Amphibious Plants and Phylogenetics

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Introduction

Many plants have adapted to seasonal flooding and unpredictable aquatic conditions by rapidly changing their leaf morphology, allowing them to live in either terrestrial or aquatic environments, such as the two leaf morphs displayed in figure 1. Such environmentally mediated changes in leaf morphology are known as heterophylly. Heterophylly in angiosperms is found in many diverse taxa. (Goliber and Feldman, 1990; Kuwabara et al., 2003; Iida et al., 2016) The independent evolution of this trait amongst different unrelated species implies that it evolved as a means of increased survival within regions of varied environmental conditions.

While heterophylly occurs in a diverse range of plants, the molecular mechanisms of phenotypic plasticity within green plants appears to have some conservation with respect to the plant hormone abscisic acid (ABA) (Takezawa et al., 2011.) Research on the plant hormone ABA indicated that application of the hormone to Ludwigia arcuata (an amphibious plant) was found to display terrestrial-type leaves when grown in aqueous ABA. (Kuwabara et al., 2003) A similar phenomena was observed in water grown Potamogeton nodosus and Ranunculus trichophyllus dosed with ABA, terrestrial leaves were observed after the growth period. (Kim, et al., 2018; Anderson, 1978) ABA is a plant hormone that effects a wide array of species, from green plants to cyanobacteria.

ABA is also involved in leaf abscission, fruit development, and drought tolerance. ABA is thought to also be accumulated in plants experiencing osmotic shock, facilitating stress response, tolerance, and even gene expression response. (Yamaguchi-Shinozaki and Shinozaki, 2006; Nakashima and Yamaguchi-Shinozaki, 2013, Goda et al., 2008) Given that various amphibious plant species respond with changes in leaf morphology when ABA is applied (Kane and Albert, 1987; Goliber and Feldman, 1989) it is not unlikely that other hormones, such as ethylene, are also closely tied to heterophyllic leaf formation. The ABA signaling pathway, involved in the mechanism for terrestrial leaf formation, may also be closely tied to the development of heterophylly in unrelated species.

Figure 1: The striking differences of leaf morphology within the genus Limnophila. Differences in environmental factors, such as temperature or aquatic submergence, lead to changes in morphology. (Smith, BioSciences 2017)
In this paper, I aim to address heterophyllic leaf development within another amphibious plant species, *Limnophila sessiliflora*, by investigating how plant hormones affect leaf morphology in both submerged and terrestrial plants. Additionally, I am investigating evolutionary history of species within the *Limnophila* genus by genetic sequencing and by using phylogenetic trees.

**Materials and Methods**

*Limnophila sessiliflora* and *Limnophila aromatica* seeds were sterilized with a 0.2% solution of sodium hypochlorite solution and sown on MS medium.

**Physiological Experiment**

*L. sessiliflora* seedlings were transplanted into plant boxes filled with MS medium and inserted into the medium. Plant hormones were added into the water or directly into the medium, including Gibberelic Acid (GA$_3$) UniconazoleP (UNI, GA$_3$ inhibitor), 1-aminocyclopropane-1-carboxylic acid (ACC, ethylene precursor), AgNO$_3$ (ethylene inhibitor) and Abscisic acid (ABA), at concentrations of 10$^{-6}$ M, 10$^{-7}$ M, and 10$^{-8}$ M. Plants were allowed to grow for 3 weeks at 23°C, under the light condition 60 μmol m$^{-2}$ s$^{-1}$ and long day growing conditions (16 hours daylight and 8 hours darkness). Following the 3 week incubation, whole plants were scanned to document leaf morphology. Plants were then fixed with FAA Fixative (50% ethyl alcohol, 2.5% glacial acetic acid and 2.5% formalin). Following vacuum treatment, leaf samples were scanned. Leaves were also wet mounted, cleared by chloral hydrate solution, and three cell layers, Adaxial epidermis, palisade mesophyll, and abaxial epidermis were photographed using a differential interference contrast microscope DM4500 (Leica Microsystems) at 40X.

For each of the various hormone treatments 10 palisade mesophyll cells were chosen at random and their cell area and aspect ratio were measured for each photograph using the FIJI program.

