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

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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.

Introduction

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

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

in response to submergence likely evolved independently in each lineage.

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

In this study, we analyzed leaf formation in *Callitriche* plants (known as water starworts). Members of the genus *Callitriche* are small flowering plants with a broad 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 *Calltriche* 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

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

137 The directional expansion of plant cells is usually associated with the
138 directional orientation of cell wall cellulose microfibers, which is controlled by the
139 orientation of cortical microtubules (cMTs) (Preston, 1974; Green, 1980; Gunning and
140 Hardham, 1982; Lloyd, 1991). We observed that the cMTs of the palisade cells in young
141 (approximately 1 mm long) submerged leaves were oriented perpendicular to the axis of
142 cell elongation, whereas they were not well organized in the palisade cells of young
143 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 $AgNO_3$, 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 GA_3 (Figure 2G, H), implying GA production is required for the

166 elongated leaf form. Additionally, plants grown in water containing GA_3 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 $AgNO_3$ and GA_3 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 $AgNO_3$ and ABA treatment drastically increased stomata density to the extent of normal
174 aerial leaves, whereas uniconazole P and the combination of $AgNO_3$ and GA_3 led to
175 slight increases. In the other GA_3 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 $AgNO_3$, ABA, and
181 $AgNO_3$ + GA_3, the epidermal pavement cells and palisade cells were similar in shape to
182 those of aerial leaves, although the cells in the $AgNO_3$ + GA_3-treated leaves expanded
183 much more than the cells of leaves treated with $AgNO_3$ 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 GA_3 and
190 uniconazole P + GA_3 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 GA_3 was added to the medium, the plants elongated and developed

leaves that were longer than those of the control plants, but GA_3 did not induce the formation of normal submerged leaves (Figure 3C, E). Similar phenotypes were also induced by spraying plants with a GA_3 solution (Supplemental Figure 3).

Finally, the plants were grown in medium containing both ACC and GA_3 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 GA_3, 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

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

239 Regarding gene expression analyses, we extracted RNA from leaf primordia
240 shorter than 500 µm, which were collected from three individuals grown under either
241 aerial or submerged conditions. This growth stage was recently confirmed to precede or
242 coincide with the initiation of cell differentiation under both conditions (Koga et al.,
243 2020). We obtained single-read sequences (Supplemental Data Set 1) and found 4,275
244 differentially expressed genes (DEGs) between developing aerial and submerged leaves
245 (false discovery rate [FDR] < 0.05 and expression-level $\log_2$(fold-change) > 1). We
246 further analyzed gene expression patterns in the primordia submerged in water
247 containing $AgNO_3$, uniconazole P, or ABA, which inhibit the formation of submerged
248 leaves. A total of 2,933, 5,508, and 16,517 genes were identified as DEGs in the
249 $AgNO_3$-, ABA-, and uniconazole P-treated leaf primordia, respectively, relative to the
250 corresponding expression levels under normal submerged conditions (Figure 4).
251 Because the leaves resulting from these treatments were significantly shorter and wider
252 than the leaves formed under normal submerged conditions, we speculated that there is
253 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 $AgNO_3$ and ABA, but not uniconazole P, produced leaves that were almost the same as aerial leaves, implying the DEGs common to the $AgNO_3$-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*

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

Because submergence did not induce drastic changes in the *C. terrestris* leaf 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 μm 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 *PYR1* 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 $AgNO_3$ 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 $AgNO_3$-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 GA_1 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.

Previous research on the molecular response to submergence proved that a subfamily of ERF TFs (i.e., group VII ERFs) contributes to the viability or the morphological changes of some plants under submerged condition (VEEN et al., 2014; Fukao et al., 2006; Xu et al., 2006; Licausi et al., 2011; Hattori et al., 2009). In Arabidopsis, the HRE1/2 and RAP2.12 TFs in this group function under low-oxygen stress conditions (i.e., hypoxia) to enhance flooding tolerance (Licausi et al., 2010). We detected three orthologous group VII ERF TF genes in the *Callitriche* transcriptomes. Of the three group VII ERF TF genes in *C. palustris*, two (*RAP2.2* and *RAP2.3*) were almost constitutively expressed under all conditions like *RAP2.12* of Arabidopsis, whereas the expression of the other orthologous gene (*HRE2*) was upregulated under submerged conditions, although not in the $AgNO_3$-treated primordia (Figure 7A). The same tendency was observed for the orthologs in *C. terrestris* (Figure 7A).

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

386 genes encoding these TFs in several *Callitriche* species offers additional evidence of
387 their functions in stomatal development (Doll et al., 2021). Among the other stomatal
388 development-related genes, *EPIDERMAL PATTERNING FACTOR* (*EPF*) *1/2* orthologs
389 and *TOO MANY MOUTHS* (*TMM*) genes were identified as DEGs in leaf primordia
390 (Figure 7A). Additionally, the expression of the *EPFL9/STOMAGEN* ortholog was
391 downregulated in submerged leaves. These genes were more highly expressed in the
392 samples treated with $AgNO_3$ and ABA than in the submerged control, although the
393 differences were not always significant (Figure 7A).

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

402

403 **Candidate gene set for the regulation of heterophylly**

404 Finally, we compared transcriptome data to extract a set of genes that may be
405 important for the differential development of *C. palustris* leaves. Of the 287 genes
406 extracted from the comparisons of *C. palustris* experimental conditions, we excluded 22
407 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

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

Discussion

Heterophylly in *Callitriche* and other aquatic plants

In this study, we focused on *Callitriche* species to analyze the plasticity of leaf development. Recent studies clarified the molecular basis of heterophylly in several distant lineages of aquatic plants. In *Rorippa aquatica* and *Hygrophila difformis*, the compound leaf forms of submerged plants are associated with the transcriptional activation of class I KNOX genes in leaves (Nakayama et al., 2014; Li et al., 2017). However, orthologs of these genes are not expressed in the simple leaves of *C. palustris* leaves under submerged conditions. Hence, these genes were not in our candidate gene set. An earlier study confirmed that abaxialization resulting from changes in the expression of genes related to dorsiventral polarity is important for heterophylly in *Ranunculus trichophyllus* (Kim et al., 2018). In contrast, our analysis of KANADI and HD-ZIP III TF gene expression in *C. palustris* leaves did not produce evidence of abaxialization (Figure 7A). These genes were also not in the candidate gene set. In *R. trichophyllus*, the activation of ethylene signaling and repression of ABA signaling contribute to the upregulated and downregulated expression of KANADI and HD-ZIP III genes, respectively. Thus, similar hormonal changes, but varying downstream gene expression, are presumed to occur in *C. palustris*. The differences in the mechanisms regulating heterophylly reflect the independent evolution of heterophylly in these plant 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 *CLV1* 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 *CLV1* promoter is active in
490 leaf mesophyll cells (Kawade et al., 2013), although the effect of *CLV1* 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 GA_3 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

