Transition From Proto-Kranz-Type Photosynthesis to HCO$_3^-$ Use Photosynthesis in the Amphibious Plant *Hygrophila polysperma*

**Genki Horiguchi**$^1$, **Kaori Matsumoto**$^2$, **Kyosuke Nemoto**$^1$, **Mayu Inokuchi**$^{2,3}$ and **Naoki Hirotsu**$^{1,2,*}$

$^1$ Graduate School of Life Sciences, Toyo University, Gunma, Japan, $^2$ Faculty of Life Sciences, Toyo University, Gunma, Japan, $^3$ Department of Aquatic Bioscience, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo, Japan

*Correspondence:* Naoki Hirotsu
hirotsu@toyo.jp

**Specialty section:**
This article was submitted to Plant Physiology, a section of the journal Frontiers in Plant Science

**Received:** 03 March 2021  
**Accepted:** 26 April 2021  
**Published:** 16 June 2021

*Hygrophila polysperma* is a heterophyllous amphibious plant. The growth of *H. polysperma* in submerged conditions is challenging due to the low CO$_2$ environment, increased resistance to gas diffusion, and bicarbonate ion (HCO$_3^-$) being the dominant dissolved inorganic carbon source. The submerged leaves of *H. polysperma* have significantly higher rates of underwater photosynthesis compared with the terrestrial leaves. 4,4′-Diisothiocyanatostilbene-2,2′-disulfonate (DIDS), an anion exchanger protein inhibitor, and ethoxyzolamide (EZ), an inhibitor of internal carbonic anhydrase, repressed underwater photosynthesis by the submerged leaves. These results suggested that *H. polysperma* acclimates to the submerged condition by using HCO$_3^-$ for photosynthesis. *H. polysperma* transports HCO$_3^-$ into the leaf by a DIDS-sensitive HCO$_3^-$ transporter and converted to CO$_2$ by carbonic anhydrase. Additionally, proteome analysis revealed that submerged leaves accumulated fewer proteins associated with C4 photosynthesis compared with terrestrial leaves. This finding suggested that *H. polysperma* is capable of C4 and C3 photosynthesis in the terrestrial and submerged leaves, respectively. The ratio of phosphoenol pyruvate carboxylase to ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) in the submerged leaves was less than that in the terrestrial leaves. Upon anatomical observation, the terrestrial leaves exhibited a phenotype similar to the Kranz anatomy found among C4 plants; however, chloroplasts in the bundle sheath cells were not located adjacent to the vascular bundles, and the typical Kranz anatomy was absent in submerged leaves. These results suggest that *H. polysperma* performs proto-Kranz type photosynthesis in a terrestrial environment and shifts from a proto-Kranz type in terrestrial leaves to a HCO$_3^-$ use photosynthesis in the submerged environments.

**Keywords:** bicarbonate use, proto-Kranz anatomy, carbon concentrating mechanism, amphibious plant, Acanthaceae, submergence
INTRODUCTION

Carbon assimilation is essential for plant growth. In stress conditions emanating from high temperature, high light intensity, salt (Chen et al., 2015), or drought (Flexas and Medrano, 2002), stomata are closed to suppress water loss, causing decreases in gas exchange and increases in photorespiration. Higher plants evolved C4 photosynthesis and crassulacean acid metabolism (CAM) to maintain carbon assimilation rates under conditions limiting carbon acquisition. C4 photosynthesis and CAM concentrate CO₂ around ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) to avoid limiting carbon assimilation condition such as when the stomata are closed. C4 photosynthesis concentrates CO₂ by physically separating primary CO₂ fixation by phosphoenolpyruvate carboxylase (PEPC) in mesophyll cells (MCs) from secondary fixation by Rubisco in bundle sheath cells (BSCs) (Hatch, 1971; Kanai and Edwards, 1973). In contrast, CAM plants fix CO₂ using PEPC during the night and conduct secondary fixation by Rubisco during the day (Black et al., 2003). C4 photosynthesis and CAM are adaptations allowing photosynthesis to continue in limiting CO₂ conditions (Sage et al., 2012).

C4 photosynthesis and CAM are found not only in land plants but also in aquatic plants (Yin et al., 2017). In submerged conditions, gas diffusion is 10⁴ times slower compared with the terrestrial condition, thereby limiting dissolved inorganic carbon (DIC) and oxygen availability (Maberly and Madsen, 2002). Moreover, the relative distribution of DIC constituents (CO₂, HCO₃⁻, and CO₃²⁻) depends mainly on the pH of the aqueous environment (Pedersen et al., 2013). Bicarbonate (HCO₃⁻) is the dominant DIC form between pH 7.0 and pH 10.0. Most freshwater lakes have a pH value that range from 6 to 8 (Hasler et al., 2018). HCO₃⁻ is the dominant DIC form in nature. Submerged environments usually have low CO₂ levels; thus, C4 photosynthesis and CAM have the advantage. Some aquatic plants switch photosynthetic pathways with changing external environments. Egeria densa and Hydrilla verticillata (Hydrocharitaceae), aquatic monocots, typically perform C₃ photosynthesis but induce single-cell C₄ photosynthesis under carbon-limiting conditions (Casati et al., 2000; Bowes et al., 2002). Ottelia alismoides (Hydrocharitaceae), a freshwater monocot, exhibits C₄ characteristics (Zhang et al., 2014; Shao et al., 2017; Han et al., 2020) and increases CAM activity under low CO₂ conditions (Zhang et al., 2014). Isoetes howelli and I. sinensis (Isoetaceae), amphibious lycophytes, increase CAM activity in leaves that develop in the submerged condition but not in leaves that develop in the terrestrial condition (Keely, 1983; Yang and Liu, 2015). Leaves with CAM activity have a higher net photosynthetic rate and lower apparent photorespiration in the submerged environment (Pedersen et al., 2011). Eleocharis species, amphibious monocots, use diverse photosynthetic carbon assimilation pathways (C₃, C₃–C₄, C₄-like, and C₄) in their terrestrial and submerged leaves (Murphy et al., 2007). Interestingly, Eleocharis species are unlike other plants that can switch their photosynthetic metabolism; the terrestrial leaves of Eleocharis vivipara exhibit C₄ characteristics, whereas submerged leaves exhibit C₃ characteristics (Ueno et al., 1988; Murphy et al., 2007). The alteration of photosynthetic type in E. vivipara is confirmed not only in enzymatic localization and activities but also in anatomical structure as the Kranz anatomy is absent in submerged leaves.

Leaf anatomy reflects the photosynthetic capacities for both CO₂ and light. To concentrate CO₂ in BSCs, larger BSCs are needed that contain more abundant organelles (Lundgren, 2020). Aquatic plants typically have reduced leaf thicknesses, cuticles, and stomatal densities compared with terrestrial plants (Veening and Sadidharan, 2019). Leaves that are thin or have a thin cuticle have lower gas diffusion resistance properties (Frost-Christensen et al., 2003; Mommer et al., 2005). The presence of chloroplasts in epidermal cells is a common feature in aquatic plants (Rascio, 2002; Borum et al., 2016; Han et al., 2020). Differences in cell shape and in the chloroplast content of the two mesophyll cell types, palisade and spongy tissues, are important factors in allocating light energy within leaves (Terashima and Saeki, 1983). Leaf anatomical features, especially chloroplast positioning, can influence the internal light environment and light use efficiency (Xiao et al., 2016).

