A molecular basis behind heterophylly in an amphibious plant, *Ranunculus trichophyllus*

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**Abstract**

*Ranunculus trichophyllus* is an amphibious plant that produces thin and cylindrical leaves if grown under water but thick and broad leaves if grown on land. We found that such heterophylly is widely controlled by two plant hormones, abscisic acid (ABA) and ethylene, which control terrestrial and aquatic leaf development respectively. Aquatic leaves produced higher levels of ethylene but lower levels of ABA than terrestrial leaves. In aquatic leaves, their distinct traits with narrow shape, lack of stomata, and reduced vessel development were caused by *EIN3*-mediated overactivation of abaxial genes, *RtKANADI*s, and accompanying with reductions of *STOMAGEN* and *VASCULAR-RELATED NAC-DOMAIN7* (*VDN7*). In contrast, in terrestrial leaves, *ABI3*-mediated activation of the adaxial genes, *RIHD-ZIPIII*s, and *STOMAGEN* and *VDN7* established leaf polarity, and stomata and vessel developments. Heterophylly of *R. trichophyllus* could be also induced by external cues such as cold and hypoxia, which is accompanied with the changes in the expression of leaf polarity genes similar to aquatic response. A closely-related land plant *R. sceleratus* did not show such heterophyllic responses, suggesting that the changes in the ABA/ethylene signaling and leaf polarity are one of key evolutionary steps for aquatic adaptation.

**Author summary**

Evolutionary adaptation into aquatic environment is widely observed in diverse clades of land plants. To understand the molecular basis behind such adaptation, we analyzed *Ranunculus trichophyllus*, an amphibious plant producing different leaf shape depending on the growth conditions. Aquatic leaves of this plant produce higher levels of ethylene, which causes overactivation of genetic circuits composed of *EIN3*, an ethylene signaling transducer, and abaxial genes that suppress genes regulating stomata and xylem development. In contrast, terrestrial leaves produce higher levels of ABA, which activates adaxial genes and causes activation of stomata and xylem developments. Such changes in the ABA/ethylene signaling and leaf polarity after submergence were not observed in the
Introduction

Since plants are sessile organisms, specific adaptations to their given environments are critical for their survival. Thus, compared to animals, plants show higher levels of phenotypic plasticity, differential phenotypic alterations exhibited in the same species depending on their surrounding environments [1, 2]. One of the most dramatic plant plasticity is heterophylly, an ability to produce morphologically different types of leaves depending on the growth environments [3]. Amphibious plants produce different shapes of leaves when grown under water compared to terrestrial growth; they usually produce thin and slender leaves in aquatic conditions but produce thick and stout leaves in terrestrial conditions [3]. Currently, the molecular mechanisms behind such heterophylly of amphibious plants are not well known.

Plant hormones participate in various plant developments so that plant architecture is shaped by the accurate regulation of the hormones [4, 5]. Plasticity by water adaptation is also regulated by plant hormones. Abscisic acid (ABA), auxin, ethylene, and gibberellin (GA) were proposed to mediate perception and responses to submergence into water [6, 7]. For example, auxin influences hyponastic growth and development of adventitious roots in submerged condition [6, 8]. Ethylene also regulates adventitious roots and rapid shoot growth when submerged, especially in deep water rice [7, 9]. Exogenous ABA treatment induces terrestrial leaf development in many aquatic plants whereas exogenous GA induces aquatic leaf development in some aquatic plants [3].

Leaf, as a photosynthetic organ, is a major plant organ showing plastic development depending on the environments [10, 11]. Leaves are developed from the shoot apical meristem as lateral organs and the leaf development is coordinated through three axes, a proximo-distal axis, an adaxial-abaxial (dorso-ventral) axis and medio-lateral axis. Adaxial-abaxial polarity has been well studied at the molecular level because establishment of this axis is critical for leaf morphogenesis [12]. Recent studies have identified several families of transcription factor genes determining adaxial and abaxial cell fate in the leaf [12, 13]. For example, KANADI (KAN) and YABBY (YAB) families and two AUXIN RESPONSE FACTOR genes (ARF3/ETTIN and ARF4) play critical roles in determination of abaxial cell fate whereas class III homeodomain-leucine zipper (HD-ZIPIII) genes, ARP (ASYMMETRIC LEAVES1, ROUGH SHEATH2, and PHANTASTICA) class Myb genes, and a LOB domain transcription factor, ASYMMETRIC LEAVES2 (AS2), determine adaxial cell fate [14–18]. In addition, the expression domains of leaf polarity genes are finely delimited by small RNA; i.e., miR165/166 degrades HD-ZIPIII transcripts in abaxial side and tasiRNA erases the transcripts of ARF3 and ARF4 in adaxial side of the leaves [14, 19]. Likewise, abaxial fate-determining genes act antagonistically with adaxial fate-determining genes. For example, the HD-ZIPIII genes are ectopically expressed in abaxial side of the leaves in kan1 kan2 double mutant, thus causing adaxialization [15]. In addition, overexpression of KAN2 causes reduced expression of PHB, a HD-ZIPIII gene [15], indicating that KAN genes suppress the expression of HD-ZIPIII. In contrast, gain-of-function of HD-ZIPIII causes adaxialization whereas loss-of-function of HD-ZIPIII genes like phb phv rev triple mutant causes abaxialization, indicating that HD-ZIPIII genes antagonistically suppress KANs [13, 20]. Interestingly, both abaxialization and adaxialization cause partial radialization of the leaves.
Land plants have evolved from aquatic plants, algae, in Silurian period, ca. 400 million years ago [21]. Afterwards, they have developed various traits for land adaptation such as vascular structure, stomata and seed development [22, 23]. In addition, they have evolved a plant hormone, abscisic acid (ABA), and ABA signaling to endure dehydrated environments [24]. During the evolutionary process, diverse families of plants re-colonized water and turned into aquatic plants [25, 26]. Although derived from diverse clades of land plants, many submerged plants share common phenotypes such as thin and cylindrical leaves [3]. As submerged plants are subjected to the same selection pressure, there may be a common evolutionary developmental (evo-devo) mechanism modified from the genetic circuits present in the terrestrial plants. Such evo-devo mechanisms have yet to be disclosed.

In this study, we delved into the potential evo-devo adaptive mechanism of an amphibious plant, *Ranunculus trichophyllus* var. *kadzusensis*, which is an endangered species in Korea that lives in rice pad. We hypothesized that amphibious plants are an evolutionary bridge between land and aquatic plants, thus, the elucidation of adaptive molecular mechanism in *R. trichophyllus* would provide an insight how land plants re-adapted to aquatic environments. *Ranunculus* is a widespread genus containing hundreds of species adapted to various habitats in the northern hemisphere [27]. Many *Ranunculus* species live near the water, and some species adapted to aquatic environments [27, 28]. Therefore, *Ranunculus* genus is a good model system to investigate how land plants recolonized aquatic environments.

