The molecular framework of heterophylly in *Callitriche palustris* L. differs from that in other amphibious plants

Hiroyuki Koga¹, Mikiko Kojima², Yumiko Takebayashi², Hitoshi Sakakibara^{2,3} and Hirokazu Tsukaya¹

1. Graduate School of Science, The University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo, 113-0033, Japan

2. RIKEN Center for Sustainable Resource Science, 1-7-22, Suehirocho, Tsurumi-ku, Yokohama, Kanagawa, 230-0045, Japan

3. Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya, Aichi, 464-8601, Japan

Corresponding Author: Hiroyuki Koga (hiro1224koga@gmail.com)

Abstract

Heterophylly refers to the development of different leaf forms in a single plant depending on the environmental conditions. It is often observed in amphibious aquatic plants that can grow under aerial and submerged conditions. Although heterophylly is well recognized in aquatic plants, the associated developmental mechanisms and the molecular basis remain unclear. In this study, we analyzed heterophyllous leaf formation in an aquatic plant, *Callitriche palustris*, to clarify the underlying developmental and molecular mechanisms. Morphological analyses revealed extensive cell elongation and the rearrangement of cortical microtubules in the elongated submerged leaves of *C. palustris*. Our observations also suggested that gibberellin, ethylene, and abscisic acid regulate the formation of submerged leaves. However, the perturbation of one or more of the hormones was insufficient to induce the formation of submerged leaves under aerial conditions. Finally, we analyzed gene expression changes during aerial and submerged leaf development and narrowed down the candidate genes controlling heterophylly via transcriptomic comparisons, including a comparison with a closely related terrestrial species. We revealed that the molecular mechanism regulating heterophylly in *C. palustris* is associated with hormonal changes and diverse transcription factor gene expression profiles, suggesting differences from the corresponding mechanisms in previously investigated amphibious plants.

34 **Introduction**

35 Plants have a remarkable variety of leaf forms (Tsukaya, 2018). How such diverse leaf
36 forms evolved is a central question related to the evolution of land plants. Although leaf
37 forms generally vary between species or sometimes between populations, in some
38 species, individual plants can produce completely different leaf forms depending on the
39 environmental conditions the shoot is exposed to (Arber, 1920; Allsopp, 1967;
40 Sculthorpe, 1967). The ability to form different types of leaves depending on the
41 environment is called heterophylly. Because an individual heterophyllous plant has
42 distinct leaf developmental activities, it is useful for studying various modes of leaf
43 development in a single genetic background.

44 Amphibious plants that can grow under aerial and submerged conditions are
45 often used to investigate heterophylly, which is quite extensive in these species (Arber,
46 1920; Sculthorpe, 1967). Heterophylly is thought to be an important adaptive feature of
47 amphibious plants, which usually grow under conditions in which water levels fluctuate
48 seasonally or unexpectedly (Bradshaw, 1965). Amphibious plants often bear different
49 types of leaves when their shoot apex is underwater to either rapidly escape from the
50 submerged state or to adapt to underwater conditions. In the latter case, the leaves
51 produced under submerged conditions are generally thin, filamentous, and sometimes
52 highly branched, with a long and narrow leaf blade and/or a higher surface area relative
53 to volume (Wells and Pigliucci, 2000). These characteristics are thought to be
54 advantageous for surviving underwater. Because aquatic plants emerged from various
55 lineages of land plants (Cook, 1999; Du and Wang, 2014), the subsequent heterophylly

56 in response to submergence likely evolved independently in each lineage.

57 Previous studies revealed that phytohormones are involved in controlling
58 heterophylly in aquatic plants (Wanke, 2011; Nakayama et al., 2017; Li et al., 2019). In
59 many aquatic plants, gibberellic acid (GA) and ethylene promote the formation of
60 submerged leaves, whereas abscisic acid (ABA) stimulates the formation of aerial
61 leaves. Although the morphological basis of heterophylly has been well studied in
62 various aquatic plants, the underlying molecular mechanisms remained unknown until
63 recently. Specifically, Nakayama et al. (2014) clarified the molecular development
64 related to heterophylly in the aquatic plant *Rorippa aquatica* (Brassicaceae), which
65 forms branched dissected leaves when submerged. They revealed that GA negatively
66 regulates dissected leaf development, which is correlated with both submergence and
67 low temperatures, possibly by altering the expression patterns of class I KNOTTED1-
68 LIKE HOMEODOMAIN (KNOX) genes in leaf primordia (Nakayama et al., 2014). In
69 another study, in which *Ranunculus* species (Ranunculaceae) were used, heterophyllous
70 leaf development was determined to be regulated by ethylene and ABA signaling that
71 modifies the expression of leaf polarity genes, including those encoding KANADI and
72 HD-ZIP III transcription factors (TFs) (Kim et al., 2018). These studies suggest that the
73 molecular basis of heterophylly in aquatic plants varies among species. Hence, studying
74 diverse species is important for elucidating plant adaptations to aquatic environments.

75 In this study, we analyzed leaf formation in *Callitriche* plants (known as water
76 starworts). Members of the genus *Callitriche* are small flowering plants with a broad
77 global distribution. It is a member of the Plantaginaceae, to which the well-studied

78 snapdragon (*Antirrhinum majus*) belongs. *Callitriche* species are typically amphibious,
 79 able to grow both on land and in water (e.g., in freshwater ponds, streams, and rivers)
 80 (Erbar and Leins, 2004). This genus also comprises some terrestrial species that usually
 81 grow in moist soil. Although the sister genus of *Callitriche* is an amphibious genus,
 82 *Hippuris*, it was recently proposed that *Callitriche* has a terrestrial ancestor because the
 83 basal clade of the genus was revealed a terrestrial lifestyle (Ito et al., 2017). The other
 84 terrestrial species in the genus were likely derived from an amphibious ancestor. Thus,
 85 the evolution toward aquatic habitats and reversal of the terrestrial habitat may have
 86 occurred in the *Callitriche* lineage (Philbrick and Les, 2000; Philbrick and Jansen,
 87 1991). Accordingly, *Callitriche* plants are suitable materials for studying the
 88 evolutionary mechanisms mediating adaptations to aquatic environments.

89 The significant heterophylly of some *Callitriche* species has long been
 90 recognized (e.g., Schenck, 1887), and many classic developmental and physiological
 91 studies have been conducted using *Callitriche* species to understand the mechanisms of
 92 aquatic adaptation. Regarding leaf development, several descriptive works and
 93 physiological experiments have been performed. For example, Jones described the
 94 development of leaves under both aerial and submerged conditions using *C. intermedia*
 95 (= *C. hamulata*), *C. stagnalis*, and *C. obtusangula* (Jones, 1952, 1955). More recently,
 96 Deschamp and Cooke reported dimorphic leaf development in *C. heterophylla* at the
 97 cellular level and suggested the involvement of phytohormones and turgor pressure in
 98 the control of heterophylly in this plant (Deschamp and Cooke, 1984, 1983, 1985).
 99 Besides, we recently reported that *C. palustris*, a close relative of *C. heterophylla*,

showed extensive heterophylly in response to submergence even in the laboratory (Koga et al., 2020). We documented the aerial and submerged leaf developmental processes in this species, and defined the stages of leaf development on the basis of advances in research regarding vascular and stomatal development and cell proliferation activities (Koga et al., 2020). We revealed that submerged leaf formation in *C. palustris* is characterized by differential cell proliferation (in terms of direction), extensive cell elongation, and decreases in the number of stomata and veins as well as in cuticle thickness. However, the mechanistic aspects of heterophylly, such as hormonal and genetic controls, were not analyzed. Here, we observed the cellular changes, hormonal effects, and changes in gene expression associated with heterophylly in this plant. We also report a potential regulatory gene set for dimorphic leaf formation in this species identified through comparative transcriptomic analyses among plants exposed to pharmacological treatments, as well as with the closely related terrestrial species *C. terrestris*.

