Submergence-Induced Morphological, Anatomical, and Biochemical Responses in a Terrestrial Species Affect Gas Diffusion Resistance and Photosynthetic Performance

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Gas exchange between the plant and the environment is severely hampered when plants are submerged, leading to oxygen and energy deficits. A straightforward way to reduce these shortages of oxygen and carbohydrates would be continued photosynthesis under water, but this possibility has received only little attention. Here, we combine several techniques to investigate the consequences of anatomical and biochemical responses of the terrestrial species *Rumex palustris* to submergence for different aspects of photosynthesis under water. The orientation of the chloroplasts in submergence-acclimated leaves was toward the epidermis instead of the intercellular spaces, indicating that underwater CO_2 diffuses through the cuticle and epidermis. Interestingly, both the cuticle thickness and the epidermal cell wall thickness were significantly reduced upon submergence, suggesting a considerable decrease in diffusion resistance. This decrease in diffusion resistance greatly facilitated underwater photosynthesis, as indicated by higher underwater photosynthesis rates in submergence-acclimated leaves at all CO_2 concentrations investigated. The increased availability of internal CO_2 in these ''aquatic'' leaves reduced photorespiration, and furthermore reduced excitation pressure of the electron transport system and, thus, the risk of photodamage. Acclimation to submergence also altered photosynthesis biochemistry as reduced Rubisco contents were observed in aquatic leaves, indicating a lower carboxylation capacity. Electron transport capacity was also reduced in these leaves but not as strongly as the reduction in Rubisco, indicating a substantial increase of the ratio between electron transport and carboxylation capacity upon submergence. This novel finding suggests that this ratio may be less conservative than previously thought.

Complete submergence severely inhibits gas exchange between the plant and the environment because gas diffusion in water is approximately 10^4 slower than in air. This can lead to oxygen deficiency in the plant and, concomitantly, energy deficits due to hampered aerobic metabolism (Crawford and Brändle, 1996). Well-known adaptations of plants to submergence include elongation of shoot organs to restore contact with the atmosphere (Voesenek et al., 2004) and the ability to switch to anaerobic metabolism to generate ATP in the absence of O_2 (Perata and Alpi, 1993). Another, yet overlooked, phenomenon to reduce the shortages of oxygen and carbohydrates might be the potential for sustained photosynthesis under water (He et al., 1999; Vervuren et al., 1999).

The poor gas diffusion under water, however, severely limits inorganic carbon supply for photosynthesis. To reduce this limitation, specialized aquatic plant species have developed CO_2-concentrating mechanisms (Bowes et al., 2002), or can use HCO_3^- (Maberly and Madsen, 2002) or utilize sediment CO_2 (Pedersen et al., 1995) as a carbon source. In nonspecialized plants, however, underwater photosynthesis rates and affinity for CO_2 are expected to be low due to the high gas diffusion resistance of the leaves (Maberly and Madsen, 1998; Sand-Jensen and Frost-Christensen, 1999; Madsen and Maberly, 2003).

Recently, we observed that *Rumex palustris* plants submerged in water with ambient O_2 and CO_2 concentrations had subambient O_2 concentrations in their leaves in darkness. However, O_2 concentrations were much closer to ambient in leaves that were acclimated to submerged growth conditions (Mommer et al., 2004). This suggests increased diffusion of oxygen from the water column into the leaves and, thus, reduced gas diffusion resistance as result of acclimation to submergence. This finding therefore encouraged us to investigate the photosynthetic consequences of acclimation to submergence in this terrestrial species, which is a well-explored model species for terrestrial plant responses to submergence (Visser et al., 1996; Cox et al., 2003; Voesenek et al., 2003).

Photosynthetic acclimation to submergence has hardly been investigated in terrestrial species, but more is known from work on amphibious plant species. These plants grow in the transition from

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land to water and develop both specialized terrestrial and aquatic leaves. Sand-Jensen et al. (1992) and Frost-Christensen and Sand-Jensen (1995) showed that aquatic leaves of these species have increased underwater CO_2 assimilation rates compared to their terrestrial counterparts, as a result of the reduced gas diffusion resistance of the leaves. Frost-Christensen et al. (2003) also showed that this reduced gas diffusion resistance in aquatic leaves of amphibious plant species originates from reduced cuticle thickness and its reduced resistance for gases such as O_2. Photosynthesis rates may, however, not only be hampered by diffusional resistances (Long and Bernacchi, 2003) but also by biochemical limitations (Centritto et al., 2003). Reduced Rubisco activities have been observed in aquatic leaves of amphibious species relative to their terrestrial leaves, and the aquatic leaves therefore have a reduced carboxylation capacity (Farmer et al., 1986; Beer et al., 1991).

Here, we investigate if the terrestrial plant *R. palustris* shows responses to submergence that may be expected from the analogy with amphibious species, including reduced cuticle thickness in submergence-acclimated leaves and, consequently, higher underwater photosynthesis rates. Furthermore, we explore novel responses of photosynthesis in response to submergence. An aspect that has not received much attention in amphibious plants species is that under water the internal CO_2 concentration (C_i) is relatively low compared to internal O_2 concentration, which favors oxygenation reactions of Rubisco over carboxylation (Ogren, 1984). Underwater photosynthesis is therefore predicted to be characterized by high photorespiration rates (Lloyd et al., 1977; Salvucci and Bowes, 1982). Here, we report the first in vivo estimates of photorespiration rates in higher plants under water. This allows investigation of the effect of submergence acclimation on photorespiration, since decreased gas diffusion might alter the ratio between internal CO_2 and O_2 concentrations. Another consequence of an aquatic environment is the relatively low availability of electron sinks relative to photon absorption (MacKenzie et al., 2004), which may impose a large excitation pressure on the electron transport system (Niyogi, 2000). Increased C_i as a result of acclimation to submergence might lower the excitation pressure and thus reduce potential photoinhibition. Additionally, we investigate if, next to reduced diffusion resistance, submergence acclimation also involves changes at the biochemical level of photosynthesis. If acclimation to submergence in terrestrial species resembles differences between the leaf types of amphibious species (Beer et al., 1991), we would expect reduced amounts of Rubisco protein in submergence-acclimated leaves of *R. palustris*. As different processes of the photosynthetic machinery are highly coordinated and kinetic properties are tuned to each other (von Caemmerer, 2000), electron transport capacity was expected to show the same response to submergence as described for carboxylation capacity.

RESULTS

Leaf Morphology and Anatomy Change as a Result of Submergence

Leaf morphological and anatomical features of *R. palustris* showed strong plasticity in response to submergence. The leaves developed during submergence (referred to as "aquatic leaves") were elongated and thin in comparison with "terrestrial leaves" developed completely in air, but still contained well-developed air spaces (Table I; Fig. 1, a and b). Furthermore, the epidermal cell wall and cuticle thickness were greatly reduced in aquatic leaves compared to terrestrial leaves (Fig. 2).

Leaf mass per area (LMA) was considerably lower in aquatic leaves compared to terrestrial leaves (Table I). This difference was largely caused by starch accumulation in the terrestrial leaves (Table I). Soluble sugar contents were not significantly different between the leaf types. When LMA was corrected for these nonstructural carbohydrates, the difference between the leaf types was strongly reduced, but a significant difference still remained (Table I).

