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C_4 photosynthetic modifications in the evolutionary transition from land to water in aquatic grasses

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Abstract Cladistic analysis supports the conclusion that the Orcuttieae tribe of C_4 grasses reflect evolution from a terrestrial ancestry into seasonal pools. All nine species in the tribe exhibit adaptations to the aquatic environment, evident in the structural characteristics of the juvenile foliage, which persist submerged for 1–3 months prior to metamorphosis to the terrestrial foliage. Aquatic leaves of the least derived or basal genus *Neostapfia* have few morphological and anatomical characteristics specialized to the aquatic environment and have retained full expression of the C_4 pathway, including Kranz anatomy. *Orcuttia* species have many derived characteristics and are more specialized to the aquatic environment. These latter species germinate earlier in the season and persist in the submerged stage longer than *Neostapfia* and evidence from the literature indicates length of submergence is positively correlated with fitness components. Aquatic leaves of *Orcuttia* species lack Kranz or PCR bundle sheath anatomy, yet ^{14}C-pulse chase studies indicate >95% malate + aspartate as the initial products of photosynthesis and these products turn over rapidly to phosphorylated sugars, indicating a tight coupling of the C_4 and C_3 cycles. Presence of the C_4 pathway is further supported by enzymological data. Contemporary dogma that Kranz anatomy is a *sine qua non* for operation of the C_4 pathway is contradicted by the patterns in *Orcuttia*; however, it is unknown whether the pathway acts as a CO_2 concentrating mechanism in these aquatic plants.

Key words C_4 grasses · Orcuttiae · Kranz anatomy · Aquatic grasses · Cladistic analysis

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Introduction

Comparative biology places structural and functional relationships in a phylogenetic context, thus providing insights into the relationship between physiology, anatomy, ecology and evolution (Monson 1996). An area well suited to this approach is the study of photosynthetic pathways in plants, as great strides have been made in understanding the role of C_3, C_4, and CAM photosynthesis in the ecological distribution of plants (Ehleringer and Monson 1993). Clades comprising species of diverse habitats should exhibit interesting patterns of photosynthetic diversification (Moore 1982), and those illustrating the evolutionary transition from land to water may be particularly promising. Here I present a study of photosynthetic pathways in a small tribe of C_4 grasses that is clearly rooted in a terrestrial ancestry but has radiated into an amphibious environment, where part of the life cycle is spent as a submerged aquatic.

Orcuttieae is a tribe of Poaceae comprising nine annual species endemic to shallow rain-filled seasonal pools in the mediterranean-climate region of the southwestern United States (Stone et al. 1988). All are rare and most are restricted to California "vernal pools" and are government-listed as endangered or threatened (Skinner and Pavlik 1994). Germination occurs in spring while seeds are submerged, and in some species germination is cued by hypoxic conditions (Griggs 1976; Keeley 1988). Indeed, in years when precipitation is insufficient to fill pool basins, seeds remain dormant (Crampton 1959; Holland 1987). These grasses seldom establish above the high water mark, suggesting they are unable to compete with upland grasses. Inundation eliminates upland species and this ecological release is evident in the fact that one or more of these Orcuttieae grasses often dominate pool basins for a month or more after pools dry. In order to capitalize on these ephemeral growing conditions, germination is initiated under water and plants establish as submerged aquatics for 1–3 months.

The juvenile foliage that develops underwater is replaced by terrestrial foliage upon pool dry-down in late spring. The latter leaves have Kranz anatomy, indicating C_4 photosynthesis, which likely contributes to these grasses surviving well into the summer drought, often reaching anthesis in summer on the dry, cracked substrates in the pool basins. These grasses are of interest because they spend a significant part of their life cycle as submerged aquatics, an unusual habitat for C_4 plants (Ehleringer and Monson 1993). Also, other studies of amphibious species have shown switches in photosynthetic pathways, for example, during the transition from an aquatic to a terrestrial existence there is a switch from CAM to C_3 in *Isoetes howellii* (Keeley 1996) and a switch from C_3 to C_4 in *Eleocharis vivipara* (Ueno et al. 1988).

The purpose of this study was to investigate the morphological, anatomical, physiological, and biochemical characteristics of species representative of the three Orcuttieae genera, *Neostapfia*, *Tuctoria*, and *Orcuttia*. The primary focus was to contrast the juvenile aquatic leaves and the adult terrestrial leaves, and in particular, the development of Kranz anatomy and operation of the C_4 pathway. Coupling these data with a phylogenetic analysis illustrates the structural and functional trade-offs in the transition from land to water.

Methods

Leaf morphology, anatomy and habitat specialization

Collections of seeds and substrate from several vernal pools in California included *Neostapfia colusana*, *Tuctoria greenei*, *Orcuttia viscida*, and *O. californica* (nomenclature according to Reeder 1982). These plants were grown outdoors in Los Angeles by submerging seeds and substrate in deionized water in December. Pools were maintained with deionized H_2O (plus natural precipitation) through spring and allowed to begin drying in late April. Studies reported here used fresh leaf material from these artificial vernal pools, except stable carbon isotope measurements for other species in the tribe were either collected in the field and dried or were from herbarium specimens.

Fresh leaves from aquatic and terrestrial foliage were cut in small sections in the fixative 2% paraformaldehyde-3% glutaraldehyde in 25 mmol l^{-1} potassium phosphate buffer, followed by a change to fresh fixative after 1 h, and left overnight at 4°C. Tissue was rinsed in buffer, post-fixed in 1% aqueous OsO_4 for 2 h, then rinsed in 50% ethanol and dehydrated in a graded ethanol series. Leaf samples were infiltrated overnight at 70°C in Spurr's resin followed by dehydration through an ethanol series and embedded in Spurr's resin. Sections 10 μm thick were cut with an ultra-microtome (Sorvall MT2-B) and stained with 1% toluidine or methylene blue, or iodine. Light microscopy was used to determine presence of Kranz anatomy and stomata, chloroplast position in the bundle sheath cells, chloroplast shape, type of bundle sheath outline and interveinal count of mesophyll cells separating two bundle sheaths. A computer planimeter digitizer was used for cross-sectional area and perimeter, interveinal area, airspace area, plus density, size and perimeter of mesophyll cells, epidermal cells, and vascular bundles, parameters of particular interest in studies of C_4 anatomy (Dengler et al. 1994). All measurements presented are the mean of $n = 3$. Also, samples treated as described above were viewed in a Hitachi HU 11 A transmission electron microscope.