Results

Differential cellular expansion in the heterophylly of *C. palustris*

A previous study reported that *C. palustris* exhibits extensive heterophylly in response to submergence (Schenck, 1887), and we showed that this heterophylly is reproducible under scalable experimental conditions (Koga et al., 2020). The plant forms ovate leaves when the shoots are exposed to aerial conditions, whereas it forms ribbon-like leaves when the shoots are submerged in water (Figure 1A, B). Additionally, the

heterophylly of *C. palustris* is characterized by a decrease in the number of veins and the suppression of stomatal development in submerged leaves. The significantly narrower and longer submerged leaves were suggested to be the result of differential cellular arrangement and differential cell expansion (Koga et al., 2020). Because of the differential cell expansion, the aerial leaves were composed of jigsaw puzzle-like pavement cells and round palisade cells, whereas the cells of both the epidermal and mesophyll layers in submerged leaves were elongated along the proximal–distal axis (Figure 1C–F). A quantitative analysis of cell size and shape indicated that the pavement cells of submerged leaves had lower solidity values or were less lobed (Figure 1H; Supplemental Figure 1) and were significantly more elongated (Figure 1I) than the corresponding cells of aerial leaves. Similarly, the palisade cells of submerged leaves were less circular and much more elongated than the palisade cells of the aerial leaves (Figure 1K, L; Supplemental Figure 1). Despite the apparent differences in cell shape, the projected cell areas on the paradermal plane of both epidermal and palisade cells differed only slightly between the leaf forms (Figure 1G, J).

The directional expansion of plant cells is usually associated with the directional orientation of cell wall cellulose microfibrils, which is controlled by the orientation of cortical microtubules (cMTs) (Preston, 1974; Green, 1980; Gunning and Hardham, 1982; Lloyd, 1991). We observed that the cMTs of the palisade cells in young (approximately 1 mm long) submerged leaves were oriented perpendicular to the axis of cell elongation, whereas they were not well organized in the palisade cells of young aerial leaves (Figure 1M, N). Therefore, the differences in cell shapes are likely due to

144 the regulation of cMTs and the subsequent cellulose fiber orientation, resulting in
145 directional cell expansion in submerged leaves.

146

147 **Effects of hormones on the formation of submerged leaves**

148 As summarized in the Introduction, previous studies in diverse aquatic plants have
149 revealed that phytohormones are generally involved in controlling heterophylly in
150 response to submergence. In *C. heterophylla*, dimorphic leaf development is reportedly
151 related to GA and ABA contents (Deschamp and Cooke, 1983). Ethylene is also a key
152 regulator of the submergence response in various aquatic plants (Cox et al., 2004;
153 Jackson, 2008). To confirm the effects of these phytohormones in *C. palustris*, we
154 conducted hormone perturbation experiments.

155 After determining the effective concentrations of inhibitors or hormones
156 (Supplemental Figure 2), we examined the phenotypes of mature leaves from
157 submerged plants treated with these chemicals at the minimal effective concentrations
158 (Figure 2, Supplemental Figure 4). When the plants were grown in water containing
159 AgNO₃, which inhibits ethylene signaling, they formed aerial-like leaves of a slightly
160 shorter length (Figure 2B, H). After adding uniconazole P, an inhibitor of GA synthesis,
161 to the water, the plants also produced leaves that were shorter and broader than typical
162 submerged leaves (Figure 2C, H). A similar tendency was also observed when a
163 different GA synthesis inhibitor, paclobutrazol, was added to the water (Supplemental
164 figure 2). The inhibition of submerged-type leaf development by uniconazole P was
165 recovered by adding GA₃ (Figure 2G, H), implying GA production is required for the

166 elongated leaf form. Additionally, plants grown in water containing GA₃ exhibited an
167 enhanced submerged leaf phenotype, with much longer and narrower leaves than
168 normal, regardless of whether plants were also treated with uniconazole P (Figure 2E,
169 G, H). On the other hand, combined treatment with AgNO₃ and GA₃ did not induce
170 complete submerged-type leaves, although the leaves were longer and narrower than
171 aerial leaves (Figure 2 F, H). Furthermore, the formation of submerged leaves was also
172 inhibited when plants were grown in water containing ABA (Figure 2D, H). Both
173 AgNO₃ and ABA treatment drastically increased stomata density to the extent of normal
174 aerial leaves, whereas uniconazole P and the combination of AgNO₃ and GA₃ led to
175 slight increases. In the other GA₃ treatment conditions, stomatal densities remained at
176 the level of normal submerged leaves (Figure 2J).

177 Regarding cell shape analyses, we observed and measured several cellular
178 indices (i.e., cell area, perimeter, circularity, aspect ratio of the best fit ellipse [AR],
179 solidity, width, and length; Supplemental Figure 1) to discriminate shapes among states
180 (Figure 2K, L, Supplemental Figures 5, 6). In the leaves treated with AgNO₃, ABA, and
181 AgNO₃ + GA₃, the epidermal pavement cells and palisade cells were similar in shape to
182 those of aerial leaves, although the cells in the AgNO₃ + GA₃-treated leaves expanded
183 much more than the cells of leaves treated with AgNO₃ alone (Figure 2I, K, L). These
184 cells had a lower aspect ratio and shorter length than the cells of plants grown under
185 control conditions (Supplemental figure 5), presumably representing their complex,
186 jigsaw puzzle-like cellular shape. In uniconazole P-treated leaves, highly circular and
187 relatively simple pavement cells were detected, indicating that the cells were less

188 complex than those of aerial leaves, but they were not elongated, unlike the
189 corresponding cells of submerged leaves (Figure 2I, K, L). Following the GA₃ and
190 uniconazole P + GA₃ treatments, the leaf cells were highly expanded, and their shapes
191 were similar to those of control leaf cells (Figure 2I, K, L). These observations suggest
192 that inhibiting GA signaling alone is insufficient to block submerged-type cell
193 differentiation, resulting in an intermediate leaf phenotype.

194

195 **Effects of hormones on the formation of aerial leaves**

196 We next evaluated the effects of hormone applications on leaves under aerial conditions
197 to determine whether GA or ethylene signals alone or combined are sufficient to trigger
198 the formation of submerged leaves (Figure 3, Supplemental Figures 4, 5). Because an
199 inhibitor of ethylene signaling prevented submerged leaves from forming, we grew the
200 plants in medium containing ACC, which is an ethylene precursor, thereby enabling the
201 activation of ethylene signaling even under aerial conditions. In this case, the plants
202 exhibited a dwarf phenotype, which suggests strong inhibition of plant growth by
203 ethylene signaling (Supplemental Figure 3). This phenotype was seemingly the opposite
204 of that of submerged plants. Meanwhile, the plants produced slightly narrower leaves
205 than controls, but it was insufficient for the development of submerged leaves under
206 aerial conditions (Figure 3B, E, Supplemental Figure 4). To verify these results, we
207 exposed plants to ethylene gas, which produced the same results as those obtained for
208 the plants grown in ACC-containing medium (Supplemental Figure 3).

209 When GA₃ was added to the medium, the plants elongated and developed

leaves that were longer than those of the control plants, but GA₃ did not induce the formation of normal submerged leaves (Figure 3C, E). Similar phenotypes were also induced by spraying plants with a GA₃ solution (Supplemental Figure 3).

Finally, the plants were grown in medium containing both ACC and GA₃ to activate ethylene and GA signaling under aerial conditions. However, this treatment resulted in slightly narrower leaves, but failed to induce the formation of submerged leaves (Figure 3D, E, Supplemental Figure 4). Stomatal densities decreased in response to GA₃, which suggests that the GA signal negatively regulates stomatal formation (Figure 3G). However, the stomatal density was still higher than that of the submerged leaves. Substantial cellular changes in the aerial and submerged leaves were not observed following the application of hormones, although slight elongations and narrowing were detected that were consistent with the leaf forms (Figure 3F, H, I, Supplemental Figures 5, 6). Previous research on other aquatic plants proved that some submerged-like leaf phenotypes, such as a significantly narrow or compounded leaf form or extensive decreases in stomatal density, can be induced by a simple hormonal perturbation in many cases (Kuwabara et al., 2001; Sato et al., 2008; Nakayama et al., 2014; Kim et al., 2018; Horiguchi et al., 2019; Li et al., 2017). Therefore, our results suggest that the extent of the hormonal contribution to heterophylly differs between *C. palustris* and previously investigated amphibious plants.

Gene expression patterns associated with different leaf developments

To address the molecular aspects of heterophylly in *C. palustris*, we performed a

comprehensive mRNA sequencing analysis. Because the genome of the species has not been sequenced, we first reconstructed the transcriptome using RNA extracted from whole plants grown under aerial and submerged conditions (Supplemental Table 1). The data revealed many more genes in *C. palustris* than in known model species, possibly because the incomplete assembly resulted in the fragmentation of single genes and because of genome duplication events in *C. palustris*, which is supposedly a tetraploid species ($n = 10$, $x = 5$) (Philbrick, 1994; Prančl et al., 2014).