In contrast to aquatic leaves of many amphibious plants, the aquatic leaves of *R. palustris* contained stomata, even more than their terrestrial counterparts (Table I; Fig. 1, g and h). The pavement cells in the epidermis of terrestrial leaves were clearly "jigsaw" shaped, whereas the epidermal cells of the aquatic leaves were more rectangular (Fig. 1, g and h), as has been observed in elongated organs (Glover, 2000). The

Table I. *Morphological and anatomical characteristics of terrestrial and aquatic leaves of R. palustris*

NSC-free LMA is leaf mass per area minus the weight contribution of nonstructural carbohydrates (NSC); #Stomata is the number of stomata per unit leaf area; and #Epidermis is the number of epidermis cells per unit leaf area. Samples were taken from 44-d-old plants. Values are means ± SE. Data were analyzed with Student's *t* tests, and ln-transformation was applied if discrepancies from homogeneity of variance were found. **, $P < 0.01$; ***, $P < 0.001$; n.s., not significant.

Parameter	Terrestrial Leaves	Aquatic Leaves	*n*	*P*
Leaf length (cm)	9.0 ± 0.1	16.7 ± 0.7	6	***
Leaf width (cm)	2.7 ± 0.0	2.0 ± 0.1	6	***
Leaf area (cm^2)	20.1 ± 0.8	14.4 ± 0.7	10	***
Leaf thickness (μm)	222 ± 4	178 ± 8	3	**
Aerenchyma content (%; v:v)	21.0 ± 0.6	18.5 ± 1.2	6	n.s.
LMA (g m^{-2})	47.8 ± 1.46	20.0 ± 0.7	10	***
Starch (mg g^{-1} DW)	415.7 ± 29.7	31.6 ± 6.0	8	***
Suc (mg g^{-1} DW)	30.2 ± 2.9	24.8 ± 1.4	8	n.s.
NSC-free LMA (g m^{-2})	26.5 ± 0.8	18.9 ± 0.7	8	***
#Stomata (mm^{-2})				
Adaxial	29 ± 2	73 ± 6	5	***
Abaxial	47 ± 5	85 ± 6	5	**
#Epidermis (mm^{-2})				
Adaxial	224 ± 24	923 ± 33	5	***
Abaxial	271 ± 23	943 ± 28	5	***
Chlorophyll (μmol m^{-2})	288 ± 17	212 ± 9	6	**

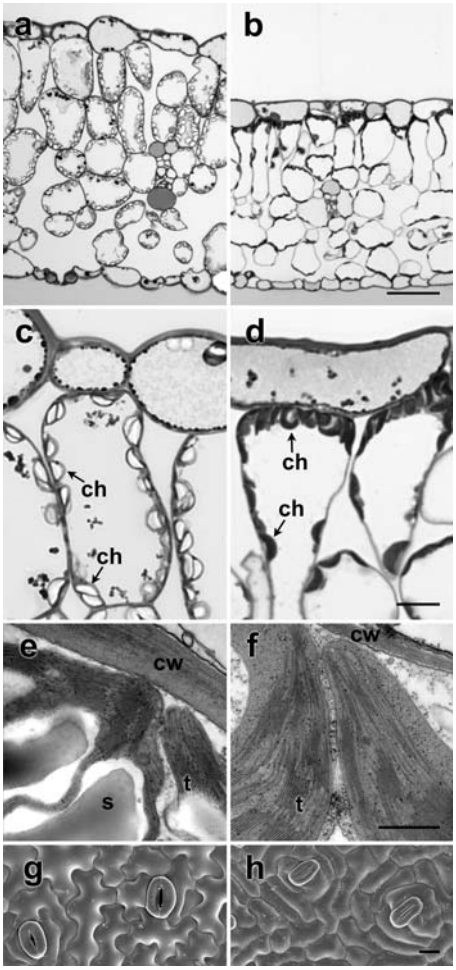

Figure 1. Anatomy of terrestrial and aquatic leaves of *R. palustris*. a and b, LM image of a transverse leaf section of terrestrial (a) and aquatic leaf (b; 250×; scale bar represents 50 μm). c and d, Detail of the adaxial epidermis and mesophyll cells showing the position of chloroplasts (ch) in terrestrial (c) and aquatic leaf (d; 1,000×; scale bar represents 10 μm). e and f, TEM image of chloroplast with thylakoid structure (t). Terrestrial leaf (e) showing starch deposition (s) and aquatic leaf (f; 11,000×; scale bar represents 500 nm) are shown. g and h, SEM image of leaf surface showing stomata and epidermis cells of terrestrial (g) and aquatic leaf (h; 300×; scale bar represents 20 μm). Samples were taken from 44-d-old plants.

pavement cells of the elongated aquatic leaves were smaller than those of the terrestrial leaves, as shown by the larger number of cells per mm^2 and per leaf lamina in aquatic leaves (Table I). The chloroplasts in the aquatic leaves were clearly oriented toward the epidermis as opposed to the intercellular orientation in terrestrial leaves (Fig. 1, c and d).

Acclimation to Submergence Leads to Higher Underwater CO_2 Assimilation Rates

To investigate the functional consequences of these morphological and anatomical acclimation responses, underwater oxygen evolution was measured in both leaf types. Aquatic leaves already had a net positive assimilation rate (A_n) under water at 10 μM free CO_2 (equilibrium pressure at 25 Pa CO_2, which is slightly below atmospheric equilibrium), whereas in the terrestrial leaves the minimal CO_2 concentration for positive A_n was between 100 and 250 μM CO_2 (Fig. 3a). The slope of the underwater CO_2 response curves was steeper in aquatic leaves than in terrestrial ones (Fig. 3a), indicating a higher affinity for CO_2. Maximum assimilation rates under water were also higher in aquatic leaves than in terrestrial leaves, even when CO_2 concentrations in the water were increased to 2,000 μM.

Aerial gas exchange measurements also revealed differences between the two leaf types in response to CO_2 (Fig. 3b), but these were opposite to those under water (Fig. 3a) since A_n was lower in aquatic leaves compared to terrestrial leaves at ambient CO_2 concentrations in air (Fig. 3b).

In terrestrial leaves, A_n were much lower in water than in air, indicating that CO_2 is limiting photosynthesis under water, even at the extremely high CO_2 concentration of 2,000 μM (Fig. 4, a and b). The highest CO_2 concentrations both in water and air yielded similar A_n in aquatic leaves (Fig. 4, a and b), but electron transport rates (J_F; calculated from chlorophyll

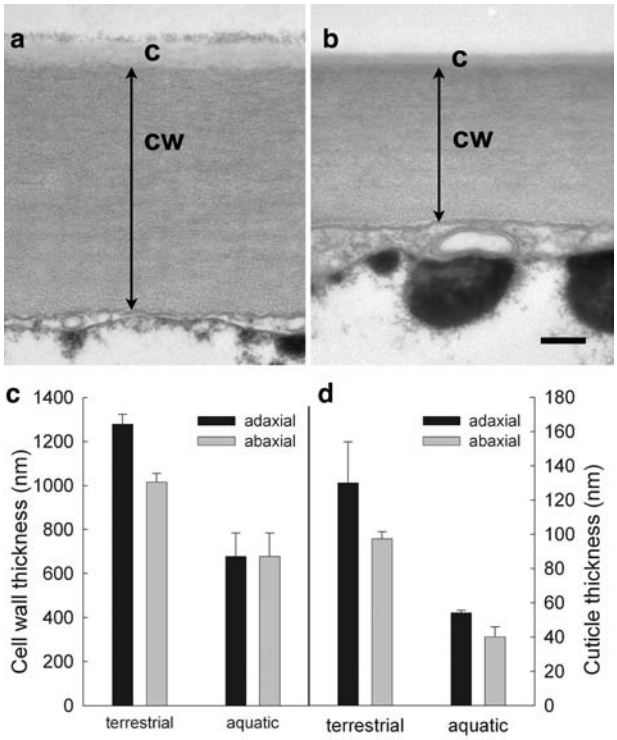

Figure 2. Cell wall and cuticle thickness of epidermis cells of terrestrial and aquatic leaves of *R. palustris*. A and b, TEM image of a transverse leaf section showing the adaxial epidermal cell wall (cw) with a cuticle (c). a, Terrestrial leaf; b, aquatic leaf (36,300×; scale bar represents 200 nm). c and d, Graphs show the difference in thickness of cell wall (c) and cuticle (d). Samples were taken from 44-d-old plants. Data are means ± SE.

