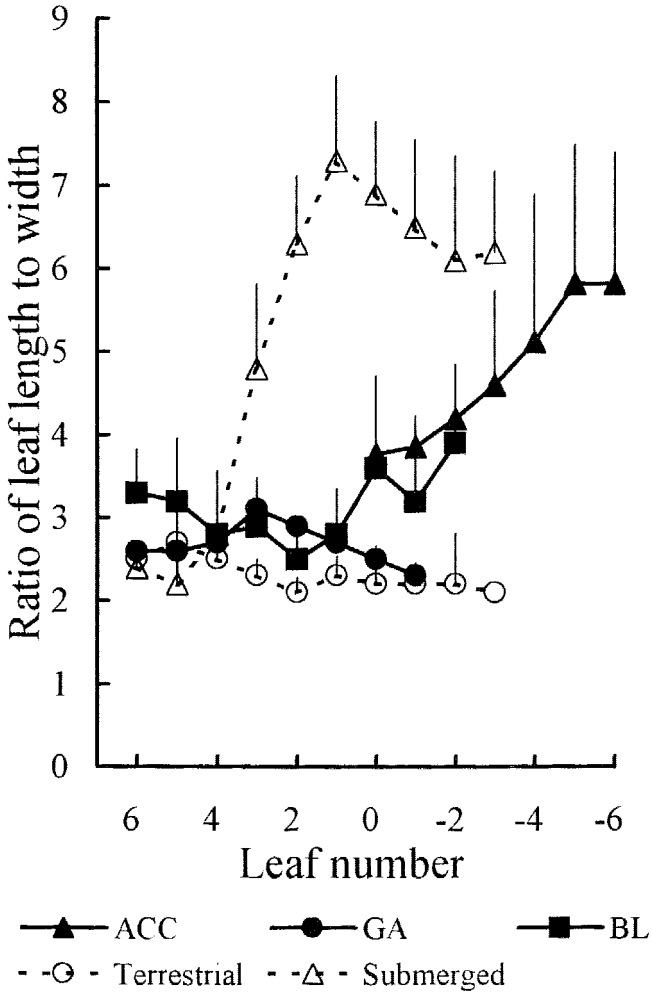

Fig. 6 Effects of plant hormones on the shape of leaves in *L. arcuata*. The ratio of length to width of leaves on terrestrial shoot that had been treated with various plant hormones for three weeks are shown for various leaves (LN 6 to LN-6). The ratios of leaf length to width for terrestrial and submerged controls are also shown. Terrestrial shoots were treated with 10^{-4} M ACC (ACC), 10^{-6} M BL (BL), and 10^{-5} M GA_3 (GA_3) for three weeks. Symbols and bars represent means ± SD. For each measurement 20 leaves were collected from different plants of the clone.

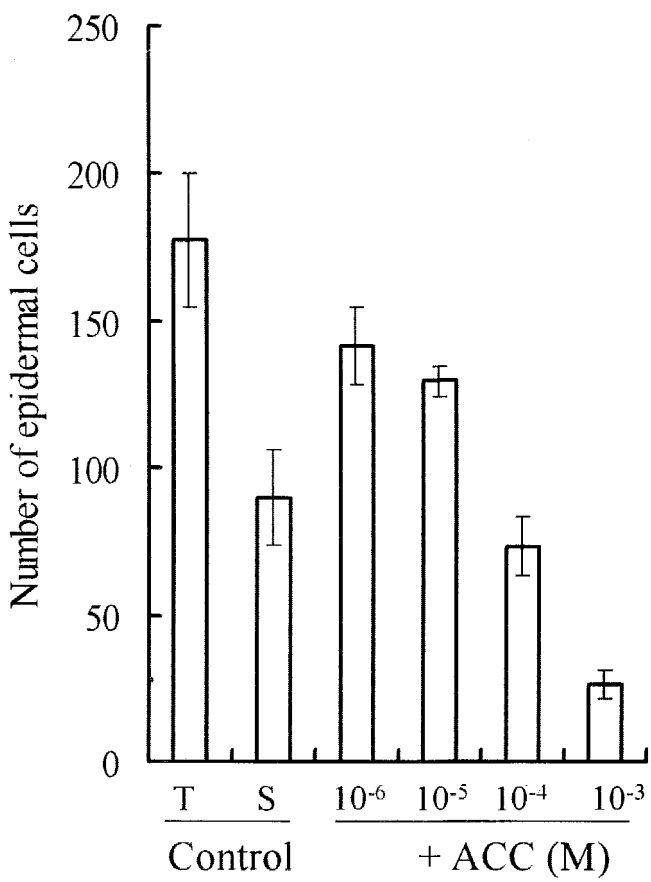

Fig. 7 Dependency of the number of adaxial epidermal cells aligned in transverse sections under terrestrial condition upon different concentration of ACC. After *L. arcuata* was treated with 10^{-6} M, 10^{-5} M, and 10^{-4} M ACC for 3 weeks, cell numbers in the transverse sections in the comparable leaves were compared. However, in the case of 10^{-3} M, 4 weeks are required for such observations. Data of terrestrial leaves (T) and submerged leaves (S) are shown. Data represent means ± SD. For each measurement 20 leaves were collected from different plants of the clone.

