Tansley insight

Shape shifting by amphibious plants in dynamic hydrological niches

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Contents

Summary 79  IV. Conclusions and future challenges 83
I. Introduction 79  Acknowledgements 83
II. Leaf morphological transitions 80  References 83
III. Underwater photosynthesis 82

Summary

Amphibious plants thrive in areas with fluctuating water levels, partly as a result of their capacity to make specialized leaves when submerged or emerged. The tailor-made leaves improve gas exchange underwater or prevent aerial desiccation. Aquatic leaves are thin with narrow or dissected forms, thin cuticles and fewer stomata. These traits can combine with carbon-concentrating mechanisms and various inorganic carbon utilization strategies. Signalling networks underlying this plasticity include conserved players like abscisic acid and ethylene, but closer inspection reveals greater variation in regulatory behaviours. Moreover, it seems that amphibious leaf development overrides and reverses conserved signalling pathways of their terrestrial counterparts. The diversity of physiology and signalling makes plant amphibians particularly attractive for gaining insights into the evolution of signalling and crop improvement.

I. Introduction

Hydrological gradients are a strong determinant of plant species distribution, and species occupying the riparian side of these gradients experience fluctuating water levels and high flooding risks (Silvertown et al., 2015; Sarneel et al., 2019). For plants that are used to terrestrial life, inundation has dramatic consequences (Loreti et al., 2016). In the aquatic environment, gas diffusion is c. 10,000 times slower, which has grave consequences for oxygen (O₂) and carbon dioxide (CO₂) availability (Nobel 2009). Combined with potential reductions in light availability underwater, photosynthesis will be severely hampered. The resulting energy and carbon crisis is perhaps the greatest challenge for flooded plants. In illuminated conditions, the reduction in photosynthesis also generates oxidative stress as a result of an imbalance between light-harvesting and diffusion-limited carbon fixation (Horiguchi et al., 2019). For plants that typically inhabit the aquatic niche, the sudden aerial exposure is also not without risk. The lack of a thick cuticle makes their leaves prone to desiccation and the sudden exposure to light and high amounts of O₂ lead to excessive reactive oxygen species formation (Yeung et al., 2018).

Amphibious plants can successfully occupy the terrestrial–aquatic environmental interface. They often propagate via tubers and rhizomes, and/or time their life cycle to coincide with periods of favourable water levels (Sosnova et al., 2010). During shallow flooding, elongation of shoot organs can facilitate re-establishment of aerial contact and is typically combined with aerenchyma formation to improve internal aeration (Pierik et al., 2008; Herzog & Pedersen 2014).
Despite possessing leaves with a slightly higher specific leaf area (Box 1), species from the water’s edge do not have better underwater photosynthesis than those from higher elevation levels (Winkel et al., 2016). However, many species living in this transition zone do have the capacity to form new leaves adapted to either the new aquatic or aerial conditions. This drastic alteration of leaf form in response to environmental changes is termed heterophylly.

Aquatic leaves, compared with those formed aerially, usually show a higher amount of dissection or they retain the simple leaf shape with a more narrow, elongated form. Additional changes in aquatic leaves include a minimal or even absent cuticle, and fewer or absent stomata. Besides the remarkable display of leaf plasticity, some amphibious plants utilize carbon-concentrating mechanisms (CCMs) and/or bicarbonate (HCO$_3^-$) uptake systems to improve underwater photosynthesis and facilitate the amphibious dual life. A multitude of internal and external signals are used to sense air–water transitions and trigger these dramatic changes. Here we highlight the current understanding of shoot plasticity and photosynthesis physiology, and their adaptive significance for an amphibious lifestyle. We also call for increased leveraging of wild species for broadening our knowledge on mechanisms of plasticity in variable environments.

II. Leaf morphological transitions

When confronted with a sudden change in environment, existing leaves have limited capacity to undergo drastic morphological changes. Therefore, the initiation of the development of either a terrestrial or an aquatic leaf is established early on in development at the shoot apex. Here we consider three main developmental changes from the terrestrial to aquatic transition perspective (Fig. 1), either the formation of narrow leaves or the formation of dissected leaves. Both leaf forms typically lack stomatal development.