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

In some aquatic plants, ethylene positively regulates the formation of submerged leaves. For example, when *Ludwigia arcuate* (Onagraceae) is treated with ethylene gas or ACC under aerial growth conditions, the plants produce submerged-type leaves, indicating that ethylene signaling almost sufficiently induces submerged-type leaf development in this species (Kuwabara et al., 2003; Sato et al., 2008). Similarly, treating the aerial parts of *R. trichophyllus* and *Hygrophila defformis* plants with ethylene induces drastic changes in some leaf phenotypes, which are similar to those observed in submerged leaves to some extent (Kim et al., 2018; Li et al., 2017; Horiguchi et al., 2019). The involvement of ethylene is reasonable because, under submerged conditions, ethylene spontaneously accumulates in the plant body because of the limited gas exchange in water (Jackson, 1985). Indeed, substantial increases in ethylene levels have been reported for various plants growing under submerged conditions (Voesenek and Sasidharan, 2013) for a review). In *C. palustris*, an exposure to $AgNO_3$, which inhibits the perception of ethylene, induced the production of aerial-type leaves under submerged conditions, which suggests that ethylene signaling must be 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 GA_3 and ACC. The inhibition of ethylene signaling resulted in
571 aerial-like cellular morphologies even with GA_3 (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 GA_3,
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 Abscisic 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 $AgNO_3$- 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

606 the candidate gene set (Figure 7B, Supplemental Data Set 4). The downregulated
607 expression of *CpPYR1* in submerged leaves can be explained by reductions in the
608 number of guard cells and the amount of vascular tissue because *PYR1* gene is
609 specifically expressed in these tissues in Arabidopsis (Gonzalez-Guzman et al., 2012).
610 The *PYL4* expression levels were markedly upregulated in the submerged primordia of
611 both *C. palustris* and *C. terrestris*, implying changes in the expression of these genes
612 are not associated with the morphological changes. However, CpPYL4 may contribute
613 to physiological changes in the submerged form by modulating the sensitivity to ABA
614 or inducing differential responses downstream of the ABA signaling pathway.
615
616 **Requirement of additional factors for the formation of submerged leaves**
617 The results of our hormone perturbation experiments suggest that factor(s) other than
618 ethylene and GA are also required to induce the development of submerged leaves
619 under aerial conditions. Our results of hormonal measurements suggested a reduction of
620 ABA amount is one of the requirements. Because the submerged condition is a
621 drastically different environment from the aerial condition, various factors can be
622 assumed, such as changes in light quality, photoperiod, and temperature. These factors
623 have previously been demonstrated to induce differential leaf formation in other aquatic
624 plants (Wells and Pigliucci, 2000). One such factor to be considered may be high turgor
625 pressure under submerged conditions (Deschamp and Cooke, 1983). Low-oxygen stress
626 is another potential factor. Indeed, in *R. trichophyllus*, hypoxia can partly induce the
627 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

694 manually traced the contours of 30 pavement cells and 30 subepidermal palisade cells
695 per leaf. The analyzed cells were randomly selected, but we avoided choosing pavement
696 cells adjacent to guard cells and hair cells because their shapes were distorted. We then
697 examined the cell size and other shape-related indices using the "Area," "Shape
698 descriptors," and "Perimeter" measurement functions of ImageJ/Fiji (Supplemental
699 Figure 1; see ImageJ documents for more details). The cellular length and width were
700 measured as the long and short side lengths of the bounding smallest rectangle,
701 respectively (Supplemental Figure 1). Statistical analyses and visualizations were
702 performed using the following packages of the R software (version 4.0.3): multcomp
703 (version 1.4-16) (Hothorn et al., 2008), tidyverse (version 1.3.0) (Wickham et al., 2019),
704 and ggforce (version 0.3.3; https://CRAN.R-project.org/package=ggforce), and
705 ggfortify (version 0.4.11) (Tang et al., 2016).
706
707 **Immunofluorescence**
708 The immunofluorescence of microtubules was analyzed as previously described
709 (Pasternak et al., 2015), with slight modifications. Briefly, the leaves were fixed in 2%
710 PFA/0.5% glutaraldehyde prepared in a microtubule-stabilizing buffer, after which the
711 central region was dissected. After permeabilizing with methanol, cell wall enzymes,
712 and a 3% (v/v) IGEPAL/10% (v/v) DMSO solution, the samples were incubated with a
713 1:1,000 primary antibody solution (T5168; Sigma-Aldrich, St. Louis, MO, USA) and
714 then with a 1:500 Alexa Fluor 488-conjugated secondary antibody solution (A-11017;
715 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 $AgNO_3$, 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

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

Comparative expression analyses

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

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

Hormonal amount measurement

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

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

Accession numbers

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

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Figure legends

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

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 $AgNO_3$, (C)10^{-7} M uniconazole P, (D) 10^{-7} M ABA, (E) 10^{-6} M GA_3, (F) 10^{-6} M $AgNO_3$ + 10^{-6} M GA_3, and (G) 10^{-7} M uniconazole P + 10^{-6} M GA_3. (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 μm.

Figure 3. Effects of ACC and GA_3 on *C. palustris* leaf development under aerial conditions. (A–D) Leaf forms of the plants treated with phytohormones under aerial conditions: (A) control, (B) 10^{-5} M ACC, (C) 10^{-5} M GA_3, and (D) 10^{-5} M ACC + 10^{-5} M GA_3. (E) Length–width plot of mature leaves. Each point represents one leaf. 25-30 mature leaves collected from 3 biological replicates were measured for each treatment. Points denoted by "Sub" represent the untreated submerged leaves shown in Figure 2 and are included for comparison purposes. (F) Images of the adaxial epidermis (top) and sub-epidermal palisade layer (bottom) of the mature leaves. (G) Plots of stomatal density. Crosses represent the means. Different letters denote significant differences among treatments (Tukey's test, $P < 0.05$, n=4-8). (H, I) Principal component analysis plots of adaxial pavement cells (H) and palisade cells (I). Each point represents one cell (n = 180 for each treatment). Data were collected for six leaves from three biological replicates. Loading plots are overlapped on the graphs. Data for the leaves or the cells of the submerged control in Figure 2 (denoted as Sub) are included in every plot for 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, $AgNO_3$-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.

