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ORIGINAL ARTICLE

Gibberellic acid induces non-Kranz anatomy with C_4-like biochemical traits in the amphibious sedge *Eleocharis vivipara*

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Abstract

Main conclusion **Gibberellic acid induces photosynthetic tissues with non-Kranz anatomy and C_4-like biochemical traits in terrestrial-form plants of *Eleocharis vivipara*. This suggests that the structural and biochemical traits are independently regulated.**

Abstract The amphibious leafless sedge, *Eleocharis vivipara* Link, develops culms (photosynthetic organs) with C_4-like traits and Kranz anatomy under terrestrial conditions, and C_3 traits and non-Kranz anatomy under submerged conditions. The conversion from C_3 mode to C_4-like mode in *E. vivipara* is reportedly mediated by abscisic acid. Here, we investigated the effects of gibberellic acid (GA) on the differentiation of anatomical and photosynthetic traits because GA is involved in heterophylly in aquatic plants. When 100 µM GA was sprayed on terrestrial plants, the newly developed culms had non-Kranz anatomy in the basal part and Kranz-like anatomy in the upper part. In the basal part, the mesophyll cells were well developed, whereas the Kranz (bundle sheath) cells were reduced and contained few chloroplasts and mitochondria. Stomatal frequency was lower in the basal part than in the upper part. Nevertheless, these tissues had abundant accumulation and high activities of C_4 photosynthetic enzymes and had C_4-like $\delta^{13}C$ values, as seen in the culms of the terrestrial form. When submerged plants were grown under water containing GA-biosynthesis inhibitors (uniconazole or paclobutrazol), the new culms had Kranz anatomy. The culms developed under paclobutrazol had the C_3 pattern of cellular accumulation of photosynthetic enzymes. These data suggest that GA induces production of photosynthetic tissues with non-Kranz anatomy in terrestrial plants of *E. vivipara*, without concomitant expression of C_3 biochemical traits. The data also suggest that the differentiation of C_4 structural and biochemical traits is regulated independently.

Keywords Amphibious plant · C_4 development · C_4 photosynthesis · C_3 photosynthesis · *Eleocharis vivipara* · Gibberellic acid · Kranz anatomy

Introduction

Photosynthesis is the most important physiological process for growth and survival of plants, so it is a matter of vital importance for plants to fix atmospheric CO_2 efficiently. In C_3 photosynthesis, which is widespread among land plants, atmospheric CO_2 is fixed by ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco), and then synthesized into carbohydrates through the Calvin–Benson (C_3) cycle in mesophyll cells. Rubisco is a bifunctional enzyme that catalyzes both carboxylation and oxygenation of ribulose 1,5-bisphoshate. In the oxygenation reaction, 2-phosphoglycolate is produced and this compound is metabolized in the photorespiratory (glycolate) cycle. In ordinary air, one-fourth of the photosynthetically fixed CO_2 is lost by

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photorespiration, reducing photosynthetic efficiency (Sage et al. 2012; Leegood 2013). If the concentration of CO_2 decreases, photorespiration accelerates.

C_4 plants are thought to have evolved from C_3 plants in response to the reduced atmospheric CO_2 levels that occurred about 30 million years ago (Christin et al. 2008; Sage et al. 2012). In contrast to C_3 plants that perform photosynthesis within a single cell, most C_4 plants photosynthesize using two types of photosynthetic cells, i.e., mesophyll cells and Kranz (bundle sheath) cells (Edwards and Voznesenskaya 2011; Langdale 2011). In C_4 plants, atmospheric CO_2 is initially fixed as C_4 acids by phosphoenolpyruvate carboxylase (PEPC) in the mesophyll cells. The C_4 acids are transferred to adjacent Kranz cells, where they are decarboxylated to release CO_2 for refixation by Rubisco. This process, known as the C_4 cycle, concentrates CO_2 at the Rubisco sites, thereby reducing photorespiration and enhancing photosynthesis (Hatch 1987; Leegood 2013).

Although the anatomical, biochemical, and physiological traits of C_4 plants have been well characterized (Hatch 1987; Edwards and Voznesenskaya 2011; Leegood 2013), the genetic and developmental aspects remain to be elucidated (Langdale 2011; Nelson 2011; Sedelnikova et al. 2018; Schlüter and Weber 2020). Recently, the improvement of crop yields has become extremely important, and enhancement of photosynthesis and yield of C_3 crops by introduction of the C_4 cycle has been attempted. Combining the Kranz anatomical structure and the C_4 biochemical traits in C_3 leaves by genetic engineering might improve C_3 crop yields (Schuler et al. 2016; Ermakova et al. 2020). Although some of the regulators involved in the differentiation of bundle sheath cells and the vein patterning of C_4 leaves have been found (Roth et al. 1996; Slewinski et al. 2012, 2014; Sedelnikova et al. 2018), further study is needed to properly understand the regulatory mechanisms of Kranz-type leaf development.

In general, plants exploit only a single mode of photosynthesis in their leaves. However, some plants can change the photosynthetic mode in response to changes in environmental conditions. It is well known that some succulent plants can shift their photosynthetic mode from C_3 metabolism to crassulacean acid metabolism (CAM) and vice versa, when water availability changes (Cushman 2001; Taybi et al. 2002; Winter 2019). However, a conversion between C_3 and C_4 mode seldom occurs, even when growth conditions change. One reason for this may be that the conversion between C_3 and CAM modes requires little structural change in the leaves, whereas conversion from C_3 to C_4 mode requires cellular differentiation together with the biochemical changes. The exceptions are some submerged aquatic plants with single-cell C_4 photosynthesis such as *Hydrilla verticillata* (L.F.) Royle (Bowes 2011).

The freshwater amphibious leafless sedge, *Eleocharis vivipara* Link, is a unique plant because its photosynthetic traits alternate between C_3 mode and C_4-like mode, depending on the growth conditions (Ueno et al. 1988; Ueno 2001). *E. vivipara* lacks the leaf blade, and the culm is the photosynthetic organ, as in other *Eleocharis* species. The culms of the terrestrial form have C_4-like photosynthetic traits of NAD-malic enzyme (NAD-ME) type with Kranz anatomy, whereas those of the submerged form have C_3 photosynthetic traits with non-Kranz anatomy (Ueno 1996a, b; Ueno and Ishimaru 2002). This conversion is achieved by the regulation of gene expression of C_4 photosynthetic enzymes accompanied by differentiation of photosynthetic cells in response to the growth conditions (Agarie et al. 1997, 2002; Ueno 2001). When the terrestrial form is immersed in water, the plant gradually develops new culms with intermediate states over several months of transition and finally changes its photosynthetic mode from C_4-like to C_3 mode. When the submerged form is exposed to air, its culms die due to desiccation, and the plant rapidly produces new culms with C_4-like mode (Ueno et al. 1988; Uchino et al. 1998; Ueno 2001). The culms (primary culms) often form new culms (secondary culms) at the tip by proliferation despite the terrestrial and submerged forms (Ueno 1996a). When the tips of submerged culms reach the water surface, they often proliferate new aerial culms with C_4-like mode, even though the submerged culms on which they grow maintain C_3 mode (Agarie et al. 2002). This makes *E. vivipara* an intriguing system to study the regulatory mechanism responsible for C_3 and C_4 differentiation in response to environmental changes. Recently, comparative transcriptomic studies have been undertaken for *E. vivipara* (Harada et al. 2018) and the closely related species *Eleocharis baldwinii* (Torr.) Chapm. (Chen et al. 2011, 2014); the latter species changes from C_4-like mode in terrestrial form to C_3–C_4 intermediate mode in submerged form (Ueno and Ishimaru 2002; Ueno 2004). Transcription of the components of the glycolysis pathway, citrate acid metabolism, and protein synthesis, and transporters of metabolites and ions, together with those of the C_4 core pathway, changes during C_4 induction (Chen et al. 2011, 2014; Harada et al. 2018).

