Planta (2003) 217: 880–887
DOI 10.1007/s00425-003-1062-z

ORIGINAL ARTICLE

Asuka Kuwabara · Keiichi Ikegami · Tomokazu Koshiba
Toshiyuki Nagata

Effects of ethylene and abscisic acid upon heterophylly in *Ludwigia arcuata* (Onagraceae)

Received: 10 February 2003 / Accepted: 15 May 2003 / Published online: 3 July 2003

Abstract In this study, we examined the effects of ethylene and abscisic acid (ABA) upon heterophyllous leaf formation of *Ludwigia arcuata* Walt. Treatment with ethylene gas resulted in the formation of submerged-type leaves on terrestrial shoots of *L. arcuata*, while treatments with ABA induced the formation of terrestrial-type leaves on submerged shoots. Measurement of the endogenous ethylene concentration of submerged shoots showed that it was higher than that of terrestrial ones. In contrast, the endogenous ABA concentration of terrestrial shoots was higher than that of submerged ones. To clarify interactions of ethylene and ABA, simultaneous additions of these two plant hormones were examined. When *L. arcuata* plants were treated with these two plant hormones, the effects of ABA dominated that of ethylene, resulting in the formation of terrestrial-type leaves. This suggests that ABA may be located downstream of ethylene in signal transduction chains for forming heterophyllous changes. Further, ethylene treatment induced the reduction of endogenous levels of ABA in tissues of *L. arcuata*, resulting in the formation of submerged-type leaves. Thus the effects of ethylene and ABA upon heterophyllous leaf formation are discussed in relationship to the cross-talk between signaling pathways of ethylene and ABA.

Keywords Abscisic acid · Ethylene · Heterophylly
Ludwigia · Plant hormonal interactions · Submergence

A. Kuwabara (✉) · T. Nagata
Department of Biological Sciences,
Graduate School of Science,
The University of Tokyo, 7-3-1, Hongo,
Bunkyo-ku, 113-0033 Tokyo, Japan
E-mail: asuka@biol.s.u-tokyo.ac.jp
Fax: +81-5684-2063

K. Ikegami · T. Koshiba
Department of Biological Sciences,
Graduate School of Science,
Tokyo Metropolitan University, Hachioji-shi,
192-0397 Tokyo, Japan

Abbreviations ABA: abscisic acid · ACC: 1-aminocyclopropane-1-carboxylic acid · L/W ratio: ratio of leaf length to width · LN: leaf number · GAs: gibberellins

Introduction

Plants that thrive at the waterside often exhibit heterophylly; the leaf form of submerged leaves is very different from that of terrestrial ones. Round-shaped and thick leaves with stomata are observed in dry upland conditions, while thin and elongated leaves with no or few stomata are formed under submerged conditions. Such dimorphic changes of leaves are interpreted as a kind of adaptive strategy of these aquatic and semi-aquatic plants to environmental changes, since they have to withstand two different environments of terrestrial conditions and submergence. Heterophylly is observed in various phylogenetically unrelated species, from water ferns such as *Marsilea quadrifolia* (Liu 1984) to widespread families of angiosperms: *Ranunculus flabellaris* (Bruni et al. 1996), *Callitriche heterophylla* (Deschamp and Cooke 1983), *Hippuris vulgaris* (Goliber and Feldman 1990), *Potamogeton nodosus* (Anderson 1978). However, studies on physiological mechanisms that regulate the heterophyllous changes remain largely to be resolved (Kuwabara and Nagata 2002).

From the anatomical point of view, the thin and elongated shapes of submerged leaves are commonly accompanied by elongated epidermal cells (Schmidt and Millington 1968; Deschamp and Cooke 1983; Young et al. 1987; Goliber and Feldman 1990). In one case, gibberellins (GAs) are likely to be involved as an endogenous factor that induces the formation of submerged leaves with elongated epidermal cells (Deschamp and Cooke 1983). However, we did not observe the elongation of epidermal cells during the formation of narrow-shaped submerged leaves of *L. arcuata*, a process which is common in other heterophyllous plants

(Kuwabara et al. 2001). Instead, a decrease in the number of epidermal cells aligned in the transverse direction was observed in this plant (Kuwabara et al. 2001). Furthermore, we suggested that this change may be mediated by ethylene, as the application of 1-aminocyclopropane-1-carboxylic acid (ACC), an ethylene biosynthesis precursor, caused the formation of submerged-type leaves on terrestrial shoots (Kuwabara et al. 2001).

In this study, we attempted to clarify the involvement of ethylene in the formation of submerged-type leaves. Terrestrial shoots that were treated with ethylene gas formed submerged-type leaves. In addition, the results of other physiological experiments, in which an inhibitor of ethylene perception was applied to submerged shoots and endogenous ethylene levels of shoots were measured, supported the hypothesis that ethylene is actually involved in the formation of submerged-type leaves of *L. arcuata*.

On the other hand, it has been reported in many heterophyllous plants that treatment with abscisic acid (ABA) induces the formation of terrestrial-type leaves on submerged shoots, implying that ABA is a key factor in the formation of terrestrial-type leaves (Anderson 1978; Mohan Ram and Rao 1982; Deschamp and Cooke 1983; Young and Horton 1985; Kane and Albert 1987). In fact, we confirmed that addition of ABA caused the formation of terrestrial-type leaves on submerged shoots of *L. arcuata*. Furthermore, endogenous levels of ABA in terrestrial shoots were higher than those of submerged shoots. Since ethylene and ABA functioned in opposite directions, the interaction between ethylene and ABA upon heterophyllous changes of *L. arcuata* became a focus of this study. Outcomes from these experiments are discussed in relationship to the formation of submerged-type leaves of *L. arcuata* upon submergence.