Figure 5. Submergence response of the terrestrial species *Callitriche terrestris*. (A) Images of shoots grown under aerial (left) and submerged (right) conditions. (B, C) Leaves from aerial (B) and submerged (C) shoots. (D) Length–width plots of mature leaves from both conditions. (E) Plots of length/width ratios for the mature leaves (n = 44 each, from 3 biological replicates). The *P* value was calculated by Welch's *t*-test. (F–I) Images of the epidermal (E) and palisade layer (F) cells of a leaf from an aerial plant and the epidermal (G) and palisade layer (H) cells of a leaf from a submerged plant. (J) Plots of stomatal densities of mature leaves (n=13, 10, from 3 biological replicates). The *P* value calculated by Welch's *t*-test and Hedge's *g* calculated to indicate the effect size are provided. (K) MA plot for transcriptome analyses of the *C. terrestris* developing leaves. Red and blue points represent the downregulated and upregulated DEGs, respectively, under submerged conditions (FDR < 0.05 and $\log_2$(fold-change) > 1). The numbers of upregulated and downregulated DEGs are indicated. (L, M) Venn diagrams 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 $\log_2$(fold-change) values, relative to the corresponding
888 levels in the submerged controls. Asterisks indicate significant differences (FDR < 0.05,
889 expression-level $\log_2$(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) GA_1, and (E) GA_4 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.

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

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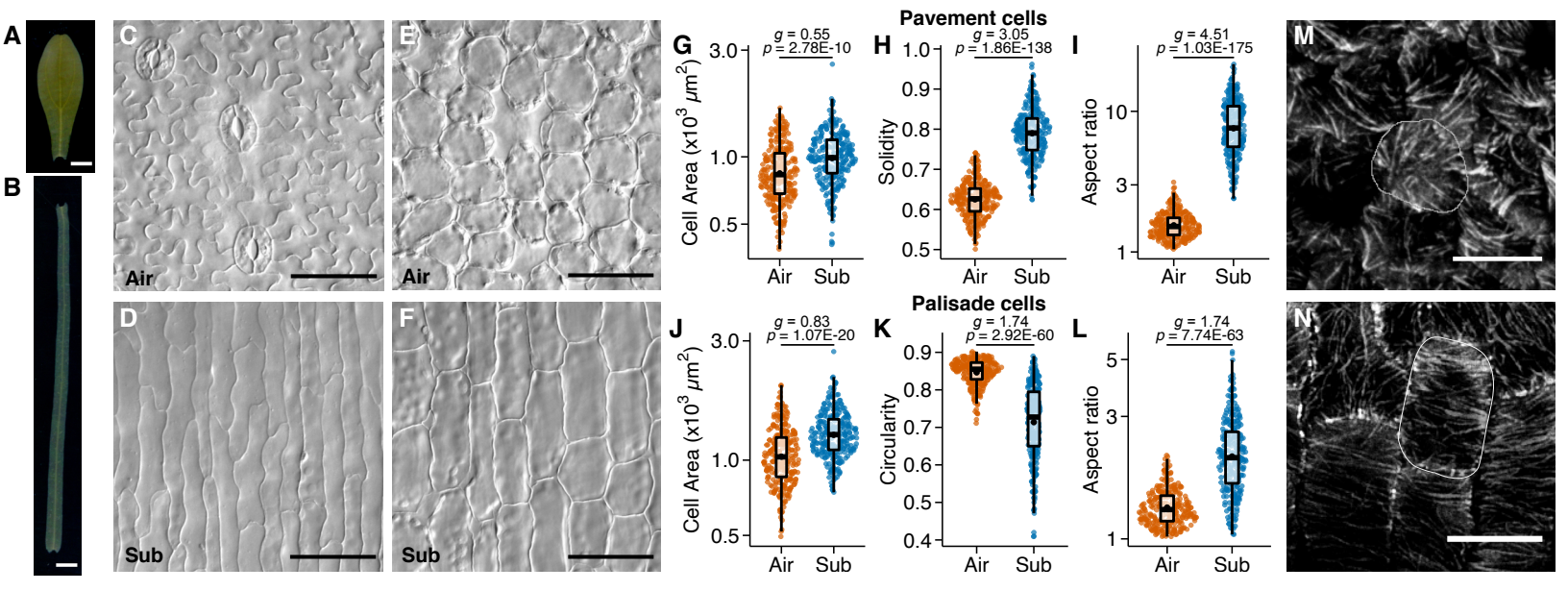
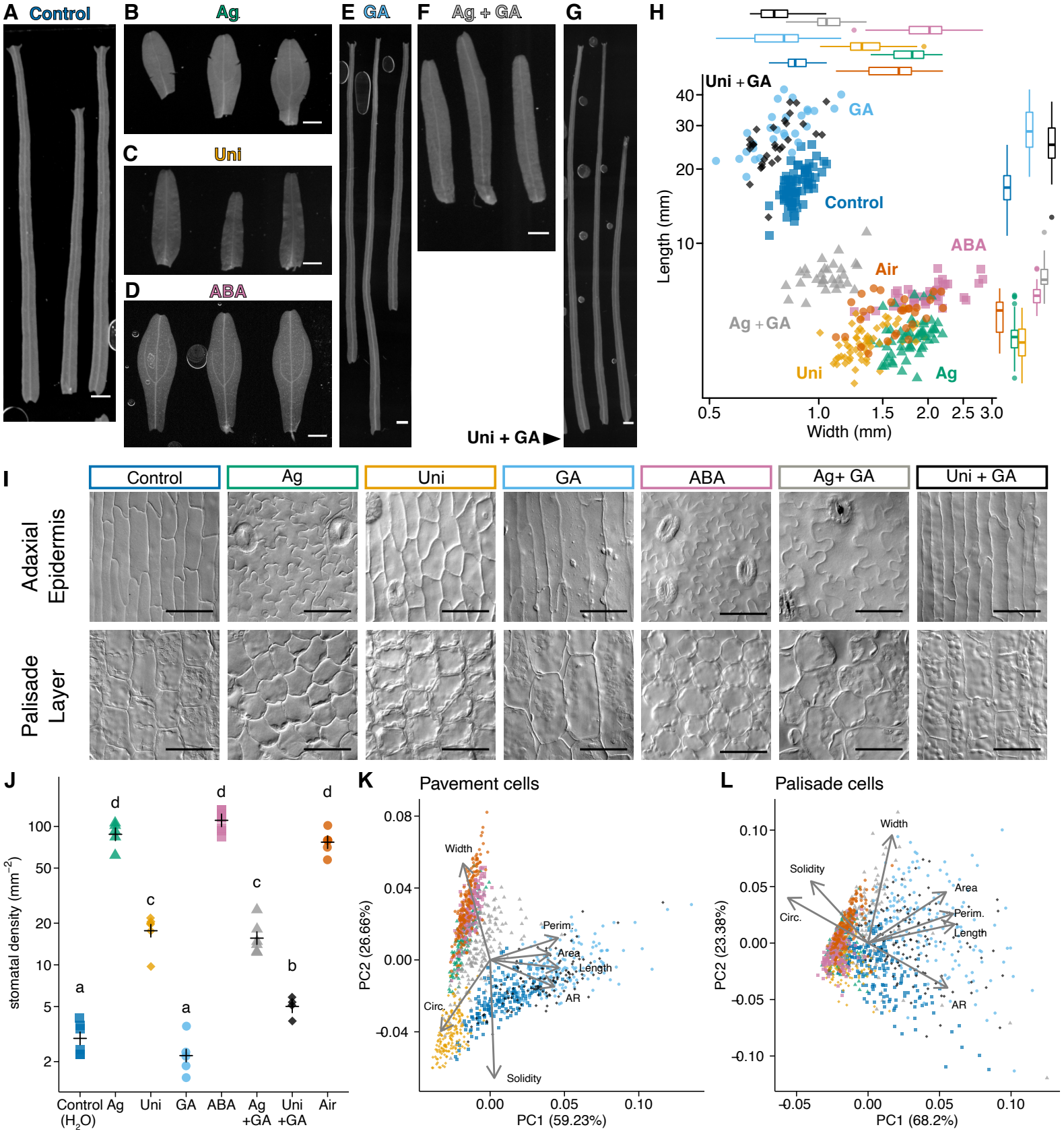