**Sequencing Experiment**

Five different plant species, including *L. sessiliflora*, *L. aromatica*, *L. indica*, *L. aquatica*, and *Bacopa monnieri* were analyzed for DNA sequencing.

The young growing tip shoot, or the whole seedling, of each plant was removed and placed in liquid nitrogen. Frozen plant shoots were homogenized, then genomic DNA was extracted by DNeasy plant mini kit (QIAGEN).

PCR was performed by Primestar GXL (takara), using Primers for rbcL and trnS-trnG region. PCR results by electrophoresis with 1% agarose gel, and purified by AMpureXP beads (Beckman Coulter).
The sequencing reaction was performed with BigDye terminator v3 (Thermo Fisher Scientific) using the PCR products as templates and rbcL and trnS-trnG primers were selected. (Shaw et al, 2005) After purification by CleanSeq (Beckman Coulter), samples were sequenced by ABI3130 sequencer (Thermo Fisher Scientific).

Following sequencing, samples were corrected and edited using 4 peaks software and CLC sequence viewer. Sequence data was used to generate phylogenetic trees using MEGA software.

Results and Discussion

Mechanism Experiment

UNI (GA₃ inhibitor) treated seedlings developed a distinct morphology from the control group, including stem and leaf shortening.

GA₃ hormone addition to *L. sessiliflora* plants caused stem

Figure 3: Whole plant scans of control water *L. sessiliflora* (right) and terrestrial (left)

Figure 5: GA₃ treated terrestrial *L. sessiliflora*

Figure 6: Leaf scan of submerged *L. sessiliflora* treated with UNI (GA₃ inhibitor)

UNI (GA₃ inhibitor) addition in submerged *L. sessiliflora* (figure 6) induced leaf shortening compared to the control group, characteristic of the terrestrial leaf-form.

Figure 7: Single leaf scan of submerged control *L. sessiliflora*

GA₃ was also observed to cause changes to leaf morphology within terrestrial *L. sessiliflora*, (figure 9) causing leaf elongation, typical of the aquatic leaf-form.

Figure 8: Single leaf scan of terrestrial *L. sessiliflora* treated with GA₃
These morphological changes induced by GA$_3$ and UNI may indicate that heterophylly in this species is partially determined by GA$_3$ concentration. Although GA$_3$ and UNI were found to induce significant morphological change within this species, it should be noted that *L. sessiliflora* tends to show some inconsistencies in overall plant morphology and leaf form with respect to the submerged and terrestrial morphs. For example, within the ABA sample group, both terrestrial and submerged morphology were observed.

Leaf palisade mesophyll cell area measurements (figure 10) indicated that cell area decreased in submerged UNI-treated leaves, submerged Ag$+^+$-treated leaves, and submerged ABA treated leaves while increasing in terrestrial GA$_3$ leaves. This data further supports that GA$_3$ may be involved in aquatic leaf formation, as larger palisade mesophyll cells are characteristic of the submerged form.

In contrast to palisade mesophyll cell area, the aspect ratio is similar for both control terrestrial and control submerged plant cells. The aspect ratio of the cells remained relatively constant regardless of treatment with plant hormone, including...
both the control test groups, indicating that aspect ratio does not differ between the submerged and terrestrial leaf forms. This contrasts with other amphibious plants, such as Callitriche heterophylla, which changes cell shape drastically in the submerged leaf form. (Deschamp and Cooke, 1985)

**Sequencing Experiment**

Phylogenetic analysis of the 5 different plant species was performed for two different primer sets, rbcL and trnS-trnG region. The trnS-trnG dataset (figure 12) indicates that *L. indica* and *L. aquatica* are the most closely related species within this group with *Bacopa monnieri* (M on figures 12 and 13) used as an outgroup.

This is in contrast with the phylogenetic tree generated by rbcL sample data, which shows different evolutionary relationships, where *L. aquatica* represents a more ancient lineage of the genus *Limnophila*.

It is important to note that *L. aromatica* has lost the amphibious plant morph, being isolated to growing within aquatic environments only. This trait shows that the absence of the amphibious plant trait in this species may be related to evolutionary divergence, as shown in higher base pair changes in the rbcL gene in *L. aromatica*. While this does offer more insight into the evolutionary history of the genus *Limnophila*, additional tests on other genetic regions are needed to validate greater genetic divergence in *L. aromatica*.