Aquatic phototrophs have carbon-concentrating mechanisms (CCMs) to acclimate to low CO₂ conditions. Algal transport of HCO₃⁻ from the external environment to carboxysomes or pyrenoids relies on transporters such as BCT1 (Oma et al., 2019), SbtA (Shibata et al., 2002), BicA (Price et al., 2004), HLA3 (Yamano et al., 2015), and SLC4 (Nakajima et al., 2013), or channels such as LCIA (Yamano et al., 2015). Carboxysomes contain multiple forms of carbonic anhydrase (CA), including CaC and CMCM, whereas pyrenoids contain theta CA (Duanmu et al., 2009; Kikutani et al., 2016; Gee and Niyogi, 2017), and Rubisco, where HCO₃⁻ is converted to CO₂. Algal CCMs are referred to as biophysical CCMs, whereas C₄ photosynthesis and CAM are referred to as biochemical CCMs. Having biophysical or biochemical CCMs to increase CO₂ availability is advantageous for submerged plants. In higher plants, HCO₃⁻ utilization mechanisms likely include biophysical CCMs. Potamogeton lucens and Elodea canadensis exhibit a light-induced polar pH reaction (Prins et al., 1979; Elzenga and Prins, 1989). In these plants, HCO₃⁻ is taken and converted to CO₂ in the lower side, and OH⁻ is excreted from the upper side. Posidonnia oceanica, a seagrass, transports HCO₃⁻ into the cell by a HCO₃⁻/H⁺ symporter (Rubio et al., 2017). The submerged leaves of Hygrophiella difformis, an amphibious eudicot, have a HCO₃⁻ utilization mechanism that is hypothesized to transport HCO₃⁻ across the plasma membrane by a means other than H⁺ symport (Horiguchi et al., 2019). O. alismoides has two pathways, passive diffusion of CO₂ that is converted to HCO₃⁻ using an external α-CA and transport of HCO₃⁻ into cells by SLC4 (Huang et al., 2020).

Higher plants have diverse acclimation strategies for low CO₂ environments, including submergence; however, the photosynthetic acclimation strategies used by submerged plants remain unclear in eudicots. In this study, we investigated how the heterophyllous amphibious eudicot Hygrophiella polysperma acclimated to low CO₂ availability in a submerged condition by comparing the properties of terrestrial and submerged leaves. We hypothesized that H. polysperma uses not only CO₂ but also...
HCO$_3^-$ as a photosynthetic carbon source and/or increases CO$_2$ availability by morphological and functional change.

MATERIALS AND METHODS

Plant Growth Conditions

Clonal seedling cuttings of _H. polysperma_, a heterophyllous amphibious plant, were used. Three plants were planted into each vinyl pot (220 ml) containing soil (Leaf Pro Soil normal, Leaf Corporation, Gunma, Japan). Ten pots were placed into each of two glass tanks (W30 cm × D30 cm × H40 cm). The pots were partially submerged in tap water such that the water level was kept below the soil surface. Plants were grown at 25°C and illuminated with a 150-W metal halide lamp (Funnel 2 8000K, Kamihata, Hyogo, Japan) with a photosynthetic photon flux density (PPFD) of 200 µmol m$^{-2}$ s$^{-1}$, 8-h light and 16-h dark. After 2 weeks of initial growth, plants were separated into two environmental treatments: terrestrial and submerged. The terrestrial treatment maintained the initial growing condition. The submerged treatments were achieved by adding 25 L of tap water to the tank and maintaining the same light and temperature conditions as described for the terrestrial treatment. The temperature, pH, and alkalinity in the submerged treatment at day time were 25°C, 7.38, and 0.3 mEq L$^{-1}$, respectively. Leaves that developed after the terrestrial and submerged treatments were regarded as terrestrial and submerged leaves, respectively. The uppermost fully expanded leaves from both terrestrial and submerged treatments were used for subsequent experiments.

Underwater Photosynthesis Measurement

Underwater oxygen evolution was monitored using a liquid phase oxygen electrode (OXYG-1, Hansatech, Norfolk, United Kingdom). Four leaf disks (6-mm diameter, total projected area of 113 mm$^2$) were placed in an oxygen electrode chamber (DW1/AD, Hansatech, Norfolk, United Kingdom) containing measurement buffer whose composition was modified for every experiment. The temperature inside the oxygen electrode chamber was maintained at 25°C by submerging the chamber in a low-temperature water bath (NCB-1,200, EYELA, Tokyo, Japan). The DIC response curve for the net oxygen evolution rate was obtained for NaHCO$_3$ values between 10 and 1,650 µM. In addition to NaHCO$_3$, the measurement buffer contained 0.1 mM phosphate buffer (pH 6.3), 1.5 mM KCl, 1.0 mM NaCl. Photosynthesis was started by illumination at an irradiance of 285 µmol photons m$^{-2}$ s$^{-1}$ after dark acclimation for over 15 min. The DIC concentration increased immediately after injecting NaHCO$_3$. The light response curve of the net oxygen evolution rate was measured for PPFD values from 0 to 820 µmol photons m$^{-2}$ s$^{-1}$ in the presence of 10 µM NaHCO$_3$. The initial slopes were calculated in low DIC (10–150 µM NaHCO$_3$) and light (0–125 µmol photons m$^{-2}$ s$^{-1}$) regions. After measuring the underwater photosynthetic rate, leaves were collected to analyze the chlorophyll content.

Measurement of HCO$_3^-$ Use

To investigate the DIC form used for photosynthesis, the photosynthetic oxygen evolution rate was measured at pH 6.3 and 8.3. DIC constituents (CO$_2$ and HCO$_3^-$) present in the media at pH 6.3 or 8.3 existed as a level of 1:1 or HCO$_3^-$ only, respectively. The pH of the measurement buffer (10 mM NaHCO$_3$, 1.5 mM KCl, and 1.0 mM NaOH) was adjusted by adding 1 M HCl or 1 M NaOH. The inhibitor experiment was performed by a previously described procedure (Horiguchi et al., 2019). For inhibitor experiments, stock solutions (10 mM) of acetazolamide (AZ) and ethoxyzolamide (EZ) were prepared by dissolution in 50 mM NaOH. AZ and EZ inhibit the external CA and internal CA, respectively. Tris(hydroxymethyl)aminomethane (TRIS), an inhibitor of HCO$_3^-/H^+$ symport, and 4,4′-diisothiocyanatostilbene-2,2′-disulfonate (DIDS), an anion exchange protein inhibitor, were prepared daily. The photosynthetic oxygen evolution rate was measured in the HCO$_3^-$ condition (pH 8.3) in the presence of 0.1 mM AZ, 0.1 mM EZ, 50 mM TRIS, or 0.3 mM DIDS.

Measurement of Phosphoenol Pyruvate Carboxylase, Rubisco, and Carbonic Anhydrase Activities

Terrestrial and submerged leaves were harvested 2 h after the onset of the light period and were stored at −80°C until enzymatic activities were measured. The frozen samples were homogenized in an ice-cold extraction buffer [50 mM Tris (hydroxymethyl) aminomethane (Tris), 15 mM MgCl$_2$, 5 mM dithiothreitol (DTT), 0.1 mM ethylenediaminetetraacetic acid (EDTA), 10% (w/w) polyvinylpolypyrrolidone, 5% (w/v) polyvinylpyrrolidone, and 10% (v/v) glycerol (pH 8.0)] with quartz sand using a cold mortar and pestle. Part of the homogenate was used to measure the chlorophyll content; the remainder was centrifuged at 12,000 × g, 15 min at 4°C. The supernatant liquid (the crude extract) was kept on ice during the measurements.

Phosphoenol pyruvate carboxylase activity was determined as described previously (Zhang et al., 2014). Briefly, PEPC activity in the crude extract was measured in 1 mL of reaction mixture [50 mM Tris, 15 mM MgCl$_2$, 0.1 mM EDTA, 20 mM bicarbonate, 0.2 mM NADH, 1 mM phosphoenol pyruvate (PEP), and 5 U malate dehydrogenase (pH 8.0)]. The reaction was started by adding PEP after preincubating the reaction mixture and sample for 5 min at 25°C. The absorbance at 340 nm was recorded for 5 min, and the activity was calculated from the change in absorbance as a function of time.

