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Permanently open stomata of aquatic angiosperms display modified cellulose crystallinity patterns

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ABSTRACT

Most floating aquatic plants have stomata on their upper leaf surfaces, and usually their stomata are permanently open. We previously identified 3 distinct crystallinity patterns in stomatal cell walls, with angiosperm kidney-shaped stomata having the highest crystallinity in the polar end walls as well as the adjacent polar regions of the guard cells. A numerical bio-mechanical model suggested that the high crystallinity areas are localized to regions where the highest stress is imposed. Here, stomatal cell wall crystallinity was examined in 4 floating plants from 2 different taxa: basal angiosperms from the ANITA grade and monocots. It appears that the non-functional stomata of floating plants display reduced crystallinity in the polar regions as compared with high crystallinity of the ventral (inner) walls. Thus their guard cells are both less flexible and less stress resistant. Our findings suggest that the pattern of cellulose crystallinity in stomata of floating plants from different families was altered as a consequence of similar evolutionary pressures.

Stomata are crucial for plant functioning because of their fundamental role in the regulation of gas exchange between the plant and its surrounding environment. It is evident that the morphology, distribution, orientation and development of stomata have diversified since they first evolved ~400 million years ago.[1,2] Stomatal cell walls are uniquely strong and flexible, enabling repeated opening and closing of the stomatal pore multiple times every day; interestingly the triggers for stomatal opening (i.e. light and CO_2) appear to be similar among the different plant groups whereas those for stomatal closing differ.[3] There is significant ongoing debate regarding stomatal evolution, the differences in stomatal function between taxonomic groups, and how they impact plant performance, partially driven by disparities between evidence from different sources, for example ABA-responsiveness and the presence and localization of ABA-signaling pathway components.[4-8] Although relatively few studies focus on the effect of cell wall composition and structure on stomatal function, wall properties are known to directly affect the structure and mechanical properties of guard cell walls and therefore stomatal function.[1,9-11] In our previous work[12] we found 3 distinct, taxonomic group-dependent crystallinity patterns in stomatal cell walls, with angiosperm kidney-shaped stomata having the highest crystallinity in the polar end walls and in the adjacent polar regions of the guard cells (Fig. 1A–C). Our Finite Elements model indicated that the highly crystalline areas might serve a biomechanical purpose by strengthening the cell wall in areas of high stress.[12]

However, this work omitted to investigate the numerous different highly-modified types and behaviors of stomata that exist within extant plant groups.[3] For instance, there are several examples of non-functional stomata, i.e., those that do not have the ability to open and close[13] such as the stomata present in parasitic plants, flowers or fruits. While probably the most interesting example of non-functional stomata are the permanently open stomata of aquatic plants, or macrophytes. Non-functional stomata are considered to be an advanced character of aquatic plants, while the ability to open (and close) the stomata is a vestigial trait of the terrestrial ancestry.[14]

Floating aquatic plants have independently evolved multiple times in ferns, liverworts and angiosperms and can be found in most freshwater habitats globally.[15] They appear to have evolved once in liverworts (Ricciaceae) and at least twice in ferns (*Ceratopteris* in the Pteridaceae and *Azolla* and *Salvinia* in the Salviniaceae). In angiosperms, several families contain aquatic plants with floating leaves. It is likely that floating plants arose independently at least 13 times in angiosperms and they are found in the ANITA grade (Nymphaeaceae), eudicots (Polygonaceae, Lythraceae, Plantaginaceae, Ranunculaceae, Gentianaceae), and monocots (Butomaceae, Hydrocharitaceae, Potamogetonaceae, Araceae, Aponogetonaceae, Typhaceae, Gramineae).[15] Floating plants often exhibit similar adaptations to the aquatic habitat, providing an interesting example of convergent evolution. They remain buoyant on water by the means of large air spaces[15,16] in their floating
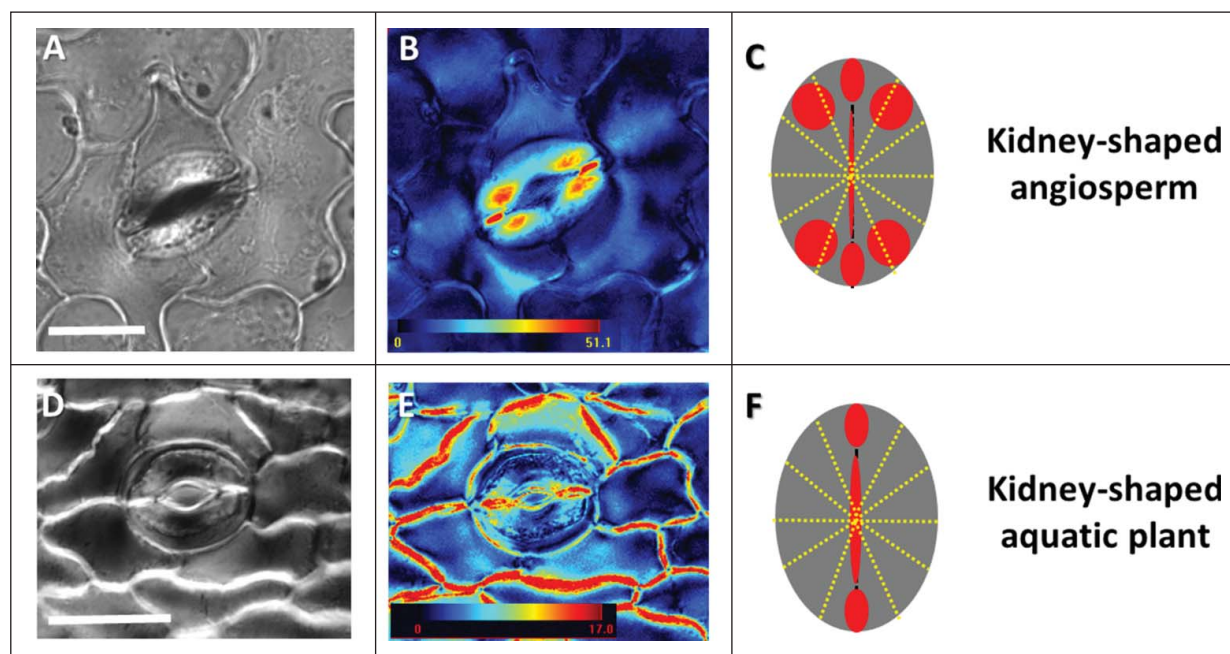
Figure 1. Floating aquatic plants have stomata with an altered pattern of cellulose crystallinity. Crystallinity patterns in the land plant *Cyclamen persicum* (A,B); and aquatic plant *Nuphar lutea* (D,E) shown by liquid crystal polarized light microscopy (LC-PolScope). A schematic representation of the regular crystallinity pattern in angiosperm kidney-shaped stomata (C) and the altered pattern in the stomata of floating plants (F). The retardance color scale bar codes the retardance range. Scale: 25 μm.