^{14}C pulse-chase studies

Fresh leaf samples were cut to *c.* 4–6 cm and the cut end placed in a water-filled plastic pipette tip and sealed with putty. Aquatic leaf samples were immersed in a 25-ml serum vial filled with 25 mmol l^{-1} MES buffer (pH 6.0) (this and all other solutions were made fresh daily with H_2O purified through a Barnstead Nanopure II filtration system). Terrestrial leaf samples were placed in a high humidity chamber and both types of samples were kept in the light for 15 min prior to the experiment. Irradiance during the incubation, pulse, and chase was 500–700 μmol m^{-2} s^{-1}. The pulse was initiated by injecting 5.5 Mbq of ^{14}C-sodium bicarbonate (2 Gbq $mmol^{-1}$) in the buffer solution with aquatic leaves or into 1 ml of 1.0 mol l^{-1} HCl in the bottom of a 25-ml serum vial with terrestrial leaf samples suspended above. Aquatic leaf samples were given a 5-s pulse and either killed or chased by washing with buffer and then placing in fresh buffer for 15, 60, 180 or 300 s. Terrestrial leaves were pulsed for 2 s and chases were done in a ^{14}C-free atmosphere. All samples were killed by immersing in liquid nitrogen. This pulse-chase experiment was repeated three times for each species.

Samples were ground in a Ten Broech glass grinder with 10% acetic acid in ethanol (95%) and centrifuged at 116.4 km s^{-1} for 10 min. After decanting the supernatant, the pellet was re-suspended in 95% ethanol and centrifuged and the procedure repeated once more with a water wash of the pellet. The first supernatant was combined with the two washes and placed in a glass beaker in front of a bright light until photo bleached. The supernatant and pellet samples were dried down at 80°C and re-suspended in 1.0 ml of distilled water. The supernatant was centrifuged in a microcentrifuge tube for 10 min at low speed on a table top centrifuge and then decanted into a "Microtainer" serum separator tube and centrifuged again at 116.4 km s^{-1} for 10 min. A 0.1-ml aliquot was removed and placed in 4.0 ml Bray's solution for ^{14}C counting on a LKB 1214 Rackbeta scintillation counter, and the remainder of the sample was retained for separation. An aliquot of suspended pellet was likewise taken for scintillation counting and the remainder of the pellet discarded.

Two-dimensional separation was on 20 × 20 cm thin layer cellulose plates with a combination of electrophoresis followed by chromatography (Schürman 1969). Approximately 5,000–10,000 DPM (usually 25–50 μl sample) were slowly spotted in one corner of the plate and placed on cooling plates maintained at 15°C in an LKB Multiphor II unit. Electrophoretic separation along the first dimension was at 900 V and 70–75 mA for 50 min with pyridine: glacial acetic acid: water (2:9:189) solvent. After drying over night, separation along the second axis was in upright enclosed chromatography chambers with a sec-butanol:formic acid:water (6:1:2) solvent. After the front had migrated 8 cm above the first axis, plates were dried overnight and then run a second time with fresh solvent to a height of 15 cm.

Autoradiographs were made by incubating 20 × 25 cm film sheets (Kodak X-OMAT) held against the plates in the dark for 1–2 weeks. After developing, the negatives were used to make a tracing paper paradigm of radioactive spots on the plate. Identification of these spots was made by comparing coordinates with a catalogue of standards run in our laboratory under identical conditions. The radioactivity in each spot was determined by scraping the cellulose into a vial with Bray's solution and scintillation counting was made against an automatic quench curve for the cellulose. Percentage distribution of label was obtained by dividing these counts by the total counts taken from the plate, with the counts from the pellet factored in.

Enzyme assays

For all assays, fresh leaf material was ground with the appropriate buffer in a glass grinder kept on ice and centrifuged for 5 min at 116.4 km s^{-1} and 4°C and assayed immediately (at 25°C) without further purification. Aliquots for chlorophyll determination (Sestak et al. 1971) were stored for up to 30 min on ice in the dark. Activities presented are the mean of $n = 3$ assays.

RuBP carboxylase and PEP carboxylase were assayed from the same extract of leaves ground in 50 mmol l^{-1} TRIS-HCl, 10 mmol L^{-1} $MgCl_2$, 0.1 mmol l^{-1} EDTA, 5 mmol l^{-1} isoascorbate, 1% w/v PVP-400 at pH 8.0. Both enzymes were assayed using $^{14}CO_2$ fixation techniques and liquid scintillation counting of acid-stable products. RuBP carboxylase was assayed in the active form as described by Lorimer et al. (1977). Experiments were initiated by addition of RuBP substrate and terminated after 1 min by the addition of 6 mol l^{-1} HCl. PEP carboxylase was assayed according to the procedure of Van et al. (1976) with a 2-min incubation. Experiments were initiated by addition of PEP substrate and terminated by addition of 6 mol l^{-1} HCl saturated with 2,4-dinitrophenylhydrazine, which was required to stabilize OAA and made it necessary to use a separate quench curve.

Other important C_4 enzymes were assayed spectrophotometrically. NAD- and NADP- malic enzyme were assayed according to the procedure of Hatch and Kagawa (1974) as modified by Ueno et al. (1986). The ATP-dependent decarboxylating reaction of PEP carboxykinase was measured according to Hatch (1973) with modifications by Ueno et al. (1986). Activities of aspartate and alanine aminotransferases were determined by the procedure of Hatch and Mau (1973). Pyruvate, P_i dikinase activity was determined after Ashton et al. (1990).

For comparison, assays were run on well-known species either grown from seed (*Zea mays*), purchased as fresh leaves from the grocery (*Spinacia oleracea*), or as potted plants from a nursery (*Ananas comosus, Kalanchoe daigremontiana*).

$^{13}C/^{12}C$ isotopes

Plant samples were dried at 70°C and ground before mass spectrometer analysis in laboratories at either UCLA or University of Utah. $\delta^{13}C$ isotope ratios were expressed relative to the Pee Dee Belemnite standard and were transformed to discrimination (Δ) values as

$$\Delta = (\delta_m + \delta_p)/(1 + \delta_p),$$

where δ_p is the measured isotope ratio for leaf samples and δ_m is the isotope ratio for the growing medium; for terrestrial leaves this was assumed to be $-8‰$ for the atmosphere (Farquhar et al. 1989) and for aquatic leaves it was measured for water collected at the time of leaf collection. Water samples were collected by syringe and injected into vacuum tubes and stored in the refrigerator until sampled (Osmond et al. 1981; Keeley and Sandquist 1992). CO_2 gas was isolated according to the procedure by Games and Hayes (1976). Terrestrial leaves were based on a single sample and the aquatic leaves and water samples represent the mean of three samples each.

Phylogenetic analysis

Twenty-four characters based on vegetative, floral, embryonic and chromosomal data (Reeder 1982; Watson and Dallwitz 1992), were used to evaluate the phylogenetic relationships within the tribe. Character polarity was determined by the outgroup comparison method (Humphries and Funk 1984). Traits were assigned a ranking of presence or absence and this data matrix was used by PAUP Version 3.1.1 (Swofford 1993) to provide phylogenetic placement of the Orcuttieae genera.