Regarding gene expression analyses, we extracted RNA from leaf primordia shorter than 500 μm , which were collected from three individuals grown under either aerial or submerged conditions. This growth stage was recently confirmed to precede or coincide with the initiation of cell differentiation under both conditions (Koga et al., 2020). We obtained single-read sequences (Supplemental Data Set 1) and found 4,275 differentially expressed genes (DEGs) between developing aerial and submerged leaves (false discovery rate [FDR] < 0.05 and expression-level $\log_2(\text{fold-change}) > 1$). We further analyzed gene expression patterns in the primordia submerged in water containing AgNO_3 , uniconazole P, or ABA, which inhibit the formation of submerged leaves. A total of 2,933, 5,508, and 16,517 genes were identified as DEGs in the AgNO_3 -, ABA-, and uniconazole P-treated leaf primordia, respectively, relative to the corresponding expression levels under normal submerged conditions (Figure 4). Because the leaves resulting from these treatments were significantly shorter and wider than the leaves formed under normal submerged conditions, we speculated that there is a key gene set that promotes the formation of submerged leaves among the common

DEGs in the four comparisons. Interestingly, the whole transcriptome profiles of the treated primordia differed from that of the aerial leaf primordia despite their similar appearance (Supplemental Figure 7), indicating that the number of key gene set for differential development could be relatively a small fraction of the whole DEGs. Indeed, a total of 200 genes were identified as common DEGs among the four comparisons (Figure 4, red). The submerged plants treated with AgNO₃ and ABA, but not uniconazole P, produced leaves that were almost the same as aerial leaves, implying the DEGs common to the AgNO₃-treated, ABA-treated, and aerial leaf primordia, but not the uniconazole P-treated leaf primordia, include genes that are important for the development of submerged leaves. As such category, 87 genes were identified (Figure 4, magenta). Taken together, we narrowed down the important candidate genes for differential leaf development to 287 genes.

To determine whether the genes were differentially expressed only in developing leaves, we also compared the transcriptomes of mature aerial and submerged leaves. Of the 287 candidate genes, 153 were developmental stage-specific DEGs, whereas 134 were identified as DEGs in both developing and mature leaves (Figure 4). Although both types of DEGs may be important for heterophylly, those common to developing and mature leaves are likely related to general functions, including responses to environmental cues or signaling, whereas the DEGs specific to developing leaves are likely related to the regulation of cell division and differentiation.

Analyses of non-heterophyllous *C. terrestris*

276 Although most *Callitriche* species are aquatic or amphibious, some are terrestrial,
 277 including *C. terrestris*, which grows in a relatively dry environment (Fassett, 1951).
 278 Phylogenetic analyses indicated that *C. terrestris* belongs to a sister clade of an
 279 amphibious clade that includes *C. palustris* and *C. heterophylla* (Philbrick and Les,
 280 2000). Because the outer clade of this group also comprises amphibious species, *C.*
 281 *terrestris*, and other terrestrial species in this clade are believed to have an amphibious
 282 ancestor (Philbrick and Les, 2000). Thus, heterophylly in response to submergence
 283 likely weakened or was lost in these species during the transition to terrestrial habitats.
 284 To test this assumption, we collected *C. terrestris* plants and established a culturing
 285 system in our laboratory. The *C. terrestris* plants grew well in a growth chamber in
 286 either soil or Murashige and Skoog (MS) medium under sterile conditions. More
 287 specifically, in MS medium, the plants grew under both aerial and submerged
 288 conditions, but there were no major differences in leaf forms (Figure 5A–C). Compared
 289 with the aerial leaves, the submerged leaves were slightly shorter and narrower, which
 290 resulted in a decrease in the leaf area, but not in the leaf index (Figure 5D, E).
 291 Additionally, there were no differences in the leaf cell shapes (Figure 5F–I). Although
 292 stomatal density was rather increased in the submerged condition, considering the
 293 decrease of leaf area of the submerged plants, stomatal development was apparently not
 294 significantly affected by submergence. Accordingly, we concluded that *C. terrestris*
 295 does not exhibit heterophylly in response to submergence, unlike *C. palustris*.

296 Because submergence did not induce drastic changes in the *C. terrestris* leaf
 297 form, a comparison of gene expression patterns between *C. terrestris* and *C. palustris*

was expected to provide relevant information regarding the key regulators of heterophylly. Therefore, we also analyzed the *C. terrestris* transcriptome. We first reconstructed the *C. terrestris* transcriptome using RNA-seq data for whole plants under both aerial and submerged conditions (Supplemental Table 1). To compare the gene expression patterns of *C. terrestris* with those of *C. palustris*, we analyzed orthologous relationships among putative coding genes. Because *C. terrestris* is a diploid species ($n = 5$; Philbrick 1994), we expected a one-to-many relationship with the orthologous genes in the tetraploid *C. palustris*. We identified 19,645 *C. terrestris* genes that were orthologous to 34,083 *C. palustris* genes (Supplemental Table 1). More precisely, 9,396 *C. terrestris* genes had a one-to-one relationship with *C. palustris* genes, whereas 10,249 genes were associated with 24,687 *C. palustris* genes having a one-to-multi relationship (Supplemental Figure 8). We subsequently sequenced the RNA extracted from the young leaves ($< 500 \mu\text{m}$ long) collected from *C. terrestris* plants grown under aerial or submerged conditions. A total of 2,177 genes were differentially expressed between the developing leaves of *C. terrestris* plants grown under aerial and submerged conditions (Figure. 5K). Notably, the enriched gene ontology (GO) terms for the *C. palustris* and *C. terrestris* DEGs between the aerial and submerged leaf primordia were largely unshared (Supplemental Figure 9; Supplemental Data Set 2). Consistent with this result, we detected relatively few overlapping DEGs (Figure 5L, M).

Expression-level changes in genes involved in hormone signaling

To examine the hormonal contribution to heterophylly from a gene expression aspect,

we extracted the expression profiles of orthologous genes related to ethylene, GA, and ABA biosynthesis, inactivation, and signal transduction from the RNA-seq data (Figure 6A, B). Additionally, we quantified the contents of ABA, GAs, and other hormones in the shoots of *C. palustris* with hormone/inhibitors treatment as well as in the shoots of *C. terrestris* (Figure 6C-E, Supplemental Data Set 3). In *C. palustris*, the genes encoding proteins related to ABA biosynthesis (ABA1–3, AAOs, and NCEDs) tended to be highly expressed in mature aerial leaves, but not in primordia. The expression of genes encoding some ABA-inactivation enzymes (CYP70As) was also repressed in the *C. palustris* mature submerged leaves. Regarding *C. terrestris*, for which only the primordia were examined, there were no drastic changes in the expression of ABA biosynthesis genes like in the *C. palustris* primordia. Meanwhile, the ABA content decreased substantially in the submerged shoots (Figure 6C), consistent with the downregulation expression of NCED-encoding genes in the mature leaves. Similar results were obtained for the *C. terrestris* shoots. Among the signaling-related genes, there were notable changes in the expression of ABA receptor orthologs. Specifically, the expression of a *PYRI* ortholog was downregulated in the submerged leaves of *C. palustris*, whereas the expression of *PYL4–6* orthologs was upregulated in the submerged leaves of both species (Figure 6B).

Of the ethylene biosynthesis genes, the expression levels of the *ACO4* orthologs were clearly downregulated in the submerged control, relative to the corresponding levels in the aerial, uniconazole P-treated, and ABA-treated *C. palustris* and *C. terrestris* samples, suggesting ethylene biosynthesis was repressed. Considering

342 this downregulated expression was not observed following the AgNO₃ treatment, it
343 might be the result of activation of ethylene signaling (i.e., negative feedback). Ethylene
344 cannot diffuse effectively from plant tissues underwater. Hence, ethylene signaling was
345 likely activated in the plants, even if the related biosynthesis genes were expressed at
346 low levels. The upregulated expression of *ERF1* orthologs in the submerged primordia,
347 but not in AgNO₃-treated primordia, may reflect the activation of ethylene signaling
348 (Solano et al., 1998).

349 Considering GA signaling is required for submerged leaf formation (Figure 2),
350 it was surprising that we were unable to detect the activation of GA biosynthesis based
351 on gene expression levels (Figure 6A). Instead, the expression of some orthologous
352 genes related to GA deactivation (*GA2OX* genes) was upregulated under submerged
353 conditions. Additionally, we did not detect active forms of GAs in *C. palustris* shoots
354 under any condition, indicative of a lack of drastic changes in GA contents (Figure 6D,
355 E). In contrast, in *C. terrestris*, the GA₁ content decreased in submerged shoots (Figure
356 6D). Assuming that *GA2OX* expression levels are affected by the negative feedback
357 regulation of GA signaling like in *Arabidopsis* (Thomas et al., 1999), GA signaling may
358 be activated in submerged plants even at an extremely low GA level, possibly because
359 of increased sensitivity. However, *GID1* receptor genes were similarly expressed under
360 aerial and submerged conditions (Figure 6B).