Materials and methods

Plant growth conditions

Ludwigia arcuata Walt. (Onagraceae) was purchased from a local market in Tokyo, and was taxonomically identified by Dr. Ching-I. Peng (Academia Sinica, Taipei, Republic of China). Experimental materials were prepared from the stock cultures that were vegetatively propagated every week under the terrestrial conditions (Kuwabara et al. 2001). Both stock and experimental cultures were carried out in a growth chamber at 27 °C under continuous illumination of 80 μmol photons m^{-2} s^{-1} by fluorescent tubes.

In order to discriminate leaves that were newly developed under experimental trials from the pre-existing ones, leaf number (LN) was assigned in each shoot before the start of experiments, as described previously (Kuwabara et al. 2001). At the beginning of experiments, the youngest primordium was numbered as LN 1, and older leaves were numbered basipetally from LN 1; leaves that emerged after LN 1 were designated LN 0, LN −1, LN −2, as shown in Fig. 4a. To discriminate the location of respective leaves, 2-mm-long tips of leaves of LN 6 or LN 8 were cut off as LN markers (Fig. 4a).

Approximately 4-cm-long apical cuttings that were obtained from stock cultures were immersed in 3% sodium hypochlorite solution (effective Cl^- concn. >5%; Kokusan Chem. Works, Tokyo, Japan) for 20–30 min. During this treatment, LN markers were given, and axillary buds, if there were any, were removed with a fine tweezers. Subsequently, the shoots were immersed in the second solution containing 16.7% sodium hypochlorite and 0.02% Tween 20 (ICN Biochemicals, Cleveland, OH, USA) for 5 min. After the shoots were washed with sterile deionized water, stems and leaves below the lowest node were removed from each sterilized shoot with a razor. Five shoots were cultivated in a 300-ml Erlenmeyer flask that contained 80 ml of sterilized basal medium containing 65%-strength Murashige and Skoog (1962) basal salt mixture (Sigma) and 1% sucrose, being solidified with 0.9% agar (Aldrich) after autoclaving. After 1 day of pre-culture, whole experiments were conducted under sterile conditions. During this period, emergence of a new leaf primordium (LN 0) was not detected even under a dissecting microscope. Openings of flasks were covered with layers of aluminum foils during the pre-treatments and subsequent submergence. Upon ethylene treatment, openings were sealed with silicone plugs, while upon growth under terrestrial conditions, openings were covered with perforated silicone plugs. Submerged conditions were made by pouring 220 ml sterilized deionized water into flasks. Plants were cultivated for 2–3 weeks, until leaves of LN −2 or LN −3 were completely expanded.

Treatment with plant hormones and inhibitors

A mixture of ABA isomers (Wako Pure Chem. Ind., Osaka, Japan) was added to basal medium before autoclaving. The concentrations of ethylene gas (GL Sciences, Tokyo, Japan) added to flasks were confirmed by measurements with a gas chromatograph (GC 390B; GL Sciences). A solution of 1 μM $AgNO_3$ was used for inhibiting ethylene perception.

Quantification of heterophyllous changes

The change in leaf shape was assessed by the ratio of leaf length to width (L/W ratio). One leaf from a pair of leaves at each node was used for assessment of leaf shape, while the other leaf was used for anatomical analysis. To measure the length and width of leaves, leaves were fixed in FAA (5% formaldehyde, 5% acetic acid, and 45% ethanol) and then they were flattened on a petri dish, because fresh leaves of *L. arcuata* often showed conspicuous epinasty and tended to wither easily. Then 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 in a solution of 7.2% formaldehyde, 10% dimethyl sulfoxide and 0.1% Nonidet-P40 (Sigma) in 50 mM sodium phosphate buffer (pH 7.0).

Measurement of endogenous ethylene concentrations

Endogenous ethylene concentrations in *L. arcuata* plants were determined by the vacuum-extraction technique basically according to Beyer and Morgan (1970). In brief, 25 shoots of *L. arcuata* were gently removed from solidified agar medium, and immediately put into the solution containing 0.1% (v/v) Tween 20 and saturated $(NH_4)_2SO_4$. Under submergence in this solution, roots and plant parts that had been immersed in the agar medium were discarded. Subsequently, each shoot was cut under submergence into two to three pieces, which were quickly transferred into a collection flask containing saturated $(NH_4)_2SO_4$ solution. The exposure of tissues to air during cutting and extraction was limited to a maximum of ca. 2–3 s per experiment. After gases in the plant tissue were extracted under vacuum, each gas sample (0.2 ml) was taken with a syringe from the collection flask. Ethylene concentrations were measured with a gas chromatograph (GC 390B; GL Sciences) equipped with a stainless column [3 mm i.d., 2 m long; packed with Al_2O_3 (60–80 mesh)] and a flame ionization detector. The injector and the detector were used at 120 °C, while the column was maintained at 100 °C.