Rubisco activity was determined by modifying a previously described procedure (Suzuki et al., 2007). The Rubisco activity in
crude extracts was activated by incubation with 20 mM NaHCO₃ at 25°C for 5 min. The activated sample was injected into a reaction mixture [50 mM Tris, 15 mM MgCl₂, 0.1 mM EDTA, 10 mM bicarbonate, 0.2 mM NADH, 0.6 mM ribulose 1,5-bisphosphate, 5.0 mM phosphocreatine, 20 U of glyceraldehyde-3-phosphate dehydrogenase, 10 U of phosphoglyceric acid kinase, and 1 U of creatine phosphokinase (pH 8.0)] that was bubbled with N₂ gas. The activity was calculated from the decreasing absorbance at 340 nm as a function of time.

The CO₂ hydration and HCO₃⁻ dehydration activities of CA were determined by monitoring pH changes after the addition of substrate (Wilbur and Anderson, 1948; Kikutani et al., 2016). The CO₂ hydration reaction was initiated by the addition of ice-cold CO₂ saturated water to 20 mM Tris–HCl buffer (pH 8.4) containing the crude extract. The time required for the pH to drop from 8.3 to 8.0 was measured. The HCO₃⁻ dehydration reaction was initiated by the addition of ice-cold 50 mM NaHCO₃ to 50 mM MES buffer (pH 5.5) containing the crude extract. The time required for the pH to increase from 5.7 to 6.0 was measured. The CA activity was calculated as the Wilbur and Andersson unit (WAU): WAU = T₀/T − 1, where T₀ and T are the times required for the pH change in the absence and the presence of the sample, respectively.

Chlorophyll in leaf homogenates was extracted in the dark for 15 min with 80% (v/v) acetone on ice. The extract was centrifuged at 20,400 × g for 10 min at 25°C. The absorbance of the supernatant liquid was measured at 750, 663.6, and 646.6 nm to calculate the chlorophyll concentration (Porra et al., 1989).

Leaf Malate Content

Malate was extracted as previously described (Yang and Liu, 2015) and quantified by an enzymatic method. Leaves were sampled at dusk (the end of the light period) or dawn (the end of the dark period). Samples were ground in ice-cold perchloric acid using an ice-cold mortar and pestle. The homogenate was kept on ice before analysis. Malate in the leaf extracts was detected by an enzymatic method using an F kit (#139068, Roche Diagnostics, K.K., Tokyo, Japan).

Anatomical and Morphological Analyses

Leaf segments (3 mm × 3 mm) added to 2% (v/v) glutaraldehyde and 2% (w/v) paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) were placed in a desiccator connected to an aspirator and vacuum infiltrated to −0.09 Pa for 5 min. Infiltration was repeated until the segments were fully submerged, followed by fixation at 4°C for 3 h. The leaf segments were rinsed in 0.1 M phosphate buffer and dehydrated by an ethanol series (50, 70, 80, 90, 95, and 99.5%) followed last with acetic. The samples were infiltrated with the graded infiltration resin series, Spurr resin (Spurr Low Viscosity Embedding kit, Polysciences, Inc., PA, United States): acetone = 1:2, 1:1, 2:1 for 1.5 h, and Spurr’s resin overnight. The leaf segments were embedded in Spurr's resin at 70°C overnight. Samples (1 µm) for light microscopy were sectioned using an ultramicrotome (Leica EM UC7, Leica, Wetzlar, Germany) at room temperature. Leaf cross sections were stained with 0.1% (w/v) toluidine blue in 0.1 M phosphate buffer (pH 7.0) and were observed using a light microscope (BX41, Olympus, Tokyo, Japan). Quantitative characteristics of leaf structure were measured using Image J ver. 1.45 s (National Institutes of Health, Bethesda, MD, United States).

Leaf morphological characteristics, including leaf area, leaf length, leaf width, and stomatal density were measured. The area, length, and width of the leaves were measured from leaf images scanned using Image J. The leaf thickness was measured from leaf cross section using Image J. Stomatal density was determined using the same microscope.

Proteome Analysis

Proteomic analysis was performed as described previously (Perera et al., 2019). Briefly, three terrestrial (88 mg) and three submerged leaves (68 mg) were harvested. The leaves were immediately frozen in liquid N₂ and stored at −80°C. Soluble and membrane proteins were extracted from the frozen leaves (Perera et al., 2019). Samples were analyzed using Nano liquid chromatography-mass spectrometry (LC–MS/MS) with an UltiMate 3,000 (Dionex, Tokyo, Japan) and Q-Exactive Plus (Thermo Fisher Scientific, Tokyo, Japan). LC-MS/MS data were analyzed using the search engine Mascot Server v2.5.1. Spectral data were identified against NCBI. Statistical analysis of protein spectral counts was conducted using the proteome software Scaffold v4.8.7 (Proteosome Software, United States).

Statistical Analyses

The data were expressed as the mean ± standard deviation (SD). Student’s t test was performed to detect differences between the two groups using Microsoft Excel 2013 (Microsoft, WA, United States). One-way analysis of variance (ANOVA) and Tukey’s honestly significant difference (HSD) test were calculated using JMP statistical software v9.0.2 (SAS Institute, NC, United States) and used for multiple comparisons. The initial slopes of the DIC or light response curves were generated by the Levenberg–Marquardt method and compared using the Prism software v6.07 (GraphPad Software, CA, United States). We evaluated the significance of the effect of leaf, measurement DIC concentration, or light interactions on photosynthetic rate by using two-way ANOVA; multiple comparisons were performed by the Holm–Sidak test in the Prism software v6.07. Data were determined to be statistically different when p < 0.05.

RESULTS

Morphological Leaf Traits and Underwater Photosynthesis

The morphology of H. polysperma leaves that developed under terrestrial or submerged environments was strikingly different...
Horiguchi et al. Photosynthesis in Amphibious Plant

10 mM NaHCO₃ ± submerged leaves of *H. polysperma* under water photosynthesis per chlorophyll content in terrestrial and submerged leaves of *H. polysperma* that developed under terrestrial (left) or submerged (right). The scale bar is 10 mm.

**FIGURE 1** | Leaf morphology, dissolved inorganic carbon (DIC), and light-response curves of underwater photosynthesis in terrestrial and submerged leaves of *Hygrophila polysperma*. (A) Heterophyllous leaves of *H. polysperma* that developed under terrestrial (left) or submerged environments (right). The scale bar is 10 mm. (B) The DIC response curve for underwater photosynthesis per chlorophyll content in the terrestrial and submerged leaves of *H. polysperma* (*n* = 3). DIC concentration increased from 10 to 1,650 µM by injected NaHCO₃. Measurement light intensity was 285 µmol m⁻² s⁻¹, and the pH was 6.3. (C) Light-response curve of underwater photosynthesis per chlorophyll content in the terrestrial and submerged leaves of *H. polysperma* (*n* = 3). Photosynthetic photon flux density increased from 0 to 820 µmol photons m⁻² s⁻¹ in the presence of 10 mM NaHCO₃ at pH 6.3. The results shown in panels (B) and (C) are expressed as means ± SD. Significance was analyzed by two-way ANOVA with Holm–Sidak test (*p* < 0.05).

Submerged leaves were significantly narrower and thinner compared with terrestrial leaves (Table 1). *H. polysperma* has stomata on both the abaxial and adaxial leaf surfaces. The adaxial stomatal density of submerged leaves was significantly lower than that of terrestrial leaves; however, adaxial stomatal density was not significantly different between terrestrial leaves and submerged leaves (Table 1). Submerged leaves had a significantly lower chlorophyll a and b content compared with terrestrial leaves, but the ratios of chlorophyll a to chlorophyll b (a/b) were similar (Table 1).