In freshwater aquatic and amphibious plants, plant hormones are known to be involved in the development of morphologically and anatomically distinct leaves on the same plant, called heterophylly (Minorsky 2003; Wanke 2011; Li et al. 2019). Although *E. vivipara* lacks leaf blades, heterophylly occurs between the culms of the terrestrial and submerged forms. Ueno (1998) found that abscisic acid (ABA), a stress hormone, induces the formation of culms with Kranz anatomy and C_4-like biochemical traits in the submerged form of *E. vivipara*. Gibberellic acid (GA) is involved in the expression of heterophylly in some aquatic plants (McComb 1965; Kane and Albert 1982, 1987; Deschamp and Cooke

1984). Interestingly, McComb (1965) and Deschamp and Cooke (1984) reported that GA treatment elicits the formation of water-form leaves, an opposite response to the ABA treatment. It is not known whether GA is involved in the structural and biochemical changes in photosynthesis of *E. vivipara*.

In this study, the effects of GA on the expression of structural and photosynthetic traits in the terrestrial form of *E. vivipara* were investigated. The results showed that GA induced the formation of photosynthetic tissue with non-Kranz anatomy and the development of C_4-like photosynthetic traits under aerial conditions, suggesting that the structural and biochemical traits of C_4 photosynthesis are regulated independently.

Materials and methods

Plant materials and growth conditions

Eleocharis vivipara plants were originally collected from a creek in the vicinity of Tampa, Florida, USA (Ueno et al. 1988) and have been cultivated since collection in a greenhouse at Kyushu University, Fukuoka, Japan. Small shoots of the terrestrial form were transplanted into 500 mL pots filled with a commercial soil mix formulated for growing vegetables (Iseki Co. Ltd, Tokyo, Japan), and were grown in a growth chamber at 25 °C and 70% relative humidity under natural sunlight (12–13 h photoperiod) for one month. The maximum photon flux density was approximately 1000 µmol m^{-2} s^{-1}. The plants were watered daily and half-strength standard Hoagland nutrient solution was applied weekly. The submerged form was induced by pruning off the culms from terrestrial plants and submerging the pots under 50 cm of water in aquaria. These plants were grown in the aquaria for at least 5 months. Tap water was added to the aquaria at a slow rate to overflow continuously, to prevent the growth of epiphytes. The pH of the water in the aquarium was ~ 7.8.

Treatment with GA

The culms of the terrestrial form grown in the growth chamber were cut off at 2–3 cm above the soil surface. For the GA treatments, a stock solution of 10 mM GA in ethanol was diluted to 10, 50, 100, and 200 µM with distilled water. The pruned plants were sprayed (10 mL per pot) every morning with GA (GA_3, Wako Pure Chemical Industries, Osaka, Japan). Water without GA but containing 2% ethanol was also sprayed as control. The GA-treated plants were grown in a growth chamber under the same conditions as described above. After 3–4 weeks of GA treatment, the new primary culms were excised at ground level, divided into basal and upper parts, and their anatomy and physiology were evaluated.

Treatment with GA-biosynthesis inhibitors

Plants were grown on solid culture medium in glass bottles, because epiphytes proliferate on the plant surface under still water. Small shoots generated at the tops of the primary culms of the terrestrial form were excised and sterilized by immersion in 70% ethanol for 30 s and then in 5% (*v*/*v*) HClO for 10 min. The shoots were then grown in 900 mL glass bottles containing 100 mL of standard Murashige and Skoog medium (Duchefa Biochemie B.V., Haarlem, The Netherlands; pH 6.0), supplemented with 2% (*w*/*v*) sucrose, and solidified with 0.2% (*w*/*v*) gellan gum after autoclaving. To permit air exchange, several air holes were made in the lid of the glass bottle and covered with sun-biofilter material (Sumitomi Industries Co. Ltd, Tokyo, Japan). The culture bottles were placed in the same growth chamber under conditions used for the GA treatment. After several weeks, the developed new culms were excised, and the plants were then submerged in sterilized water containing 0 (control), 10, 100, or 500 µM uniconazole or paclobutrazol (both from Wako Pure Chemical Industries, Osaka, Japan); these compounds are known as inhibitors of GA biosynthesis (Lenton et al. 1994; Kataoka et al. 2008). The new culms that developed under submerged conditions were examined.

Anatomy and ultrastructure

Samples taken from the middle of each upper and basal parts of culms were fixed in 3% (*v*/*v*) glutaraldehyde in 50 mM sodium phosphate buffer (pH 6.8) at room temperature for 1.5 h. Subsequently, they were washed with sodium phosphate buffer and then postfixed in 2% (*w*/*v*) OsO_4 in phosphate buffer for 2 h. The samples were dehydrated through an acetone series and embedded in Quetol resin (Nisshin EM Co. Ltd., Tokyo, Japan). The embedded samples were cut transversely with glass or diamond knives on an ultramicrotome (Porter-Blum MT-2B, Sorvall Inc., Norwalk, Connecticut, USA). Semi-thin sections (1 µm thickness) were stained with toluidine blue O and observed under a light microscope (Eclipse Ci-L, Nikon Instech Co. Ltd., Tokyo, Japan). Ultrathin sections were stained with TI blue (Nisshin EM, Tokyo, Japan; Inaga et al. 2007) and lead citrate, and observed under an electron microscope (Model JEM-100CX, JEOL, Tokyo, Japan) at 75 kV. To determine the size of chloroplasts in the mesophyll and Kranz cells of the basal part of culms, long axes of chloroplasts were measured on electron micrographs. Means of 10–15 measurements per plant were calculated.

A portion of the samples fixed in 3% (*v*/*v*) glutaraldehyde was used to prepare sections containing chlorophyll

(Chl). The samples were washed with distilled water, sectioned with a razor blade using a Micro Slicer (DTK-1000 N, Dosaka EM, Kyoto, Japan), and the sections were observed under the light microscope to assess the presence of chloroplasts in cells.

Stomatal frequency was determined using transverse sections of culms of similar diameter, and was defined as the number of stomata per section, since it was difficult to estimate exactly the surface area of culms. Stomatal length was measured on longitudinal sections of culms. Means of 10 measurements per plant were calculated.

Antisera used

The following antisera were used for immunolocalization: maize leaf PEPC and pyruvate Pi dikinase (PPDK) antisera (courtesy of T. Sugiyama, RIKEN, Yokohama, Japan), pea leaf Rubisco large subunit (LSU) antiserum (courtesy of S. Muto, Nagoya University, Nagoya, Japan), and *Amaranthus tricolor* leaf NAD-ME antiserum (courtesy of H. Nakamoto, Saitama University, Urawa, Japan). These were the same antisera as used by Ueno (1996b, 1998). Antiserum dilutions were 1:500 for PEPC and PPDK and 1:1000 for Rubisco LSU and NAD-ME.