Results

Leaf morphology and habit

Seed and natural substrate were submerged in December and *Orcuttia viscida* and *O. californica* germinated in late January, followed by *Tuctoria greenei* in February and by *Neostapfia colusana* in March. Thus, duration of leaf inundation ranged from less than 1 month for *Neostapfia* to 3 months for *Orcuttia*. In all species, juvenile leaves produced underwater were terete and basal. *Neostapfia* produced only a couple such leaves whereas *Orcuttia* species produced a distinct "isoetid"-like rosette of five to eight basal leaves, and *Tuctoria* was intermediate in leaf number. After approximately a month of submergence, both *Orcuttia* species initiated basal leaves that elongated and, upon reaching the surface, changed from terete to laminate and floated on the water surface. Upon pool drying all species underwent a metamorphosis (Fig. 1), juvenile foliage withered and was replaced by the terrestrial foliage with laminate caulescent leaves.

Leaf anatomy – terrestrial

N. colusana, T. greenei, O. viscida, and O. californica were laminate and amphistomatous with well-developed Kranz anatomy of the XyMs+ type and centrifugal chloroplasts (Fig. 2). Starch was restricted to these

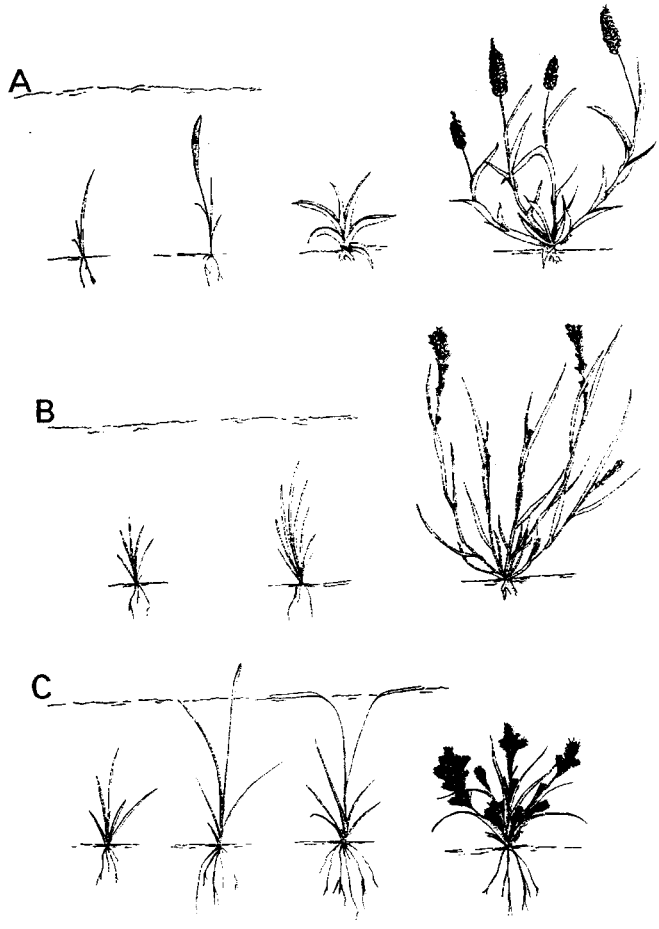

Fig. 1 Stages of phenological development in the aquatic and terrestrial foliage of **A** *Neostapfia colusana*, **B** *Tuctoria greenei*, and **C** *Orcuttia viscida* (Drawing by Melanie Baer-Keeley.)

