

# Convergent evolution of desiccation tolerance in grasses

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## Abstract

Desiccation tolerance has evolved repeatedly in plants as an adaptation to survive extreme environments. Plants use similar biophysical and cellular mechanisms to survive life without water, but convergence at the molecular, gene, and regulatory levels remains to be tested. Here, we explore the evolutionary mechanisms underlying the recurrent evolution of desiccation tolerance across grasses. We observed substantial convergence in gene duplication and expression patterns associated with desiccation. Syntenic genes of shared origin are activated across species, indicative of parallel evolution. In other cases, similar metabolic pathways are induced, but using different gene sets, pointing towards phenotypic convergence. Species-specific mechanisms supplement these shared core mechanisms, underlining the complexity and diversity of evolutionary adaptations to drought. Our findings provide insight into the evolutionary processes driving desiccation tolerance and highlight the roles of parallel and convergent evolution in response to environmental challenges.

## 38 Introduction

39 Anhydrobiosis, or life without water, is rare but widely distributed across life, spanning  
40 microbial, animal, and plant lineages. Plants that can tolerate desiccation in their vegetative  
41 tissues are known as resurrection plants due to their dramatic ability to revive from an extremely  
42 dry state (water potential < -100 MPa or relative water content < 10%)<sup>1</sup>. Desiccation tolerance  
43 likely arose in plants during the Ordovician period and is thought to have played a critical role in  
44 facilitating the transition from aquatic to terrestrial environments by early land plants<sup>2</sup>. These  
45 ancestral mechanisms of anhydrobiosis were retained in many non-seed plants (e.g., mosses,  
46 liverworts, ferns, and fern allies) and there is a high frequency of vegetative desiccation  
47 tolerance among extant bryophytes and pteridophytes<sup>3</sup>. In contrast, vegetative desiccation  
48 tolerance was lost, or suppressed, in the common ancestor of seed plants, presumably in a  
49 tradeoff for other systems of drought avoidance and escape, such as annual life histories, water  
50 transport, and retention mechanisms including stomata, vasculature, and roots<sup>4</sup>. Desiccation  
51 tolerance then re-evolved convergently in a subset of vascular plants, likely through the rewiring  
52 of ancestral anhydrobiosis pathways maintained in seeds, spores, and pollen<sup>5-7</sup>. The retention  
53 and re-evolution of desiccation tolerance seems to have been driven by a combination of  
54 selective pressures in habitats with extreme water limitation, seasonal drought, and sporadic  
55 water availability<sup>8</sup>. Consequently desiccation tolerance is more common in some lineages than  
56 others, but diverse species of resurrection plants can often be found co-occurring in tightly  
57 intertwined communities on rocky outcroppings in arid tropical and subtropical regions across  
58 the world<sup>3,9</sup>.

59 Despite more than 500 million years of evolution and divergence across extant  
60 resurrection plants, multiple biochemical and physiological mechanisms of desiccation tolerance  
61 are shared across distantly related species. For example, all surveyed resurrection plants  
62 accumulate small non-reducing sugars and other osmoprotectants to vitrify the cytoplasm and  
63 safeguard macromolecules during drying<sup>10</sup>. Dramatic shifts in carbohydrate and lipid  
64 metabolism as well as the protection (or in some cases degradation) of photosynthetic apparatus  
65 are also observed in all resurrection plants during drying<sup>11-14</sup>. All surveyed desiccation tolerant  
66 plants leverage robust anti-oxidant scavenging systems, mobilize numerous intrinsically  
67 disordered and protective proteins, and have specialized cell wall properties that maximize  
68 flexibility and mitigate the mechanical strain of shrinkage<sup>3,14,15</sup>. These broad features of  
69 anhydrobiosis are largely shared across organisms and tissues, but the specific metabolic  
70 pathways, regulatory networks, and activated genes are notably complex and variable among  
71 species<sup>3,10,16</sup> and tissues<sup>17</sup>.

72 The recurrent evolution of desiccation tolerance offers an exciting opportunity to  
73 understand how complex traits evolve independently across both broad and narrow  
74 phylogenetic distances. The evolution of complex traits can occur via multiple pathways<sup>18,19</sup>,  
75 and it is often assumed that when closely related taxa evolve the same trait independently, they  
76 do so by leveraging the same genetic pathways (parallelism) due to internal constraints within  
77 that lineage<sup>20</sup>. In contrast, when distantly related taxa evolve the same trait independently they  
78 are expected to leverage divergent pathways and genes (convergence), due to contrasting  
79 genetic starting points<sup>19,21</sup>. However, these patterns are not always observed in nature, and  
80 contradictory examples exist, where distantly related taxa exhibit independent but identical  
81 mutations and closely related taxa do not<sup>21</sup>. The recurrent evolution of desiccation tolerance at

82 multiple phylogenetic scales provides an ideal system to untangle the mechanisms of  
83 convergent and parallel evolution. An important first step towards decoding the evolutionary  
84 pathways to desiccation tolerance is characterizing the extent of shared genetic adaptations,  
85 overlapping pathways, and lineage specific processes across resurrection plants .

86 Desiccation tolerance has received growing research attention in recent years and  
87 several resurrection plants have emerged as models for understanding this remarkable trait <sup>22</sup>.  
88 Desiccation tolerance is found in at least ten angiosperm families, and is most common in  
89 Poaceae, where it evolved independently at least six times across three subfamilies and is  
90 found in dozens of grass species<sup>3</sup>. Thus, the grasses are an excellent system to test if the same  
91 pathways, regulatory modules, and mechanisms were recruited during the recurrent evolution of  
92 desiccation tolerance. Most genomic studies of resurrection plants have investigated only a  
93 single species in isolation <sup>17,23–27</sup> or tolerant and sensitive taxon comparison <sup>7,28,29</sup>, but none  
94 have identified core responses shared among independent lineages of resurrection plants.  
95 Here, we quantify the extent of shared mechanisms of anhydrobiosis across resurrection  
96 grasses and investigate the roles of parallel mutation and convergent pathway adaptation in the  
97 evolution of desiccation tolerance. We present highly contiguous genome assemblies of three  
98 resurrection grasses native to Sub-Saharan Africa coupled with comprehensive gene  
99 expression datasets and supporting physiological data. We leveraged comparative genomic and  
100 transcriptomic approaches to investigate the evolution of desiccation tolerance in these three  
101 species. We also extend these analyses to other desiccation tolerant and sensitive grasses to  
102 describe a core signature that defines desiccation tolerance.

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## 105 **Results**

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### 107 ***Comparative genomics of desiccation tolerant grasses***

108 We searched for signatures of convergent evolution across three grasses in two  
109 Chloridoideae subtribes representing at least two independent origins of desiccation tolerance:  
110 *Microchloa caffra* Nees. in subtribe Eleusininae and *Oropetium capense* Stapf. and *Tripogon*  
111 *minimus* Steud. in the Tripogoninae subtribe. These three species have overlapping  
112 distributions and tend to co-occur in shallow soils on rocky outcroppings, locally known as  
113 ruwari, across Sub-Saharan Africa (Figure 1a). *Microchloa caffra*, commonly known as  
114 pincushion grass, is distributed from Uganda to South Africa and is the largest of the three  
115 species. *Oropetium capense* is smaller and grows as densely packed tufts on exposed rock  
116 surfaces. *Tripogon minimus*. is a small but loosely tufted grass that occurs in shallow soils in  
117 both western and southern Africa (Figure 1a). *Microchloa caffra* plants were collected from  
118 Buffelskloof Private Nature Reserve in Mpumalanga and *O. capense* and *T. minimus* were  
119 collected from Swebe Swebe Private Wildlife Reserve in Limpopo, South Africa.

120 We generated reference genome assemblies for each of the three grasses using PacBio  
121 HiFi data. *O. capense* and *T. minimus* are diploid with haploid genome sizes of ~195 Mb based  
122 on flow cytometry, and *M. caffra* is hexaploid with a 1.25 Gb haploid genome. Sequencing reads  
123 were assembled using Hifiasm <sup>30</sup>, producing near complete reference assemblies for *O.*  
124 *capense* and *T. minimus* and a highly contiguous draft assembly of *M. caffra* (Table 1). Six and  
125 nine of the ten chromosomes were assembled telomere-to-telomere for *T. minimus* and *O.*

126 *capense* respectively, and the remaining chromosomes were split into two contigs. The *M.*  
127 *caffra* genome assembly was more fragmented, with 118 contigs spanning 968 Mb and a contig  
128 N50 of 16 Mb. The monoploid genome size of *M. caffra* is 322 Mb, which is roughly 30% larger  
129 than *O. capense* and *T. minimus* (237 and 223 Mb, respectively), and this expansion was driven  
130 largely by DNA transposons. All three species have a similar proportion of long terminal repeat  
131 retrotransposons (22-27%), but 27% of the *M. caffra* genome is composed of DNA transposons  
132 compared to 12% in *O. capense* and 16% in *T. minimus* (Table 1). Despite this expansion of  
133 transposons in *M. caffra*, the three Chloridoid grasses have very compact genomes compared  
134 to most grasses<sup>31</sup>. We used the MAKER-P pipeline to annotate these three genome  
135 assemblies, with RNAseq data and protein homology as evidence. The *O. capense* and *T.*  
136 *minimus* genome assemblies have 28,826 and 26,527 gene models respectively, which is  
137 comparable to the well-annotated model resurrection plant *Oropetium thomaeum* (L.f.) Trin  
138 (28,835)<sup>24,32</sup>. The *M. caffra* genome assembly has 85,245 gene models, which matches the  
139 expectations for a hexaploid genome (Table 1). We assessed annotation quality using the land  
140 plant (Embryophyta) dataset of Benchmarking Universal Single-Copy Orthologs (BUSCO) and  
141 found between 95.3-97.1% complete proteins across the three grasses, suggesting the genome  
142 assemblies are largely complete and well-annotated (Table 1).

143 We leveraged comparative genomic approaches to identify evolutionary signatures  
144 associated with desiccation tolerance and enable cross-species comparisons of gene  
145 expression data. The three grass genomes are largely collinear with *O. thomaeum*, and have  
146 considerable conserved gene content despite some notable structural rearrangements. Seven  
147 pairs of *O. thomaeum* and *O. capense* chromosomes have near perfect synteny, with  
148 chromosomes 8 and 9 showing a few large-scale inversions, and a telomeric translocation on  
149 chromosome 2 (Supplemental Figure 1). *Tripogon minimus* has similar macrosynteny with *O.*  
150 *thomaeum*, but has no rearrangements in chromosome 8. Synteny between *M. caffra* and *O.*  
151 *thomaeum* is more fragmented because of phylogenetic divergence and each *O. thomaeum*  
152 region has between 2-4 homeologous regions in *M. caffra* (Supplemental Figure 2). We  
153 calculated the synonymous substitution rates (Ks) between homeologous gene pairs within *M.*  
154 *caffra* to date the polyploid event(s). We observed a single Ks peak of 0.13 across all  
155 homeologous gene pair combinations, suggesting the autohexaploidy event occurred ~4 million  
156 years ago from rapidly successive polyploidy events (Supplemental Figure 2d). Using MCSan  
157 with *O. thomaeum* as an anchor, we identified 18,428 syntenic orthologs (syntelogs) shared  
158 among the three grasses, as well as previously published tolerant grasses *Eragrostis nindensis*  
159 Ficalho & Hierr<sup>28 33</sup>. These syntelogs were used to identify patterns of gene duplication  
160 associated with desiccation tolerance across grasses and as anchor points to compare  
161 expression of conserved genes across species.

