Report

Current Biology

Loss of ancestral function in duckweed roots is accompanied by progressive anatomical reduction and a re-distribution of nutrient transporters

Highlights

- Duckweed roots have undergone progressive anatomical reduction
- Duckweed roots are required for neither growth nor nutrient uptake
- Expression bias of nutrient transporters has shifted from roots to aerial tissues
- Duckweed roots have lost their ancestral function and can be considered vestigial

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In brief

Organ loss is a reoccurring feature during evolution. Ware et al. show that in adapting to the aquatic environment, duckweed roots underwent progressive anatomical simplification and are no longer required for their once salient function of nutrient uptake. This research positions duckweed roots as an ideal model to study vestigiality in plants.

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Report

Loss of ancestral function in duckweed roots is accompanied by progressive anatomical reduction and a re-distribution of nutrient transporters

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SUMMARY

Organ loss occurs frequently during plant and animal evolution. Sometimes, non-functional organs are retained through evolution. Vestigial organs are defined as genetically determined structures that have lost their ancestral (or salient) function.[1–3] Duckweeds, an aquatic monocot family, exhibit both these characteristics. They possess a uniquely simple body plan, variably across five genera, two of which are rootless. Due to the existence of closely related species with a wide diversity in rooting strategies, duckweed roots represent a powerful system for investigating vestigiality. To explore this, we employed a panel of physiological, ionomic, and transcriptomic analyses, with the main goal of elucidating the extent of vestigiality in duckweed roots. We uncovered a progressive reduction in root anatomy as genera diverge and revealed that the root has lost its salient ancestral function as an organ required for supplying nutrients to the plant. Accompanying this, nutrient transporter expression patterns have lost the stereotypical root biased localization observed in other plant species. While other examples of organ loss such as limbs in reptiles[4] or eyes in cavefish[5] frequently display a binary of presence/absence, duckweeds provide a unique snapshot of an organ with varying degrees of vestigialization in closely related neighbors and thus provide a unique resource for exploration of how organs behave at different stages along the process of loss.

RESULTS AND DISCUSSION

Duckweed root anatomy is highly reduced and supports progressive between-genera reduction in number of cortical cell layers

Duckweeds (*Lemnaceae*) are highly morphologically reduced free-floating angiosperms lacking many of the key organs common in flowering plants. The entire plant body is reduced to a flattened frond that sits at the air-water interface, sometimes subtended by a root.[6] The family comprises 5 genera divided into 2 subfamilies, Lemnoideae and Wolffioideae (Figure 1). Within these genera, there is an evolutionary trajectory in number of roots consistent with root vestigialization: the earliest-diverging genera, *Spirodela* and *Landoltia*, possess multiple roots; the later diverging genus *Lemna* possesses a single root; and the most recently diverging lineages are rootless (*Wolffia* and *Wolffiella*) (Figure 1).

Vestigiality affects function and anatomy in tandem.[2] We hypothesized that if duckweed roots were vestigial, then they would most likely be universally simplified, and additionally that there might be progressive reduction of anatomy in later-diverging genera. We built upon previous reports looking into a handful of individual species[8–12] by surveying the root anatomy of a global collection of specimens including representatives of almost all rooted duckweed species, which we compared to the outgroup *Pistia stratiotes*. Like duckweeds, *Pistia* species are free-floating members of the Araceae and share many morphological and ecological similarities. Fossil evidence[13,14] and phylogenetic analyses[15] suggest that duckweeds and *Pistia* independently colonized aquatic habitats, with the duckweed colonization event being considerably earlier.[16]

While lateral roots were clearly present in *Pistia*, similar to previous reports,[6] we observed that roots of duckweeds neither branch nor form root hairs. Consistent with this, An et al.[8] have reported loss of developmental genes associated with lateral root and root hair formation in *Spirodela*. Anatomical analysis revealed that roots are consistently reduced in both cell number and morphological complexity compared with *Pistia* (Figures 2A and S1; Table S1). Typical patterning observed included 3–5 cortical cell layers (CCLs), a highly reduced vasculature consisting of 16–18 cells enclosed by an endodermis (Figure 2B). The vascular bundle composed a single central xylem that is typically surrounded by a small number (7–10) of what

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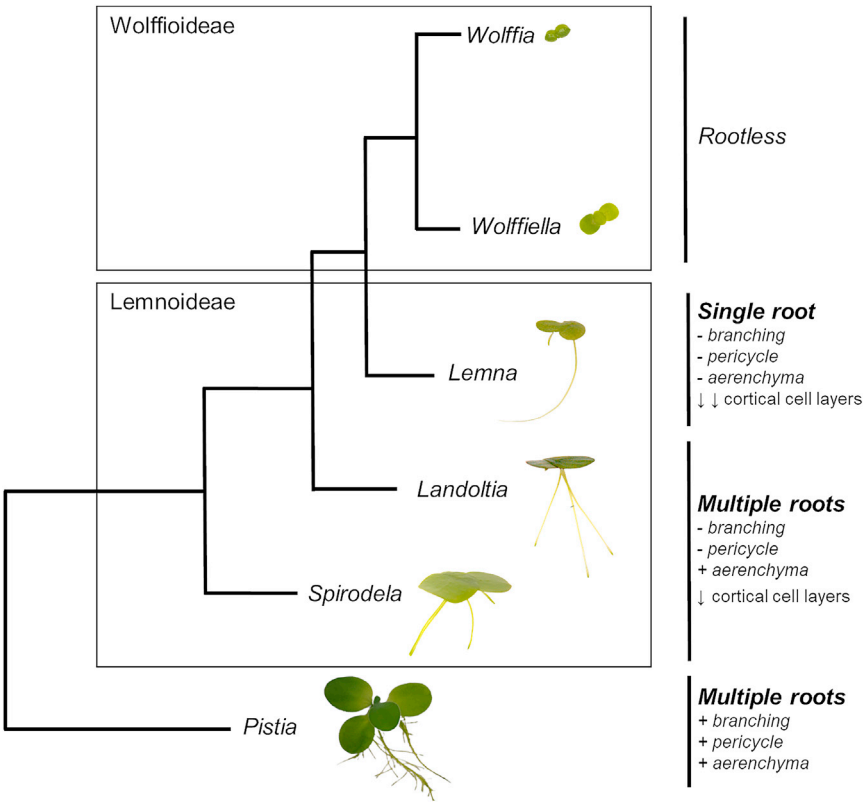

