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DATA INSIGHT

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Phylotranscriptomics provides a treasure trove of flood-tolerance mechanisms in the Cardamineae tribe

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Abstract

Flooding events are highly detrimental to most terrestrial plant species. However, there is an impressive diversity of plant species that thrive in flood-prone regions and represent a treasure trove of unexplored flood-resilience mechanisms. Here we surveyed a panel of four species from the Cardamineae tribe representing a broad tolerance range. This included the flood-tolerant *Cardamine pratensis*, *Rorippa sylvestris* and *Rorippa palustris* and the flood-sensitive species *Cardamine hirsuta*. All four species displayed a quiescent strategy, evidenced by the repression of shoot growth underwater. Comparative transcriptomics analyses between the four species and the sensitive model species *Arabidopsis thaliana* were facilitated via de novo transcriptome assembly and identification of 16 902 universal orthogroups at a high resolution. Our results suggest that tolerance likely evolved separately in the *Cardamine* and *Rorippa* species. While the *Rorippa* response was marked by a strong downregulation of cell-cycle genes, *Cardamine* minimized overall transcriptional regulation. However, a weak starvation response was a universal trait of tolerant species, potentially achieved in multiple ways. It could result from a strong decline in cell-cycle activity, but is also intertwined with autophagy, senescence, day-time photosynthesis and night-time fermentation capacity. Our data set provides a rich source to study adaptational mechanisms of flooding tolerance.

KEYWORDS

adaptation, *Arabidopsis*, *Cardamine*, *Rorippa*, submergence

1 | INTRODUCTION

Some plant species can thrive in extreme environments. Underwater conditions are especially challenging to plant life since low gas-diffusion rates hamper oxygen and carbon dioxide (CO_2) exchange with the environment. This restricts mitochondrial respiration and photosynthesis, respectively, potentially resulting in a severe energy and carbon shortage. Most terrestrial plant species are very flood-sensitive and cannot survive extended periods of flooding. However, the plant kingdom has evolved a variety of adaptive strategies to circumvent these challenges.

One strategy involves plant morphological and anatomical modifications that enhance the aeration of submerged plant parts.

Dedication: This work is dedicated to Rens (L. A. C. J.) Voesenek, who was an inspirational scientist and pioneer in the field, and a generous mentor to all of us.

This includes the development of adventitious roots, aerenchyma formation in stems, petioles and roots, the development of root barriers preventing radial oxygen loss to the anoxic soil and shoot elongation to re-establish aerial contact (Combs-Giroir & Gschwend, 2024; Lin et al., 2024; Pedersen et al., 2021). In contrast, the quiescence strategy involves restricted growth and metabolism under water until the floods recede, thus conserving valuable resources for regrowth after desubmergence (Combs-Giroir & Gschwend, 2024; Voesenek & Bailey-Serres, 2013). Among crops, rice cultivars provide the best-studied examples of these contrasting flood-adaptive responses (Hattori et al., 2009; Xu et al., 2006).

Plants that use the above strategies tend to perform best under terrestrial conditions. For heterophyllous species, the favoured environment is less clear. Aerial-formed leaves of these species are like those of terrestrial plants, but those formed underwater are thin (high specific leaf area), have a negligible cuticle, are void of stomates, and exhibit either high dissection or a narrow, elongated morphology. Aerial and aquatic leaves can also vary in CO_2 and bicarbonate usage and carbon fixation pathways (C_3, C_4, CAM). Heterophylly in amphibious plants optimizes photosynthesis for underwater conditions and prevents desiccation when grown aerially (Van Veen & Sasidharan., 2021). These species have been invaluable in unravelling the environmental influence on adaptive leaf development, for example, *Ranunculus trichophyllus* (Kim et al., 2018), *Potamogeton octandrus* (He et al., 2018), *Callitriche palustris* (Koga et al., 2021), *Hygrophila difformis* (Li et al., 2022), *Rorippa aquatica* (Ikematsu et al., 2023).

Most of these flood-adaptive morphological and anatomical acclimation responses are mediated by the plant hormone ethylene (Leeggangers et al., 2023; Pedersen et al., 2021; Sasidharan & Voesenek, 2015). Being a gas, it quickly accumulates to high concentrations in flooded tissues and is an important flooding stress cue (Voesenek & Sasidharan, 2013). However, ethylene is also a key signal that stimulates senescence, which might not always be beneficial (Rankenberg et al., 2024). Accordingly, species in permanently deluged conditions have lost the ability to produce and/or sense ethylene (Ma et al., 2024; Olsen et al., 2016; Summers et al., 1996; Voesenek et al., 2015).

Most current crops are flood-sensitive with tolerance traits being lost in breeding cycles focused on yield parameters. As climate change exacerbates the frequency and unpredictability of flooding events, it is imperative to intensify our efforts to explore naturally stress-resilient plants. Flood-tolerant plant species serve as valuable models for identifying and understanding tolerance mechanisms, species divergence and adaptation. The Brassicaceae family is particularly interesting in this context, including a range of model, wild and crop species varying in their tolerance to different flooding regimes. Within the Brassicaceae family, a wide range of adaptations to specific water-rich habitats exist (Nikolov & Tsiantis, 2017). Most Brassicaceae are sensitive to excess water availability, including the model species *Arabidopsis thaliana* (Lee et al., 2011; Mustroph et al., 2009; Vashisht et al., 2011) or the oil crop *Brassica napus* (Wittig et al., 2021). However, this family also includes species that are flood-adapted. Especially one clade, the tribe Cardamineae, contains many flooding-tolerant members, namely from the genera *Cardamine*, *Rorippa*, and *Nasturtium* (Cook, 1999).

Our previous work characterized flood responses in *Nasturtium officinale*, watercress, which has adapted to growth in small rivers (Howard & Lyon, 1952). This species uses the escape strategy to avoid internal oxygen deficiency conditions (Müller et al., 2021). Within the genus *Cardamine*, the newly emerging model plant species *Cardamine hirsuta* (Hajheidari et al., 2019; Kierzkowski et al., 2019) is not described as flooding-tolerant, but some of its close relatives prefer wet growth conditions (Akiyama et al., 2021; Kantor et al., 2023; Shimizu-Inatsugi et al., 2017). The genus *Rorippa* is also known to prefer wet environments (*Rorippa sylvestris*, *R. amphibia*; Akman et al., 2014; Sasidharan et al., 2013), and one member even shows pronounced heterophylly (*R. aquatica*, Ikematsu et al., 2023; Nakamasu et al., 2014).

In this work, we built on our previous knowledge to further explore flooding resilience mechanisms in Brassicaceae species. We selected two flood-sensitive species, *A. thaliana* and *C. hirsuta*, and the flood-tolerant *Cardamine pratensis*, *R. sylvestris* and *Rorippa palustris* (Supporting Information S1: Figure 1). Despite the variety of adaptive mechanisms, all five species phenotypically exhibited the quiescent strategy. Through a comparative transcriptomic analysis of the shoot response to submergence, we were able to distinguish key players associated with a successful quiescence when flooded. The narrow phylogenetic range, but wide diversity in tolerance found within the Brassicaceae allowed us to quantitively compare species at high genetic resolution. We found distinct routes towards submergence tolerance, which are associated with either a strong down-regulation of the cell cycle, or with minimized transcriptomic reconfiguration when under water.

2 | MATERIALS AND METHODS

2.1 | Plant material, growth conditions and stress treatment

Seeds for *A. thaliana*, ecotype Col-0, were raised in house. Seeds of *C. hirsuta* were obtained from Miltos Tsiantis. *R. sylvestris* was propagated clonally from plants originally collected in the Netherlands (Akman et al., 2012; Stift et al., 2008). Seeds for the other two species were bought from commercial seed suppliers (*C. pratensis*, www.templiner-kraeutergarten.de; *R. palustris*, www.wildblumen.at).

All plant species were grown on a soil mixture (*A. thaliana* until the 10-leaf-stage, all other species until the 6-leaf stage) about 3–4 weeks after germination, according to established protocols (Müller et al., 2021), under short-day conditions (8 h light, 16 h darkness), 100 µmol photons × m^{-2} × s^{-2} and23°C.

Submergence stress was applied to the plants by immersing them in big, transparent boxes (about 50 L volume) filled with 40 L of tap water 24 h before start of the treatment. Treatment was started 2 h after start of illumination. For survival experiments, plants were kept

under water for up to 10 weeks. At each time point, eight plants were removed from the boxes and were kept under normal growth conditions for another 2 weeks. Surviving plants were counted, while survival was defined as the ability to form new green leaves within the 2-week-recovery period. For molecular and biochemical analysis, the youngest leaves including the meristem were collected after 24 and 48 h of stress treatment, together with aerated controls and frozen immediately in liquid nitrogen.

For measuring growth of the leaf, the length of the youngest leaf was measured with a digital calliper. Measurements were done at the start of the submergence treatment and at three subsequent days. For each leaf, the daily length increment was calculated.

2.2 | Biochemical parameters

Frozen tissue (young leaves including meristems) was ground to fine powder, and subsequently used for extraction of metabolites and measurement of soluble sugars by established methods previously described (Riber et al., 2015). Proteins were extracted and activity of alcohol dehydrogenase (ADH) was determined as previously described (Gasch et al., 2016).

