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ORIGINAL ARTICLE

Salt stress induces Kranz anatomy and expression of C_4 photosynthetic enzymes in the amphibious sedge *Eleocharis vivipara*

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Abstract

Eleocharis vivipara Link is a unique amphibious leafless plant of the Cyperaceae. The terrestrial form develops culms with Kranz anatomy and C_4-like traits, while the submerged form does culms with non-Kranz anatomy and C_3 traits. The submerged form develops new culms with C_4-like mode when exposed to air or exogenous abscisic acid. In this study, we investigated whether salt stress (0.05–0.3 M NaCl) has a similar effect. When the submerged form was grown for one month in solutions of 0.1 M NaCl and more, culm growth was strongly suppressed. However, these plants slowly developed new culms that had Kranz anatomy with chloroplast-abundant Kranz bundle sheath cells. Although the culms of the submerged form had only few stomata, culms grown in the NaCl solution had many stomata. The NaCl-grown culms also accumulated large amounts of C_4 photosynthetic enzymes (phosphoenolpyruvate carboxylase and pyruvate Pi dikinase), and the cellular localization patterns of these enzymes and ribulose 1,5-bisphosphate carboxylase/oxygenase were similar to those in terrestrial culms. Accumulation of C_4 enzymes increased in mature culms of the submerged form (with non-Kranz anatomy) when exposed to 0.2 M NaCl solution for one week. These results suggest that salt stress induces development of Kranz anatomy and expression of C_4 photosynthetic enzymes in the submerged C_3 form of *E. vivipara,* whereas the anatomical and biochemical traits of C_4 photosynthesis appear to be regulated independently.

Keywords C_4 development · C_4 photosynthesis · C_3 photosynthesis · *Eleocharis vivipara* · Kranz anatomy · Salt stress

Abbreviations

ABA	Abscisic acid
BS	Bundle sheath
GA	Gibberellic acid
LSU	Large subunit
MS	Mestome sheath
PEPC	Phosphoenolpyruvate carboxylase
PPDK	Pyruvate Pi dikinase
PS	Parenchyma sheath
Rubisco	Ribulose 1,5-bisphosphate carboxylase/oxygenase

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Introduction

Efficient photosynthesis is critical for the growth and survival of plants. In C_3 photosynthesis, the prevalent photosynthetic system, atmospheric CO_2 is fixed by ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) in mesophyll cells. Under the atmospheric conditions of low CO_2, however, the efficiency of C_3 photosynthesis is reduced by the high oxygenase activity of Rubisco, known as photorespiration (Bauwe 2011; Sage et al. 2012). In contrast, C_4 photosynthesis is adapted to relatively low atmospheric CO_2, such that the CO_2 is primarily fixed into C_4 acids by phosphoenolpyruvate carboxylase (PEPC) in the mesophyll cells. The C_4 acids are moved to adjacent bundle sheath (BS) cells, where they are decarboxylated

and the released CO_2 is re-fixed by Rubisco. This biochemical process concentrates CO_2 around Rubisco, suppressing the oxygenase activity (Hatch 1987; von Caemmerer and Furbank 2003; Langdale 2011; Schlüter and Weber 2020). C_4 plants have higher photosynthetic capacity than C_3 plants under high light, high temperature and water deficiency, i.e., in conditions which tend to accelerate photorespiration (Sage et al. 2018).

While C_4 photosynthesis generally involves cooperation of the two types of photosynthetic cells, C_3 and CAM photosynthesis each occurs within a single photosynthetic cell. This difference may influence the flexibility of the photosynthetic mode in response to changing environments. There are many facultative CAM plants, which shift from C_3 to CAM mode in response to reduced soil water availability (Lüttge 2004; Winter 2019). However, plants that shift from C_3 to C_4 mode are very rare, likely because the change requires substantial anatomical and biochemical changes in the leaves. The exceptions are some submerged aquatic plants, such as *Hydrilla verticillata* (L. F.) Royle, that perform C_4 photosynthesis in a single cell, and can shift from C_3 to C_4 mode under CO_2-limited water conditions (Bowes 2011).

Aquatic and amphibious plants can develop either land- or water-form leaves, depending on whether the shoot apex is emergent from or submerged in water. This phenomenon is called heterophylly (Minorsky 2003; Li et al. 2019). The amphibious leafless sedge, *Eleocharis vivipara* Link, expresses C_4-like mode under terrestrial conditions and C_3 mode under submerged conditions, accompanied by pertinent structural changes in the photosynthetic cells (Ueno et al. 1988; Ueno 2001). Since *E. vivipara* lacks leaf blades, the culm functions as the photosynthetic organ, and it is the culm that exhibits heterophylly (Ueno et al. 1988). The culms of the terrestrial form have Kranz anatomy, characterized by well-developed Kranz BS cells and tightly arranged mesophyll cells; the culms of the submerged form have non-Kranz anatomy with poorly developed Kranz cells containing few organelles and relatively well-developed mesophyll cells (Ueno et al. 1988; Ueno 1996a). The culms of the terrestrial form have high activities of C_4 enzymes and show $^{14}CO_2$-fixation pattern of C_4 mode (Ueno et al. 1988). However, the following studies suggested that functionally active Rubisco distributes in the mesophyll cells as well as the Kranz cells, differing from true C_4 mode (Ueno 1996b; Ueno and Ishimaru 2002). Thus, the terrestrial form performs C_4-like mode of photosynthesis (Ueno and Ishimaru 2002) that is situated as a stage in C_4 evolution just before full C_4 photosynthesis (Cheng et al. 1988; Sage et al. 2012; Tashima et al. 2021). In contrast, the culms of the submerged form have only lower activities of C_4 enzymes and show $^{14}CO_2$-fixation pattern of C_3 mode (Ueno et al. 1988). The change of photosynthetic metabolism in *E. vivipara* is accomplished by cellular regulation of the expression of C_3 and C_4 photosynthetic enzymes, accompanied by anatomical modifications (Agarie et al. 1997, 2002; Ueno 2001).

Osmotic stress is the main environmental factor causing heterophylly in amphibious plants (Deschamp and Cooke 1984; Goliber and Feldman 1989; Li et al. 2019), and expression of heterophylly is triggered in this manner in *E. vivipara*. However, *E. vivipara* is quite unique because the conversion of photosynthetic mode occurs together with anatomical changes of the photosynthetic organ. Plant hormones including abscisic acid (ABA) and gibberellic acid (GA) are involved in the expression of heterophylly (Anderson 1978; Hsu et al. 2001; Minorsky 2003; Wanke 2011; Li et al. 2019). Treatment of the submerged C_3 form of *E. vivipara* with ABA induces development of new culms with C_4-like mode and Kranz anatomy (Ueno 1998). Treatment of the terrestrial form with GA induces the formation of unusual culms with non-Kranz anatomy but C_4-like biochemical traits (Suizu et al. 2021). Thus, *E. vivipara* provides an intriguing system to examine the regulatory mechanisms of C_3 and C_4 development (Ueno 2001). Recently, transcriptome analyses have been used to elucidate the regulatory mechanism of C_3/C_4 conversion in *E. vivipara* (Harada et al. 2018) and in the related species, *Eleocharis baldwinii* (Torr.) Chapm. (Chen et al. 2014). These studies have suggested that transcription of the components of the glycolysis pathway, citrate acid metabolism, protein synthesis, and transporters as well as those of the C_4 pathway is modified during the C_4 development.