Figure 1. Representative phylogeny of the duckweed genera highlighting the progressive loss of roots and loss of individual root traits as genera diverge

Pistia (an aquatic free-floating Aroid outside the Lemnaceae) serves as an outgroup. Presence of individual root traits is indicated by + and −; arrows next to cortical cell layers indicate the progressive reduction in layer number. The phylogeny was redrawn based on original data in Tippery and Les.[7] Representative images (not to scale) of species from each genus are shown.

appear to be phloem parenchyma cells. Fuchsin staining for lignin confirmed the presence of the Casparian strip within the endodermis in representative species of each genus; however, we noted less extensive lignification of the central xylem in *Landoltia* and *Lemna* (Figures 2A and S1). The pericycle, which would typically surround the vascular bundle, was absent in all duckweeds surveyed, and this most likely explains the absence of lateral roots. *Pistia*, conversely, has a greater number of CCLs, multiple xylem files, a considerably larger stele containing approximately 100 cells, and a pericycle (Figures 2C and S1).

After sectioning roots of our duckweed collection (encompassing 14 of the 16 extant rooted species), we conducted a principal component analysis (PCA) on a variety of anatomical parameters to survey trends (Figures 2B and S2A). Each point in this analysis represents the data captured from a root section of a separate individual, with 22 input variables (Figure S2B). The PCA displays 5 distinct clusters, broadly consistent with phylogenetic groupings (Figure 2C).

Pistia primary roots are mostly separated from duckweed roots along PC1, which correlates with traits corresponding to the vascular cylinder, endodermis diameter, xylem/phloem cell number, and outermost CCLs (Figure 2C). Duckweed genera primarily separate along PC2, which correlates most strongly with cortical cell size, the number of cells within cortex layers 2 and 3, and their diameters (Figure 2C). *Pistia* lateral roots fall within the *Lemna* cluster. It is feasible that this cluster represents or is approaching an anatomical "minimum," below which it would not be possible to form a root.

Given the correlation of PC2 with CCLs, we measured the number of CCLs between *Spirodela* spp., *Landoltia* spp., and *Lemna* spp. (Figure S2C). Three out of six *Spirodela* accessions display 5 CCLs, while eleven out of twelve later-diverging *Lemna* accessions show 3 CCLs. Consequently, the mean number of CCLs is progressively reduced through the genera (Figures 2B and S2C). Several duckweed species also have large extracellular air spaces within the cortex, possibly schizogenous aerenchyma.[17] This feature appears more frequently in *Spirodela* (5 out of 6 lines), in 1 out of 2 of the *Landoltia* lines, and in only 2 out of 12 of the *Lemna* lines (*yungensis* and to a lesser extent *valdiviana*) (Figures 2A and S1). Given that the duckweed genera broadly cluster morphologically within their own genetic groups, and we see a continuous quantitative reduction in numbers of CCLs and aerenchyma *from* Spirodela *to Lemna*, we propose that in tandem with reduction in root number, root anatomy is progressively reduced in more recently derived duckweed lineages (annotated onto the phylogeny in Figure 1).

Continuous root removal does not compromise growth in duckweed, unlike in *Pistia stratiotes*

Our anatomical analyses clearly established a progressive reduction in root anatomical complexity across all duckweed species correlating with more derived overall body plans. For an organ to be considered vestigial, it must also have lost its salient function. Defining salient functions for an individual organ can be challenging. However, for almost all angiosperms, a primary function of roots is that they are required to supply water and nutrients to the growing plant, sustaining and facilitating growth of aboveground tissues.[18] Since as early as the mid-1800s, botanists have pondered over duckweed root function. Hegelmaier[19] noted that *Lemna gibba* without roots can sometimes be found in natural habitats. Gorham[20] observed that coating the underside of *Lemna minor* fronds with a hydrophobic wax reduced growth rate. Muhonen and colleagues[21] also noted that *Spirodela polyrhiza* could still grow following a single root excision event. While these studies imply that roots may not be required for normal growth, they do not evaluate systematically or directly test their capacity for resource capture. Indeed, more recent studies show that both roots and fronds have the capacity to acquire N in both *Lemna minor* and *Landoltia*