Elongated narrow aquatic leaves

When submerged, many amphibious species form new leaves that are longer and narrower. Sometimes the leaves are also pointed at the proximal end, forming an oblanceolate shape. Additionally, these leaves have a higher SLA, are thinner, lack stomata and have minimal cuticle development (Nakayama et al., 2017). The advantage of producing a thin leaf without a cuticle is the reduction of the distance for inward diffusion of O$_2$ and CO$_2$ required for respiration and underwater photosynthesis. The exact importance of the aquatic leaf shape remains unclear, but a narrower leaf would have a thinner diffusive boundary layer (Box 1), further enhancing gas exchange with the environment. Detailed investigations in Rumex palustris estimated a 38-fold reduction in CO$_2$ diffusion resistance in aquatic leaves (compared with unacclimated terrestrial leaves) associated with higher photosynthesis rates and reduced photorespiration (Mommer et al., 2005, 2006).

Amongst amphibious plants, abscisic acid (ABA) has emerged as a major regulator of leaf morphological alterations (Nakayama et al., 2017). In Marsilea quadrifolia, the elongated aquatic leaf form requires low ABA conditions, and terrestrial leaves were created when submerged in water containing ABA. Subsequently, specific transcriptional ABA responses could already be observed at the shoot apex (Hsu et al., 2001). The correlation between elevated ABA concentrations and the terrestrial leaf form was also found in Potamogeton wrightii. Here, even salinity stress-induced ABA triggered terrestrial leaf formation underwater (Iida et al., 2016). Such ABA-dependent heterophyllous changes were also observed in Ludwigia arculata where ABA concentrations were downregulated by ethylene accumulating in submerged tissues, as frequently observed in wetland species (Kuwabara et al., 2003; Benschop et al., 2005).

The amphibious Ranunculus trichophyllus produces extremely thin, rounded aquatic leaves with enhanced abaxial and retarded adaxial development, in contrast to the thick, wide terrestrial leaf form (Kim et al., 2018). Interestingly, its terrestrial relative, Ranunculus sceleratus, does not display such heterophyllly. A transcriptome analyses of R. trichophyllus aquatic and terrestrial leaves revealed strong repression of genes associated with wax biosynthesis required for cuticle formation, and secondary cell wall and vascular development. Heterophyllic leaf development was determined by hormonal regulation of gene families involved in leaf polarity control, namely HD-ZIP IIIIs and KANADI. Submergence-induced ethylene accumulation stimulated KANADIIs required for abaxial development (Kerstetter et al., 2001), whilst HD-ZIP IIII-mediated adaxial development (McConnell et al., 2001) was retarded via a submergence-
induced loss of ABA stimulation. *Ranunculus sceleratus* lacked these hormonal and transcriptional responses, suggesting that the changes in ABA/ethylene signalling and leaf polarity control are key evolutionary steps for aquatic adaptation (Kim et al., 2018).

Leaf morphology is also strongly regulated by light quality cues (Momokawa et al., 2011). The red : far-red ratio (R : FR) rises with increasing water depth. Accordingly, low R : FR triggered terrestrial leaf formation in submerged *Rotella hippuris*, with the converse being true for aquatic leaves upon emersion. Interestingly, R : FR values indicative of proximity to the water surface or aerial conditions required high blue light to facilitate underwater terrestrial leaf formation. At high R : FR values typical of deep flooding, blue light had no effect. Thus the integration of light quantity and quality can be critical in detecting water-level fluctuations (Momokawa et al., 2011).

**Dissected aquatic leaves**

An extreme form of heterophylly is the formation of highly dissected leaves underwater, with reduced stomatal density and cuticle thickness. A narrow and dissected leaf might also facilitate better water flow around and through it, and so prevent mechanical stress. An increase in dissection index is found in many species across a wide range of phylogenetic lineages. The underlying mechanisms have recently been extensively investigated for species such as *Hygrophila difformis* and *Rorippa aquatica* (Nakayama et al., 2014; 6, Kim et al. (2018); 7, Nakayama et al. (2014); 8, Horiguchi et al. (2019); 9, Li et al. (2017); 10, Casati et al. (2000); 11, Rao et al. (2002); 12, Ueno (1998). ABA, abscisic acid; GA, gibberellin.