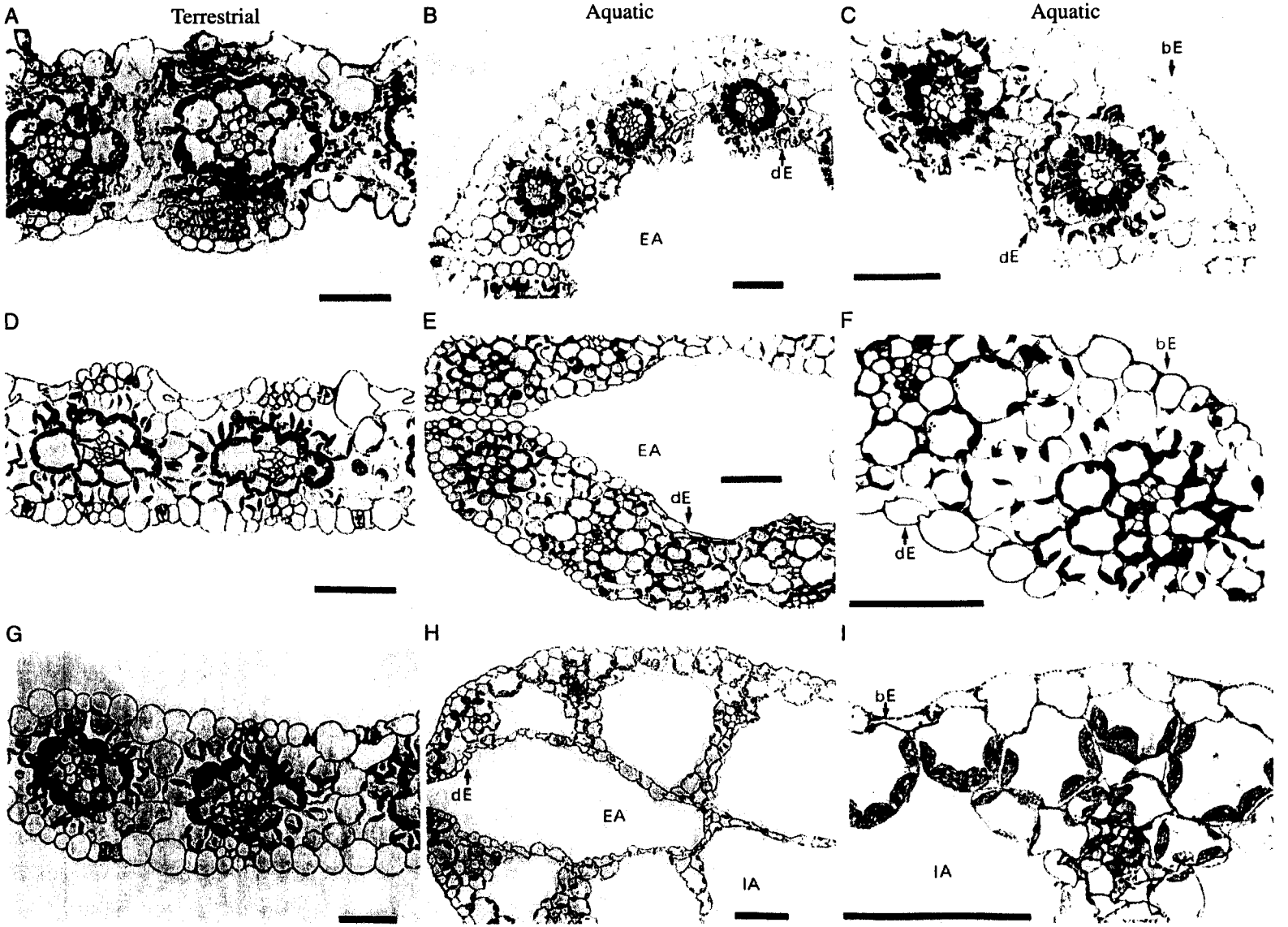

Fig. 2 Transverse sections of terrestrial and aquatic leaves of **A, B, C** *N. colusana*, **D, E, F** *T. greenei* and **G, H, I** *O. viscida* (*bar* = 50 μm, *EA* extra-cellular airspace, *IA* intra-celulllar airspace, *bE* abaxial epidermis, *dE* adaxial epidermis). Folding of aquatic leaves (**D–I**) was due to a greater proliferation of cells on the abaxial than on the adaxial surface; ratios of abaxial/adaxial perimeter for aquatic leaves were 39% in *Orcuttia*, 69% in *Tuctoria*, and 82% in *Neostapfia*, vs. 90–92% in terrestrial leaves

bundle sheath cell chloroplasts, indicating that this was the site of the photosynthetic carbon reduction (PCR) cycle. Stomata were equally abundant on both adaxial and abaxial surfaces in all species.

Leaf anatomy – aquatic

Juvenile aquatic leaves were distinct from terrestrial laminate leaves in forming a terete leaf by folding of the leaf so the margins were nearly touching (Fig. 2).

Extensive lacunae were evident in aquatic *Orcuttia* leaves, resulting in 75% of cross-sectional area as air space, but lacunae were not present in the other two genera and air space was only 10–11% (Fig. 2). Stomata were absent from *O. viscida* and *O. californica*, present only on the adaxial surface of *Neostapfia*, and, although present on both surfaces in *Tuctoria*, density was lower than in terrestrial leaves (Fig. 2).

Kranz anatomy was well developed in both aquatic and terrestrial leaves of *Neostapfia*, although there were major structural differences. Aquatic leaves had bundle sheath cells perpendicular to the veins with elongate centripetal bundle sheath chloroplasts (Fig. 2) and, as evident under TEM, were lacking grana (Fig. 3); in contrast the terrestrial leaves had parallel bundle sheath cells with somewhat oval centrifugal chloroplasts (Fig. 2) and well-developed grana (Fig. 3). Additionally, the outer walls of bundle sheath cells were about 2.25 times thicker in terrestrial than in aquatic leaves. *Tuctoria* also had Kranz-type bundle sheath anatomy in the aquatic leaves, although the contrast in chloroplast density between bundle sheath cells and mesophyll cells was markedly less than in terrestrial leaves (Fig. 2).

Kranz anatomy was absent from the aquatic leaves of *O. viscida* and *O. californica* and chloroplasts were concentrated in the outer mesophyll cells, on the inside wall or the centripetal position (Fig. 2) and were sites of starch production. Relative to terrestrial leaves, there was a significant ($P < 0.01, n = 30$, with 2-tailed t-test) reduction in the vascular bundle area and size of epidermal cells. Aquatic *Orcuttia* had five to seven cells between vascular bundles, in contrast to one to three for aquatic *Neostapfia* or *Tuctoria*, or terrestrial leaves of all species.

Floating leaves (not shown) of both *Orcuttia* species lacked lacunae and were laminate with well developed Kranz anatomy, with abundant adaxial stomata.

Photosynthetic pathway

Pulse-chase radioisotope experiments indicate operation of the C_4 pathway in terrestrial and aquatic leaves of all species (Fig. 4); the preponderance of carbon is initially fixed into the organic acids malate and aspartate and these turn over rapidly into phosphorylated sugars and other products such as starch. In all terrestrial leaves and aquatic leaves of *Neostapfia* and *Orcuttia*, 95–100% of the initial carbon fixation products were C_4 products, whereas this was slightly lower for aquatic *Tuctoria*. Comparing *Neostapfia*, *Tuctoria* and *Orcuttia* there appears to be a trend of increasing importance of aspartate as a primary photosynthetic product. The rapid turnover of organic acids, coupled with an increase in phosphorylated compounds, indicates transfer of carbon from the C_4 to the C_3 cycle and points to the presence of a functional C_4 pathway in both aquatic and terrestrial leaves.

Fig. 3 Ultra structure of bundle sheath cell chloroplasts from **A** aquatic and **B** terrestrial leaves of *Neostapfia colusana* (bar = 1 μm)