Measurement of endogenous ABA concentrations

Before the extraction of ABA, roots and portions of plants that had been immersed under the medium were removed as for the case of ethylene extractions. After 7 days of culture, the immersed portions were composed of stems and leaves below the nodes with leaves to LN 9, unless otherwise specified. The extraction and measurement of ABA were carried out as described in Cheng et al. (2002). Shoots (approx. 0.5 g) were homogenized in 80% (v/v) acetone containing 0.1 mg ml^{-1} 2,6-di-tert-butyl-4-methylphenol. After adding [^{13}C]ABA (Asami et al. 1999) as an internal standard, the homogenate was shaken for 1 h on ice in darkness and then centrifuged at 4 °C. The precipitate was re-extracted, and the combined supernatant was evaporated to remove acetone. ABA was partially purified from the residual aqueous solution by partitioning using hexane and ether and then applied for HPLC. ABA was methylated with diazomethane and analyzed by GC–selected ion monitoring–MS by monitoring 192 fragments of [^{13}C]ABA and 190 fragments of endogenous ABA, respectively. GC–MS analysis was performed with a mass spectrometer (GCMSQP5050; Shimadzu, Kyoto, Japan) coupled to a gas chromatograph (GC-17A; Shimadzu) with a DB-1 capillary column (0.25 mm i.d., 30 m long, 0.25 μm film thickness; J&W Scientific, Folsom, CA, USA).

Anatomy

For the observation of epidermis, whole leaves were fixed in a mixture of ethanol and acetic acid (7:1, v/v), and cleared with an aqueous solution of chloral hydrate as described in Kuwabara et al. (2001). The number of epidermal cells aligned in the transverse direction was counted on the transverse sections of leaves at the site of maximum width. Leaves were fixed in FAA, and were embedded in Technovit 7100 resin (Kulzer & Co., Wehrheim, Germany) and sectioned as described by Tsukaya et al. (1993). The sections were stained with a 0.1% (w/v) solution of toluidine blue in 100 mM sodium phosphate buffer (pH 7.0) for 1 min at 65 °C. All samples were observed under a light microscope (BX51; Olympus Optical Co., Tokyo, Japan).

Results

Ethylene as an endogenous factor for forming submerged-type leaves

When terrestrial shoots of *L. arcuata* were exposed to ethylene gas (100 μl l^{-1}) for 2–3 weeks, newly developed leaves displayed narrow submerged-type shapes (Fig. 1c). Notably, the morphology of these leaves and shoots looked very much like those observed upon submergence (Fig. 1b, c). In contrast, when terrestrial shoots were treated with ACC (100 μM), suppression of the growth of apical buds and consequently the overgrowth of axillary buds was observed (Fig. 1e). Such a change was observed in shoots submerged under natural conditions or in the terrestrial shoots that were treated with ethylene gas (Fig. 1b, c, e). Figure 2a shows that terrestrial shoots that were treated with >50 μl l^{-1} ethylene produced leaves with a higher L/W ratio, ranging from 5 to 6, which was comparable to that of submerged leaves (see Fig. 4b). Leaves produced under 10 μl l^{-1} ethylene showed a lower L/W ratio than submerged leaves. When a higher L/W ratio was displayed under the submerged conditions, this was accompanied by two other anatomical changes (Kuwabara et al. 2001): reduced numbers of epidermal cells aligned transversely, and a lower stomatal density. Accordingly, these two anatomical parameters were examined in the newly developed submerged-type leaves under 100 μl l^{-1} ethylene treatments. Actually, the reduction in the epidermal cell numbers aligned transversely was 46% (Fig. 3a), while the reduction in numbers of stomata on the abaxial and adaxial sides was 76% and 83%, respectively (Fig. 3b). Similar changes in anatomy to those found in newly developed submerged-type leaves after ethylene treatment were observed under submergence (see columns S and E in Fig. 3a, b). These results showed that the submerged-type leaves that were induced by the ethylene treatment shared common morphological characteristics with the submerged leaves. It should be noted that the curve of the dependency of L/W ratio upon LN shifted to the left of that displayed by simple submergence of *L. arcuata*, implying that the response of leaves to ethylene in changing leaf shape occurred slightly earlier than their response to submergence (Fig. 4b). This suggested also that leaves that perceived submergence would accumulate ethylene, resulting in directing the formation of submerged-type leaves. It was inferred from these results that exogenously applied ethylene caused the formation of submerged-type leaves under terrestrial conditions.

To prove that ethylene is an endogenous factor for producing submerged-type leaves upon submergence, it is necessary to examine whether ethylene accumulates in submerged shoots of *L. arcuata*. When we measured the endogenous levels of ethylene by gas chromatography, ethylene levels in plants that were submerged for 7 days were 10-fold higher than those of plants terrestrially grown for 7 days (Fig. 5a). On the other hand, when the effect of Ag^{+}, which is a well-known inhibitor of ethylene perception (Beyer 1976), on the heterophyllous changes under the submerged conditions was tested, submerged shoots that were treated with Ag^{+} failed to produce narrow submerged-type leaves (Fig. 1d). This implies that there should exist a signaling pathway downstream from ethylene perception, resulting in the formation of submerged-type narrow leaves. Thus these results collectively indicated that ethylene was an endogenous factor for the formation of submerged-type leaves of *L. arcuata* under the submerged conditions.