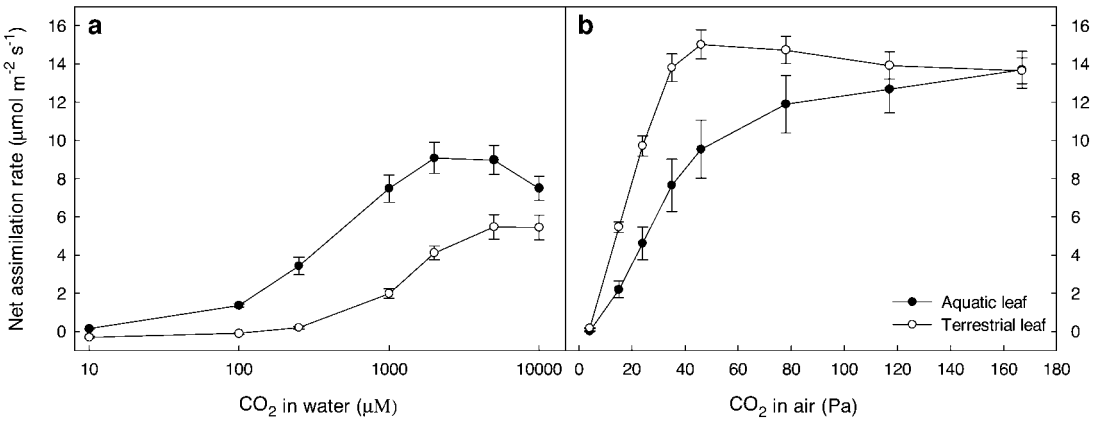

Figure 3. CO_2 response curves of terrestrial and aquatic leaves of *R. palustris* under water (a; in O_2 production) and in air (b; in CO_2 uptake) at PPFDs of 400 and 1,000 μmol m^{-2} s^{-1}, respectively. CO_2 concentrations in the water (a) and partial pressure (b) are plotted on the *x* axes. Measurements were performed on leaves of 44-d-old plants. Data are means ± SE; $n = 6$.

fluorescence) were higher in air than in water in these leaves, which might be explained by a difference in photosynthetic photon flux density (PPFD) between the measurements in air and water.

It appeared that A_n of terrestrial leaves was constant between two batches of plants of the same age (Figs. 3b and 4b) but that A_n of aquatic leaves in air was more variable. The variable A_n of aquatic leaves shortly after desubmergence may be due to variation in stomatal opening, but we could not measure that due to uncertainties about cuticular transpiration. Still, the relative responses to submergence were identical between the two batches of plants.

The underwater gas exchange measurements together point to a significant decrease in diffusion resistance in response to submergence, which is indicated by higher A_n of aquatic leaves at low CO_2 concentrations in water.

Acclimation to Submergence Results in Decreased Photorespiration Rates Under Water

To investigate if the reduced diffusion resistance in aquatic leaves affected photorespiration rates, we determined the difference between photosynthetic electron transport rate divided by 4 ($J_F/4$) measured by means of chlorophyll fluorescence and gross assimilation rate ($A_g = A_n + R_D$), based on gas exchange. This difference is considered to be representative for in vivo electron transport involved in oxygenation (V_O) and, consequently, photorespiratory CO_2 release (R_P), together referred to as photorespiration rate. Terrestrial

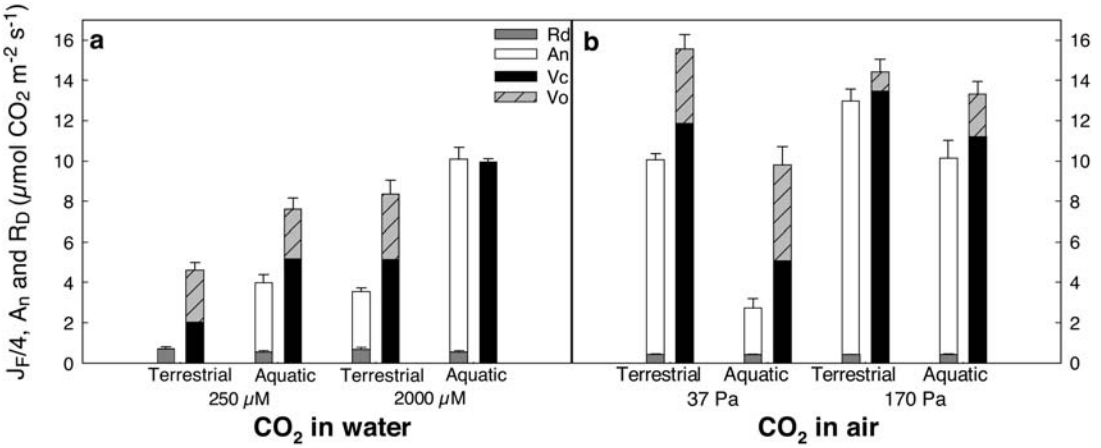

Figure 4. Electron transport rate ($J_F/4 = V_c + V_o$, carboxylation rate + oxygenation rate) measured with chlorophyll fluorescence and gross photosynthesis rate ($A_g = A_n + R_D$, net assimilation rate + dark respiration rate) measured with gas exchange in terrestrial and aquatic leaves of *R. palustris* under water (a) and in air (b). PPFDs were 400 and 1,000 μmol m^{-2} s^{-1} for the measurements under water and in air, respectively. J_F was corrected for the J_F/J_C ratio measured in the absence of photorespiration. The difference between the two bars (J_F and A_g) within a leaf type × treatment combination reflects the process of photorespiration. The contribution of V_o to the J_F was estimated as 1.5 times the difference between $J_F/4$ and A_g. Measurements were performed on leaves of 44-d-old plants, grown from a different batch of plants than presented in Figure 3. Data are means; for J_F, A_n, and R_D SEs are given; $n = 6$.

leaves had substantial J_F under water at 250 μM CO_2 but no net CO_2 assimilation (Fig. 4a). At this CO_2 concentration, which is 15 times higher than in air-saturated water, the electron transport rate involved in carboxylation (V_C) was apparently compensated by an equally large R_P plus a small share of respiration rate (R_D; respiration rate in the dark), assuming that no other quantitatively important electron acceptors are active.

Both J_F and A_g increased considerably in both leaf types by increasing the CO_2 concentration in the water from 250 to 2,000 μM (Fig. 4a), indicating that the system under water was limited by electron acceptors at low CO_2 concentration. There was a clear net O_2 production at 2,000 μM in terrestrial leaves, and J_F was also higher than at the low CO_2 concentration, but photorespiration rates were still substantial in these leaves as indicated by the difference between J_F and A_g (Fig. 4a). At this high CO_2 concentration, J_F and A_g were, however, identical in the aquatic leaves, indicating that photorespiration in these leaves was so low that it could not be detected anymore.