162 To test for convergent evolution we characterized patterns of expansion and duplication  
163 in gene families with important roles in desiccation tolerance. The genomes of all sequenced  
164 resurrection plants have large tandem arrays of early light induced proteins (ELIPs)<sup>34</sup>, and we  
165 observed this same pattern across the desiccation tolerant grasses investigated here.  
166 *Oropetium capense*, *T. minimus*, and *M. caffra* all have massive tandem arrays of 39, 31, and  
167 58 ELIPs respectively, compared to an average of 4 in the genomes of desiccation sensitive  
168 grasses<sup>34</sup>. This expansion of ELIPs is similar to other chlorophyll retaining (homiochlorophyllus)  
169 resurrection plants and is generally higher than chlorophyll degrading (poikiochlorophyllus)

170 species. ELIPs are universally highly expressed in the diploid resurrection grasses *O. capense*  
171 and *T. minimus* during drying, desiccation, and early rehydration, but only a subset of the ELIPs  
172 in the *M. caffra* tandem arrays have desiccation induced expression (Supplemental Figure 3).  
173 We used CAFE<sup>35</sup> to test for changes in the dynamics of ELIP copy number evolution across  
174 land plants. We found significant increases in the rate of ELIP expansion in all desiccation  
175 tolerant lineages of plants (Figure 2c). Within the grass family, ELIP expansion occurred  
176 independently in subtribes Eleusininae, Sporobolinae, Eragrostidinae, and Tripogonae, but  
177 *Oropetium* and *Tripogon* share a single origin of desiccation tolerance (Figure 2c). Other gene  
178 families with well-characterized roles in desiccation tolerance such as late embryogenesis  
179 abundant (LEAs) and heat shock proteins (HSPs) show no expansion in resurrection plants  
180 based on OrthoFinder and or CAFE (Supplemental Figures 4-7).

181 We identified the origin of duplicated ELIPs to test if the same or different ancestral  
182 copies were duplicated in each lineage using a synteny based approach. Tandem duplication of  
183 ELIPs within the Tripogoninae occurred on Chromosome 8, and the Eleusininae and  
184 Eragrostidinae subtribe species have no syntenic ELIPs in this region, despite otherwise high  
185 collinearity (Figure 2a). Most ELIPs in Eleusininae and Eragrostidinae species are found in large  
186 tandem arrays on Chromosome 7, compared to 4-5 ELIPS within Tripogoninae (Figure 2b).  
187 Together, phylogenetic and comparative genomics analyses suggest these grass lineages  
188 duplicated ELIPs independently, supporting the convergent evolution of desiccation tolerance  
189 within Chloridoideae.

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### 192 **Searching for overlapping signatures of desiccation tolerance**

193 We collected dehydration and rehydration timecourses of *O. capense*, *T. minimus*, and  
194 *M. caffra* plants under similar conditions in a climate controlled growth chamber. Plants reached  
195 desiccation after ~17-20 days of natural drying, with a relative water content (RWC) < 10% and  
196 photosystem II efficiency ( $F_v/F_m$ ) approaching 0.0 (Figure 1b-d). RWC and  $F_v/F_m$  recovered  
197 within 12 hours of rehydration in *O. capense* and *T. minimus*, but  $F_v/F_m$  took longer to recover in  
198 *M. caffra* (Figure 1b). We collected gene expression data (RNAseq) at six comparable  
199 timepoints of drying and recovery for each of the three species. We quantified RNA abundance  
200 and gene expression patterns across the dehydration-rehydration timecourse in each species  
201 individually. RNAseq reads were pseudo-aligned to the respective genomes using Salmon (v  
202 1.9.0)<sup>36</sup> and normalized counts were used for all downstream analyses. In general, gene  
203 expression profiles were tightly associated with the hydration status of the plants. Correlation  
204 matrices and principal component analysis (PCA) show tight clustering of samples by hydration  
205 status, with hydrated, desiccated, and rehydrated samples forming distinct clusters for each  
206 species (Supplemental Figure 8).

207 Using RWC as a covariate, we identified genes that were significantly up- and down-  
208 regulated during dehydration and rehydration processes. Both dehydration and rehydration  
209 induced substantial changes in gene expression in all three desiccation tolerant grasses, with  
210 35-52% of genes showing differential abundance during dehydration and 23-47% during  
211 rehydration (Figure 3a and Supplemental Figure 9). *Microchola caffra* had significantly more  
212 differentially expressed (DE) genes (Supplemental Figure 9) given its hexaploidy, but a lower  
213 proportion of DE genes compared to the other two grasses (Figure 3a). Broadly, desiccation



214 and rehydration had inverse expression profiles, and most genes that increased in abundance  
215 during dehydration, dissipated during rehydration and vice versa (Supplemental Figure 9).  
216

217 To enable comparisons across species, we leveraged the 18,428 conserved syntelogs  
218 and searched for overlapping patterns in the expression of these shared genes. There was  
219 considerable overlap in gene expression across the three focal resurrection grasses, with ~18-  
220 24% of all DE syntelogs showing similar expression across species (Figure 3b and  
221 Supplemental Figure 10a). The proportions of DEGs shared across the three resurrection  
222 grasses for both up- and down-regulated genes was considerably more than observed in  
223 previous studies or expected due to chance. In order to differentiate between desiccation  
224 tolerance mechanisms and more general drought responses, we identified the extent of shared  
225 syntelog expression between these resurrection grasses and the desiccation sensitive species  
226 *E. tef*, which was sampled along a similar dehydration timecourse in a previous study<sup>28</sup>. There  
227 was considerable overlap in syntelog expression between the resurrection grasses and *E. tef*  
228 (Supplemental Figure 11), reflecting deeply conserved mechanisms of drought tolerance in  
229 grasses. We also detected a large set of genes that were expressed exclusively in the  
230 resurrection grasses, which likely play desiccation specific roles to survive anhydrobiosis.  
231 Species-specific expression patterns are also evident, particularly for *E. tef*.

232 Dimensionality reduction and co-expression analyses also point towards parallel  
233 mechanisms of desiccation tolerance in resurrection grasses. Samples clustered primarily by  
234 hydration status and secondarily by species in PCA (Figure 3c,d and Supplemental Figure 12).  
235 We defined co-expression modules for each species and screened for shared network level  
236 responses within co-expressed genes. High confidence modules were defined for each species,  
237 and we grouped these into three broad classes based on the expression pattern of each  
238 module: 1) elevated expression in hydrated conditions, 2) elevated expression during  
239 dehydration, and 3) elevated expression during rehydration (Figure 4d). We identified  
240 substantial overlap in gene module conservation with elevated expression during dehydration,  
241 but less overlap in modules with high expression during rehydration (Figure 4a). We then  
242 identified enriched gene ontology (GO) terms for each co-expression module and performed  
243 hierarchical clustering on the enrichment p-values of GO terms. Modules clustered by their  
244 expression profile rather than species identity, suggesting that hydration status is more  
245 predictive of gene expression than species identity (Figure 4c) and pointing towards a shared  
246 signature of desiccation tolerance in resurrection grasses.

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### 249 ***Functional characterization of the shared signatures of desiccation tolerance***

250 Our analyses of syntelog expression tested for ancestral conservation and parallelism,  
251 but it is also possible that different lineages of resurrection plants may utilize similar metabolic  
252 strategies for achieving desiccation tolerance but through divergent genes and pathways. To  
253 investigate this possibility, we used the Kyoto Encyclopedia of Genes and Genomes (KEGG) to  
254 assign each gene to a predicted enzymatic function and metabolic pathways and compared the  
255 overlap in these functional predictions across species. We detected substantially higher overlap  
256 in KEGG terms across species (~30-40%) compared to DE syntelogs (only 18-24%) (Figure 5b  
257 and Supplemental Figure 10c). The increased similarity at a metabolic level suggests that while

258 these species do not always leverage parallel gene copies, they induce similar metabolic  
259 mechanisms to survive anhydrobiosis, providing evidence of convergence across species.

260 We further investigated the functional roles of shared gene expression via GO  
261 enrichment and KEGG analyses. We found that many hallmarks of desiccation tolerance were  
262 shared across the three resurrection grasses, including the controlled downregulation of  
263 photosynthesis and rapid induction of protective mechanisms. Enriched GO terms during  
264 dehydration were related primarily to signaling and stress responses (e.g., stress perception  
265 and ROS scavenging activities), developmental regulation (e.g., photoperiodism and  
266 germination processes), cellular reorganization (e.g., lipid droplet formation, vesicle fusion,  
267 endocytosis), and modifications to transcription and translation (e.g., RNA modifications,  
268 splicing, and protein degradation). In contrast, enriched GO terms during rehydration are related  
269 to photosynthesis and metabolism (e.g., fructose biosynthesis, cellulose biosynthesis, and light  
270 harvesting), pigment metabolism (e.g., chlorophyll biosynthesis and anthocyanin metabolism),  
271 protein modification (e.g., protein phosphorylation and proteolysis), and some residual stress  
272 response (e.g., response to cold and non photochemical quenching) (Figure 5a). Hierarchical  
273 clustering of enriched GO terms also highlighted the inverse relationship between dehydration  
274 and rehydration process (Figure 5a and Supplemental Figure 10d).

275 To differentiate between desiccation tolerance mechanisms and more typical drought  
276 tolerance responses, we compared the enriched GO terms for DE syntelogs uniquely induced in  
277 the resurrection grasses vs. those shared with desiccation sensitive *E. tef* (Supplemental Figure  
278 11). Many of the classic stress response terms were shared across all species, reflecting deeply  
279 conserved responses to water deprivation. For example, all species showed metabolic arrest  
280 during drying with a particular emphasis on photosynthetic shut down. All species exhibited an  
281 increase in classic stress response terms such as response to heat, response to water  
282 deprivation, response to hydrogen peroxide, and sucrose metabolic process. These processes  
283 represent core mechanisms of water deficit tolerance that likely form the foundation of  
284 desiccation tolerance. Building on this foundation, resurrection grasses appear to activate  
285 additional processes that enable more extreme resilience. For example, the resurrection  
286 grasses showed unique activation of nucleic acid processes including mRNA export, regulation  
287 of chromosome condensation, and mRNA transcription by RNA polymerase II suggesting  
288 greater overall regulation of transcription and translation. Several terms associated with the  
289 circadian rhythm and hormonal signaling were also uniquely upregulated in the resurrection  
290 grasses, indicating a central role of circadian clock processes in preparing for desiccation. The  
291 resurrection grasses exhibited a unique downregulation of tissue and cellular developmental  
292 processes, implying a tightly regulated cessation of metabolism at later stages of drying. Taken  
293 together, this suggests that resurrection grasses build on a shared foundation of drought  
294 tolerance to achieve desiccation tolerance via a highly organized shift in cellular processes.