We measured the net photosynthetic O₂ evolution rate (Pn) relative to the chlorophyll content and leaf area under submerged conditions when DIC or light intensity was varied. Submerged leaves generally had a higher underwater Pn at pH 6.3 in response to an increased DIC content than terrestrial leaves at DIC concentrations above 150 µM NaHCO₃ (Figure 1B). Furthermore, the initial slopes of the DIC response curves from 10 to 150 µM NaHCO₃ were 0.09 ± 0.02 and 0.26 ± 0.04 µmol O₂ mg⁻¹ h⁻¹ µM⁻¹ for terrestrial and submerged leaves, respectively (mean ± SD, *p* < 0.001). Submerged leaves had a higher underwater Pn, in response to increased light intensity compared with terrestrial leaves (Figure 1C). The initial slopes of the light response curves from 0 to 125 µmol photons m⁻² s⁻¹ were 0.29 ± 0.06 and 0.60 ± 0.07 µmol O₂ mg⁻¹ h⁻¹ PPFD⁻¹ for terrestrial and submerged leaves, respectively (mean ± SD, *p* = 0.003). In contrast, underwater Pn per unit leaf area responded to DIC and light differently than underwater Pn expressed relative to the chlorophyll content. Terrestrial leaves had a larger underwater Pn on a per leaf area basis compared with submerged leaves at high DIC levels and high light intensities (Supplementary Figure 1). There were no significant differences in the initial slopes of underwater Pn in response to DIC content or light intensity between terrestrial and submerged leaves.

**Estimation of HCO₃⁻ Use**

To investigate the HCO₃⁻ affinity of *H. polysperma*, we measured the underwater Pn at pH 6.3 at which the concentrations

| TABLE 1 | Leaf morphological characteristics in terrestrial and submerged leaves of *Hygrophila polysperma*. |
|----------|-------------------------------------------------|
| Leaf length (mm) | 28.8 ± 1.5 | 37.1 ± 3.7 | ** |
| Leaf width (mm) | 11.8 ± 0.9 | 9.7 ± 0.6 | ** |
| Length-width ratio | 2.4 ± 0.2 | 3.8 ± 0.4 | ** |
| Leaf area (mm²) | 230.0 ± 10.3 | 271.8 ± 23.7 | * |
| Leaf thickness (µm) | 149.7 ± 21.6 | 89.3 ± 10.2 | * |
| Abaxial stomatal density (no. mm⁻²) | 92 ± 5 | 26 ± 3 | ** |
| Adaxial stomatal density (no. mm⁻²) | 32 ± 4 | 24 ± 3 | n.s. |
| Chlorophyll a (mg m⁻²) | 184.9 ± 10.3 | 68.0 ± 10.1 | ** |
| Chlorophyll b (mg m⁻²) | 66.2 ± 2.5 | 24.3 ± 2.4 | ** |
| Chl a/b | 2.8 ± 0.05 | 2.8 ± 0.1 | n.s. |

All values are means ± SD (*n* = 3–4). Significance was analyzed with Student's *t*-test (n.s., not significant, *p* < 0.05, **p** < 0.01).
of CO$_2$ and HCO$_3^-$ are equivalent and at pH 8.3 at which the concentration of HCO$_3^-$ is dominant. The underwater Pn relative to the Chl content was highest in the submerged leaves at pH 6.3 and lowest in the terrestrial leaves at pH 8.3 (Figure 2A). The submerged leaves had a significantly higher underwater Pn compared with the terrestrial leaves in both DIC conditions. The Pn at pH 6.3 was significantly higher than at pH 8.3 in both leaf types. The HCO$_3^-$ affinity values calculated as the ratio of the Pn at pH 8.3 to the Pn at pH 6.3 were 0.3 ± 0.06 and 0.72 ± 0.11 for terrestrial and submerged leaves, respectively (mean ± SD, $p = 0.001$).

We also determined the CO$_2$ hydration and HCO$_3^-$ dehydration reactions from CA activity in terrestrial and submerged leaves. The CO$_2$ hydration reaction in terrestrial leaves was significantly higher than the CO$_2$ hydration reaction in submerged leaves or the HCO$_3^-$ dehydration reaction in submerged and terrestrial leaves (Supplementary Figure 2). There was no difference between the CA activities other than the CO$_2$ hydration reaction in terrestrial leaves. The HCO$_3^-$ dehydration/CO$_2$ hydration ratio in the submerged leaves was significantly higher compared with that in the terrestrial leaves (Figure 2B).

Because submerged leaves had an increased HCO$_3^-$ affinity, we examined the mechanism of HCO$_3^-$ use by an inhibitor experiment. EZ, an inhibitor of internal CA, and DIDS, an inhibitor of the SLC family of anion exchangers, significantly inhibited the photosynthesis of submerged leaves (Figure 2C). The underwater Pns of submerged leaves was not sensitive to AZ and or TRIS, both of which inhibit external CA and HCO$_3^-$/H$^+$ symport, respectively.

Proteome Analysis

A total of 3,239 proteins were identified by proteomic analysis of which 186 proteins were differentially expressed between terrestrial and submerged leaves. One hundred proteins were significantly downregulated in submerged leaves compared with the terrestrial leaves, and 86 proteins were significantly upregulated (Supplementary Tables 1, 2). In this study, a protein identified as a HCO$_3^-$ transporter in algae was not detected in *H. polysperma*. Among seven CA proteins detected in *H. polysperma* (Table 2), the amount of chloroplast β-CA was significantly lower in the submerged leaves compared with the terrestrial leaves.

The downregulated proteins included proteins associated with biochemical CCMs: β-CA, PEPC, aspartate aminotransferase (AspAT), NAD-malate dehydrogenase (NAD$^+$-MDH), NADP-malic enzyme (NADP-ME), and alanine aminotransferase (AlaAT) (Figure 3A). Additionally, proteins related to the photosynthetic electron transport system were present in lower amounts in submerged leaves compared with terrestrial leaves (Supplementary Table 1). In contrast, the upregulated proteins included proteins associated with the Calvin cycle: Rubisco, phosphoglycerate kinase (PGK), fructose-bisphosphate aldolase (ALDO), transketolase (TK), and phosphoribulokinase (PRK) (Figure 3B). The Rubisco large subunit and glyceraldehyde-3-phosphate dehydrogenase
sections of terrestrial leaves but not in submerged leaves (Figures 5A, B); however, chloroplasts in BSCs were not located near the vascular bundles as is typical for the Kranz anatomy in some C4 plants.

The analysis of cross sections from terrestrial and submerged leaves identified significant differences in leaf structure (Figure 6). The MC size was significantly higher in submerged leaves than in terrestrial leaves (Figure 6A). The BSC and epidermal cell sizes were significantly smaller in the submerged leaves compared with the terrestrial leaves (Figures 6B, C). Therefore, the submerged leaves had a significantly higher MC/BSC size ratio compared with terrestrial leaves (Figure 6D). On the other hand, there were no differences in the percent of tissue coverage of a cross section between terrestrial and submerged leaves (Supplementary Table 3).