Interestingly, measurements on the two leaf types in air revealed patterns opposite to those found in underwater measurements. The difference between A_g and J_F in terrestrial leaves measured in air at 37 Pa CO_2 was 29%, indicating substantial photorespiration rates at ambient conditions (Fig. 4b). This difference disappeared when the CO_2 concentration was increased to 170 Pa. Photosynthesis was low in aquatic leaves when measured in air at 37 Pa CO_2, as indicated by low A_g and J_F as compared to terrestrial leaves. Moreover, the difference between these parameters was large (Fig. 4b), indicating high photorespiration rates in the aquatic leaves in air. A_g increased much more than J_F in these aquatic leaves at high CO_2 concentration, and photorespiration consequently decreased but was still evident. To summarize, aquatic leaves had higher net underwater CO_2 assimilation rates combined with lower photorespiration rates than terrestrial leaves, when measured under water.

Aquatic Leaves Have Lower Nonphotochemical Quenching Under Water

Chlorophyll fluorescence measurements showed the excitation pressure on the electron transport chain and, thus, the risk of photodamage. The partitioning of absorbed photons was calculated over inherent inefficiency (*L*), photosynthetic electron transport (*P*), heat dissipation (*D*), and to processes that may potentially damage the plant (excitation pressure or excess energy, *E*). This partitioning depends on environmental cues such as light and temperature, but also on the availability of electron sinks such as CO_2 and O_2 (MacKenzie et al., 2004). The latter was different in terrestrial and aquatic leaves because of their differences in diffusion resistance. In general, the leaf type with treatment combinations with the highest J_F also showed the highest photochemical quenching (q_P) and, thus, also the highest partitioning to photosynthetic electron transport (*P*). The effect of the increased availability of electron sinks on partitioning was illustrated by the increased q_P in both leaf types with increasing CO_2 concentrations. Nonphotochemical quenching (q_N) including proportions of absorbed photons directed to heat dissipation (*D*) and excess energy (*E*) was, as expected, highest in the situations with the lowest CO_2 availability; i.e. the terrestrial leaf at low CO_2 had the largest *E* fraction among the measurements in water, and the aquatic leaf at low CO_2 had the largest *E* fraction among the measurements in air (Table III; Fig. 5, a and b). Furthermore, we observed a slight reduction of the maximum quantum

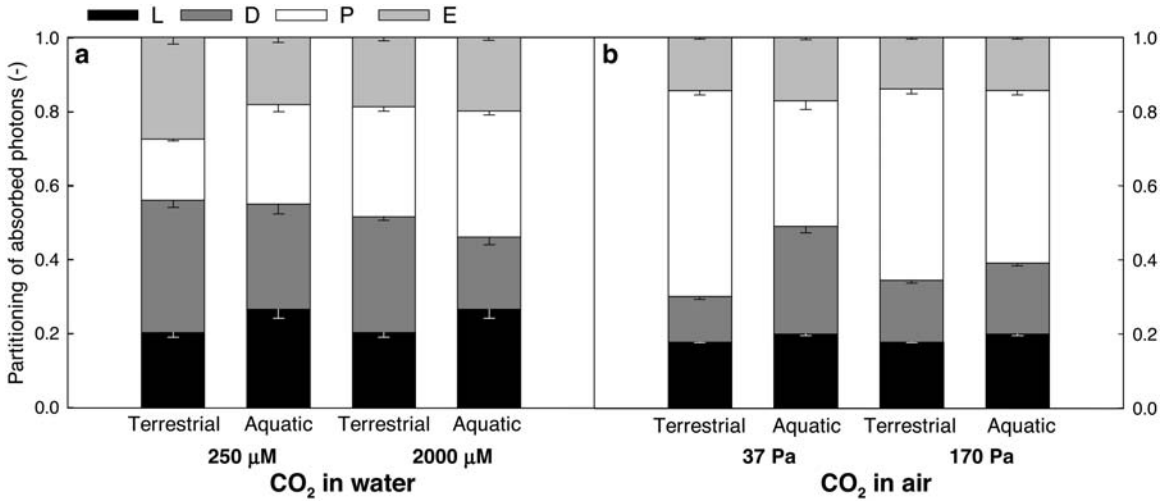

Figure 5. Partitioning diagrams of total absorbed photons of terrestrial and aquatic leaves of *R. palustris* at different CO_2 concentrations under water (a) and in air (b). Partitioning parameters are inherent inefficiency in dark (*L*), thermal dissipation (*D*), electron transport (*P*), and excess energy (*E*). PPFDs were 400 and 1,000 μmol m^{-2} s^{-1} for the measurements in water and air, respectively. Measurements were performed on leaves of 44-d-old plants. Data are means ± SE; $n = 6$.

yield of PSII (F_v/F_m) when leaves were measured in water compared to air, and this decrease was stronger for aquatic leaves than for terrestrial leaves (Table III), indicating some down-regulation of PSII efficiency when PPFD exceeds 200 μmol m^{-2} s^{-1} under water.

The fact that the aquatic leaves showed higher q_P and lower q_N under water than did the terrestrial leaves indicates that acclimation to submergence reduced excitation pressure on the photosystems under water (E) and, therefore, the risk of photodamage.

Aquatic Leaves Have a Higher Electron Transport Capacity Relative to Carboxylation Capacity

According to the model described for C_3 photosynthesis (von Caemmerer, 2000), carboxylation capacity (V_{Cmax}) can be derived from the slope of the aerial CO_2 response curve. However, the reduced slope of the aerial CO_2 response curve in aquatic leaves of *R. palustris* (Fig. 3a) may reflect not only a biochemical limitation in these leaves but also diffusional limitation at the lower CO_2 concentrations. Since C_i in aquatic leaves cannot be calculated accurately due to possible cuticular transpiration in these leaves, the calculation of the V_{Cmax} from gas exchange measurements was precluded. An alternative way to investigate if V_{Cmax} was reduced as a result of submergence acclimation is to measure the amount of Rubisco protein, which reflects in vivo Rubisco activity and, thus, V_{Cmax} at saturating light intensities (Woodrow and Berry, 1988) when Rubisco is fully activated.

The Rubisco analyses showed that aquatic leaves of *R. palustris* contained nearly 2 times less Rubisco protein on a leaf area basis than terrestrial leaves (Table II). Since photosynthetic reactions are generally highly coordinated, with carboxylation capacity and electron transport capacity tuned to each other, we expected the reduced Rubisco in aquatic leaves to be accompanied by a similar reduction of the electron transport capacity. Electron transport capacity (J_{Fmax}; calculated from chlorophyll fluorescence) was estimated from chlorophyll fluorescence at a PPFD of 1,000 μmol m^{-2} s^{-1} and a CO_2 concentration of 170 Pa, which was saturating (data not shown). J_{Fmax} was reduced in aquatic leaves compared to terrestrial leaves of *R. palustris*, irrespective of the scenario used for the calculation (Table II). However, the reduction of J_{Fmax} was less than the reduction in Rubisco content. Consequently, the higher J_{Fmax}/Rubisco ratio in the aquatic leaves (Table II) suggests that submergence-acclimated leaves have a considerably higher photosynthetic electron transport capacity relative to carboxylation capacity compared to nonacclimated leaves.

DISCUSSION

Leaves of the terrestrial plant species *R. palustris* show pronounced morphological, anatomical, and biochemical acclimation to submerged conditions. This resulted in differences in photosynthetic performance under water between the terrestrial and aquatic leaves. The underwater photosynthesis measurements show the immense capacity of this flooding-tolerant species to adjust its morphology and physiology to submerged conditions.