361

362 **Gene expression patterns related to heterophylly**

363 We subsequently focused on the genes likely associated with heterophylly.

364 Previous research on the molecular response to submergence proved that a subfamily of
365 ERF TFs (i.e., group VII ERFs) contributes to the viability or the morphological
366 changes of some plants under submerged condition (VEEN et al., 2014; Fukao et al.,
367 2006; Xu et al., 2006; Licausi et al., 2011; Hattori et al., 2009). In Arabidopsis, the
368 HRE1/2 and RAP2.12 TFs in this group function under low-oxygen stress conditions
369 (i.e., hypoxia) to enhance flooding tolerance (Licausi et al., 2010). We detected three
370 orthologous group VII ERF TF genes in the *Callitriche* transcriptomes. Of the three
371 group VII ERF TF genes in *C. palustris*, two (*RAP2.2* and *RAP2.3*) were almost
372 constitutively expressed under all conditions like *RAP2.12* of Arabidopsis, whereas the
373 expression of the other orthologous gene (*HRE2*) was upregulated under submerged
374 conditions, although not in the AgNO₃-treated primordia (Figure 7A). The same
375 tendency was observed for the orthologs in *C. terrestris* (Figure 7A).

376 We also determined that the expression patterns of some TF genes were
377 consistent with the phenotypes. The expression levels of the orthologs of the bHLH TF
378 genes *SPEECHLESS* (*SPCH*), *MUTE*, and *FAMA* were downregulated in developing
379 submerged leaves, in which fewer stomata differentiated than in the aerial leaves
380 (Figure 7A). In Arabidopsis, these genes are explicitly expressed in the guard cell
381 lineage to promote stomatal differentiation (Ohashi-Ito and Bergmann, 2006;
382 MacAlister et al., 2007; Pillitteri et al., 2008). Because these TFs are involved in
383 stomatal development in many other land plants, including monocots and even mosses
384 (Liu et al., 2009; Chater et al., 2016), it is likely they are also involved in stomatal
385 development in *Callitriche* species. The stomatal lineage-associated expression of the

genes encoding these TFs in several *Callitriche* species offers additional evidence of their functions in stomatal development (Doll et al., 2021). Among the other stomatal development-related genes, *EPIDERMAL PATTERNING FACTOR (EPF) 1/2* orthologs and *TOO MANY MOUTHS (TMM)* genes were identified as DEGs in leaf primordia (Figure 7A). Additionally, the expression of the *EPFL9/STOMAGEN* ortholog was downregulated in submerged leaves. These genes were more highly expressed in the samples treated with AgNO₃ and ABA than in the submerged control, although the differences were not always significant (Figure 7A).

A narrow, needle-like terete leaf form may result from the expansion of the abaxial region of a primordium (Waites and Hudson, 1995). This mechanism of narrowing was confirmed to be involved in the heterophylly of an aquatic plant species from the family Ranunculaceae (Kim et al., 2018). Thus, we also analyzed the expression of HD-ZIP III and KANADI TF genes, which determine the dorsiventral polarity (Figure 7A). Although some of the *KANADI* family genes were expressed at low levels in the submerged primordia, the overall expression profiles of the dorsiventral genes were not indicative of abaxialization.

Candidate gene set for the regulation of heterophylly

Finally, we compared transcriptome data to extract a set of genes that may be important for the differential development of *C. palustris* leaves. Of the 287 genes extracted from the comparisons of *C. palustris* experimental conditions, we excluded 22 genes that were orthologous to DEGs in *C. terrestris* because the consistent differential

expression in *C. terrestris* and *C. palustris* implies that they may not be critical for differential leaf development (Figure 5L, M). Another 15 genes were also excluded because their expression levels were downregulated in the developing submerged leaves of *C. palustris*, but their orthologs were not expressed in *C. terrestris*, suggesting that the downregulated expression of these genes does not affect leaf formation. Thus, 250 genes, including 145 genes lacking an identified ortholog, were retained to form the candidate gene set for heterophylly regulation and differential development (Figure 7B; Supplemental Data Set 4). Of these genes, the coding regions were predicted for 208 genes.

The general importance of TFs in various developmental processes and environmental responses suggests that changes in TF gene expression levels are crucial for heterophylly. Therefore, we identified possible TF genes using PlantTFDB 4.0 (Jin et al., 2017) and via manual annotations. Among 80,437 predicted protein-coding genes, 2,489 encoded TFs. We identified 19 potential TF genes among the 208 candidate genes with predicted coding regions. The expression of five TF genes was upregulated in submerged leaves (Figure 7C). Of the 19 identified TF genes, 13 were developmental stage-specific DEGs. In addition to the TF genes, the candidate gene set included receptor-like protein (RLP) genes, such as a *CLAVATA1* (*CLV1*) ortholog and *BARELY ANY MERISTEM* (*BAM*) 1/2/3 homologs (Figure 7A, B), and 12 histone-like genes with expression levels that were upregulated in submerged primordia (Figure 7B; Supplemental Data Set 4). Moreover, a GO enrichment analysis revealed many of the candidate genes encoded transporters with increased abundance in submerged primordia

430 as well as TFs (Figure 7B; Supplemental Data Set 5).

431

432 **Discussion**

433 **Heterophylly in *Callitriche* and other aquatic plants**

434 In this study, we focused on *Callitriche* species to analyze the plasticity of leaf
 435 development. Recent studies clarified the molecular basis of heterophylly in several
 436 distant lineages of aquatic plants. In *Rorippa aquatica* and *Hygrophila difformis*, the
 437 compound leaf forms of submerged plants are associated with the transcriptional
 438 activation of class I KNOX genes in leaves (Nakayama et al., 2014; Li et al., 2017).
 439 However, orthologs of these genes are not expressed in the simple leaves of *C. palustris*
 440 leaves under submerged conditions. Hence, these genes were not in our candidate gene
 441 set. An earlier study confirmed that abaxialization resulting from changes in the
 442 expression of genes related to dorsiventral polarity is important for heterophylly in
 443 *Ranunculus trichophyllus* (Kim et al., 2018). In contrast, our analysis of KANADI and
 444 HD-ZIP III TF gene expression in *C. palustris* leaves did not produce evidence of
 445 abaxialization (Figure 7A). These genes were also not in the candidate gene set. In *R.*
 446 *trichophyllus*, the activation of ethylene signaling and repression of ABA signaling
 447 contribute to the upregulated and downregulated expression of KANADI and HD-ZIP
 448 III genes, respectively. Thus, similar hormonal changes, but varying downstream gene
 449 expression, are presumed to occur in *C. palustris*. The differences in the mechanisms
 450 regulating heterophylly reflect the independent evolution of heterophylly in these plant
 451 species.

452

453 **Molecular background of dimorphic leaf formation in *C. palustris***

454 On the basis of transcriptome comparisons, we included 250 genes in the candidate
 455 gene set that regulates heterophylly in *C. palustris*. Although we currently do not know
 456 the development-related functions of these genes in *C. palustris*, some of the genes had
 457 expression patterns that are consistent with those in previous studies of genes in model
 458 organisms, including genes involved in stomatal development. We detected
 459 downregulated expression of *bHLH*, *EPF1/2*, and *TMM* genes in submerged leaves. In
 460 Arabidopsis, these genes are expressed almost exclusively in the stomatal lineage cells
 461 (Ohashi-Ito and Bergmann, 2006; MacAlister et al., 2007; Hara et al., 2007, 2009;
 462 Pillitteri et al., 2008), with only the *TMM* genes also expressed in the surrounding
 463 epidermal cells (Nadeau and Sack, 2002). Thus, the expression patterns for these genes
 464 in the current study may be due to a decrease in the number of stomatal lineage cells in
 465 submerged leaves. In addition to the genes expected to be expressed in the stomatal
 466 lineage cells, the expression levels of *EPFL9/STOMAGEN* orthologs were also
 467 downregulated in submerged primordia. In Arabidopsis, *EPFL9/STOMAGEN* is
 468 expressed in mesophyll cells and positively controls stomatal development in epidermal
 469 cells (Sugano et al., 2010). The external application of EPFL9/STOMAGEN leads to
 470 increased stomatal density, whereas knocking down *EPFL9/STOMAGEN* via RNA
 471 interference has the opposite effect (Sugano et al., 2010; Tanaka et al., 2013). Thus, the
 472 downregulated *EPFL9* expression potentially explains the decreased stomatal density in
 473 *C. palustris*. The suppression of *EPFL9/STOMAGEN* expression in submerged leaves

474 was also observed in *R. trichophyllus*, likely because of activation of ethylene signaling
475 and decreased ABA levels and the resulting inhibition of HD-ZIP III (Kim et al., 2018).
476 In *C. palustris*, however, although the ABA content decreased in the submerged
477 condition, HD-ZIP III gene expression was not repressed (Figure 7A). Thus, it is
478 possible that stomatal control differs between *C. palustris* and *R. trichophyllus* leaves.
479 Genetic analyses of stomatal regulation in *C. palustris*, including the involvement of
480 ethylene signaling, are required to enable comparisons of the mechanisms.