A
B
C
Air
E
Air
D
Sub
F
Sub
Pavement cells
G
g = 0.55
p = 2.78E-10
Cell Area (x10^3 μm^2)
3.0
1.0
0.5
Air
Sub
H
g = 3.05
p = 1.86E-138
Solidity
1.0
0.9
0.8
0.7
0.6
0.5
Air
Sub
I
g = 4.51
p = 1.03E-175
Aspect ratio
10
3
1
Air
Sub
M
Palisade cells
J
g = 0.83
p = 1.07E-20
Cell Area (x10^3 μm^2)
3.0
1.0
0.5
Air
Sub
K
g = 1.74
p = 2.92E-60
Circularity
0.9
0.8
0.7
0.6
0.5
0.4
Air
Sub
L
g = 1.74
p = 7.74E-63
Aspect ratio
5
3
1
Air
Sub
N


A Control
B Ag
C Uni
D ABA
E GA
F Ag + GA
G
Uni + GA
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Uni + GA
GA
Control
ABA
Air
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Uni
Ag
Length (mm)
Width (mm)
I
Control
Ag
Uni
GA
ABA
Ag+ GA
Uni + GA
Adaxial Epidermis
Palisade Layer
J
stomatal density (mm^{-2})
Control (H$_2$O)
Ag
Uni
GA
ABA
Ag +GA
Uni +GA
Air
K
Pavement cells