Immunohistochemistry

Intercellular localization of C_3 and C_4 enzymes in the culms of the terrestrial and submerged forms and in the GA-induced culms was investigated by immunohistochemistry under the light microscope. Samples taken from the middle of the basal part of culms were fixed in 3% (*v*/*v*) paraformaldehyde with 0.2% (*v*/*v*) glutaraldehyde in 50 mM sodium phosphate buffer (pH 6.8) on ice for 5 h, and then washed in sodium phosphate buffer. The fixed samples were dehydrated using an ethanol-tertiary butyl alcohol series and embedded in Paraplast Plus (Sigma-Aldrich Inc., St Louis, Missouri, USA). Transverse sections (10 µm thickness) were cut on a rotary microtome (PR-50, Yamato Kohki Industrial Co. Ltd., Saitama, Japan) and mounted on poly-L-lysine (Sigma-Aldrich Inc.) coated slides. The slides were dried for 1 day at 42 °C and then immunostained for PEPC, NAD-ME, PPDK, and Rubisco LSU as described by Hatakeyama and Ueno (2016), using the antiserum specific to each enzyme.

Immunogold electron microscopy

Immunogold labeling and electron microscopy were performed to observe intracellular localization of PEPC and Rubisco LSU in the culms developed under water containing 100 µM paclobutrazol. Samples taken from the middle of the basal and upper parts of the culms were fixed in 3% (*v*/*v*) glutaraldehyde in 50 mM sodium phosphate buffer (pH 6.8) on ice for 4 h and washed in sodium phosphate buffer. The samples were dehydrated in an ethanol series on ice, gradually infiltrated with Lowicryl K4M resin (Polysciences Europe GmbH, Eppelheim, Germany) at −20 °C, and then polymerized under ultraviolet light at −20 °C for 1–2 days. Ultrathin transverse sections were cut with a diamond knife. Immunolabeling for PEPC and Rubisco LSU proteins was performed as described by Ueno (1996b), using the above antisera.

Fresh weight/dry weight ratio and chlorophyll content

The fresh culm samples (0.2 g) were either air-dried at 80 °C for one day and weighed, or immersed in 80% acetone for about two days at 4 °C in the dark, until the culm pieces became colorless. The Chl content in the acetone was determined by the method of Porra et al. (1989).

Enzyme assay

Culms (0.25 g fresh weight) were ground on ice with 0.5 g of sea sand, 25 mg Polyclar AT, and 1 mL of extraction solution, which contained 50 mM Hepes–KOH (pH 7.5), 0.2 mM EDTA-2Na, 2.5 mM $MgCl_2$, 2.5 mM $MnCl_2$, 5 mM DTT, 0.2% (*v*/*v*) Triton X-100, and 0.7% (*w*/*v*) BSA. Homogenates were filtered through a double layer of gauze, and the filtrates were centrifuged at 12,000×*g* for 5 min at 4 °C. The supernatant was used to assay enzyme activity. For Rubisco, the supernatant was pre-incubated in the presence of 10 mM $NaHCO_3$ and 10 mM $MgCl_2$ at 25 °C for 10 min to obtain maximum activation. Activities of PEPC, NAD-ME, and Rubisco were assayed spectrophotometrically in 1 mL reaction mixture at 25 °C, using the methods described by Ueno (1998).

Carbon isotope ratio

Culms from three plants (0.1 g fresh weight per plant) were dried at 60 °C for 2 days, and ground with a mortar and pestle. A 2 mg aliquot of the ground sample was used to measure ^{12}C and ^{13}C content, as described by Yorimitsu et al. (2019). The isotope ratio of the sample was expressed as $\delta^{13}C$ (‰) relative to the isotope ratio of the Pee Dee Belemnite standard (Ehleringer and Osmond 1989).

Statistical analyses

Data were presented as means of three plants ± SE. These data were analyzed using Statce14 software (OMS Publishers, Tokorozawa, Saitama, Japan). The significance of differences in the fresh weight/dry weight ratio, Chl content, chloroplast size, stomatal length, stomatal frequency,

and enzyme activities were tested by ANOVA, followed by Tukey and Kramer post hoc tests. *P* values less than 0.05 were considered statistically significant.

Results

The development of secondary culms was especially conspicuous in the submerged form, and the gross morphology of the submerged and terrestrial forms differed markedly (Fig. 1a, b). In general, the diameter of the secondary culms of *E. vivipara* is much smaller than that of the primary culms (Ueno 1996a). Therefore, primary culms from the terrestrial and submerged form that had similar diameter were used for the experiments.

Effects of GA concentration on the terrestrial form

Newly developed culms of the terrestrial-form *E. vivipara* plants sprayed with 10 µM or 50 µM GA had various anatomical features. Most culms were intermediate between those of the terrestrial and submerged forms (Supplementary Fig. S1), although some were similar to the culms of the terrestrial form. This may be attributable to the difficulty in applying the GA spray uniformly to plants having caespitose morphology. The plants sprayed with 200 µM GA grew slowly and were chlorotic, and most of them died. Plants sprayed with 100 µM GA remained healthy and developed new culms (Fig. 1c). Plants sprayed with water without GA but containing 2% ethanol developed quite similar culms to the terrestrial form (data not shown). Based on these results, the 100 µM GA treatment was used in further experiments.

Morphological and anatomical features

The terrestrial form of *E. vivipara* had firm, stiff culms (Fig. 1a), whereas the submerged form had soft, weak culms (Fig. 1b). New culms developed under 100 µM GA treatment of terrestrial plants were slightly weaker and paler than the culms of the terrestrial form (Fig. 1c).

In the culms of terrestrial and submerged forms, the chlorenchyma and vascular bundles surrounded central, translucent aerenchyma (Fig. 2a, b). The vascular bundles were smaller and the air cavities were larger in the submerged form than in the terrestrial form. The culms of the terrestrial form had unusual Kranz-type anatomy, called fimbristyloid anatomy; it is characterized by three layers of bundle sheath cells, an innermost Kranz sheath, a middle colorless mestome sheath, and an outermost parenchyma sheath (PS) (Fig. 2d). The Kranz cells contained abundant chloroplasts, whereas, the PS cells had few chloroplasts (Fig. 2d; Ueno 1996a). The mesophyll cells were elongated and tightly arranged (Fig. 2a, d). There was little difference in anatomical structure of the basal and upper parts of the culms of the terrestrial form (Fig. 2g, j). The culms of the submerged form also had three layers of BS cells, but the Kranz cells were smaller than those of the terrestrial form (Fig. 2e), and the mesophyll cells were round and much larger than the Kranz cells (Fig. 2b, e), so Kranz anatomy was not apparent. The upper part (Fig. 2h) had somewhat larger vascular bundles and somewhat more elongated mesophyll cells than those of the basal part (Fig. 2k). In the GA-induced culms, the difference between the basal and upper parts was more distinct than in the culms of the submerged form (Fig. 2i, l). The basal part had anatomical features similar to those of the submerged form: round mesophyll cells were well developed, whereas the Kranz cells were small (Fig. 2c, f). However, the upper part retained the anatomical features of the terrestrial form (Fig. 2i).