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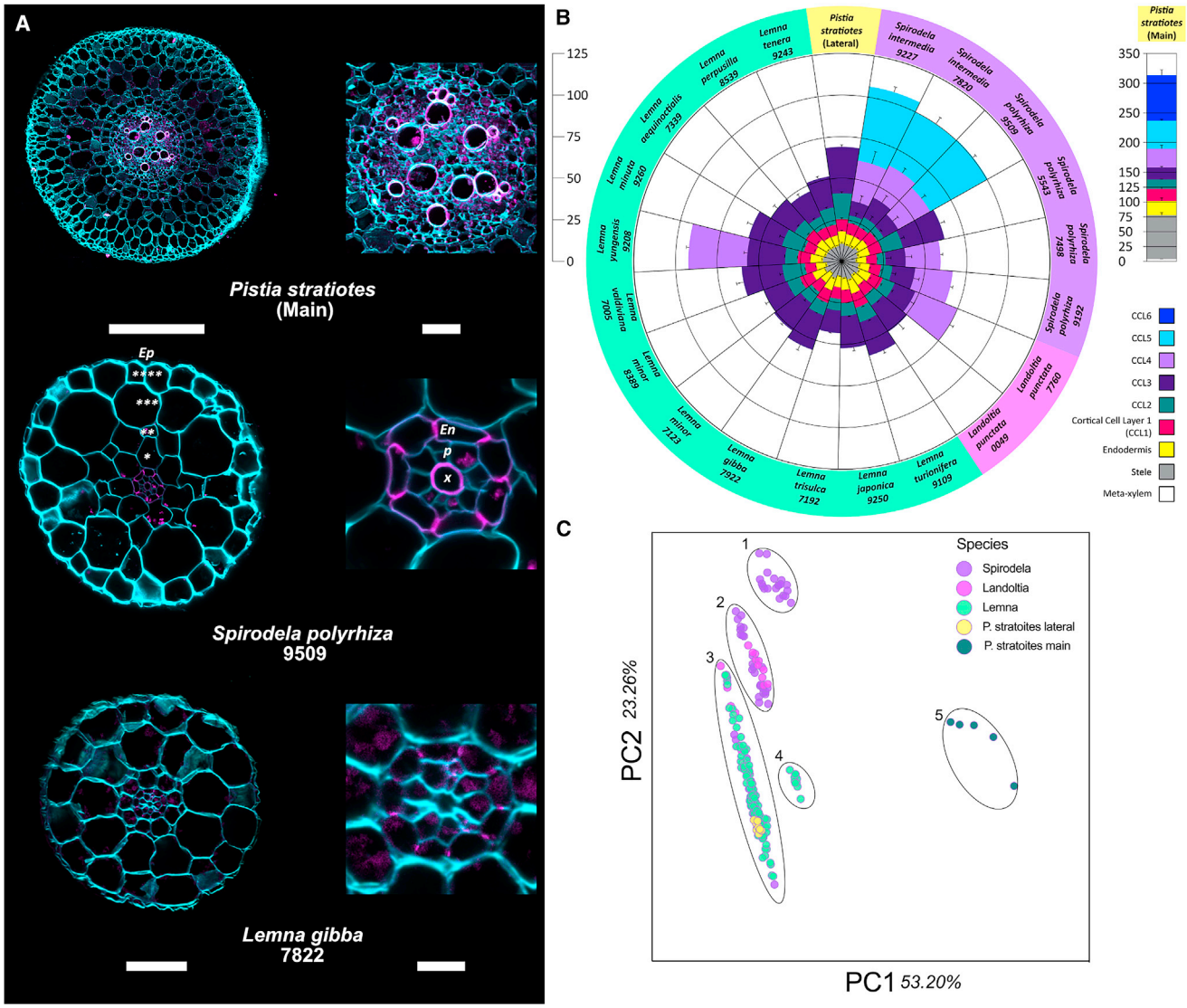

Figure 2. Comparison of root anatomical traits across a panel of almost all extant duckweeds reveals a highly reduced anatomy

(A) Representative images of root sections from *Spirodela polyrhiza* and *Lemna gibba* alongside the main root of *Pistia stratiotes*. Images were obtained via fresh tissue sectioning and confocal imaging following staining with calcofluor (cellulose/cyan) and basic fuchsin (lignin/magenta). Scale bars for *Pistia stratiotes*, 500 μm (top left) and 100 μm (top right). Scale bars for *Spirodela polyrhiza* and *Lemna gibba*, 50 μm (bottom left) and 10 μm (bottom right). x, central xylem; p, phloem parenchyma; En, endodermis; Ep, epidermis; consecutive cortex cell layers are shown with asterisks. See also Figure S1 for additional species.

(B) Rose diagram displaying the root diameter at each cell layer. Main roots of *Pistia stratiotes* are shown in a separate bar chart for clarity. Background color underlying the species labels represents genera: yellow represents *Pistia*, purple *Spirodela*, pink *Landoltia*, green *Lemna*. Color coded key to the different cell layers is displayed on the rose diagrams. n = 10, apart from *Pistia* (main root) and *Lemna trisulca*, where n = 5. Error bars ± SD. See also Table S1 for data.

(C) PCA based on 22 variables, with 210 rows (Figure S2), derived from an anatomical analysis of fresh root sections from 20 duckweed lines, encompassing 14 species, and main and lateral roots of *Pistia stratiotes*. Clusters have been manually highlighted and numbered for ease of further discussion. Percentage of variance explained by each PC is indicated on the relevant axis.

punctata.[22,23] We hypothesized that the observed reduction in root anatomical complexity would be mirrored by a reduced requirement of roots for plant growth.

To test this hypothesis, we compared growth rate in response to root removal in six representative duckweed species, *Spirodela polyrhiza* (9509), *Landoltia punctata* (0049), *Lemna minor* (8389), *Lemna tenera* (9020), *Lemna yungensis* (9208), and *Lemna turionifera* (9109), alongside the outgroup *Pistia stratiotes*. Root removal was conducted daily, minimizing growth of new root material. Frond (or aerial tissue) area was measured concurrently, normalized to the initial area value (Figure 3A). We observed considerable increase (5- to 10-fold) in frond area for all duckweed species with intact root systems. Growth of root-removed groups generally did not significantly differ from that of the control (i.e., intact root systems), and the only 2 significant time points were transitory (Figure 3A). In contrast, root removal markedly and significantly reduced the growth rate of *Pistia stratiotes* (Figure 3A). We also measured dry weight

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of frond or aerial tissue at the end of the growth series. For *Spirodela polyrhiza*, *Lemna minor*, and *Lemna yungensis*, we did not observe a difference in dry weight in rooted versus root excised samples. For *Landoltia punctata* and *Lemna tenera*, there was a slight reduction in frond mass at the end of the time series ($p \leq 0.05$), and for both *Pistia* and *Lemna turionifera* we observed a larger reduction in frond mass ($p \leq 0.01$), suggesting some variation among duckweeds (Figure 3B). Taken alongside the growth curves, these results support the hypothesis that duckweed roots are not required to sustain growth in laboratory conditions, but *Pistia* roots have an important role in driving growth.

Root removal does not impair the ability of duckweed species to absorb macro- and micronutrients

To directly test the capacity of roots for nutrient uptake, we leveraged an ionomics platform in tandem with N content analysis, which permitted a survey of the elemental landscape of duckweed fronds and *Pistia* aerial tissue following the root removal growth series. Every element present in our growth media was quantified.

We first considered the acquisition of N and P, the major root-acquired nutrients regulating aquatic plant growth.[24] In no instances did root removal in duckweed impact N or P levels in the frond, while both elements were significantly reduced by root excision in *Pistia* (Figures 3C and 3D). Additionally, no other elements included in our analysis (0 out of 13) exhibited reduced accumulation in *Spirodela polyrhiza*, *Landoltia punctata*, or *Lemna yungensis* grown without roots. *Lemna minor* and *turionifera* both exhibited reduced accumulation of a single element—Cu and Na, respectively—after root removal, while *Lemna tenera* showed reduced accumulation of K and Mo. This suggests that the frond itself can acquire a breadth of mineral elements. Conversely, in *Pistia*, 7 of the 14 elements quantified exhibited reduced accumulation in shoot tissues following root removal, with N, P, S, K, Fe, Mn, and Zn all being significantly reduced (Figures 3C and 3D; Table S2).