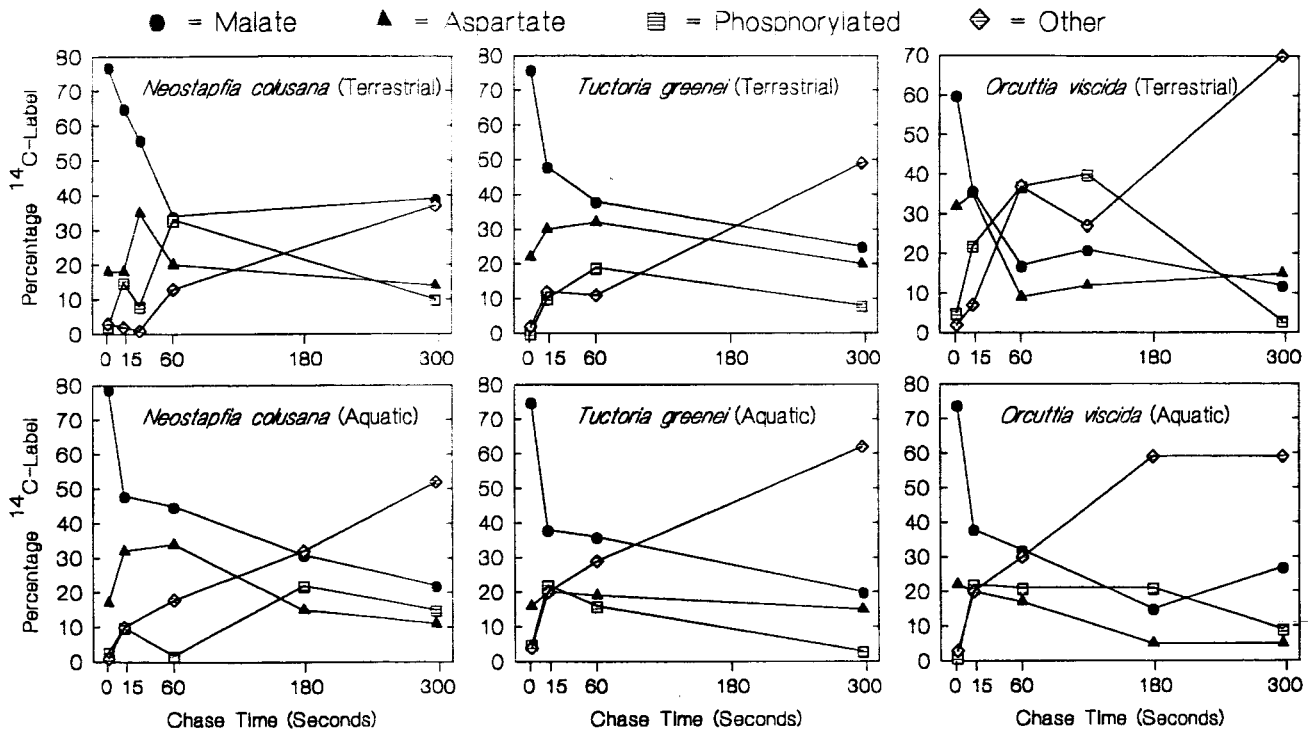

Fig. 4 Pulse-chase experiments in the light for *N. colusana*, *T. greenei*, and *O. viscida* ($n = 3$). Patterns were nearly identical between aquatic *O. viscida* and *O. californica* (not shown)

Presence of the C_4 pathway is particularly surprising for aquatic *O. viscida* and *O. californica*, both of which lack Kranz (PCR bundle sheath) anatomy (Fig. 2).

Photosynthetic enzymes

Substantial RuBP carboxylase and PEP carboxylase activity was recorded from both terrestrial and aquatic leaves of all three species (Table 1). The ratio of RUBISCO:PEP carboxylase activity in terrestrial leaves was comparable between *Neostapfia* and corn and was slightly higher for *Tuctoria* and higher still for *Orcuttia*. Aquatic leaves exhibited a similar RUBISCO:PEP carboxylase pattern: corn < *Neostapfia* < *Tuctoria* < *Orcuttia*, although aquatic *Orcuttia*, with a ratio of 5.3, was still substantially lower than a typical C_3 plant such as spinach with 16.0 (Table 1). The floating leaves of *Orcuttia* (not shown) had a ratio of 5.0, similar to the submerged foliage.

One of the three decarboxylating enzymes, PEP carboxykinase, was not detectable (Table 1). *Neostapfia colusana* had comparable levels of both NAD- and NADP-malic enzyme, but, the other two species had substantially greater NADP-malic enzyme activity. Presence of both malate and aspartate in the initial C_4 assimilation products is consistent with the presence of both decarboxylases. Relative to corn, both aspartate and alanine aminotransferase activities were substantial in terrestrial and aquatic leaves of all three species (Table 1). Pyruvate, P_i dikinase is required for the C_4 pathway, but typically absent from C_3 plants such as spinach (Table 1). Activity of this enzyme was similar in both terrestrial and aquatic leaves of *Neostapfia*, *Tuctoria*, and *Orcuttia*.

$^{13}C/^{12}C$ isotopes

Terrestrial foliage of all species in the Orcuttieae had $\delta^{13}C$ values between −11.4 and −13.9 (‰), within the range of C_4 plants and, since it was assumed they were in similar atmospheres, discrimination values (Δ) were comparable (Table 2). Aquatic foliage $\delta^{13}C$ ranged from −14.8 for *Neostapfia* to −19.1 (‰) for *Orcuttia*; however, discrimination values were highest for *Tuctoria*, and all aquatic leaf Δ values were more similar to terrestrial C_4 than C_3 plants (Table 2).

Phylogenetic analysis

Three genera are recognized in the Orcuttieae tribe, the monotypic *Neostapfia*, three species of *Tuctoria* (two are narrow endemics restricted to a single pool), and five *Orcuttia* species (Reeder 1982). Placement within the Poaceae has been problematical due to the lack of any clear alliances with other genera (Reeder 1965; Renvoize and Clayton 1992). It is one of four tribes in the Chloridoideae, a subfamily of approximately 1400 species, apparently all but one having C_4 photosynthesis (Watson and Dallwitz 1992), suggesting Orcuttieae was likely derived from a C_4 ancestor. Within this subfamily it

Table 1 Activities of carboxylases RuBP carboxylase (RUBISCO) and PEP carboxylase, decarboxylases NAD^+- and NADP-malic enzyme and PEP carboxykinase, and two other important C_4 enzymes in representative species of the three Orcuttieae genera for aquatic and terrestrial leaves. To compare crude extract assays in our lab with other reports, at least one well-documented species was included as a control for each enzyme assay. For all enzymes $n = 3$ (*nd* not detectable, *nt* not tested)

Species	(μmol mg^{-1} Chl h^{-1})								
	RUBISCO	PEP carboboxylase	RUBISCO: PEP carboxylase	Malic Enzyme		PEP carboxy-kinase	Aminotransferase		Pyruvate, P_i di-kinase
				NAD^+	NADP		Aspartate	Alanine	
Neostapfia colusana									
Aquatic	583	434	1.3	10	25	nd*	1342	4278	221
Terrestrial	458	668	0.7	63	60	nd	1874	2496	138
Tuctoria greenii									
Aquatic	288	117	2.5	6	25	nd	781	1242	106
Terrestrial	374	230	0.7	9	63	nd	2113	6217	99
Orcuttia viscida									
Aquatic	285	54	5.3	8	78	nd	765	1536	134
Terrestrial	339	179	1.9	7	78	nd	1253	4084	181
O. californica									
Aquatic	183	34	5.4	4	47	nd	175	317	67
Terrestrial	299	157	1.9	8	41	nd	890	3407	160
Zea mays									
(Corn, C_4)	462	842	0.5	nt	–	–	643	421	289
Spinacia oleracea									
(Spinach, C_3)	865	54	16.0	–	–	–	–	–	nd
Ananas comosus									
(Pineapple, CAM)	–	–	–	–	83	908	–	–	–
Kalanchoe daigremontiana									
(CAM)	–	–	–	96	73	–	–	–	–

Table 2 Stable carbon isotope ratios in terrestrial and aquatic leaves

Species	Terrestrial leaves		Aquatic leaves	
	$\delta^{13}C$ (‰)	Δ (‰)	$\delta^{13}C$ (‰)	Δ (‰)
Neostapfia colusana	−13.7	5.8	−14.8	8.6
Tuctoria greenei	−13.4	5.5	−18.2	9.2
T. fragilis	−12.5	4.6	–	–
T. mucronata	−13.3	5.4	–	–
Orcuttia californica	−13.9	6.0	–	–
O. inaequalis	−11.4	3.4	–	–
O. pilosa	−13.4	4.6	–	–
O. tenuis	−12.3	4.4	–	–
O. viscida	−12.9	4.9	−19.1	4.9
Spinacia oleracea[a]	−30.1	22.8	–	–

[a] Spinach is a C_3 plant

is most closely aligned with the Chlorideae, a tribe comprising 151 genera.

