Comparative transcriptomics reveals lineage specific evolution of cold response in Pooideae

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Abstract

Background: Understanding how complex traits evolve through adaptive changes in gene regulation remains a major challenge in evolutionary biology. Over the last ~50 million years, Earth has experienced climate cooling and ancestrally tropical plants have adapted to expanding temperate environments. The grass subfamily Pooideae dominates the grass flora of the temperate regions, but the role of cold-response gene regulation in the transitioning from tropical to temperate climate remains unexplored.

Results: To establish if molecular responses to cold are conserved throughout the phylogeny, we assembled the transcriptomes of five Pooideae species spanning early to later diverging lineages, and compared short- and long-term cold responsive genes using 8633 high confidence ortholog groups with resolved gene tree topologies. We found that a majority of cold responsive genes were specific to one or two lineages, an observation that we deem incompatible with a cold adapted Pooideae ancestor. However, all five species shared short-term cold response in a small set of general stress genes as well as the ability to down-regulate the photosynthetic machinery during cold temperatures.

Conclusions: Our observations indicate that the different Pooideae lineages have assembled cold response programs in parallel by taking advantage of a common potential for cold adaptation.

Key words: regulatory evolution, cold response, comparative transcriptomics, Pooideae, adaptation

Background

Adaptation to a changing climate is essential for long term evolutionary success of plant lineages. During the last ~50 million years of climate cooling (Fig. 1c), several plant species adapted to temperate regions. A key step in this transitioning was the integration of novel temperate climate cues, such as seasonal fluctuations in temperature, in the regulatory network controlling cold stress responses. Here we used the temperate grass subfamily Pooideae as a model system for studying gene regulatory evolution of cold stress.

The temperate grass flora is dominated by members of the subfamily Pooideae [1], and the most extreme cold environments are inhabited by Pooideae species. The ancestors of this group were, however, most likely adapted to tropical or subtropical climates [2, 3]. Many Pooideae species experience cold winters (Fig. 1b) and although a recent study inferred adaptation to cooler environments at the base of the Pooideae phylogeny [4], it is still not known whether the Pooideae's most recent common ancestor (MRCA) already was adapted to cold stress, or if adaptation to cold evolved independently in the Pooideae lineages.

Pooideae is a large subfamily comprising 4200 species [5], amongst them economically important species such as wheat and barley. Given the commercial importance of this group, various aspects of adaptation to temperate climate such as flowering time, cold acclimation, and frost and chilling tolerance have been studied (reviewed by [6–13]). These studies are, however, confined to a handful of species in the species rich, monophyletic clade "core Pooideae" [14] and recently also to its sister clade, containing the model grass *Brachypodium distachyon* [15–17]. It is thus unknown how adaptation to temperate climate evolved in earlier diverging Pooideae lineages to promote the success of this subfamily in temperate regions.

Environmental stress is assumed to be a strong evolutionary force, and the colonization of temperate biomes by Pooideae was likely accompanied by adaptation to cold conditions. A MRCA already adapted to cold (the ancestral hypothesis) offers a plausible basis for the ecological success of the Pooideae subfamily in the northern temperate regions [1]. However, paleoclimatic reconstructions infer a generally warm climate, and a very limited abundance of temperate environments, during the time of Pooideae emergence, around 50 million years ago (Mya) [18–22]. Indeed, it was not before ca. 33.5 Mya, during the Eocene-Oligocene (E-O)

66 transition, that the global climates suddenly began to cool [23, 24] (Fig. 1c). Climate cooling at
67 the E-O transition coincided with the emergence of many temperate plant lineages [25] and may
68 have been an important selection pressure for improved cold tolerance in Pooideae [26, 27]. If
69 the E-O cooling event has been the major evolutionary driving force for cold adaptation in
70 Pooideae grasses, those findings lend support for lineage specific evolution of cold adaptation
71 (the lineage specific hypothesis), as all major Pooideae lineages had already emerged by the time
72 of the E-O transition [2, 28] (Fig. 1a).