**DISCUSSION**

**HCO\(_3^−\)** Uptake and Conversion to CO\(_2\)

HCO\(_3^−\) use mechanisms are distinguished by inhibitor sensitivity in underwater photosynthesis. In a previous study, four inhibitors of HCO\(_3^−\) utilization (AZ, TRIS, EZ, and DIDS) were used to distinguish HCO\(_3^−\) use mechanisms in aquatic phototrophs (Beer et al., 2002; Hellblom and Axelsson, 2003; Fernández et al., 2014; Rubio et al., 2017; Tsuji et al., 2017). In marine diatoms, DIDS, an anion exchanger inhibitor, suppressed high-DIC-affinity photosynthesis in the pennate diatom *P. tricornutum* and the centric diatom *Chaetoceros muelleri*, but AZ, an external CA inhibitor, did not affect high-DIC-affinity photosynthesis (Tsuji et al., 2017). Tsuji et al. (2017) also reported that AZ reduced HCO\(_3^−\) affinity in the pennate *Cylindrotheca fusiformis* and the centric *Thalassiosira pseudonana*, but DIDS did not affect HCO\(_3^−\) affinity. The DIDS-sensitive group converted HCO\(_3^−\) to CO\(_2\) after transport of HCO\(_3^−\) into cells; the DIDS-insensitive group facilitated CO\(_2\) diffusion by external CA. Submerged leaves of *H. polysperma* had an enhanced affinity for HCO\(_3^−\) compared with terrestrial leaves (Figure 2A). The ratio of HCO\(_3^−\) dehydration to CO\(_2\) hydration by CA was higher in the submerged leaves than in the terrestrial leaves (Figure 2B). Furthermore, EZ, internal CA inhibitor, and DIDS inhibited the underwater Pn of submerged leaves, AZ and TRIS; HCO\(_3^−\)/H\(^+\) symporter inhibitor did not (Figure 2C). These results suggest that *H. polysperma* transports HCO\(_3^−\) into...
leaves by a DIDS-sensitive transporter and converts HCO$_3^-$ to CO$_2$ by an intercellular CA. *H. polysperma* leaves grown under submerged conditions achieved a higher photosynthetic rate by a biophysical CCM in low DIC levels (Figure 1B); therefore, submerged plants accumulated more proteins associated with the Calvin cycle compared with plants grown under terrestrial conditions (Figure 3B). HCO$_3^-$ use mechanism in *H. polysperma* is an effective way to acclimate to submerged environment. 4,4′-Diisothiocyanatostilbene-2,2′-disulfonate inhibits HCO$_3^-$ uptake and/or underwater photosynthesis in plants ranging from algae to higher plants (Nakajima et al., 2013;...
Fernández et al., 2014; Huang et al., 2020). Huang et al. (2020) first reported the presence of direct HCO$_3^-$ uptake via a DIDS-sensitive SLC4 transporter in the higher plant O. alismoides. Here, we report that the underwater Pn of H. polysperma-submerged leaves was also inhibited by DIDS; this is the first report of DIDS inhibiting the photosynthetic gas exchange of a higher plant. DIDS inhibits not only the SLC4 family of transporters but also members of the SLC26 gene family in mammals (Soleimani et al., 2001). HCO$_3^-$ transporters associated with biophysical CCMs have been identified in both the SLC4 and SLC26 gene families; SLC4 is present in a marine diatom (Nakajima et al., 2013) and an aquatic angiosperm (Huang et al., 2020), and BicA, a member of the SLC26/SulfP transporter family, is present in cyanobacteria (Price and Howitt, 2011). Nevertheless, neither SLC4 nor SLC26 anion exchangers annotated as HCO$_3^-$ transporter were identified in our proteomics analysis without regard to the expression levels. These results suggest that H. polysperma may move HCO$_3^-$ into leaves via a novel and as yet unidentified DIDS-sensitive HCO$_3^-$ transporter.

In algae, inorganic carbon uptake within the cells consumes light energy (Tchernov et al., 2003; Burnap et al., 2015; Wang et al., 2015). To photosynthesize in MCs, H. polysperma transports HCO$_3^-$ into leaves via the epidermal cells. Because H. polysperma does not have chloroplasts in the epidermal cells (Figure 5), energy for HCO$_3^-$ uptake may have to be supplied by MCs. Submerged leaves had higher initial slopes of their photosynthetic light response curves on a chlorophyll basis, indicating more efficient light use (Figure 1C). The chlorophyll content per leaf area in the submerged leaves was significantly higher than that in the terrestrial leaves, but the light use efficiency on the basis of leaf area was constant (Table 1 and Supplementary Figure 1B). These results imply that the light use efficiency of submerged leaves was high to provide sufficient light energy for HCO$_3^-$ uptake.

In algae, CA is a key component of the biophysical CCMs. In algae, CA mutants have lower photosynthetic DIC affinity and growth in low CO$_2$ conditions than wild type (Karlsson et al., 1998; Kikutani et al., 2016). These CAs are localized in...
the thylakoid lumen. Chloroplast CAs that are localized in the thylakoid lumen are assumed to supply CO$_2$ to the Calvin cycle. CA contributes not only biophysical CCMs but also biochemical CCMs. In C4 plants, CO$_2$ hydration activity of CA plays an essential role in primary CO$_2$ fixation by HCO$_3^-$ production. Some studies revealed the CA contributions of MC and BSC to total CA activity. *Amaranthus cruentus* leaves and C4 eudicots, were isolated two CAs (Guliev et al., 2003). One associated with the chlorophyll of BSCs is responsible for 8% of the total leaf CA activity; the other, found in the MC cytoplasmic fraction, represents 62% of the total leaf CA activity (Guliev et al., 2003). In corn (*Zea mays*, C4 grass) and green foxtail millet (*Setaria viridis* (L) P. Beauv., C4 grass), CO$_2$ assimilation rates in transgenic lines of loss of β-CA(s) were unchanged at ambient CO$_2$ but decreased at low CO$_2$ (Studer et al., 2014; Osborn et al., 2017). The β-CA mutant of *S. viridis* decreases growth and photosynthesis at ambient CO$_2$ (Chatterjee et al., 2021). On the other hand, pineapple (*Ananas comosus*), a CAM plant, increases the expression levels of three copies of CA at night time (Ming et al., 2015). Cytosolic β-CA activity may be partial to CO$_2$ hydration reaction contrary to chloroplast CA. The submerged leaves of *H. polysperma* increased the HCO$_3^-$ dehydrogenation/CO$_2$ hydration ratio of CA activity compared with that of terrestrial leaves (Figure 2B); however, the terrestrial leaves attained higher HCO$_3^-$ dehydrogenation and CO$_2$ hydration CA activities than those of the submerged leaves (Supplementary Figure 2). Seven differentially regulated proteins were identified as CAs by proteomic analysis (Table 2). The amount of chloroplast β-CA protein was significantly lower in the submerged leaves than in the terrestrial leaves, whereas the levels of other CAs did not change. In the case of other aquatic plants, *P. lucens* lack external CA (Staal et al., 1989); CA activity of *E. canadensis* is not influenced by the CO$_2$ concentration (Elzenga and Prins, 1988). In *O. alismoides*, external CA activity was greater in leaves acclimated to low CO$_2$ aquatic environment compared with that in leaves acclimated to high CO$_2$ aquatic environment; however, the expression of the four isoforms of putative α-CA1 was not significantly different between two CO$_2$ conditions (Huang et al., 2020). Our results suggest that the reduction in CO$_2$ hydration activity in the submerged leaves is caused by a decrease in the amount of β-CA, resulting in an increased HCO$_3^-$ dehydrogenation/CO$_2$ hydration ratio of CA activity in the submerged leaves.