ABA as an endogenous factor for inducing the formation of terrestrial-type leaves

It has been reported in several heterophyllous plant species that ABA induces the formation of terrestrial-type leaves under submerged conditions (Kane and Albert 1987 and references therein). Accordingly, we examined whether ABA is a factor that induces the formation of terrestrial-type leaves in submerged shoots of *L. arcuata*. When we added ABA (1 μM) to submerged plants of *L. arcuata* for 3 weeks, terrestrial-type leaves were formed with a lower L/W ratio ranging from

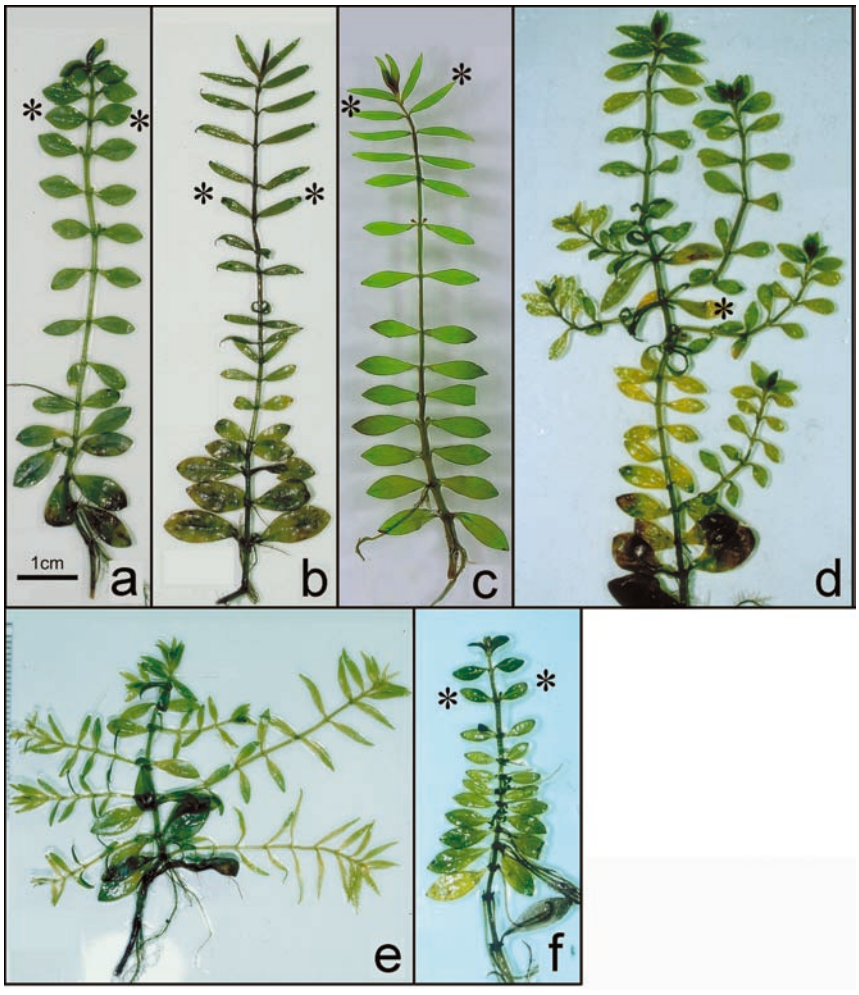

Fig. 1a–f Gross morphology of *Ludwigia arcuata* grown under various experimental conditions. All shoots were grown under sterile conditions for 2–3 weeks with continuous light. **a** A terrestrial shoot with terrestrial-type leaves. **b** A submerged shoot grown for 2 weeks after transfer from terrestrial conditions. **c** A terrestrial shoot treated with 100 μl l^{-1} ethylene. **d** A submerged shoot treated with 1 μM Ag^{+} (an inhibitor of ethylene perception). **e** A terrestrial shoot treated with 100 μM ACC. **f** A submerged shoot treated with 1 μM ABA. *Asterisks* indicate the leaves of LN 0 (see the text)

2 to 3 under submergence (Figs. 1f, 4b). When we added >1 μM ABA to submerged plants of *L. arcuata*, the L/W ratio of the newly developed leaves was around 2, which was similar to that of terrestrial leaves (Figs. 2b, 4b). The application of <0.1 μM ABA induced the formation of leaves with a higher L/W ratio than the terrestrial leaves (Figs. 2b, 4b). When submerged shoots were treated with >10 μM ABA, newly developed leaves showed a terrestrial-type round shape; however, severe growth inhibition was observed. When, in addition to the L/W ratio, two other morphological parameters, the number of epidermal cells aligned in the transverse direction and stomatal density, were examined in newly developed terrestrial-type leaves induced by 1 μM ABA, ABA treatments were found to have induced a 46% increase in the number of epidermal cells aligned in the transverse sections over the untreated control (Fig. 3a). The stomatal density of these ABA-treated leaves was 2-fold higher on the adaxial side, and 3-fold higher on the abaxial side, compared with untreated submerged leaves (Fig. 3b). Thus, ABA-induced terrestrial-type leaves produced under submerged conditions were morphologically almost identical to those produced under terrestrial conditions.

To examine whether there is any correlation between the amount of ABA in tissues and the formation of terrestrial-type leaves, we used GC/MS to measure the amount of ABA extracted from shoots, as described in Cheng et al. (2002). After subculture, the ABA content of terrestrial shoots at the initial stage was 60 ng g FW^{-1} (Fig. 5b). After these plants were cultured under submerged-conditions for 7 days, the ABA level decreased to 5–8 ng g FW^{-1}, whereas when they were cultured under the terrestrial conditions as before, the ABA level decreased only slightly to 35–48 ng g FW^{-1}. These results indicated that in *L. arcuata*, ABA was involved in the formation of terrestrial-type leaves under terrestrial conditions.