The ultrastructure of the Kranz cells in the basal parts of the culms of the terrestrial form included numerous chloroplasts and mitochondria (Fig. 3a, d). The grana of chloroplasts of the Kranz cells were well developed (Fig. 3d), as in those of mesophyll cells. These structural features are typical of the Kranz (bundle sheath) cells in NAD-ME type C_4 plants (Hatch 1987; Ueno 1996a). However, the Kranz cells of the submerged form contained only a few small chloroplasts and mitochondria (Fig. 3b, e). The GA-induced culms were similar to those of the submerged form, with few organelles (Fig. 3c, f). The chloroplasts of

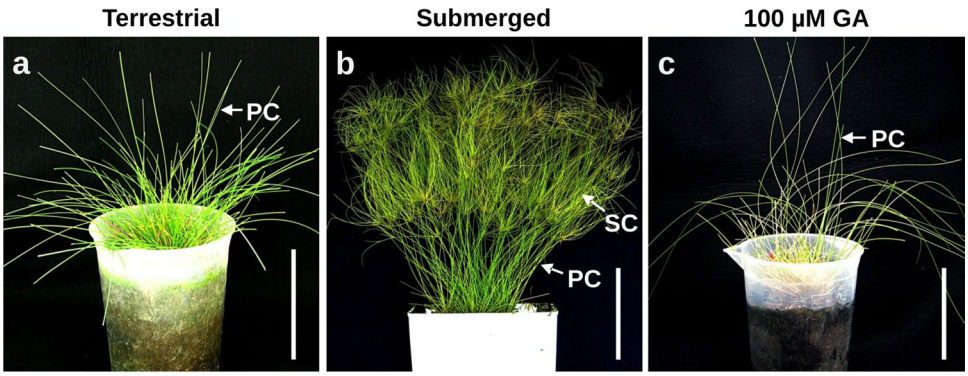

Fig. 1 Gross morphology of *Eleocharis vivipara*. **a** The terrestrial form. **b** The submerged form, induced from the terrestrial form by complete submergence and with well-developed secondary culms present on the primary culms. **c** Plant with culms induced by spraying 100 µM GA under aerial (terrestrial) conditions. *PC* primary culm, *SC* secondary culm. *Bars* = 10 cm

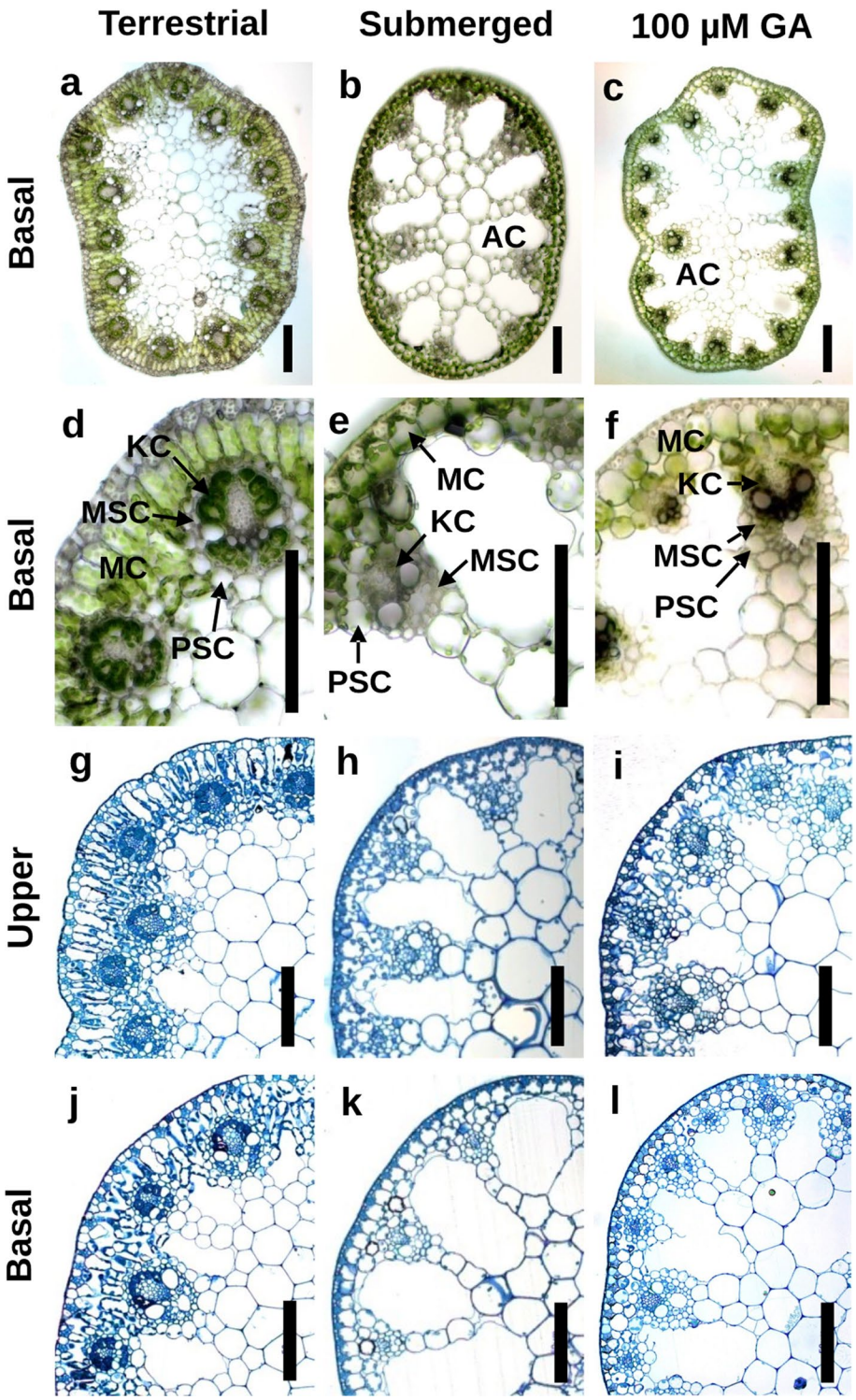

Fig. 2 Anatomical structures of culms of the terrestrial and submerged forms and 100 µM GA-induced culms of *Eleocharis vivipara*. **a**–**f** Hand-cut transverse sections of basal parts of fresh culms. **g**–**l** Transverse sections of the upper and basal parts of culms embedded in Quetol resin and stained with toluidine blue O. **a**, **d**, **g**, **j** The terrestrial form. **b**, **e**, **h**, **k** The submerged form. **c**, **f**, **i**, **l** 100 µM GA-induced culms. *AC* air cavity, *KC* Kranz cell, *MC* mesophyll cell, *MSC* mestome sheath cell, *PSC* parenchyma sheath cell. *Bars* = 100 µm

the Kranz cells in the submerged form and the GA-induced culms had well-developed grana (Fig. 3e, f), as in those of mesophyll cells. The chloroplasts of the Kranz cells was smaller in the culms of the submerged form and the GA-induced culms than in the culms of the terrestrial form (Table 1). With regard to the size of chloroplasts of the mesophyll cells, there was no significant difference among the three culms (Table 1).