A surprising result was an increased accumulation of certain nutrients in duckweed fronds following root excision. This included B, S, Mg, Ca, Fe, Mn, Co, and Mo (Figures 3C and 3D). A potential hypothesis is that duckweed roots could be being neofunctionalized for the storage, sequestration, or efflux of excess nutrients. Indeed, raphides (calcium oxalate crystals) are present in *Lemna minor* and have been shown to localize within roots.[25,26]

Together, these data suggest that while roots are no longer required for growth and nutrient uptake in duckweeds, *Pistia* roots still play an important role in nutrient acquisition. However, given that they are not absolutely required for growth, it may be that *Pistia* is en route to root vestigiality, albeit at a considerably less advanced stage than the duckweeds.

Vestigiality in the duckweed root corresponds to shifted spatial expression of nutrient transporters

Given that duckweed roots are not required for nutrient uptake, we questioned whether this might involve changes in nutrient transporter expression. We generated bulk RNA sequencing datasets to determine the expression pattern of nutrient transport genes in *Spirodela*, *Pistia*, and *Arabidopsis* comprising the mature frond or leaf and mature root.

Because of their well-evidenced roles in N and P uptake via the root, we queried the *NRT1*, *NRT2*, *PHT*, *PHO*, and *AMT* gene families. These families contain members responsible for acquiring and transporting N and P from the soil, such as NRT1.5, NRT2.1, and PHT1.1, but not all members serve this function.[27–30] We took a gene-family-level approach and identified all members of each gene family in *Pistia* and *Spirodela* using *Arabidopsis* as a reference, ensuring no members were missed. Protein domain structure-based ortholog identification revealed a substantial contraction in the quantity of members of these families in *Spirodela polyrhiza* relative to other species, consistent with a previous report,[31] although our methodology further pruned the quantity of PHT transporters to 3.

In *Arabidopsis*, the majority of N and P transporters are significantly enriched in either the root or shoot, with more being abundant in roots rather than shoots, consistent with roots playing a specialized role in nutrient uptake (Figures 4A and 4B). In *Spirodela*, the majority of N and P transporters show no bias in root versus shoot expression, suggesting that both organs may be equally capable of acquiring N and P (Figures 4A and 4B). This is consistent with the frond being entirely able to compensate for root excision in terms of nutrient acquisition in our previous experiments. The percentage of transporters enriched in the root in *Pistia* is somewhat intermediate between *Arabidopsis* and *Spirodela*, in keeping with the idea that *Pistia* roots are in the early stages of vestigiality.

Transporters such as *PHT1;1* are often responsive to environmental conditions and induced under low phosphate.[32] We hypothesized that if *Spirodela* roots were not required for nutrient uptake, then we would not expect to see phosphate transporters specifically induced in roots. We therefore examined the spatial expression of phosphate transporters in *S. polyrhiza* after 24 h of phosphate-limiting conditions. In this dataset, the *PHO1* and *PHO2* orthologs were the only ones to be differentially expressed under inductive conditions; both showed higher induction in the frond than root (Figure S3A). Following transfer to phosphate-limiting conditions, we also observed a higher number of differentially expressed genes in the frond with a specific enrichment for transporters (Figures S3B and S3C). Collectively, these data suggest that alongside root vestigialization, there has been both a contraction in the number of members in N and P transporter families and a shift in the expression patterns so that these transporters are no longer predominantly expressed in roots.

Conclusions

Our results support a model of progressive vestigiality of roots across the duckweeds. Subtle anatomical differences between genera and species exist, opening the door to this organ's utilization as a model for deepening our understanding of vestigiality and organ loss. It has been speculated that duckweed roots may have acquired novel functions, such as acting as a sea anchor.[8,10] In this paper we show that duckweed roots are not required for nutrient uptake. We predict that the role of duckweed roots in nutrient assimilation has become gradually diminished over millions of years and that this is an ongoing process. We caution that our results should not be interpreted to suggest that duckweed roots play no role in nutrient uptake. Indeed, as we show transporters are still expressed in *S. polyrhiza* roots, it is probable that these roots still retain some capacity for nutrient