PC2 (26.66%)
PC1 (59.23%)
Width
Perim.
Area
Length
AR
Circ.
Solidity
L
Palisade cells
PC2 (23.38%)
PC1 (68.2%)
Width
Solidity
Area
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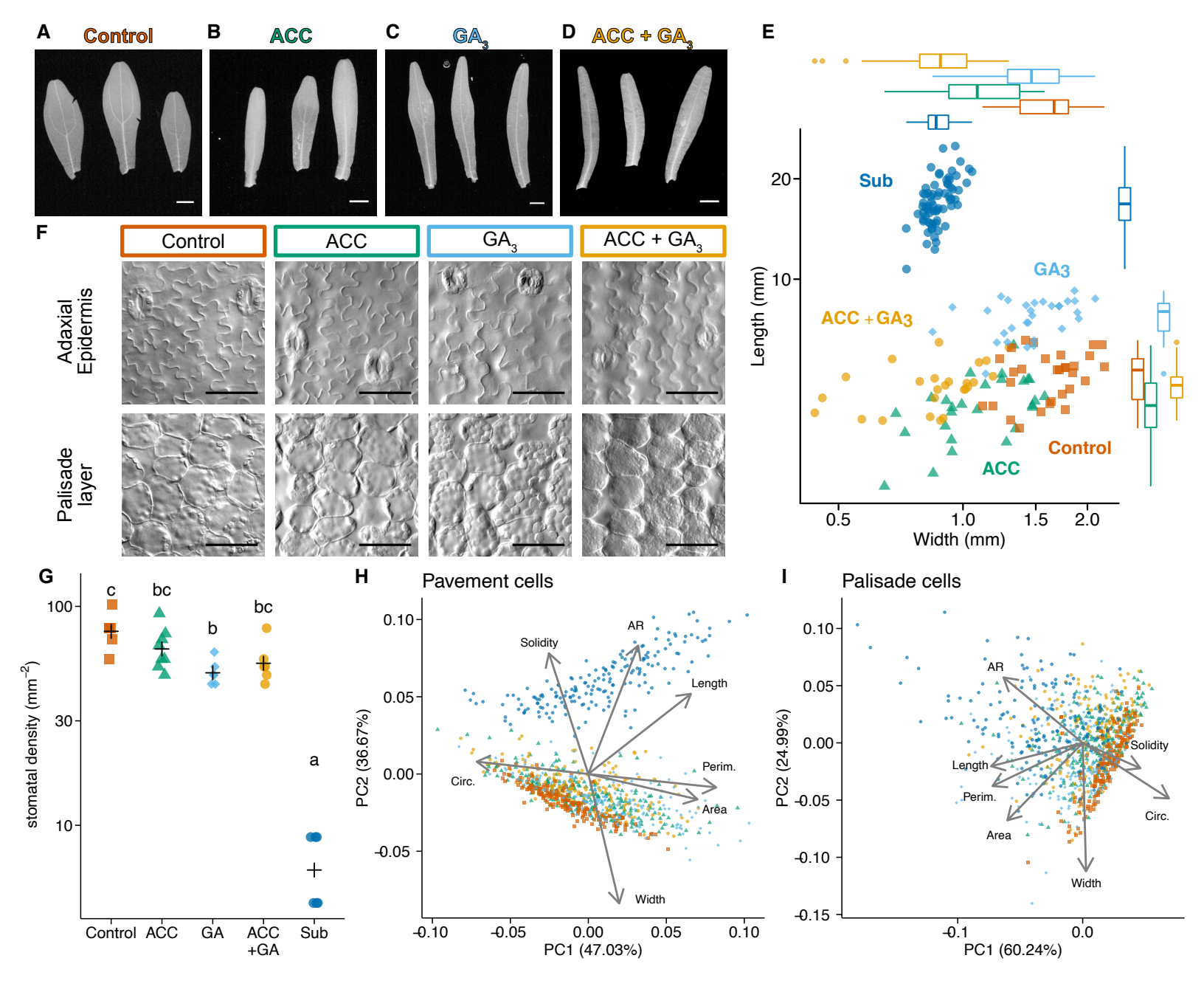
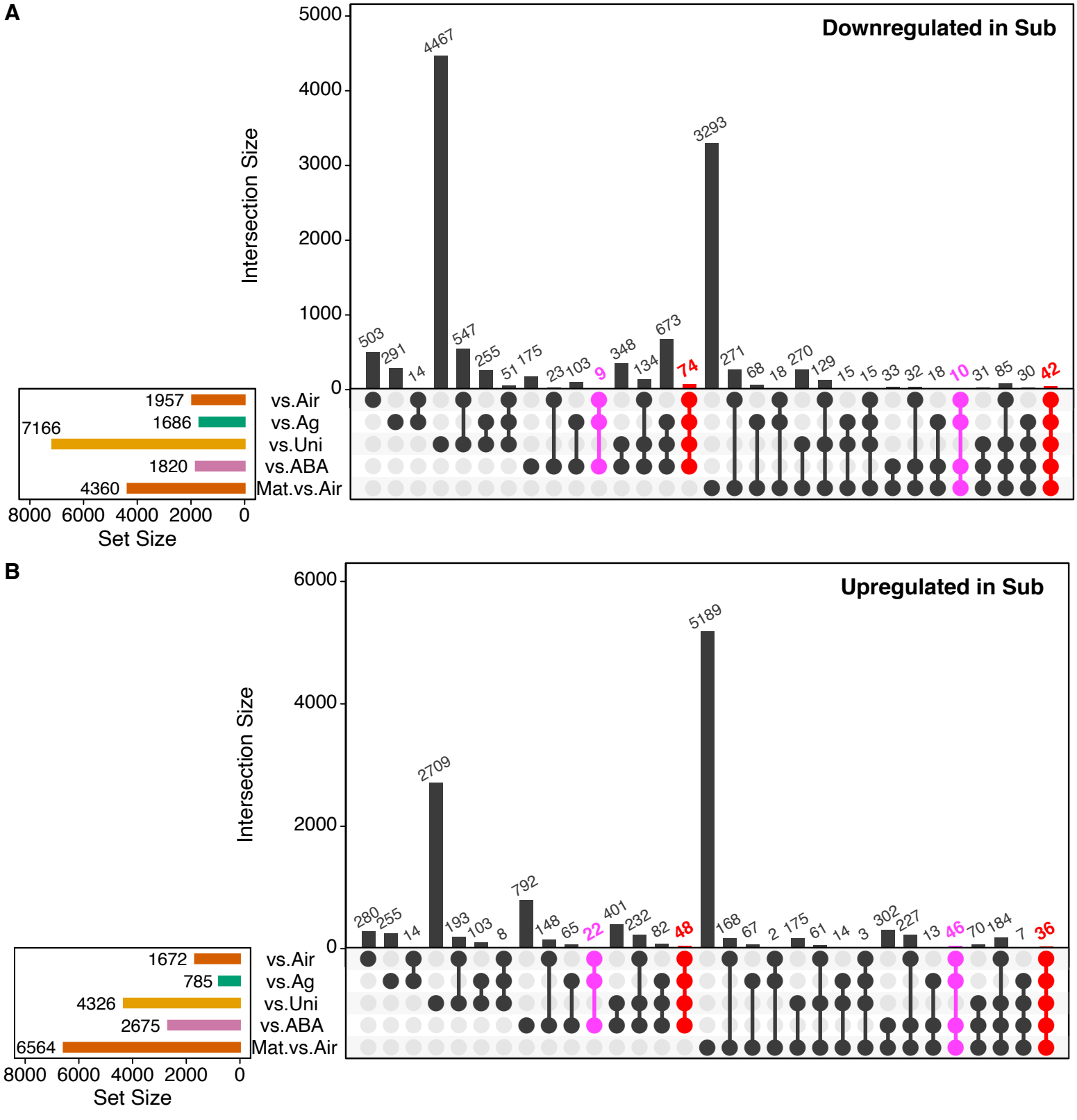
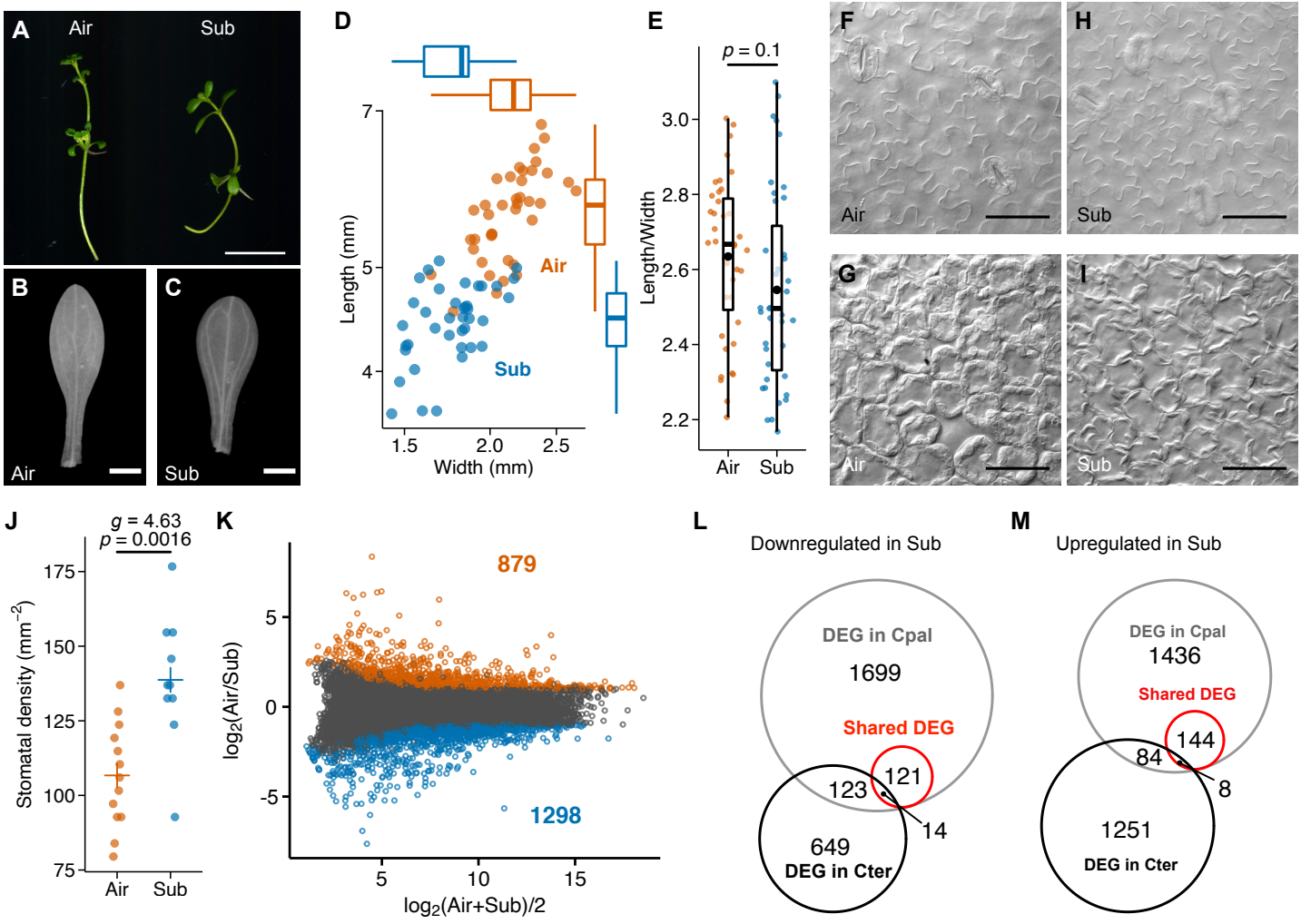
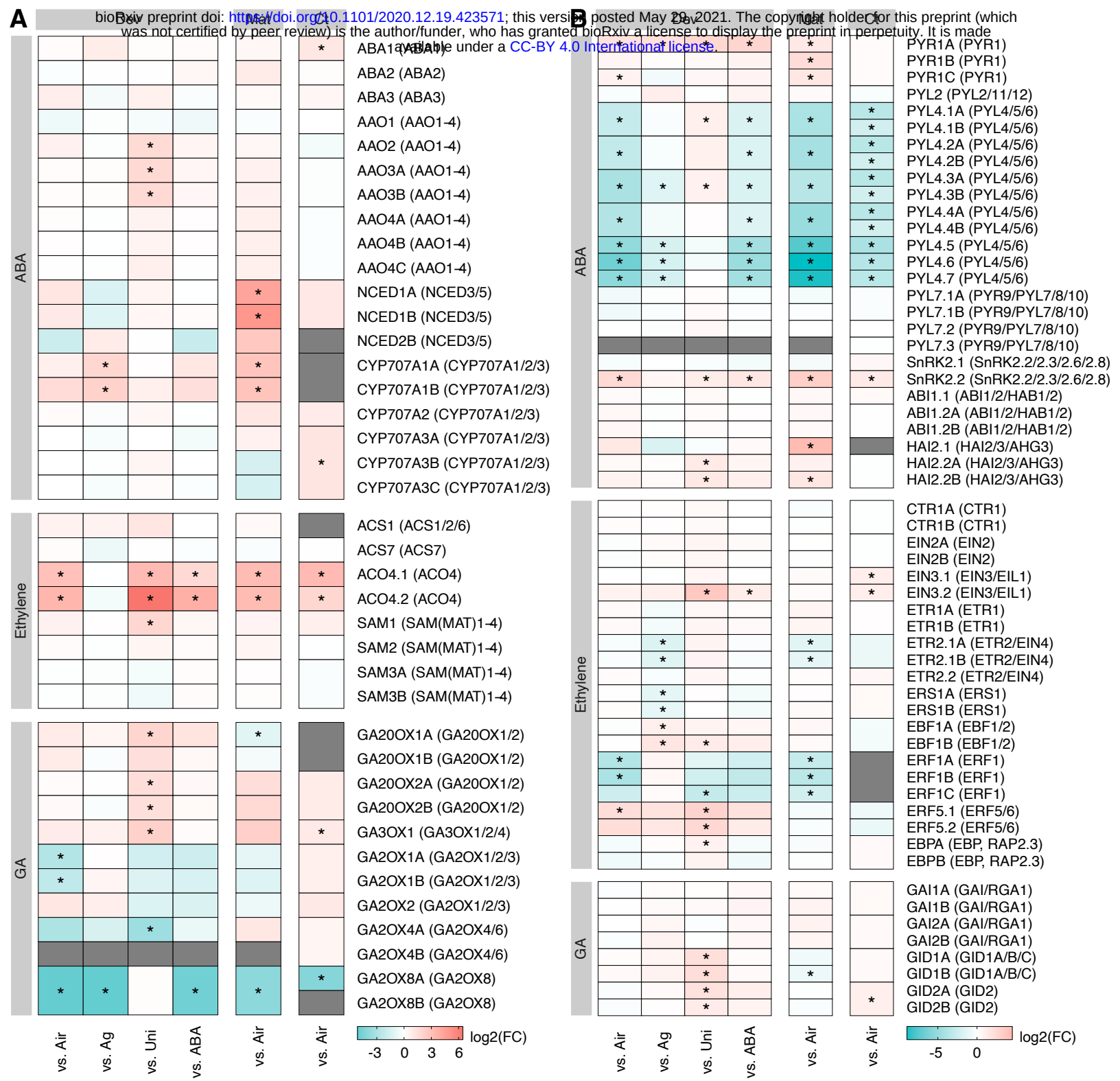
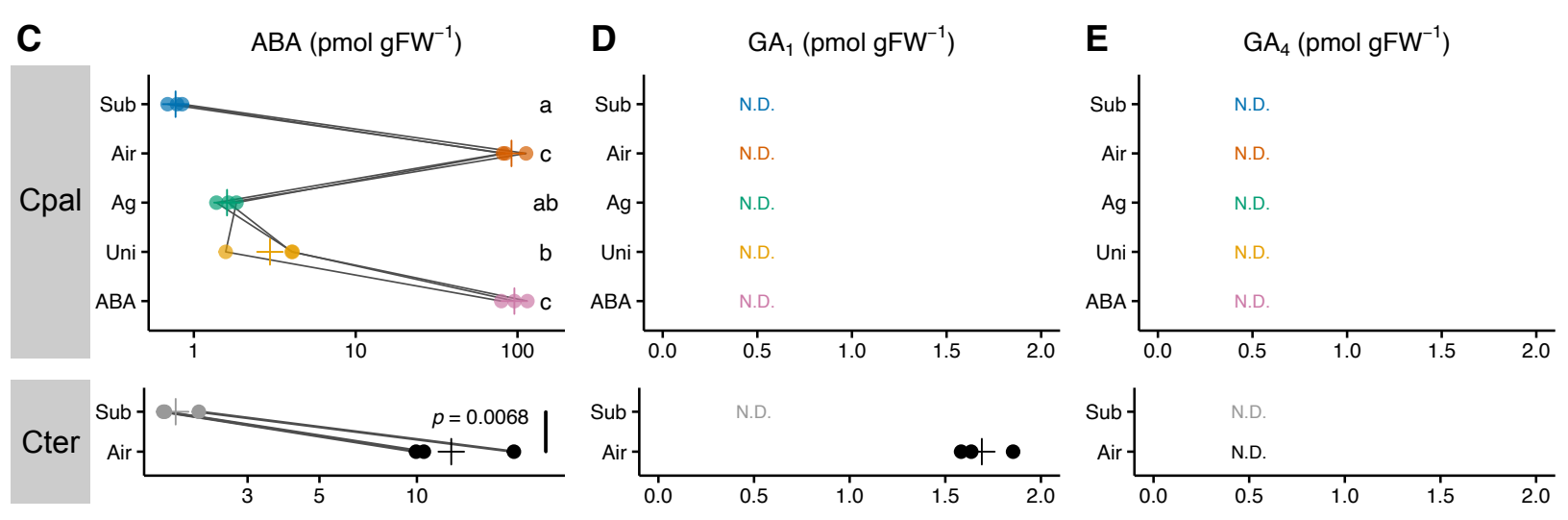
Perim.
Length
AR