The culms of the terrestrial form had many stomata, but culms of the submerged form were almost without stomata (Table 1; Ueno 1996a). In the GA-induced culms, the stomatal frequency of the upper part was very similar to that

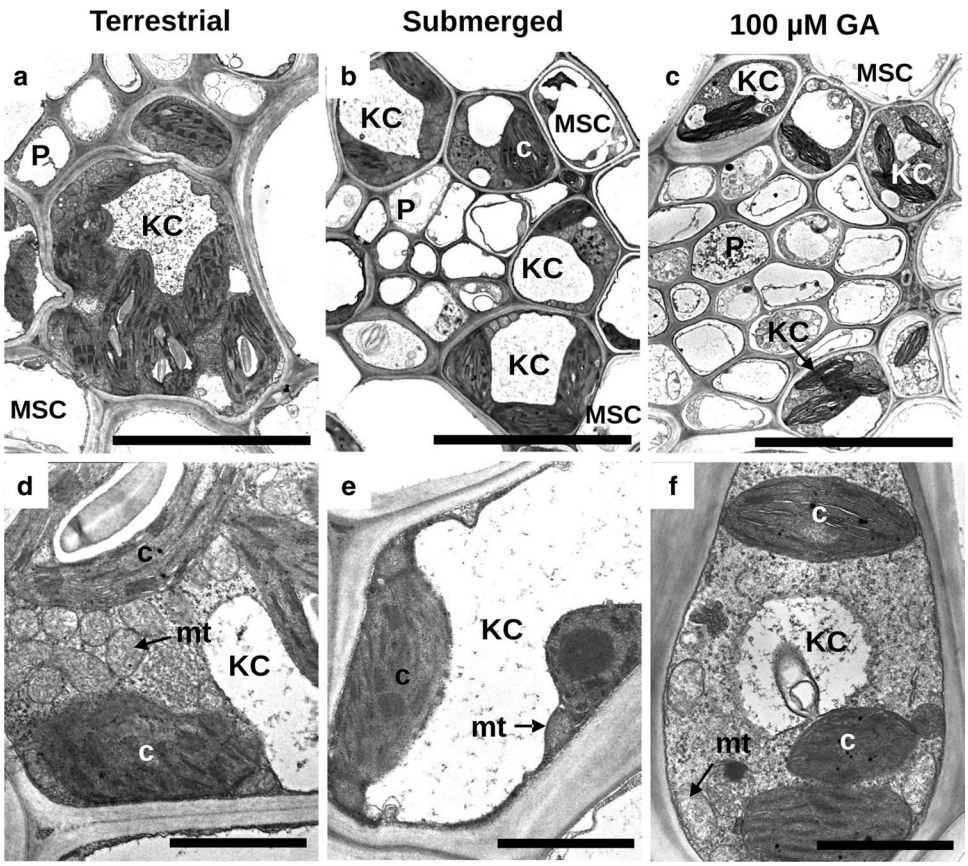

Fig. 3 Ultrastructure of Kranz cells in the basal parts of culms of the terrestrial and submerged forms and 100 μM GA-induced culms of *E. vivipara*. **a**, **d** The terrestrial form. **b**, **e** The submerged form. **c**, **f** 100 μM GA-induced culms. *KC* Kranz cell, *MSC* mestome sheath cell, *c* chloroplast, *mt* mitochondrion, *P* phloem. *Bars* = 10 μm (**a**–**c**), 1 μm (**d**–**f**)

Table 1 Anatomical, biochemical, and physiological characteristics of culms from terrestrial and submerged forms of *E. vivipara* and culms induced by application of 100 μM GA

Characteristic	Terrestrial form		Submerged form		GA-induced culms	
	Basal	Upper	Basal	Upper	Basal	Upper
Chloroplast length						
Mesophyll cells (μm)	5.5 ± 0.1a	–	5.3 ± 0.3a	–	5.3 ± 0.5a	–
Kranz cells (μm)	5.3 ± 0.5a	–	3.4 ± 0.2b	–	3.4 ± 0.2b	–
Stomatal frequency (no. per section)	9.3 ± 1.1a	9.8 ± 1.1a	0.3 ± 0.2bc	0.2 ± 0.1c	4.4 ± 1.2b	10.9 ± 1.5a
Stomatal length (μm)	394 ± 0.4ab	428 ± 1.3a	325 ± 0.8d	355 ± 0.8cd	360 ± 1.2bd	370 ± 0.8bc
Fresh weight/dry weight (g g^{-1})	5.53 ± 0.08a	5.34 ± 0.14ab	4.95 ± 0.07c	5.16 ± 0.08bc	4.96 ± 0.03c	5.02 ± 0.04bc
Chl content (mg g^{-1} FW)	1.03 ± 0.08a	1.08 ± 0.03a	0.73 ± 0.03bc	0.63 ± 0.05c	0.64 ± 0.06c	0.94 ± 0.11ab
PEPC activity (μmol mg^{-1} Chl h^{-1})	2116 ± 215b	3320 ± 125a	35 ± 5d	37 ± 4d	1273 ± 164c	2271 ± 150b
NAD-ME activity (μmol mg^{-1} Chl h^{-1})	98 ± 13c	223 ± 9a	ND	ND	88 ± 16c	168 ± 17b
Rubisco activity (μmol mg^{-1} Chl h^{-1})	390 ± 25a	324 ± 26ab	99 ± 13d	98 ± 20d	213 ± 13c	260 ± 34bc
Activity ratio of Rubisco/PEPC	0.2 ± 0.0b	0.1 ± 0.0b	2.8 ± 0.1a	2.7 ± 1.3a	0.2 ± 0.0b	0.1 ± 0.0b
$\delta^{13}C$ (‰)	− 17.5	− 17.5	− 32.6	− 32.3	− 17.9	− 17.9

All values except $\delta^{13}C$ are means of 3 plants ± SE. Within a row, different lower-case letters indicate significant difference at $P < 0.05$; *ND* not detectable

of the terrestrial culms; stomatal frequency of the basal part did not differ significantly from that of the basal part of the submerged form (Table 1). The stomatal length of both parts was greater in the culms of the terrestrial form than in those of the submerged form (Table 1). In both parts of the GA-induced culms, the stomatal length was intermediate between these two forms (Table 1).

Immunolocalization of C_3 and C_4 enzymes

The intercellular localization of the C_3 and C_4 enzymes in the basal part of the GA-induced culms was compared to those in the terrestrial and submerged forms (Fig. 4). In the culms of the terrestrial form (Fig. 4a) and in the GA-induced culms (Fig. 4c), PEPC was densely stained in the mesophyll and PS cells but not in the mestome sheath or the Kranz cells. The staining was stronger in the PS cells than in the mesophyll cells. In the culms of the submerged form, little to no staining for PEPC was observed (Fig. 4b). In all three types of culms, staining for NAD-ME was observed only in the Kranz cells. However, the staining was much stronger in the culms of the terrestrial form (Fig. 4d) than in the culms of the submerged form (Fig. 4e). The degree of staining in the GA-induced culms was intermediate between these two forms (Fig. 4f). In the culms of the terrestrial form (Fig. 4g) and in the GA-induced culms (Fig. 4i), PPDK was densely stained only in the mesophyll and PS cells, but little or no staining was observed in the culms of the submerged form (Fig. 4h). In all three types of culms, Rubisco LSU was densely stained in all photosynthetic cells, i.e., in the mesophyll, PS, and Kranz cells (Fig. 4j–l). Thus, the intercellular localization patterns of the C_3 and C_4 enzymes in the basal part of the GA-induced culms were similar to those of the terrestrial form.