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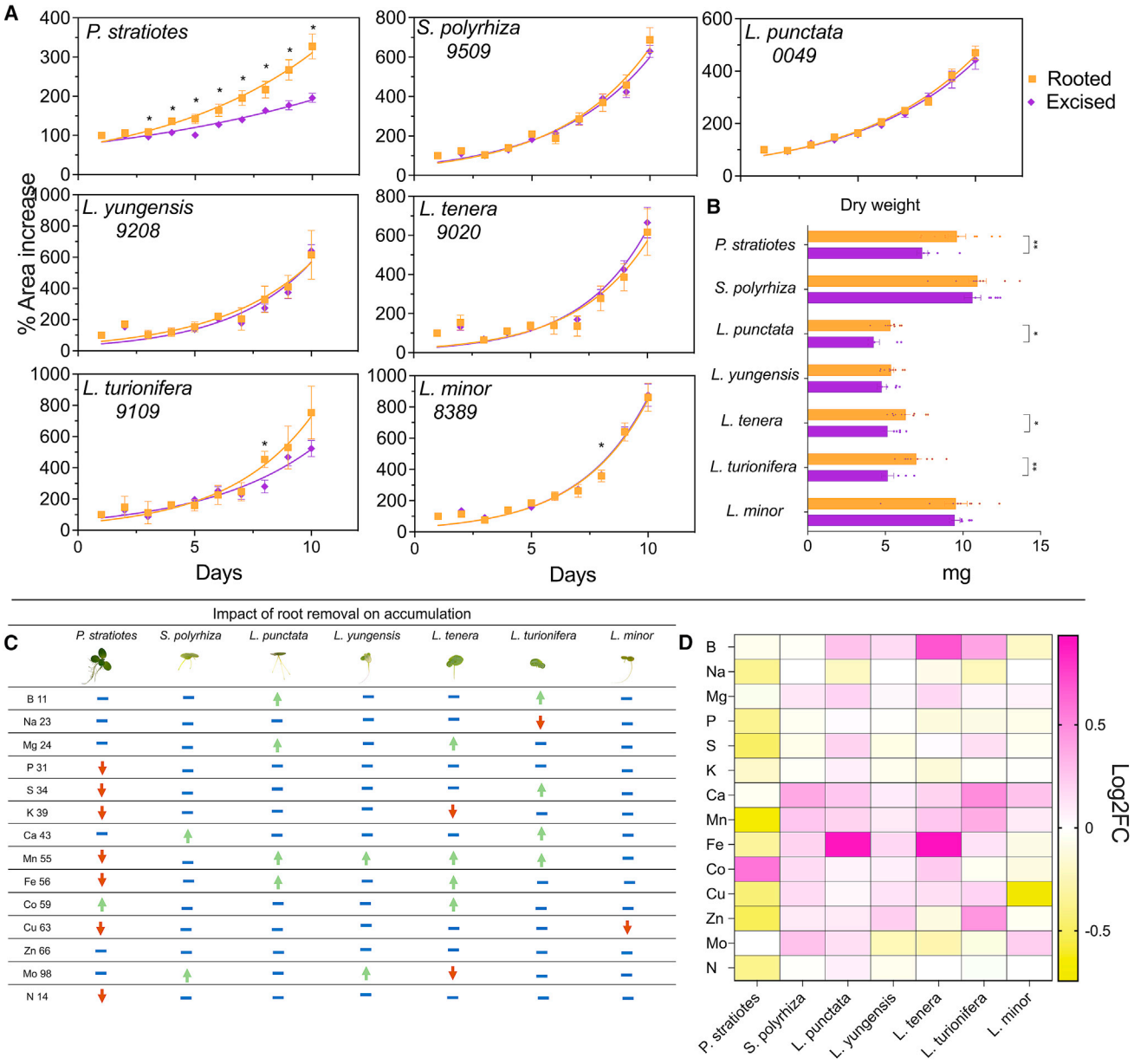

Figure 3. Growth and nutrient uptake of 6 duckweed species are not strongly impacted by continual root removal, unlike the aroid *Pistia stratiotes*

Plants were subjected to continuous root removal, and growth and elemental composition of fronds was compared to untreated controls.

(A) Growth measured as area of fronds (or aerial tissues for *Pistia*), derived from daily imaging from beneath, and plotted as a percentage increase relative to the initial (day 1) area value. Species are indicated on the graphs. n = 10 flasks, each initially seeded with 3 colonies for duckweed and 1 plant for *Pistia*. Asterisks show statistically significant ($p \leq 0.05$) differences as assessed by two-way repeated-measures ANOVA followed by Šidák's multiple comparisons. Error bars = 95% CI. Lines show the best fit of an exponential (Malthusian) growth curve.

(B) Dry weights of frond or aerial tissue harvested from the final time point of the growth series shown in (A). Asterisks show statistically significant (*$p < 0.05$, **$p < 0.01$) differences as assessed by an unpaired t test with two-tailed p value. Error bars = SE.

(C) Table indicating whether root removal results in statistically significant increased accumulation (green upward arrow), decreased accumulation (red downward arrow), or no significant change (blue hyphen). Significance ($p < 0.05$) was determined by one-way ANOVA followed by Holm-Šidák's multiple comparisons test. n = 5 flasks, each initially seeded with 3 colonies for duckweeds; n = 7 flasks, each initially seeded with 1 plant for Pistia.

(D) Heatmap showing the log2 fold change of elemental concentrations in the frond for rooted versus rootless treatments for each species. See also Table S2 for data.

uptake. The fact that duckweed roots are not required for nutrient uptake has presumably allowed the organ to atrophy and lose features such as lateral roots and root hairs, and in the case of *Wolffia* for the root to disappear completely. Indeed, the loss of root hairs and lateral roots may go hand in hand with the reduced capacity of roots for nutrient uptake, as

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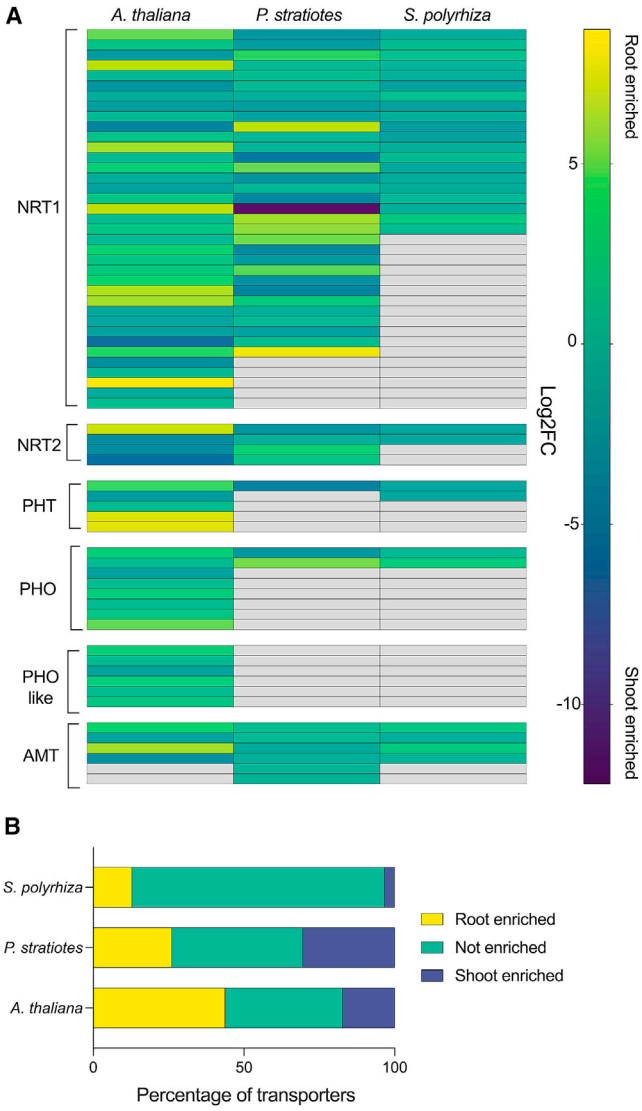

Figure 4. Expression of nutrient transporter gene families shows a reduction in root bias in *Spirodela polyrhiza* compared to *Pistia stratiotes* and *Arabidopsis thaliana*

Heatmap showing log2 fold change (Log2FC) for expressed members of the *PHT*, *NRT1*, *NRT2*, *PHO*, and *AMT* gene families as identified by domain aware ortholog analysis in each species for roots contrasted with leaf/frond tissue. Yellow, positive Log2FC = upregulated in root; blue, negative Log2FC = upregulated in shoot. Where there are fewer total transporters, squares are grayed out.