leaf blades, petioles or roots. Floating leaves have their lower (abaxial) surface completely submerged in water, while the upper (adaxial) surface is exposed to the atmosphere. Unsurprisingly, floating plants usually have epistomatous leaves (in which only the upper surface has stomata).[14,15] Therefore, gas exchange occurs mainly from the adaxial leaf surface. Often aquatic plants have non-functional i.e., permanently open stomata that cannot regulate water loss.[14] In aquatic plants, CO_2-exchange is not limited by water availability. Thus, as a floating leaf has no need to conserve water, closing the stomatal pore is not necessary and losing the ability to do so would likely have no associated negative selection pressure(s). Indeed, many floating plants have relatively high photosynthetic capacities.[17] Furthermore, large-scale deletion of genes from the stomata developmental pathway has been seen to accompany the loss of stomata in marine angiosperms.[18]

Only a few works discuss the subject of non-functional stomata mechanics. In *Salvinia herzogii* it was proposed that the guard cells are physically unable to close because of peculiar, probably cuticular, extensions near the pore.[19] In *Lemna minor* the guard cells are apparently dead, and thus unable to move.[14] In *Nymphaea* and *Nuphar* the guard cells are intact, and Ziegler suggested that there is no substomatal cavity, which possibly prevents the guard cells movement[14]; although, the stomata of *Nyphaea violacea* and several other members of the genus have since clearly been shown to possess substomatal cavities.[20,21] It is interesting that the reasons considered to be involved in control of stomatal closure and therefore the generation of non-functional stomata are largely thought to be anatomic in light of discussions that stomatal closure may be controlled by different triggers in non-vascular compared with vascular plants.[3] Plant cell wall composition is also known to differ between different plant groups[22] and it is now known that cell wall

composition contributes to stomatal function.[3,4,6] However, as far as we are aware, there are no studies that have investigated the cell wall composition and structure of the non-functional stomata of floating plants.

In the current study, stomatal cell wall crystallinity was investigated in 4 floating plants: *Nymphaea alba* and *Nuphar lutea* (Nymphaeaceae, ANITA grade; one of the earliest diverging lineages of Angiosperms), *Alisma plantago-aquatica* and *Limnobium laevigatum* (Alismataceae, monocots). *Alisma* apparently has partially functional stomata,[23] while in *Nymphaea*,[14] *Nuphar*,[14] and probably *Limnobium*, stomata are completely non-functional. We focused our study on angiosperms to reduce the effects of differences that may exist in stomatal function due to, for example, differences in ABA-responsiveness between ferns and angiosperms.[4]