Here we show that the heterophylly of an amphibious plant, *R. trichophyllus*, is widely controlled by two plant hormones, ethylene and ABA. The protoplast transfection assays, at least in cellular level, demonstrated our hypothesis indicating that ethylene, increased at aquatic conditions, induces ETHYLENE INSENSITIVE3 (EIN3)-mediated overactivation of abaxial genes, KANs, and suppression of STOMAGEN (STO) and VASCULAR-RELATED NAC-DOMAIN7 (VDN7), which cause cylindrical leaf morphology, lack of stomata, and reduced xylem development, three hallmarks of aquatic plants. In contrast, ABA, increased at terrestrial conditions, establishes leaf polarity through ABSCISIC ACID INSENSITIVE3 (ABI3)-mediated activation of adaxial genes, HD-ZIPIIIIs, in adaxial side of the leaves, and induces STO and VDN7 for the development of stomata and vessel elements. In addition, we show that molecular changes have occurred in the expressions of ABA biosynthetic gene and leaf polarity genes in aquatic *R. trichophyllus* compared to a land plant relative, *R. sceleratus*.

**Results**

**Morphological traits showing heterophylly of *R. trichophyllus***

*Ranunculus trichophyllus* is an amphibious plant that grows both on land and under water. Depending on their growth environments, they develop morphologically different types of leaves (Fig 1). Under terrestrial conditions, *R. trichophyllus* develops thick and broad leaves whereas under aquatic conditions it produces thin and cylindrical leaves (Fig 1A). The leaf index, a leaf length-to-width ratio, in aquatic leaves was approximately 10-fold higher than that in terrestrial leaves (Fig 1Ai), which is an indicative of slender appearance of aquatic leaves. Microscopic analyses showed that terrestrial leaves have well-developed stomata, particularly on adaxial surfaces, whereas aquatic leaves completely lack stomata (Fig 1Ab, c, f and g). When comparing cell structure, terrestrial leaves showed stout and irregular-shaped epidermal cells, whereas aquatic leaves showed slender and rectangular epidermal cells. We also observed that terrestrial leaves have a higher number of developed vessel elements than aquatic leaves (Fig 1Ad and h). In contrast, a close sister species *R. sceleratus*, which lives near the waterside, does not show such heterophylly (Fig 1B). The leaf index and leaf morphology of *R. sceleratus*, were not affected by 1 week submersion. In addition, stomata density and the number of
Fig 1. Heterophyllic leaf developments depending on environments are shown in *R. trichophyllus* but not in sister species, *R. sceleratus*. Seedling morphologies and microscopic images of *R. trichophyllus* (A) and *R. sceleratus* (B) grown under aerial vs aquatic environments. Seeds of *R. trichophyllus* or *R. sceleratus* were germinated on solid MS media for 1 week, then transferred to aerial or aquatic environments. The true leaves produced at 7 days after transference were used for analysis. (a-d) terrestrial and (e-h) aquatic/submerged plants, (b, c) cell shapes of terrestrial leaves and (f, g) those of aquatic/submerged leaves, (d) vasculature of terrestrial and (h) that of aquatic leaves. (i-k) Statistical analyses of leaf indices (i), stomatal densities (j), and number of vessel elements (k) in terrestrial and aquatic/submerged leaves. Data are collected from 24 individual samples and presented as means ± SD from three biological replicates. Black arrowheads denote stomata and white arrowheads denote individual vessel element.

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vessel elements were little changed by submergence (Fig 1B). Moreover, *R. scleratus* showed severe growth retardation by 3 weeks of long-term submergence, which is similar to *Arabidopsis thaliana* (S1 Fig). It suggests that the heterophyllic response of *R. trichophyllus* is evolutionarily adaptive trait for long-term submergence.

**Transcriptomic analysis of *R. trichophyllus***

To understand the molecular basis of heterophylly in *R. trichophyllus*, we performed quantitative whole gene expression analysis using terrestrial leaves vs aquatic leaves by RNA sequencing. A total of 77,459 transcripts were analyzed, and ca. 15.8% of transcripts were up- or down-regulated in aquatic leaves compared to terrestrial leaves (Fig 2A). In general, the genes involved in the response to internal and external stimuli and stress-response genes showed significant up-regulation in aquatic plants (Fig 2B). Among the Gene Ontology (GO) terms related to stress response, ‘response to hypoxia’ (GO:0001666), ‘defense response’ (GO:0006952), ‘response to osmotic stress’ (GO:0006970) were prominent for up-regulation in aquatic plants, which may have evolved to protect plants from environmental stimuli and hypoxia stress in aquatic environments. We also found that genes involved in stomata and vascular developments were considerably down-regulated in aquatic leaves, which reflect lack of stomata and underdeveloped vessel elements. Moreover, well-known pathogen-resistance genes were up-regulated and genes for wax biosynthesis were down-regulated (Fig 2C). More importantly, the transcriptome analysis clearly pointed out that the two plant hormones, ethylene and ABA, are related to heterophyllic leaf development (Fig 2D). Such transcriptional changes may be required for evolutionary adaptation into aquatic environments. To test this hypothesis, we analyzed the effects of submergence on the expressions of the orthologous genes from *R. scleratus*. In contrast to *R. trichophyllus*, the orthologous genes from *R. scleratus* showed no significant differential expression in response to submergence (S2 Fig).

**Hormonal regulation of heterophyllic development**

In addition to our transcriptome analysis, there are studies showing that ethylene, and GA can cause land-grown amphibious plants to develop an aquatic leaf-like morphologies [29, 30]. Thus, we wondered if any of the plant hormones effect on the heterophyllic development of *R. trichophyllus* seedlings (Fig 3). We found that exogenous ethylene treatment of terrestrial plants caused an increase of the leaf index, reduced number of stomata and vessel elements, whereas treatment of the aquatic leaves with silver nitrate (AgNO₃), an inhibitor of ethylene biosynthesis, caused the opposite effects such that decreased leaf index and increased the number of stomata and vessel elements (Fig 3A). In contrast, when aquatic plants were treated with ABA, the leaf index was dramatically reduced whereas the numbers of stomata and vessel elements were increased (Fig 3B). GA treatment on the terrestrial plants did not reduce the number of stomata and vessel elements (Fig 3D and 3E). Likewise, paclobutrazol (PBZ), an inhibitor of GA biosynthesis, did not affect to stomata and vasculature development even though the leaf index was decreased (Fig 3C–3E). In addition, auxin and brassinosteroid (BR) treatments caused almost no effect (S3 Fig). The results suggest that aquatic leaf morphologies of *R. trichophyllus* are dependent on ethylene whereas terrestrial ones are dependent on ABA. GA, auxin, and BR do not appear to be involved in the heterophylly of *R. trichophyllus*. In contrast to *R. trichophyllus*, *R. scleratus* did not show any morphological changes in response to ethylene and ABA (S4 Fig), indicating that ethylene and ABA signaling could control leaf development in *R. trichophyllus* but not in *R. scleratus*. 
ABA and ethylene mediate heterophyllic development

Since the treatments of plant hormones indicated that ABA and ethylene mediates heterophyllic leaf development of *R. trichophyllus*, we analyzed the contents of ABA and ethylene in...
terrestrial and aquatic leaves (Fig 4A and 4B). As expected, terrestrial leaves contained 3 times higher level of ABA than aquatic leaves whilst aquatic leaves contained 4 times higher level of ethylene than terrestrial leaves. Then, we checked if expressions of any specific genes encoding the enzymes involved in the critical steps of ABA and ethylene biosynthetic pathways are
Fig 4. Differential expression of ethylene and ABA-related genes under terrestrial and aquatic environments is specific to amphibious *R. trichophyllus*. (A and B). Contents of ethylene (A) and ABA (B) in terrestrial vs aquatic leaves. (C and D). Comparison of transcript levels of ethylene- (A) and ABA- (B) biosynthesis and responsive genes following submergence into water. Plants harvested at 7 hours (7h), 1 day (1d), and 2 days (2d) after submergence were compared with terrestrial and aquatic plants for expressions. (E) Effects of submergence on the expression levels of AAO3 genes in *R. trichophyllus*, *Arabidopsis*, and *R. sceleratus*. For submergence, two weeks old plants grown on solid MS media were submerged into water for 5 days for RNA extraction. The data represent means ± standard error from three biological and two technical replicates.