295 KEGG annotations revealed characteristic desiccation tolerance mechanisms shared  
296 across resurrection grasses. Metabolic pathways associated with photosynthetic energy  
297 metabolism were significantly down regulated in all three grasses. Interestingly, we observed an  
298 increase of malate to pyruvate catalysis with concomitant regeneration of NADPH, which could  
299 be related to NADPH's REDOX potential for antioxidant enzymes such as glutathione  
300 reductase. We also detected noticeable changes to carbohydrate and energy metabolism,  
301 including a shift towards the production of raffinose and stachyose under dehydrating conditions  
302 as seen in other resurrection plants (reviewed in <sup>10</sup>). Central carbohydrate metabolism appeared

303 operational suggesting that at low water contents, other solvents, such as natural deep eutectic  
304 solvents within the mitochondria may facilitate glycolysis, the TCA, and electron transport<sup>37</sup>.  
305 Amino acid metabolism favored degradative pathways with an increase in endoplasmic  
306 reticulum-mediated ubiquitination and proteolysis, which could be serving a glucogenic role by  
307 converting amino acids to pyruvate, or by generating an available amino acid pool for the rapid  
308 assembly of thermo- and osmoprotective proteins. While amino acid metabolic pathways were  
309 generally down-regulated, a few important pathways including glutathione metabolism were up-  
310 regulated. Reduced glutathione (GSH) exerts numerous effects in the cell<sup>38</sup> from interaction  
311 with hormones to acting as direct ROS quencher, and maintaining a steady supply of GSH is a  
312 feature all three resurrection grasses share. Lipid metabolism showed a shift towards the  
313 production of glycerolipids and glycerophospholipids which likely supports triacylglycerol  
314 phosphatidylcholine production. The accumulation of phosphatidylcholine may further lead to  
315 phosphatidic acid synthesis, which has been implicated in numerous plant processes from  
316 signaling to storage<sup>39-41</sup>. Pathways involved in the transcription and translation of genetic  
317 information also showed an up-regulation of transcription factors, RNA polymerase, and  
318 spliceosome activity, suggesting that active transcription and RNA processing are still occurring.  
319 However, we observed substantial downregulation of ribosome activity, suggesting that RNA is  
320 either differentially translated or delayed. Upon rehydration, up-regulated processes involved in  
321 overall resumption of normal metabolic activity such as several photosystem I and II proteins,  
322 light harvesting complexes, starch synthesis, and cell wall remodeling such as xyloglucan O-  
323 acetyltransferase, expansin, and pectinesterase were observed.

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### 325 ***Desiccation tolerance mechanisms are broadly conserved across grasses***

326 Desiccation tolerance evolved independently in at least four subtribes of Chloridoideae  
327 (Eleusininae, Eragrostidinae, Sporobolinae, and Tripogoninae; Figure 2c), and we integrated  
328 comparable desiccation and rehydration expression datasets from additional species to test for  
329 patterns of convergence across grasses more broadly. Building on our detailed comparisons  
330 across the three study species, we expanded our analysis to include publicly available RNAseq  
331 samples from desiccation tolerant *O. thomaeum*<sup>42</sup> and *E. nindensis*<sup>28</sup>, leveraging syntelogs for  
332 cross-species comparisons. Similar to the three species comparisons described above,  
333 dimensionality reduction across the five species generally separated samples by hydration  
334 status along PC1 and PC2 (Supplemental Figure 12). While PCA provided some degree of  
335 separation; residual heterogeneity, experimental differences, noise, or species level differences  
336 in the datasets might have obscured underlying conserved biology. To account for this, we  
337 employed a topological data analysis (TDA) approach to discern the underlying structure of the  
338 expression datasets. We utilized the Mapper algorithm, which condenses the dataset into a  
339 scalable, navigable representation. The Mapper algorithm is particularly well-suited for genome  
340 scale analyses, as the underlying datasets are often characterized by high dimensionality and  
341 sparsity<sup>43</sup>. For our gene expression data, we constructed Mapper graphs using a "stress lens"  
342 with the well-watered condition as a reference point. This model represents the baseline for  
343 gene expression and we quantified the residuals or deviation of each sample from the baseline,  
344 which represent the degree of water stress or recovery.

345 The resultant Mapper graph illustrates a clear topological shape that delineates  
346 desiccation processes across grasses (Figure 6). Each node on the graph represents a cluster



347 of similar RNAseq samples, and the node color depicts the identity of samples within that  
348 cluster. Connections between nodes signify shared samples among the intersecting clusters.  
349 These graphs reveal a compelling topological depiction of the gene expression variations  
350 induced by water stress across different species. Similar topology was observed for both  
351 targeted comparison of the three focal species (Figure 6a and b) and for the larger dataset  
352 including *E. nindensis* and *O. thomaeum* (Figure 6c and d). In both instances, clear delineation  
353 between samples of different hydration statuses are evident, while the species are intermixed.  
354 We then added the desiccation sensitive sister species *E. tef* in a final analysis (Supplemental  
355 Figure 13), which revealed a similar topology across all species with notable gaps in *E. tef*.  
356 Broadly, this supports our finding that similar ancestral mechanisms are being recruited for  
357 foundational drought tolerance mechanisms, which are enhanced in resurrection plants via the  
358 independent recruitment of specific desiccation tolerance pathways.

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### 361 ***Species specific mechanisms underlying desiccation tolerance***

362 Despite the considerable overlap in gene expression across all three focal species,  
363 species-specific processes were also evident. In *M. caffra*, unique antioxidant responses were  
364 induced including glutathione biosynthetic processes, glutamate decarboxylation, and L-  
365 ascorbic acid biosynthesis. Other processes enriched uniquely in *M. caffra* included seed-  
366 related terms such as seed oil body biogenesis and seed maturation. Several GO terms  
367 associated with phytohormones were also uniquely induced in *M. caffra*, including overall  
368 ethylene responses such as S-adenosylmethionine metabolic process, ethylene-activated  
369 signaling pathway, and response to 1-aminocyclopropane-1-carboxylic acid, suggesting that  
370 hormonal regulation might be exerting an effect on the partial breakdown of thylakoids and  
371 photosynthetic machinery as seen in classical senescence<sup>44</sup>. *Microchloa caffra* also exhibited  
372 unique lipid, sphingolipid, riboflavin, and selenocompound metabolism, as well as  
373 sesquiterpenoid and tripenoid biosynthesis. *Microchloa caffra* was the only species to have  
374 multiple pathways involved in signal transduction up-regulated including phospholipase D and  
375 calcium signaling. Uniquely down-regulated processes in *M. caffra* appear to center around  
376 arresting growth and development, such as phototropism, gravitropism, leaf and root  
377 morphogenesis, cell wall biogenesis, and regulation of auxin polar transport. There was also a  
378 down-regulation of general amino acid-tRNA aminoacylation and nitrogen fixation and  
379 assimilation.

380 *Oropetium capense* had fewer uniquely enriched processes compared to *M. caffra*, but  
381 some notable patterns were detected. Uniquely upregulated processes in *O. capense* centered  
382 around histone H3 and H4 acetylation, histone H3-K9 demethylation, and histone H2B  
383 ubiquitination. The relative degree of acetylation of histones is directly related to the openness  
384 of chromatin which impacts transcription in specific drought-responsive genes<sup>45</sup>. Histone  
385 demethylation<sup>46</sup> and H2B ubiquitination also regulate drought responsive genes<sup>47</sup>.  
386 Interestingly, terms associated with chloroplast mRNA processing, poly(A)+ mRNA export from  
387 the nucleus, ribosome assembly, and regulation of translation were also upregulated in *O.*  
388 *capense*, suggesting continued translation and active processing of mRNA from both the  
389 chloroplast and nucleus, presumably through increased transcriptional regulation due to histone  
390 modifications. *Oropetium capense* exhibited unique up-regulation of C5-branched dibasic

391 metabolism and down-regulation within galactose metabolism. Uniquely down-regulated  
392 processes in *O. capense* were minimal, but included regulation of salicylic acid metabolic  
393 process and auxin polar transport. Monoterpenoid biosynthesis was up-regulated for *O.*  
394 *capense* and fatty acid degradation and steroid hormone biosynthesis was down-regulated.  
395 Ferroptosis, an iron-dependent form of programmed cell death, was exclusively down-regulated  
396 in *O. capense*.

397 *Tripogon minimus* also had fewer species-specific processes compared to *M. caffra*.  
398 Uniquely up-regulated processes were centered around response to oxidative stress,  
399 peroxisome organization, and removal of superoxide radicals. *Tripogon minimus* was the only  
400 species to show upregulation of anthocyanin-containing compound biosynthetic process which  
401 is a typical response seen in the homoiochlorophyllous resurrection plants<sup>48</sup>. Similar to the  
402 other two species, regulation of auxin-mediated signaling pathways were down-regulated as  
403 was cellular response to salicylic acid stimulus. Other processes centered around mismatch  
404 repair, chloroplast RNA processing, ribosome biogenesis, and plastid transcription.  
405 Phosphonate and phosphinate, taurine and hypotaurine, and D-amino acid metabolism were  
406 exclusively down-regulated in *T. minimus* whereas retinol metabolism was up-regulated.

407 Despite the unique pathways identified in each of the focal species, all three species  
408 appear to respond to drought and desiccation stress by leveraging similar mechanisms. The  
409 processes uniquely activated in each species are consistently centered around defense  
410 mechanisms, the induction of quiescence, and reduction of normal growth and metabolism  
411 under desiccated conditions. While nuanced variation in metabolism and defense responses are  
412 evident, all species exhibit well known mechanisms of desiccation tolerance. Taken together,  
413 the three species appear to share a core set of conserved mechanisms which are then  
414 supplemented with convergent species-specific modules.