Interactions between ABA and ethylene upon the formation of heterophyllous leaves

The above-mentioned results revealed that ethylene was an endogenous factor for the formation of submerged-type leaves, while the factor that induced the formation of terrestrial-type leaves was ABA. As ABA and ethylene acted in opposite directions, it was quite intriguing

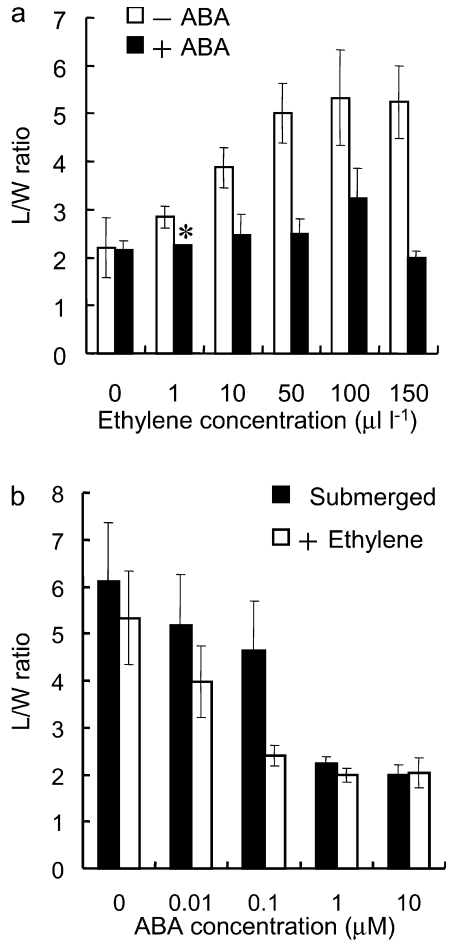

Fig. 2a, b Effects of different concentrations of ethylene (**a**) and ABA (**b**) on the shapes of newly developed leaves of *L. arcuata*. **a** L/W ratios of newly developed leaves on terrestrial shoots that were treated with various concentrations of ethylene in the presence of 10^{-6} M ABA (*solid bars*) or absence of ABA (*open bars*). **b** L/W ratios of leaves that emerged after treatment with various concentrations of ABA. Leaves on submerged shoots (*solid bars*) and those on terrestrial shoots that were treated with 100 μl l^{-1} ethylene (*open bars*) are shown. The bar with an *asterisk* indicates a single measurement. L/W ratios of leaves on terrestrial shoots that were treated with and without ABA were (means ± SE) 2.5 ± 0.05 and 2.2 ± 0.06, respectively

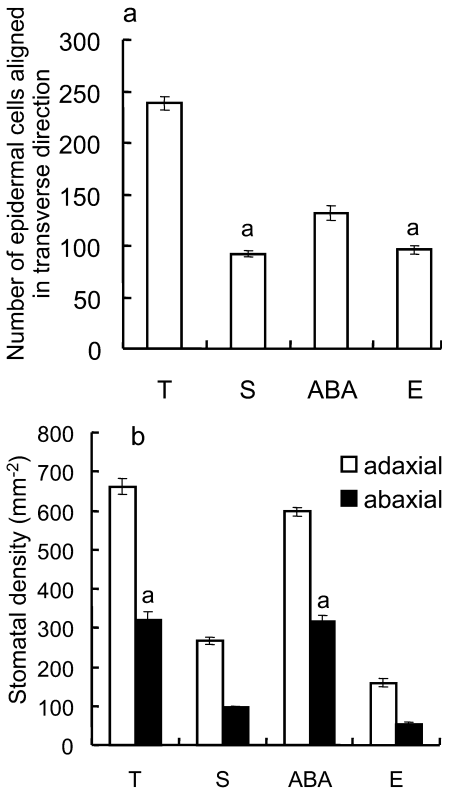

Fig. 3a, b Comparison of the morphological characteristics among terrestrial, submerged, ABA-induced terrestrial-type, and ethylene-induced submerged-type leaves of *L. arcuata*. **a** The number of epidermal cells aligned in transverse directions was compared among newly developed leaves on terrestrial shoots (*T*), submerged shoots (*S*), submerged shoots that were treated with 1 μM ABA (*ABA*), and terrestrial shoots that were treated with 100 μl l^{-1} ethylene (*E*). **b** Stomatal densities of the abaxial (*open bars*) and adaxial (*solid bars*) sides of leaves. Stomatal densities were compared among newly developed leaves on terrestrial shoots (*T*), submerged shoots (*S*), submerged shoots that were treated with 1 μM ABA (*ABA*), and terrestrial shoots that were treated with 100 μl l^{-1} ethylene (*E*). Bars marked with letter *a* are statistically comparable according to the Student's *t*-test ($P < 0.05$), and are significantly different from the unmarked bars. Bars represent means ± SE of 20 leaves from different individuals

to examine the antagonistic interactions between these two factors.

When the terrestrial shoots were treated with various concentrations of ethylene in the presence of 1 μM ABA, newly developed leaves displayed terrestrial-type leaves with lower L/W ratios (Fig. 2a), implying that the effect of ethylene that could have induced the formation of submerged-type leaves was completely suppressed by the addition of ABA. On the other hand, when terrestrial shoots were treated with various concentrations of ABA in the presence of an excess of ethylene gas (100 μl l^{-1}), the shape of newly developed leaves showed dependency on the concentrations of added ABA (Fig. 2b).

It is inferred from these results that upon simultaneous treatments with ethylene and ABA, leaf shapes of newly developed leaves showed dependency basically on the ABA levels, but not on ethylene levels. This implied that in the signaling pathway of heterophyllous leaf formation, ABA seems to be located downstream from the action of ethylene.