2.3 | RNA extraction, sequencing, and expression analysis

Frozen tissue (young leaves including meristems) was ground and further processed as described in Müller et al. (2021). Subsequent RNA extraction and library preparation was also done exactly as in our previous analysis. Raw reads were processed through adapter trimming of the FASTQ files using the BBDuk algorithm. Detailed approaches of the analysis are described in the Supporting Information Data. In short, de novo transcriptomes were assembled with Trinity version 2.6.6 (Grabherr et al., 2011; Haas et al., 2013) for *C. pratensis*, *R. palustris* and *R. sylvestris*. A genome-guided approach was used for *C. hirsuta* with HISAT2 mapped reads to the genome (Gan et al., 2016; Kim et al., 2019). Sets of orthologous genes between the studied species (Orthogroups) were identified through an all-versus-all discontiguous megablast (Camacho et al., 2009), followed by Orthofinder (Emms & Kelly, 2019). Read mapping for expression quantification was done with Kallisto (Bray et al., 2016). Fold changes upon submergence, differences between timepoints and phylogenetic and tolerance-specific effects were determined with the EdgeR package (Robinson et al., 2010). Gene Ontology (GO) enrichment was done with GOseq and the Arabidopsis annotation (Carlson, 2019a, 2019b, Young et al., 2010).

The raw data for this study have been deposited at EMBL-EBI in the European Nucleotide Archive under accession number PRJEB73992 (https://www.ebi.ac.uk/ena/browser/view/PRJEB73992) and in ArrayExpress under accession number E-MTAB-13910 (https://www.ebi.ac.uk/biostudies/arrayexpress/studies/E-MTAB-13910).

3 | RESULTS

3.1 | Brassicaceae species vary in their tolerance to complete submergence

To characterize submergence tolerance, the five rosette species were fully submerged at the 10- or 6-leaf developmental stage (vegetative stage ~3-4 weeks after germination). Plant mortality was periodically checked based on the ability to recover within 10 weeks. *A. thaliana* was the most sensitive surviving only 3–4 weeks (Figure 1a, Supporting Information S1: Figure 2). The two *Cardamine* species occupy a wetter niche (Table 1, Ellenberg & Leuschner, 2010), and accordingly showed better submergence survival. *C. hirsuta* survived about 6 weeks of submergence, while *C. pratensis* survived up to 10 weeks. Both *Rorippa* species survived the 10-week submergence period used here (Figure 1a, Supporting Information S1: Figure 2). Extensive algal growth prevented longer submergence periods in our system. Overall, the performance of the five species corresponded to their Ellenberg ecological niche indicator values for moisture levels (Table 1). The survival data indicated a tolerance gradient from a mildly sensitive *A. thaliana* to the moderately tolerant *C. hirsuta*, to the extremely tolerant *C. pratensis*, *R. palustris* and *R. sylvestris*.

Next, we determined the underwater growth strategy in the five species by monitoring the youngest leaf during submergence. All five species lacked an escape response. Four species, *A. thaliana*, *C. hirsuta*, *R. palustris* and *R. sylvestris* had a significantly reduced leaf growth rate underwater during the first 3 days of submergence, while *C. pratensis* had similar growth rates under control and submerged conditions (Figure 1b).

The growth data was supplemented with the analyses of several relevant flood-tolerance related biochemical parameters. ADH activity was measured as a proxy for fermentation ability in response to any hypoxia experienced underwater. Though illuminated submergence does not cause hypoxia in the shoot (*Arabidopsis*, Lee et al., 2011; rapeseed, Wittig et al., 2021; watercress, Müller et al., 2021), at night local and subcellular oxygen deficiency might occur in submerged leaves. The two sensitive species (*A. thaliana*, *C. hirsuta*) did not enhance ADH activity after 24 or 48 h of submergence (Figure 1c). In contrast, *C. pratensis* and both *Rorippa* species had significantly higher ADH activities after 24 or 48 h of submergence (Figure 1c)

Soluble carbohydrates are the major substrate for glycolysis, which is the key energy source under hypoxia. The soluble carbohydrate content decreased in all five species within 24 and 48 h of submergence, but with some differences between species (Figure 1d). The sugar levels in control plants of *C. pratensis* and *R. sylvestris*, the two perennial species, were significantly higher compared with the other three species. After 24 and 48 h, the sugar levels were very low in most species, except for *R. sylvestris*, which showed significantly higher sugar levels compared with other species even after 48 h of submergence (Figure 1d).

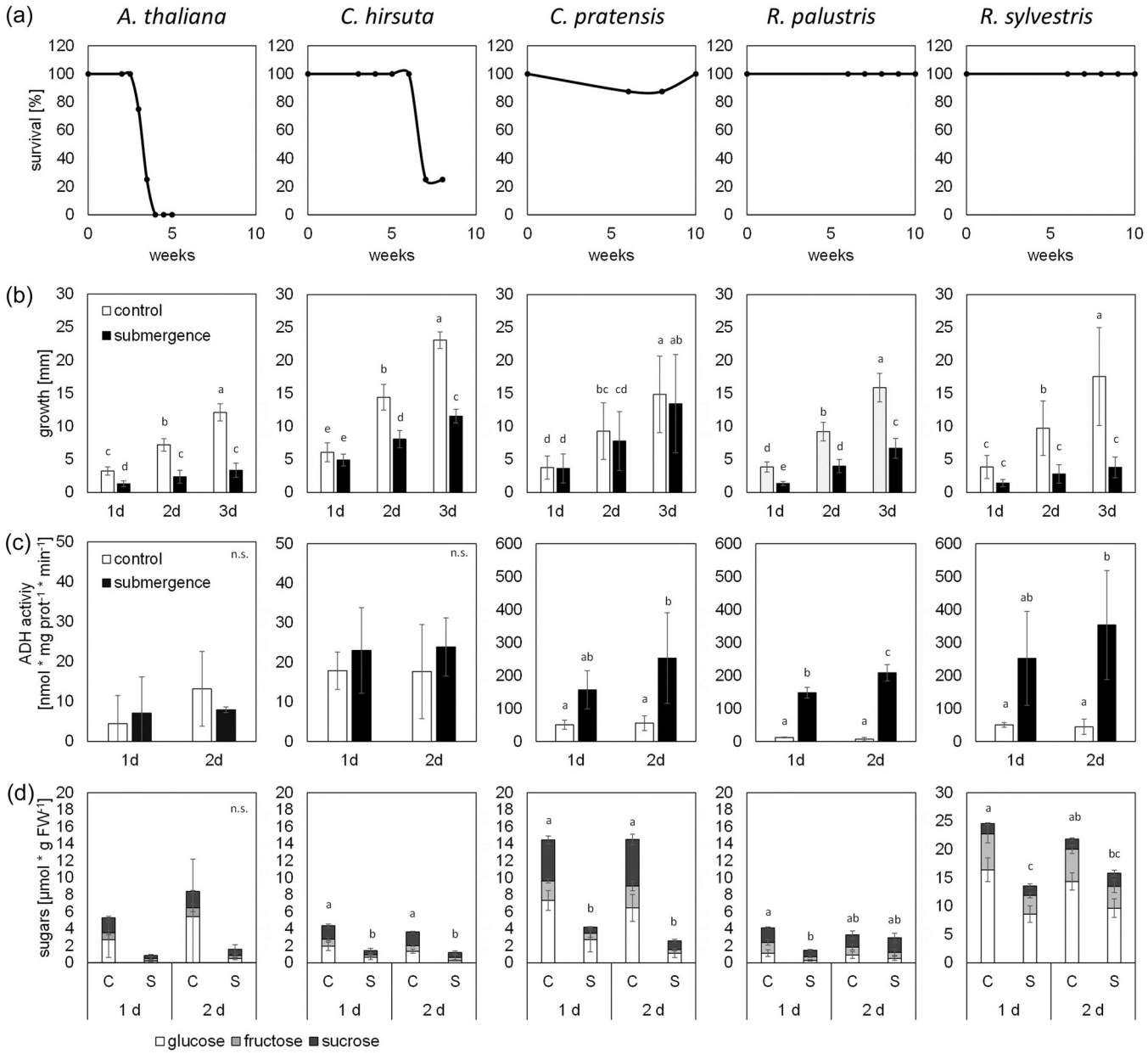

FIGURE 1 Characterization of submergence tolerance in Brassicaceae. (a) Survival rates under illuminated submerged conditions with regular day–night (8:16 h) rhythm. (b) Growth activity of leaves underwater. Plants with 6 true leaves (*Cardamine hirsuta*, *Cardamine pratensis*, *Rorippa palustris*, *Rorippa sylvestris*) or 10 leaves (*Arabidopsis thaliana*) were submerged under short-day conditions (black bars) and compared with control plants in air (white bars). The length of the youngest leaf was measured just before the treatment and after 1, 2 and 3 days. The increase in growth is shown with means ± SD from three independent experiments with five plants per replicate ($n = 15$). (c) ADH activity under submergence. 3- to 4-week-old plants were submerged under short-day conditions (black bars). After 1 and 2 days of treatment, young leaves were harvested together with leaves from aerated controls (white bars). ADH activities are shown as mean ± SD from three to four biological replicates. (d) Carbohydrate dynamics in Brassicaceae under submergence. Plants were stressed and harvested as in (c). Soluble sugar levels (glucose, fructose, sucrose) are shown as mean ± SD from three biological replicates. (b–d) Different letters indicate significant differences at $p < 0.05$ (one-way ANOVA, Tukey's HSD test). ADH, alcohol dehydrogenase; ANOVA, analysis of variance; HSD, honestly significant difference; ns, not significant.

3.2 | Flooding-sensitive Brassicaceae species have slower transcriptomic reconfiguration upon submergence

Biochemical and anatomical traits related to tolerance have a molecular origin. Furthermore, among tolerant species there is still considerable morphological variation, indicating a significant role for molecular tolerance. We therefore conducted transcriptome profiling to identify genes and key processes associated with flood tolerance, and potential novel tolerance regulators. To circumvent the lack of genome information for *C. pratensis*, *R. palustris* and *R. sylvestris* we made de novo-assembled transcriptomes for these species

TABLE 1 Ellenberg moisture values and characteristics of the studied species.