415

416

## 417 Discussion

418 Our data suggest that the repeated evolution of desiccation tolerance within grasses  
419 occurred via both parallel adaptations in the same ancestral genes and complementary  
420 modifications to analogous pathways. We find evidence that core mechanisms of desiccation  
421 tolerance are shared across resurrection grasses, and are supplemented with species-specific  
422 adaptations. Many of these mechanisms overlap with typical drought responses and it is likely  
423 that the evolution of anhydrobiosis builds on deeply conserved responses to water deficit shared  
424 across all plants. Phenotypic and metabolic similarities in anhydrobiosis mechanisms have been  
425 observed for decades, but the evolutionary pathways of convergence and parallelism have been  
426 obscured by a lack of systems-level data and inconsistencies in experimental procedures<sup>49</sup>.  
427 Here, we leveraged large scale genomic and transcriptomic datasets in a replicated and  
428 standardized framework to characterize signatures underlying the recurrent evolution of  
429 desiccation tolerance within chloridoid grasses.

430 The adaptations required for desiccation tolerance appear to be sufficiently narrow, such  
431 that not any organism can, or will, evolve desiccation tolerance<sup>4</sup>. The physiological changes  
432 that occur during the final stages of desiccation are dramatic and specialized biochemistry and  
433 molecular mechanisms are required to protect the cellular macromolecules for life without water.  
434 Achieving anhydrobiosis requires tight coordination and orchestration of multiple physiological

435 processes, and there may be only a few trajectories to evolve this trait. However, desiccation  
436 tolerance mechanisms overlap considerably with typical drought responses and many plants  
437 possess the basic cellular machinery required to achieve desiccation tolerance<sup>28</sup>. Desiccation  
438 tolerance is likely an ancestral adaptation in plants that evolved during terrestrialization,  
439 subsequently formed the basis of seed pathways, and was later rewired again in vegetative  
440 tissues<sup>5,25,42,50</sup>. While previous studies have found surprisingly little overlap in gene expression  
441 across desiccation tolerant plants<sup>51,52</sup>, our data suggest that the repeated evolution of specific  
442 genetic, biochemical, and physiological traits required for anhydrobiosis, are highly convergent  
443 and build on more broadly conserved water deficit responses.

444 Convergence is thought to be driven primarily by exposure to external selective  
445 pressures that lead to the same emergent phenotype, while parallelism is thought to be  
446 impacted more by internal constraints of the system<sup>18</sup> through independent mutations in the  
447 same ancestral gene<sup>19,21</sup>. Because anhydrobiosis has evolved independently in both distantly  
448 and closely related taxa, it is an ideal system in which to explore the roles of convergent and  
449 parallel evolution. Numerous other independently evolved traits such as C4 and CAM  
450 photosynthesis are highly complex, making their repeated evolution surprising<sup>53</sup> and difficult to  
451 characterize. In the case of C4 photosynthesis, both mutations in the same genes and  
452 recruitment of unique pathways occurred in distantly related lineages to enable the emergent C4  
453 phenotype<sup>54</sup>. Desiccation tolerance is similarly complex, involving the synchronized  
454 orchestration of numerous pathways and genes, and it is likely that both external pressures (e.g.  
455 selection in extremely xeric habitats) and internal constraints (lineage specific predispositions)  
456 play a role in the recurrent evolution of desiccation tolerance. Here, we detected signatures of  
457 both processes and identified far more overlap in gene expression across resurrection grasses  
458 than expected by chance or detected in previous studies<sup>29,51</sup>. The observed expansion of ELIP  
459 tandem arrays coupled with activation of similar metabolic pathways driven by different gene  
460 sets, suggests that both parallel and convergent processes contribute to the recurrent evolution  
461 of desiccation tolerance in grasses.

462 Our systems-level analyses add to the growing literature on the mechanisms of  
463 desiccation tolerance, and many of the patterns observed here corroborate previous findings  
464<sup>17,25,29,34,51</sup>. We show that desiccation induces a major and reversible shift in gene expression  
465 where normal growth and development are halted and numerous protective mechanisms are  
466 induced<sup>13,14,55–57</sup>. Gene expression coalesced around a signature desiccation response during  
467 drying with all three species initiating parallel processes<sup>58</sup>. The resumption of species specific  
468 processes related to growth and development was evident upon rehydration. The shared  
469 pathways of anhydrobiosis observed in these grasses pull on the deeply conserved architecture  
470 of drought tolerance coupled with convergent and parallel mutations that provide the necessary  
471 protection to survive extreme desiccation. This reflects the relatively narrow set of regulatory  
472 networks and pathways in plants that can enable the evolution of desiccation tolerance, but also  
473 hints as multiple evolutionary paths to anhydrobiosis

474

475

## 476 **Methods**

477

478 *Field collections, plant growth, and maintenance*

479 Plants for the current study were collected from two research sites in South Africa:  
480 Buffelskloof Nature Reserve in Mpumalanga (-25.30229 S, 030.50631 E) (*Microchloa caffra*)  
481 and Swebe Swebe Private Wildlife Reserve in Limpopo (-23.7949 S, 028.0705 E) (*Oropetium*  
482 *capense* and *Tripogon minimus*). Voucher specimens of each species were collected, pressed,  
483 and deposited at the National Herbarium of South Africa in Pretoria (specimen numbers:  
484 PRE1004810-0, PRE1004793-0, and PRE1004794-0). Seeds of each species were also  
485 collected and transported to Michigan State University under United States Department of  
486 Agriculture (USDA) permit #537-22-37-10071 and according to the specifications in a Material  
487 Transfer Agreement established between Drs. Jill M. Farrant, Robert VanBuren, and Rose A.  
488 Marks. Seeds were cold stratified at 4°C for two weeks and then germinated on our standard  
489 propagation mix (50:50 sure-mix:redi-earth) and grown in a climate controlled growth chamber  
490 with a 16 hour photoperiod and internal temperature of 28/18°C. Six weeks after germination,  
491 individual seedlings were transplanted into separate pots and grown to maturity and a single  
492 plant (genetic line) of each species was selected for downstream experimentation. Seeds of  
493 each genetic line were collected, cold stratified, and ~70 seedlings from each species were  
494 germinated. One seedling from each species was used for genome sequencing and was  
495 transplanted into a larger pot. The remaining seedlings were used for the desiccation and  
496 rehydration timecourses experiments and three seedlings were transplanted into 4" pots. The  
497 three plants in each pot were pooled during sampling and treated as a single biological  
498 replicate. These plants were grown for another two weeks prior to experimental treatments,  
499 during which time they were maintained in constantly hydrated conditions in a growth chamber  
500 set to the conditions described above.

501  
502 *Dehydration treatment and sample collection*

503 After ~8 weeks of growth, plants were subjected to dehydration treatment. Prior to  
504 treatment, any emerging reproductive tissues (e.g., panicles) were removed from plants. To  
505 initiate dehydration treatment, plants were watered to full soil saturation and each pot was  
506 weighed to ensure consistency across replicates. Water was then withheld until plants became  
507 completely desiccated (between 2 and 3 weeks depending on the species). Plants were  
508 sampled at targeted hydration states during the process of dehydration, including well watered,  
509 partially dehydrated, fully desiccated, and rehydrated. We used visual cues to direct our  
510 sampling and sampled plants at the first signs of visible leaf curling, partial pigmentation, deep  
511 pigmentation, and full desiccation and validated the hydration status of tissues by measuring  
512 relative water content (RWC). Plants were then rehydrated through a combination of watering  
513 from the base and misting the aerial portions to simulate natural rainfall and sampled 24 and 48  
514 hours post rehydration. We aimed to sample plants at biologically relevant water contents and  
515 therefore directed our sampling by use of visual cues rather than a set number of hours. This  
516 allowed us to compensate for different drying rates across species and plants due to subtle  
517 variation in size, water use efficiency, and relative humidity in the growth chamber.

518 At each timepoint, we measured the photosynthetic efficiency ( $F_v/F_m$ ) and RWC, and  
519 harvested tissue for RNAseq. Briefly,  $F_v/F_m$  was measured on dark adapted leaves using a Opti-  
520 Sciences OS30p+ chlorophyll fluorometer with the default test parameters. Relative water  
521 content was measured using a set of 10-15 representative leaves from each pot / biological  
522 replicate. Leaf mass was weighed immediately after collection (fresh weight), again after 48

523 hours submerged in dH<sub>2</sub>O in darkness at 4°C (turgid weight), and finally after 48 hours in a 70°C  
524 drying oven (dry weight). RWC was calculated as (fresh weight - dry weight)/(turgid weight - dry  
525 weight). Tissue for RNAseq was collected by harvesting all the vegetative tissue from each pot  
526 and flash freezing in liquid nitrogen. Tissue samples were stored in a -80°C freezer prior to  
527 downstream processing.

528

#### 529 *RNA extraction and sequencing*

530 Frozen leaf tissue was ground to a powder by hand in a mortar and pestle with liquid  
531 nitrogen. RNA was extracted from each sample using Spectrum Plant Total RNA kit according  
532 to the manufacturer instructions. Total RNA was then cleaned to remove impurities and  
533 contaminants using Zymo Clean & Concentrator kit. DNase treatment was carried out during  
534 clean and concentration steps according to manufacturer instructions. Sample concentration  
535 was assessed on a qubit using the RNA broad range reagent set, purity was assessed with a  
536 nanodrop, and RNA integrity was visualized on an agarose gel. RNAseq libraries were  
537 constructed by Novogene following a standard polyA+ enrichment strategy including  
538 fragmentation and cDNA synthesis. The resulting libraries were sequenced on an Illumina  
539 HiSeq 4000 under 150 bp paired end mode

540

#### 541 *High molecular weight DNA extraction, and sequencing*

542 Tissue for whole genome sequencing was collected from a single mature plant of each  
543 species. Healthy green tissue was harvested and flash frozen in liquid nitrogen. Tissue was  
544 ground by hand in a mortar and pestle for >20 minutes to liberate nuclei. Pure, high molecular  
545 weight genomic DNA was extracted by first isolating nuclei with the Circulomics Nuclei Isolation  
546 kit and then extracting DNA with the Circulomics Nanobind Plant Nuclei Big DNA kit. HiFi  
547 libraries were constructed from the Genomic DNA and sequenced at the University of Georgia  
548 Sequencing Core on a PacBio Sequel II machine.

549

#### 550 *Genome assembly*

551 We used flow cytometry to estimate genome sizes (2C DNA values) for the three  
552 grasses. Healthy leaf tissue was harvested from each genotype. Nuclei were isolated and  
553 stained according to standard protocols. The stained nuclei were then run on a BD Accuri™ C6  
554 Plus Flow Cytometer at Plantploidy.com. *Hosta plantaginea* was used as an internal reference.  
555 We built reference genomes for each species using high fidelity (HiFi) PacBio long read data. In  
556 total, 70.1 Gb of HiFi reads were generated for *M. caffra*, 15.9 Gb for *O. capense*, and 20.2 Gb  
557 for *T. minimus*, representing 56, 82, and 103 x genome coverage for each species respectively.  
558 K-mer analysis revealed that *O. capense* and *T. minimus* have low within genome  
559 heterozygosity and *M. caffra* is a highly heterozygous autopolyploid<sup>59</sup>. PacBio reads were  
560 assembled using hifiasm (v 0.18)<sup>30,60</sup> with default settings for *O. capense* and *T. minimus* and  
561 the number of haplotypes was set to 6 for *M. caffra* (flag: --n-hap 6). The resulting assemblies  
562 were highly contiguous with six and nine of the ten chromosomes assembled telomere to  
563 telomere for *T. minimus* and *O. capense* respectively, and 118 contigs across 968 Mb with an  
564 N50 of 16 Mb for *M. caffra* (Table 1). Raw assemblies were filtered for non-plant contigs using a  
565 representative microbial database with BLAST<sup>61</sup>. Full length chloroplast and mitochondrial



566 genomes were identified and retained, and any additional partial or rearranged organelle  
567 genomes were removed.