Monophyly for Orcuttieae genera is supported by the absence of a ligule, a synapomorphy shared by all Orcuttieae (Reeder 1982) and a characteristic not found in the rest of the subfamily Chloridoideae or most all Poaceae (Watson and Dallwitz 1992). Outgroup selection is problematical due to the wide separation between Orcuttieae genera and other genera in the Chlorideae. *Distichlis* and *Eragrostis* have been suggested as candidates for the sister group (J. Travis Columbus, Rancho Santa Ana Botanical Garden, Claremont, California). The former was selected because of its extremely reduced ligule, wide representation in the Americas, and propensity for open habitats and seashores (Watson and Dallwitz 1992), although conclusions would be unchanged if *Eragrostis* were chosen (data not shown).

Character polarity for cladistic analysis is shown in Table 3. Exhaustive tree searches using maximum parsimony analyses generated three trees. The most parsimonious was obtained in 25 steps and showed *Neostapfia* basal to the family (Fig. 5). This conclusion was supported 100% by bootstrap analysis with $n = 1000$.

Aquatic specialization

The juvenile foliage of each of the Orcuttieae genera was scored for characters commonly modified by aquatic environments (Sculthorpe 1967). Seedlings and juvenile *Orcuttia* exhibited far greater specialization to the aquatic environment than *Tuctoria* or *Neostapfia* (Table 4).

Discussion

Adaptive value of C_4 photosynthesis in the terrestrial stage

In these vernal pools, C_4 grasses comprise 63% of the grass flora, in contrast to 9% in the adjacent grasslands (J. Keeley, unpublished work). High incidence of C_4

Table 3 Character state polarity for cladistic analysis. Despite their potential phylogenetic value, characters used to construct the aquatic index (Table 4) were excluded from the cladistic analysis

	Distichlis	*Neostapfia*	*Tuctoria*	*Orcuttia*
1. Ligules (0 = present, 1 = absent)	0	1	1	1
2. Multicellular glands (0 = absent, 1 = present)	0	1	1	1
3. Malate deposition in leaf glands (0 = present, 1 = absent)	0	0	0	1
4. PCR centripetal chloroplasts (0 = present, 1 = absent)	0	0	1	1
5. NADP-ME decarboxylase type (0 = absent, 1 = present)	0	0	1	1
6. Sclerenchyma bundles associated with all veins (0 = present, 1 = absent)	0	0	1	1
7. Aneuploidy addition or reduction (0 = absent, 0.5 = absent or present, 1 = present)[a]	0	0	0.5	1
8. Spikelets all alike, all florets bisexual with hermaphrite florets (0 = absent, 1 = present)	0	0	1	1
9. Spikelets distichous (0 = absent, 1 = present)	0	0	0	1
10. Spikelets (sub)sessile (0 = absent, 1 = present)	0	0	1	1
11. Spikelets compressed laterally (0 = present, 1 = absent)	0	1	0	0
12. Glumes (0 = present, 1 = absent)	0	1	0	0
13. Glumes similar (0 = absent, 1 = present)	0	(–)[b]	1	1
14. Glumes ≥ 9 nerved (0 = absent, 1 = present)	0	(–)	1	1
15. Palea keel winged (0 = present, 1 = absent)	0	1	1	1
16. Palea broad (0 = present, 1 = absent)	0	0	1	1
17. Lemma many nerved (0 = absent, 1 = present)	0	1	1	1
18. Lemma apex (0 = ± entire, 1 = 5-toothed)	0	0	0	1
19. Lemma hairy (0 = absent, 1 = present)	0	0	0	1
20. Lodicule reduction (0 = well developed 0.5 = highly reduced, 1 = absent)	0	0	0.5	1
21. Lodicules fused (0 = absent, 1 = present)	0	0	1	(–)
22. Epiblasts (0 = present, 1 = absent)	0	0	0	1
23. Caryopsis compressed laterally (0 = absent, 1 = present)	0	1	1	1
24. Caryopsis elongate (0 = absent, 1 = present)	0	0	1	1

[a] Basic chromosome number is $x = 10$, a euploid value of $2n = 40$ is reported for *Distichlis*, *Neostapfia*, and two *Tuctoria* species
[b] Character not present

Table 4 Aquatic index for juvenile foliage of three Orcuttieae genera

	Terrestrial Orcuttieae	*Neostapfia*	*Tuctoria*	*Orcuttia*
1. Sclerenchyma bundles (0 = present, 5 = absent)	0	5	5	5
2. Cylindrical (permanently folded) leaf (0 = absent, 5 = present)	0	5	5	5
3. Duration of submergence (0–5)	0	1	3	5
4. Isoetid-type rosette (0–5)	0	0	3	5
5. Floating leaves (0 = absent, 5 = present)	0	0	0	5
6. Lacunal development (0–5)	0	0	0	5
7. Stomata (0 = present, 5 = absent)	0	0	0	5
8. Disarticulation of caryopsis after wetting (0 = absent, 5 = present)	0	0	0	5
Relative Aquatic Index Sum[a]	0	0.28	0.40	1.00

[a] Relative scale from 0 to 1, with 1 being characteristic of aquatic foliage, according to criteria in Sculthorpe (1967) and Keeley (1990)

plants is likely tied to the fact that the pool basins retain moisture longer into the spring and summer growing season and thus, relative to surrounding grasslands, growing season temperatures are higher (Teeri and Stowe 1976; Ehleringer and Monson 1993). The adaptive value of the C_4 pathway in this habitat is illustrated by the fact that vernal pool C_4 grasses flower and reach anthesis in late spring and early summer, long after the disappearance of standing water and under far more xeric and warmer conditions than the rest of the largely C_3 pool flora, most of which will have withered a month or more earlier.