### Structural Changes in Terrestrial and Submerged Leaves

Aquatic plants have different leaf anatomical characteristics compared with land plants: thinner leaves, cuticles, and cell walls, a few or no stomata, and epidermal cells with chloroplasts or chloroplasts facing the epidermis in MCs (Veen and Sasidharan, 2019). The thin leaves of aquatic plants improve gas exchange when leaves are submerged (Frost-Christensen et al., 2003). The submerged leaves of *H. polysperma* were thinner than the terrestrial leaves, and the stomatal density on the abaxial surface was lower than that of the terrestrial leaves (Table 1). The reduction in leaf thickness was achieved by decreasing the thickness of the MC layer (Figures 5A,B). Considering the results, *H. polysperma* appears to follow an acclimation mechanism for aquatic plants that facilitates passive CO$_2$ diffusion. Furthermore, both molecular size and diffusion resistance of HCO$_3^-$ are larger than that of CO$_2$, thinner leaves, especially smaller epidermal cells (Figure 6C), are effective not only in CO$_2$ diffusion but also in HCO$_3^-$ uptake. The submerged leaves had large MCs compared with terrestrial leaves (Figures 5A,B, 6A). These MCs in submerged leaves are expected to equip with big vacuoles. In submerged leaves of *H. polysperma*, some lower MCs were observed the absence of chloroplasts (Figure 5B). The submerged leaves may separate function between upper and lower MCs as in the cases of *E. canadensis* and *P. lucens*. For instance, upper MCs, which contain chloroplasts, perform photosynthesis using HCO$_3^-$; lower MCs, which have no or few chloroplasts, excrete or store OH$^-$ to maintain pH homeostasis.

### Terrestrial *Hygrophiila polysperma* Had Proto-Kranz-Type Photosynthetic Characteristics

In this study, we found that the submerged leaves of *H. polysperma* accumulated lower levels of proteins associated with biochemical CCM compared with the terrestrial leaves (Figure 3A). A result of the proteomics analysis implies that the terrestrial leaves use a biochemical CCM, and the submerged leaves do not. Biochemical CCMs include C4 photosynthesis and CAM, two pathways that require specific enzymes. Therefore, we measured the diel changes in malate content to confirm CAM activity in the leaves of *H. polysperma*. Terrestrial and submerged leaves did not accumulate malate at dawn (Table 3), suggesting that the terrestrial leaves did not perform CAM. Modified C4 photosynthetic pathways are identified as C3–C4 and C4-like photosynthesis in some plant species such as *Flaveria* (Mckown and Dengler, 2007), *Eleocharis* (Murphy et al., 2007), *Heliotropium* (Muhaidat et al., 2011), *Moricandia* (Schütler et al., 2017), and *Chenopodium* (Yorimitsu et al., 2019). C3–C4 intermediate plants have extended BSCs and lower CO$_2$ compensation point. In *Eleocharis*, C3–C4 intermediate species exhibit weak C4 cycles that means that there is a higher rate of PEPC to Rubisco than that of the C3 species and localization of enzymes between MC and BSC (Murphy et al., 2007). In *Heliotropium* and *Chenopodium* C3–C4 intermediate species, abundant mitochondria and localization of glycine decarboxylase in extended BSCs suggested that they capture, concentrate, and re-assimilate CO$_2$ released by photorespiration (Muhaidat et al., 2011; Yorimitsu et al., 2019). This simple CCM is called C2 photosynthesis, glycine shuttle, and photosynthetic CO$_2$ pump (Lundgren, 2020). Interestingly, some *Heliotropium* and *Chenopodium* species exhibit a proto-Kranz type of photosynthesis that is functionally C3 but involves BSCs. Proto-Kranz plants have larger BSC size than MC size like the biochemical CCM plant; however, enzyme activities related to C4 photosynthesis are the same levels as C3 plants (Muhaidat et al., 2011; Yorimitsu et al., 2019). The biochemical and anatomical characteristics of *H. polysperma*
leaves had unique features that do not differentiate between photosynthetic types. The ratio of PEPC to Rubisco increases with the transition from C3 to C4, leading to enhanced C4 activity. In the terrestrial leaves of *H. polysperma*, the ratio of PEPC to Rubisco was significantly higher than that in the submerged leaves (Figure 4C). However, the ratios of PEPC to Rubisco were 0.29 and 0.14 in the terrestrial and submerged leaves, a range in values that is typical for C3 plants (Murphy et al., 2007; Zhang et al., 2014). Anatomical analysis of *H. polysperma* found vascular bundles surrounding BSCs that contained few chloroplasts (Figures 5A, B). Nevertheless, the ratio of MC/BSC in a cross sectional area was 4.5 in the terrestrial and submerged leaves (Supplementary Table 3), a value that is identical to that in *Flaveria*, a C3–C4 plant (Mckown and Dengler, 2007), *Heliotropium* (Muhaidat et al., 2011), and the Kranz-like plant *Chenopodium* (Yorimitsu et al., 2019). In *H. polysperma*, the cell size ratios of MC/BSC were significantly different between terrestrial and submerged leaves (Figure 6D) at 0.5 and 1.3, respectively. These ratios are within the range for proto-Kranz- and non-Kranz-type plants (Yorimitsu et al., 2019).

Our results imply that under terrestrial conditions, *H. polysperma* conducts incomplete C4 photosynthesis, likely proto-Kranz, and alters its photosynthetic metabolism to C3 photosynthesis when leaves are underwater.

**Conclusion**

In summary, we report a comprehensive photosynthetic mechanism to acclimate *H. polysperma*, a heterophyllous amphibious plant to a submerged environment that has limited CO2. Leaves that developed in the submerged condition adopted two DIC uptake strategies: (i) passive CO2 diffusion without external CA and (ii) active HCO3− transport by a DIDS-sensitive HCO3− transporter-like member of the SLC gene family. Furthermore, our results suggest that *H. polysperma* can alter its photosynthesis from a proto-Kranz type in terrestrial leaves to a C3 type in submerged leaves. To improve photosynthetic performance for increasing crop yields, many researchers have attempted to introduce a biophysical CCM or a C4 pathway into C3 crop plants (Price et al., 2013; Ermakova et al., 2020).
Further characterization and understanding of the acclimation mechanisms to extreme stress conditions in *H. polysperma* will provide novel resources for improving stress resistance and photosynthesis in eudicot crop plants.

**DATA AVAILABILITY STATEMENT**

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: ProteomeXchange repositories. The names of the repository/repositories and accession number(s) can be found below: ProteomeXchange (PXD024779) and jPOST (JPST001108).

**AUTHOR CONTRIBUTIONS**

GH and NH conceived the research project and designed the study. GH, KM, and KN performed the experiments and analyzed the data. MI supported the anatomical analysis. GH and NH wrote manuscript. All authors contributed to the article and approved the submitted version.

**FUNDING**

This work was supported by the INOUE ENRYO memorial grant, Toyo University.

**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.675507/full#supplementary-material

**REFERENCES**

Beer, S., Bjork, M., Hellblom, F., and Axelsson, L. (2002). Inorganic carbon utilization in marine angiosperms (seagrasses). *Funct. Plant Biol.* 29:349. doi: 10.1071/PP01185

Black, C. C., Barry, C., Vickery, H., Walker, D., and Winter, K. (2003). Crassulacean acid metabolism photosynthesis: “working the night shift.” *Photosynth. Res.* 76:329.