In this context, it is necessary to examine whether the addition of one type of plant hormone affects the endogenous levels of the other type of plant hormone in *L. arcuata*. To this aim, when the endogenous level of ethylene was measured in the presence or absence of ABA after 7 days of submergence, it was confirmed that the ethylene level was not significantly affected by the presence of ABA (Fig. 5a), excluding the possibility that ABA affects the endogenous level of ethylene under submergence. On the other hand, when the endogenous levels of ABA in terrestrial shoots was measured in the presence of an excess of ethylene (100 μl l^{-1}) after 7 days of culture, ABA levels were notably reduced to 20% of

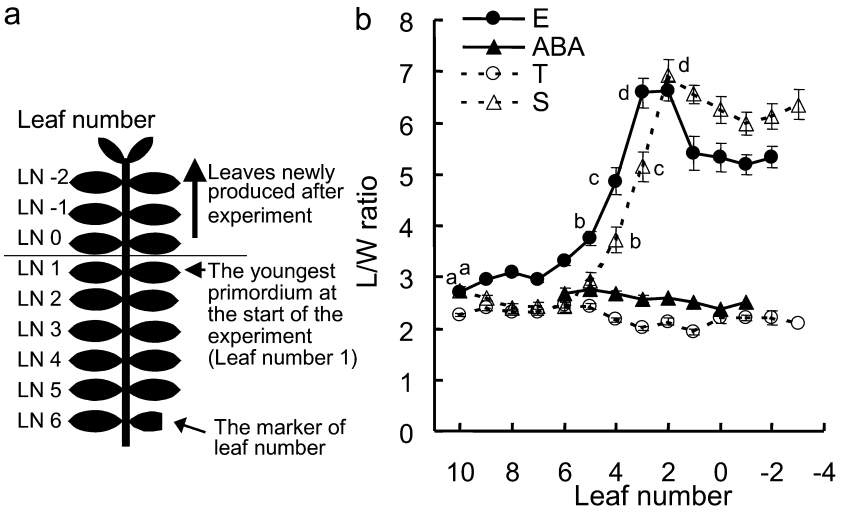

Fig. 4a, b Dependency of L/W ratios upon the leaf age in shoots of *L. arcuata*. **a** Diagram showing the designation of LN. The youngest leaf primordium is designated LN 1. The older leaves were numbered basipetally from LN 1, and leaves that emerged after LN 1 were designated LN 0, LN −1, LN −2. **b** Quantitative assessment of heterophyllous changes; L/W ratios for LNs of terrestrial leaves (*open circles*), submerged leaves (*open triangles*), terrestrial leaves produced under ethylene treatment (*filled circles*), and submerged leaves produced under ABA treatment (*filled triangles*) are shown. L/W ratios marked by the same letters (*a–d*) are statistically comparable according to the Student's *t*-test ($P < 0.05$). Symbols and bars represent means ± SE of 20 leaves from different plants of the same clone

that of the terrestrial control, a reduction comparable to that observed in submerged shoots (Fig. 5b). In fact, excess ethylene induced the formation of submerged-type leaves on terrestrial shoots (Fig. 1c). Thus, high levels of ethylene reduced endogenous ABA levels under terrestrial conditions.

Regarding the heterophyllous leaf formation of *L. arcuata*, the effect of Ag^+ should be considered further, as in the presence of Ag^+, an inhibitor of ethylene perception, terrestrial-type leaves were formed under submergence. Measurements of endogenous ABA levels of Ag^+-treated shoots showed a lower level of ABA, which was comparable to that of simply submerged shoots. It seems that interception in the ethylene signaling pathway could affect heterophyllous leaf formation in *L. arcuata*; the significance of this will be interpreted in the Discussion.

Discussion

In this study, several lines of evidence revealed that ethylene is an endogenous factor for forming submerged-type leaves; application of ethylene gas induced submerged-type leaves on terrestrial shoots of *L. arcuata* and in fact endogenous levels of ethylene were higher in the submerged shoots. From this point of view, it is worth interpreting the leftward shift of the L/W ratio with respect to LN that was displayed by the ethylene-treated shoots in comparison with simply submerged shoots (Fig. 4b). Under submergence, first ethylene would accumulate in the tissue, which could cause the formation of submerged leaves. However, the direct exposure of terrestrial shoots to ethylene would cause the ethylene-accumulation step to be skipped, so that the changes in leaf form would occur earlier than in the case of submerged plants. Although LN 5 and LN 6 leaves responded to ethylene treatment, they did not respond to submergence. This difference may reflect the possibility that, in the latter case, the level of accumulated ethylene was insufficient to change shapes of LN 5 and LN 6 leaves.

On the other hand, the addition of ABA to the submerged shoots of *L. arcuata* caused the formation of terrestrial-type leaves (Fig. 1f), as has been observed in other plant species (Kane and Albert 1987 and references therein). In fact, measurement of ABA by GC/MS revealed that the endogenous ABA level of terrestrial shoots was higher than that of submerged shoots, as is the case for *Hippuris vulgaris* (Goliber and Feldman 1989). Thus, there is a correlation between a higher level of ABA and the formation of terrestrial-type leaves in *L. arcuata*.

The results described above prompted us to examine the antagonistic interactions between ethylene and ABA upon heterophyllous changes in *L. arcuata*. The benefit of this experimental system using *L. arcuata* is the easy assessment of leaf forms enabling the interactions of ethylene and ABA to be readily examined, an advantage that does not occur in other heterophyllous plant species. In fact, results from the simultaneous addition of these two plant hormones to terrestrial shoots revealed that the change in leaf shape was basically dependent on the applied ABA concentrations. In addition, it was demonstrated that exogenously applied ABA did not affect the endogenous ethylene levels, while the application of ethylene reduced the endogenous levels of ABA (Fig. 5).