Aquatic specialization

Characteristics widely associated with aquatic foliage include loss of stomata, extensive lacunal development

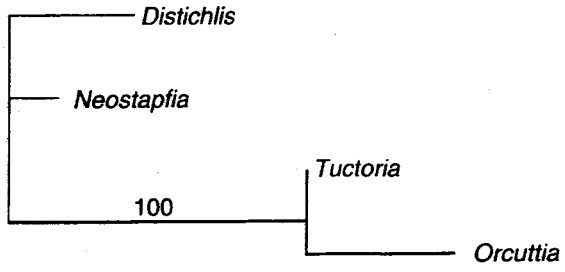

Fig. 5 Phylogenetic tree for Orcuttieae and sister genus *Distichlis* with minimal number of steps of 25 and consistency index of 1.00 and retention index of 1.00 (two other trees were generated with a maximum number of steps of 33). Separation of *Tuctoria* and *Orcuttia* from *Neostapfia* is supported 100% by bootstrap analysis with 1000 replicates

and floating leaves (Sculthorpe 1967). Seasonal pools and other oligotrophic environments select for other traits, including terete leaves arranged in an isoetid-type rosette (Keeley 1990, 1991). Based upon the distribution of these characteristics in the aquatic foliage of the three Orcuttieae genera, *Orcuttia* species have specialized far more to the aquatic environment than others in the tribe (Table 4). Although not all of the nine species in the tribe have been studied in the detail reported here, it is known that aquatic specializations such as floating leaves are produced by all *Orcuttia* species, but not by the other two *Tuctoria* species (Reeder 1982). *Neostapfia* exhibits the least specialization to the aquatic environment, though the terete leaf morphology of the aquatic foliage is quite distinct from terrestrial foliage (Fig. 2). This leaf morphology is typical of the aquatic stage in all three Orcuttieae genera and may reflect paedomorphosis, since related genera such as *Distichlis* and *Eragrostis* have embryonic leaves that are rolled with leaf margins meeting (Watson and Dallwitz 1992). *Neostapfia* exhibits another distinguishing feature separating aquatic and terrestrial foliage; BSC chloroplasts are markedly different in orientation (Fig. 2) and grana formation is different (Fig. 3), although it is unknown whether or these characteristics confer advantage under aquatic conditions.

The radical difference between inundated vs aerial conditions has selected for metamorphosis of foliage between juvenile aquatic and adult terrestrial leaves. Such adaptive change, when mapped on the phylogenetic tree indicated by cladistic analysis, supports the conclusion that specialization to the aquatic environment represents the derived condition, a conclusion supported by the fact that the designation "aquatic plants" is reserved for *Orcuttia* and is not used to describe other members of the Chloridoideae (Watson and Dallwitz 1992). Early germination in *Orcuttia* results in a substantially longer aquatic stage. The selective force behind increasing tolerance to inundation is reflected in the significant correlation between seed output (and other measures of fitness in *Orcuttia tenuis*) and duration of inundation (Griggs 1980).

Transition from land to water

Vernal pool origins are tied to initiation of the mediterranean climate in mid-Pleistocene, as summer-rains gave way to summer drought (Axelrod 1973), and this date is consistent with geomorphological dating of vernal pool landscapes (Stone 1990). It has been suggested that much of the vernal pool flora has evolved since that time (Raven and Axelrod 1978). As proposed for Orcuttieae, systematic studies of *Navarettia* (Crampton 1954; Spencer and Riesberg 1997) and *Lasthenia* (Ornduff 1966) also suggest that vernal pool endemics were derived from upland ancestors. In these C_3 genera the very close relationship between upland and pool taxa is consistent with a relatively recent origin (*c.* 100,000 years) for the pool species. In contrast, all nine species in the Orcuttieae are vernal pool endemics and have no close terrestrial relatives. Indeed, the extraordinarily unique synapomorphy of eligulate leaves suggests they are distantly related to modern Chloridoideae, and this is further supported by the presence of NADP-ME catalyzed decarboxylation (Table 1), an unusual characteristic in the subfamily (Hattersley and Watson 1976; Watson and Dallwitz 1992). Outside of the subfamily, NADP-ME is correlated with C_4 plants in more mesic environments and is coupled with reduced oxygen generation in the PCR cells (Hatch 1987), features consistent with an adaptive advantage in seasonal pools.

Based on the modern-day distribution of terrestrial C_4 plants, in regions of high growing season temperature (Ehleringer and Monson 1993), the divergence from a terrestrial to an amphibious life cycle is likely to have sprung from an ancestor existing under a summer-rain climate. Thus, Orcuttieae may have originated in the Pliocene or earlier (Axelrod 1973). If so, the Orcuttieae would pre-date the origin of vernal pools and may have arisen along the shores of the extensive Tertiary sea that dominated the Great Central Valley of California (Raven and Axelrod 1978). Consistent with this scenario is the fact that the putative sister group *Distichlis* (Table 3) and the basal group *Neostapfia* are often distributed in saline-alkaline pools, which contrasts with *Orcuttia*'s preference for neutral to acidic pools (Stone et al. 1988).

Aquatic C_4 photosynthesis

Presence of fully expressed C_4 photosynthesis in the aquatic stage of these species is unique, as this pathway has not been reported from the aquatic environment (Ehleringer and Monson 1993). Aquatic leaves of both *Neostapfia* and *Tuctoria* have Kranz-type bundle sheath anatomy, 90–95% of initial light-fixation products in malate and aspartate and rapid turnover of these products into phosphorylated sugars (Fig. 3) (aquatic leaves of *Orcuttia* are similar except they lack Kranz-type bundle sheath cells; this will be discussed in the next section). Some aquatic macrophytes such as *Hydrilla verticillata* fix C_4 products in the light; however, only

about 50% of the initial products are C_4 and there is a slow turnover of these products during the first few minutes of pulse-chase studies, and *Hydrilla* lacks Kranz anatomy (Bowes and Salvucci 1984; Magnin et al. 1997). Similar patterns have been noted for *Eleocharis baldwinii* (Uchino et al. 1995).

The question remains whether the unusual presence of C_4 plants in this aquatic environment is adaptive, or is the result of phylogenetic inertia, as suggested for C_4 *Euphorbia* species in shaded understory forests of Hawaii (Pearcy and Calkin 1983; Pearcy et al. 1987). Arguing against a neutral role is the fact that molecular studies show the relatively complex developmental and biochemical modifications in the C_4 pathway are controlled by a few regulatory genes, which would make evolutionary changes in pathway expression relatively easy (Monson 1996; Ehleringer and Monson 1993; Ku et al. 1996). This is supported by studies showing changes in primary photosynthetic products with age in the same plant (Kennedy and Laetsch 1973), the production of C_3 and C_4 leaves in the same species (Ellis 1974), C_4 and CAM in the same plant (Koch and Kennedy 1980), and the switch from CAM to C_3 in the same leaf (Ting and Rayder 1982).