It appears that for the aquatic plants investigated, although belonging to different families and taxonomic groups, cell wall crystallinity pattern in the guard cells is similarly altered, as compared with the pattern typically observed for angiosperm kidney-shaped stomata (Fig. 1). All the plants examined had high crystallinity near the pore. *Nymphaea*, *Nuphar* and *Limnobium* seem to share a very similar crystallinity pattern having high crystallinity in the polar end walls and lacking crystalline areas in the adjacent polar regions (Figs. 2A, B and C). In contrast, *Alisma* (having partially functional stomata), possess crystallinity pattern in the polar end walls and adjacent polar-regions, similar to that previously observed for angiosperm kidney-shaped stomata (Fig. 2D). All 4 species displayed similar patterns of cellulose orientation in their stomata, identical to all kidney-shaped stomata (data not shown). It is important to mention, that usually the stomata close after the epidermis is peeled. However, non-functional stomata are unable to do so and hence remain open; potentially impacting stomatal geometry.
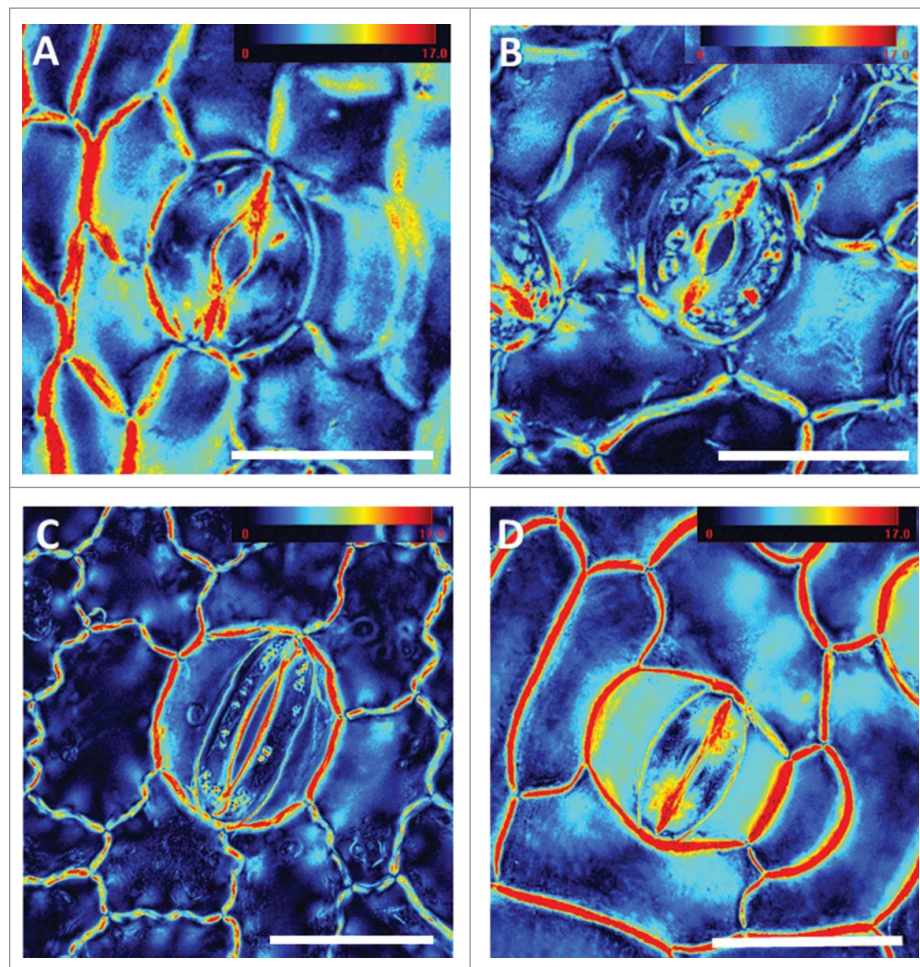
Figure 2. Stomatal crystallinity patterns of floating aquatic plant species. Liquid crystal polarized light microscopy (LC-PolScope) images of the adaxial leaf epidermis from the early-diverging angiosperms *Nuphar lutea* (A), *Nymphaea alba* (B), and the monocotyledons *Limnobium laevigatum* (C) and *Alisma plantago-aquatica* (D). The retardance color scale bar codes the retardance range. Scale: 50 μm.

Interestingly, the ventral wall (cell wall at the pore margins) of all 4 plant species examined displayed high crystallinity. The ventral wall has to remain flexible to allow the guard cells to elongate and shorten during the repeated cycles of stomata opening and closing which occur as part of plant adaptation to changing environment. High crystallinity in this area is assumed to be associated with increased cell wall stiffness and therefore might interfere with stomatal movement. These observations suggest that the altered pattern of cell wall crystallinity in floating plants is probably associated with the loss of stomatal function, though more research is needed to come to definite conclusions. However, the observation that in the partially functional stomata of *Alisma* the crystallinity in the polar-regions is not significantly reduced strengthens this hypothesis.

Aquatic floating plants have specific adaptations to their unique habitat. Their permanently open stomata display similar alterations in cellulose crystallinity pattern, presumably as a consequence of similar environmental pressure(s) yielding another fascinating example of convergent evolution. It would be very interesting to know whether other cell wall constituents (such as pectins, lignins, phenols, etc.) are modified in this unique context. Better understanding of the biomechanical impacts of key changes in cell wall composition in response to environmental adaptation could extend our knowledge of how different plant species may respond to climate- and anthropologically-induced environmental changes including drought and salinity.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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