Species	Ellenberg moisture value	Ploidy	Fertilization	Lifestyle
Arabidopsis thaliana	4	Diploid	Selfing	Annual
Cardamine hirsuta	5	Diploid	Selfing	Annual
Cardamine pratensis	6–9 (uncertain)	Tetraploid	Nonselfing	Perennial
Rorippa palustris	8=	Tetraploid	Selfing	Annual
Rorippa sylvestris	8=	Tetraploid	Nonselfing	Perennial

(Supporting Information S1: Figure 3). For optimal read mapping for *C. hirsuta* we created a genome-guided assembly to use as a reference based on the published *C. hirsuta* genome (Gan et al., 2016). Alignment success for expression quantification ranged from 70% to 87% between the species (Supporting Information S1: Figure 4).

For interspecies transcriptomic comparisons, it is crucial to accurately identify orthologs. Therefore, we performed an all-versus-all blast with representative transcripts for each gene of the assembled transcriptomes (*C. hirsuta*, *C. pratensis*, *R. palustris*, *R. sylvestris*) and the primary transcripts of *A. thaliana* (Cheng et al., 2017) and *R. islandica* (Brassicales Map Alignment Project, Department of Energy's Joint Genome Institute, http://bmap.jgi.doe.gov/). By clustering the resulting graph network of related sequences, we identified discrete groups of orthologous sequences, known as orthogroups (Emms & Kelly, 2019, Supporting Information S2: Data 1). We optimized the strength of grouping such that we maximized the number of orthogroups that cover all species (Supporting Information S1: Figure 5). This resulted in the identification of 16 902 universal orthogroups that were represented by five or six species (Figure 2a). For the species with available genome information (*A. thaliana*, *C. hirsuta* and *R. islandica*) relatively few genes could not be placed in a group of sequences from other species, compared to the de novo transcriptome-based assemblies (Figure 2b). Most orthogroups were represented by no gene or only one gene per species. However, the tetraploid species (*C. pratensis*, *R. sylvestris*, *R. palustris*) were frequently (~35%) present with two genes in an orthogroup (Figure 2c). When considering only the universal orthogroups, the vast majority of these orthogroups contained one gene for the diploid species (*C. hirsuta*, *A. thaliana*, *R. islandica*) and two genes for the tetraploid species (*C. pratensis*, *R. palustris*, *R. sylvestris*) (Figure 2d), which indicated good separation of homologous sequences into the smallest viable orthogroups containing most species.

To compare the transcriptomic reprogramming of the five species, we summarized orthogroup expression as the sum of all included transcripts as done previously for closely related species (Bräutigam et al., 2011; Van Veen et al., 2013). Overall, orthogroup expression was a reliable predictor of the expression level of the individual genes within that orthogroup, where for the separate species the estimated fold changes of the orthogroups explained 79%–89% of the variation found for individual genes (Supporting Information S1: Figure 6).

To check whether there were differences in the statistical strength to detect differential expression we compared the fold changes of differentially expressed genes to their overall sequencing depth. This showed a similar power to detect differential expression across species and timepoints (Supporting Information S1: Figure 7). Per species we fitted a full factorial model to retrieve the submergence response after 24, 48 h and an interaction effect to highlight temporal effects (Figure 3a). Additionally, we estimated the mean response to submergence over both timepoints by the main submergence effect in an additive model (Supporting Information S2: Data 2 and 3). Overall, the transcriptomic reconfiguration did not differ drastically between the five species. The number of differentially expressed orthogroups (DEOGs) after 24 or 48 h ranged from around 500 to 1600 across all species (Figure 3a). The small number of orthogroups with a significant time × treatment interaction effect (Figure 3a) and a strong correlation between the timepoints (Figure 3b) indicated highly similar responses on both days. However, the more flood-sensitive *A. thaliana* and *C. hirsuta* had a delayed response in the downregulation of a considerable portion of their negatively regulated transcriptome.

To functionally characterize the general transcriptomic response, a GO-term enrichment analysis was performed on the DEOGs per species (Supporting Information S2: Data 4). This revealed among induced genes many GO terms associated with stress response (e.g., response to stimulus, response to stress, response to abiotic stimulus, response to light) and GO terms expected to be enriched under submergence, such as 'response to hypoxia' and 'response to ethylene'. The down-regulated genes had fewer commonalities in their function, mainly related to synthesis of secondary metabolites.

3.3 | Interspecies comparison of ortholog behaviour reveals phylogenetic- and tolerance-dependent variation in the severity of transcriptomic responses

The above qualitative assessment provides a global picture of the transcriptomic behaviour in the five species. To quantitatively compare and pinpoint key sets of orthologous genes that can explain variation in flood tolerance or represent a conserved set of transcriptomic responses, we leveraged the established orthology (Figure 2) to directly contrast transcriptomic reconfiguration between species.

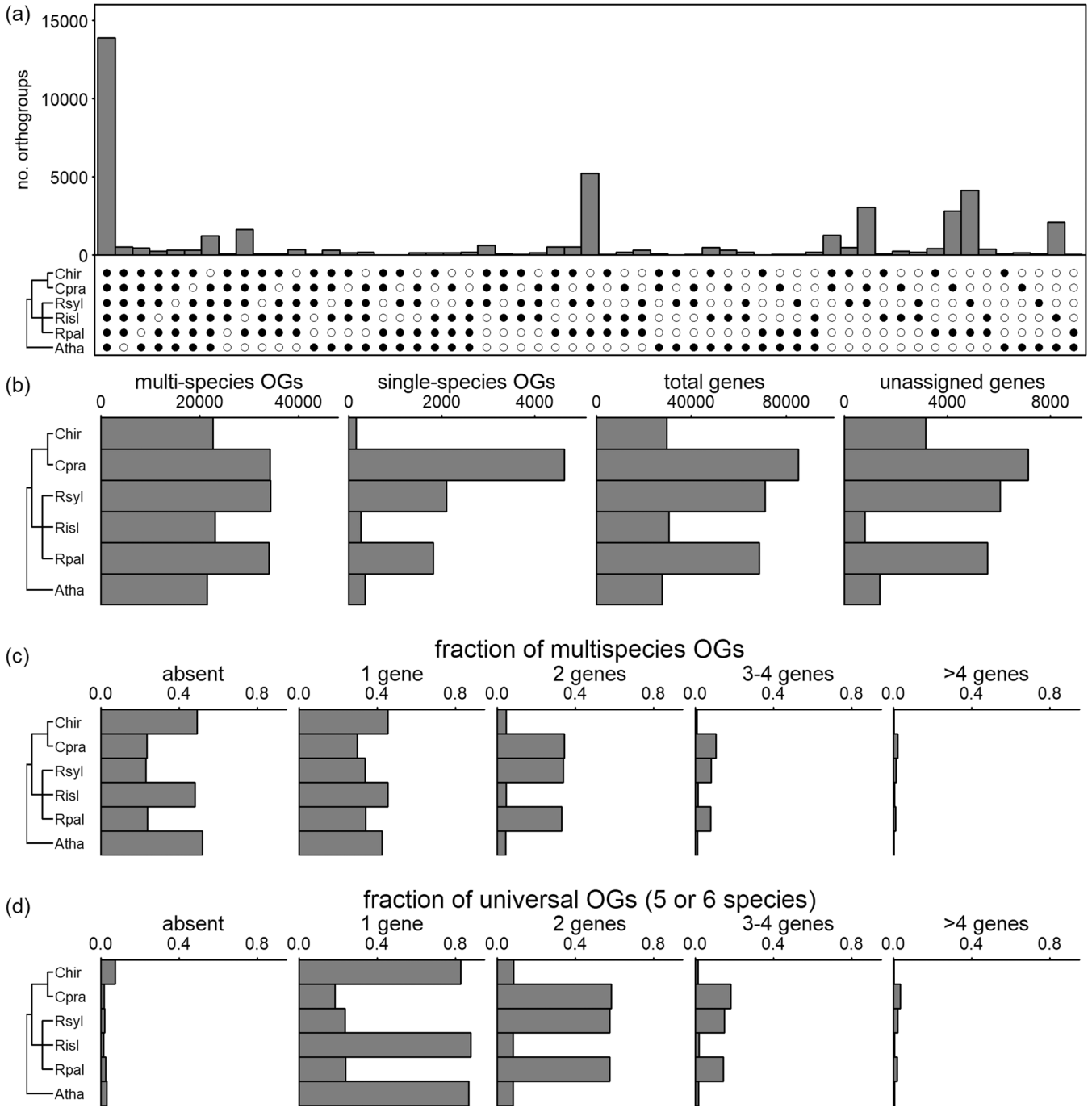

FIGURE 2 Identification of groups of orthologous sequence, orthogroups (OGs). (a) The number of OGs identified with representative sequences of the species indicated by filled circles. (b) Summary statistics of the genomic scope of the species considered for orthology analysis. Here, multispecies OGs include sequences from at least two species; single-species OGs include two or more sequence from a single species that are grouped together because of high homology; total genes is the number of sequences on which orthology analysis was performed. An unassigned gene had insufficient homology to other sequences to be placed in a group with other sequences and so forms an OG by itself. (c) The proportion of OGs that contain zero to many sequences of a particular species. (d) As (c), but then only considering OGs that have sequences from at least five species. *Atha*, *Arabidopsis thaliana*; *Chir*, *Cardamine hirsuta*; *Cpra*, *Cardamine pratensis*; *Rpal*, *Rorippa palustris*; *Rsyl*, *Rorippa sylvestris*.