481 Our candidate gene set included many genes encoding RLPs. These proteins
482 are important for stress responses (e.g., defense against bacteria and viruses), but they
483 also function as receptors for hormones and small peptide ligands, which influence
484 various growth and developmental processes. An ortholog of *CLVI* and orphan *BAM*-
485 like genes were also included in the candidate gene set (Figure 7A). The functions of
486 the encoded proteins related to the regulation of the stem cell lineage in the shoot apical
487 meristem are well known (DeYoung et al., 2006; Clark et al., 1997; DeYoung and Clark,
488 2008), but, in Arabidopsis, these genes are broadly expressed in the plant body,
489 including leaves (Klepikova et al., 2016). For example, the *CLVI* promoter is active in
490 leaf mesophyll cells (Kawade et al., 2013), although the effect of *CLVI* expression in
491 leaves remains unreported. On the other hand, *BAM* genes are involved in xylem
492 development in Arabidopsis (Qian et al., 2018). Therefore, the downregulated
493 expression of the *BAM* orthologs in submerged *C. palustris* primordia may be
494 associated with a decrease in the number of leaf veins (Koga et al., 2020). Although the
495 changes in the expression of these receptor-like genes may be the result of changes in

cellular state, rather than the drivers of cell differentiation, functionally characterizing these receptor-like genes may help elucidate the molecular basis of differential leaf development.

In the context of developmental biology, it is worthwhile to focus on TF genes in the candidate gene set. Because heterophylly of *C. palustris* is accompanied by significant changes in cell shape, it is likely that distinct differentiation processes mediate the formation of each leaf type. In Arabidopsis, some candidate TF gene homologs affect organ elongation. Both *OBP4L1* (Cluster-23900.0) and *OBP4L2* (Cluster-57510.0), which are Dof-type TF genes with downregulated expression in submerged primordia, are homologs of Arabidopsis *OBP4*, which is reportedly a negative regulator of cell growth (Xu et al., 2016; Rymen et al., 2017). Additionally, *PRE1A* (Cluster-74746.0) and *PRE1B* (Cluster-74746.1) expression levels were upregulated in submerged primordia. Their Arabidopsis homologs, *PACLOBUTRAZOL RESISTANCE* genes (*PRE1-6*), promote cell elongation in the hypocotyls, stems, and leaf petioles in a process controlled by GA signaling (Shin et al., 2019; Lee et al., 2006; Zhang et al., 2009). On the basis of the Arabidopsis phenotypes resulting from the disruption of these genes, it is unlikely that the expression-level changes in only one of these genes can substantially induce leaf blade elongations in *C. palustris*. We speculate that the coordinated effects of these genes and possibly other functionally unknown TF genes are responsible for the specific differentiation of elongated leaf cells in submerged leaf primordia. The molecular mechanism regulating heterophylly will be more precisely elucidated by studies focusing on these genes and how the hormones

518 regulate their expression.

519

520 **Hormonal control of heterophylly**

521 In a previous study on *C. heterophylla*, a GA application under aerial conditions
522 induced the development of submerged-type leaves (Deschamp and Cooke, 1983).

523 However, we were unable to replicate this result. Instead, in our scalable experimental
524 system, the medium-cultured plants produced slightly elongated leaves in response to
525 the GA₃ application under aerial growth conditions. In these leaves, the cells were still
526 highly lobed in the epidermis and had a relatively low aspect ratio in both layers
527 (Supplemental Figures 5, 6), suggesting that GA signaling is insufficient to induce
528 extensive cell elongation and the formation of submerged-type leaves. Thus, undesirable
529 environmental factors may have affected the conditions that the soil-cultured plants
530 were exposed to in the earlier study. Nevertheless, GA biosynthesis, as well as the
531 subsequent activation of GA signaling, are likely required for the formation of
532 submerged-type leaves. Notably, the reorientation of cMTs was associated with this
533 phenomenon because GA signaling promotes cell elongation through the reorientation
534 of cMTs and the cellulose microfibers (Mita and Shibaoka, 1984; Takeda and Shibaoka,
535 1981; Lloyd, 1991). Indeed, GA signaling was possibly more activated under
536 submerged conditions than under aerial conditions, with upregulated *GA2OX* expression
537 levels except in uniconazole P-treated primordia (Figure 6). Because our quantitative
538 analysis of hormones failed to detect increases in GA contents, this activation may be
539 caused by an undetectable increase in GA levels or increased sensitivity to GA (Figure

540 6). However, although GA signaling is required to promote cell elongation during the
541 formation of submerged leaves in *C. palustris*, it alone seemed insufficient for
542 modulating cMT orientations to the extent necessary to produce a submerged leaf.
543 Considering uniconazole P was unable to completely block submerged-type cell
544 formation, it is likely that GA signaling is not required for determining cellular fate, but
545 is needed to promote general cell expansion regardless of the condition (Figure 8).

546 In some aquatic plants, ethylene positively regulates the formation of
547 submerged leaves. For example, when *Ludwigia arcuate* (Onagraceae) is treated with
548 ethylene gas or ACC under aerial growth conditions, the plants produce submerged-type
549 leaves, indicating that ethylene signaling almost sufficiently induces submerged-type
550 leaf development in this species (Kuwabara et al., 2003; Sato et al., 2008). Similarly,
551 treating the aerial parts of *R. trichophyllus* and *Hygrophila defformis* plants with
552 ethylene induces drastic changes in some leaf phenotypes, which are similar to those
553 observed in submerged leaves to some extent (Kim et al., 2018; Li et al., 2017;
554 Horiguchi et al., 2019). The involvement of ethylene is reasonable because, under
555 submerged conditions, ethylene spontaneously accumulates in the plant body because of
556 the limited gas exchange in water (Jackson, 1985). Indeed, substantial increases in
557 ethylene levels have been reported for various plants growing under submerged
558 conditions (Voesenek and Sasidharan, 2013) for a review). In *C. palustris*, an exposure
559 to AgNO₃, which inhibits the perception of ethylene, induced the production of aerial-
560 type leaves under submerged conditions, which suggests that ethylene signaling must be
561 activated for the formation of submerged leaves. However, this mechanism seems to be

562 insufficient for the formation of submerged leaves in *C. palustris* because the
 563 application of ethylene or ACC did not induce the formation of submerged leaves.
 564 Regarding cellular morphology, the activation of ethylene had minimal effects and did
 565 not lead to submerged leaf phenotypes (Figure 3; Supplemental Figure 5). This is
 566 consistent with the inhibition of submerged leaf formation by uniconazole P under
 567 submerged conditions, in which ethylene should spontaneously accumulate. This
 568 implies that the formation of submerged leaves is a GA-dependent process.
 569 Nevertheless, we were unable to induce the formation of submerged leaves through the
 570 combined application of GA₃ and ACC. The inhibition of ethylene signaling resulted in
 571 aerial-like cellular morphologies even with GA₃ (Figure 2), implying that ethylene
 572 signaling has a greater role than GA signaling in submerged-type cell differentiation.
 573 Collectively, these results indicate that *C. palustris* requires ethylene signaling for
 574 submerged-type cell differentiation, but it is nearly insensitive to ethylene under aerial
 575 conditions. Additional factors associated with submergence are likely needed for
 576 ethylene signaling to trigger the formation of submerged-type leaves.

577 In the current study, the application of ACC or ethylene, even with GA₃,
 578 induced seemingly normal stress responses commonly observed in terrestrial plants
 579 (e.g., strong growth inhibition and chlorosis) (Supplemental Figure 3). This ethylene
 580 response was inconsistent with the healthy growth of the submerged plants.
 581 Interestingly, in experiments conducted by Musgrave *et al.* (1972), applying ethylene
 582 gas to the floating rosettes of *Callitriche platycarpa* plants enhanced stem elongation,
 583 but the leaf forms were not thoroughly examined. Additionally, an effective amount of

584 ethylene for promoting stem elongation accumulated in the submerged plants. These
585 results suggest that at least in the floating shoots, where the basal part of the plant is
586 submerged, the plant reacts to ethylene with an elongation response, unlike in the fully
587 aerial condition (Musgrave et al., 1972). The mechanism mediating the contradictory
588 effects of ethylene signaling under submerged and aerial conditions remains unclear,
589 because the genetic basis of ethylene signaling in *C. palustris* has not been
590 characterized. Thus, molecular investigations of the ethylene responses are required to
591 fully elucidate the heterophylly of *C. palustris*.