Decreased Diffusion Resistance Is Beneficial for Underwater CO_2 Assimilation

The functionality of the response to submergence was evident from the higher maximum underwater photosynthesis rates in aquatic leaves than in terrestrial leaves of *R. palustris* (Fig. 3a). Another benefit of the acclimation to submergence was the higher net underwater assimilation rate (A_n) at low CO_2 concentrations in aquatic compared to terrestrial leaves of

Table II. *Biochemical and photosynthetic characteristics in terrestrial and aquatic leaves of R. palustris*

J_{max} and V_{Cmax} are calculated according to von Caemmerer (2000). J_{Fmax} is electron transport capacity obtained from chlorophyll fluorescence measurements at PPFD 1,000 μmol m^{-2} s^{-1} and 170 Pa CO_2. J_{FmaxC} is electron transport capacity corrected for deviation from J_C at low O_2 (1 kPa) and high CO_2 (170 Pa) concentrations. The data presented in this table were obtained from a set of slightly younger plants than used elsewhere in this article: measurements were performed on the fourth just fully expanded leaf of terrestrial and aquatic plants of 31 and 35 d old, respectively. Values are means ± SE; $n = 6$. Data were analyzed with Student's t tests, and J_{Fmax}/Rubisco data were ln-transformed to obtain homogeneity of variance. *, $P < 0.05$; ***, $P < 0.001$.

Parameter	Terrestrial Leaves	Aquatic Leaves	P
Chlorophyll (μmol m^{-2})	373 ± 6	271 ± 4	***
Soluble protein (g m^{-2})	4.1 ± 0.2	2.9 ± 0.2	***
Rubisco protein (μmol m^{-2})	2.3 ± 0.1	1.2 ± 0.1	***
J_{max}/V_{Cmax}	1.55 ± 0.05	Not determined	
J_{Fmax} (μmol e^- m^{-2} s^{-1})	78.8 ± 2.0	59.6 ± 2.3	***
J_{Fmax}/Rubisco (mol e^- [mol Rubisco]$^{-1}$ s^{-1})	35.2 ± 2.2	49.3 ± 3.7	***
J_{FmaxC} (μmol e^- m^{-2} s^{-1})	84.5 ± 2.3	59.8 ± 3.0	***
J_{FmaxC}/Rubisco (mol e^- [mol Rubisco]$^{-1}$ s^{-1})	37.2 ± 1.9	49.4 ± 3.5	*

R. palustris (Fig. 3a), reaching values (<10 μM) that are ecologically relevant and comparable to those of aquatic leaves of amphibious plants (Sand-Jensen and Frost-Christensen, 1999). Affinity for CO_2 under water was higher in aquatic than in terrestrial leaves, as indicated by a steeper slope of the CO_2 response curve of the aquatic leaves (Fig. 3a), which is commonly observed in amphibious plants (Maberly and Spence, 1989; Sand-Jensen and Frost-Christensen, 1999). These differences in underwater gas exchange characteristics are clearly the result of a decreased diffusion resistance in response to submergence in this terrestrial species. This is most obvious from the underwater photosynthetic performance at low CO_2 since affinity for CO_2 is the combined result of all diffusional resistances over the leaf, although it can also be affected by biochemical limitations (Centritto et al., 2003). Under water, cuticle resistance is considered to be an important factor determining CO_2 affinity (Frost-Christensen et al., 2003). Cuticle resistance is determined by its thickness and the chemical composition of waxes and cutin (Lequeu et al., 2003). Cuticle thickness per se is not necessarily associated with resistance for water transport and gas diffusion, since no clear correlations between these parameters have been observed in several drought-tolerant species (Kerstiens 1996a, 1996b; Schreiber and Riederer, 1996). However, Frost-Christensen et al. (2003) showed that aquatic leaves of five amphibious species had a reduced cuticle thickness compared to terrestrial leaves and an accompanying reduced diffusional resistance for gases. We therefore conclude that it is very likely that the difference in underwater CO_2 affinity between the leaf types of *R. palustris* (Fig. 4a) will mainly be explained by a difference in cuticle thickness (Fig. 2, a, b, and d) and its putative decrease in resistance (Frost-Christensen et al., 2003). The differences in CO_2 affinity between the leaf types were not related to boundary layer effects, since boundary layers were minimal and similar in the leaf discs in the well-stirred cuvette. It is not likely that stomata will contribute significantly to CO_2 uptake under water since most stomata were closed under water, as checked with cryo scanning electron microscopy (SEM; Fig. 1, g and f). Internal resistance for diffusion in the mesophyll is considered to be negligible as compared to the strong barrier formed by the cuticle.

Chloroplast Orientation as Evidence for Epidermal Diffusion of CO_2

The marked orientation of the chloroplasts of the aquatic leaves provides evidence for the importance of gas diffusion through the epidermis and its cuticle layer under water. The chloroplasts of the terrestrial leaves of *R. palustris* were oriented around the intercellular air spaces and absent from walls adjoining neighboring cells (Fig. 1c), which is a universal phenomenon (Psaras et al., 1996). In contrast, the chloroplasts of the aquatic leaves were directed toward the adaxial and abaxial epidermal layers and positioned against cell walls that do not border intercellular spaces (Fig. 1d). This effect of submergence on chloroplast orientation has also been described for the amphibious species *Ranunculus flabellaris* (Bruni et al., 1996) but could not be detected in *Mentha aquatica* (Sand-Jensen and Frost-Christensen, 1999).

A factor known to regulate chloroplast movement is light intensity (Wada et al., 2003; Kondo et al., 2004), but in this study the differential orientation of chloroplasts in the leaf types cannot be caused by this factor since light intensities were similar in both treatments. We therefore suggest this to be an orientation toward CO_2 supply, as has been proposed for the chloroplast orientation around the CO_2-containing intercellular air spaces in terrestrial plants (Evans and Loreto, 2000). In terrestrial leaves, CO_2 enters through the stomata and then readily diffuses through intercellular airspaces, finally reaching the chloroplast. The epidermal orientation of the chloroplasts in the aquatic leaves would, from this perspective, indicate the entire epidermal layer to be the principle site of entry for CO_2 under water, with the cuticle layer being the major diffusion barrier. Interestingly, in true aquatic macrophytes, chloroplasts may develop in the epidermal cells (Rascio et al., 1999) even closer to the CO_2 source.

The clustering of chloroplasts in aquatic leaves might have decreased light absorptance (Evans and Vogelmann, 2003), thus causing a slight overestimation of electron transport J_F when the terrestrial chloroplast orientation would be reestablished quickly in air (see equation in ''Materials and Methods''). The partitioning of electrons to photorespiration would then be slightly overestimated, but we consider this effect to be subordinate to the other differences between the two leaf types.

Reduced Diffusion Resistance Results in Decreased Photorespiration

Indications for the occurrence of high photorespiration rates under water have been derived in the past from CO_2 compensation points (Van et al., 1976; Lloyd et al., 1977; Salvucci and Bowes, 1981), combined with in vitro determinations of the activity of enzymes from the photorespiratory and C_4 metabolism pathways (Van et al., 1976; Salvucci and Bowes, 1982, 1983; Spencer et al., 1996). Here, we use gas exchange and chlorophyll fluorescence measurements on plants under water to estimate in vivo V_O and, consequently, R_P, further referred to as photorespiration rate.