568

### 569 *Genome annotation*

570 A library of repetitive elements was constructed for each of the three grass genomes  
571 using the EDTA package (v2.0.0)<sup>62</sup>. EDTA comprehensively identifies DNA-based transposable  
572 elements using HelitronScanner<sup>63</sup>, and LTR retrotransposons using LTR\_FINDER<sup>64</sup> and  
573 *LTRharvest*<sup>65</sup>. Protein coding genes were annotated using the MAKER-P pipeline (v2.31.10)<sup>66</sup>  
574 with the following sets of input data for training. Transcript evidence was generated using the  
575 dehydration-rehydration timecourse RNAseq data from leaf tissue of each species described  
576 below. Raw RNAseq reads were quality trimmed using fastp (v 0.23)<sup>67</sup> and aligned to the  
577 unmasked genomes using the splice aware alignment program STAR (v2.6)<sup>68</sup>. A set of non-  
578 overlapping transcripts was identified from the aligned data using StringTie (v1.3.4)<sup>69</sup> with  
579 default parameters. The resulting gff files were used as transcript evidence for MAKER. The  
580 same protein evidence was used as training for each of the three grasses and this includes the  
581 full annotations of *Oryza sativa*<sup>70</sup>, *Arabidopsis thaliana*<sup>71</sup>, *Oropetium thomaeum*<sup>24,32</sup>, and  
582 *Eragrostis tef*<sup>33</sup>. These datasets were used as input for MAKER and we utilized SNAP<sup>72</sup> and  
583 Augustus (version 3.0.2)<sup>73</sup> for *ab initio* gene prediction, performing two rounds of iterative  
584 training to refine our models. To filter out repetitive element-derived proteins, we used BLAST  
585 using a non-redundant transposase library against the raw gene models produced by MAKER.  
586 We assessed the completeness of our assembly using the plant-specific embryophyte set of  
587 Benchmarking Universal Single-Copy Orthologs (BUSCO v.2)<sup>74</sup>. These high-confidence gene  
588 models were used for all downstream analyses.

589

### 590 *Comparative genomics*

591 The three desiccation tolerant grass genomes were compared to each other and other  
592 Chloridoid grasses using the MCScan toolkit (v1.1)<sup>75</sup> implemented in python  
593 [[https://github.com/tanghaibao/jcvi/wiki/MCscan-\(Python-version\)](https://github.com/tanghaibao/jcvi/wiki/MCscan-(Python-version))]. Syntenic orthologs were  
594 identified across the three focal species, *E. nindensis*, *E. tef*, and *Oropetium thomeaum* using  
595 the chromosome-scale *O. thomeaum* genome as an anchor. Syntenic blocks were identified  
596 using gene models aligned using LAST with a minimum of five overlapping syntenic genes. The  
597 macrosyntenic dot plots, histograms of depth, and microsynteny plots were generated using the  
598 python version of MCScan. A set of 18,428 conserved syntenic orthologs across all six  
599 desiccation tolerant grasses was created and used for downstream comparative genomic and  
600 cross-species transcriptomic analyses. We identified orthologous genes across a subset of 33  
601 land plant species to search for patterns of gene family expansion in desiccation tolerant  
602 lineages as well as for downstream comparative genomic analyses. We included the following  
603 species with desiccation tolerant species highlighted: *Ananas comosus*, *Arabidopsis thaliana*,  
604 *Brachypodium distachyon*, *Eleusine coracana*, *Eragrostis curvula*, *Eragrostis nindensis* (DT),  
605 *Eragrostis pilosa*, *Eragrostis tef*, *Hordeum vulgare*, *Lindernia brevidens* (DT), *Lindernia*  
606 *subracemosa*, *Microchloa caffra* (DT), *Marchantia polymorpha* (DT), *Medicago truncatula*,  
607 *Oropetium capense* (DT), *Oryza sativa*, *Oropetium thomaeum* (DT), *Physcomitrium patens*  
608 (DT), *Sorghum bicolor*, *Setaria italica*, *Selaginella lepidophylla* (DT), *Solanum lycopersicum*,  
609 *Selaginella moellendorffii*, *Sporobolus pyramidalis*, *Sporobolus stapfianus* (DT), *Setaria viridis*,

610 *Triticum aestivum*, *Tripogon minimus* (DT), *Vitis vinifera*, *Xerophyta viscosa* (DT), *Zostera*  
611 *japonica*, *Zostera marina*, and *Zea mays*. Proteins were clustered into orthologous groups using  
612 Orthofinder (v2.2.6)<sup>76</sup> with default parameters. For the orthogroup enrichment analysis, we  
613 calculated a Z-score for each species within each orthogroup, compared it to a normal  
614 distribution to obtain a p-value, and then adjust these p-values using the Benjamini and  
615 Hochberg procedure to get q-values. We then searched for statistically enriched orthogroups  
616 across all of the sequenced desiccation tolerant grasses. Using this approach, we identified  
617 between 486 and 8,863 enriched orthogroups in the 33 species we included in our analysis, and  
618 found none that are conserved across all desiccation tolerant grasses outside of ELIPs.

619

#### 620 *ELIP gene family evolution*

621 To test the hypothesis that the ELIP gene family expansions are associated with the  
622 evolution of desiccation tolerance, we used CAFÉ (v 5.1)<sup>35</sup>, which analyzes changes in gene  
623 family size in a phylogenetic framework. The input tree was created from the amino acid  
624 sequences from 36 land plant species, with a focus on Chloridoid grasses (ELIPs count  
625 phylogeny figure; Supplemental Table 1). Sequences were first clustered using Orthofinder (v  
626 2.4.1)<sup>76</sup>, filtered to remove any orthogroups that did not contain all taxa, and aligned using  
627 MAFFT (v 7.305b)<sup>77</sup>. No single copy orthologs were found containing all taxa for species tree  
628 construction. Instead, we pruned gene trees and alignments to the largest subtree containing  
629 unique taxa using PhyloPyPruner (v 1.2.4) (<https://gitlab.com/fethalen/phylopypruner>); where  
630 paralogs were monophyletic within a species, we randomly pruned all but one sequence prior to  
631 extracting the largest subtree. The resulting pruned gene trees and alignments were further  
632 filtered to remove any trees no longer containing at least 19 taxa. This final set of 195  
633 alignments were concatenated and used to construct a phylogeny using IQ-TREE (v 2.3.0)<sup>78</sup>  
634 and time calibrated fast least-squares dating<sup>79</sup>.

635 ELIP gene family counts per haploid genome for non-focal taxa were done using  
636 BLASTP with the *Arabidopsis thaliana* (L.) Heynh. ELIP1 amino acid sequence as query for the  
637 remaining proteomes. We further investigated two other gene families with known roles in  
638 desiccation tolerance – heat shock proteins (HSPs) and late embryogenesis abundant proteins  
639 (LEAs) – along with 20 random selected orthogroups, to contextualize the tempo of ELIP  
640 evolution. These count data and the time calibrated phylogeny were used as input for CAFÉ  
641 under a single lambda model.

642

#### 643 *Transcriptomic analyses*

644 RNA sequencing reads were processed following a pipeline developed by the VanBuren  
645 Lab (<https://github.com/pardojer23/RNAseqV2>). Briefly, sequence read quality was assessed  
646 with fastQC (v 0.23) and reads were trimmed with trimmomatic (v 0.38)<sup>80</sup> to remove adapters  
647 and low quality bases. Trimmed reads were sudo-aligned to reference genomes using Salmon  
648 (v 1.9.0)<sup>36</sup>, and the resulting quantification files were processed with tximport (v 3.18)<sup>81</sup> and to  
649 generate normalized expression matrices of transcripts per million (TPM). A Principal  
650 Component Analysis (PCA) was used to visualize replicate and sample relationships within  
651 each species using the respective TPM expression values. A cross-species PCA was performed  
652 using the TPM matrix of conserved syntenic orthologs across all species. To effectively quantify  
653 gene expression while acknowledging the complexities introduced by polyploidy, we summed

654 the expression levels of all homeologs in *E. nindensis*, *E. tef*, and *M. caffra* to obtain a single  
655 gene expression value to enable interspecies comparisons. This approach is grounded in the  
656 logic that a unified expression value not only simplifies the analysis but also encapsulates  
657 potential functional diversifications among homeologs. This methodology has been applied and  
658 validated in our previous research<sup>17,29,82,83</sup>. We then computed the Z-score of the TPM for each  
659 syntenic ortholog in each sample and ran PCA on the matrix of Z-scores of each syntenic  
660 ortholog across all timepoints and species.

661

#### 662 *Differentially expressed genes*

663 Differentially expressed genes (DEGs) were identified independently for each species  
664 with DESeq2 R package (v 1.42.0)<sup>84</sup>. Briefly, transcript abundance estimates from Salmon were  
665 imported into DESeq2 using tximport to generate counts matrices. Count matrices were  
666 normalized, and hierarchical clustering was conducted for basic quality control and visualization  
667 of relationships across experimental timepoints and biological replicates. We tested multiple  
668 models for differential expression in DESeq2, including models that identified DEGs by pairwise  
669 comparisons of each timepoint against well-watered, and models that used the continuous  
670 variables of RWC or  $F_v/F_m$  as covariates. DEGs identified by pairwise comparisons were  
671 summarized into a nonredundant list of up and down regulated genes during dehydration and  
672 rehydration. DEGs identified using the continuous variables are based on a significant linear  
673 association (positive or negative) with RWC or  $F_v/F_m$ . When identifying DEGs, we included the  
674 term “process” in our model to differentiate between dehydration and rehydration processes. To  
675 select the best performing model, we quantified similarities and differences in the number and  
676 identity of DEGs defined by each model. There was a high degree of overlap in genes identified  
677 by all three models. Ultimately, we selected the model based on RWC because it performed  
678 well and is easily comparable across experiments regardless of sampling time, consistency  
679 across replicates, or differences in experimental design. These analyses produced species-  
680 specific lists of DEGs during dehydration and rehydration with significant (FDR adjusted P-value  
681 <0.05) associations with RWC. Log2foldchange values are calculated for one unit change in  
682 RWC.