73 A restricted number of plant lineages successfully transitioned into the temperate region,
74 emphasizing the difficulties in evolving the coordinated set of physiological changes needed to
75 withstand low temperatures [29]. During prolonged freezing, plants need to maintain the
76 integrity of cell membranes to avoid osmotic stress [30]. Cold and freezing tolerance is
77 associated with the ability to cold acclimate, which is achieved through a period of extended,
78 non-freezing cold triggered by the gradually lower temperature and day-length in the autumn.
79 During cold acclimation, a suite of physiological changes governed by diverse molecular
80 pathways results in an increase in the sugar content of cells, change in lipid composition of
81 membranes and synthesis of anti-freeze proteins [13, 31]. Also, low non-freezing temperatures
82 may affect plant cells by decreasing metabolic turnover rates, inhibiting the photosynthetic
83 machinery and decreasing stability of biomolecules (e.g. lipid membranes) [10, 12]. Several
84 studies have used transcriptomics to compare cold stress response, however, they focused on
85 closely related taxa or varieties within model species [17, 32–36]. As such, these studies were
86 not able to investigate evolutionary mechanisms underlying adaptation to cold climates of entire
87 clades.

88 Here, we used *de novo* comparative transcriptomics across the Pooideae phylogeny to study the
89 evolution of cold adaptation in Pooideae. Specifically, we aim to establish if molecular responses
90 to cold are conserved in the Pooideae subfamily or if they are the result of lineage specific
91 evolution. The transcriptomes of three non-model species (*Nardus stricta*, *Stipa lagascae* and
92 *Melica nutans*), which belong to early diverging lineages, were compared to the transcriptomes
93 of the model grass *Brachypodium distachyon* and the core Pooideae species *Hordeum vulgare*
94 (barley). 8633 high confidence ortholog groups with resolved gene tree topologies were used to
95 identify cold-response genes. We found that only a small number of genes were cold responsive