The combination of underwater gas exchange and chlorophyll fluorescence measurements showed that photorespiration rates can be very high under water, since the difference between A_g (sum of A_n and R_L) and J_F was up to 80% and 50% of the total electron transport (J_F) in terrestrial and aquatic leaves at 250 μM CO_2, respectively (Fig. 4a). The relative partitioning of electron transport to photorespiration was higher in

terrestrial leaves compared to aquatic leaves under water, and, therefore, we conclude that acclimation to submergence considerably reduces photorespiration. The actual photorespiratory carbon losses in submerged terrestrial leaves, however, will be limited. Respired CO_2 will be refixated immediately in a system where diffusion of CO_2 from the water column into the leaf is rate limiting.

Apparently, the reduced photorespiration in submerged aquatic leaves does not result from reduced O_2 build up, as internal oxygen concentrations in these leaves at external CO_2 concentrations of 250 μM are 60% higher, rather than lower, than in submerged terrestrial leaves of *R. palustris* (Mommer et al., 2004). We therefore suggest that it is the decreased gas diffusion resistance, resulting in much higher C_i, that is responsible for the lower photorespiration rate.

An assumption in these estimates of photorespiration is that the proportion of *J* partitioned to alternative electron sinks is similar in the different conditions. If any, the most likely change in the relative contribution of underwater electron sinks would be an increase of the Mehler ascorbate peroxidase pathway (water-water cycle), reducing O_2 directly at PSI. However, evidence for the Mehler ascorbate peroxidase pathway playing a substantial role in terrestrial plants and algae is controversial (Badger et al., 2000; Foyer and Noctor, 2000; Ort and Baker, 2002). Direct proof is lacking (Asada, 1999; Ruuska et al., 2000; Heber, 2002), and the only measurements that may imply an altered sink under water originate from measurements on drought-acclimated plants (Biehler and Fock, 1996).

Reduced Diffusion Resistance Results in Reduced Excitation Pressure

The excitation pressure of the electron transport system depends on the amount of electrons that cannot be directed to photosynthetic electron transport or heat dissipation (Niyogi, 2000), i.e. high excess energy (*E*; Fig. 5a) or high nonphotochemical quenching (q_N in Table III). The proportion potentially damaging energy was relatively high in the terrestrial leaves at the lowest CO_2 concentrations under water compared to aquatic leaves, since the proportions of photons directed to photosynthetic electron transport (*P*) was low and safe heat dissipation (*D*) was probably operating at maximum capacity. In the aquatic leaves, higher proportions of energy could be used in photosynthesis and photorespiration (high *P*), leaving less energy for *D* or *E*. Acclimation to submergence thus apparently reduced the pressure on the electron transport chain, thereby reducing the potential for photodamage. Although light intensities may be low in the turbid main channel of larger rivers during a natural flooding event (Vervuren et al., 2003), there may still be potential for photodamage to occur in plants such as *R. palustris* that grow at sites of the floodplains where still, clear waters remain for substantial periods and underwater light intensities are high.

Table III. *Chlorophyll fluorescence parameters of terrestrial and aquatic leaves of R. palustris, measured under water and in air at different CO_2 concentrations*

Under water, PPFD was 400 μmol m^{-2} s^{-1} and O_2 concentration 110 to 170 μM (40%–60% of air saturation). In air, PPFD was 1,000 μmol m^{-2} s^{-1} and O_2 pressure 21 kPa. Measurements were performed on leaves of 44-d-old plants. Values are means ± SE; $n = 6$. Data were analyzed with Student's *t* tests. *, $P < 0.05$; ***, $P < 0.001$; n.s., not significant.

Measurement in:	Parameter	Terrestrial Leaves	Aquatic Leaves	*P*
Water	F_v/F_m	0.795 ± 0.013	0.754 ± 0.014	*
250 μM CO_2	q_P	0.381 ± 0.022	0.597 ± 0.032	***
	q_N	0.840 ± 0.015	0.785 ± 0.014	*
2,000 μM CO_2	q_P	0.615 ± 0.021	0.634 ± 0.013	n.s.
	q_N	0.812 ± 0.011	0.663 ± 0.015	***
Air	F_v/F_m	0.821 ± 0.002	0.798 ± 0.005	***
37 Pa CO_2	q_P	0.796 ± 0.009	0.661 ± 0.022	***
	q_N	0.488 ± 0.042	0.752 ± 0.025	***
170 Pa CO_2	q_P	0.789 ± 0.010	0.766 ± 0.008	n.s.
	q_N	0.620 ± 0.027	0.592 ± 0.012	n.s.

Submergence Affects the Ratio between Carboxylation and Electron Transport Capacity

If there has been any biochemical limitation for CO_2 affinity under water, next to the differences in diffusion resistance it will have been in the aquatic rather than the terrestrial leaves, since in the first Rubisco contents were lower (Table II) whereas underwater photosynthesis rates were higher (Fig. 3a). This again shows the limitation of CO_2 supply under water and, thus, also the importance of reduced gas diffusion resistance.

The reduced Rubisco contents in the aquatic compared to the terrestrial leaves of *R. palustris* per se were not surprising (Table II), since this has been observed before in some but not all amphibious plants investigated so far (Farmer et al., 1986; Beer et al., 1991). Novel, however, is the finding that J_{Fmax} was not reduced to the same extent. Submergence increased the ratio between the more conservative estimate of J_{FmaxC} (with the empirically derived J_C/J_F ratio) and Rubisco content 1.3 times, from 37 in terrestrial to 49 in aquatic leaves (Table II). An increase of this ratio has been reported only very recently for drought-stressed plants (Kitao et al., 2003; Bota et al., 2004). We, however, observed a stronger change of this ratio. The two almost opposite stresses have in common that C_i values are low, but it remains uncertain if these responses are convergent.

Conclusion

The orientation of the chloroplasts toward the epidermis in the aquatic leaves of *R. palustris* indicates diffusion of CO_2 through the epidermis and the cuticle layer under water. The decreased thickness and most likely also the decreased resistance of the cuticle in submergence-acclimated leaves reduced the gas

diffusion resistance considerably, resulting in higher underwater assimilation rates at low CO_2 concentrations. Moreover, the reduced gas diffusion resistance also resulted in decreased photorespiration rates and excitation pressures in the submergence-acclimated leaves under water. The biochemical level of the photosynthetic machinery was also affected by acclimation to submergence, as both Rubisco content and electron transport capacity were reduced in the submergence-acclimated leaves. However, these two factors did not change to the same extent, and the data suggest an increase of the ratio between electron transport and carboxylation capacity. Acclimation to submergence thus largely consists of a reduced gas diffusion resistance, leading to a higher C_i and, thus, higher net underwater assimilation rates, a reduced fraction of photorespiration, and reduced excitation pressure on the electron transport chain.

MATERIALS AND METHODS

Plant Material and Growth Conditions

For all experiments and measurements, except those concerning Rubisco content and its relationship with electron transport capacity (see below), *Rumex palustris* Sm. seeds were germinated for 10 d in a petri dish on moistened filter paper at temperatures of 22°C during daytime (PPFD 20 μmol m^{-2} s^{-1}) and 10°C at night. The seedlings were transplanted to pots of 0.3 L, containing a sieved sand/potting soil mixture (1:1, v:v), and grown for another 24 d in a growth chamber (16 h light [200 μmol m^{-2} s^{-1}], 8 h dark, 20°C). The plants were watered once a week with one-quarter-strength Hoagland nutrient solution. To investigate the responses to submergence, one group of plants was completely submerged, whereas the other group was kept drained. The submerged plants, hereafter referred to as plants with "aquatic leaves," were submerged in basins (80 × 60 × 70 cm) filled with tap water, which was circulated with a flow rate of 3 L min^{-1}. Plants of the drained treatment (having "terrestrial leaves") were placed in a similar basin as described above, but without it being filled with water. In both treatments, PPFD was 200 μmol m^{-2} s^{-1}, the day/night cycle was 16/8 h, and the temperature was 20°C. The treatments lasted 10 d, and the plants developed at least two new leaves (six and seventh developed leaves) during the treatments. The apical halves of these new leaves were used in the analyses.