683 To gain insight into possible similarities and differences among the study species, we  
684 looked at the overlap in DE syntenic orthologs. To do so, we used venn diagrams to identify the  
685 shared syntelogs in up- and down-regulated genes during both dehydration and rehydration  
686 across the three study species. We then compared the observed proportion of overlapping  
687 DEGs in each category to the proportion of genes expected to overlap by chance (assuming  
688 independent draws), and tested if these were significantly different using Fisher’s exact test.  
689 This analysis was then extended to include DEGs identified in the desiccation sensitive sister  
690 species *E. tef* to distinguish between typical drought vs. pure desiccation responses. We then  
691 conducted targeted analyses to look at the functional roles of DE syntelogs that were uniquely  
692 shared across the three resurrection species vs. those that were common with *E. tef*. We also  
693 investigated the functional signatures of differentially abundant transcripts that were unique to  
694 each species. To do so we pulled the lists of DE syntelogs that were only found in one of the  
695 focal species.

696

#### 697 *Functional annotation of DEGs*

698 We annotated DE syntelogs with KEGG and GO terms to describe generalized  
699 metabolic and cellular processes responses shared across in the three study species. KEGG  
700 annotations were generated for each species using BLASTKoala  
701 (<https://www.kegg.jp/blastkoala/>) on the complete set of annotated peptide sequences. These  
702 KEGG terms were then assigned to syntenic orthologs and DE KEGG terms that were shared  
703 across all three species during dehydration and rehydration were identified and plotted using  
704 venn diagrams. These shared DE KEGG terms were used to generate metabolic pathway maps  
705 using the reconstruct function of the KEGGmapper tool  
706 (<https://www.genome.jp/kegg/mapper/color.html>) for up- and down-regulated terms in  
707 dehydration and rehydration. This returned a list of syntelogs per metabolic pathway and Brite  
708 descriptions. The difference between the number of syntelogs assigned to each pathway for up  
709 and down regulated genes was computed. The list was then sorted to determine pathways that  
710 were primarily up or down regulated. Next, the assigned KO numbers were paired with  
711 quantitative data on gene expression to identify which pathways were active at various  
712 timepoints. To summarize patterns, genetic information processes (transcription, translation,  
713 folding, sorting, and degradation, replication and repair, and processing in viruses) were  
714 grouped together. Similarly Environmental information processing (membrane transport and  
715 signal transduction) and cellular processes (transport and catabolism, cell growth and death,  
716 cellular community- prokaryote and eukaryotes, and cell motility) were grouped together.  
717 Pathways assigned to Organismal systems and Human disease were ignored. KEGG  
718 annotation is not without its limits as single KEGG identifiers can be present in multiple  
719 pathways.

720 GO terms were assigned through homology with the well annotated genome of sister  
721 species *O. thomaeum*<sup>26</sup>. This was done through a BLASTP (v 2.14.0)<sup>85</sup> search of all *O.*  
722 *thomaeum* protein sequences against the protein sequences of each study species. Parameters  
723 were set to return the single best match for each peptide and an e-value cutoff of 1e-10. We  
724 assigned the GO terms from *O. thomaeum* to the homologous genes in our target species. We  
725 then used TopGO R package (v 2.54.0) to identify significantly enriched GO terms (P-  
726 value<0.05) within sets of DEGs for up- and down-regulated genes during dehydration and  
727 rehydration in each target species and for the different sets of overlapping and unique syntelogs  
728 identified via cross-species comparisons.

### 729 *Co-expression analyses*

731 To complement the above analyses, we generated co-expression networks using  
732 Weighted Gene Co-expression Network Analysis (WGCNA) R package (v1.7)<sup>86</sup>. While DE  
733 analyses can be informative to identify and describe overarching patterns and large shifts in the  
734 data, more nuanced patterns of gene expression can be obscured. To investigate the more  
735 subtle temporal changes in gene expression, we used co-expression analyses to identify  
736 modules of co-expressed genes for each species.

737 For each species, we created a signed co-expression network using WGCNA. Each  
738 dataset was filtered to remove genes with no expression. To construct a weighted co-  
739 expression network, we determined a soft thresholding power for each dataset. This power was  
740 chosen to satisfy WGCNA's assumption that a weighted co-expression network is scale-free. An  
741 adjacency matrix, representing the strength of connections between genes in the network, was



742 constructed for each network using the soft thresholding power. For module detection, this  
743 matrix was then converted to a topological overlap matrix (TOM) and hierarchal clustering was  
744 used on the TOM to group genes into modules based on similar expression patterns.  
745 Additionally, we calculated connectivity of each gene within its network and its assigned module  
746 using WGCNA's network analysis functions.

747 We identified shared and species-specific co-expressed genes using UpSet plots<sup>87</sup>. For  
748 all co-expressed genes, we identified the syntenic orthologs and computed the overlap across  
749 species. We combined all modules for a species that showed increased expression during  
750 dehydration, during rehydration, and under non-stressed conditions. We identified the sets of  
751 shared syntelogs as well as those that were only found in a single species. We then ran GO  
752 enrichment analysis of these sets of shared and unique co-expressed genes.

### 753 754 *Topological data analysis*

755 We employed a topological data analysis (TDA) approach following the pipeline  
756 described at <https://github.com/PlantsAndPython/plant-evo-mapper> to discern the underlying  
757 structure of the expression datasets. We utilized the Mapper algorithm, which condenses the  
758 dataset into a scalable, navigable representation. The Mapper algorithm is particularly well-  
759 suited for genome scale analyses, as the underlying datasets are often characterized by high  
760 dimensionality and sparsity. For our gene expression data, we constructed Mapper graphs  
761 using a "stress lens" formulated by applying a linear model using the well-watered condition as  
762 a reference point. This model represents the baseline for leaf expression and we quantified the  
763 residuals or deviation of each sample from the baseline, which represents the degree of water  
764 stress or recovery. We generated three different mapper graphs, one was constructed using the  
765 syntelog expression matrix from just the three focal resurrection grasses; *M. caffra*, *O. capense*,  
766 and *T. minimus*. The second mapper graph was constructed using the syntelog expression  
767 matrix that included two additional resurrection grasses (*E. nindensis* and *O. thomaeum*) and  
768 the third graph included the desiccation sensitive species *E. tef*. For the mapper graph, we  
769 specified different intervals and overlap for the 3 species comparisons and the 5 species  
770 comparisons. For the three species, we specified 110 intervals with a 90% overlap and for the 5  
771 species comparison we specified 120 intervals with 95% overlap.

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781  
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783 RAM, LVP, JS, ISG, and RV conducted data analyses and contributed to data interpretation and  
784 conceptual framing of the manuscript. RAM and RV drew the figures. RAM, LVP, and RV wrote  
785 the manuscript. All authors edited and reviewed the manuscript.

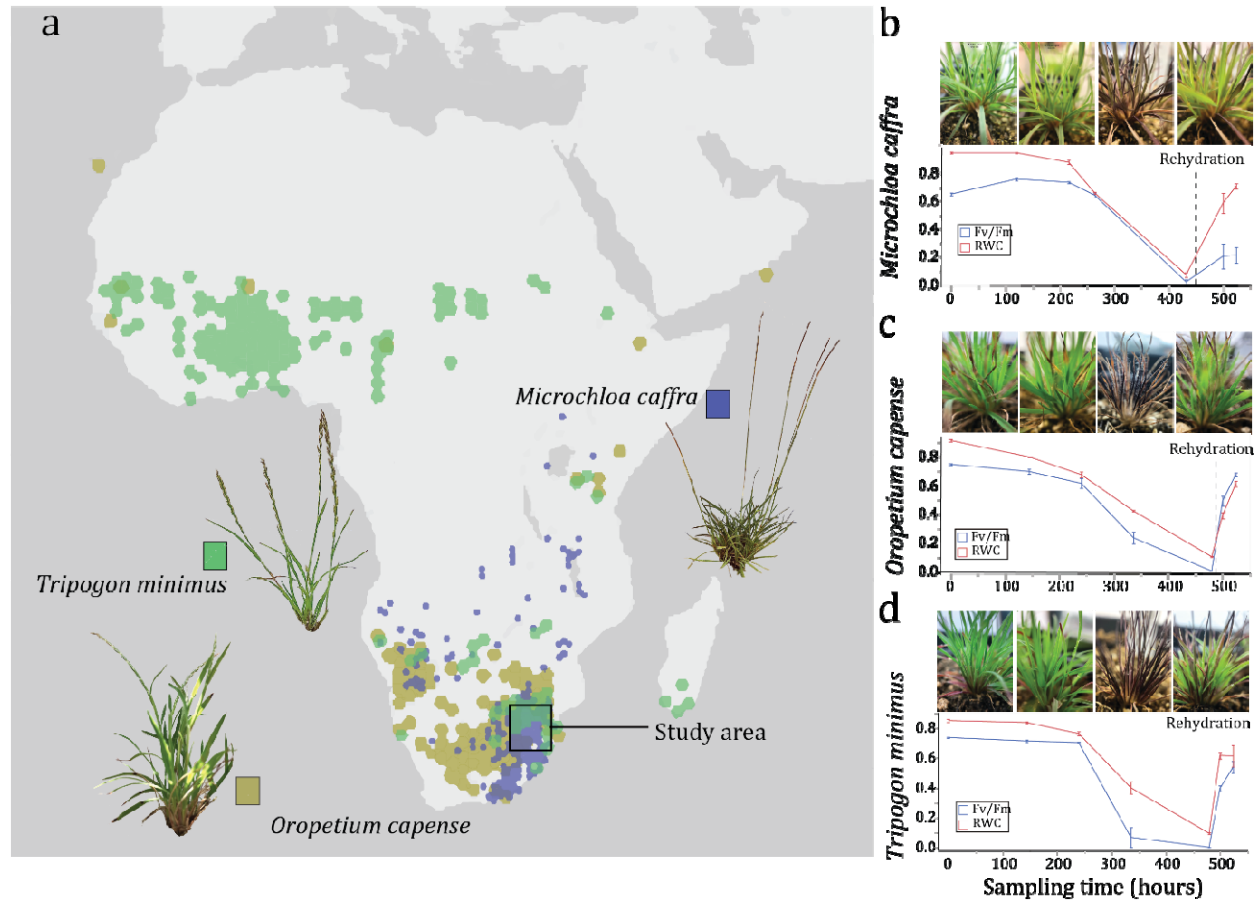


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787 **Data availability:** Sequence data associated with this study are deposited at NCBI under  
788 BioProject PRJNA1044305 and BioSamples SAMN38380430-92. Genome assemblies are  
789 hosted on CoGe (<https://genomeevolution.org/>) under the following IDs: 65089 (*T. minimus*),  
790 65046 (*O. capense*), and 64494 (*M. caffra*). Metadata and other data summaries associated  
791 with this study are provided at Dryad <https://doi.org/10.5061/dryad.kh18932c4>.