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differentially regulated according to environments. The gene encoding enzyme for critical step of ABA biosynthesis is ABA Aldehyde Oxidase (AAO) and that for ethylene biosynthesis is ACC Oxidase (ACO) [31, 32]. Thus, we cloned the orthologs of AAO and ACO from R. trichophyllus and compared the expression levels depending on the growth condition (Fig 4C and 4D). Consistent with the hormonal contents, terrestrial plants showed higher expression of RtAAO than aquatic plants whereas aquatic plants showed higher expression of RtACO than terrestrial plants in general. For ethylene biosynthesis, RtACO4B and RtACO4C showed remarkable increase in aquatic plants compared to terrestrial plants (Fig 4C). To address if submergence of land plants into water causes rapid changes in the expression of ABA/ethylene biosynthesis genes, we checked dynamic expressions of the genes at 7 hours, 1 day, and 2 days after submergence. The results showed that ethylene-biosynthesis genes, RtACO4B and RtACO4C, showed rapid increase within 7 hours, then slow increase until 2 days after submergence (Fig 4C). In case of ABA-biosynthesis genes, RtAAO1 and RtAAO3, the transcript levels were highly decreased within 7 hours of submergence but slowly increased afterwards (Fig 4D). We also checked if genes responsive to ABA and ethylene are increased in terrestrial and aquatic leaves of R. trichophyllus respectively. As expected, aquatic leaves showed higher expression of ethylene-responsive genes whereas terrestrial leaves showed higher expression of ABA-responsive genes (Fig 4C and 4D). Moreover, dynamic expression patterns of ABA/ethylene responsive genes after submergence showed rapid changes within 7 hours after submergence. Such results suggest that aquatic condition triggers in vivo ethylene signaling cascades and suppresses ABA signaling pathway.

In addition, we found that although submergence of R. trichophyllus into water rapidly downregulates expression of the ABA biosynthesis gene, RtAAO3 (ABA-aldehyde oxidase), expression of AtAAO3, an Arabidopsis ortholog, is not reduced, instead increased by submergence, perhaps due to hypoxic stress (Fig 4E). In R. sceleratus, a waterside plant, the expression of ortholog, RsAAO3, was reduced relatively weakly by submergence (Fig 4E). This result suggests that the suppression of ABA biosynthesis in aquatic environments is an evolutionary adaptation developed in amphibious R. trichophyllus.

**Differential expression of leaf polarity genes according to growth environments**

To address the molecular mechanism behind heterophyllic leaf development, we explored the roles of several leaf development genes in the differential leaf morphologies in aquatic and terrestrial environments. Since the leaf structure and morphology is mainly governed by leaf polarity genes, we cloned three KAN and three HD-ZIPIII homologs, which determine abaxial and adaxial identity, respectively [14–16]. We named these genes KANa, -b, and -c, and HD-ZIPIIIa, -b, and -c (S5 Fig). Overexpression of RtKANa and RtHD-ZIPIIIa in Arabidopsis caused narrow or curling leaf morphology, which phenocopied the transgenic lines overexpressing Arabidopsis homologs (S5C Fig) [14, 16, 33]. The expression of the abaxial genes, RtKANs, was much higher in aquatic than in terrestrial leaves, suggesting that RtKANs are overexpressed in aquatic environments. In contrast, expression of adaxial genes, RtHD-ZIPIIIis, was significantly reduced in aquatic leaves (Fig 5A and 5B). In situ hybridization showed that RtKANa expression is mainly detectable around the midvein and abaxial side, but is not detectable in the adaxial side of terrestrial leaves (Fig 5F and S6 Fig). However, strong expression of RtKANa throughout aquatic leaves was observed (Fig 5H). In contrast, the expression domain of RtHD-ZIPIIIa was confined to the adaxial side of terrestrial leaves and was barely detectable in aquatic leaves (Fig 5J and 5L). These findings strongly support the hypothesis that the axial expressions of RtKANs and RtHD-ZIPIIIis are perturbed in aquatic leaves. In contrast to
Ranunculus trichophyllus, Arabidopsis and R. sceleratus did not show any such alteration of the polarity gene expression following submergence (S7 Fig), indicating that these traits are acquired during re-adaptation to water.

Next, we investigated whether the expressions of leaf polarity genes are affected by ethylene and ABA. Under the terrestrial condition, treatment of plants with ethylene resulted in increased expression of the abaxial genes, \( \text{RtKANs} \), thus, phenocopying aquatic leaves. However, the expression of adaxial genes, \( \text{RtHD-ZIPIIIs} \), were not significantly affected (Fig 5C). Under aquatic condition, treatment of plants with ABA and AgNO\(_3\) led to increased expression of \( \text{RtHD-ZIPIII} \) genes, although no remarkable decrease in \( \text{RtKAN} \) expression was observed (Fig 5D). These results support the hypothesis that ethylene activates \( \text{RtKANs} \), whereas ABA activates \( \text{RtHD-ZIPIIIs} \) as shown in the model (Fig 5E). The RNA expression pattern observed by \textit{in situ} hybridization also supported these results: ethylene treatment increased the expression domain of \( \text{RtKAN} \) in terrestrial leaves whereas ABA treatment increased that of \( \text{RtHD-ZIPIIIa} \) in aquatic leaves (Fig 5I and 5M).

**Hormonal signaling cascades to leaf polarity genes**

To address if ABA and ethylene directly regulate leaf polarity genes, \( \text{RtHD-ZIPIIIa} \) and \( \text{RtKANs} \), we developed a \textit{Ranunculus} protoplast transient expression assay using seedlings grown on solid MS media. The promoters of \( \text{RtKANa} \) and \( \text{RtHD-ZIPIIIa} \) were fused to the...
luciferase reporter gene (LUC) and tested for their response to ethylene and ABA in transiently transfected protoplasts. As expected, RtKANa promoter was rapidly induced by ethylene but was not affected much by ABA whereas the RtHD-ZIPIIIa promoter was strongly induced by ABA but was not significantly affected by ethylene (Fig 6A and 6D). This indicates that ethylene and ABA directly regulate the promoters of RtKANa and RtHD-ZIPIIIa respectively.

Then, we searched for candidate transcription factors that might mediate the ethylene and ABA signaling by directly acting on the promoters of RtKANs and RtHD-ZIPIIIIs. Interestingly, when RtEIN3 is cotransfected with proKANa-LUC into protoplasts, it caused strong activation of luciferase activity. Similarly, when RtABI3 is cotransfected with proHD-ZIPIIIa-LUC, it caused strong activation of luciferase activity (Fig 6B and 6E). This indicates that RtEIN3 and RtABI3 directly activate RtKANs and RtHD-ZIPIIIIs respectively. Consistently, transfected RtEIN3 increased expressions of all three endogenous RtKAN genes and transfected RtABI3 increased all of RtHD-ZIPIIIIs (Fig 6C and 6F), which supports the model shown in Fig 4E.