(B) Bar chart displaying percentage of genes that are root enriched, shoot enriched, or not enriched in their expression between the 2 tissues. Significant enrichment is considered to be log2FC > 2/−2 with an adjusted p value below 0.05. All significant genes are grouped into a single color matching the maximum values on the heatmap. See also Data S1 for transporter expression in phosphate-limiting conditions.

both root hairs and lateral roots have been shown to have high expression of many transporters including the PHTs.[33]

Collectively, these results lay a foundation for the use of duckweed roots as a model system for investigating vestigiality. Particularly relevant to these goals is the current aggressive drive to reposition duckweed as a model species, with a multitude of new resources either available or imminent, including genomes and transformation protocols.[34] We demonstrate that duckweed roots are vestigial with respect to their salient role as organs required for nutrient uptake. Coupled with this we see increasing anatomical reduction across the more derived genera and a shift in expression bias of nitrate and phosphate transporters away from the root in *S. polyrhiza*.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.cub.2023.03.025.

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AUTHOR CONTRIBUTIONS

A.W., B.M.C.K., and A.B. designed the project. A.W. and D.H.J. performed anatomical analyses and monitored root function with support from J.A.A. P.F. performed the ionomic profiling. E.C. performed RNA-seq in *Arabidopsis*. K.E.S. aided with sampling. A.W. performed RNA-seq in *Spirodela* and *Pistia*, and A.W. and R.B. conducted bioinformatic analyses. L.Y. and D.M.W. supervised parts of the project. A.W. and A.B. wrote the manuscript, with input from all authors.

DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Spirodela intermedia	Landolt Duckweed Collection, Zurich	9227
Spirodela intermedia	Landolt Duckweed Collection, Zurich	7820
Spirodela polyrhiza	Landolt Duckweed Collection, Zurich	7498
Spirodela polyrhiza	Landolt Duckweed Collection, Zurich	5543
Spirodela polyrhiza	Landolt Duckweed Collection, Zurich	9192
Landoltia punctata	Landolt Duckweed Collection, Zurich	7760
Landoltia punctata	Landolt Duckweed Collection, Zurich	0049
Lemna turionifera	Landolt Duckweed Collection, Zurich	9109
Lemna japonica	Landolt Duckweed Collection, Zurich	9250
Lemna trisulca	Landolt Duckweed Collection, Zurich	7192
Lemna gibba	Landolt Duckweed Collection, Zurich	7922
Lemna minor	Landolt Duckweed Collection, Zurich	7123
Lemna minor	Landolt Duckweed Collection, Zurich	8389
Lemna valdiviana	Landolt Duckweed Collection, Zurich	7005
Lemna yungensis	Landolt Duckweed Collection, Zurich	9208
Lemna minuta	Landolt Duckweed Collection, Zurich	9260
Lemna aequinoctalis	Landolt Duckweed Collection, Zurich	7339
Lemna perpusilla	Landolt Duckweed Collection, Zurich	8539
Lemna tenera	Landolt Duckweed Collection, Zurich	9243
Chemicals, peptides, and recombinant proteins		
Fluorescent Brightener 28	Sigma	F3543
Basic Fuchsin	BDH Stains	CI42510
SeaKem LE Agarose	Lonza	50001
Nitric Acid 68% d = 1.42, Primar Plus, for Trace Metal Analysis, Fisher Chemical	Fischer Scientific	10456463
RNeasy Mini kit	Qiagen	74104
Trizol Reagent	Ambion	15596018
Deposited data		
Spirodela polyrhiza root and frond phosphate starvation RNA-Seq	GEO	GEO: GSE226143
Arabidopsis Shoot versus Root transcriptome	GEO	GEO: GSE226144
Pistia Leaf and Root transcriptome	GEO	GEO: GSE225923
Software and algorithms		
R Studio	Posit	https://posit.co/products/open-source/rstudio/
Graphpad Prism	Graphpad Software	https://www.graphpad.com/scientific-software/prism/
Salmon v1.10	Carl Kingsford Lab, Carnegie Mellon	https://combine-lab.github.io/salmon/
Hisat v 2.0.5	Daehwan Kim Lab, Cancer Prevention Research Institute of Texas (CPRIT)	http://daehwankimlab.github.io/hisat2/
FeatureCounts	Shi Lab, Olivia Newton-John Cancer Research Institute	https://bioconductor.org/packages/release/bioc/html/DESeq2.html
R packages: Deseq2	https://doi.org/10.18129/B9.bioc.DESeq2	https://bioconductor.org/packages/release/bioc/html/DESeq2.html

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
ClusterProfiler	https://doi.org/10.18129/B9.bioc.clusterProfiler	https://bioconductor.org/packages/release/bioc/html/clusterProfiler.html
ImageJ	NIH Image	http://rsbweb.nih.gov/ij/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Anthony Bishopp (anthony.bishopp@nottingham.ac.uk).

Materials availability

There were no transgenic plant seeds or new materials generated in this study.