Direct pairwise comparison of DEOGs between species revealed a correlation in submergence responses. However, given a model where the transcriptional response would be identical between pairs of species, there remained a fair amount of unexplained variation with an average R^2 value of 0.25 and extremes of 0.07 and 0.55 (Supporting Information S1: Figures 8 and 9). These pairwise comparisons showed specific biases that indicated differences in the strength of up- or downregulation when submerged. To systematically portray these biases, we analysed the per-species deviation from the average submergence response across all five

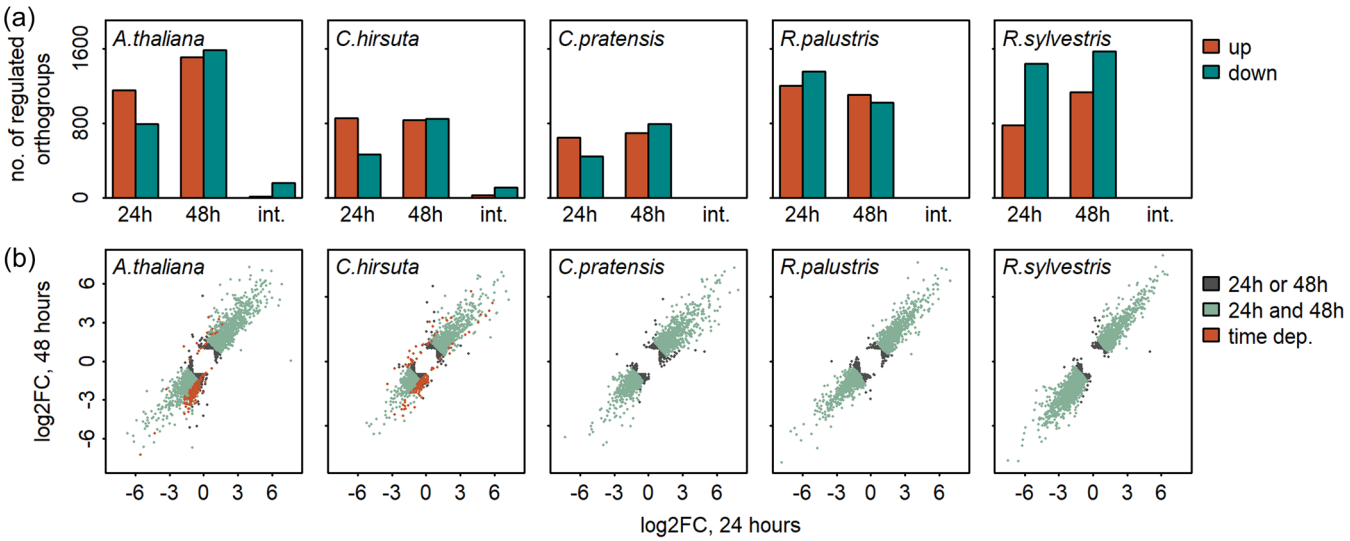

FIGURE 3 (a) The number of differentially expressed orthogroups after 24 and 48 h of submergence in the light compared to control after similar duration under aerated conditions, and whether the response at 48 h differs from the response at 24 h (time × treatment interaction: int.). Differential expression concerns a $p_{adj.} < 0.01$ and a $|\log_2 FC| > 1$. (b) Scatterplot depicting the relationship between transcriptomic differences at 24 and 48 h. Only orthogroups that are differentially expressed (24 h, 48 h or int.) are shown. *A. thaliana*, *Arabidopsis thaliana*; *C. hirsuta*, *Cardamine hirsuta*; *C. pratensis*, *Cardamine pratensis*; FC, fold change; *R. palustris*, *Rorippa palustris*; *R. sylvestris*, *Rorippa sylvestris*.

species (Figure 4). A sliding window across this mean response allowed us to assess biases (weaker or stronger than the mean response) in the strength of transcriptome reconfiguration. Regarding downregulation, for the tolerant *R. palustris* and *R. sylvestris* significantly more orthogroups showed a stronger downregulation after 24 h, with *R. sylvestris* sustaining this trend even after 48 h. In contrast, the downregulation of orthogroups in *C. hirsuta* and *C. pratensis* was weak, both at 24 and 48 h. *A. thaliana* over the whole had an average response after 24 h, but after 48 h down-regulated genes had a stronger suppression and upregulated genes a stronger induction than the average of the species we assessed. Overall, this analysis combined with the number of observed DEOGs indicates that when genes are down-regulated in the tolerant *Rorippa* species, the magnitude of regulation is strong. In comparison, the *Cardamine* species tend towards milder gene expression downregulation.

To zoom in further on specific contrasting regulatory patterns between the species or highly conserved responses, we searched for orthogroups that showed significant regulation dependent on the species, genus or tribe by testing for a significant interaction of the phylogeny with treatment at either timepoint in three separate models (Figure 5, Supporting Information S2: Data 5). Most between-species variation in transcriptomic reconfiguration, based on the number of significant orthogroups, was explained by individual species (species × treatment interaction; Figure 5a), with a vast amount being retained when species were nested into their corresponding genus. However, by nesting all Cardamineae species, relatively few orthogroups were found with phylogenetic-dependent regulation based on the tribe × treatment interaction. This indicates that at least between the three genera studied here the flood responses are quite divergent. This reiterates the observation (Supporting Information S1: Figures 8 and 9) that the genera were diverse in their submergence responses.

Furthermore, we tested which orthogroups were significantly associated with the identified tolerance groups (Figure 1) and were regulated in line with a tolerance gradient from the sensitive *A. thaliana*, the moderately tolerant *C. hirsuta*, to the extremely tolerant *C. pratensis*, *R. palustris* and *R. sylvestris* (Figure 5d). Very few orthogroups had an expression profile that significantly matched the tolerance gradient (*Rsyl*, *Rpal* and *Cprat* ≥ *Chir* ≥ *Atha*). The tolerant Cardamineae tribe is not only a phylogenetic factor, by also provides a distinction in flood tolerance to further expand the candidate tolerance orthogroups.

The above test provided a platform to identify the highly conserved responses, transcriptomic adjustments with a phylogenetic effect or those orthogroups with an expression associated with the observed tolerance gradient. First, to highlight the truly conservatively regulated genes that do not vary between the species, we clustered the orthogroups without a species–specific effect ($p_{species \times treatment} > 0.05$, $p_{treatment,\ mean\ across\ species} < 0.01$ and $|\log_2 FC_{\ mean\ across\ species}| > 0.5$). These 1095 DEOGs were split into four distinct clusters (Figure 6, Supporting Information S2: Data 6) where the majority of orthogroups showed a mild up- or downregulation upon submergence (Cluster 3, 515 DEOGs; Cluster 1, 414 DEOGs), and 53 orthogroups that were strongly upregulated (Cluster 4) or 113 orthogroups with moderate downregulation (Cluster 2). GO categories that were enriched among the mildly induced orthogroups included biotic defence responses, indole glucosinolate metabolism and autophagy (Supporting Information S2: Data 7). GO enrichment among mildly down-regulated orthogroups were concerned mostly with mitochondrial respiration and electron transport chain, and possibly cell division via spindle formation. The strongly induced or down-regulated clusters had only poor GO enrichment, partly indicative of the smaller cluster size, but also a functionally diverse set of orthogroups. However, the strongly