Prior to this report, clear evidence of the C_4 pathway, complete with Kranz anatomy, has not been reported for aquatic plants. In vernal pools, the selective value of C_4 lies in the fact that it is a CO_2-pumping mechanism, which concentrates CO_2 around the active site of the PCR enzyme RUBISCO, maintaining a high CO_2:O_2 ratio and eliminating photorespiration (Ehleringer and Monson 1993). Habitats conducive to a low CO_2:O_2 ratio around the active site of RUBISCO will provide a selective advantage to C_4 plants. Seasonal pools generate such conditions as they are shallow, densely vegetated, have high light and high temperatures, and as a consequence through much of the day they are CO_2-depleted and supersaturated with O_2 (Keeley and Busch 1984), and do favor other CO_2-concentrating mechanisms, e.g., CAM photosynthesis (Keeley 1996, 1998). Such conditions, however, are not unique to vernal pools, as other aquatic habitats generate low CO_2:O_2 ratios (Van et al. 1976; Spencer et al. 1994). Lack of fully-expressed C_4 plants in other aquatic habitats may be tied to the absence of an available C_4 ancestor or evolution of other physiological mechanisms for overcoming low CO_2:O_2 ratios (Salvucci and Bowes 1983), or considerations suggested below.

Uncoupling the C_4 pathway from Kranz anatomy: case of the aquatic *Orcuttia*

Contemporary dogma that Kranz anatomy is a *sine qua non* for operation of the C_4 pathway is contradicted by the biochemical patterns in *Orcuttia*. Aquatic leaves lack Kranz-type bundle sheath anatomy (Fig. 2), yet pulse chase studies provide unequivocal evidence of the C_4 biochemical pathway (Fig. 3); C_4 acids comprise >95% of the primary photosynthetic products and these turn over rapidly to phosphorylated sugars (see also Table 1).

Terrestrial species that lack complete expression of the C_4 syndrome, are commonly lumped in a category known as C_3–C_4 intermediates (Edwards and Ku 1987; Monson 1989; Monson and Moore 1989). This is a somewhat artificial grouping as the primary criterion for inclusion is the failure to meet all typological criteria at the structural, physiological, and biochemical level of either the C_3 or the C_4 syndrome. The vast majority of C_3–C_4 intermediates are quite unlike aquatic *Orcuttia* in that they lack the C_4 biochemical pathway but possess unusual organelle distribution or non-photosynthetic enzymes that generate C_4-like physiology. Those with C_4-assimilation mostly have partial development of Kranz anatomy, and incomplete intercellular separation of PEP carboxylase and RUBISCO, leading to intermediate biochemical and physiological responses (Monson 1989; Brown and Hattersley 1989).

In terms of carbon assimilation pathway, aquatic *Orcuttia* are not C_3–C_4 intermediates. They have fully expressed C_4-assimilation, which is tightly coupled to the C_3 cycle. However, lack of Kranz anatomy, and presence of starch in all mesophyll chloroplasts – indicating PCR function in all chlorenchymous cells – suggests there is no intercellular separation of the C_4 or PEP-carboxylase-catalyzed carbon assimilation (PCA) pathway and the C_3 or RUBISCO-catalyzed carbon reduction (PCR) pathway. In terrestrial plants the tight link between C_4-assimilation and inter-cellular separation of PCA and PCR resulting from Kranz anatomy appears to be essential for effective operation of a CO_2-pump. Perhaps this aquatic environment has selected for a different spatial arrangement of carboxylation events.

A hypothesis worth exploring is whether or not conditions in the aquatic environment allow uncoupling of these two characteristics and still maintain a CO_2-pumping mechanism. A similar uncoupling of behaviors, previously thought to be mandatory for proper functioning of CAM, has been demonstrated for vernal pool *Isoetes*; in the absence of functional stomata, the high diffusive resistance of water alone appears to inhibit outward diffusion of CO_2 during daytime decarboxylation, thus contributing to an efficient CO_2-pumping mechanism (Keeley 1996, 1998). More specific to *Orcuttia* is the model of Bowes and Salvucci (1984) and Reiskind et al. (1989). *Hydrilla verticillata* under stressful conditions exhibits partial C_4-assimilation, and although lacking Kranz anatomy does maintain intracellular spatial separation of cytosolic PEP carboxylase and chloroplastic RUBISCO (Reiskind et al. 1989), leading to the chloroplastic concentration of carbon, well above ambient levels (Reiskind et al. 1997) and is dependent upon C_4 fixation (Spencer et al. 1996).

For such a pump to be effective it is necessary that there be an equilibrium between CO_2 leakage from the C_3 cycle and velocity of the C_4 cycle. To accomplish this there must be a diffusion barrier that reduces CO_2

leakage from the C_3 cycle, or in other words a disequilibrium between the CO_2 pool at the active site of Rubisco and the CO_2 pool in the ambient environment (Edwards and Walker 1983; Monson 1989; Dai et al. 1993; Evans and von Caemmerer 1996). The centripetal arrangement of mesophyll chloroplasts in aquatic *Orcuttia* leaves (Fig. 2) places chloroplasts on the interior side of the cells, and thus, the cytosol (likely site of C_4-PCA) is poised between the C_3-PCR cycle and the ambient environment. It is hypothesized that PCR leakage of CO_2 from the chloroplasts would be recaptured by cytosolic PEP carboxylase, and the extremely high diffusive resistance of water (relative to air) would assist by slowing the outward diffusion of CO_2 (Parkhurst 1994). Consistent with this pathway is the high RUBISCO:PEP carboxylase ratio in aquatic foliage (Table 1). Inward diffusion could also occur into the lacunae, but CO_2 diffusion may be greatly inhibited because of the CO_2 concentration gradient; the atmosphere of the lacunae (with their substantial cross-sectional airspace and a relatively short path length) may approach that of the sediment with 4.2 mol m^{-3} CO_2 and 0.054 mol m^{-3} O_2 (Keeley and Sandquist 1991).

Conclusions

Evolution in Orcuttieae has proceeded towards greater specialization to the aquatic environment. *Neostapfia colusana* is basal to the tribe and exhibits the least specialization to the aquatic environment. Other than terete leaf morphology and elimination of abaxial stomata the aquatic foliage has retained Kranz anatomy and is a fully expressed C_4 species. Aquatic foliage in *Orcuttia* exhibit numerous characteristics specialized to the aquatic environment and assimilate carbon through the C_4 pathway, but in the absence of Kranz anatomy. This suggests either they are in a "transistion" state from C_4 to C_3 photosynthesis or there are characteristics of the aquatic environment that provide selective advantage to intra-cellular separation of C_4-PCA and C_3-PCR pathways, as opposed to inter-cellular separation. Further research is required to determine whether intra-cellular separation of PCA and PCR pathways is sufficient to act as a CO_2 concentrating mechanism. Other physiological specializations to the aquatic mileau may be the substantially elevated RUBISCO:PEP carboxylase levels observed for *Orcuttia*. This potentially could reflect greater specialization to the higher carbon levels of the aquatic milieu, since under a doubling of atmospheric CO_2, terrestrial CAM plants show a similar reduction in PEP carboxylase activity, despite increased C_4 fixation (Nobel et al. 1996).

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