Finally, we wondered if ethylene and ABA signalings directly control heterophyllic leaf development through transcriptional cascades. Thus, we transfected upstream transcription factors, EIN3, ABI3, and HD-ZIPIII into protoplasts, then checked the expression of two key regulators controlling stomata and vessel developments; STO, encoding a peptide protein turning on stomatal development[34], and VDN7, encoding a NAC domain transcription factor controlling vascular development.[35] RtSTO and RtVND7 were down-regulated in aquatic leaves, which are consistent with the lack of stomata and reduced number of vessel elements in aquatic leaves (Fig 6G). Protoplast transfection assays showed that transient overexpression of RtABI3 or RtHD-ZIPIIIa increases transcript levels of RtSTO and RtVND7, suggesting that stomata and vessel developments in terrestrial leaves are controlled by an ABI3-RtHD-ZIPIIIa regulatory module (Fig 6H). In contrast, overexpression of RtEIN3 decreases transcript levels of RtSTO and RtVND7. Taken together, ethylene and ABA signaling control leaf polarity, stomata development and vascular development, the three hallmarks of heterophyllic development in R. trichophyllus.

Cold and hypoxia induce aquatic leaf development

It has been reported in some species of amphibious plants that certain environmental conditions such as cold cause aquatic leaf development mimicking aquatic condition [36]. Thus, we checked if any environmental conditions cause aquatic leaf development in R. trichophyllus (Fig 7). We found that 4°C cold temperature and hypoxia (less than 1% O2 concentration) caused significant increase of leaf index. In addition, the plants grown at cold temperature showed lack of stomata and decrease of vessel numbers, indicating that cold temperature mimics the aquatic condition well. However, hypoxia caused reduced number of stomata and vessel elements, suggesting that hypoxia mimics aquatic condition partially (Fig 7A). Then, we checked if cold and hypoxia effect on the expressions of leaf polarity genes similar to aquatic condition (Fig 7B and 7C). As expected, expression of KAN genes was higher whereas that of HD-ZIPIII genes was lower in the plants grown under cold temperature compared to room temperature. Consistent with the phenotypic effect, hypoxia caused less effects on the expression of both polarity genes than cold temperature. This result suggests that environmental cues inducing aquatic leaf development also cause similar molecular changes in R. trichophyllus.

Discussion

Although embryophytes, land plants, have evolved from water to land by acquiring land adaptation such as vascular development and broad-leaf morphology, diverse plant species from
Fig 6. Effects of ethylene and ABA signalings on the promoter activation of leaf polarity genes and genes critical for stomata and vascular developments. (A and B) Luminescence analysis of proRtKANa::LUC when added with ACC, an ethylene precursor, or ABA in protoplast solution (A), control; without chemical treatment, and when cotransfected with CFP-RtEIN3 in protoplasts (B), control; cotransfected with CFP construct. (C) Effects of CFP-RtEIN3 transfection on the relative expressions of endogenous KAN genes in protoplasts, control; transfected with CFP construct. (D and E) Luminescence analysis of proHD-ZIPIIIa::LUC when added with ACC or ABA in protoplast solution (D), control; without chemical treatment, and when cotransfected with CFP-RtABI3 in protoplasts (E), control; cotransfected with CFP construct. (F) Effects of CFP-RtABI3 transfection on the relative expressions of endogenous HD-ZIPIII genes in protoplasts, control; transfected with CFP construct. (G) Relative transcript levels of RtSTO and RtVDN7, encoding critical regulators of stomata and vascular developments, when transfected with Rt-EIN3, Rt-ABI3, and RtHD-ZIPIIIa fused with CFP coding sequence. Control; transfected with CFP construct. (H) Comparison of transcript levels of RtSTO and RtVDN7 between terrestrial and aquatic leaves of *R. trichophyllus*. *P < 0.05; **P < 0.01; ***P < 0.001 (unpaired Student’s t-test).
different phylogenetic clades have returned to aquatic environments, indicating that water re-adaptation is quite common [21, 37]. *R. trichophyllus* is an amphibious plant, an evolutionary bridge between land and aquatic plants, and produces typical aquatic leaves if grown under water. In this report, we show that the heterophyllic leaf development of this plant is mainly determined by ABA and ethylene signalings which regulate leaf polarity genes. In aquatic environments, ethylene level is increased and the ethylene signaling overactivates the expression of abaxial genes, *RtKAN* genes, which antagonistically suppresses adaxial genes, *RtHD-ZIPIII* genes.

![Table A](https://doi.org/10.1371/journal.pgen.1007208.g007)

| Condition           | Leaf index | Stomatal distribution (mm²) | Vessel numbers |
|---------------------|------------|-----------------------------|----------------|
| Room Temp. (22°C, 20% O₂) | 5.47±0.47  | 71.4±9.61                   | 4.56±1.01      |
| Cold (4°C)          | 48.4±13.0  | 0.00±0.0                    | 1.67±0.49      |
| Hypoxia (1% O₂)     | 17.6±4.94  | 0.61±1.59                   | 2.17±0.51      |

![Fig 7](https://doi.org/10.1371/journal.pgen.1007208.g007)

Fig 7. Cold and hypoxia induce aquatic leaf development in *R. trichophyllus*. (A) Heterophylly induced by cold and hypoxia. 1 week-old seedlings after germination on the MS media were transferred to cold chamber (4°C) for 1 month or hypoxia chamber (1% O₂) for 2 weeks. The column Room Temp is a control at 22°C with 20% O₂. (B and C) Gene expression analyses of *KAN* genes (B), and *HD-ZIPIII* genes (C) in the leaves after cold for 1 month and hypoxia for 2 weeks treated. *P < 0.05; **P < 0.01 (unpaired Student's t-test).”

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overexpression of abaxial genes is most likely the molecular mechanism behind the cylindrical shape of aquatic leaves. In contrast, in terrestrial environments, ABA level is increased and ABA signaling activates the expression of adaxial genes, *RtHD-ZIPIII*, which establishes adaxial-abaxial polarity and causes broad leaf development (Fig 4E).

ABA is a well-known stress hormone in plants; it is accumulated by various abiotic and biotic stresses and confers resistance against them [38, 39]. Since flooding is also a stress to land plants, it is plausible that myriad of land plants show increased levels of ABA after submergence [40, 41]. Such ABA accumulation seems to be evolutionarily adaptive to land plants because it renders the plants to adopt ‘stunt strategy’; enduring the flooding period by inducing growth retardation which restricts energy consumption [42]. However, submergence-tolerant species such as deepwater rice and *Rumex palustris* have evolved differently. They show the opposite response to submergence in which ABA contents decreased [6, 43]. It indicates that reducing ABA level is adaptive to aquatic environments in some plant species. Consistent with this, ABA contents and the expression levels of *RtAAO*, a gene involved in a critical step of ABA biosynthesis, are decreased in *R. trichophyllus* in aquatic condition. It is likely that the suppression of ABA biosynthesis under water is widely occurred among submergence-tolerant plants and *R. trichophyllus* has also adopted similar evo-devo mechanism during evolution for aquatic adaptation.