To determine Rubisco content and its proportional relationship with electron transport capacity (J_{Fmax}), younger plants were used than described above because extraction efficiency of Rubisco was low in older plants, possibly due to the high amounts of secondary metabolites in this species. For this experiment, seeds were germinated for 10 d as described above. The seedlings were transplanted to pots of 0.3 L, containing a sieved sand/potting soil mixture (1:1, v:v), and grown for another 17 d (16 h light [150 μmol m^{-2} s^{-1}], 8 h dark, 20°C, and further treated as described above) until they had formed three leaves and the fourth was about to develop. Thereafter, one group of plants was kept drained for another 4 d at the same conditions, whereas the second group was completely submerged in basins of 20 × 15 × 20 cm for 8 d (16 h light [150 μmol m^{-2} s^{-1}], 8 h dark, 20°C). The duration of the growth period previous to the treatment and of the treatment itself were chosen in such a way that plants reached a comparable developmental stage at the end of each treatment, allowing proper evaluation of all parameters on the fully grown fourth leaf.

Leaf Anatomy and Morphology

Leaf anatomy was investigated with light microscopy (LM) and transmission electron microscopy (TEM). Samples were taken from the top of the leaf and fixed immediately in 2% glutaraldehyde in 0.1 M phosphate buffer at pH 7.2 for 2 h at room temperature, followed by postfixation in 1% (w/v) osmium tetroxide in the same buffer for 1 h. Handling of the materials was fast and identical for all samples such that the harvesting and fixing procedure could not differentially affect anatomical features of the samples. The tissue was dehydrated through an ethanol series with steps of 10% and via propylene oxide embedded in Spurr's resin (Spurr, 1969). For LM, sections of 1 μm were stained with toluidine blue (0.1% in 1% borax) and viewed with a Leitz Ortoplan microscope. For TEM, thinner sections (20 nm) were poststained with uranyl acetate and lead citrate according to standard procedures and viewed with a transmission electron microscope (JEOL JEM 100CX II). Thicknesses of the leaves (four counts within sample, $n = 3$), outer cell walls of epidermis cells, and cuticles (20 counts within sample, $n = 2$) were measured with digital software.

Cryo-field emission SEM techniques were used for determination of three dimensions of the leaf surface. Immediately after the plants had been taken out of the treatment, leaf discs, originating from the top of the leaf, were glued onto a stub with colloidal carbon adhesive and frozen in slushy liquid N_2. The samples were transferred in a transfer holder under vacuum into a cryo-preparation chamber of −180°C (Oxford Alto 2500; Gatan). Samples were then freeze-dried for 4 min at −90°C and sputter-coated with 4 nm gold palladium and conveyed under high vacuum to the cold stage of a scanning electron microscope (FESEM-JSM 6330; JEOL).

Other shoot parameters measured included leaf length and width and LMA. LMA was calculated from leaf area (Li-3000; LI-COR) and dry mass (determined after drying for 48 h at 80°C) of the lamina at which measurements were conducted. Nonstructural carbohydrate-free LMA was calculated by subtraction of the mean weight contribution of nonstructural carbohydrates from the overall LMA replicates. Aerenchyma content was measured according to the buoyancy-based method as described in Visser and Bögemann (2003).

Gas Exchange Measurements in Air and Under Water

CO_2 assimilation rates in air ($n = 6$) were measured on terrestrial and aquatic leaves with an open gas exchange measurement system where air was led through leaf chambers. Differences in CO_2 and water partial pressure between air flows entering and leaving the leaf chamber were measured with an infrared gas analyzer (LI-COR L6262). The leaf chambers contained a window (67 × 69 mm) through which the light beam of a lamp mounted in a slide projector was transmitted (for details, see Pons and Welschen, 2002). PPFD at leaf level was 1,000 μmol m^{-2} s^{-1}, which was enough to saturate photosynthesis; leaf temperature was 20°C; and vapor pressure difference between leaf and air was between 0.4 and 0.5 kPa. Submerged plants were taken out of the water 1 h before the measurement started and placed in a plastic bag to maintain turgor of the leaves. Only the measured part of the leaf was released from the bag during the measurement. This treatment did not result in water deficit.

Underwater gas exchange was measured as oxygen evolution in water with different free CO_2 concentrations ($n = 6$). Oxygen release and uptake of squared leaf parts (1 cm^2) were recorded using a Clark-type oxygen electrode positioned at the bottom of a cuvette (Chlorolab 1; Hansatech Instruments; Delieu and Walker, 1972). The cuvette was filled with 1.5 mL of 50 mM MES-BPT buffer, pH 6.5, containing 5 mM $CaCl_2$, 1.5 mM KCl, 1 mM NaCl, and $NaHCO_3$ concentrations ranging from 23 μM to 23 mM (resulting in a free CO_2 concentration of 10 μM to 10 mM, respectively). The CO_2 concentration in the water was calculated from the pH-dependent CO_2-HCO_3^--CO_3^{-2} equilibrium, with pKa values of 6.38 and 10.32, respectively (Mackereth et al., 1978). PPFD under water was 400 μmol m^{-2} s^{-1}. The leaf parts were fixed in the cuvette so that light distribution was homogeneous. Oxygen concentrations were kept between 110 and 170 μM (40%–60% of air saturation) to limit variation in diffusion gradients between the leaf parts and the buffer. A magnet, driven by a custom stirrer unit, provided circulation of the buffer in the cuvette, thereby minimizing boundary layers. A cylinder around the cuvette that contained circulating thermostatted water using a thermostatic water bath (Haake DC 50) maintained the water temperature at 20°C.

Measurement of Chlorophyll Fluorescence in Combination with Gas Exchange

Photorespiration rates were estimated in terrestrial and aquatic leaves by combining gas exchange measurements with chlorophyll *a* fluorescence measurements. The measurements in air were performed with the aerial gas exchange system as described above, except that modified Parkinson cuvettes were used (ø 18 mm; PP Systems; Pons and Welschen, 2002). Holders for the fiber optics of a portable PAM2000 chlorophyll fluorometer (Walz GmbH)

were placed on these chambers, without shading the leaf (Pons and Welschen, 2003). Measurements were performed at two CO_2 concentrations (ambient and saturating, 37 and 170 Pa CO_2, respectively) and two O_2 concentrations (ambient [21 kPa] and 1 kPa O_2; $n = 6$). R_D and F_v/F_m were measured after 30 min in the dark. The saturating pulse and measuring light were maximal for determining maximum fluorescence (F_m) and revealed appropriate signals, also for minimal fluorescence (F_0). Net assimilation rates accompanying quantum yield measurements ($\Delta F/F_m'$) were recorded after 30 min in saturating light (PPFD 1,000 μmol m^{-2} s^{-1}) and replicated three times with 5-min intervals and averaged for each CO_2 concentration. Minimum fluorescence yield after induction (F_0') was determined after a far-red light pulse following immediately after the light was switched off.

After these aerial measurements, the plants were returned to their respective treatment basin overnight. Underwater gas exchange and chlorophyll fluorescence measurements were performed on the same leaves the next morning.