Borum, J., Pedersen, O., Kotula, L., Fraser, M. W., Statton, J., Colmer, T. D., et al. (2016). Photosynthetic response to globally increasing CO$_2$ of co-occurring temperate seagrass species. *Plant Cell Environ.* 39, 1240–1250. doi: 10.1111/pce.12658

Bowes, G., Rao, S. K., Estavillo, G. M., and Reiskind, J. B. (2002). C4 mechanisms in aquatic angiosperms: comparisons with terrestrial C4 systems. *Funct. Plant Biol.* 29, 379–392. doi: 10.1071/PP01219

Burnap, R. L., Hagemann, M., and Kaplan, A. (2015). Regulation of CO$_2$ concentrating mechanism in cyanobacteria. *Life* 5, 348–371. doi: 10.3390/life5010348

Casati, P., Lara, M. V., and Andreo, C. S. (2000). Induction of a C4-like mechanism of CO$_2$ fixation in *Egeria densa*, a submerged aquatic species. *Plant Physiol.* 123, 1611–1621. doi: 10.1104/pp.123.4.1611

Chatterjee, J., Coe, R. A., Acebron, K., Thakur, V., Yennamalli, R. M., Danila, F., et al. (2021). A low CO$_2$-responsive mutant of *Setaria viridis* reveals that reduced carbondioxide anhydrase limits C4 photosynthesis. *J. Exp. Bot.* 72, 3122–3136. doi: 10.1093/jxb/erab039

Chen, T. W., Kahlen, K., and Stützel, H. (2015). Disentangling the contributions of osmotic and ionic effects of salinity on stomatal, mesophyll, biochemical and light limitations to photosynthesis. *Plant Cell Environ.* 38, 1528–1542. doi: 10.1111/pce.12504

Duanmu, D., Wang, Y., and Spalding, M. H. (2009). Thylakoid lumen carbonic anhydrase (CAH3) mutation suppresses air-die-gerontic phenotype of LCIB mutant in *Chlamydomonas reinhardtii*. *Plant Physiol.* 149, 929–937. doi: 10.1104/pp.108.132456

Elzenga, J., and Prins, H. (1988). Adaptation of *Eloaea* and *Potamogeton* to different inorganic carbon levels and the mechanism for photosynthetic bicarbonate utilisation. *Funct. Plant Biol.* 15:727. doi: 10.1071/PP980727

Elzenga, J. T., and Prins, H. B. (1989). Light-induced polar pH changes in leaves of *Eloea canadensis*: I. Effects of carbon concentration and light intensity. *Plant Physiol.* 91, 62–67. doi: 10.1104/pp.91.1.62

Ermakov, M., Danila, F. R., Furbank, R. T., and von Caemmerer, S. (2020). On the road to C4 rice: advances and perspectives. *Plant J.* 101, 940–950. doi: 10.1111/tpj.15462

Fernández, P. A., Hurd, C. L., and Roldea, M. Y. (2014). Bicarbonate uptake via an anion exchange protein is the main mechanism of inorganic carbon acquisition by the giant kelp *Macrocystis pyrifera* (Laminariales, Phaeophyceae) under variable pH. *J. Phycol.* 50, 998–1008. doi: 10.1111/jpy.12247

Flexas, J., and Medrano, H. (2002). Drought-inhibition of photosynthesis in C3 plants: stomatal and non-stomatal limitations revisited. *Ann. Bot.* 89, 183–189. doi: 10.1093/aob/mcf027

Frost-Christensen, H., Jørgensen, L. B., and Floto, F. (2003). Species specificity of resistance to oxygen diffusion in thin cuticular membranes from amphibious plants. *Plant Cell Environ.* 26, 561–569. doi: 10.1046/j.1365-3040.2003.00986.x

Gee, C. W., and Niogyi, K. K. (2017). The carbonic anhydrase CAH1 is an essential component of the carbon-concentrating mechanism in *Nannochloropsis oceanica*. *Proc. Natl. Acad. Sci. U.S.A.* 114, 4537–4542. doi: 10.1073/pnas.170013911

Guliev, N. M., Babaev, G. G., Bairamov, S. M., and Aliev, D. A. (2003). Purification, properties, and localization of two carbonic anhydrases from *Amaranthus cruentus* leaves. *Russ. J. Plant Physiol.* 50, 213–219. doi: 10.1023/A:1022925214846

Han, S., Maberly, S. C., Gontero, B., Xing, Z., Li, W., Jiang, H., et al. (2020). Structural basis for C4 photosynthesis without Kranz anatomy in leaves of the submerged freshwater plant *Ottelia alismoides*. *Ann. Bot.* 125, 869–879. doi: 10.1093/aob/mca005

Hasler, C. T., Jeffrey, J. D., Schneider, E. V. C., Hannan, K. D., Tix, J. A., and Suski, C. D. (2018). Biological consequences of weak acidification caused by elevated carbon dioxide in freshwater ecosystems. *Hydrobiologia* 806:3332. doi: 10.1007/s10750-017-3332-y

Hatch, M. D. (1971). The C4-Pathway of photosynthesis. Evidence for an intermediate pool of carbon dioxide and the identity of the donor C4-dicarboxylic acid. *Biochem. J.* 125, 425–432. doi: 10.1042/bj1250425

Hellblom, F., and Axelsson, L. (2003). External HCO$_3^-$ dehydration maintained by acid zones in the plasma membrane is an important component of the photosynthetic carbon uptake in *Ruppia cirrhosa*. *Photosynth. Res.* 77, 173–181. doi: 10.1023/A:1025809415048

Horiguchi, G., Nemoto, K., Yokoyama, T., and Hirotsu, N. (2019). Photosynthetic aclimation of terrestrial and submerged leaves in the amphibious plant *Hygrophila difformis*. *AqB Plants* 11:plz009. doi: 10.1093/aobpla/plz009

Huang, W., Han, S., Jiang, H., Gu, S., Li, W., Gontero, B., et al. (2020). External α-carboxyl anhydrase and solute carrier 4 are required for bicarbonate uptake in a freshwater angiosperm. *J. Exp. Bot.* 71, 6004–6014. doi: 10.1093/jxb/eraa351

Kanai, R., and Edwards, G. E. (1973). Enzymatic separation of mesophyll protoplasts and bundle sheath cells from C4 plants. *Naturwissenschaften* 60, 157–158. doi: 10.1007/BF00509479

Karlsson, J., Ciarke, A. K., Chen, Z. Y., Huggins, S. Y., Park, Y. II, Husic, H. D., et al. (1998). A novel α-type carbonic anhydrase associated with the thylakoid
membrane in *Chlamydomonas reinhardtii* is required for growth at ambient CO₂. *EMBO J.* 17, 1208–1216. doi: 10.1093/embj/17.5.1208

Keeley, J. E. (1983). Crassulacean acid metabolism in the seasonally submerged aquatic *Isoetes howellii*. *Oecologia* 58, 57–62. doi: 10.1007/BF00384542

Kikutani, S., Nakajima, K., Nagasato, C., Tsuji, Y., Miyatake, A., and Matsuda, Y. (2016). Thylakoid luminal Ω-carbonic anhydrase critical for growth and photosynthesis in the marine diatom *Phaeodactylum tricornutum*. *Proc. Natl. Acad. Sci. U.S.A.* 113, 9828–9833. doi: 10.1073/pnas.1603112113

Lundgren, M. R. (2020). C2 photosynthesis: a promising route towards crop improvement? *New Phytol.* 228, 1734–1740. doi: 10.1111/nph.16494

Mckown, A. D., and Dengler, N. G. (2007). Key innovations in the evolution of Kranz anatomy and C4 vein pattern in *Flaveria* (Asteraceae). *Am. J. Bot.* 94, 382–389. doi: 10.3732/ajb.94.3.382

Ming, R., VanBuren, R., Wai, C. M., Tang, H., Schatz, M. C., Bowers, J. E., et al. (2007). Increased Rubisco content in transgenic rice transformed with the ‘Sense’ rbcS gene. *Plant Physiol.* 148, 626–637. doi: 10.1103/pcp.90.3.1035

Price, G. D., Woodger, F. J., Badger, M. R., Howitt, S. M., and Tucker, L. (2004). Identification of a SULP-type bicarbonate transporter in marine cyanobacteria. *Proc. Natl. Acad. Sci. U.S.A.* 101, 18228–18233. doi: 10.1073/pnas.0405211101

Price, G. D., and Howitt, S. M. (2011). The cyanobacterial bicarbonate transporter BicA: its physiological role and the implications of structural similarities with human SLC26 transporters. *Biochem. Cell Biol.* 89, 178–188. doi: 10.1139/O10-158

Price, G. D., Pengelly, J. J. L., Forster, B., Du, J., Whitney, S. M., von Caemmerer, S., et al. (2013). The cyanobacterial CCM as a source of genes for improving photosynthetic CO₂ fixation in crop species. *J. Exp. Bot.* 64, 753–768. doi: 10.1093/jxb/ers257