Increase of ethylene level by flooding is also observed among various plant taxa, which is achieved by the enhancement of biosynthesis or local entrapment by submergence [7]. In addition, in wide range of plants from moss to Arabidopsis, the treatment of exogenous ethylene mimics submerged growth [44, 45]. Therefore, it is likely that the activation of ethylene signaling is a widely conserved response to submergence in plants. Consistently, ethylene biosynthesis and signaling are increased in *R. trichophyllus* by submergence. Interestingly, the antagonistic interaction between ABA and ethylene found in heterophylly of *R. trichophyllus* is also observed in many developmental processes in plants. For example, hyponastic growth of leaf in submerged *R. palustris* is regulated by the antagonistic interaction of ABA and ethylene [8]. Therefore, heterophylly of *R. trichophyllus* seems to be evolved from the common mechanism of ABA/ethylene interaction observed in other land plants.

Based on the roles of leaf polarity genes known in *A. thaliana*, differential expressions of leaf polarity genes in *R. trichophyllus* seem to lead to the three developmental changes which are required for adaptation to aquatic environments. That is, cylindrical shape leaves and reduced numbers of stomata seem to be caused by overactivation of the abaxial genes, *RtKAN*s, as ectopic expression of KAN1 or KAN2 in Arabidopsis throughout the leaf primordia results in abaxialized radial organs, with a concomitant loss of *HD-ZIPIII* expression [14, 16]. Subsequently, the loss of *RtHD-ZIPIII* seems to cause reduced number of vessel elements, similar to loss-of-function of *HD-ZIPIII* genes in Arabidopsis showing reduced xylem [14]. Recent reports showed that a KNOX-GA module is critical for the heterophylic development of *Rhipippa aquatica* [36], which is a different mechanism than that we have found in *R. trichophyllus*. KNOX-GA module seems not to be a main mechanism for the heterophyll in *R. trichophyllus*. First, morphological pattern of heterophylly is not similar in the two species (S8A Fig). Heterophyll of *R. aquatica* is achieved by deep serration of leaves, thus it changes simple leaves to dissected compound leaves. In contrast, in case of *R. trichophyllus*, leaf complexity is increased in both terrestrial and aquatic leaves during growth and leaf branching pattern is not significantly different between terrestrial and aquatic leaves (S8A Fig). Instead, heterophyll of *R. trichophyllus* is achieved by leaf elongation and radialization rather than leaf serration. Second, GA has little effect on the heterophyll of *R. trichophyllus* although it is a main participant of the heterophyll in *R. aquatica* (Fig 3D and 3E). Thus, the heterophylic developments in *R.
trichophyllus and *R. aquatica* have adopted different mechanisms, indicating that convergent evolution has occurred.

In spite of such differences in overall architecture, there is some convergent point between *R. trichophyllus* and *R. aquatica*. Like *R. aquatica*, aquatic leaves were generated by cold environment in *R. trichophyllus* (Fig 7). It is still unclear why amphibious plants induce aquatic leaf development in response to cold stress. Wells and Pigliucci (2000) proposed an “anticipatory plasticity hypothesis” in which plants can show similar phenotypic plasticity in response to different external cues coming together in nature. For example, submergence into water in nature causes combination of changes in diverse environmental cues, e.g. humidity, temperature, changes in light quality, etc. Thus, cold and hypoxia-induced molecular changes in the expression of leaf polarity genes in *R. trichophyllus* seem to be supportive to our hypothesis that changes in leaf polarity drive the evolution of amphibious adaptation in *R. trichophyllus*. The more direct evidence will be obtained through the analyses of mutants and transgenic plants which show defects in the heterophyllic development. In that sense, the recent report suggesting *Hygrophilia difformis* as a model plant to study heterophyll of amphibious plant is interesting [46]. *H. difformis* is easy to grow and propagated vegetatively well and above all, it can be easily transformed by *Agrobacterium tumefaciens*. However, the molecular basis of heterophyll of *H. difformis* is similar to that of *R. aquatica* but is different with that of *R. trichophyllus*. For example, in *H. difformis*, GA is a major regulator determining heterophyll and aquatic leaf development is achieved by deep serration of leaves, thus changing simple leaves to dissected compound leaves. Such characteristics are very similar with those of *R. aquatic* but dissimilar to those of *R. trichophyllus*. Therefore, *H. difformis* as a model plant for amphibious plants is limiting.

From comparative studies using two land plants, *A. thaliana* and *R. sceleratus*, and one amphibious plant, *R. trichophyllus*, we found that at least two molecular changes have occurred in *R. trichophyllus* during evolutionary adaptation to aquatic environments; the suppression of ABA biosynthesis and abaxialization of leaf development. Since ABA signaling components and the regulatory mechanism of HD-ZIPIII transcription factors seem to have evolved during land colonization by plants [24, 47], such molecular changes observed in *R. trichophyllus* are suggestive of evolutionary trend rearranging pre-existing gene networks instead of generating novel one [48]. Since many aquatic plants share similar morphological traits observed in submerged *R. trichophyllus*, further analysis of this plant will provide deep insight into the understanding of convergent evolution occurred in aquatic plants.

### Methods

#### Plant materials and growth conditions

Seeds of *Ranunculus trichophyllus* var. *kadzusensis* were collected from its native habitat at Ganghwa Island, South Korea. Seeds of *Ranunculus sceleratus*, collected from Namyangju City, were donated from the Korea National Arboretum. Seeds were sterilized with 70% ethanol and with 1% NaOCl and 0.5% Tween-20 solution. Seeds were sown on half-strength Murashige-Skoog (MS) medium containing 50 μM carbenicilin, 75 μM cefotaxim, and 1% agar. Seeds of *R. trichophyllus* and *R. sceleratus* were germinated on solid MS media for 1 week, at that time root radicles were just protruded. Then germinated seedlings are transferred to aerial or aquatic/submerged environments. The true leaves produced at 7 days after transference were used for morphological analysis and RNA expression analysis. For transcriptome analysis, the plants 10 days after transference were used for RNA expression. In case of Arabidopsis, 4 days-old seedlings after germination were transplanted, then submerged into water for 2 weeks. For
Arabidopsis thaliana, Col-0 seeds were used. The growth room was maintained at 22°C, 60 ± 10% relative humidity in long day photoperiod (16h light/8h dark).

RNA sequencing analyses

cDNA libraries were obtained using 1 μg of total RNA extracted from whole plant tissues of *R. trichophyllus*. 100 base pair paired-end libraries were sequenced by Illumina HiSeqTM 2000. The libraries were quantified according to the qPCR Quantification Protocol Guide and qualified using an Agilent Technologies 2100 Bioanalyzer. RNA-seq reads were de novo assembled and mapped using Trinity and TopHat programs and the relative transcript levels were calculated by FPKM (Fragments Per Kilobase of exon per Million fragments mapped) using Cufflinks software. Excluded transcripts were filtered with 1 FPKM value and transformed to logarithm scale. They are normalized by quantile normalization method. Transcripts were assigned a putative function, then gene ontology analysis was performed by using DAVID tool (http://david.abcc.ncifcrf.gov/).

Hormone treatments

One-week-old seedlings of *R. trichophyllus* were used. The concentrations of hormones used were 10 μM NAA (1-naphthaleneacetic acid, Duchefa, N0903), 50 μM ACC (1-aminocyclopropane-1-carboxylic acid, Sigma Aldrich, A3903), 1 μM EBL (epi-brassinolide, Sigma Aldrich, E1641), 50 μM bikinin (Sigma Aldrich, SML0094) and 10 μM gibberellin (GA, bio-World, 714248), respectively. For submerged treatment, abscisic acid (ABA, Sigma Aldrich, A0149), silver nitrate (AgNO₃, Sigma Aldrich, S8157), and paclobutrazol (PBZ, Sigma Aldrich, 46046) were added into the aquatic media. The concentrations used were 1 μM ABA, 10 μM AgNO₃, and 10 μM PBZ, respectively. After 10 days of growth, the first true leaves from the seedlings were analyzed.