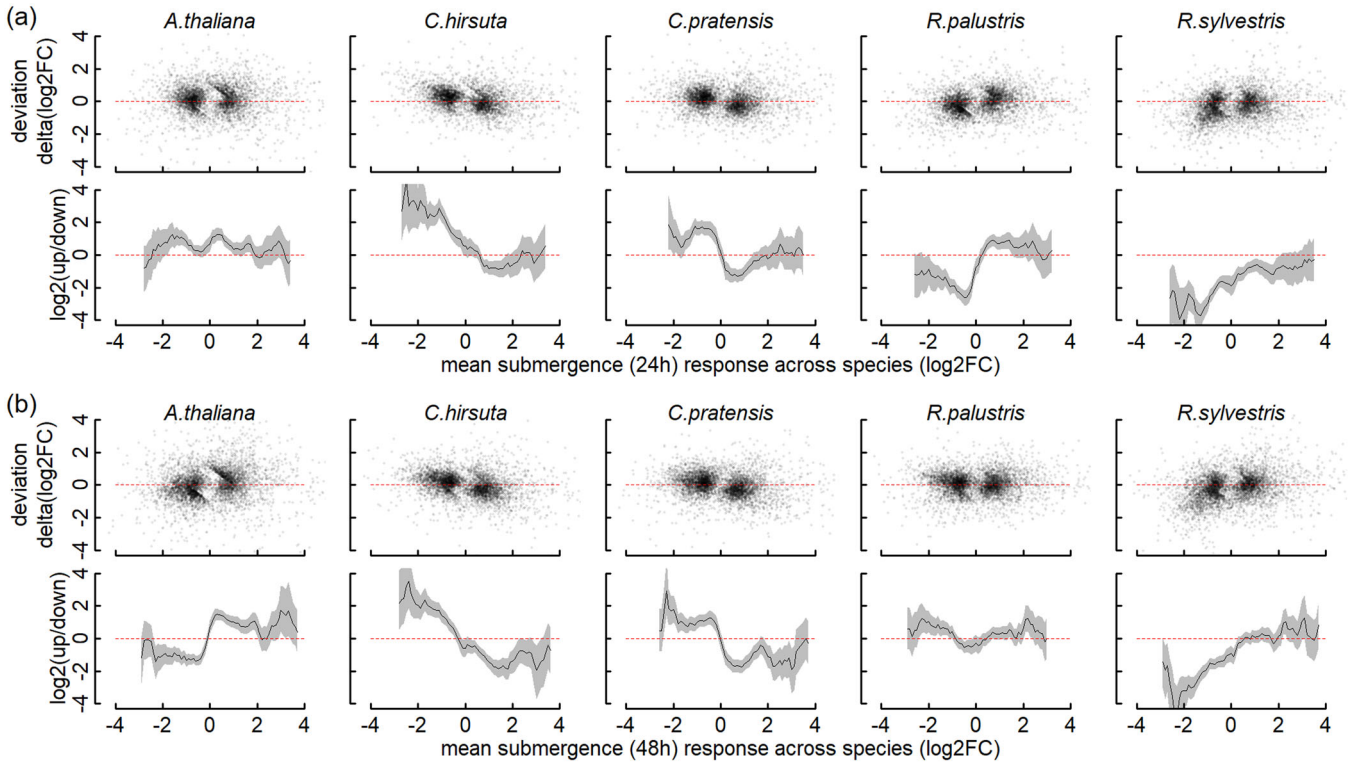

FIGURE 4 The response of individual species compared to average response across all species at 24 (a) and 48 h (b). The deviation from the mean response considers the absolute distance of the log2FCs, where the red dashed line indicates a transcriptomic response identical to the species average (*x*-axis). The Log2 of the ratio of the number of positively and negatively deviating orthogroups (|delta(log2FC)| > 0.5) captures the bias of that species at any mean submergence response in a sliding window (width = 0.5). The grey band considers the 95% confidence interval (exact binomial test), and the dashed red line indicates no deviation from the average. Orthogroups with $p_{adj.} < 0.001$ and $|\log_2 FC| > 1$ in at least one species were considered for the analysis. *A. thaliana*, *Arabidopsis thaliana*; *C. hirsuta*, *Cardamine hirsuta*; *C. pratensis*, *Cardamine pratensis*; FC, fold change; *R. palustris*, *Rorippa palustris*; *R. sylvestris*, *Rorippa sylvestris*. [Color figure can be viewed at wileyonlinelibrary.com]

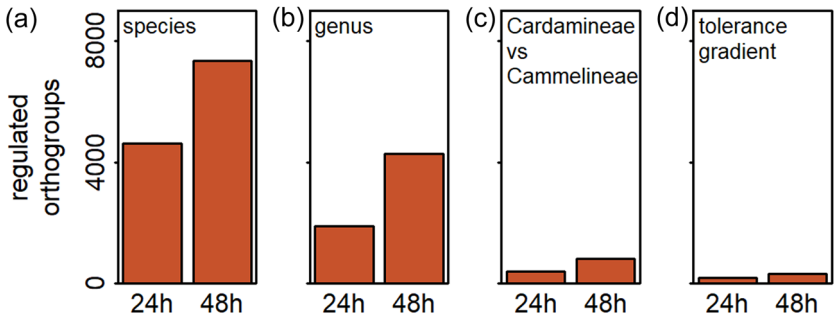

FIGURE 5 (a–c) The number orthogroups (OGs) whose regulation is dependent on either the species (a), the genus (b) or the tribe (c) (species × treatment, genus × treatment, tribe × treatment; $p_{adj.} < 0.001$). (d) The number of OGs whose magnitude of regulation only increases or decreases over successive tolerance groupings. Here, *A. thaliana* is sensitive, *C. hirsuta* is intermediate and *C. pratensis*, *R. palustris* and *R. sylvestris* are tolerant. In addition to an ordered change in magnitude of regulation these OGs require a significant effect of the grouping ($p_{adj.} < -0.001$) and considerable range in the magnitude of regulation$|\text{Log}_2FC_{\text{tolerant vs. sensitive}}| > 1$). *A. thaliana*, *Arabidopsis thaliana*; *C. hirsuta*, *Cardamine hirsuta*; *C. pratensis*, *Cardamine pratensis*; FC, fold change; OG, orthogroup; *R. palustris*, *Rorippa palustris*; *R. sylvestris*, *Rorippa sylvestris*; vs, versus. [Color figure can be viewed at wileyonlinelibrary.com]

down-regulated orthogroups were enriched in xylan metabolism and leaf development.

To highlight any phylogenetic effects in the transcriptomic reconfiguration, we also highlighted the genus-dependent flood responses ($p_{\text{genus} \times \text{treatment}} < 0.001$ and $|\log_2 FC_{\text{between any genus}}| > 1$, Figure 7a). We found genus to be the lowest phylogenetic level that still retained a strong signature of evolutionary divergence in submergence responses (Figure 5). Clustering and GO term analysis of these 2609 orthogroups revealed strong enrichment of cell cycle, DNA replication, disaccharide metabolism related terms among orthogroups that were down-regulated particularly in the *Rorippa* species (Cluster 7, 233 DEOGs, Supporting Information S2: Data 6 and 7). Regarding

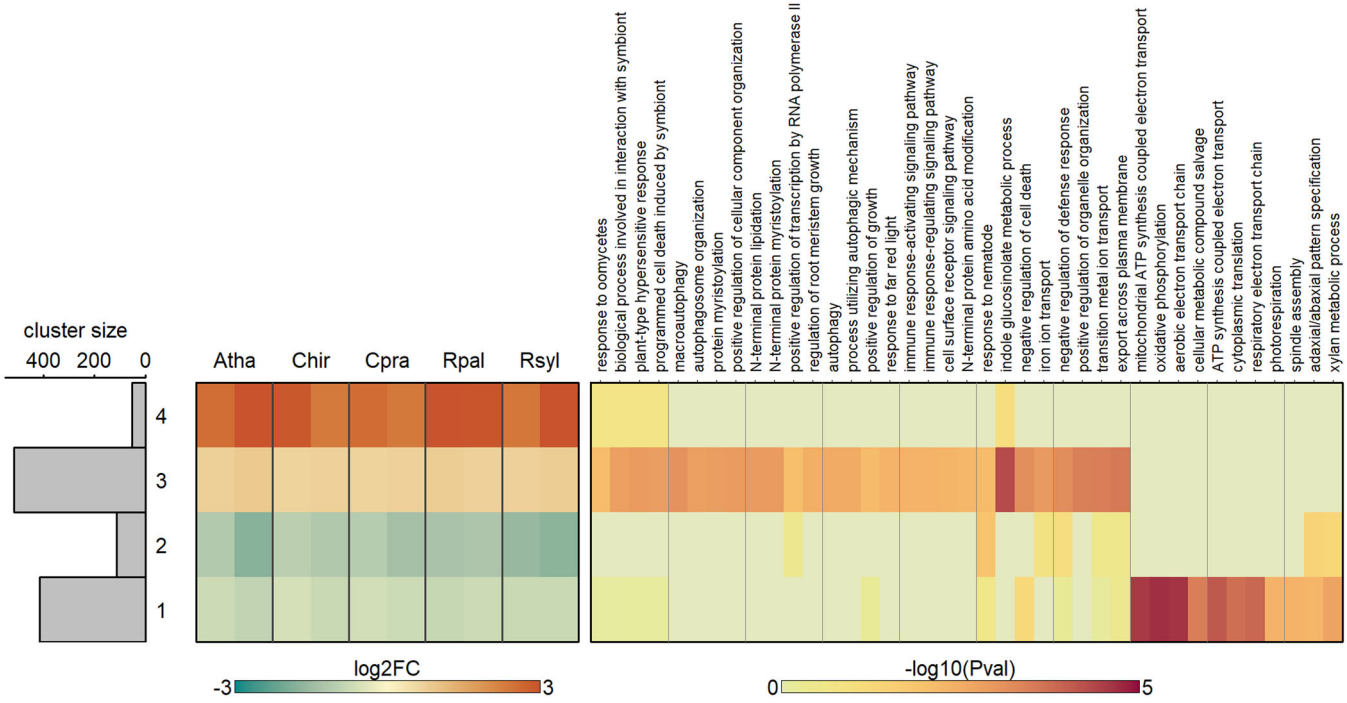

FIGURE 6 Clustering of the orthogroups without any species effect. For these no significant effect of species ($p_{adj.} > 0.05$; Figure 5a), but a significant mean response across all species ($p_{adj.} < 0.01$ and $|\log_2 FC| > 0.5$) was observed. The data was hierarchically clustered based on Euclidean distances and an agglomeration by a minimum variance method. Only GO terms with at least 80 OGs, 5 DEOGs and $p < 0.01$ are shown. For brevity only Biological Process GO terms are depicted. Cluster assignment of individual OGs and complete GO enrichment analysis can be found in Supporting Information S2: Data 6 and 7. *Atha*, *Arabidopsis thaliana*; *Chir*, *Cardamine hirsuta*; *Cpra*, *Cardamine pratensis*; DEOG, differentially expressed orthogroup; FC, fold change; GO, Gene Ontology; OG, orthogroup; *Rpal*, *Rorippa palustris*; *Rsyl*, *Rorippa sylvestris*.

orthogroups especially induced in *Arabidopsis*, chlorophyll breakdown-related terms were enriched (Cluster 6, 246 DEOGs). The *Cardamine* genus also showed unique patterns of induction or suppression. Here, anthocyanin metabolism and responses to cytokinin were enriched among orthogroups that were mildly down-regulated compared to *Rorippa* and *Arabidopsis* (Cluster 8, 46 DEOGs). Also, the *Cardamine* species had sets of orthogroups that were only poorly upregulated compared to the other species (Cluster 3, 74 DEOGs), or were only upregulated in the *Cardamine* (Cluster 4, 115). However, there was no clear enrichment of GO terms among these clusters.