Regarding the signal transduction chains downstream of ethylene perception, the application of Ag^+, an inhibitor of ethylene perception, caused the

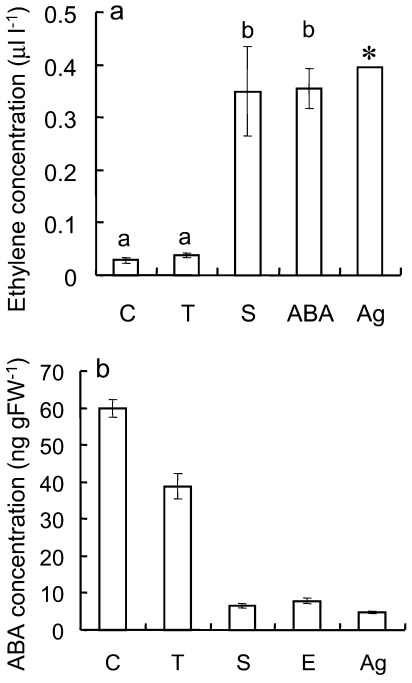

Fig. 5a, b Comparison of the endogenous concentrations of ethylene (**a**) and ABA (**b**) in shoots of *L. arcuata*. **a** Endogenous ethylene concentrations were measured in shoots that were pre-cultured for one day (*C*), in shoots grown under terrestrial conditions for 7 days (*T*), in shoots submerged for 7 days (*S*), in shoots treated with ABA (1 μM) for 7 days under submergence (ABA), and in shoots treated with Ag^+ (1 μM) for 7 days under submergence (*Ag*). Notably, ABA did not affect the endogenous ethylene levels of submerged shoots. **b** Endogenous ABA concentrations were examined in shoots that were pre-cultured for 1 day (*C*), in shoots grown under terrestrial conditions for 7 days (*T*), in shoots submerged for 7 days (*S*), in shoots treated with ethylene (100 μl l^{-1}) under terrestrial conditions for 7 days (*E*), and in shoots that were treated with Ag^+ (1 μM) under submergence (*Ag*). Ethylene concentrations marked by the same letters (*a*, *b*) are statistically comparable according to the Student's *t*-test ($P < 0.05$). The bar marked with an *asterisk* represents a single measurement. Bars represent means ± SE of three independent measurements

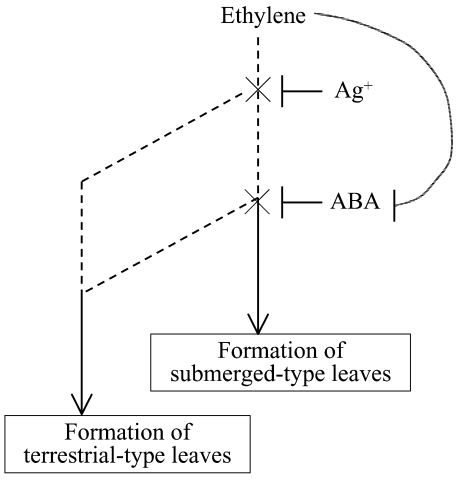

Fig. 6 Regulation of heterophyllous leaf formation in *L. arcuata*. Both ABA and Ag^+ inhibit the ethylene-signaling pathway, resulting in the formation of terrestrial-type leaves. ABA levels are negatively regulated by ethylene. Under terrestrial conditions, the ethylene-signaling pathway would not be activated, since the ethylene level is low and the high level of ABA inhibits the ethylene-signaling pathway, resulting in the formation of terrestrial leaves. On the other hand, under submerged conditions, the ethylene-signaling pathway would be activated, since the ethylene level is high and the ABA level is low, resulting in the formation of submerged-type leaves. Signaling pathways indicated by *broken lines* remain to be examined in further studies

formation of terrestrial-type leaves under submergence, although a lower level of ABA was detected in the tissues. This observation suggested that upon heterophyllous leaf formation, the signaling pathway downstream of ethylene perception might be intimately associated with heterophyllous leaf formation. As schematically illustrated in Fig. 6, the ABA level alone may not be sufficient for the formation of heterophyllous changes and the ethylene signaling pathway may regulate this process, whose mechanisms remain to be resolved by further studies. Such interactions of ABA and ethylene have been seen in other examples (see Cheng et al. 2002).

Interactions between ethylene and ABA have become hot issues in plant growth regulation studies. In the case of deepwater rice, accumulated high levels of ethylene accelerate the increase in endogenous GAs under submergence, resulting in the elongation of internodes (Hoffmann-Benning and Kende 1992). However, in this system ABA adversely affects the promoting effect of ethylene upon GAs. On the other hand, analyses of growth inhibition and seed germination with the ethylene-insensitive mutant *ein2* of *Arabidopsis thaliana* revealed that ABA synthesis has been accelerated in this mutant (Ghassemian et al. 2000). Nonetheless, growth inhibition in roots of *A. thaliana* by ABA seemed to be mediated by an ethylene-signaling pathway (Beaudoin et al. 2000). In addition, the plants' response to glucose seems to be mediated via interactions between ethylene and ABA (Cheng et al. 2002). Further, when the growth of *Galium aparine* was inhibited by high concentrations of auxin, the resultant increased level of ABA in the tissues caused the actual inhibition (Hansen and Grossman 2000). It is also reported that the decrease in water potential in maize roots caused the reduction in ethylene biosynthesis, which induced the accumulation of ABA in roots, resulting in the elongation of primary roots (Spollen et al. 2000). Such examples imply that interactions between ethylene and ABA are quite widespread. In this context, the observed interactions between ethylene and ABA in heterophyllous leaf formation in *L. arcuata* offer a good system in which to investigate their interactions at the molecular and physiological levels from the viewpoint of understanding the effects of environmental cues upon plant morphogenesis.