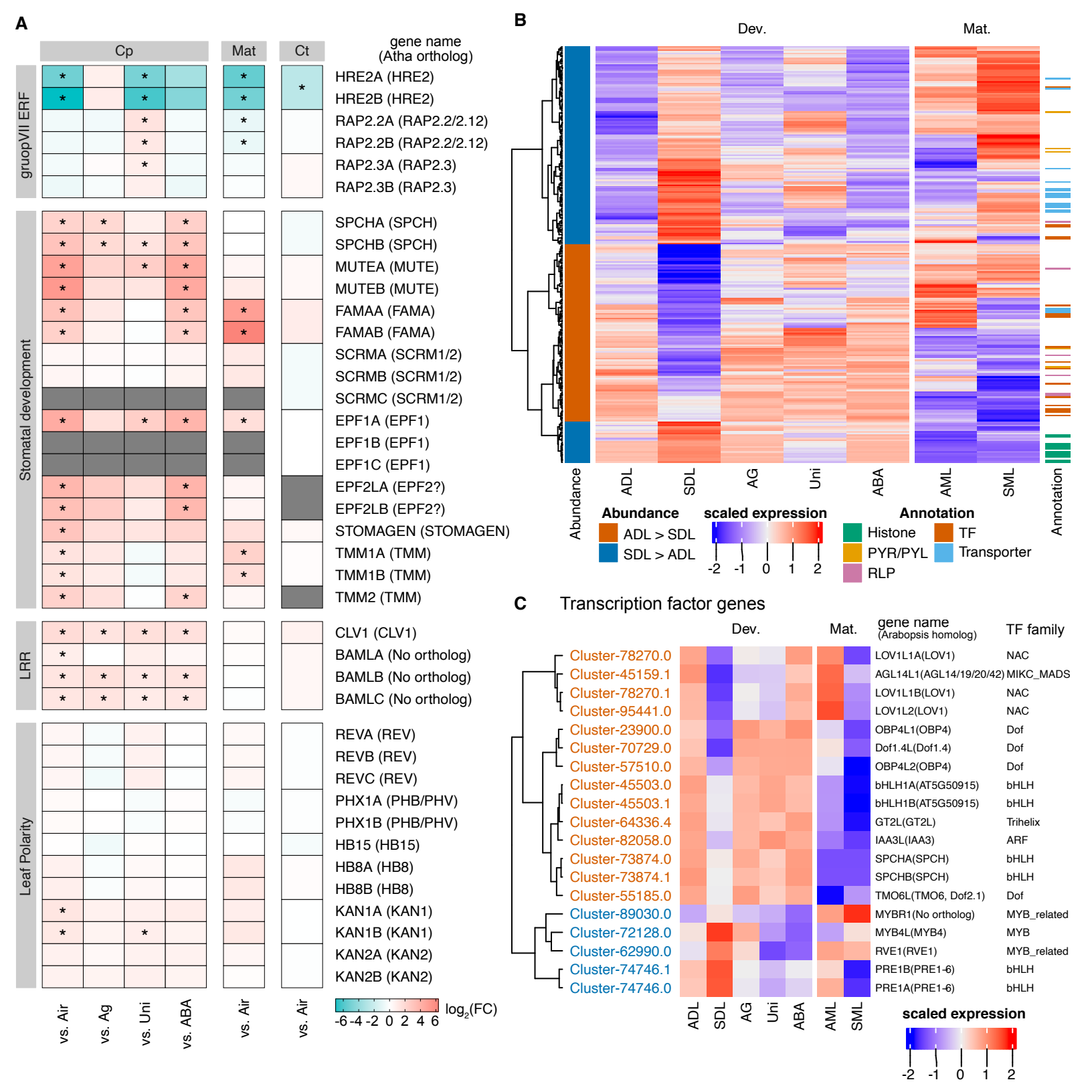

A
Control
B
ACC
C
GA3
D
ACC + GA3
E
Sub
GA3
ACC + GA3
Control
ACC
Length (mm)
Width (mm)
F
Control
ACC
GA3
ACC + GA3
Adaxial Epidermis
Palisade layer
G
stomatal density (mm−2)
c
bc
b
bc
a
Control
ACC
GA
ACC +GA
Sub
H
Pavement cells
Solidity
AR
Length
Perim.
Area
Circ.
Width
PC1 (47.03%)
PC2 (36.67%)
I
Palisade cells
AR
Solidity
Length
Perim.
Area
Circ.
Width
PC1 (60.24%)
PC2 (24.99%)