The environmental and hormonal regulation of the shift from C_3 to C_4-like mode in *E. vivipara* is reminiscent of that from C_3 to CAM mode in facultative CAM plants. Both osmotic stress and ABA induce the C_4-like mode (Ueno 1998; Agarie et al. 2002) and the CAM mode (Chu et al. 1990; Taybi et al. 2002). Salt stress can induce the shift from C_3 to CAM mode in some facultative CAM plants (Winter and von Willert 1972; Cushman et al. 1989; Winter 2019). Salt stress causes osmotic stress in the plant body, and ABA mediates this signaling pathway (Yang and Guo 2018; van Zelm et al. 2020). This suggests that salt stress may also cause the change from C_3 to C_4-like mode in *E. vivipara*.

In this study, we investigated the effects of NaCl treatment on anatomical and biochemical traits of photosynthetic tissues in newly formed and mature culms of *E. vivipara.*

Materials and methods

Plant materials and growth conditions

Eleocharis vivipara plants examined in this study were originally collected in Florida, USA (Ueno et al. 1988), and have been propagated in a greenhouse at Faculty of Agriculture, Kyushu University, Fukuoka, Japan. The methods for

cultivation and NaCl treatment are schematically shown in Supplementary Fig. S1. Small shoots of the terrestrial form of *E. vivipara* were transplanted into 500 mL pots that were filled with a commercial soil mix formulated for growing vegetables (Iseki Co. Ltd, Tokyo, Japan). The plants were grown in a growth chamber under natural sunlight (photon flux density at midday of about 1000 µmol m^{-2} s^{-1}) at 25 °C and 70% relative humidity, for about two months. They were watered daily and half-strength standard Hoagland nutrient solution was applied weekly. To induce the submerged form, the culms of the terrestrial plants were pruned off, and the pots were submerged under 50 cm of water in aquaria. These plants were grown in the aquaria for at least two months. The water in the aquaria was overflowed by addition of tap water (pH 7.8) at a slow rate to suppress the growth of epiphytes. After the culms of the submerged-form plants were cut off, the plants were grown in 8 L water tanks (2 pots per tank) in the growth chamber for about 2 months. The water in the tanks was changed weekly.

NaCl treatment

Plant growth and anatomy of newly developed culms were investigated at 0.05, 0.1, 0.2, and 0.3 M NaCl concentrations. Three plants (1 pot per tank) were examined for each treatment. All culms of submerged-form plants were cut off at 0.5–1 cm above the soil surface; the plants were submerged in 8 L tanks filled with one of the NaCl solutions and grown for one month (Supplementary Fig. S1). The NaCl solution in the tanks was changed weekly. Control plants were also grown exactly like salt-treated plants but in water. The culms of *E. vivipara* have the growing region in the basal part. Thus, after culms were cut, they often developed new tissues (elongated culms). As a result, the plant had elongated culms together with true new culms developed from the culm primordia. However, it was easy to distinguish these culms, because the elongated culms had cut end at the apex. In this experiment, true new culms were used. The same NaCl concentrations except 0.05 M were used for analyses of new culms by immunohistochemistry and western blotting. Submerged-form plants with preserved mature culms (non-Kranz anatomy) were exposed to 0.2 M NaCl solution for one week and analyzed by immunohistochemistry and western blotting.

Culm anatomy

Samples taken from the middle of culms were fixed in 3% (v/v) glutaraldehyde in 50 mM sodium phosphate buffer (pH 6.8) at room temperature for 2 h. The fixed culms were washed with distilled water and transversely sectioned with a razor blade using a Micro Slicer (DTK-1000 N, Dosaka EM, Kyoto, Japan). The Sections (10 µm thickness) were examined under a light microscope (Eclipse Ci-L, Nikon Instech Co. Ltd., Tokyo, Japan).

Stomatal frequency was determined at the midpoint of the upper and basal parts of each culm. It was difficult to exactly estimate the surface area of the culms, because transverse sections of most culms did not have an outline of complete ellipse. Therefore, the stomatal frequency was measured using transverse sections of culms that had similar diameter, and was defined as the number of stomata observed per section. Although this method of measurement does not reflect actual stomatal frequency such as stomatal number per unit surface area, it is possible to compare relative stomatal frequency in culms (Suizu et al. 2021). For stomatal frequency, the data from five culms per plant were averaged to give one value for a plant. Then, mean ± SE was calculated using the individual values from three plants.

Antisera for immunochemistry and western blots

The same antisera as in Ueno (1996b, 1998) and Suizu et al. (2021) were used: maize leaf PEPC and pyruvate Pi dikinase (PPDK) antisera (courtesy of T. Sugiyama, RIKEN, Yokohama, Japan) and pea leaf Rubisco large subunit (LSU) antiserum (courtesy of S. Muto, Nagoya University, Nagoya, Japan). Antiserum dilutions were 1:500 for PEPC and PPDK, and 1:1000 for Rubisco LSU for immunohistochemistry and western blotting.

Immunohistochemistry

Samples taken from the middle 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 slides coated with poly-L-lysine (Sigma-Aldrich Inc.). The slides were dried for 1 day at 42 °C and then immunostained for PEPC, PPDK, and Rubisco LSU as described by Hatakeyama and Ueno (2016), using the antiserum specific to each enzyme. For controls, the antiserum was replaced by non-immune serum. Anti-rabbit goat antibody conjugated with horseradish peroxidase (American Qualex, San Clemente, California, USA) was used as secondary antibody. Localization of these enzymes in the sections was visualized by use of peroxidase-stain-DAB (3,3-diaminobenzidine tetrahydrochloride) Kit (Nacalai Tesque, Inc., Kyoto, Japan) and investigated under the light microscope.