Price, G. D., Woodger, F. J., Badger, M. R., Howitt, S. M., and Tucker, L. (2004). Identification of a SULP-type bicarbonate transporter in marine cyanobacteria. *Proc. Natl. Acad. Sci. U.S.A.* 101, 18228–18233. doi: 10.1073/pnas.0405211101

Prins, H. B. A., Snel, J. F. H., Heldt, R. J., and Zanstra, P. E. (1979). Photosynthetic bicarbonate utilization in the aquatic angiosperms *Potamogeton and Elodea*. *Hydrobiol. Bull.* 13, 106–111. doi: 10.1007/BF02284714

Rascio, N. (2002). The underwater life of secondarily aquatic plants: some problems and solutions. *C.R. Crit. Rev. Plant Sci.* 21, 401–427. doi: 10.1080/0735-26091042992

Rubio, L., García, D., García-Sánchez, M. J., Xavier Niell, F., Felle, H. H., and Fernández, J. A. (2017). Direct uptake of HCO₃⁻ in the marine angiosperm *Posidonia oceanica* (L.) Delile driven by a plasma membrane H⁺ economy. *Plant Cell Environ.* 40, 2820–2830. doi: 10.1111/pce.13057

Sage, R. F., Sage, T. L., and Kocacinar, F. (2012). Photorespiration and the evolution of C4 photosynthesis. *Annu. Rev. Plant Biol.* 63, 19–47. doi: 10.1146/annurev-plant-042811-105311

Schütler, U., Bräutigam, A., Gowik, U., Melzer, M., Christin, P. A., Kurz, S., et al. (2017). Photosynthesis in C3-C4 intermediate *Moriaindia species*. *J. Exp. Bot.* 68, 191–206. doi: 10.1093/jxb/erw391

Shao, H., Gontero, B., Mabler, S. C., Jiang, H. S., Cao, Y., Li, W., et al. (2017). Responses of *Ottelia alismoides*, an aquatic plant with three CCMs, to variable CO₂ and light. *J. Exp. Bot.* 68, 3985–3995. doi: 10.1093/jxb/erx064

Shibata, M., Katoh, H., Sonoda, M., Ohkawa, H., Shimoyma, M., Fukuzawa, H., et al. (2002). Genes essential to sodium-dependent bicarbonate transport in cyanobacteria: function and phylogenetic analysis. *J. Biol. Chem.* 277, 18658–18664. doi: 10.1074/jbc.M112468200

Staal, M., Elzenga, J. T. M., and Prins, H. B. A. (1989). ¹⁴C fixation by leaves and leaf cell protoplasts of the submerged aquatic angiosperm *Potamogeton lucens*: Carbon dioxide or bicarbonate? *Plant Physiol.* 90, 1035–1040. doi: 10.1104/pp.90.3.1035

Studer, A. J., Gandin, A., Kolbe, A. R., Wang, L., Cousins, A. B., and Brutnell, T. P. (2014). A limited role for carbonic anhydrase in C4 photosynthesis as revealed by a *ca1ca2* double mutant in maize. *Plant Physiol.* 165, 608–617. doi: 10.1104/pp.114.237605

Soleimani, M., Greeley, T., Petrovic, S., Wang, Z., Amlal, H., Kopp, P., et al. (2001). Pendrin: an apical Cl⁻/OH⁻/HCO₃⁻ exchanger in the kidney cortex. *Am. J. Physiol. Ren. Physiol.* 280, F356–F364. doi: 10.1152/ajprenal.2001.280.2.F356

Suzuki, Y., Ohkubo, M., Hatakeyama, H., Ohashi, K., Yoshizawa, R., Kojima, S., et al. (2007). Increased Rubisco content in transgenic rice transformed with the ‘Sense’ rbcS gene. *Plant Cell Physiol.* 48, 626–637. doi: 10.1093/pcp/pcm035

Tchernov, D., Silverman, J., Luz, B., Reinhold, L., and Kaplan, A. (2003). Massive light-dependent cycling of inorganic carbon between oxygenic photosynthetic microorganisms and their surroundings. *Photosynth. Res.* 77, 95–103. doi: 10.1023/A:1025869600935

Terashima, I., and Saeki, T. (1983). Light environment within a leaf I. optical properties of paradermal sections of camellia leaves with special reference to differences in the optical properties of palisade and spongy tissues. *Plant Physiol.* 24, 1493–1501. doi: 10.1093/jxp/24.6.1493

Tsuji, Y., Maharikada, A., and Matsuda, Y. (2017). Evolutionarily distinct strategies for the acquisition of inorganic carbon from seawater in marine diatoms. *J. Exp. Bot.* 68, 3949–3958. doi: 10.1093/jxb/erx102

Ueno, O., Samejima, M., Muto, S., and Miyachi, S. (1988). Photosynthetic characteristics of an amphibious plant, *Elodea virivaria*: expression of C4 and C3 modes in contrasting environments. *Proc. Natl Acad. Sci. U.S.A.* 85, 6733–6737. doi: 10.1073/pnas.85.18.6733

Veen, H., and Sasidharan, R. (2019). Shape shifting by amphibious plants in dynamic hydrological niches. *New Phytol.* 229, 79–84. doi: 10.1111/nph.16347

Wang, Y., Stessman, D. J., and Spalding, M. H. (2015). The CO₂ concentrating mechanism and photosynthetic carbon assimilation in limiting CO₂: how *Chlamydomonas reinhardtii* works against the gradient. *Plant J.* 82, 429–448. doi: 10.1111/pj.12829

Wilbur, K. M., and Anderson, N. G. (1948). Electrometric and colorimetric determination of carbonic anhydrase. *J. Biol. Chem.* 176, 147–154

Xiao, Y., Tholen, D., and Zhu, X.-G. (2016). The influence of leaf anatomy on the internal light environment and photosynthetic electron transport rate: exploration with a new leaf ray tracing model. *J. Exp. Bot.* 67, 6021–6035. doi: 10.1093/jxb/erw359
Yamano, T., Sato, E., Iguchi, H., Fukuda, Y., and Fukuzawa, H. (2015). Characterization of cooperative bicarbonate uptake into chloroplast stroma in the green alga *Chlamydomonas reinhardtii*. *Proc. Natl. Acad. Sci. U. S. A.* 112, 7315–7320. doi: 10.1073/pnas.1501659112

Yang, T., and Liu, X. (2015). Comparing photosynthetic characteristics of *Isoetes sinensis* Palmer under submerged and terrestrial conditions. *Sci. Rep.* 5:17783. doi: 10.1038/srep17783

Yin, L., Li, W., Madsen, T. V., Maberly, S. C., and Bowes, G. (2017). Photosynthetic inorganic carbon acquisition in 30 freshwater macrophytes. *Aquat. Bot.* 140, 48–54. doi: 10.1016/j.aquabot.2016.05.002

Yorimitsu, Y., Kadosono, A., Hatakeyama, Y., Yabiku, T., and Ueno, O. (2019). Transition from C3 to proto-Kranz to C3–C4 intermediate type in the genus *Chenopodium* (Chenopodiaceae). *J. Plant Res.* 132, 839–855. doi: 10.1007/s10265-019-01135-5

Zhang, Y., Yin, L., Jiang, H. S., Li, W., Gontero, B., and Maberly, S. C. (2014). Biochemical and biophysical CO₂ concentrating mechanisms in two species of freshwater macrophyte within the genus *Ottelia* (Hydrocharitaceae). *Photosyn. Res.* 121, 285–297. doi: 10.1007/s11120-013-9950-y

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2021 Horiguchi, Matsumoto, Nemoto, Inokuchi and Hirotsu. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.