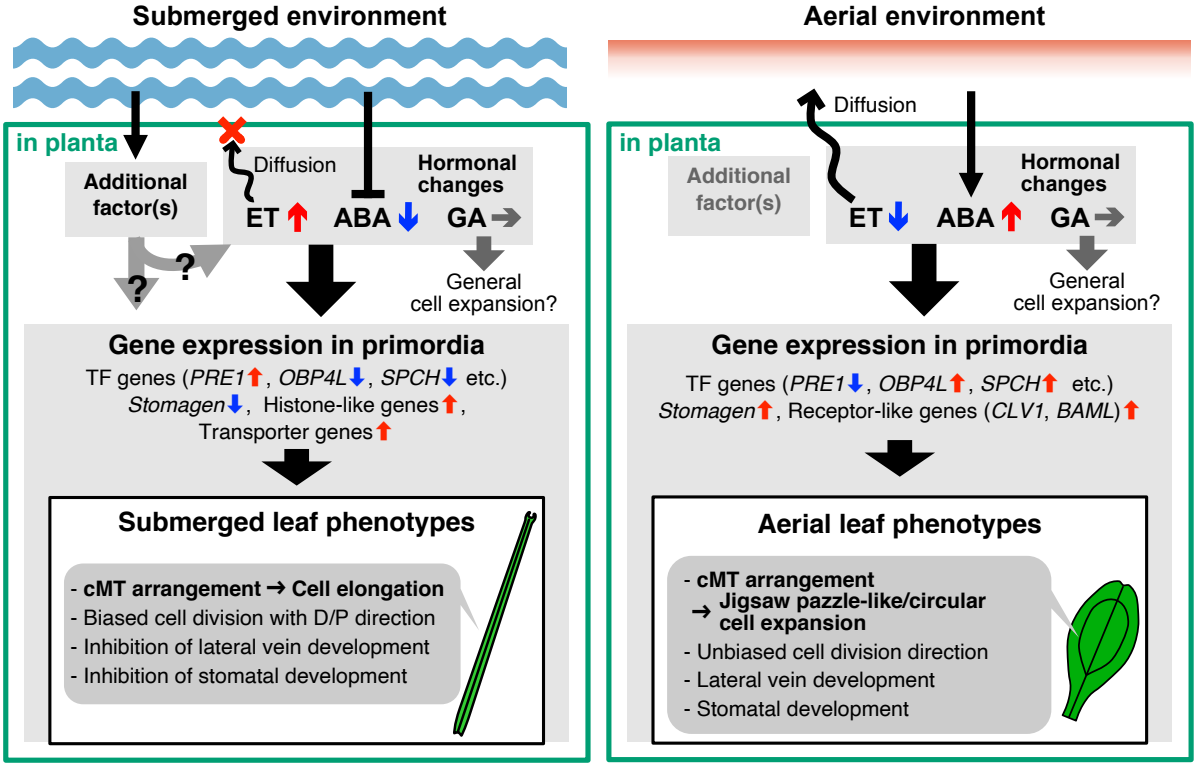

A
Downregulated in Sub
Intersection Size
5000
4000
3000
2000
1000
0
4467
3293
503
291
14
547
255
51
175
23
103
9
348
134
673
74
271
68
18
270
129
15
15
33
32
18
10
31
85
30
42
1957
1686
7166
1820
4360
vs.Air
vs.Ag
vs.Uni
vs.ABA
Mat.vs.Air
8000 6000 4000 2000 0
Set Size
B
Upregulated in Sub
Intersection Size
6000
4000
2000
0
5189
2709
280
255
14
193
103
8
792
148
65
22
401
232
82
48
168
67
2
175
61
14
3
302
227
13
46
70
184
7
36
1672
785
4326
2675
6564
vs.Air
vs.Ag
vs.Uni
vs.ABA
Mat.vs.Air
8000 6000 4000 2000 0
Set Size


A
Air
Sub
B
Air
C
Sub
D
Length (mm)
Width (mm)
Air
Sub
E
p = 0.1
Length/Width
Air
Sub
F
Air
H
Sub
G
Air
I
Sub
J
g = 4.63
p = 0.0016
Stomatal density (mm−2)
Air
Sub
K
879
1298
log2(Air/Sub)
log2(Air+Sub)/2
L
Downregulated in Sub
DEG in Cpal
1699
Shared DEG
123
121
14
649
DEG in Cter
M
Upregulated in Sub
DEG in Cpal
1436
Shared DEG
144
84
8
1251
DEG in Cter






A
Cp
Mat
Ct
gene name
(Atha ortholog)
gruopVII ERF
HRE2A (HRE2)
HRE2B (HRE2)
RAP2.2A (RAP2.2/2.12)
RAP2.2B (RAP2.2/2.12)
RAP2.3A (RAP2.3)
RAP2.3B (RAP2.3)
Stomatal development
SPCHA (SPCH)
SPCHB (SPCH)
MUTEA (MUTE)
MUTEB (MUTE)
FAMAA (FAMA)
FAMAB (FAMA)
SCRMA (SCRM1/2)
SCRMB (SCRM1/2)
SCRMC (SCRM1/2)
EPF1A (EPF1)
EPF1B (EPF1)
EPF1C (EPF1)
EPF2LA (EPF2?)
EPF2LB (EPF2?)
STOMAGEN (STOMAGEN)
TMM1A (TMM)
TMM1B (TMM)
TMM2 (TMM)
LRR
CLV1 (CLV1)
BAMLA (No ortholog)
BAMLB (No ortholog)
BAMLC (No ortholog)
Leaf Polarity
REVA (REV)
REVB (REV)
REVC (REV)
PHX1A (PHB/PHV)
PHX1B (PHB/PHV)
HB15 (HB15)
HB8A (HB8)
HB8B (HB8)
KAN1A (KAN1)
KAN1B (KAN1)
KAN2A (KAN2)
KAN2B (KAN2)
vs. Air
vs. Ag
vs. Uni
vs. ABA
vs. Air
vs. Air
log2(FC)
-6 -4 -2 0 2 4 6
B
Dev.
Mat.
Abundance
ADL
SDL
AG
Uni
ABA
AML
SML
Annotation
Abundance
ADL > SDL
SDL > ADL
scaled expression
-2 -1 0 1 2
Annotation
Histone
TF
PYR/PYL
Transporter
RLP
C
Transcription factor genes
Dev.
Mat.
gene name
(Arabopsis homolog)
TF family
Cluster-78270.0 LOV1L1A(LOV1) NAC
Cluster-45159.1 AGL14L1(AGL14/19/20/42) MIKC_MADS
Cluster-78270.1 LOV1L1B(LOV1) NAC
Cluster-95441.0 LOV1L2(LOV1) NAC
Cluster-23900.0 OBP4L1(OBP4) Dof
Cluster-70729.0 Dof1.4L(Dof1.4) Dof
Cluster-57510.0 OBP4L2(OBP4) Dof
Cluster-45503.0 bHLH1A(AT5G50915) bHLH
Cluster-45503.1 bHLH1B(AT5G50915) bHLH
Cluster-64336.4 GT2L(GT2L) Trihelix
Cluster-82058.0 IAA3L(IAA3) ARF
Cluster-73874.0 SPCHA(SPCH) bHLH
Cluster-73874.1 SPCHB(SPCH) bHLH
Cluster-55185.0 TMO6L(TMO6, Dof2.1) Dof
Cluster-89030.0 MYBR1(No ortholog) MYB_related
Cluster-72128.0 MYB4L(MYB4) MYB
Cluster-62990.0 RVE1(RVE1) MYB_related
Cluster-74746.1 PRE1B(PRE1-6) bHLH
Cluster-74746.0 PRE1A(PRE1-6) bHLH
ADL
SDL
AG
Uni
ABA
AML
SML
scaled expression
-2 -1 0 1 2


Submerged environment
in planta
Additional factor(s)
Diffusion
ET
ABA
GA
Hormonal changes
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
Diffusion
in planta
Additional factor(s)
Hormonal changes
ET
ABA
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 pazzle-like/circular cell expansion
- Unbiased cell division direction
- Lateral vein development
- Stomatal development