Stomata and vessel elements

For whole mount clearing, first true leaves were soaked in clearing solution (2.5 g chloral hydrate; 0.3 ml 100% glycerol; 0.7 ml distilled water), then incubated for 3 h at 55°C. The epidermis and xylem elements were observed using an Axio Imager A1 microscope (Carl Zeiss) under DIC optics. Images were captured using an AxioCam HRc camera (Carl Zeiss).

Hormonal contents

Seedlings (using about 20 individual seedlings) were grown in MS with or without 150 ml distilled water. Using 3 ml disposable syringe, we harvested capped air in headspace containing ethylene, then sealed by parafilm. Using Hamilton syringe, 100 μl gas was extracted from sealed air, then feeding to gas chromatography with flame ionization detector (Agilent 7890B GC). We used HP-5 column (#19091J-413, Agilent). Ethylene production was normalized by seedling weight. For measuring ABA contents, we used ABA ELISA kit (CSB-E09159Pl). Intensity of 450 nm fluorescence was determined by using Plate reader-Powerwave X (Bio-Tek). ABA production was normalized by sample weight. The measurements were performed from three biological replicates and two technical replicates each.

Sequencing of orthologous genes

Candidate genes were selected using information of *A. thaliana* and the TAIR database (www.arabidopsis.org). The Arabidopsis sequences were used to search for orthologous genes from Aquilegia coerulea, for which the genome database (http://www.phytozome.net/search.php?)
method=Org_Acoerulea) is available. *Aquilegia coerulea* is the closest relative to the Ranunculus genus among plants that have an available sequence database.

**Gene expression studies using real-time PCR**

For real-time qPCR, total RNA was isolated from leaves using TRI reagent (Sigma Aldrich, T9424) and RNeasy Plant Mini Kit (Qiagen, 74904). cDNA was generated using 4 μg of total RNA, 5 unit of reverse transcriptase (Fermentas, EP0442), 4 μl of 2.5 mM dNTP, 2 μl of 50 mM oligo(dT), and ddH2O to 40 μl. For real-time qPCR, 0.3 μl of synthesized cDNA was mixed with 2 μl of 5 μM primers and 10 μl of SYBR Green qPCR Master Mix (Bio-Rad), and ddH2O to 20 μl. Real-time qPCR analysis was performed by CFX96 Real-Time PCR system (Bio-Rad). The relative transcript levels were calculated according to the ΔΔCt method. [49]

**Protoplasts transfection assay**

Leaflets of *R. trichophyllus* which was grown on short days (8 h light/16 h dark, 22˚C) were used for the isolation and transfection of protoplasts. The method of transfection was based on previously described.[50] For transfection, we used 10% PEG final concentration. After 1 day of incubation, the protoplasts were harvested for real-time qPCR and luciferase activity assays. For determining promoter activity, we used luciferase assay system (Promega, E1500) and microplate luminometer (Berthold).

**Phylogenetic analyses**

Multiple alignment of amino acid sequences was performed using the ClustalX2.1 program (http://www.clustal.org/download/current/), which generates aligned phy format files. These aligned files were passed through the PHYLIP program (version 3.69) for phylogenetic analyses (http://evolution.genetics.washington.edu/phylip.html). In the PHYLIP software, SEQBOOT, PROTDIST, NEIGHBOR, and CONSENSE programs were run sequentially to generate draft unrooted phylogenetic trees and to obtain bootstrap values. The phylogenetic tree was drawn using the TreeView program (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

**Whole mount in situ hybridization**

All in situ hybridization experiments were performed as described previously.[51] For signal detection using NBT/BCIP (Roche, 11681451001), 100 ng of DIG-labelled RNA probes per mL of ALP buffer was used for hybridization. The images were obtained by light microscopy.

For fluorescence detection using HNPP (2-hydroxy-3-naphtoic acid-2’-phenylanilide phosphate, Roche, 1758888001), leaves were hybridized with DIG-labelled RNA probes, then stained with a mixture of 10 μl HNPP and 0.25 mg Fast Red TR solution per mL in ALP buffer containing 2 mM levamisole for 30 min at room temperature. Leaves were washed in distilled water for 10 min and incubated with 0.2 μg per mL DAPI (4,6-diamidino-2-phenylindole) for 10 min at RT for nuclear counter-staining. Fluorescence was detected by confocal laser scanning microscopy (LSM700, Carl Zeiss).

**Statistical analysis**

Statistical analyses were performed using an unpaired Student’s *t*-test. For multiple comparisons, we used a one-way ANOVA and post-hoc test. We considered *P* < 0.05 as statistically significant. All statistical analyses were performed using the statistical package R.[52]
Supporting information

S1 Fig. Effects of long-term submergence on the three plants, *R. trichophyllus*, *R. sceleratus*, and Arabidopsis. (A) Cartoon depicting habitats of *R. trichophyllus*, *R. sceleratus*, and *A. thaliana*. (B) Effects of long-term submergence on the plant growth. For *R. trichophyllus* and *R. sceleratus*, 1 week-old seedlings after germination on the MS media were transferred to aquatic condition for 3 weeks. For Arabidopsis, 4 day-old seedlings after germination were transferred to aquatic condition for 2 weeks.

(TIF)

S2 Fig. Comparison of gene expressions between terrestrial vs aquatic/submerged plants of *R. trichophyllus* (A) and *R. sceleratus* (B). Differential expressions of genes affiliated to GO terms for defense, wax, stomata, and vasculature were compared. The ortholog of *RtPDF1.4* for *R. sceleratus* could not be cloned. For submergence, two weeks old plants grown on solid MS media were submerged into water for 5 days for RNA extraction.

(TIF)

S3 Fig. Effects of auxin and brassinosteroid on the leaf index of terrestrial leaves of *R. trichophyllus*. (A) Effect of auxin agonist, NAA, on the leaf index. (B) Effects of brassinosteroid inhibitor, EBL, and an agonist, bikinin, on the leaf index. The data are presented as means ± SD from three biological replicates.

(TIF)

S4 Fig. Effects of ethylene and ABA on the leaf morphologies of *R. sceleratus*. (A) Land-grown plants treated with (+: lower panel) or without (control: upper panel) ethylene precursor ACC. 1 week-old seedlings were treated with ethylene, then analyzed after 7 days. (B) Submerged plants treated with (+: lower panel) or without (control: upper panel) ABA. 1 week-old seedlings were submerged into ABA-containing water, then analyzed after 10 days. Images from left to right, seedling morphologies, microscopic structure of cell shapes, vessel elements in petiole. Black arrowhead denotes stomata, white arrowhead denotes vessel elements.

(TIF)

S5 Fig. Phylogenetic trees of orthologs of KAN and HD-ZIPIII gene families. (A and B) The families of KAN (A) and HD-ZIPIII (B) genes from *R. trichophyllus* and *R. sceleratus* were aligned with those of *A. thaliana* based on amino acid sequences. *AtPSR1* and *AtML1* were used as out-groups. Bootstrap values are denoted beside branch nodes. Only values greater than 75 are presented. (C) Overexpression lines of *RtKAN* and *RtHD-ZIPIII* genes can provoke abnormal leaf formation. 2 weeks-old seedlings were pictured.