from 3 to 4 after the treatment with BL and from 4 to 6 after treatment with ACC. The ratio of leaf length to width of terrestrial leaves, which served as controls, remained at 2 (Figs. **1C, D, 6**). When leaves had been treated with BL, petiole-like structures were associated with fully expanded leaves, but such structures were not found on submerged shoots (Figs. **1B, D**). S-type narrow leaves, defined by a higher ratio of leaf length to width, were only induced by treatment of terrestrial shoots with ACC (Figs. **1B, C, 6**). Therefore, we investigated the effects of ACC on the terrestrial shoots of *L. arcuata* in greater detail.

Effects of ACC

Treatment for three weeks of terrestrial leaves with ACC at more than 10^{-5} M induced a decrease in the width of leaves and, correspondingly, in the number of adaxial epidermal cells aligned in transverse sections (Figs. **1C, 4C–E, 7**). The ratio of leaf length to width of leaves LN-2 treated with 10^{-6} M ACC was about 3.6, this value was 1.6-fold higher than those of terrestrial leaves, and was 1/2 that of submerged leaves. When terrestrial shoots were treated with ACC at concentrations above 10^{-4} M, the growth of apical buds was suppressed and many lateral shoots were produced (Fig. **1C**). Thus, we measured leaves on lateral shoots only after plants had been treated with 10^{-4} M ACC. S-type leaves, induced by this treatment, had a higher ratio of leaf length to width, which ranged from 4 to 5, while the ratio of leaf length to width of typical terrestrial leaves was close to 2 (Fig. **6**). The density of stomata on these S-type leaves was lower than on typical terrestrial leaves. The treatment with 10^{-4} M ACC did not affect the shapes of epidermal cells on either side of the leaf (data not shown). When terrestrial shoots were treated with 10^{-3} M ACC, leaf shape was changed dramatically. Transverse sections of leaves were ellipsoidal, and these leaves did not develop a flattened leaf blade (Fig. **4E**). Moreover, the dorsiventral axis of vascular bundles was abnormally oriented in the leaves (Fig. **4F**). We also tried to treat terrestrial leaves with ethylene gas released from

500 mg/l of 2-chloroethylphosphonic acid (Ethephon Standard) at neutral pH. The concentration of ethylene gas released from 500 mg/l of Ethephon Standard was 84.3 ± 18.9 ppm under this experimental condition, a day after addition. The changes in leaf form caused by the treatment with Ethephon Standard were comparable to those of treatments with 10^{-4} M ACC.

Discussion

Characteristics of terrestrial and submerged leaves

The leaves of *L. arcuata* showed distinct changes in shape upon submergence. Leaves produced on submerged shoots were much narrower than those on terrestrial shoots (Figs. **1A,B**). Such heterophyllous changes have been observed in other species, such as *C. heterophylla* (Deschamp and Cooke, 1983[7], 1984[8], 1985[9]), *C. intermedia* (Jones, 1955[15]), and *H. vulgaris* (Bodkin et al., 1980[4]; Goliber and Feldman, 1990[13]). In these latter cases, the changes in leaf shape have been ascribed to changes in the shape of epidermal cells. In contrast, our work revealed that, in *L. arcuata,* the shape of epidermal cells is similar in submerged leaves and terrestrial leaves (Figs. **3A,B**). However, the number of epidermal cells aligned in transverse sections was significantly lower in typical S-type leaves than in typical T-type leaves (Table **1**, Figs. **4A,B**). Thus, in *L. arcuata,* the changes in leaf shape were not caused by changes in the shape of epidermal cells but by changes in the number of epidermal cells aligned in transverse sections. The leaves LN 1 through 4 changed shape upon submergence (Fig. **2B**), while leaves LN 5 and LN 6 did not change shape, indicating that leaf primordia at stages earlier than P4 have more plasticity in terms of leaf morphogenesis, while the morphological fates of leaf primordia at stages later than P5 have already been determined. Our developmental analysis clearly showed that P5 is the critical stage with respect to susceptibility to the effects of submergence in terms of the number of epidermal cells in the lamina (Fig. **5**). Increases in the numbers of epidermal cells aligned in transverse sections of typical T-type leaves continued even after P5, whereas such increases in typical S-type leaves stopped at P5 (Fig. **5B**).