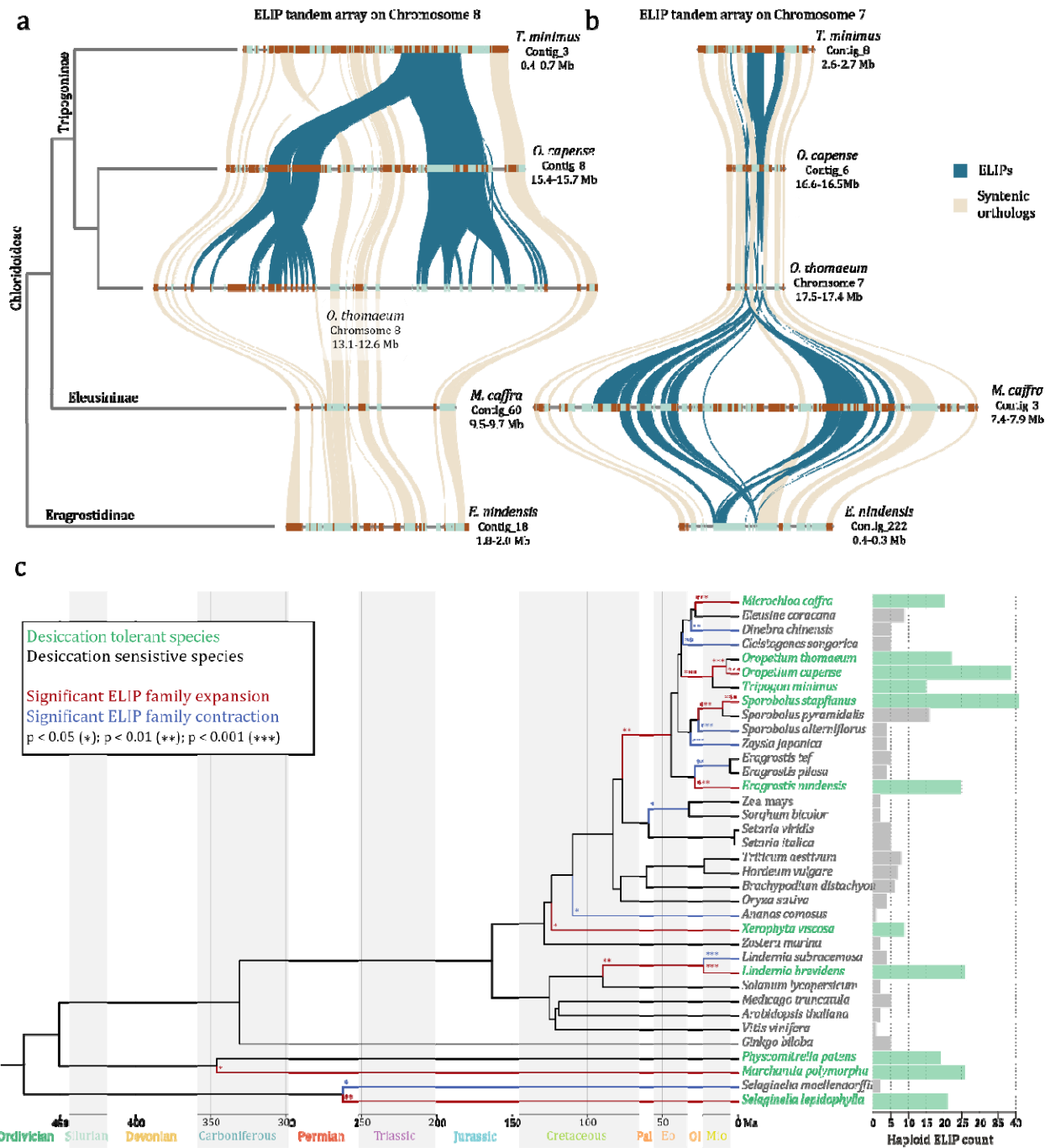
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796 **Figure 1. Overview of species distribution and experimental design to test for convergent evolution in**  
797 **grasses.** (a) Estimated distribution of the three desiccation tolerant grasses *Microchloa caffra*, *Oropetium capense*,  
798 and *Tripogon minimus*. Distribution data were taken from GBIF.org (21 November 2023) GBIF Occurrence Download  
799 <https://doi.org/10.15468/dl.5jf47y>. Collections for the current study were made in Mpumalanga and Limpopo  
800 provinces of South Africa. Relative water content and  $F_v/F_m$  of plants during dehydration and rehydration timecourses  
801 for (b) *M. caffra*, (c) *O. capense*, and (d) *T. minimus*. Three biological replicates were sampled at each timepoint for  
802 each species. Error bars represent standard error of the mean.  
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**Figure 2. Independent tandem gene duplication of ELIPs in different resurrection grass lineages.**

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Microsyntenic regions of the Chromosome 8 (a) and Chromosome 7 (b) ELIP tandem arrays is shown for resurrection

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grasses in the Tropogoninae (*T. minimus*, *O. thomaeum*, and *O. capense*), Eleusininae (*M. caffra*), and

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Eragrostidinae (*E. nindensis*) subtribes of Chloridoideae. Syntenic orthologs between the species are shown in beige

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and the ELIPs are highlighted in blue. Only a single syntenic region for autopolyploids *M. caffra* (hexaploid) and *E.*

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*nindensis* (tetraploid) is shown for simplicity, but each of the other haplotypes contain the same gene content in these

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regions. (c) Evolutionary dynamics showing significant changes in the rates of gene family expansion (red) and

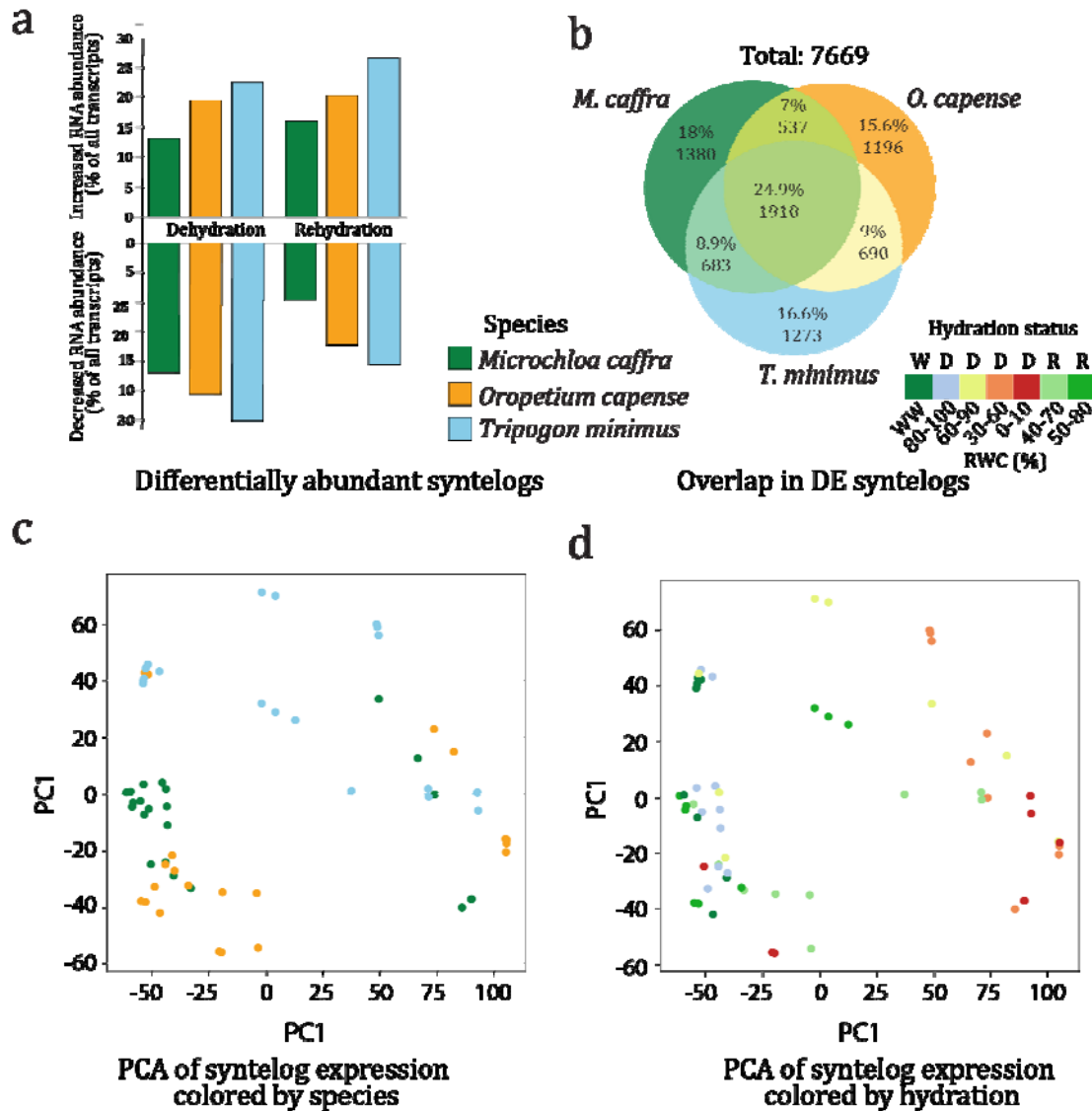
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contraction (blue) of ELIPs inferred by CAFE. The haploid normalized number of ELIPs are plotted for desiccation

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tolerant (green) and sensitive (gray) species.