Relatively few genus-specific orthogroups showed a pattern that would support a unified approach to achieve tolerance, that is, up or down in all tolerant species. However, we ultimately sought to identify the specific molecular components that could be instrumental in conferring submergence tolerance or sensitivity among the five selected species. We thus selected the orthogroups that had a tribe-specific effect, since all Cardamineae are at least moderately tolerant (Figure 1, $p_{tribe \times treatment} < 0.001$ and $|\log_2 FC_{tribe \times treatment}| > 1$). And we selected those with a regulatory pattern that follows the tolerance gradient, to address the weaker submergence performance of *C. hirsuta* compared to the other Cardamineae species (Figure 1, $p_{tol \times treatment} < 0.001$ and *Rsyl*, *Rpal* and *Cpra* ≥ *Chir* ≥ *Atha*). The resulting 675 orthogroups were placed into seven clusters of similar regulation patterns (Figure 7b, Supporting Information S2: Data 6 and 7), which showed that the tolerant species mostly lacked or had a reduced up- and downregulation compared to the sensitive *A. thaliana* and moderately tolerant *C. hirsuta*. Among these upregulated orthogroups several GO terms associated with chlorophyll breakdown (Cluster 2, 157 DEOGs) and secondary metabolism were enriched (Cluster 3, 224 DEOGs), whereas among the down-regulated orthogroups enrichment was in terms associated with anthocyanin and glucosinolate metabolism (Cluster 7, 84 DEOGs). The orthogroups that did show a stronger regulation, either up or down, in the tolerant species had GO enrichment for terms associated with protein complex oligomerization and the tricarboxylic acid cycle for upregulated orthogroups (Cluster 5, 66 DEOGs). Down-regulated orthogroups were overrepresented by cutin biosynthesis-related terms, shade avoidance and oxygen perception (Cluster 4, 115 DEOGs).

3.4 | Hypoxic responses are minor, while carbon starvation and ethylene-regulated transcripts are the most responsive in the submergence transcriptome

Hypoxia, ethylene and carbon starvation are strongly associated with submergence. Indeed, these aspects surfaced in the unbiased transcriptome analysis and phenotypic characterization of the five species. Given their importance, we also investigated transcripts associated with these processes in a targeted approach.

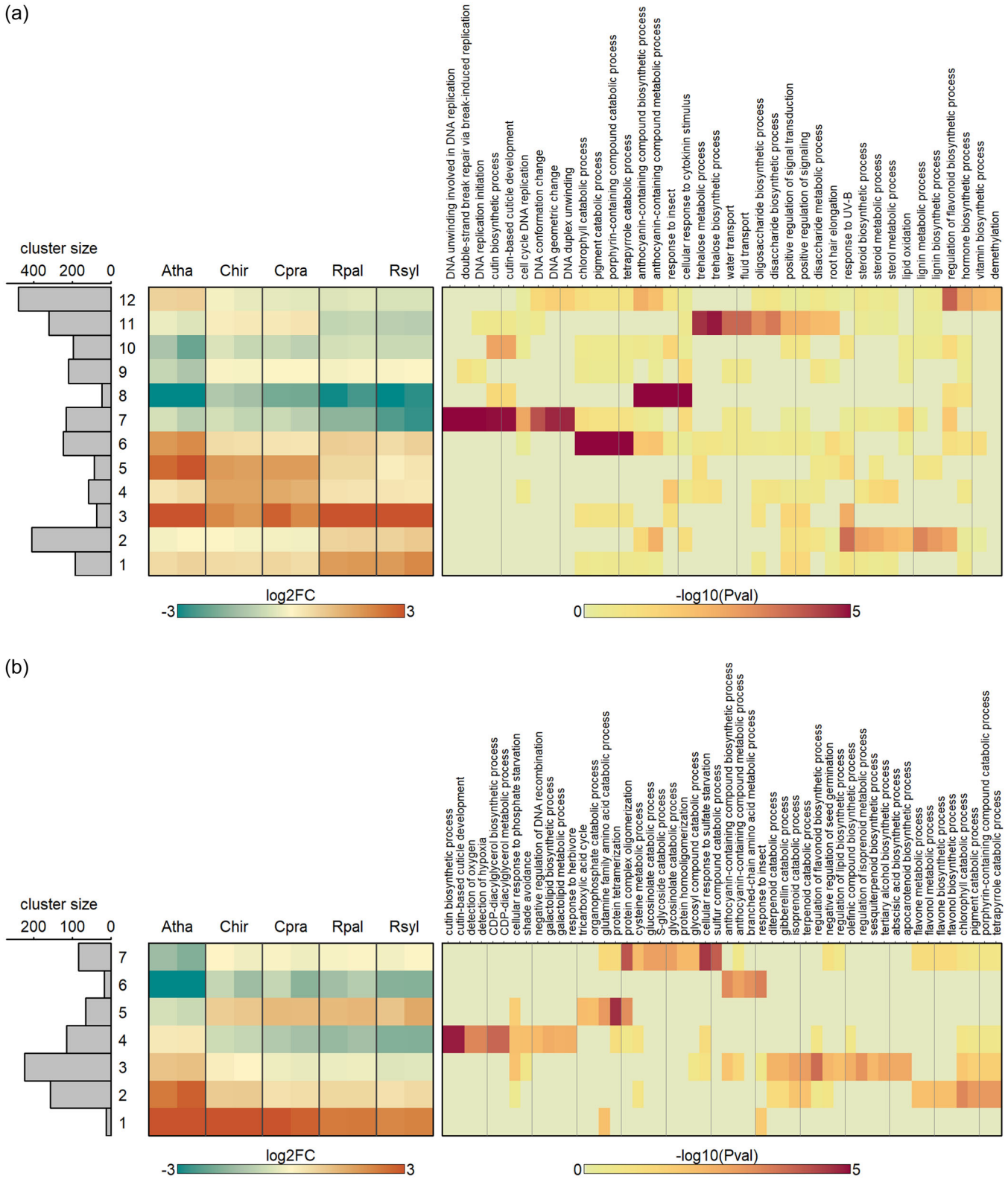

FIGURE 7 Clustering of the OGs with divergent transcriptional response between species. (a) Clustering and GO enrichment of OGs whose transcriptional response depends on the genus, thus OGs where a $p_{adj.} < 0.001$ (Figure 5b) and $|\log_2 FC_{between\ any\ genus}| > 1$ was observed. (b) transcriptional responses associated with tolerance are clustered. These are the OGs where the transcriptional response depends on the tribe, thus with a $p_{adj.} < 0.001$ (Figure 5c) and a $|\log_2 FC_{Cardamineae\ vs.\ A.\ thaliana}| > 1$), and also those OGs that followed the tolerance gradient (Figure 5d). The data were hierarchically clustered based on Euclidean distances and an agglomeration by a minimum variance method. Only GO terms with at least 80 OGs, 5 DEOGs and $p < 0.01$ are shown. For brevity, only biological process GO terms are depicted. Cluster assignment of individual OGs and complete GO enrichment analysis can be found in Supporting Information S2: Data 6 and 7. *Atha*, *Arabidopsis thaliana*; *Chir*, *Cardamine hirsuta*; *Cpra*, *Cardamine pratensis*; DEOG, differentially expressed orthogroup; FC, fold change; GO, Gene Ontology; OG, orthogroup; *Rpal*, *Rorippa palustris*; *Rsyl*, *Rorippa sylvestris*; vs, versus.

Mustroph et al. (2009) identified a subset of genes induced upon hypoxia regardless of tissue and cell type in *A. thaliana*, which are widely considered a core set of hypoxia responsive genes (HRGs). HRGs responded similarly in the five tested species in response to submergence (Figure 8a, Supporting Information S2: Data S8), but were not consistently upregulated. Only *HUP54*, *ACHT5*, *PP2-A13*, and an unknown protein were strongly induced. The HRGs typically considered as highly reliable hypoxia-response indicators, namely