Acknowledgements We thank Dr. Richard Thompson (INRA, Dijon, France) for his critical reading of the manuscript. Thanks are also due to Prof. Noriaki Kondo of the University of Tokyo and Dr. Nobuyoshi Nakajima of National Institute for Environmental Studies, Tsukuba, Japan, for their valuable advice in exposing plants to ethylene gas and allowing us to use their gas chromatographs and other equipment. Some of the experiments were

technically assisted by Mr. Kenro Imamura and Ms. Erika Oka of the University of Tokyo. This work was supported in part by a grant from the Japan Society for the Promotion of Science (T.N.).

References

Anderson LWJ (1978) Abscisic acid induces formation of floating leaves in the heterophyllous aquatic angiosperm *Potamogeton nodosus*. Science 201:1135–1138

Asami T, Sekimata K, Wang JM, Yoneyama K, Takeuchi Y, Yoshida S (1999) Preparation of (±)-[1,2-$^{13}C_2$] abscisic acid for use as a stable and pure internal standard. J Chem Res (Synop) 11:658–659

Beaudoin N, Serizet C, Gosti F, Giraudat J (2000) Interactions between abscisic acid and ethylene signaling cascades. Plant Cell 12:1103–1115

Beyer EM Jr (1976) A potent inhibitor of ethylene action in plants. Plant Physiol 58:268–271

Beyer EM Jr, Morgan PW (1970) A model for determining the concentration of ethylene in the gas phase of vegetative plant tissues. Plant Physiol 46:352–354

Bruni NC, Young JP, Dengler NG (1996) Leaf developmental plasticity of *Ranunculus flabellaris* in response to terrestrial and submerged environments. Can J Bot 74:823–837

Cheng W-H, Endo A, Zhou L, Penney J, Chen H-C, Arroyo A, Leon P, Nambara E, Asami T, Seo M, Koshiba T, Sheen J (2002) A unique short-chain dehydrogenase/reductase in *Arabidopsis* glucose signaling and abscisic acid biosynthesis and functions. Plant Cell 14:2723–2743

Deschamp PA, Cooke TJ (1983) Leaf dimorphism in aquatic angiosperms: significance of turgor pressure and cell expansion. Science 219:505–507

Ghassemian M, Nambara E, Cutler S, Kawaide H, Kamiya Y, McCourt P (2000) Regulation of abscisic acid signaling by the ethylene response pathway in *Arabidopsis*. Plant Cell 12:1117–1126

Goliber TE, Feldman LJ (1989) Osmotic stress, endogenous abscisic acid and the control of leaf morphology in *Hippuris vulgaris* L.. Plant Cell Environ 12:163–171

Goliber TE, Feldman LJ (1990) Developmental analysis of leaf plasticity in the heterophyllous aquatic plant *Hippuris vulgaris*. Am J Bot 77:399–412

Hansen H, Grossman K (2000) Auxin-induced ethylene triggers abscisic acid biosynthesis and growth inhibition. Plant Physiol 124:1437–1448

Hoffmann-Benning S, Kende H (1992) On the role of abscisic acid and gibberellin in the regulation of growth in rice. Plant Physiol 99:1156–1161

Kane ME, Albert LS (1987) Abscisic acid induces aerial leaf morphology and vasculature in submerged *Hippuris vulgaris* L.. Aquat Bot 28:81–88

Kuwabara A, Nagata T (2002) Views on developmental plasticity of plants through heterophylly. Recent Res Dev Plant Physiol 3:45–59

Kuwabara A, Tsukaya H, Nagata T (2001) Identification of factors that cause heterophylly in *Ludwigia arcuata* Walt. (Onagraceae). Plant Biol 3:98–105

Liu LBL (1984) Abscisic acid induces land form characteristics in *Marsilea quadrifolia* L. Am J Bot 71:638–644

Mohan Ram HY, Rao S (1982) In-vitro induction of aerial leaves and of precocious flowering in submerged shoots of *Limnophila indica* by abscisic acid. Planta 155:521–523

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco cultures. Physiol Plant 15:473–497

Schmidt BL, Millington WF (1968) Regulation of leaf shape in *Proserpinaca palustris*. Bull Torrey Bot Club 95:264–286

Spollen WG, LeNoble ME, Samuels TD, Bernstein N, Sharp RE (2000) Abscisic acid accumulation maintains maize primary root elongation at low water potentials by restricting ethylene production. Plant Physiol 122:967–976

Tsukaya H, Naito S, Redei GP, Komeda Y (1993) A new class of mutations in *Arabidopsis thaliana*, *acaulis*1, affecting the development of both inflorescences and leaves. Development 118:751–764

Young JP, Horton RF (1985) Heterophylly in *Ranunculus flabellaris*: the effect of abscisic acid. Ann Bot 55:899–902

Young JP, Dengler NG, Horton RF (1987) Heterophylly in *Ranunculus flabellaris*: the effect of abscisic acid on leaf anatomy. Ann Bot 60:117–125