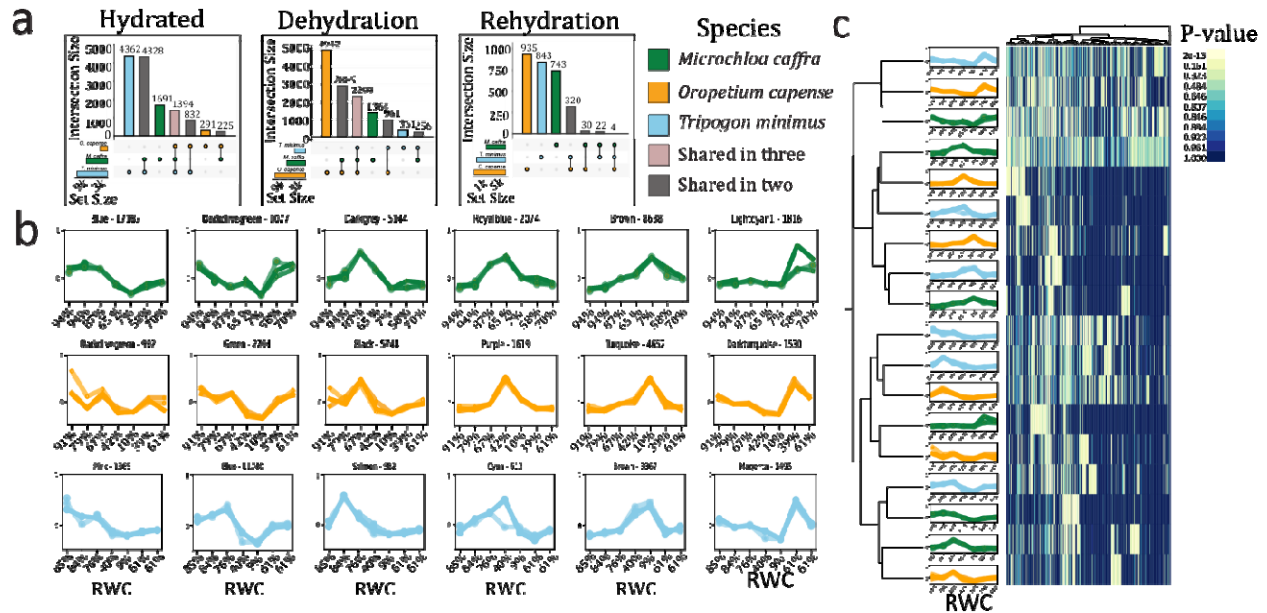
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**Figure 3. Overlapping expression dynamics of conserved genes across species.** (a) Barplot showing the percentage of DEGs in each species for up- and down-regulated genes in dehydration and rehydration conditions. (b) Venn diagram showing the number of syntenic orthologs that increased in abundance during dehydration and overlap across species. The percentage and number of genes in each set are shown. (c,d) Principal component analysis of z-score transformed expression values for conserved syntelogs across all three species. Samples are colored by hydration status in (c) and by species identity in (d).

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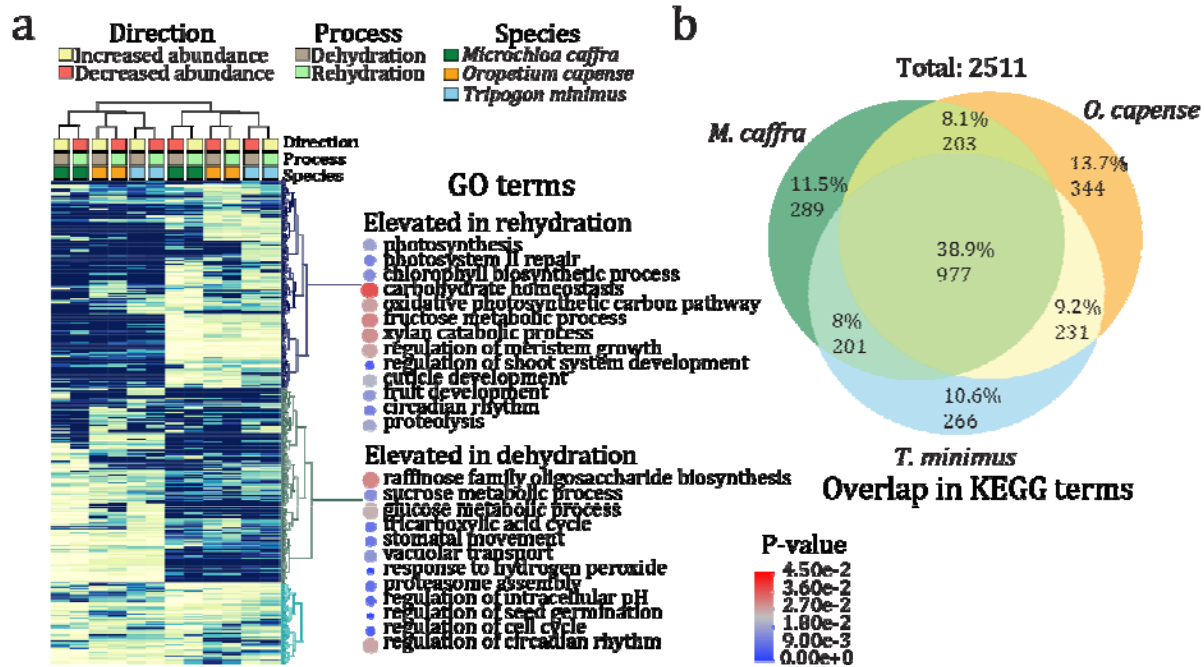
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**Figure 4. Comparative co-expression network dynamics across resurrection grasses.** (a) UpSet plots showing the number of shared and unique syntenic orthologs among co-expression modules characterized by elevated expression in hydrated, dehydrated, or rehydrated conditions. (b) Co-expression modules for each species. The X-axis shows the approximate relative water content (RWC) of samples at each timepoint. The module name and total number of genes in the module are listed above. (c) Hierarchical clustering of enriched GO terms for each co-expression module. Secondary clustering performed on modules shows that modules are organized by expression profile rather than species.



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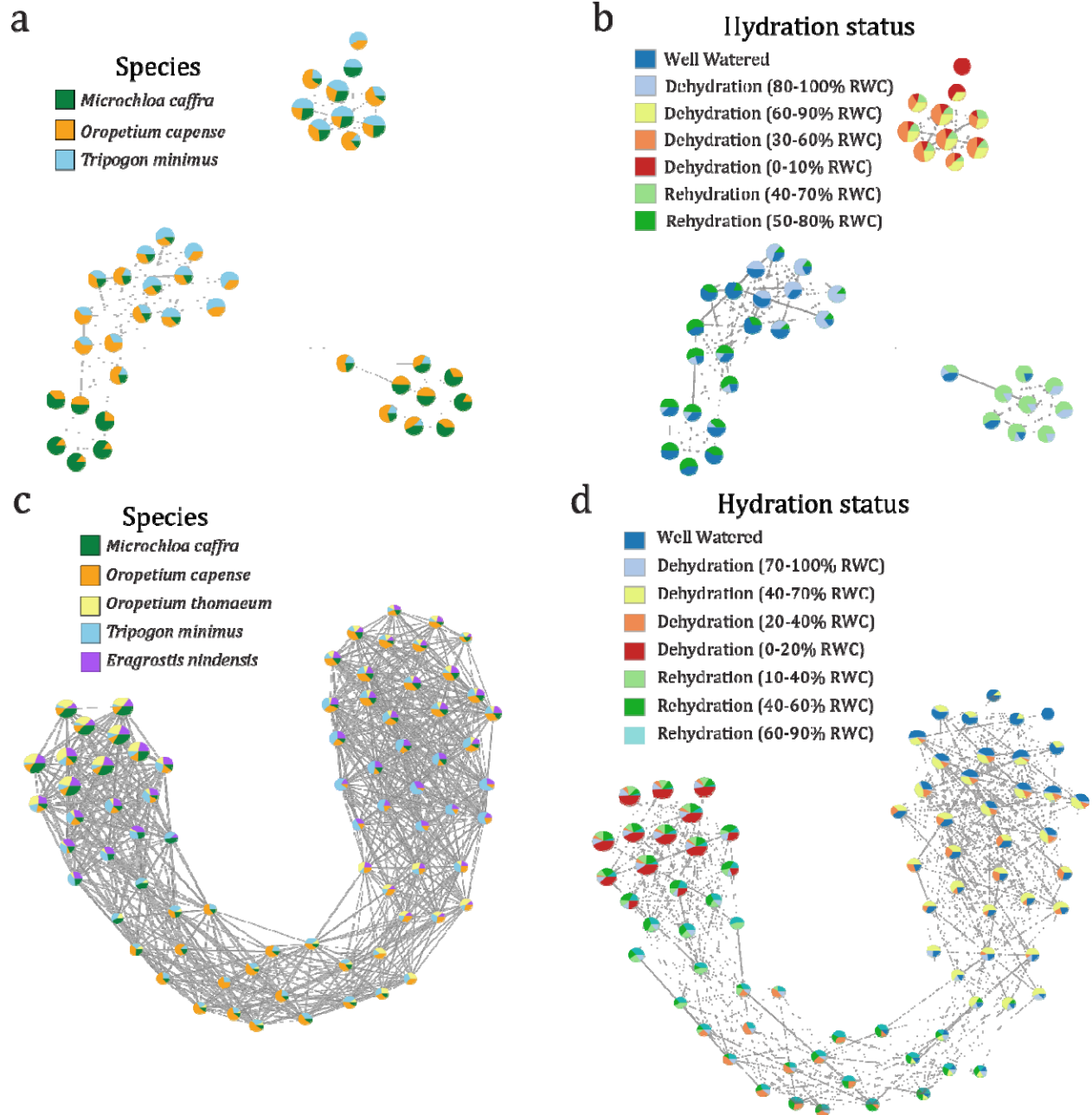
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**Figure 5. Overlapping gene functions during desiccation and rehydration in resurrection grasses.** (a) Hierarchical clustering of p-values for enriched GO terms for each species and condition. Selected GO terms are highlighted for genes that increased in abundance during dehydration (decreased in abundance during rehydration) and genes that increased in abundance during rehydration (decreased in abundance during dehydration). Points are colored by the average enrichment p-value across all species and sized by the number of genes assigned to that GO term. (b) Venn diagram showing the number of overlapping KEGG terms that increased in abundance during dehydration. The percentage and number of KEGG terms in each set are shown.



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846 **Figure 6. Mapper graphs showing the topological shape of desiccation induced gene expression across**  
847 **species.** Nodes within the graph represent clusters of RNAseq samples that are akin to one another, with the node  
848 color indicating the identity of the samples contained within. Edges, or the connections between nodes, delineate  
849 shared samples across intersecting clusters. Mapper graphs for the three species comparisons are shown in (a) and  
850 (b), and Mapper graphs for the five species comparisons are shown in (c) and (d). Nodes within the graph are colored  
851 by species (a and c) or hydration status (b and d).

852 **Table 1. Assembly stats of the three resurrection grasses**

<b>Assembly stats</b>	<b><i>O. capense</i></b>	<b><i>T. minimus</i></b>	<b><i>M. caffra</i></b>
<b>Ploidy</b>	diploid	diploid	hexaploid
<b>Total assembly size (Mb)</b>	237	223	968
<b>Number of contigs</b>	14	57	118
<b>Contig N50</b>	27,924,228	19,548,099	16,141,787
<b>Contig L50</b>	4	5	22
<b>Number of genes</b>	28,826	26,527	85,245
<b>Complete BUSCO (%)</b>	97.1	95.3	96.2
<b>LTR elements (% of genome):</b>	27.6	22.4	27.6
<b>Ty1/Copia (%)</b>	4.0	5.2	5.5
<b>Gypsy/DIRS1 (%)</b>	21.4	13.2	15.1
<b>DNA transposons (%)</b>	12.2	15.9	27.6
<b>Total repeats (%)</b>	41.7	39.4	56.1

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