Western blot analysis

Culms were sampled, frozen immediately in liquid nitrogen, and stored at − 80 °C. These culms (0.3 g fresh weight) were ground using a pestle and mortar with 0.25 g sea sand and 25 mg insoluble polyvinylpyrrolidone in 1 ml of grinding medium on ice. The grinding medium contained 50 mM Hepes–KOH (pH 7.5), 5 mM dithiothreitol, 0.2 mM Na_2-EDTA, 1 mM phenylmethylsulfonylfluoride, 0.1 mM leupeptin, 0.2% (v/v) Triton X-100. Homogenates were filtered through gauze, and the filtrates were centrifuged at 10,000 g for 10 min at 4 °C. The quantity of soluble protein in the supernatants was determined by the method of Bradford (1976). Western blot analysis was performed as described by Ueno (1992). Polypeptides were separated by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose sheets. The nitrocellulose sheets were probed with antisera and horse radish peroxidase-conjugated goat anti-(rabbit)IgG antibodies (Bio-Rad Laboratories Inc., California, USA). Soluble proteins (10 µg for PEPC and PPDK and 2.5 µg for Rubisco LSU) were loaded in each lane. The SDS–polyacrylamide gels were stained with 0.25% (w/v) Coomassie brilliant blue.

Statistical analyses

The data of stomatal frequency were analyzed using Statce14 software (OMS Publishers, Tokorozawa, Saitama, Japan). The significance of differences was tested by one way analysis of variance (ANOVA), followed by Tukey and Kramer post hoc tests. *P* values less than 0.05 were considered statistically significant.

Results

Effects of salt stress on growth of submerged form

Eleocharis vivipara plants developed secondary slender culms on the tips of the primary thick culms by proliferation, which were especially conspicuous in the submerged form (Fig. 1a, b; Ueno 1996a). NaCl treatment of the submerged form inhibited the growth of culms, and very few secondary culms were formed (Fig. 1c). Therefore, only primary culms of the terrestrial, submerged forms, and NaCl-treated plants were used in further analyses. Inhibition was weakest at 0.05 M NaCl (data not presented). Mature culms on the submerged form gradually became chlorotic when exposed to 0.2 M NaCl, and died within several weeks. New culms developed in NaCl solution did not show such symptom.

Anatomical structure of culms

The culms of the terrestrial form had Kranz anatomy (Fig. 2a, d) and those of the submerged form had non-Kranz anatomy (Fig. 2b, e). In the terrestrial culms, the vascular bundles were surrounded by three layers of BS cells: an innermost layer of Kranz cells, a middle layer of mestome sheath (MS) cells, and an outermost layer of parenchyma sheath (PS) cells (Fig. 2d). This unusual Kranz type-anatomy is called fimbristyloid anatomy (Ueno 1996a; Edwards

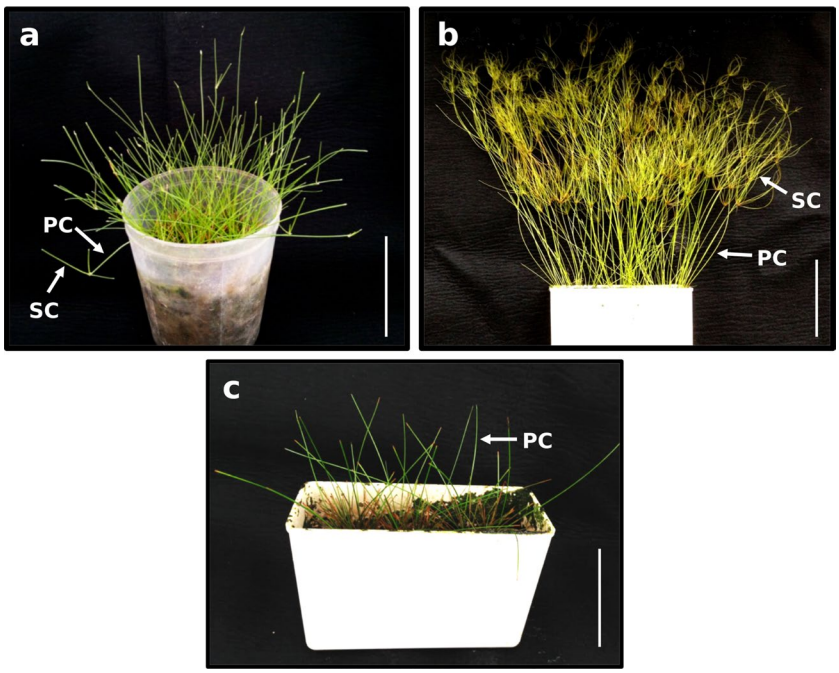

Fig. 1 Gross morphology of *Eleocharis vivipara*. **a** The terrestrial form; **b** the submerged form; **c** plant that developed in 0.1 M NaCl solution. *PC* primary culm, *SC* secondary culm. Bars = 10 cm

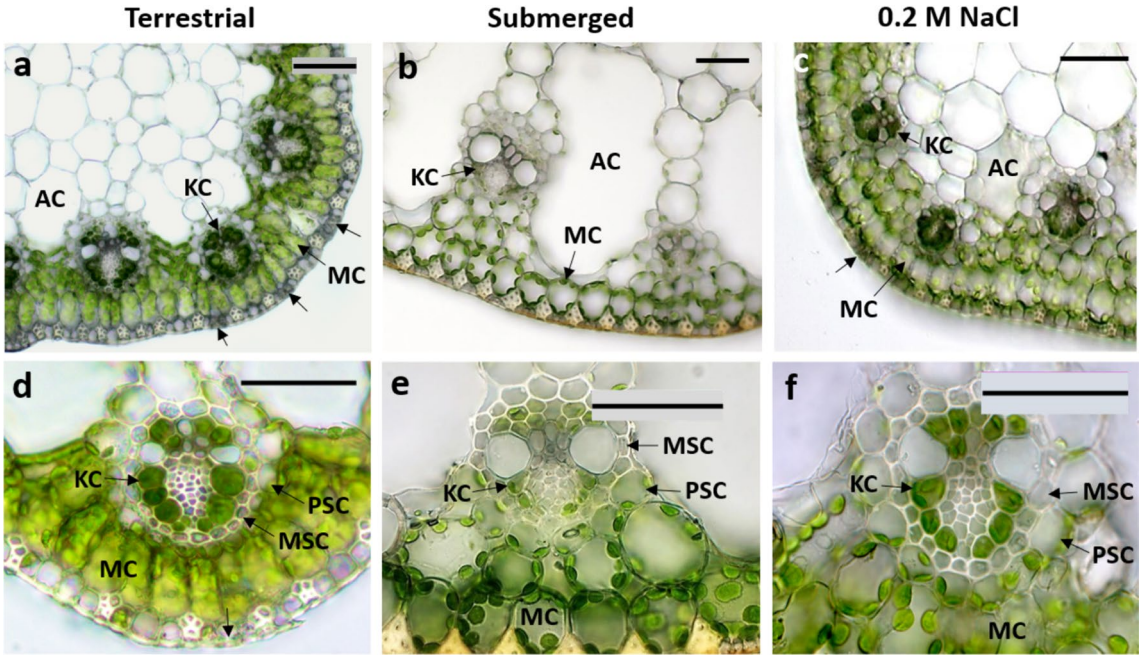

Fig. 2 Anatomical structure of culms of *Eleocharis vivipara.* **a** and **d** The terrestrial form; **b** and **e** the submerged form; **c** and **f** new culms produced by submerged-form plants in 0.2 M NaCl solution. Unlabeled arrows in (**a**, **c**, **d**) indicate stomata. *AC* air cavity, *KC* Kranz cell, *MC* mesophyll cell, *MSC* mestome sheath cell, *PSC* parenchyma sheath cell. Bars = 50 μm