For these underwater measurements, the fluorescence fiber probe was inserted in the water cylinder of the Chlorolab1 cuvette, under such an angle that the light beam of the projector was not impeded. The measurements under water were again performed on leaf parts (1 cm^2), following the same protocol as described above. CO_2 concentrations were 250 and 2,000 μM, which have been shown before to be distinctive for the CO_2 response of the leaf types (Mommer et al., 2004). PPFD under water was 400 μmol m^{-2} s^{-1} since higher PPFD resulted in inaccurate $\Delta F/F_m'$ in the terrestrial leaves at the lowest CO_2 concentrations. R_D measurements were performed at an oxygen concentration of 230 μM (80% of air saturation), which does not limit respiration in Rumex species (Laan et al., 1990).

Calculations and Assumptions

Electron transport rate based on aerial gas exchange measurements (J_C) was calculated from measurements at low O_2 (1 kPa) and high CO_2 (170 Pa), where photorespiration is negligible. We assumed that four electrons are required per assimilated CO_2, so that J_C is equal to 4 times A_g (von Caemmerer, 2000). A_g was calculated from A_n and R_D, assuming that mitochondrial respiration in light is not different from R_D.

Electron transport rate was also calculated from chlorophyll fluorescence measurements (J_F) following (Genty et al., 1989):

$$J_F = 0.5 \times \text{abs} \times \text{PPFD} \times \Delta F/F_m',$$

where 0.5 is a factor that accounts for partitioning of excitation energy between PSI and PSII (Genty et al., 1989). Leaf absorptance (abs) was calculated from chlorophyll concentrations (Table I or II, depending on the experiment) according to Evans and Poorter (2001). This resulted in absorption coefficients of 0.79 and 0.76 for terrestrial and aquatic leaves of the 44-d-old plants and 0.82 and 0.79 for terrestrial and aquatic leaves of the younger set of plants, respectively.

J_F and J_C were compared under nonphotorespiratory conditions of low O_2 (1 kPa) and high CO_2 (170 Pa) in air only, because internal CO_2 and O_2 concentrations could not be sufficiently controlled in water. The ratio J_C/J_F was smaller than unity in both leaf types, 0.72 ± 0.04 and 0.73 ± 0.05 for terrestrial and aquatic leaves of 44-d-old plants and 0.71 ± 0.02 and 0.78 ± 0.02 for terrestrial and aquatic leaves of the younger set of plants, respectively. The J_F values measured at ambient O_2 concentrations in air and those measured in water were multiplied by this empirically obtained ratio according to Epron et al. (1995) and Pons and Welschen (2003). The thus corrected J_F values represent electron transport associated with CO_2 assimilation, assuming that the processes causing the $J_F - J_C$ difference remain constant between measurement conditions. Such processes could be unequal partitioning of excitation energy between the two photosystems or the activity of alternative electron acceptors or others, but that was not investigated in this study. We used for each replicate plant the corresponding J_C/J_F ratio.

Electron transport capacity (J_{Fmax}) was obtained by aerial gas exchange measurements at saturating CO_2 concentrations (170 Pa) and ambient O_2 (21 kPa). Results for J_{Fmax} in the experiments concerning Rubisco content and its proportional relationship with electron transport capacity (J_{Fmax}) are shown with and without the use of the J_C/J_F ratio. To show that terrestrial leaves of *R. palustris* have a normal ratio of electron transport capacity (J_{max}) over V_{Cmax}, these parameters were also calculated from the photosynthesis (A_n)-intercellular CO_2 (C_i) relationship (von Caemmerer, 2000). The parameters needed for this calculation (CO_2 compensation point in the absence of respiration [Γ*] and Michaelis-Menten constants for Rubisco) originate from von Caemmerer et al. (1994) and are corrected for temperature (20°C) and atmospheric pressure (101.3 kPa).

Measurements of J_F, A_n, and respiration rate (R_D) were used to estimate the partitioning of electrons over carboxylation (V_C), oxygenation (V_O), and photorespiration (R_P). The difference between electron transport rate ($J_F/4 = V_C + V_O$) and gross photosynthesis rate ($A_g = A_n + R_D$) was used as an estimate of electron transport involved in oxygenation (V_O) and, consequently, R_P. Since $A_g = V_C - 0.5\,V_O$, where $0.5\,V_O = R_P$, the $J_F/4 - A_g$ difference was considered to represent $1.5\,V_O$ (von Caemmerer, 2000).

q_P and q_N were calculated from chlorophyll fluorescence measurements (van Kooten and Snel, 1990). Partitioning of absorbed photons in PSII was calculated by dividing total absorbed energy over inherent inefficiency in dark ($L = 1 - F_v/F_m$), thermal dissipation ($D = F_v/F_m - F_v'/F_m'$), photosynthetic electron transport ($P = \Delta F/F_m'$), and excess energy ($E = 1 - L - D - P$; Demmig-Adams et al., 1996; Kato et al., 2003).

Biochemical Analyses

Chlorophyll content was determined spectrophotometrically (UV1250; Shimadzu) after extraction of the chlorophyll pigments with dimethylformamide for 7 d in the dark at 4°C in samples of the leaves on which photosynthetic measurements were performed. Equations of Inskeep and Bloom (1985) were used to calculate the chlorophyll *a* and *b* pigment concentrations.

Determination of carbohydrate content of the leaves was based on the assay described by Colmer et al. (2001). Leaves were harvested between 9 and 10 AM (i.e. 3 to 4 h after the light had switched on), quickly frozen in N_2, and stored in a freezer at −80°C until further processing. After freeze-drying, 10 mg of fine-cut material was used to measure nonstructural carbohydrates. All soluble sugars (hexose units) were extracted in 4 mL of ethanol (80%) during 30 min in a shaking water bath at 80°C, and this step was repeated with 2 mL of ethanol after centrifugation. Insoluble sugars (starch) were analyzed by boiling the residue in 3.5 mL of demineralized water for 3 h and then incubating the solution for 24 h at 37°C with 1 mL of Na^+ acetate buffer containing amyloglucosidase (75 U mg^{-1}, EC 3.2.1.3), pH 4.5. Both soluble sugars in the ethanol extraction and the Glc released from the hydrolysis of starch were measured with a Shimadzu UV1250 spectrophotometer using anthrone as a color reagent (Yem and Willis, 1954).

For soluble protein and Rubisco analysis, frozen samples of approximately 6 cm^2 were ground in Eppendorf tubes in an extraction buffer containing 200 mM Tris, pH 7.8, 20 mM $MgCl_2$, 150 μM $NaCO_3$, 20% glycerol, 1 mM EDTA, 10 μM dithiothreitol, 0.5% Triton X-100, 8 mM amino-*n*-caproic acid, 1.6 mM benzamidine, and 3% (w/v) polyvinylpolypyrrolidone. Samples were centrifuged at 14,000*g* for 10 min at 4°C and the pellet was discarded. Salt solutions were added to the supernatant to create a final concentration of 10 mM $NaHCO_3$ and 20 mM $MgCl_2$. Protein samples were run on SDS-PAGE gels (17%) for 4 h at 100 V (Westbeek et al., 1999). Five bovine serum albumin samples with different concentrations were also loaded on the gels, serving as a calibration series. After staining the gels with Coomassie Brilliant Blue for 30 min and subsequent destaining overnight, gels were scanned using custom-built image analysis. Rubisco content was calculated from the large subunit (molecular mass of 55 kD). Total soluble protein content was measured using the Bradford reagents (Bradford, 1976).

Data Analysis

Differences between leaf types were analyzed with Student's *t* tests. Levene's test was used to check the homogeneity of variances. Ln-transformation was applied if deviation from homogeneity of variance was found.

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