592 Absciscic acid is also known as an inhibitory hormone of submerged leaf
593 formation in broad aquatic plants, including ferns (Wanke 2011). Regarding *Callitriche*
594 species, an ABA treatment under submerged conditions reportedly leads to the
595 formation of aerial-like leaves (Deschamp and Cooke 1983). In the present study, we
596 proved that the ABA content decreases substantially in the submerged shoot, possibly
597 because of the downregulated expression of NCED-encoding genes in mature leaves.
598 Our leaf and cellular measurements confirmed that the application of exogenous ABA
599 under submerged conditions resulted in leaves that were morphologically identical to
600 aerial leaves, indicating that ABA levels must decrease before submerged leaves can
601 form. However, because the ABA content also decreased in the AgNO₃- and
602 uniconazole P-treated *C. palustris* shoots, a decrease in ABA levels alone is insufficient
603 for inducing submerged leaf formation. Notably, the ABA receptor orthologs *PYR1* and
604 *PYL4/5/6* were differentially expressed between the heterophyllous leaf primordia
605 (Figure 6B). Moreover, three *C. palustris*-specific *PYL4/5/6*-like genes were included in

the candidate gene set (Figure 7B, Supplemental Data Set 4). The downregulated expression of *CpPYRI* in submerged leaves can be explained by reductions in the number of guard cells and the amount of vascular tissue because *PYRI* gene is specifically expressed in these tissues in Arabidopsis (Gonzalez-Guzman et al., 2012). The *PYL4* expression levels were markedly upregulated in the submerged primordia of both *C. palustris* and *C. terrestris*, implying changes in the expression of these genes are not associated with the morphological changes. However, CpPYL4 may contribute to physiological changes in the submerged form by modulating the sensitivity to ABA or inducing differential responses downstream of the ABA signaling pathway.

Requirement of additional factors for the formation of submerged leaves

The results of our hormone perturbation experiments suggest that factor(s) other than ethylene and GA are also required to induce the development of submerged leaves under aerial conditions. Our results of hormonal measurements suggested a reduction of ABA amount is one of the requirements. Because the submerged condition is a drastically different environment from the aerial condition, various factors can be assumed, such as changes in light quality, photoperiod, and temperature. These factors have previously been demonstrated to induce differential leaf formation in other aquatic plants (Wells and Pigliucci, 2000). One such factor to be considered may be high turgor pressure under submerged conditions (Deschamp and Cooke, 1983). Low-oxygen stress is another potential factor. Indeed, in *R. trichophyllus*, hypoxia can partly induce the development of submerged leaf phenotypes under aerial conditions (Kim et al., 2018).

628 Group VII ERFs, which are activated in Arabidopsis by hypoxic conditions, influence
629 the morphological responses of submerged rice plants (Hattori et al., 2009; Fukao et al.,
630 2006; Xu et al., 2006), and may affect the viability of *Rumex palustris* and *Rorippa*
631 species under submerged conditions (Licausi et al., 2011; VEEN et al., 2014). The
632 expression patterns of the group VII ERFs imply the hypoxic response is probably
633 activated in both species under submerged conditions, with the underlying mechanism
634 controlled by ethylene signaling in *C. palustris* (Figure 7A). However, the upregulated
635 expression of the genes encoding the group VII ERFs was not correlated with leaf
636 shapes. Thus, this pathway is insufficient for the formation of submerged leaves in *C.*
637 *palustris*, or it may not be involved at all.

638 Considered together, our findings confirm that the heterophylly of *C. palustris*
639 is controlled by a complex mechanism involving phytohormones and possibly
640 additional factors (Figure 8A). Expression-level changes in relatively few genes,
641 including diverse TF genes, might be primarily involved in modulating cellular states
642 for submerged-type leaf phenotypes (e.g., rearrangement of cMT orientation and
643 subsequent cell elongation and inhibition of stomatal development). The considerable
644 cell elongation in submerged leaves requires GA signaling, but GA contents do not
645 increase following submergence. Ethylene signaling is also required for the cell
646 elongation in submerged leaves. In contrast, the abundance of ABA, which promotes
647 aerial leaf formation, must decrease prior to submerged leaf formation (Figure 8A). In
648 the aerial condition, the ABA signaling is activated, but the ethylene signaling is not as
649 it can volatilize to the atmosphere immediately, leading to aerial-type leaf phenotypes

(e.g., jigsaw puzzle-like or circular cell expansion, and stomatal and vein development; Figure 8B). These hormones are similarly involved in the heterophylly of amphibious plants from different lineages, but the changes to genetic architectures downstream of these hormone signals likely differ among the lineages. In *C. palustris*, the KNOX genes (Nakayama et al 2014, Li et al, 2017) as well as the ethylene–KANADI and ABA–HD-ZIP III modules (Kim et al., 2018) may not be involved in heterophylly. It is also notable that *C. palustris* is only slightly responsive to hormone applications under aerial conditions. In many amphibious plants, hormone applications drastically alter leaf phenotypes, although not always resulting in a completely submerged leaf form, suggesting that the extent of the contributions of the same hormones to heterophylly varies among lineages. These observations shed light on the variety of evolutionary trajectories of plant adaptations to aquatic environments.

Materials and Methods

Plant culture

Callitriche palustris L. and *Callitriche terrestris* Raf. plants were originally collected in Hakuba, Nagano, Japan (NH1 strain), and Nishinomiya, Hyogo, Japan, respectively. The plant culture system used in this study was recently described by Koga *et al.* (2020). Briefly, the plant material from a sterile culture was transplanted onto solid half-strength MS medium after the visible shoot tips were removed. A culture jar was filled with 200 mL sterile distilled water to produce submerged growth conditions. For the chemical treatments under submerged conditions, the chemical to be tested was diluted

672 in 200 mL sterile distilled water. The specific concentrations for each experiment are
673 indicated in the corresponding figures and/or figure legends. For chemical treatments
674 under aerial conditions, the chemical was added to the medium in advance or sprayed to
675 the shoots every 2 or 3 days. For the chemical treatments under aerial conditions, the
676 chemical was added to the medium in advance or was applied by spraying the shoots
677 every 2 or 3 days. Regarding the ethylene gas treatment, the plants were transplanted
678 into airtight flasks containing the growth medium, after which they were exposed to
679 ethylene gas every two days.

680

681 **Measurement of leaves and cells**

682 Before measuring the leaves and cells, the plants were treated with a formalin–acetic
683 acid–alcohol fixative. The leaves from the fixed shoots were dissected and then
684 examined. Images of the leaves were captured with a scanner or a camera attached to a
685 microscope. The length of the main vein was recorded as the leaf length, whereas the
686 length of the widest line that vertically crossed the main vein was recorded as the leaf
687 width. ImageJ/Fiji software was used to analyze the scanned images to measure each
688 index. The examined leaves were collected from two or three sibling plants as
689 biological replicates for each experiment. For cellular observations, the fixed leaves
690 were cleared in a chloral hydrate solution (Tsuge et al., 1996). The leaf cells were
691 observed using a DM4500 differential interference contrast microscope (Leica
692 Microsystems, Wetzlar, Germany). We obtained cellular images from 4–8 leaves
693 collected from two or three sibling plants as biological replicates. Using ImageJ/Fiji, we

manually traced the contours of 30 pavement cells and 30 subepidermal palisade cells per leaf. The analyzed cells were randomly selected, but we avoided choosing pavement cells adjacent to guard cells and hair cells because their shapes were distorted. We then examined the cell size and other shape-related indices using the “Area,” “Shape descriptors,” and “Perimeter” measurement functions of ImageJ/Fiji (Supplemental Figure 1; see ImageJ documents for more details). The cellular length and width were measured as the long and short side lengths of the bounding smallest rectangle, respectively (Supplemental Figure 1). Statistical analyses and visualizations were performed using the following packages of the R software (version 4.0.3): multcomp (version 1.4-16) (Hothorn et al., 2008), tidyverse (version 1.3.0) (Wickham et al., 2019), and ggforce (version 0.3.3; <https://CRAN.R-project.org/package=ggforce>), and ggfortify (version 0.4.11) (Tang et al., 2016).