and Voznesenskaya 2011). The Kranz cells were dense green due to abundant chloroplasts, whereas the MS cells lacked chloroplasts and the PS cells contained only a few chloroplasts (Fig. 2d). Elongated mesophyll cells tightly surrounded the BS cells and vascular bundles (Fig. 2a, d). However, the mesophyll cells were absent on the xylem side. Although the vascular bundles of the submerged form were also surrounded by the three layers of BS cells, the Kranz cells were much smaller than those in the terrestrial form, and contained only a few chloroplasts (Fig. 2e). The mesophyll cells were round and well developed in comparison with the Kranz cells, and formed a continuous layer beneath the epidermis (Fig. 2b). Air cavities were more developed in culms of the submerged form than in culms of the terrestrial form (Fig. 2a, b).

In approximately half of the new culms developed in 0.05 M NaCl solution, the non-Kranz-like anatomy was similar to that of the submerged form but Kranz cells were somewhat larger (Supplementary Fig. S2a). The remaining culms had an intermediate anatomy between the non-Kranz and Kranz types (Supplementary Fig. S2b). In 0.1 M NaCl solution, new culms with Kranz anatomy and those with intermediate anatomy were found. In 0.2 M NaCl (Fig. 2c, f) and 0.3 M NaCl (data not shown) solutions, most culms had Kranz anatomy. Culms with Kranz anatomy had large Kranz cells containing abundant chloroplasts (Fig. 2f), as seen in the terrestrial form (Fig. 2d). The arrangement of mesophyll cells was similar to that of the terrestrial form. However, the mesophyll cells were essentially round in culms grown in the NaCl solution (Fig. 2c). The size of air cavities in culms grown in the NaCl solution (Fig. 2c) was similar to those in the terrestrial form (Fig. 1a). *E. vivipara* plants had elongated culms together with true new culms developed under NaCl treatment, as mentioned in Materials and Methods. The elongated culms also showed similar anatomical responses for NaCl treatment (data not shown).

The culms of the terrestrial form had many stomata, but culms of the submerged form had only few stomata (Figs. 2, 3). In the terrestrial form, the stomatal frequency was higher in the upper part than in the basal part of culms. In new culms developed in 0.1 or 0.2 M NaCl solution, the stomatal frequency in the upper part was intermediate between those in the terrestrial and submerged forms (Fig. 3). In the basal parts, the stomatal frequency was similar to that in the submerged form in culms from 0.1 M NaCl solution, but was like that in the terrestrial form in culms produced in 0.2 M NaCl (Fig. 3).

Cellular expression of photosynthetic enzymes

When the culm sections of the terrestrial and submerged forms and NaCl-treated submerged plants were incubated in non-immune serum, no staining was observed (data not shown).

In the first experiment, new culms developed in 0.2 M NaCl solution were analyzed (Fig. 4). When the culm

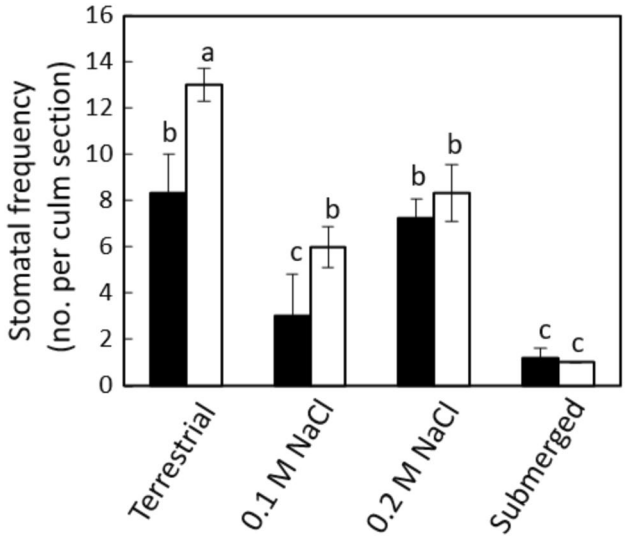

Fig. 3 Stomatal frequency of culms of the terrestrial and submerged forms of *E. vivipara* and new culms produced by submerged-form plants grown in 0.1 and 0.2 M NaCl solutions. Black bar, basal part of culms; white bar, upper part of culms. Data are means ± SE of 3 plants. The same lowercase letters above bars indicate no significant difference at $P < 0.05$

sections of the terrestrial form were incubated in the antiserum of PEPC, dense staining for PEPC was observed in the mesophyll and PS cells but not in the MS or Kranz cells (Fig. 4a). The staining was darker in the PS cells than in the mesophyll cells. In culms of the submerged form, almost no PEPC staining was observed (Fig. 4b). In new culms developed in 0.2 M NaCl solution, PEPC staining was dense in the mesophyll and even more dense in the PS cells (Fig. 4c). PPDK staining was observed in both mesophyll and PS cells in the culms of the terrestrial form (Fig. 4d), whereas it was very weak to undetectable in the culms of the submerged form (Fig. 4e). In new culms developed in 0.2 M NaCl solution, PPDK staining was dense in both mesophyll and PS cells (Fig. 4f), as in the terrestrial form. In all three types of culms, Rubisco LSU staining was dense in all photosynthetic cells; i.e., in the mesophyll, PS, and Kranz cells (Fig. 4g–i). In the culms of the submerged form and new culms developed in 0.2 M NaCl solution, the degree of staining for PPDK and Rubisco LSU more or less varied among chloroplasts (Fig. 4f, h, i). This was probably due to the difference in the amount of starch grains accumulated in the chloroplasts. Some non-specific cell staining, which is likely