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**Superscript numbers in column 1 of (c) refer to the following:**

1, van Veen et al. (2013); 2, Hsu et al. (2001); 3, Iida et al. (2016); 4, Kuwabara et al. (2003); 5, Momokawa et al. (2011); 6, Kim et al. (2018); 7, Nakayama et al. (2014); 8, Horiguchi et al. (2019); 9, Li et al. (2017); 10, Casati et al. (2000); 11, Rao et al. (2002); 12, Ueno (1998). ABA, abscisic acid; GA, gibberellin.
Fig. 2 Bicarbonate utilization routes in angiosperms. In water, the predominant form of inorganic carbon is the membrane-impermeable bicarbonate ($\text{HCO}_3^-$) ion. Successful underwater photosynthesis therefore requires bicarbonate uptake mechanisms, broadly categorized into three routes, depicted in the schematic: (1) an apoplastic carbonic anhydrase converting $\text{HCO}_3^-$ to $\text{CO}_2$; (2) apoplastic acidification by $\text{H}^+$-ATPases, which shifts the chemical equilibrium towards $\text{CO}_2$; and (3) symporters facilitating $\text{HCO}_3^-$ import into the cytosol. $\text{CO}_2$ produced via these routes can ultimately be fixed via the Calvin cycle, eventually resulting in carbohydrate biosynthesis.

increase in class II KNOX gene expression. However, compound and dissected leaves can re-enter an undifferentiated state by transient reactivation of class I KNOX genes (Bharathan et al., 2002). This undifferentiated state then allows leaflet initiation, instigated by PIN1-mediated auxin maxima (Barkoulas et al., 2008). The separation of these leaflets requires CUP SHAPED COTYLEDON (CUC3)-mediated suppression of growth between the auxin maxima, a process that is conserved across eudicots (Blein et al., 2008). Among the Brassicaceae, leaf dissection is further determined by the presence of REDUCED COMPLEXITY (RCO) which locally suppresses growth at the sides of leaves to enhance dissection (Sicard et al., 2014; Vlad et al., 2014).

In R. aquatica, the submergence-induced formation of the dissected leaf coincides with a decrease of class I KNOX and CUC3 expression, analogous to the existing knowledge of compound leaf formation. Moreover, a drop in gibberellin (GA) concentrations was observed, and KNOX genes are known to suppress GA biosynthesis. Subsequently, exogenous GA application or biosynthesis inhibition led to the reversal or exaggeration of leaf dissection, respectively (Nakayama et al., 2014). However, hormonal investigation of submergence-induced leaf dissection in H. difformis found contrasting effects of GA compared with R. aquatica (Li et al., 2017). Here leaf dissection was predominantly driven by ethylene and low ABA concentrations. Although the molecular machinery of leaf dissection is considered conserved across species, two contrasting signalling behaviours were identified here.

Development of stomatal density

The aquatic leaf has a strongly reduced stomatal density and cuticle thickness. Indeed, detailed molecular investigation in R. trichophyllus identified a strong downregulation of stomatal developmental genes underwater, some of which have been lost altogether in aquatic plants (Olsen et al., 2016; Kim et al., 2018). The underwater regulation of stomatal density and cuticle typically goes hand in hand with that of leaf shape, namely via ethylene, low ABA and/or high R : FR (Kuwabara et al., 2003; Momokawa et al., 2011; Iida et al., 2016; Kim et al., 2018). A thick cuticle in terrestrial leaves, which have higher ABA concentrations than their aquatic counterparts, agrees with the role of ABA in mediating drought responses, which includes strengthening the cuticle (Cui et al., 2016). However, the signals linked to stomatal development of amphibious heterophyll do not always align with patterns commonly observed. Stomatal density increases with high light and low CO2 availability, and is signalled through systemic leaves (Casson & Hetherington 2010). Although light intensities follow the same trend for heterophyll, the low CO2 availability underwater is not translated into high stomatal density. Likewise, low ABA concentrations and high R : FR are also known to increase stomatal density (Boccalandro et al., 2009; Tanaka et al., 2013; Jalakas et al., 2018), whereas in amphibious heterophyllous plants, low ABA and high R : FR decrease stomatal density (Kuwabara et al., 2003; Momokawa et al., 2011; Iida et al., 2016, Kim et al., 2018). Thus, the aquatic developmental programme appears to override routine terrestrial regulatory networks determining leaf formation and stomatal density.