(TIF)

S6 Fig. Whole mount in situ hybridization of *RtKANA* and *HD-ZIPIIIa* by HNPP staining. DAPI is in blue and HNPP signal for *RtKANA* or *RtHD-ZIPIIIa* is in red. The right panels show the merged fluorescence of DAPI and HNPP. A-C. Hybridization signals for *RtKANA* detected in terrestrial leaves (A), aquatic leaves (B), and terrestrial leaves treated with ethylene (C). D-F. Hybridization signals for *RtHD-ZIPIIIa* detected in terrestrial leaves (D), aquatic leaves (E), and aquatic leaves treated with ABA (F). Ab, abaxial side; Ad, adaxial side.

(TIF)

S7 Fig. Expressions of leaf polarity genes from *R. sceleratus* and *A. thaliana* are not affected by submergence. Comparison of transcript levels of KANs and HD-ZIPIIIs from *R. sceleratus* (A) and *A. thaliana* (B) before and after submergence. For submergence, two weeks old plants...
grown on solid MS media were submerged into water for 5 days for RNA extraction.

S8 Fig. Leaf architecture is similar between terrestrial and aquatic leaves. (A) Terrestrial (left panel) and aquatic leaves (right panel) from 1st true leaves. Upper row is 1st to 3rd leaves and lower row is 4th, 5th, and 6th leaves. (B) 1st leaves of terrestrial leaves (left panels) and aquatic leaves (right panels).

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References

1. de Jong M, Leyser O, editors. Developmental plasticity in plants. Cold Spring Harbor symposia on quantitative biology; 2012: Cold Spring Harbor Laboratory Press. https://doi.org/10.1101/sqb.2012.77.014720 PMID: 23250989

2. Palmer AR. Developmental plasticity and the origin of novel forms: unveiling cryptic genetic variation via “use and disuse”. JEZ-B. 2012; 318(6):466–79. https://doi.org/10.1002/jez.b.21447 PMID: 22038780

3. Wells CL, Pigliucci M. Adaptive phenotypic plasticity: the case of heterophylly in aquatic plants. PPEES. 2000; 3(1):1–18. https://doi.org/10.1078/1433-8319-00001

4. Wang Y, Li J. Molecular basis of plant architecture. Annu Rev Plant Biol. 2008; 59:253–79. https://doi.org/10.1146/annurev.arplant.59.032607.092902 PMID: 18444901

5. Wolters H, Jürgens G. Survival of the flexible: hormonal growth control and adaptation in plant development. Nat Rev Genet. 2009; 10(5):305–17. https://doi.org/10.1038/nrg2558 PMID: 19360022

6. Cox MCH, Benschop JJ, Vreeburg RAM, Wagemakers CAM, Moritz T, Peeters AJM, et al. The roles of ethylene, auxin, abscisic acid, and gibberellin in the hyponastic growth of submerged \textit{Rumex palustris} petioles. Plant Physiol. 2004; 136(2):2948–60. https://doi.org/10.1104/pp.104.049197 PMID: 15466223

7. Jackson MB. Ethylene-promoted elongation: an adaptation to submergence stress. Ann Bot. 2008; 101(2):229–48. https://doi.org/10.1002/aobc.100237 PMID: 17956854

8. Voesenek L, Benschop J, Bou J, Cox M, Groeneveld H, Millenaar F, et al. Interactions between plant hormones regulate submergence-induced shoot elongation in the flooding-tolerant dicot \textit{Rumex palustris}. Ann Bot. 2003; 91(2):205–11. https://doi.org/10.1003/aobc.1769 PMID: 12509341

9. Kende H, Van Der Knaap E, Cho H-T. Deepwater rice: a model plant to study stem elongation. Plant Physiol. 1998; 118(4):1105–10. https://doi.org/10.1104/pp.118.4.1105 PMID: 9847084
10. Nicotra AB, Leigh A, Boyce CK, Jones CS, Niklas KJ, Royer DL, et al. The evolution and functional significance of leaf shape in the angiosperms. Funct Plant Biol. 2011; 38(7):535–52. https://doi.org/10.1071/FP11057

11. Zotz G, Wilhelm K, Becker A. Heteroblasty—a review. Bot Rev. 2011; 77(2):109–51.

12. Kidner CA, Timmermans MC. Signaling sides: adaxial–abaxial patterning in leaves. Curr Top Dev Biol. 2010; 91:141–68. https://doi.org/10.1016/S0070-2153(10)91005-3 PMID: 20705181

13. Yamaguchi T, Nukazuka A, Tsukaya H. Leaf adaxial–abaxial polarity specification and lamina outgrowth: evolution and development. Plant Cell Physiol. 2012; 53(7):1180–94. https://doi.org/10.1093/pcp/pcs074 PMID: 22619472

14. Emery JF, Floyd SK, Alvarez J, Eshed Y, Hawker NP, Izhaki A, et al. Radial patterning of Arabidopsis shoots by class III HD-ZIP and KANADI genes. Curr Biol. 2003; 13(20):1768–74. https://doi.org/10.1016/S0960-9822(03)00392-X PMID: 12045274

15. Eshed Y, Izhaki A, Baum SF, Floyd SK, Bowman JL. Asymmetric leaf development and blade expansion in Arabidopsis are mediated by KANADI and YABBY activities. Development. 2004; 131(12):2997–3006. https://doi.org/10.1242/dev.01186 PMID: 15169760

16. Kerstetter RA, Bollman K, Taylor RA, Bomblles K, Poethig RS. KANADI regulates organ polarity in Arabidopsis. Nature. 2001; 411(6838):706–9. https://doi.org/10.1038/35079629 PMID: 11395775

17. Siegfried KR, Eshed Y, Baum SF, Otsuga D, Drews GN, Bowman JL. Members of the YABBY gene family specify abaxial cell fate in Arabidopsis. Development. 1999; 126(18):4117–28. PMID: 10457020

18. Xu L, Xu Y, Dong A, Sun Y, Pi L, Xu Y, et al. Novel as1 and as2 defects in leaf adaxial-abaxial polarity reveal the requirement for ASYMETRIC LEAVES1 and 2 and ERECTA functions in specifying leaf adaxial identity. Development. 2003; 130(17):4097–107. PMID: 12874130

19. Fahlgren N, Montgomery TA, Howell MD, Allen E, Dvorak SK, Alexander AL, et al. Regulation of AUXIN RESPONSE FACTOR3 by TAS3a-siRNA affects developmental timing and patterning in Arabidopsis. Curr Biol. 2006; 16(9):939–44. https://doi.org/10.1016/j.cub.2006.03.065 PMID: 16682356

20. Izhaki A, Bowman JL. KANADI and class III HD-Zip gene families regulate embryo patterning and modulate auxin flow during embryogenesis in Arabidopsis. Plant Cell. 2007; 19(2):495–508. https://doi.org/10.1105/tpc.106.047472 PMID: 17307928

21. Kenrick P, Crane PR. The origin and early diversification of land plants. A cladistic study. Smithsonian Institute Press: Washington DC.: A cladistic study. Smithsonian Institute Press; 1977.