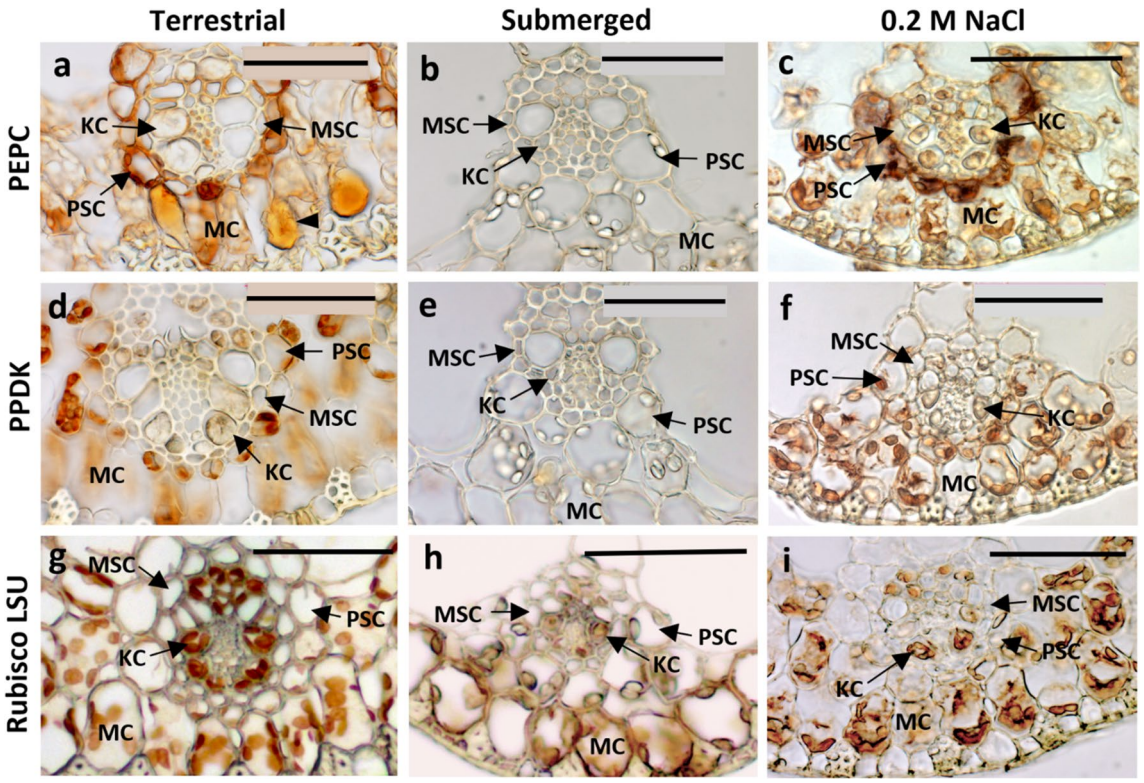

Fig. 4 Immunohistochemical localization of C_3 and C_4 photosynthetic enzymes in culms of *E. vivipara*. **a**–**c** PEP carboxylase (PEPC); **d**–**f** pyruvate Pi dikinase (PPDK); **g**–**i** large subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco LSU). **a**, **d**, and **g** The terrestrial form; **b**, **e**, and **h** the submerged form; **c**, **f**, and **i** new culms of submerged-form plants grown in 0.2 M NaCl solution. An arrowhead in (**a**) indicates non-specific cell staining. *KC* Kranz cell, *MC* mesophyll cell, *MSC* mestome sheath cell, *PSC* parenchyma sheath cell. Bars = 50 µm

attributable to accumulated polyphenolic compounds, was also observed (Fig. 4a).

In the second experiment, mature culms of the submerged form exposed to 0.2 M NaCl solution were analyzed (Fig. 5). In the culms of the submerged form grown in fresh water (control), the staining pattern of the three enzymes was similar to that found in the first experiment: almost no staining for PEPC and PPDK was observed in mesophyll, PS, and Kranz cells, whereas staining for Rubisco LSU occurred in these cells (Fig. 5a, c, e). In mature culms exposed to 0.2 M NaCl, PEPC and PPDK were densely stained in mesophyll and PS cells, but was absent in the Kranz cells (Fig. 5b, d). Staining for Rubisco LSU was observed in all photosynthetic cells (Fig. 5f). Despite their non-Kranz anatomy, these culms had a C_4-like pattern of cellular accumulation of enzymes.

Accumulation of photosynthetic enzymes

Dense bands for PEPC and PPDK were observed in culms of the terrestrial form, whereas almost no band for PEPC and a weak band for PPDK were found in culms of the submerged form (Fig. 6). In new culms developed in NaCl solutions, the density of the bands for PEPC and PPDK was higher at 0.3 M NaCl than 0.2 M NaCl. In mature culms of the submerged form that were exposed to 0.2 M NaCl solution, the PEPC band was similar to and the PPDK band was considerably weaker than those in the terrestrial form (Fig. 6). Dense bands for Rubisco LSU were observed in all of the culms tested (Fig. 6).

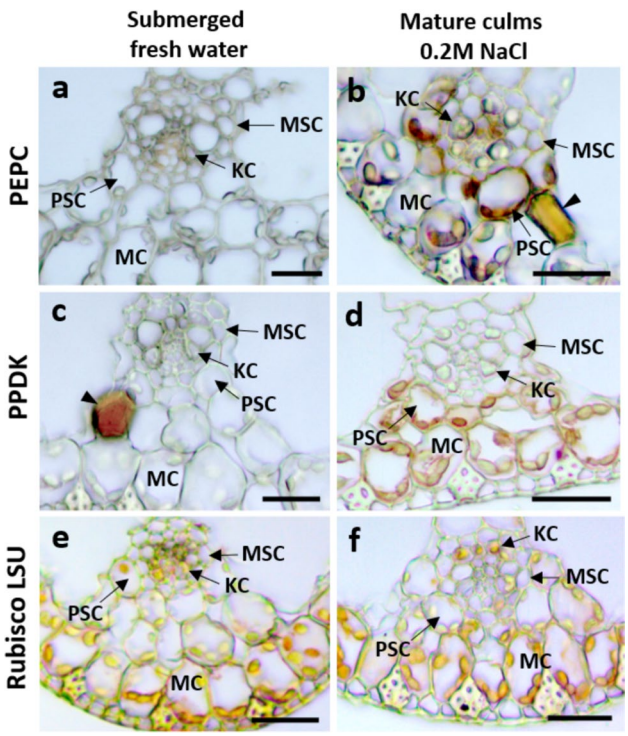

Fig. 5 Immunohistochemical localization of C_3 and C_4 photosynthetic enzymes in mature culms of the submerged-form *E. vivipara* plants that were exposed to 0.2 M NaCl solution. **a** and **b** PEPC; **c** and **d** PPDK; **e** and **f** Rubisco LSU. **a**, **c**, and **e** Culms of submerged form grown in fresh water (control); **b**, **d**, and **f** Mature culms exposed to 0.2 M NaCl. Arrowheads in (**b**, **c**) indicate non-specific cell staining. *KC* Kranz cell, *MC* mesophyll cell, *MSC* mestome sheath cell, *PSC* parenchyma sheath cell. Bars = 25 µm