Data and code availability

- All data are available in the figures, tables, and data files associated with this manuscript. Transcriptome data were deposited in GEO. This study did not result in any unique code.
- RNASeq data has been deposited on GEO and accession numbers are listed in the key resources table.
- Any additional information required to reanalyze the data reported in this work is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

All duckweed stocks employed in this experiment were obtained from the Landolt collection, ETH Zurich (http://www.duckweed.ch) except for *Spirodela polyrhiza* 9509 that was a gift from Prof Klaus Appenroth, Friedrich Schiller University Jena, Germany. Four digit Landolt accession numbers are given, and summarised in Table S1. *Pistia stratiotes* was obtained from JAM Aquatics, Wrexham, UK. Stocks were maintained on liquid N-media[35] or Schenck-Hildebrandt (SH) media at 120 μmol m^{-2} s^{-1} light and 16/8h light cycle in a Conviron growth chamber, set to 22°C with 70% RH.

METHOD DETAILS

Root cross section anatomy

Plants were grown in the same conditions as stocks. Plants were selected with roots of average or greater length, and fronds of average or greater area based on visual appraisal. For each accession, 10 plants were selected where available. Handling by the frond, plants were removed from media and gently dried on filter paper before submerging fully in a small cylindrical container of molten 4.5% agarose that had cooled to ~40°C. The containers were then placed in ice water to rapidly cool and set the agarose. Once solid, the agarose blocks were trimmed with a razor to a vertically aligned region of mature root, then were glued to the sample stages.

Stages with blocks attached were mounted in the vibratome, and for each sample 5 to 10 sections were taken , using a blade speed of 1 mm/s, a section thickness of 150μm, and a blade oscillation frequency of 65Hz. Sections were stained with fluorescent brightener 28 (calcofluor) solution at 0.3% w/v in RO water for 1 min then rinsed in RO water for 1 min. A representative sample from each genus was first stained with basic fuchsin 0.01% w/v in 50% v/v ethanol solution for 1 min, then rinsed with 50% v/v ethanol solution for 1 min twice and then rested in RO water for 1 min; these sections were then stained with calcofluor as above.

Each section was imaged using a Leica SP5 confocal microscope. Basic fuchsin stained tissue was imaged using 561 nm excitation and detected at 620–630 nm on a hybrid detector at a gain of 50%, and pinhole of 1 AU, and calcofluor stained tissue was imaged using excitation by a 405 nm diode laser at 6% power and detected at 620–630 nm on a hybrid detector at a gain of gain of 15%, and pinhole of 0.5 AU.

A representative image of a single section per plant was selected and measured using FIJI.[36] Cells were classified into layers in concentric rings from the endodermis outwards. The diameter of each layer was measured, as was the number of cells in each layer, along with the diameter of the endodermis, number of endodermal cells, and number of cells in the stele. At each layer, the diameter was measured from 5 points around the circumference of the layer, measuring the maximum distance between points on the layer, then averaged. Epidermal cells had poor dye penetration, and therefore not included in measurements.

Root removal treatments and imaging

For the root removal experiment, plants were grown in SH media. For the control treatment, no manipulation was undertaken. In the root removal treatment, all visible roots (typically less than 1mm) were removed from colonies daily using ethanol sterilised surgical

scissors. For *Spirodela polyrhiza* and *Lemna minor*, each treatment consisted of 5 individual flasks, each seeded with 3 colonies onto 100 ml of media. Individual flasks were treated as a replicate and flasks were arranged randomly in the growth cabinet and re-randomized daily. For *Pistia stratiotes*, each flask was seeded with a young individual plant with 3 emerged leaves visible to the naked eye, to a total of 7 plants/treatment. The treatment regimen was conducted for 11 consecutive days.

Plants were imaged daily in their flasks from beneath, utilising a transparent raised platform featuring a water bath in which to place the flasks to correct for optical distortion. Images were processed using FIJI to measure frond or aerial tissue area. For duckweed flasks, images were split into 8-bit RGB, and the blue channel retained. Frond tissues alone were then selected using the threshold tool and area measured. For *Pistia*, images were again split, but the red channel retained. This was then subject to gaussian blur (sigma = 7.0) and again only the aerial tissues selected using the threshold tool. In rooted samples where this alone was insufficient to separate frond and root, the select polygons tool was used to exclude any additional root captured by thresholding.

Ionomic and nitrogen content analysis

Samples were harvested immediately following the root removal experiment. Prior to harvesting, roots were removed from fronds or aerial tissues and washed for 2 min with MilliQ water 3 times. For ionomics analysis, samples were placed in pre-weighed Pyrex test tubes, and dried at 88°C for 24h. Then, dry weight was recorded, and 1 ml concentrated trace metal grade nitric acid Primar Plus (Fisher Chemicals) spiked with internal standard was added to the samples that were further digested in DigiPREP MS dry block heaters (SCP Science; QMX Laboratories) for 4 h at 115C following the method adapted from Danku.[37] After digestion, samples were diluted to 10 ml with 18.2 MΩcm Milli-Q Direct water and elemental analysis was performed using an ICP-MS, PerkinElmer NexION 2000 and 23 elements were monitored, but only the 14 elements present in our growth media were subject to downstream analysis. To correct for variation within ICP-MS analysis run, liquid reference material was prepared using pooled digested samples, and run after every 9 samples. Sample concentrations were calculated using external calibration method within the instrument software. Further data processing including calculation of final elements concentrations was performed in Microsoft Excel. For nitrogen content analysis, samples were freeze dried overnight, weighed and packed into aluminium foil pouches before analysis with a FLASH 2000 CHNS/O analyser.

RNA extraction and sequencing

Spirodela polyrhiza 9509 was seeded into 250 ml flasks containing 100 ml N-media and grown for 11 days. N-Media was removed and replaced at day 10. For the -Pi experiment, replaced media did not contain KH_2PO_4. Plants from each flask were harvested, separated into fronds and roots, pooled and snap frozen, representing a biological replicate (4 total per tissue type). *Pistia stratiotes* was seeded (1 mature individual per flask) into 500 ml flasks and grown for 10 days on 200 ml SH-media in the same conditions as *Spirodela*. Emerging plants with 3-4 leaves were selected for sampling. For the leaf sample, the largest emerged leaf was harvested from all the plants in a flask, pooled and snap frozen. For the root sample, roots with the apex and laterals removed were harvested in the same manner. Pooled tissue from a flask represented a biological replicate (4 total per tissue type). For both *Spirodela* and *Pistia*, tissues were ground in liquid nitrogen and RNA was extracted using TRIZOL followed by binding and washing with Qiagen RNeasy columns. RNA was frozen at -80°C until sequencing.