Immunofluorescence

The immunofluorescence of microtubules was analyzed as previously described (Pasternak et al., 2015), with slight modifications. Briefly, the leaves were fixed in 2% PFA/0.5% glutaraldehyde prepared in a microtubule-stabilizing buffer, after which the central region was dissected. After permeabilizing with methanol, cell wall enzymes, and a 3% (v/v) IGEPAL/10% (v/v) DMSO solution, the samples were incubated with a 1:1,000 primary antibody solution (T5168; Sigma-Aldrich, St. Louis, MO, USA) and then with a 1:500 Alexa Fluor 488-conjugated secondary antibody solution (A-11017; Thermo Fisher Scientific, Waltham, MA, USA). The stained samples were observed

716 using an FV10 confocal microscope (Olympus, Tokyo, Japan).

717

718 **RNA-seq**

719 For the transcriptome analyses, we cultured shoots from individual *C. palustris* and *C.*

720 *terrestris* plants grown under aerial or submerged conditions. After culturing for three

721 weeks, we extracted RNA from whole plants using the RNeasy Plant Mini Kit (Qiagen,

722 Hilden, Germany) and the RNase-free DNase Set (Qiagen). Sequencing libraries were

723 prepared using the TruSeq Stranded mRNA Library Prep Kit (Illumina, Inc., San Diego,

724 CA, USA), with the protocol optimized for a 300–400 bp insert size. We sequenced the

725 libraries on the HiSeq 1500 platform (Illumina) using the rapid-run mode to generate

726 150-bp paired-end reads. Additionally, for the quantitative analysis of RNA, we cultured

727 shoots from three siblings grown under aerial conditions, submerged conditions, and

728 submerged conditions with 10^{-6} M AgNO₃, 10^{-7} M uniconazole P, or 10^{-7} M ABA. We

729 extracted total RNA from 10 leaves that were shorter than 500 µm and from 4–6 mature

730 leaves using the RNeasy Micro Kit (Qiagen) and the RNeasy Plant Mini Kit (Qiagen),

731 respectively, along with the RNase-free DNase Set (Qiagen). We constructed

732 sequencing libraries with the KAPA Stranded mRNA-seq Kit (KAPA Biosystems, Inc.,

733 Wilmington, MA, USA), after which they were sequenced on the HiSeq 1500 platform

734 (Illumina) using the rapid-run mode to obtain 75-bp single-read sequences. The raw

735 reads were deposited in the DDBJ Sequence Read Archive (DRA) (Supplemental Data

736 Set 2).

737

738 **Transcriptome assembly and annotation**

739 The paired-end reads for *C. palustris* or *C. terrestris* whole plants were trimmed based
 740 on quality using Trimmomatic (version 0.36) (minimum length: 32) (Bolger et al.,
 741 2014) and assembled with Trinity (version 2.2.0) with an *in silico* normalization step
 742 (Grabherr et al., 2011). The contigs derived from rRNA were identified using
 743 RNAmmer (version 1.2.1) (Lagesen et al., 2007) and removed from the assembly.
 744 Additionally, contigs with the best matches (e-value < 1E-50) to sequences from non-
 745 Viridiplantae species (mostly bacteria, fungi, and human) following a BLASTn search
 746 of the nt database were considered as possible contaminants and eliminated. The single
 747 open reading frame (ORF) of each transcript was predicted using TransDecoder (version
 748 3.0.0) (<https://github.com/TransDecoder/TransDecoder>). The assemblies included many
 749 contigs putatively transcribed from a single gene. Because the clustering intrinsically
 750 executed by Trinity did not work properly for the tetraploid *C. palustris* transcriptome,
 751 we clustered contigs into putative genes using Corset (version 1.07) (Davidson and
 752 Oshlack, 2014). To select a representative isoform of each putative gene, the expression
 753 levels of isoforms were quantified by mapping the reads onto the assembly using
 754 Bowtie (version 1.2) and RSEM (version 2.3.0) (Li and Dewey, 2011; Langmead et al.,
 755 2009). Representative isoforms were selected based on the following criteria: having an
 756 ORF, BLASTx match to a sequence in the UniProt database, high expression level, and
 757 a relatively long transcript among the isoforms for the gene. Next, we qualitatively
 758 analyzed the assembly using BUSCO (version 4.0.1) with the Eudicot data set (Simão et
 759 al., 2015). Briefly, genes were annotated and GO terms were assigned based on the

760 BLASTx best match between representative genes and Arabidopsis protein sequences
761 (TAIR10). Orthologs were detected with OrthoFinder (version 2.1.2) (Emms and Kelly,
762 2015), using the predicted protein sequences of the species, and the protein sequences
763 from Arabidopsis, tomato (ITAG2.4), and *Mimulus guttatus* (version 2.0 in Phytozome
764 11.0) as outgroups. We subsequently identified genes with one-to-one or multi-to-one
765 relationships between tetraploid *C. palustris* and diploid *C. terrestris* as comparable
766 orthologs.

767

768 **Comparative expression analyses**

769 The reads obtained for the leaves were trimmed based on quality using Trimmomatic
770 (version 0.36), after which they were counted by RSEM by mapping them onto the
771 transcriptome assembly with Bowtie. Genes expressed at low levels (TPM < 1 in all
772 samples) were omitted from further analyses. After a normalization using the TCC
773 package (version 1.20.0) (Sun et al., 2013), the DEGs between certain sample pairs
774 were detected via multiple comparisons using edgeR (version 3.22.1) (Robinson and
775 Oshlack, 2010), with the following criteria: FDR < 0.05 and expression-level $\log_2(\text{fold-}$
776 $\text{change}) > 1$. The GO term enrichment analyses were performed using R software
777 (version 4.0.3). Specifically, we applied a hypergeometric distribution and an FDR <
778 0.05 calculated by a BH correction. Rare ontologies (< 5 in the transcriptome) were
779 omitted from the analyses. The data were visualized using the following packages of the
780 R software: tidyverse (version 1.3.0) (Wickham et al., 2019), ggfortify (version 0.4.11)
781 (Tang et al., 2016), factoextra (version 1.0.7; <https://CRAN.R->

782 project.org/package=factextra), UpSetR (version 1.4.0) (Conway et al., 2017),
 783 Vennerable (version 3.1.0.9000; <https://github.com/js229/Vennerable>), and
 784 ComplexHeatmap (version 2.5.3) (Gu et al., 2016).

785

786 **Hormonal amount measurement**

787 The shoot tips with developing and almost mature leaves were collected from plants
 788 cultured under the same conditions as the plants used for the RNA-seq experiments.
 789 Hormones were extracted and semi-purified from the frozen samples as previously
 790 described (Kojima and Sakakibara, 2012; Kojima et al., 2009).

791 The GA, ABA, salicylic acid, jasmonic acid, and auxin contents were
 792 quantified using an ultra-high performance liquid chromatography (UHPLC)-ESI
 793 quadrupole-orbitrap mass spectrometer (UHPLC/Q-Exactive; Thermo Fisher Scientific,
 794 USA) with an ODS column (AQUITY UPLC HSS T3, 1.8 μ m, 2.1 $\times$ 100 mm; Waters,
 795 Milford, MA, USA) as previously described (Kojima and Sakakibara, 2012; Shinozaki
 796 et al., 2015). Cytokinins were quantified using an ultra-performance liquid
 797 chromatography (UPLC)-electrospray interface (ESI) tandem quadrupole mass
 798 spectrometer (qMS/MS) (AQUITY UPLC System/Xevo-TQS; Waters) with an ODS
 799 column (AQUITY UPLC HSS T3, 1.8 μ m, 2.1 $\times$ 100 mm; Waters) as previously
 800 described (Kojima et al., 2009).

801

802 **Accession numbers**

803 Sequence data used in this article can be found in the DDBJ DRA with the accession
804 numbers listed in Supplemental table Data Set 2.

805

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815

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817 conducted the experiments; H.K. analyzed the data; and all authors contributed to
818 writing the manuscript.

819 **Figure legends**

820 **Figure 1. *Callitriche palustris* leaf cell morphology.** (A, B) *C. palustris* leaves grown
821 under aerial (A) and submerged conditions (B). (C) Epidermis of an aerial leaf. (D)
822 Epidermis of a submerged leaf. (E) Sub-epidermal palisade layer of an aerial leaf. (F)
823 Sub-epidermal palisade layer of a submerged leaf. (G–L) Plots of the indices for
824 pavement cells (G–I, n = 270 each) and palisade cells (J–L, n = 270 each): cell area (G,
825 J), solidity (H), circularity (K), and the aspect ratio of the best fit ellipse (I, L). Black
826 points represent the means. *P*-values calculated by Welch's *t*-test and Hedge's *g*
827 calculated to indicate the effect size are provided. Data were obtained for nine leaves
828 from three biological replicates for each condition. (M, N) Immunofluorescence images
829 of tubulin in the palisade cells of a young aerial leaf (M) and a young submerged leaf
830 (N). A representative cell is outlined. Scale bars: (A, B) 1 mm, (C–F) 50 μ m, and (M,
831 N) 10 μ m.