Fresh weight/dry weight ratio and chlorophyll content

In the upper parts, the fresh weight/dry weight ratio did not differ significantly among the three types of culms, whereas in the basal parts it was significantly lower in the submerged form and the GA-induced culms than in the terrestrial form (Table 1). The Chl content was higher in the terrestrial form than in the submerged form (Table 1). In the GA-induced culms, the Chl contents of the basal and upper parts were similar to those in both parts of the culms of the submerged and terrestrial forms, respectively.

Activities of photosynthetic enzymes

The culms of the terrestrial form had high PEPC and NAD-ME activities, although the basal parts of these culms had lower activities than the upper parts (Table 1). In the culms of the submerged form, in both the basal and upper parts, the activities of PEPC were far lower than those of the terrestrial form, and the NAD-ME activities were not detectable. The GA-induced culms had much higher activities of both

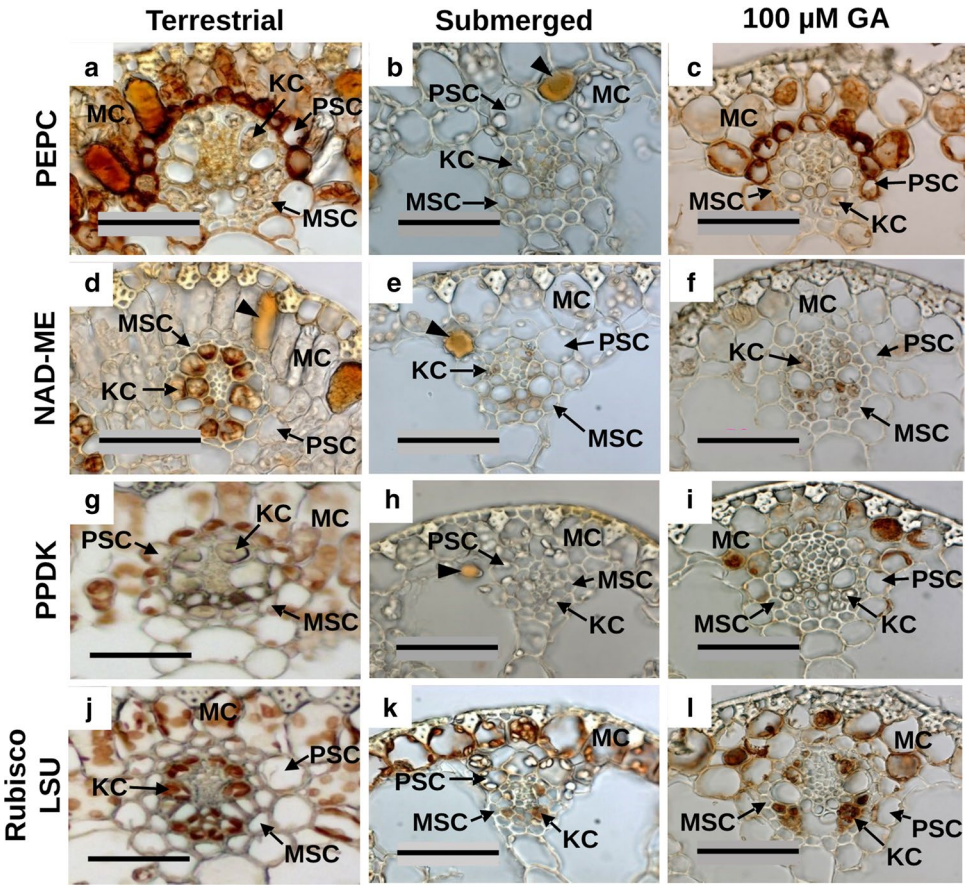

Fig. 4 Immunohistochemical localization of C_3 and C_4 photosynthetic enzymes in the basal parts of culms of the terrestrial and submerged forms and 100 µM GA-induced culms of *E. vivipara*. **a**–**c** PEP carboxylase. **d**–**f** NAD-malic enzyme. **g**–**i** Pyruvate Pi dikinase. **j**–**l** The large subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase. **a**, **d**, **g**, **j** Terrestrial form. **b**, **e**, **h**, **k** Submerged form. **c**, **f**, **i**, **l** 100 µM GA-induced culms. Arrowheads in **b**, **d**, **e**, **h** indicate non-specific cell staining, which is likely attributable to accumulated polyphenolic compounds. *KC* Kranz cell, *MC* mesophyll cell, *MSC* mestome sheath cell, *PSC* parenchyma sheath cell. *Bars* = 50 µm

PEPC and NAD-ME than the culms of the submerged form, approaching those of terrestrial form (Table 1). In addition, the activities were lower in the basal part than in the upper part of the GA-induced culms, as in the terrestrial form. The activities of Rubisco in both basal and upper parts of the culms of the terrestrial form were higher than in those of the submerged form (Table 1). Rubisco activities in the GA-induced culms were intermediate between those of the terrestrial and submerged forms. The activity ratios of Rubisco to PEPC were lower in the culms of the terrestrial form than in those of the submerged form (Table 1). No significant differences were found in the activity ratios between the GA-induced culms and those of the terrestrial form.

Carbon isotope ratio

The culms of the terrestrial form had much higher $\delta^{13}C$ values than culms of the submerged form (Table 1). The $\delta^{13}C$ values of the GA-induced culms approached those of the terrestrial form. However, there was very little difference in $\delta^{13}C$ between the basal and upper parts, within each form.

Effects of inhibitors of GA biosynthesis on the submerged form

The culms of submerged plants grown under water only (control) showed non-Kranz anatomy more clearly in the basal part than in the upper part (Fig. 5a, d). The anatomy of the culms of plants grown under water containing either 10 µM uniconazole or 10 µM paclobutrazol was similar to that of the control submerged plants (data not shown). However, plants grown under water containing either 100 µM uniconazole (Fig. 5b, e) or 100 µM paclobutrazol (Fig. 5c, f) developed culms with Kranz anatomy, often with considerable accumulation of starch grains. In general, culms showed the Kranz anatomical traits more clearly in the upper part than in the basal part. Plants grown under water containing either 500 µM uniconazole or 500 µM paclobutrazol also had Kranz anatomy but were chlorotic (data not shown). At 100 µM paclobutrazol, no immunogold labeling for PEPC was detected in either part of the culms (Fig. 6a, c), whereas, Rubisco LSU was densely labeled in the chloroplasts of both parts (Fig. 6b, d). This is consistent with the C_3 pattern of cellular accumulation of photosynthetic enzymes.