III. Underwater photosynthesis

In air, gaseous CO2 diffuses relatively easily through the leaf. But when submerged, plants need to access the dissolved inorganic carbon (DIC). Between pH 7 and 10, CO2 availability is limited and HCO3− is the dominant DIC form (Pedersen et al., 2013). This poses an additional problem for submerged plants, as HCO3−, unlike CO2, does not easily cross lipid membranes. It is not surprising, therefore, that many aquatic plants have HCO3− uptake mechanisms (Maberly & Madsen, 2002; Yin et al., 2017). Various HCO3− uptake strategies and CCMs have been characterized in cyanobacteria, algae, seagrasses and other higher plants (Poschenrieder et al., 2018). In angiosperms, three forms can generally be
distinguished (Fig. 2). First, the conversion of HCO$_3^-$ to CO$_2$ by apoplastic carbonic anhydrases (CAs). Second, an H$^+$-ATPase-mediated acidification of the apoplastic and diffusive boundary layer, which pushes the CO$_2$/HCO$_3^-$ equilibrium towards CO$_2$. Third, a symporter-mediated cotransport of HCO$_3^-$/$H^+$, and subsequent HCO$_3^-$ dehydration to CO$_2$ via cytosolic CAs. A variety of metabolic routes have been identified in aquatic plants to subsequently fix the acquired HCO$_3^-$ /CO$_2$. For example, the true aquatics (Box 1) *Hydilla verticillata* and *Egeria densa* can switch between C$_3$ and C$_4$ photosynthesis underwater and can do so even in a single cell (Casati *et al.*, 2000; Rao *et al.*, 2002).

Few studies report photosynthetic adaptation to submergence in amphibious plants. The aquatic leaves of *R. palustris* clearly had better photosynthetic capacity underwater than did terrestrial leaves (Mommert *et al.*, 2005). In the heterophyllous amphibian *H. difformis*, a combination of biochemical and anatomical leaf adaptations facilitates underwater photosynthesis (Horiguchi *et al.*, 2019). Submergence triggered the formation of highly dissected aquatic leaves with substantial O$_2$ production underwater. By contrast, submerged terrestrial leaves struggled to capture inorganic carbon, regardless of illumination. The decreased photosynthesis underwater and subsequent excess energy were linked to high oxidative stress in these leaves. Aquatic leaves had a high capacity to utilize HCO$_3^-$, which was absent in their terrestrial counterparts. Specific inhibitors were used to discern the mechanism for HCO$_3^-$ uptake in aquatic leaves. Interestingly, neither the inhibition of the apoplastic CA nor the HCO$_3^-$/$H^+$ symport affected underwater photosynthesis. Significant photosynthesis impairment was observed only when intracellular CA activity was blocked. These observations imply that submerged leaves of *H. difformis* can import HCO$_3^-$ into the cell without H$^+$ cotransport (Horiguchi *et al.*, 2019). Although common amongst true aquatic species, the extent of HCO$_3^-$ utilization amongst amphibious plants is currently unknown.

In true aquatics, CCMs such as the C$_4$ system, can be induced by a several factors, such as photoperiod, low CO$_2$ availability and ABA (Casati *et al.*, 2000; Rao *et al.*, 2002). The amphibious sedge *Eleocharis vivipara* exhibits an aquatic Kranz-less C$_3$ form and terrestrial C$_4$-like traits with Kranz anatomy (Box 1). The terrestrial form can be imposed on the aquatic leaf by ABA application and is considered as a stress signal (Ueno 1998). Interestingly, in *H. difformis* biochemical HCO$_3^-$ usage, could be mimicked by application of ethylene or prevented by blocking ethylene perception. Even existing terrestrial leaves were sensitive to ethylene and submergence and achieved an intermediary capacity of HCO$_3^-$ usage (Horiguchi *et al.*, 2019). The importance of flooding-specific cues, such as ethylene, is also apparent from work on *R. palustris*, where the morphological adaptations to submergence can also be induced by low light conditions. However, these do not yield any photosynthetic benefit, as does a true aquatic leaf (Mommert *et al.*, 2005).

IV. Conclusions and future challenges

Amphibious plants are truly shape shifters, adjusting their morphology and physiology to adapt to fluctuating environments. They have provided crucial insights into developmental regulatory networks underlying leaf plasticity. However, while some consistent regulatory factors (e.g. ABA and ethylene) are recognized, there have also been contradictions (e.g. GA regulation of leaf dissection), and much remains to be discovered regarding other cues such as light, temperature and abaxial dominance in narrow leaves. This will require a greater use of amphibious species for exploring the molecular regulation of adaptive plasticity to water extremes. Given the increased fluctuations in water stress associated with climate change, understanding such adaptations will be important if we are to engineer resilient crops (Voesehek *et al.*, 2014). The plant’s current capacity to enhance underwater photosynthesis in existing terrestrial leaves of *H. difformis* is a good sign that such traits might, at some point, be transferable to crop species.

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