832

Figure 2. Effects of phytohormone inhibitors and phytohormones on *C. palustris* leaf development under submerged conditions. (A–G) Leaf forms of the plants treated with phytohormone inhibitors and/or phytohormones under submerged conditions: (A) control, (B) 10^{-6} M AgNO₃, (C) 10^{-7} M uniconazole P, (D) 10^{-7} M ABA, (E) 10^{-6} M GA₃, (F) 10^{-6} M AgNO₃ + 10^{-6} M GA₃, and (G) 10^{-7} M uniconazole P + 10^{-6} M GA₃. (H) Length–width plot of mature leaves. Each point represents one leaf. A total of 70 (for control) or 26–40 (for the others) mature leaves collected from 3–5 biological replicates were measured for each treatment. (I) Images of the adaxial epidermis (top) and sub-epidermal palisade layer (bottom) of the mature leaves. (J) Plots of stomatal density. Crosses represent the means. Different letters denote significant differences among treatments (Tukey’s test, $P < 0.05$; $n = 4-6$). (K, L) Principal component analysis plots of adaxial pavement cells (K) and palisade cells (L). Each point represents one cell ($n = 120$ or 180 for each treatment). Data were collected for four or six leaves from three biological replicates, except for the ABA treatment (six leaves from two replicates). Loading plots are overlapped on the graphs. Data for the leaves or the cells of the aerial control in Figure 3 (denoted as Air) are included in every plot for comparison purposes. Scale bars: 50 μ m.

850 **Figure 3. Effects of ACC and GA₃ on *C. palustris* leaf development under aerial**
851 **conditions.** (A–D) Leaf forms of the plants treated with phytohormones under aerial
852 conditions: (A) control, (B) 10⁻⁵ M ACC, (C) 10⁻⁵ M GA₃, and (D) 10⁻⁵ M ACC + 10⁻⁵
853 M GA₃. (E) Length–width plot of mature leaves. Each point represents one leaf. 25–30
854 mature leaves collected from 3 biological replicates were measured for each treatment.
855 Points denoted by “Sub” represent the untreated submerged leaves shown in Figure 2
856 and are included for comparison purposes. (F) Images of the adaxial epidermis (top) and
857 sub-epidermal palisade layer (bottom) of the mature leaves. (G) Plots of stomatal
858 density. Crosses represent the means. Different letters denote significant differences
859 among treatments (Tukey’s test, $P < 0.05$, n=4–8). (H, I) Principal component analysis
860 plots of adaxial pavement cells (H) and palisade cells (I). Each point represents one cell
861 (n = 180 for each treatment). Data were collected for six leaves from three biological
862 replicates. Loading plots are overlapped on the graphs. Data for the leaves or the cells of
863 the submerged control in Figure 2 (denoted as Sub) are included in every plot for
864 comparison purposes. Scale bars: 50 μm.

865 **Figure 4. Comparative transcriptome analyses of *C. palustris*.** UpSet plots present
 866 the overlapping DEGs in five comparisons. Gene sets that exhibited consistent changes
 867 among the comparisons of aerial, AgNO₃-treated, and ABA-treated leaf primordia are
 868 colored (i.e., shared DEGs). (A) UpSet plot of DEGs downregulated in the submerged
 869 control. (B) UpSet plot of DEGs upregulated in the submerged control.

870 **Figure 5. Submergence response of the terrestrial species *Callitriche terrestris*.** (A)
871 Images of shoots grown under aerial (left) and submerged (right) conditions. (B, C)
872 Leaves from aerial (B) and submerged (C) shoots. (D) Length–width plots of mature
873 leaves from both conditions. (E) Plots of length/width ratios for the mature leaves ($n =$
874 44 each, from 3 biological replicates). The P value was calculated by Welch’s t -test. (F–
875 I) Images of the epidermal (E) and palisade layer (F) cells of a leaf from an aerial plant
876 and the epidermal (G) and palisade layer (H) cells of a leaf from a submerged plant. (J)
877 Plots of stomatal densities of mature leaves ($n=13, 10$, from 3 biological replicates). The
878 P value calculated by Welch’s t -test and Hedge’s g calculated to indicate the effect size
879 are provided. (K) MA plot for transcriptome analyses of the *C. terrestris* developing
880 leaves. Red and blue points represent the downregulated and upregulated DEGs,
881 respectively, under submerged conditions ($FDR < 0.05$ and $\log_2(\text{fold-change}) > 1$). The
882 numbers of upregulated and downregulated DEGs are indicated. (L, M) Venn diagrams
883 of *C. palustris* orthologs identified as DEGs in either *C. palustris* or *C. terrestris*.

884 **Figure 6. Hormone-related gene expression patterns and hormone contents in the**
885 **shoot tips.** (A, B) Expression-level changes to (A) putative hormone metabolism gene
886 orthologs and (B) hormone signaling gene orthologs based on the RNA-seq data. The
887 heatmaps present the mean $\log_2(\text{fold-change})$ values, relative to the corresponding
888 levels in the submerged controls. Asterisks indicate significant differences ($\text{FDR} < 0.05$,
889 expression-level $\log_2(\text{fold-change}) > 1$). Decreasing values reflect the upregulated
890 expression in the submerged control. Gray panels indicate the gene, or its expression,
891 was undetectable. Dev: comparisons among *C. palustris* leaf primordia, Mat:
892 comparison between *C. palustris* mature leaves, and Ct: comparison between *C.*
893 *terrestris* leaf primordia. The column on the right presents the names of *Callitriche*
894 genes and the Arabidopsis orthologs determined using OrthoFinder (in parentheses).
895 (C–E) Hormone contents: (C) ABA, (D) GA₁, and (E) GA₄ in the shoot tips of *C.*
896 *palustris* (top) and *C. terrestris* (bottom). Different letters indicate significant
897 differences among treatments (Tukey's test, $P < 0.05$). For comparisons involving *C.*
898 *terrestris*, the P -value was calculated by Welch's t -test. N.D.: not detected.

899 **Figure 7. Expression patterns of the notable genes and the candidate genes.** (A)
900 Heatmaps of expression-level changes to some genes of interest based on the RNA-seq
901 data. The data are presented in the same manner as in Figure 6A, B. (B) Candidate gene
902 expression patterns. The heatmaps present scaled values of normalized counts from the
903 RNA-seq data. (C) Expression patterns of 19 putative transcription factor genes picked
904 and re-clustered from panel B.

905 **Figure 8: Schematic model of heterophylly in *C. palustris*.** (A) In the submerged
 906 condition, ethylene signaling is activated, whereas ABA signaling is repressed. GA
 907 signaling may not necessarily be enhanced for submerged leaf formation. Either of the
 908 changes in hormone signaling alone is insufficient to induce the development of the
 909 submerged leaf phenotype. Additional factor(s), such as hypoxic stress, may be
 910 involved parallelly or additively with hormone signaling. The physiological changes
 911 induce gene expression changes in leaf primordia (< 500 μm long), after which leaf
 912 developmental processes associated with submerged-type cellular differentiation
 913 proceed. (B) In the aerial condition, while ethylene does not accumulate in the plant as
 914 it can diffuse outward, ABA production is promoted. Then, in the leaf primordia, many
 915 TF genes and some receptor-like genes, which are likely to be associated with the aerial
 916 leaf phenotype, are upregulated.

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1147

A **B**

A

B

L Downregulated in Sub

M Upregulated in Sub

Submerged environment

in planta

Additional factor(s)

Diffusion

ET ↑

ABA ↓

Hormonal changes

GA →

General cell expansion?

Gene expression in primordia

TF genes (*PRE1* ↑, *OBP4L* ↓, *SPCH* ↓ etc.)
Stomagen ↓, Histone-like genes ↑,
Transporter genes ↑

Submerged leaf phenotypes

- cMT arrangement → Cell elongation
- Biased cell division with D/P direction
- Inhibition of lateral vein development
- Inhibition of stomatal development

Aerial environment

in planta

Additional factor(s)

Diffusion

ET ↓

ABA ↑

Hormonal changes

GA →

General cell expansion?

Gene expression in primordia

TF genes (*PRE1* ↓, *OBP4L* ↑, *SPCH* ↑ etc.)
Stomagen ↑, Receptor-like genes (*CLV1*, *BAML*) ↑

Aerial leaf phenotypes

- cMT arrangement
→ Jigsaw puzzle-like/circular cell expansion
- Unbiased cell division direction
- Lateral vein development
- Stomatal development