Discussion

This study found that exogenously applied GA induced both non-Kranz anatomy and C_4-like biochemical traits in the culms of the terrestrial form of *E. vivipara*. As far as we know, photosynthetic tissues with mismatched anatomical and biochemical traits have not been reported previously in C_3 or C_4 plants. The responses to GA in *E. vivipara* reported here provide some insight into the regulatory mechanism

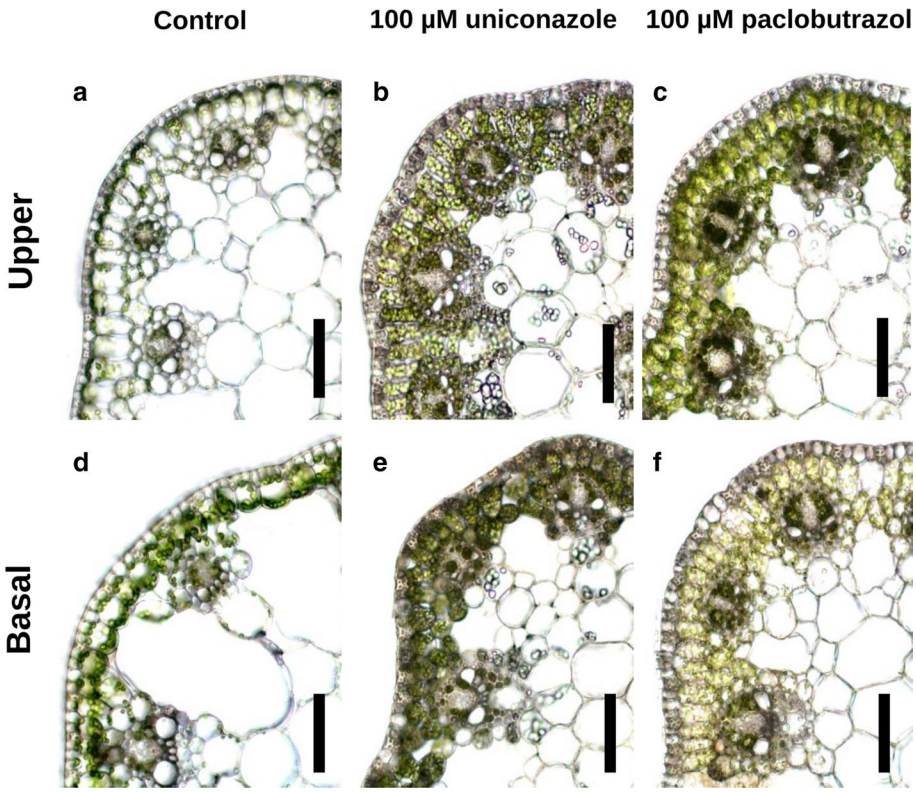

Fig. 5 Anatomical structures of culms of the submerged form of *E. vivipara* grown in a glass bottle under water alone (control) or water containing one of two GA-biosynthesis inhibitors. **a**, **d** Control. **b**, **e** 100 µM uniconazole. **c**, **f** 100 µM paclobutrazol. *Basal* basal part of culm, *Upper* upper part of culm. *Bars* = 100 µm

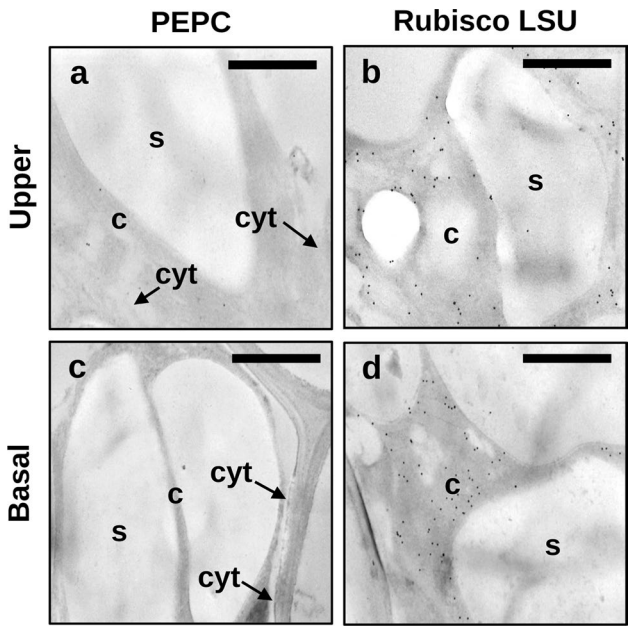

Fig. 6 Immunogold labeling of C_3 and C_4 photosynthetic enzymes in culms of *E. vivipara* developed under water containing 100 µM paclobutrazol. **a** PEPC in a parenchyma sheath cell. **b** Rubisco LSU in a mesophyll cell. **c** PEPC in a mesophyll cell. **d** Rubisco LSU in a Kranz cell. *Basal* basal part of culm, *Upper* upper part of culm, *c* chloroplast, *cyt* cytosol, *s* starch grain. *Bars* = 1 µm

of the differentiation of anatomical and biochemical traits in C_4 leaves.

Effects of GA on the development of anatomical and biochemical traits

Apart from the shoot apex being submerged in or emergent from water, heterophylly in aquatic and amphibious plants is controlled by various environmental factors, including temperature, light quality, and photoperiod. These effects are believed to be mediated by endogenous plant hormones (Maberly and Spence 1989; Minorsky 2003; Wanke 2011; Nakayama et al. 2014; Li et al. 2019), and it is not surprising that GA influenced heterophylly in *E. vivipara*. However, the effects of GA on heterophylly are quite complex. For example, in *Callitriche stagnalis* Scop. (McComb 1965) and *C. heterophylla* Pursh. (Deschamp and Cooke 1984), GA is involved in the formation of water-form leaves. In contrast, in *Proserpinaca intermedia* Mack. (Kane and Albert 1982) and *P. palustris* L. (Kane and Albert 1987), GA induces land-form leaves. Although the mechanisms responsible for these different responses are still not fully elucidated, factors other than plant hormones such as photoperiod, may also be involved. Deschamp and Cooke (1984) examined the effect of GA under long days, whereas Kane and Albert (1982, 1987) did so under short days. The work reported here was done under long days, and the anatomical responses to GA in *E. vivipara* were similar to those reported by Deschamp and Cooke (1984).

Signaling systems using plant hormones are believed to play a critical role in the expression of heterophylly (Minorsky 2003; Wanke 2011; Li et al. 2019). In the amphibious plant *Hippuris vulgaris* L., the level of endogenous ABA increases in response to the osmotic stress caused by exposure to air, and this increase promotes the development of land-form leaves (Goliber and Feldman 1989). In the aquatic fern *Marsilea quadrifolia* L., Hsu et al. (2001) characterized early genes responsive to ABA during the induction of heterophylly. In the study reported here, when the submerged form of *E. vivipara* was treated with either uniconazole or paclobutrazol, the plants produced new culms with Kranz anatomy despite being under water. This suggests that a decrease in endogenous GA due to inhibition of GA biosynthesis is responsible for the development of Kranz anatomy. In *E. vivipara*, increased endogenous GA may inhibit the regulatory network of developmental programs that lead to the formation of Kranz anatomy. This may include the ratio of Kranz (bundle sheath) to mesophyll cell volume, the structure and arrangement of mesophyll cells, and the size and number of chloroplasts and mitochondria in the Kranz cells (Roth et al. 1996; Slewinski et al. 2012, 2014; Sedelnikova et al. 2018). These data, together with the effects of ABA (Ueno 1998), imply the presence of master switches for the development of Kranz anatomy. However, the regulation of Kranz development in *E. vivipara* may also involve other hormones. Wanke (2011) has suggested that GA acts positively via ethylene in the formation of water-form leaves, while ABA antagonizes the function of both GA and ethylene. Ethylene is also involved in the development of water-form leaves in *Ludwigia arcuata* Walt. (Kuwabara et al. 2003). Further investigations are required to determine the possible effects of other hormones on heterophylly in *E. vivipara*.