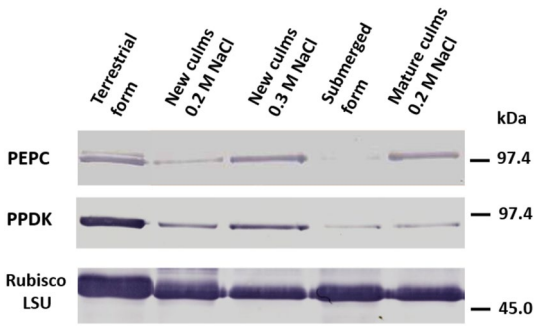

Fig. 6 Western blots of PEPC, PPDK, and Rubisco LSU in culms of the terrestrial and submerged forms and NaCl-treated submerged plants of *E. vivipara.* New culms 0.2 and 0.3 M NaCl, new culms produced by the submerged form in 0.2 and 0.3 M NaCl solutions, respectively; Mature culms 0.2 M NaCl, mature culms of the submerged form exposed to 0.2 M NaCl solution

Discussion

This study indicated that salt stress induces both Kranz anatomy and expression of C_4 photosynthetic enzymes in the submerged C_3 form of *E. vivipara*. Growth of the submerged plants was suppressed by NaCl, and the degree of suppression strengthened with increased concentrations of NaCl. In the field, *E. vivipara* usually occurs in freshwater marshes and at pond edges but has been recorded occasionally in brackish marshes (Ward and Leigh, 1975). This implies that the plant has some tolerance to salt stress.

The development of anatomical traits was affected by the concentration of NaCl. The culms of the terrestrial form had much higher stomatal frequency than culms of the submerged form (Fig. 3; Suizu et al. 2021). This reflects differences in obtaining CO_2 under terrestrial and submerged aquatic conditions (Deschamp and Cooke 1984). The new culms produced in 0.1 and 0.2 M NaCl solutions had stomatal frequency values intermediate between those of the terrestrial and submerged forms, and the stomatal frequency was higher in 0.2 M than in 0.1 M NaCl. This indicates that salt stress induces development of the structural feature of the terrestrial form.

In about half of new culms produced in 0.05 M NaCl solution, the anatomical features were intermediate between those of the terrestrial and submerged forms (Supplementary

Fig. S2). Most of the culms produced in 0.2 and 0.3 M NaCl solutions had Kranz anatomy similar to that of the terrestrial form, although the mesophyll cells remained somewhat round in shape. Kranz cells in these culms were dense green due to abundant chloroplasts, as in the terrestrial form. In *E. vivipara*, the chloroplasts of the Kranz cells are larger in the terrestrial form than in the submerged form, whereas those of the mesophyll cells show the opposite trend (Ueno, 1996a). The terrestrial form expresses C_4 biochemistry of NAD-malic enzyme type, and the Kranz cells contain abundant mitochondria (Ueno 1996b). A quantitative analysis of chloroplasts and mitochondria in the photosynthetic cells will be required for the culms developed in NaCl solution. It is interesting to note that the anatomical features of new culms that developed in 0.05 and 0.1 M NaCl solutions varied considerably, even within individual plants. Although this reason is unknown, it may be partially due to the caespitose habit of *E. vivipara* plants; i.e., culm primordia of different developmental stages were present in plants at the start of the NaCl treatment.

The pattern of cellular accumulation of C_3 and C_4 photosynthetic enzymes observed in new culms produced in 0.2 M NaCl solution was similar to that of culms of the terrestrial form (Fig. 4). Both PEPC and PPDK were localized in the mesophyll and the PS cells. Denser accumulation of PEPC in the PS cells than in the mesophyll cells has been previously observed in the terrestrial form (Ueno 1996b; Suizu et al. 2021). It is thought that this cellular pattern of PEPC accumulation may contribute to the re-fixation of CO_2 leaked from the Kranz cells (Ueno 1996b). Rubisco was present in the mesophyll, PS, and Kranz cells. In true C_4 plants Rubisco is localized only in the Kranz BS cells (Hatch 1987; Schlüter and Weber 2020), although the unusual cellular accumulation of Rubisco observed in *E. vivipara* has been found in C_4-like plants (Cheng et al. 1988; Sage et al. 2012; Tashima et al. 2021). The western blot analysis revealed that the amounts of PEPC and PPDK were higher in new culms produced in NaCl solutions than in culms of the submerged form (Fig. 6). These data imply that salt stress induces both the development of Kranz anatomy and the expression of PEPC and PPDK in newly formed culms of the submerged C_3 form.

Salt stress induces osmotic and/or ionic stress in plants (Allakhverdiev et al. 2000; Allakhverdiev and Murata 2008; Yang and Guo 2018). In land plants, salt stress is first sensed at the root surface in soil, and salts taken up by the roots are transported to the leaves (Munns and Tester 2008). In the submerged form of *E. vivipara*, salt stress would be sensed at the entire surface of the plants. Salt stress generally triggers the osmotic stress signaling and ABA pathway (Yang and Guo 2018; van Zelm et al. 2020), and the concentration of ABA in plant tissues increases under salt stress (Geng et al. 2013; Wang et al. 2017b). In amphibious plants, osmotic stress caused by exposure of the submerged form to air increases endogenous ABA concentration, resulting in the development of land-form leaves (Goliber and Feldman 1989; Hsu et al. 2001). However, the details of the signaling pathways in salt and osmotic stresses in plants are not yet fully understood (Yang and Guo 2018; van Zelm et al. 2020). The submerged form of *E. vivipara* exposed to an ABA solution produces new culms with both Kranz anatomy and C_4-like biochemical traits (Ueno 1998; Agarie et al. 2002). Salt stress could be expected to cause osmotic stress and an associated increase in endogenous ABA in the submerged form. This may trigger the regulatory network leading to development of the Kranz anatomy in culms, together with enhanced expression of C_4 photosynthetic enzyme genes. Recently, some molecular regulators involved in the development of structural traits constituting Kranz anatomy have been identified in *Zea mays* L. (Slewinski et al. 2012, 2014; Wang et al. 2017a; Sedelnikova et al. 2018). It is tempting to speculate that there may be a master switch in *E. vivipara* that leads to the formation of Kranz anatomy. On the other hand, the enhanced accumulation of PEPC and PPDK in mature C_3 culms with non-Kranz anatomy in submerged plants exposed to salt stress (Fig. 5) suggests that the anatomical and biochemical traits of C_4 photosynthesis are independently regulated in *E. vivipara*. Independent regulation of these traits in *E. vivipara* has also been observed in the transitional stage from the terrestrial form to the submerged form (Uchino et al. 1998), in mature culms of the submerged form treated with ABA (Agarie et al. 2002), and in the terrestrial form treated with GA (Suizu et al. 2021).