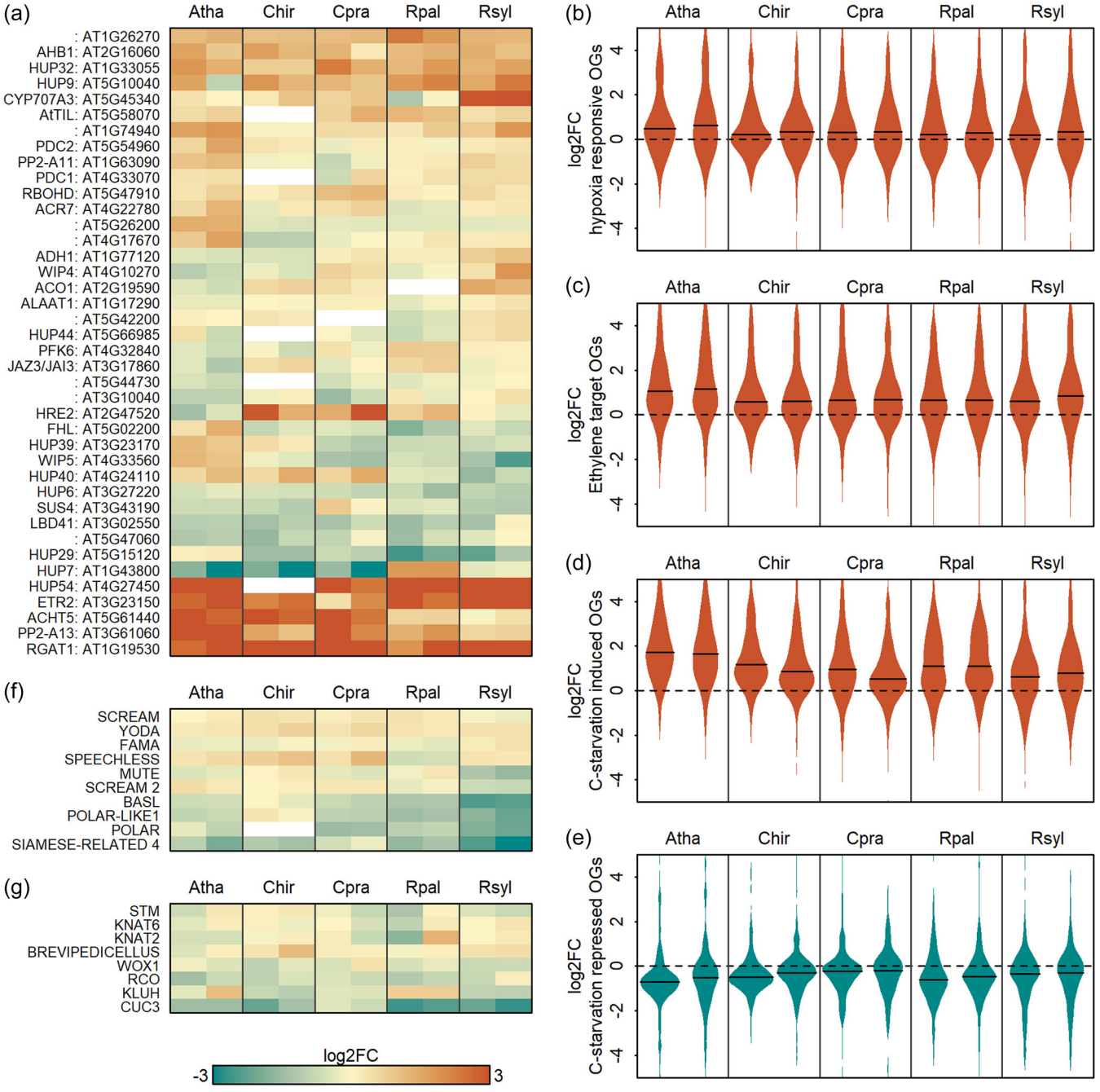

FIGURE 8 Targeted analysis of key flood adaptive responses. (a) Transcriptional response of core hypoxia responsive genes (HRGs), which are hypoxia responsive regardless of cell type, as defined by Mustroph et al. (2009). (b) Density of the response of conserved HRGs that are regulated across a wide range of plant species and hypoxia studies, as defined by Mustroph et al. (2010). (c) Density of ethylene-responsive orthogroups, which in *Arabidopsis thaliana* contain an EIN3 binding domain in their promotor and are induced by ethylene, as defined by Chang et al. (2013). (d) Density of carbon starvation-induced orthogroups as defined by Usadel et al. (2008) by combination of sugar feeding and low CO_2 studies. (e) is as (d), but with carbon starvation-repressed orthogroups. The horizontal line in (b)–(e) indicates the median log2FC of the set of orthogroups. (f) Heatmap of key players of stomata development. Induction of these genes is associated with more stomates (Smit and Bergmann, 2023). (g) Heatmap of key leaf developmental players required for leaf dissection (Bhatia et al., 2021). *Atha*, *Arabidopsis thaliana*; *Chir*, *Cardamine hirsuta*; *Cpra*, *Cardamine pratensis*; FC, fold change; OG, orthogroup; *Rpal*, *Rorippa palustris*; *Rsyl*, *Rorippa sylvestris*.

PCO1/2, *LBD41*, *ADH1* (Gasch et al., 2016; Weits et al., 2014), were not induced. Comparison of microarray data across a variety of hypoxia studies in *A. thaliana* and other species has yielded a broader set of genes that are conserved in their upregulation upon hypoxia (Mustroph et al., 2010). Many of these genes were not induced upon submergence (Figure 8b, Supporting Information S2: Data 8). The weak hypoxic signature response in this study was expected since illuminated shoots rarely experience hypoxia (Colmer & Pedersen, 2008; Lee et al., 2011; Müller et al., 2021; Vashisht et al., 2011; Van Veen et al., 2013; Wittig et al., 2021).

The gaseous hormone ethylene, rather than hypoxia, is considered a more reliable cue for submergence (Sasidharan et al., 2018). Indeed, the GO term 'Response to ethylene' was significantly enriched (p_{val}: from 5.4E−10 to 1.3E−19) among upregulated genes in all species at both timepoints (Supporting Information S2: Data 4). A focused investigation of the response of DEOGs considered to be direct targets of ethylene signalling by virtue of both an EIN3 binding domain in their promotor and induction upon ethylene treatment in *A. thaliana* (Chang et al., 2013) confirmed the presence of an ethylene signal upon submergence in our data (Figure 8c, Supporting Information S2: Data 8). Of all species, *A. thaliana* had the strongest ethylene response, which was reflected in the highest median log2FC. However, this could also be an artifact of the *A. thaliana*-biased selection of ethylene target orthogroups.

Though tolerant species maintained a better sugar status than sensitive species, we observed a drop in sugar levels and an increase in autophagy-related transcripts across all five species (Figures 1d and 6). Carbon starvation followed by sugar feeding of seedlings and low CO_2 treatment of illuminated rosettes provided a reliable set of carbon responsive genes in *A. thaliana* (Usadel et al., 2008). Taken together, these starvation-induced and -repressed genes in the five species studied here showed that all species had transcriptome reconfiguration associated with carbon starvation, and that this reconfiguration was the strongest in *Arabidopsis* (Figure 8d,e).

Hampered photosynthesis underwater, leading to carbon limitation could explain the dominant carbon starvation effects in the transcriptomes. CO_2 limitation is specifically reflected by the induction of alanine:glyoxylate aminotransferase, a key player in photorespiration, in all species apart from *C. pratensis* (OG0004610 and OG0004408, Supporting Information S2: Data 3, Liepman & Olsen, 2003; Niessen et al., 2012). Several frequently flooded plant species have evolved mechanisms to mitigate diffusion limitations. These encompass a reduction or absence of both the cuticle and stomatal abundance, and higher specific leaf area (Van Veen & Sasidharan, 2021). Since cuticle biosynthesis had the strongest downregulation in the *Rorippa* species (Figure 7), we explored the possibility of further morphological adaptations that improve underwater photosynthesis, and also explain differences in carbon status. Indeed, key players of stomatal development, such as *SCREAM2*, *BASL*, *POLAR*, *POLAR LIKE* and *SIAMESE RELATED4* (Smit & Bergmann, 2023), were down-regulated particularly in the tolerant species (Figure 8f). *R. aquatica* acclimates to underwater conditions by increasing leaf dissection (Nakayama et al., 2014). However, the known key players in the control of leaf dissection (Bhatia et al., 2021) showed no clear pattern in transcriptional reconfiguration (Figure 8g).

4 | DISCUSSION

4.1 | The Cardamineae provide a valuable resource for studying flood-tolerance traits

Several species from the Brassicaceae family have been investigated for flood resilience. Notably, the Cardamineae tribe contains several highly flood-tolerant species, including various members of the *Rorippa* genus (Akman et al., 2012; Nakayama et al., 2014; Van Veen et al., 2014) and *N. officinale* (Müller et al., 2021). These studies have yielded valuable insights into the adaptive strategies of these species. For example, regulation of the shoot escape strategy in *N. officinale* (Müller et al., 2021) bypassed the conserved ethylene-abscisic acid-gibberellin hormonal network. The formation of distinct aquatic and terrestrial leaves in *R. aquatica* has made it a model to study environmental control of leaf development (Nakayama et al., 2014).

Here, we leveraged the diverse range of flood tolerance exhibited within the Brassicaceae to study molecular responses to submergence. We extended the species palette by incorporating two *Cardamine* species and found a medium to high tolerance. By also including the sensitive *A. thaliana*, our panel represented a broad tolerance range across five species. All five species displayed a quiescent strategy underwater with submergence tolerance spanning 4 to >10 weeks. This wide survivability range surpasses the natural variation in submergence tolerance observed among natural *Arabidopsis* accessions (Vashisht et al., 2011). The selection of these quiescent species therefore provides a robust study system to explore flooding-tolerance mechanisms in plant species enabling the capture of adaptive expression profiles at a much broader genetic scale.

A notable constraint in comparisons of molecular stress responses of a wider palette of species is the capacity to compare transcriptomes effectively and accurately. In the early days, these were partly related to capacity limitations and high costs of next-generation sequencing (NGS) technologies, with most studies confined to a single or pair of contrasting species to explore environmental responses. Examples regarding flooding include escape and quiescence responses in the genus *Rumex* (Van Veen et al., 2013), aquatic leaves in waterlily (Wu et al., 2017) and heterophylly in *R. trichophyllus* (Kim et al., 2018) and *R. aquatica* (Ikematsu et al., 2023).