The responses of *E. vivipara* to emergence and submergence are quite unique, because the expression of heterophylly is accompanied by alterations in C_4 and C_3 biochemical traits. Interestingly, the GA treatment did not induce the expression of C_3 biochemical traits despite the development of non-Kranz anatomy. In the terrestrial form, the PEPC and PPDK proteins occur only in the outer PS and mesophyll cells, although they accumulate more densely in the outer PS cells adjacent to vascular bundles than in the mesophyll cells which are distant from them (Ueno 1996b). In the GA-induced culms, similar patterns of enzyme accumulation were observed, despite the differences in structure and arrangement of mesophyll cells in comparison with the terrestrial form. The accumulation of NAD-ME protein, which was localized in the Kranz cells, was intermediate between the terrestrial and submerged forms, although the activity

approached the level observed in the terrestrial form. Ueno (1998) found that ABA treatment of the submerged form of *E. vivipara* induces culms with both Kranz anatomy and C_4-like biochemical traits. However, this does not necessarily prove that there is a single ABA-responsive switch responsible for the entire differentiation of the anatomical and biochemical traits (Ueno 1998). Agarie et al. (2002) showed that ABA treatment of the submerged form increases the transcript levels of PEPC and PPDK genes in mature culms with non-Kranz anatomy, and in newly developing culms with Kranz anatomy. Uchino et al. (1998) found that when the terrestrial form was submerged, the plants developed new culms with non-Kranz anatomy and high PEPC transcript level during the transition.

The data here clearly indicate that GA affected the development of anatomical traits in *E. vivipara*, but not the biochemical traits of photosynthesis. The expression of the biochemical traits reflected the surrounding environment (i.e., terrestrial conditions). Furthermore, plants that were grown under water containing the GA inhibitor paclobutrazol developed new culms with Kranz anatomy, but with the C_3 pattern of enzyme accumulation. These data clearly suggest that the differentiation of C_4 anatomical and biochemical traits is regulated under separate signaling systems.

In the facultative CAM plant *Mesembryanthemum crystallinum* L., ABA applied exogenously to leaves induces CAM with concomitant increases in the activities of PEPC and NADP-malic enzyme (Chu et al. 1990). GA treatment also increases the activity of PEPC and the amount of PEPC protein in *M. crystallinum* with an increase in acid metabolism (Guralnick et al. 2001). However, the data in the current study did not suggest increased PEPC activity in *E. vivipara* in response to GA. Although it is well-known that CAM shares common key enzymes with C_4 photosynthesis, including PEPC, PPDK, and C_4-acid decarboxylating enzymes (Cushman 2001), the regulatory mechanisms of these enzymes in facultative CAM plants differ considerably from those in C_4 plants.

Gradient of anatomical and biochemical traits along a culm

In the GA-induced culms, the basal part showed non-Kranz anatomy, whereas the anatomy of the upper part was more similar to that of the terrestrial form. In the culms of the submerged form, this difference was also apparent in the structure of the mesophyll cells and the size of the vascular bundles. However, there were no significant differences in the stomatal traits between the basal and upper parts of submerged culms. In the GA-induced culms, the stomatal frequency was higher in the upper part than in the basal part. In both the terrestrial and GA-induced culms, activities of PEPC and NAD-ME were higher in the upper part than in the basal part. However, the activity ratio of Rubisco to PEPC and the $\delta^{13}C$ values did not differ between the two parts of these culms, suggesting that there is little difference in their photosynthetic mechanisms. In grasses the developing leaves have a maturation gradient whereby the most developed tissue is found at the tip and the least developed at the base (Wakayama et al. 2003; Nelson 2011). A developmental gradient has been also recognized in immature culms of *E. baldwinii* (Chen et al. 2011). In developing leaves of maize, the activity of C_4 enzymes and the transcript levels of their genes are higher in the tip region than in the base region (Pick et al. 2011). In *E. vivipara*, however, the differences were observed in mature culms. It is not known why these anatomical and biochemical differences occur from the tip to the base of a single culm, but there may be gradients in the distribution of endogenous hormones along the length of the culm.

Photosynthetic function in the basal part of GA-induced culms

It is interesting to consider the photosynthetic function operating in the basal part of the GA-induced culms. Since the Kranz cells were small with poorly developed chloroplasts and mitochondria, whereas the mesophyll cells were relatively well developed, it is unlikely that the C_4 cycle functioned efficiently in these tissues. Stomatal frequency in the basal part of the GA-induced culms was lower than in culms of the terrestrial form. In amphibious plants, typical waterform leaves have few or no stomata, but have thinner cuticle on the epidermal surface than that of land-form leaves (Mommer and Visser 2005). This allows absorption of CO_2 from the water via the epidermis, but it also results in low resistance to desiccation and eventually leads to death when the plants are exposed to air (Maberly and Spence 1989; Mommer and Visser 2005). However, the epidermis of the GA-induced culms was apparently well cutinized, because the culms were able to grow in air.

The Chl content in the basal part was lower in the GA-induced culms than in the culms of the terrestrial form. Although the activity of PEPC in the GA-induced culms was lower than that in the culms of the terrestrial form, the value was within the C_4 range. The activity of NAD-ME and the activity ratio of Rubisco to PEPC were similar to the terrestrial form. The intercellular localization of the C_3 and C_4 enzymes in the basal part of the GA-induced culms was also similar to that of the terrestrial form. In both the GA-induced and terrestrial form culms, large amounts of PEPC and PPDK accumulated only in the mesophyll and PS cells, whereas NAD-ME was restricted to the Kranz cells. Rubisco was observed in the mesophyll and PS cells as well as in the Kranz cells. The presence of Rubisco in the mesophyll cells is a characteristic of C_4-like plants

(Sage et al. 2012; Tashima et al. 2021). These data suggest that the basal part of the GA-induced culms developed C_4-like biochemical traits in the non-Kranz anatomy.

Although photosynthetic gas exchange was not measured, the low stomatal frequency in the basal part of the GA-induced culms may have inhibited photosynthesis due to reduced CO_2 conductance. The $\delta^{13}C$ value is an indicator of photosynthetic metabolism function in plants (Ehleringer and Osmond 1989; Cernusak et al. 2013). In both parts of the GA-induced culms, the $\delta^{13}C$ values were C_4-like, as was observed in the terrestrial form. This suggests that the C_4 cycle is dominant in both parts of the GA-induced culms. However, it is also possible that some of the carbon in the tissues of the basal part of the GA-induced culms may have been derived from storage carbon fixed prior to the GA treatment, and/or from carbon fixed by the upper part. Further analyses, including photosynthetic gas exchange and ^{14}C pulse chase labelling experiments, would enhance the understanding of the photosynthetic metabolism operating in GA-induced culms.

Author contribution statement OU and YS conceived and designed the research. YS, OU, and KT conducted the experiments and analyzed the data. OU and YS wrote the manuscript. All authors read and approved the manuscript.

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Declarations

Conflict of interest The authors declare that this study was conducted in the absence of any commercial relationships that could lead to any potential conflict of interest.

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