Some C_4 plants require small amounts of Na^+ for growth (Brownell and Crossland 1972); in particular, Na^+-coupled pyruvate transport in chloroplasts is involved in C_4 photosynthetic metabolism (Furumoto et al. 2011). However, excessive salt is generally harmful for the growth and photosynthesis of C_4 plants (Leisner et al. 2010; Omoto et al. 2012), as it is for C_3 plants (Allakhverdiev and Murata 2008; Wang et al. 2017b). Salinity is thought to be one of the environmental factors that promote the evolution of C_4 plants (Sage et al. 2012), because salt stress reduces stomatal conductance through osmotic stress, decreasing leaf intercellular CO_2 concentrations, as occurs with water deficiency (Chaves et al. 2009; Wang et al. 2017b). In the single-cell C_4 chenopod *Bienertia sinuspersici* Akhani, treatment with 0.2 M NaCl increases PEPC and PPDK amounts (Leisner et al. 2010). In maize plants, treatment with 3% (ca. 0.5 M) NaCl increases PEPC and PPDK activities (Omoto et al. 2012). It is unknown whether similar increases in the amounts or activities of the C_4 photosynthetic enzymes also occur in the terrestrial form of *E. vivipara* in response to NaCl. It is noteworthy that the submerged aquatic macrophyte *H. verticillata* shows an increase in PEPC activity when exposed to 0.5% (0.09 M) or 1.5% (0.26 M) NaCl

solution (Rout and Shaw 1998). This suggests that a signaling pathway that induces expression of C_4 photosynthetic enzymes under salt stress may also exist in *H. verticillata*. However, the reason why PEPC activity in *H. verticillata* increases in response to salt treatment is unknown (Rout and Shaw 1998). At present, it would be difficult to evaluate whether the development of Kranz anatomy and C_4 biochemical traits in *E. vivipara* by NaCl has some physiological and adaptive significance for survival under saline aquatic environments. On the other hand, some facultative CAM plants, such as *Mesembryanthemum crystallinum* L., shift from C_3 mode to CAM mode in response to salt stress (Winter and von Willert 1972; Cushman et al. 1989). In *M. crystallinum*, ABA application also enhances PEPC expression and induces CAM (Chu et al.1990; Taybi et al. 2002). Comparison of the mechanisms regulating the induction of CAM mode in the facultative CAM plants and the induction of C_4 mode in *E. vivipara* may be worthy of future study.

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Author contributions OU conceived and OU, KT, and HS designed the research. All authors conducted the experiments and analyzed the data. OU wrote the manuscript, and all authors read and approved the manuscript.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

References

Agarie S, Kai M, Takatsuji H, Ueno O (1997) Expression of C_3 and C_4 photosynthetic characteristics in the amphibious plant *Eleocharis vivipara*: structure and analysis of the expression of isogenes for pyruvate, orthophosphate dikinase. Plant Mol Biol 34:363–369

Agarie S, Kai M, Takatsuji H, Ueno O (2002) Environmental and hormonal regulation of gene expression of C_4 photosynthetic enzymes in the amphibious sedge *Eleocharis vivipara*. Plant Sci 163:571–580

Allakhverdiev SI, Murata N (2008) Salt stress inhibits photosystems II and I in cyanobacteria. Photosynth Res 98:529–539

Allakhverdiev SI, Sakamoto A, Nishiyama Y, Inaba M, Murata N (2000) Ionic and osmotic effects of NaCl-induced inactivation of photosystems I and II in *Synechococcus* sp. Plant Physiol 123:1047–1056

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

Bawue H (2011) Photorespiration: the bridge to C_4 photosynthesis. In: Raghavendra AS, Sage RF (eds) C_4 photosynthesis and related CO_2 concentrating mechanisms. Springer, Dordrecht, pp 81–108

Bowes G (2011) Single cell C_4 photosynthesis in aquatic plants. In: Raghavendra AS, Sage RF (eds) C_4 photosynthesis and related CO_2 concentrating mechanisms. Springer, Dordrecht, pp 63–80

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Brownell P, Crossland C (1972) The requirement for sodium as a micronutrient by species having the C_4 dicarboxylic photosynthetic pathway. Plant Physiol 49:794–797

Chaves MM, Flexas J, Pinheiro C (2009) Photosynthesis under drought and salt stress: regulation mechanisms from whole plant to cell. Ann Bot 103:551–560

Chen T, Zhu X-G, Lin Y (2014) Major alterations in transcript profiles between C_3–C_4 and C_4 photosynthesis of an amphibious *Eleocharis baldwinii*. Plant Mol Biol 86:93–110

Cheng SH, Moore BD, Edwards GE, Ku MSB (1988) Photosynthesis in *Flaveria brownii*, a C_4-like species. Leaf anatomy, characteristics of CO_2 exchange, compartmentation of photosynthetic enzymes, and metabolism of $^{14}CO_2$. Plant Physiol 87:867–873

Chu C, Dai Z, Ku MSB, Edwards GE (1990) Induction of crassulacean acid metabolism in the facultative halophyte *Mesembryanthemum crystallinum* by abscisic acid. Plant Physiol 93:1253–1260

Cushman JC, Meyer G, Michalowski CB, Schmitt JM, Bohnert HJ (1989) Salt stress leads to differential expression of two isogenes of phosphoenolpyruvate carboxylase during Crassulacean acid metabolism induction in the common ice plants. Plant Cell 1:715–725

Deschamp PA, Cooke TJ (1984) Causal mechanisms of leaf dimorphism in the aquatic angiosperm *Callitriche heterophylla*. Am J Bot 71:319–329

Edwards GE, Voznesenskaya EV (2011) C_4 photosynthesis: Kranz forms and single-cell C_4 in terrestrial plants. In: Raghavendra AS, Sage RF (eds) C_4 photosynthesis and related CO_2 concentrating mechanisms. Springer, Dordrecht, pp 29–61

Furumoto T, Yamaguchi T, Ohshima-Ichie Y, Nakamura M, Tsuchida-Iwata Y, Shimamura M, Ohnishi J, Hata S, Gowik U, Westhoff P, Bräutigam A, Weber APM, Izui K (2011) A plastidial sodium-dependent pyruvate transporter. Nature 476:472–475

Geng Y, Wu R, Wee CW, Xie F, Wei X, Chan PMY, Tham C, Duan L, Dinneny JR (2013) A spatio-temporal understanding of growth regulation during the salt stress response in *Arabidopsis*. Plant Cell 25:2132–2154

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

Harada D, Yamato KT, Izui K, Akita M (2018) *De novo* short read assembly and functional annotation of *Eleocharis vivipara*, a C_3/C_4 interconvertible sedge plant. Environ Cont Biol 56:81–87