The number of stomata per unit area on typical S-type leaves of *L. arcuata* was lower than that on typical T-type leaves (Table **1**). This result is coincident with previous reports on other heterophyllous aquatic angiosperms, such as *C. heterophylla* (Deschamp and Cooke, 1983[7], 1984[8], 1985[9]), *H. vulgaris* (Kane and Albert 1987 a[17]; Goliber, 1989[11]; Goliber and Feldman, 1990[13]), *R. flabellaris* (Young and Horton, 1985[31]; Young et al., 1987[32], 1990[33]), *Limnophila indica* (Mohan Ram and Rao, 1982[22]), and *Potamogeton nodosus* (Anderson, 1978[1], 1982[2]). The characteristics of S-type leaves in *L. arcuata* can be summarized as follows: higher ratio of leaf length to width, smaller numbers of epidermal cells aligned in transverse sections, due to earlier cessation of cell proliferation in the lamina; and lower densities of stomata on both surfaces.

Mechanisms responsible for heterophyllous changes

Our studies with GA_3, BL, and ACC (a precursor to ethylene) revealed that only treatment with ACC caused the production of S-type leaves with the higher ratio of leaf length to width on terrestrial shoots (Fig. **1C**). We also tried to treat terrestrial leaves with ethylene gas released from 500 mg/l of 2-chloroethylphosphonic acid (Ethephon Standard) at neutral pH, and confirmed that the results were almost the same as treatments with 10^{-4} M ACC. Higher concentrations of ACC induced narrower leaves on terrestrial shoots (Figs. **4C–E, 7**); leaves treated with 10^{-3} M ACC became extremely narrow and did not develop flattened leaf blades (Fig. **4E**). The leaves on terrestrial shoots treated with 10^{-4} M ACC had similar value of the ratio of leaf length to width to those of typical S-type leaves (Figs. **6, 7**). These leaves also had features characteristic of S-type leaves, with lower stomatal densities and reduced numbers of the epidermal cells aligned in transverse sections (Table **1**). Thus, ethylene appears to be able to change the shape of leaves from T-type to S-type, by restricting the division of epidermal cells aligned in transverse sections.

The dorsiventral axis of vascular bundles was abnormally oriented in leaves that had been formed during treatment with 10^{-3} M ACC (Fig. **4F**), suggesting that ethylene might also be involved in determination of the dorsiventral axis. However, at present, a possibility that the observed anomaly in the leaf shapes was caused by too high concentration of ACC cannot be excluded. Our studies indicate that ethylene might be involved in the plasticity of leaf development, and such a role for ethylene has not previously been proposed, to our knowledge. BL also affected leaf shape (Fig. **6**), and induced formation of petiole-like structures (Fig. **1D**), which were not observed on typical S-type leaves. BL might not be directly involved in the change in leaf shape that occurs in *L. arcuata* upon submergence.

Some semi-aquatic angiosperms, such as deepwater rice (Raskin and Kende, 1984[24]), *Rumex palustris* (Rijinders et al., 1997[25]), *Ranunculus sceleratus* (Samarakoon and Horton, 1984[26]; Smulders and Horton, 1991[27]), and *Callitriche platycarpa* (Musgrave et al., 1972[21]) do not exhibit heterophylly. They respond to flooding with elongation of internodes or petioles. In these species, ethylene that accumulates under submerged conditions promotes the elongation of internodes or petioles (Raskin and Kende, 1984[24]; Rijinders et al., 1997[25]; Samarakoon and Horton, 1984[26]; Smulders and Horton, 1991[27]). It has also been shown that, in deepwater rice, *Rumex palustris,* and *C. platycarpa,* elevated levels of GA_3 promoted by ethylene induced the elongation of internodes or petioles upon submergence (Raskin and Kende, 1984[24]; Rijinders et al., 1997[25]; Musgrave et al., 1972[21]). In *L. arcuata,* in contrast, treatment with GA_3 did not induce any appreciable changes in leaf shape (Fig. **6**) even though ethylene did induce changes in leaf shape under submerged conditions (Fig. **1C**). Thus, it is likely that ethylene affects leaf shape upon submergence, but the role of ethylene might differ among species. The underlying mechanism whereby ethylene causes heterophylly in *L. arcuata* appears to be quite different from that observed in rice and *Rumex palustris,* in which ethylene accelerates the elongation of internodes or petioles via the action of GA_3 upon submergence.

In conclusion, this study revealed that the change of leaf form in *L. arcuata* upon submergence was not ascribed to morphological change of epidermal cells, but rather to numbers of epidermal cells in transverse section. Notably, a causative factor for this change turned out most likely to be ethylene, since ad-

dition of ACC to the terrestrial shoots caused formation of submerged leaves.

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