Arabidopsis was germinated and grown on ½ MS media supplemented with 0.8% w/v agar. Seeds were ethanol sterilised and stratified at 4°C prior to growth. After 7 days, plants were removed from plates and placed in ½ MS liquid solution for 2hrs. Seedlings were placed on ½ MS plates and with a sharp scalpel roots and shoots were harvested. Material was pooled from approx. 50 roots and about 10 shoots per biological replicate (4 total per tissue type) and flash frozen in liquid nitrogen. RNA was extracted using the RNEasy Plant Mini Kit (Qiagen) as per manufacturer's instructions with on-column DNase treatment and stored at -80°C until sequencing.

RNA integrity was assessed using the RNA Nano 6000 Assay Kit on a Bioanalyzer 2100 (Agilent Technologies, CA, USA). Total RNA was used as input. Library preparations and Sequencing were performed by Novogene. mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations under elevated temperature in First Strand Synthesis Reaction Buffer (5X). First strand cDNA was synthesized using random hexamer primer and M-MuLV Reverse Transcriptase (RNase H-). Second strand cDNA synthesis was subsequently performed using DNA Polymerase I and RNase H. Remaining overhangs were converted into blunt ends via exonuclease/polymerase activities. After adenylation of 3′ ends of DNA fragments, adaptors with hairpin loop structure were ligated to prepare for hybridization. In order to preferentially select cDNA fragments of 370~420 bp in length, library fragments were purified with AMPure XP system (Beckman Coulter, Beverly, USA). Then PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index (X) Primer. PCR products were purified (AMPure XP system) and library quality was assessed on the Agilent Bioanalyzer 2100 system. Clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v3-cBot-HS (Illumina) according to manufacturer's instructions. After cluster generation, library preparations were sequenced on an Illumina Novaseq platform generating 150 bp paired-end reads.

Bionformatic analyses

For all samples, raw reads (.fastq) were processed using Perl. Clean reads were obtained by removing reads containing adapters and poly-N, and those of a low quality.

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For *Spirodela* and *Pistia,* reads from each sample were pseudoaligned and quantified against their respective transcriptomes (*Spirodela polyrhiza* 9509_oxford_v3[31]; *Pistia stratiotes* v1[38]) using Salmon[39] with k-value set at the default level -31- and the –gcbias flag passed for all samples. Entire genomes were concatenated against their transcriptomes (cds.fa) in both cases and passed to salmon as decoys. Quant.sf files output by salmon were imported into R using the package tximport and differential expression analysis was performed on the using the package DESeq2.[40]

For *Arabidopsis* and the *Spirodela* -Pi experiment, indexes for *A. thaliana* (TAIR10) and *Spirodela polyrhiza* (oxford_v3) were built using Hisat2 v2.0.5 and paired-end clean reads were aligned to the reference genome using Hisat2 v2.0.5.[41] FeatureCounts v1.5.0-p3 was used to count reads mapping to each gene. FPKM was calculated and the resulting data were subject to differential expression analysis in R using the package DESeq2.[40] Gene Ontology (GO) enrichment analysis of differentially expressed genes was implemented the R package clusterProfiler, which corrects for gene length bias. GO terms with a corrected p value < 0.05 were considered significantly enriched.

Orthology analysis

For the genome wide identification of N and P transporters in *Spirodela polyrhiza*, we used phylogeny construction framework outlined in Mutte et al.[42,43] In brief, protein sequences of known low- and high-affinity nitrate and phosphate transporters of plant model *Arabidopsis thaliana* were obtained from TAIR (https://www.arabidopsis.org). This included 9 phosphate and 52 low-affinity and 7 high-affinity nitrate transporters. *Spirodela polyrhiza* protein sequences -version 2-[44]) were downloaded from Phytozome13 (https://phytozome-next.jgi.doe.gov) and *Pistia stratiotes* protein sequences were downloaded from China National Genebank (https://db.cngb.org/). BLAST database for *A. thaliana*, *S. polyrhiza* and *P. stratoites* were generated using '*makeblastdb*' module in BLAST +v2.9.0 (https://blast.ncbi.nlm.nih.gov). Protein sequences from *Arabidopsis* were used to query *Spirodela* and *Pistia* blast databases independently for each transporter family using BLASTp. We used 0.01 as E-value cut-off to filter BLAST hits. The protein sequences of filtered BLAST hits were run through the InterproScan database (ver5.52–86.0; http://www.ebi.ac.uk/interpro/) to look for conserved domains (Data S1). Presence or absence of individual domains and their organisation were used as criterion to filter potential orthologous protein sequences. Filtered protein sequences were further tested by BLASTp search against *Arabidopsis* proteome (bi-directional blast analysis) to confirm orthology inferences. Gene IDs (converted between versions 2 and 3 of the *Spirodela polyrhiza* genomes using BLAST) encoding confirmed orthologous proteins were used for further tissue-specific expression analysis.

QUANTIFICATION AND STATISTICAL ANALYSIS

All statistical analyses were conducted in GraphPad Prism version 9.0 (https://www.graphpad.com/). For the anatomical dataset, principal coordinates analysis was conducted on 22 variables with 210 rows. Data was standardized to have a mean of 0 and an SD of 1. Principal components were selected with eigen values greater than 95% utilising parallel analysis with 1000 Monte Carlo simulations and a random seed. A list of the variables used is available in Figure S2 and the raw data is available in Table S1.

For root removal experiments, two-way repeated measures ANOVA was performed, followed with Holm-Šidák's multiple comparisons test to establish differences in growth on a per-day basis. Dry weights of rooted versus root removed samples were compared with Student's T-test with a two-tailed p-value. For nutrient concentration comparisons generated by ionomic analyses, data were compared with one-way ANOVA followed by Šidák's multiple comparisons test to establish differences in concentration between individual nutrients. Log2 fold changes generated from ionomic data and graphed in the heatmap were calculated as Log2(elemental conc. roots removed)-Log2(elemental conc. rooted). In all instances, a p-value of less than 0.05 was considered significant. In all cases, data is centred on the mean value. Error bars either display the 95% confidence interval or the standard error of the mean, as indicated in the figure legend.