Hatakeyama Y, Ueno O (2016) Intracellular position of mitochondria and chloroplasts in bundle sheath and mesophyll cells of C_3 grasses in relation to photorespiratory CO_2 loss. Plant Prod Sci 19:540–551

Hatch MD (1987) C_4 photosynthesis: a unique blend of modified biochemistry, anatomy and ultrastructure. Biochim Biophys Acta 895:81–106

Hsu TC, Liu HC, Wang JS, Chen RW, Wang YC, Lin BL (2001) Early genes responsive to abscisic acid during heterophyllous induction in *Marsilea quadrifolia*. Plant Mol Biol 47:703–715

Langdale JA (2011) C_4 cycles: past, present, and future research on C_4 photosynthesis. Plant Cell 23:3879–3892

Leisner CP, Cousins AB, Offermann S, Okita TW, Edwards GE (2010) The effects of salinity on photosynthesis and growth of the single-cell C_4 species *Bienertia sinuspersici* (Chenopodiaceae). Photosynth Res 106:201–214

Li G, Hu S, Hou H, Kimura S (2019) Heterophylly: phenotypic plasticity of leaf shape in aquatic and amphibious plants. Plants 8:420

Lüttge U (2004) Ecophysiology of crassulacean acid metabolism (CAM). Ann Bot 93:629–652
Minorsky PV (2003) Heterophylly in aquatic plants. Plant Physiol 133:1671–1672
Munns R, Tester M (2008) Mechanisms of salinity tolerance. Annu Rev Plant Biol 59:651–681
Omoto E, Taniguchi M, Miyake H (2012) Adaptation responses in C_4 photosynthesis of maize under salinity. J Plant Physiol 169:467–477
Rout NP, Shaw BP (1998) Salinity tolerance in aquatic macrophytes: probe role of proline, the enzymes involved in its synthesis and C_4 type of metabolism. Plant Sci 136:121–130
Sage RF, Sage TL, Kocacinar F (2012) Photorespiration and the evolution of C_4 photosynthesis. Annu Rev Plant Biol 63:19–47
Sage RF, Monson RK, Ehleringer JR, Adachi S, Pearcy RW (2018) Some like it hot: the physiological ecology of C_4 plant evolution. Oecologia 187:941–966
Schlüter U, Weber APM (2020) Regulation and evolution of C_4 photosynthesis. Annu Rev Plant Biol 71:183–215
Sedelnikova OV, Hughes TE, Langdale JA (2018) Understanding the genetic basis of C_4 Kranz anatomy with a view to engineering C_3 crops. Annu Rev Genet 52:249–270
Slewinski TL, Anderson AA, Zhang C, Turgeon R (2012) *Scarecrow* plays a role in establishing Kranz anatomy in maize leaves. Plant Cell Physiol 53:2030–2037
Slewinski TL, Anderson AA, Price S, Withee JR, Gallagher K, Turgeon R (2014) *Short-Root 1* plays a role in the development of vascular tissue and Kranz anatomy in maize leaves. Mol Plant 7:1388–1392
Suizu Y, Takao K, Ueno O (2021) Gibberellic acid induces non-Kranz anatomy with C_4–like biochemical traits in the amphibious sedge *Eleocharis vivipara*. Planta 254:10
Tashima M, Yabiku T, Ueno O (2021) *Coleataenia prionitis*, a C_4-like species in the Poaceae. Photosynth Res 147:211–227
Taybi T, Cushman JC, Borland AM (2002) Environmental, hormonal and circadian regulation of crassulacean acid metabolism expression. Funct Plant Biol 29:669–678
Uchino A, Sentoku N, Nemoto K, Ishii R, Samejima M, Matsuoka M (1998) C_4-type gene expression is not directly dependent on Kranz anatomy in an amphibious sedge *Eleocharis vivipara* Link. Plant J 14:565–572
Ueno O (1992) Immunogold localization of photosynthetic enzymes in leaves of *Aristida latifolia*, a unique C_4 grass with a double chlorenchymatous bundle sheath. Physiol Plant 85:189–196
Ueno O (1996a) Structural characterization of photosynthetic cells in an amphibious sedge, *Eleocharis vivipara*, in relation to C_3 and C_4 metabolism. Planta 199:382–393
Ueno O (1996b) Immunocytochemical localization of enzymes involved in the C_3 and C_4 pathways in the photosynthetic cells of an amphibious sedge, *Eleocharis vivipara*. Planta 199:394–403
Ueno O (1998) Induction of Kranz anatomy and C_4-like biochemical characteristics in a submerged amphibious plant by abscisic acid. Plant Cell 10:571–583
Ueno O (2001) Environmental regulation of C_3 and C_4 differentiation in the amphibious sedge *Eleocharis vivipara*. Plant Physiol 127:1524–1532
Ueno O, Ishimaru K (2002) Effects of an inhibitor of phosphoenolpyruvate carboxylase on photosynthesis of the terrestrial forms of amphibious *Eleocharis* species. Photosynth Res 71:265–272
Ueno O, Samejima M, Muto S, Miyachi S (1988) Photosynthetic characteristics of an amphibious plant, *Eleocharis vivipara*: expression of C_4 and C_3 modes in contrasting environments. Proc Natl Acad Sci USA 85:6733–6737
van Zelm E, Zhang Y, Testerink C (2020) Salt tolerance mechanisms of plants. Annu Rev Plant Biol 71:403–433
von Caemmerer S, Furbank RT (2003) The C_4 pathway: an efficient CO_2 pump. Photosynth Res 77:191–207
Wang P, Khoshravesh R, Karki S, Tapia R, Balahadia CP, Bandyopadhyay A, Quick WP, Furbank R, Sage TL, Langdale JA (2017a) Re-creation of a key step in the evolutionary switch from C_3 to C_4 leaf anatomy. Curr Biol 27:3278–3287
Wang Y, Stevanato P, Yu L, Ahao H, Sun X, Sun F, Li J, Geng G (2017b) The physiological and metabolic changes in sugar beet seedlings under different levels of salt stress. J Plant Res 130:1079–1093
Wanke D (2011) The ABA-mediated switch between submersed and emersed life-styles in aquatic macrophytes. J Plant Res 124:467–475
Ward DB, Leigh EM (1975) Contributions to the Flora of Florida. 8, *Eleocharis* (Cyperaceae). Castanea 40:16–36
Winter K (2019) Ecophysiology of constitutive and facultative CAM photosynthesis. J Exp Bot 70:6495–6508
Winter K, von Willert DJ (1972) NaCl-induzierter Crassulaceen-säurestoffwechsel bei *Mesembryanthemum crystallinum*. Z Pflanzenphysiol 67:166–170
Yang Y, Guo Y (2018) Elucidating the molecular mechanisms mediating plant salt-stress responses. New Phytol 217:523–539