22. Raven JA. Selection pressures on stomatal evolution. New Phytol. 2002; 153(3):371–86. https://doi.org/10.1046/j.0028-646X.2001.00534.x

23. Sperry JS. Evolution of water transport and xylem structure. JEPS. 2003; 164(S3):S115–S27. https://doi.org/10.1086/368398

24. Hauser F, Waadt R, Schroeder JI. Evolution of abscisic acid synthesis and signaling mechanisms. Curr Biol. 2011; 21(9):R346–R55. https://doi.org/10.1016/j.cub.2011.03.015 PMID: 21549957

25. Cook CDK. The number and kinds of embryo-bearing plants which have become aquatic: a survey. PPEES. 1999; 2/1:79–102. https://doi.org/10.1071/FP11057

26. Martin-Closas C. The fossil record and evolution of freshwater plants: a review. Geol Acta. 2003; 1(4):315.

27. Hörandl E, Paun O, Johannsson JT, Lehnbach C, Armstrong T, Chen L, et al. Phylogenetic relationships and evolutionary traits in Ranunculus s.l. (Ranunculaceae) inferred from ITS sequence analysis. Mol Phylogenet Evol. 2005; 36(2):305–27. https://doi.org/10.1016/j.ympev.2005.02.009 PMID: 15955512

28. Lumbreras A, Navarro G, Pardo C, Molina J. Aquatic Ranunculus communities in the northern hemisphere: a global review. Plant Biosyst. 2011; 145(sup1):118–22. https://doi.org/10.1080/11263504.2011.602728

29. Deschamp PA, Cooke TJ. Causal mechanisms of leaf dimorphism in the aquatic angiosperm Callitriche heterophylla. Am J Bot. 1984:319–29.

30. Kuwabara A, Tsukaya H, Nagata T. Identification of factors that cause heterophyll in Ludwigia arcuata Walt. (Onagraceae), Plant Biol. 2001; 3(1):98–105. https://doi.org/10.1055/s-2001-11748

31. Seo M, Koshiba T. Complex regulation of ABA biosynthesis in plants. Trend Plant Sci. 2002; 7(1):41–8. https://doi.org/10.1016/S1360-1385(01)02187-2 PMID: 11804826

32. Wang KL-C, Li H, Ecker JR. Ethylene biosynthesis and signaling networks. Plant Cell. 2002; 14(suppl 1):S131–S51. https://doi.org/10.1105/tpc.001768 PMID: 12045274

33. Eshed Y, Baum SF, Pereza JV, Bowman JL. Establishment of polarity in lateral organs of plants. Curr Biol. 2001; 11(16):1251–60. https://doi.org/10.1016/S0070-2153(01)00392-X PMID: 11525739
34. Sugano SS, Shimada T, Imai Y, Okawa K, Tamai A, Mori M, et al. Stomagen positively regulates stomatal density in *Arabidopsis*. Nature. 2010; 463(7278):241–4. https://doi.org/10.1038/nature08682 PMID: 20010603

35. Yamaguchi M, Kubo M, Fukuda H, Demura T. VASCULAR-RELATED NAC-DOMAIN is involved in the differentiation of all types of xylem vessels in Arabidopsis roots and shoots. Plant J. 2008; 55(4):652–64. https://doi.org/10.1111/j.1365-313X.2008.03533.x PMID: 1845131

36. Nakayama H, Nakayama N, Seiki S, Kojima M, Sakakibara H, Sinha N, et al. Regulation of the KNOX-GA gene module induces heterophyllic alteration in North American lake cress. Plant Cell. 2014; 26(12):4733–48. https://doi.org/10.1105/tpc.14.130229 PMID: 25516600

37. Cook CD. Aquatic plant book: Amsterdam: SPB Academic Publishing 228p.- ISBN; 1996.

38. Finkelstein RR, Gampala SS, Rock CD. Abscisic acid signaling in seeds and seedlings. Plant Cell. 2002; 14(suppl 1):S15–S45. https://doi.org/10.1105/tpc.010441 PMID: 12045268

39. Lee SC, Luan S. ABA signal transduction at the crossroad of biotic and abiotic stress responses. Plant Cell Environ. 2012; 35(1):53–60. https://doi.org/10.1111/j.1365-3040.2011.02426.x PMID: 21923759

40. Hsu F-C, Chou M-Y, Peng H-P, Chou S-J, Shih M-C. Insights into hypoxic systemic responses based on analyses of transcriptional regulation in *Arabidopsis*. PLoS One. 2011; 6(12):e28888. https://doi.org/10.1371/journal.pone.0028888 PMID: 22194941

41. Liao C-T, Lin C-H. Physiological adaptation of crop plants to flooding stress. Proc. Natl. Sci. Counc. ROC(B). 2001; 25(3):148–57. PMID: 11480770

42. Voosenek L, Bailey-Serres J. Flooding tolerance: O₂ sensing and survival strategies. Curr Opin Plant Biol. 2013; 16(5):647–53. https://doi.org/10.1016/j.pbi.2013.05.006 PMID: 23830867

43. Saika H, Okamoto M, Miyoshi K, Kusunoki T, Shinoda S, Jikumaru Y, et al. Ethylene promotes submergence-induced expression of *OsABA8ox1*, a gene that encodes ABA 8'-hydroxylase in rice. Plant Cell Physiol. 2007; 48(2):287–98. https://doi.org/10.1093/pcp/pcm003 PMID: 17205969

44. Pierik R, Tholen D, Poorter H, Visser EJ, Voosenek LA. The Janus face of ethylene: growth inhibition and stimulation. Trend Plant Sci. 2006; 11(4):176–83. https://doi.org/10.1016/j.tplants.2006.02.006 PMID: 16531097

45. Yasumura Y, Pierik R, Fricker MD, Voosenek LA, Harberd NP. Studies of *Physcomitrella patens* reveal that ethylene-mediated submergence responses arose relatively early in land-plant evolution. Plant J. 2012; 72(6):947–59. https://doi.org/10.1111/j.1365-3040.2012.02426.x PMID: 23046228

46. Li G, Hu S, Yang J, Schultz EA, Clarke K, Hou H. Water-Wisteria as an ideal plant to study heterophyll in higher aquatic plants. Plant Cell Rep. 2017; 1:1–12. https://doi.org/10.1007/s00299-017-2148-6 PMID: 28466187

47. Carlbecker A, Lee J-Y, Roberts CJ, Dettmer JB, Hesler S, Zhou J, et al. Cell signalling by microRNA165/6 directs gene dose-dependent root cell fate. Nature. 2010; 465(7296):316–21. https://doi.org/10.1038/nature08977 PMID: 20410862

48. Floyd SK, Bowman JL. The ancestral developmental tool kit of land plants. Int J Plant Sci. 2007; 168(1):1–35. https://doi.org/10.1086/509079

49. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. Methods. 2001; 25(4):402–8. https://doi.org/10.1006/meth.2001.1262 PMID: 11846609

50. Yoo S-D, Cho Y-H, Sheen J. *Arabidopsis* mesophyll protoplasts: a versatile cell system for transient gene expression analysis. Nat Protoc. 2007; 2(7):1565–72. https://doi.org/10.1038/nprot.2007.199 PMID: 17585298

51. Hejátko J, Bílilou I, Brewer PB, Fríml J, Scheres B, Benková E. *In situ* hybridization technique for mRNA detection in whole mount *Arabidopsis* samples. Nat Protoc. 2006; 1(4):1839–46. https://doi.org/10.1038/nprot.2006.333 PMID: 17487180

52. Team RC. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing; 2014. 2015.