Some studies resorted to the use of *Arabidopsis* microarrays, but the requirement for high sequence similarity restricted species choice, for example, *Rorippa* (Sasidharan et al., 2013) and *Cardamine* (Shimizu-Inatsugi et al., 2017). The advent of affordable, high-resolution NGS platforms has reduced such challenges and permits a more diverse choice of plant species driven by scientific questions rather than technological constraints. However, gene diversification among distantly related plant species still poses challenges for direct ortholog comparisons. For example, a comparative study on the gene

regulatory networks of hypoxia signalling ranging from monocots to dicots was restricted to less than 7000 shared gene families (Reynoso et al., 2019). This loss of resolution restricts findings to highly conserved genes and conserved responses. However, a study on fungal responses in the core eudicots distinguished <8000 core gene families but managed to leverage the diversification in the gene universe to explain adaptation by regulation specifically in lineage-specific gene families (Sucher et al., 2020).

In contrast, our study over a relatively narrow phylogenetic distance identified 16 902 universal orthogroups. At the same time, we identified a substantial diversity in stress responses. Orthologs between species were identified mostly on a one-to-one basis or one-to-two or two-to-two basis in the case of tetraploid species. This permitted disentanglement of responses at a high resolution and direct ortholog level comparison and clustering. This study thus goes beyond describing processes, tracing the actual genes that evolved to mediate stress adaption. The transcriptomic patterns suggest different routes towards tolerance with different identified gene sets in *Rorippa* and *Cardamine*. Also, a direct ortholog-based comparison with sharply separated gene relationships provided the scope to do a quantitative assessment of flood responses, that is, mild versus strong upregulation, rather than a qualitative resolution where gene families are classified as up, down or neither. A quantitative analysis was instrumental in identifying the attenuated regulation in *Cardamine* versus the strong downregulation in *Rorippa*, which would not have readily emerged out of a qualitative transcriptome assessment. The Cardamineae tribe thus provides an excellent study system to disentangle flood adaptions and tolerance in greater detail, for which we here provide the first step.

4.2 | Carbon starvation, autophagy and biotic defences are conserved responses to submergence

Despite considerable variation in flood tolerance, several processes were induced and suppressed equally across the five species. Of note were the activation of pathways associated with biotic stress and autophagy (Figure 6). Upregulation of the defence response during flooding has been observed in several transcriptomic studies encompassing Brassicaceae species, but also in *Rumex* (Müller et al., 2021; Van Veen et al., 2013, 2016; Wittig et al., 2021). The reason for this conserved induction of defence response remains unresolved but is potentially associated with ethylene accumulation in submerged tissues. Ethylene is an established participant in the signalling network regulating plant defence responses (Broekgaarden et al., 2015). Alternatively, defence signalling might be activated to combat elevated infection pressure following submergence (Hsu et al., 2013).

Autophagy is required to maintain cellular energy homeostasis when the substrate demand for glycolysis or respiration exceeds supply, which typically occurs during flooding (Dalle Carbonare et al., 2023). The importance of autophagy for flood tolerance is apparent from the increased flood sensitivity observed in various autophagy mutants (Chen et al., 2015). General stress-responsive GO terms and carbohydrate starvation were among the common responses (Supporting Information S2: Data 4) and have been identified in a variety of tolerant (Müller et al., 2021; Van Veen et al., 2013) and sensitive species (Van Veen et al., 2016; Wittig et al., 2021). Focused analysis of starvation marker genes indicated a more attenuated response in species from the tolerant Cardamineae tribe (Figure 8d). For the *Rorippa* genus, this matched the better maintenance of sugar levels, but this was not the case for the *Cardamine* species (Figure 1d). Sensitive rapeseed plants also had very low carbohydrate levels already after 3 h of submergence in light, with levels not rising again within 24 h of submergence (Wittig et al., 2021). The underlying causes of the strength of starvation signalling might lie in differences in underwater photosynthesis, carbon utilization rates or sensitivity to starvation cues. Regardless, all species experienced carbon starvation.

Plant hypoxia responses are associated with the characteristic upregulation of a conserved gene set (Mustroph et al., 2009, 2010). In this study, a limited number of these genes were induced (Figure 8a,b). Despite the likely absence of day-time hypoxia in our experimental system, the upregulation of these hypoxia-responsive genes could potentially be attributed to ethylene inducibility of some core hypoxic genes (Hartman et al., 2019; Van Veen et al., 2013). Illuminated submerged conditions do permit some photosynthesis, providing some energy and oxygen, which combats hypoxia. Light availability can greatly improve longevity underwater (Mommer et al., 2006; Vashisht et al., 2011). Despite a minor induction of *ADH* and *PDC* transcription (Figure 8a), we observed increased ADH activity, especially in tolerant species (Figure 1c), which might be retained from night-time hypoxia, possibly combined with minor day-time transcription driven by ethylene. These results show a positive association between the strength of hypoxic acclimation and flood tolerance. However, such relationships are not universally found (Dalle Carbonare et al., 2023). In contrast, starvation and defence responses seem more conserved transcriptomic responses to submergence.

4.3 | Phylogenetically distinct routes towards flood tolerance associate with transcriptional sensitivity and quiescence

There were many species-specific and genus-specific orthogroups, compared to conserved and tolerance-related responses (Figures 3a and 5). There was a strong phylogenetic pattern in expression, indicating distinct transcriptional reconfiguration for the *Cardamine* and *Rorippa* genus in comparison to *A. thaliana* to achieve flood tolerance. Observations of an aquatic habitat among angiosperm phylogenies suggested that an aquatic/amphibious lifestyle evolved over 200 times (Cook, 1999). Given the possibility of alternative tolerance strategies already within the narrow genetic range of the Cardamineae suggests that an amphibious lifestyle was adopted many more times.

Both *Cardamine* species had a weaker down- and upregulation, thereby maintaining a transcriptomic profile closer to that of an aerated plant. This is especially pronounced with regard to chlorophyll breakdown, compared to *Rorippa* and *Arabidopsis* (Figure 7). The advantage of a weak-response strategy is apparent from *A. thaliana* mutants retarded in senescence that perform better than wild type at nonlethal flood durations (Rankenberg et al., 2024). Similarly, an *A. thaliana* accession with superior flooding recovery had an overall weaker transcriptomic reconfiguration when flooded (Yeung et al., 2018). However, the transcriptome configuration both stems from the plant's physiology, and affects the physiology itself. The stronger transcriptional senescence response found here for *A. thaliana* compared to the tolerant species (Figure 7) could result from greater stress caused by the low flood tolerance. However, the observation that delayed senescence caused by genetic or chemical ethylene signalling inhibition improves performance (Rankenberg et al., 2024), favours a gene regulatory programme.

In contrast, an absence or a delay in transcriptomic reconfiguration has also been associated with heightened hypoxia sensitivity. Pretreatment with ethylene resulted in faster hypoxic gene induction in *Rumex palustris* and *A. thaliana*, corresponding with improved anoxia tolerance (Hartman et al., 2019; Van Veen et al., 2013). Strikingly, the two most sensitive species, *A. thaliana* and *C. hirsuta*, were the slowest in their downregulation response.

Opposite to the *Cardamine* species, both *Rorippa* species displayed strong downregulation (Figure 4). The clustering and GO enrichment indicated that this was primarily related to cell-cycle activity (Figure 7). Although leaf length measurements indicated that *Rorippa* species were not more quiescent than *A. thaliana* (Figure 1b), reduced cell-cycle activity suggests that particularly the development of new or younger leaves would be affected. Notably, shade avoidance, which affects primarily cell elongation, was also downregulated especially in the tolerant species. Activating shade avoidance is a key mechanism of the escape response of *Rumex palustris* (Van Veen et al., 2013). Overall, the observations emphasize the importance of quiescence, which might encompass more than the phenotypically discernible. With strong cell-cycle suppression, the *Rorippa* species studied here might represent a prime example of successful quiescence. This would be an effective strategy to reduce carbon and energy expenditure and is reflected in the milder sugar starvation in the two *Rorippa* species.

Heterophylly is advantageous under prolonged illuminated submerged conditions, a trait found in *R. aquatica* (Nakayama et al., 2014). A transcriptome response associated with reduced cutin biosynthesis was one of the few universally adopted by tolerant Cardamineae species (Figure 7). Furthermore, the *Rorippa* species had strongly reduced stomatal development, at least at the transcriptional level (Figure 8). These observations might simply reflect the stalling of leaf production. Nonetheless, there is strong variation in the strength of heterophylly, for example, *Rumex palustris* versus *R. trichophyllus* (Kim et al., 2018; Mommer et al., 2005), and even minor changes could improve underwater photosynthesis and tolerance.

5 | CONCLUSIONS

We show that a focus on a narrow phylogenetic range, but with numerous species and diverse phenotypic data, permits an analysis that goes beyond a qualitative inventory. The capacity to separate orthologs at a high resolution provided a platform to separate mild from strong transcriptomic reconfiguration, and to directly cluster orthologs between species. Consequently, we could separate specific genes, rather than processes, that would have changed their regulation resulting in flood tolerance. We suggest that limiting senescence contributes to tolerance. However, to maintain energy homeostasis under a negative carbon balance some senescence would be unavoidable. Strong cell-cycle suppression seems an important feature of *Rorippa* and key to maintaining energy homeostasis, at least in the early phase of submergence. Fast and strong hypoxia signalling is beneficial for hypoxia tolerance, but might not be relevant to illuminated submergence, especially here, where quiescence and limiting carbon seem imperative. Dissecting exactly which condition favours a fast, slow, weak or strong response will be a crucial challenge for future work.

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DATA AVAILABILITY STATEMENT

The raw data for this study have been deposited at EMBL-EBI in the European Nucleotide Archive (ENA) under accession number PRJEB73992 (https://www.ebi.ac.uk/ena/browser/view/PRJEB73992) and in ArrayExpress under accession number E-MTAB-13910 (https://www.ebi.ac.uk/biostudies/arrayexpress/studies/E-MTAB-13910).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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