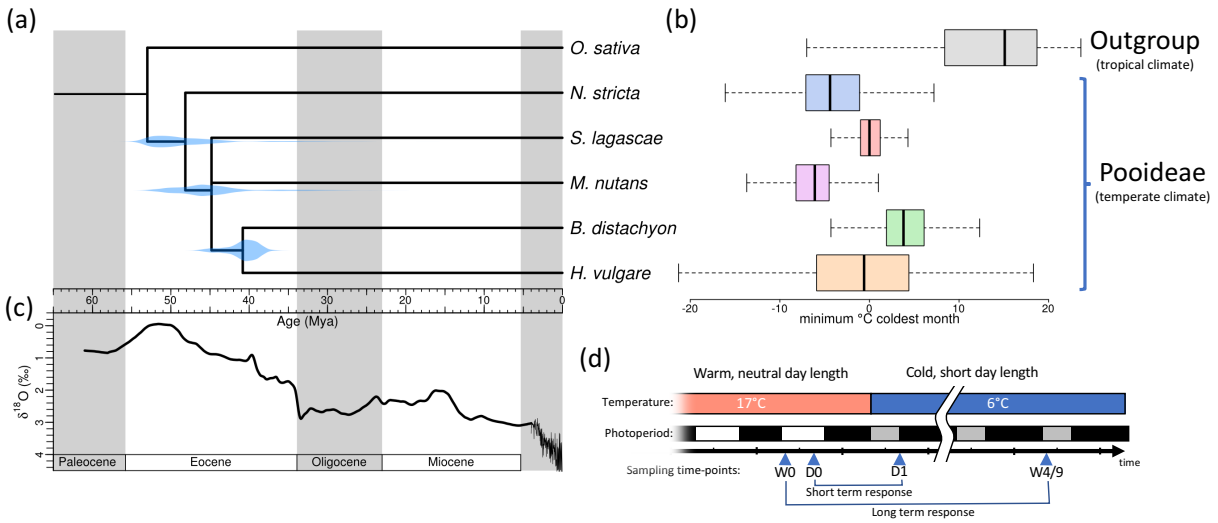

Figure 1. The Pooideae phylogeny, present and historic temperature data and the experimental design of this study. (a) *Dated phylogenetic tree of the five Pooideae investigated in this study with O. sativa as an out-species. The species phylogeny was inferred from gene trees, with the distribution of mean gene-tree node ages shown in blue. (b) The range of the minimum temperature of the coldest month (WorldClim v1.4 dataset, Bioclim variable 6, 2.5 km^2 resolution [95]) at the species geographical distribution (source: www.gbif.org). (c) Oxygen isotope ratios as a proxy for historic global temperature [18, 22] (d) Experimental design. Plants from five species of Pooideae were subjected to a drop in temperature and shorter days to induce cold response. Leaf material was sampled on the day before the onset of cold (W0 and D0), once 8 hours after cold (D1) and two times after 4 and 9 weeks (W4/W9). Short-term response was identified by contrasting gene expression in time points D0 and D1, while long-term response was identified contrasting W0 and W4/W9.*

in all the investigated Pooideae species, which suggested that their common ancestor only possessed few and possibly key, preliminary cold response mechanisms, and that evolution of cold responses evolved primarily independently in different Pooideae lineages.

Results

To investigate the evolution of cold response in Pooideae, we sampled leaf material in five species before and after subjecting them to a drop in temperature and shorter days (Fig. 1d). RNA-sequencing (RNA-Seq) was used to reveal the short and long term cold response of transcripts, and the conservation of these responses was analyzed in the context of ortholog groups.

De novo transcriptome assembly identified 8633 high confidence ortholog groups

The transcriptome of each species was assembled *de novo* resulting in 146k-282k contigs, of which 68k-118k were identified as containing coding sequences (CDS, Table S1). Ortholog groups (OGs) were inferred by using the protein sequences from the five *de novo* assemblies, as well as the reference genomes of *L. perenne*, *H. vulgare*, *B. distachyon, Oryza sativa*, *Sorghum bicolor* and *Zea mays*. The five assembled Pooideae species were represented with at least one transcript in 24k-33k OGs (Table S1).

Gene trees were generated for each OG and a set of high confidence OGs (HCOGs) was identified by filtering based on the topology of the gene trees (see Methods). This resulted in 8633 high confidence ortholog groups (HCOGs) containing transcripts from at least three of the five studied species (Table S1, Table S2).

De novo assembly followed by ortholog detection resulted in higher numbers of monophyletic species-specific paralogs than the number of paralogs in the reference genomes of *H. vulgare* and *B. distachyon*. This apparent overestimation of paralogs was almost certainly the result of the *de novo* procedure assembling alleles or alternative transcript isoforms into separate contigs. We also observed some cases where the number of paralogs were under-estimated compared to the references, which may be due to low expression of these paralogs or the assembler collapsing paralogs into single contigs. Since the *de novo* assembly procedure did not reliably assemble paralogs, we chose to represent each species in each HCOG by a single read-count value equal to the sum of the expression of all assembled paralogs. By additionally setting counts for missing orthologs to zero, we created a single cross species expression matrix with HCOGs as rows and samples as columns (Table S3).

127 *A dated species tree of the Pooideae*

128 Dated gene trees were generated using prior knowledge about the divergence times of *Oryza*-
129 Pooideae [37] and *Brachypodium-Hordeum* [28]. Based on 3914 gene trees with exactly one
130 sequence from each of the five Pooideae and rice (see Methods), a dated species tree was
131 estimated using the mean divergence times of the gene trees (Fig. 1a). In the most common gene
132 tree topology, *S. lagascae* or *M. nutans* formed a monophyletic clade, but topologies where
133 either *S. lagascae* or *M. nutans* diverged first were also common (Fig. S1). Due to this
134 uncertainty regarding the topology, *S. lagascae* and *M. nutans* branches were collapsed to a
135 polytomy in the consensus species tree.

136 *Expression clustering indicated a common global response to cold*

137 To investigate broad scale expression patterns in cold response, we clustered all samples
138 (including replicates) after scaling the expression values of each gene to remove differences in
139 absolute expression between species (see Methods, Fig. 2a). This clustering revealed the
140 differential effects of the treatments and resulted in a tree with replicates, and then time points,
141 clustering together. An exception was time points W4 and W9, which tended to cluster together
142 and by species, indicating that responses after 4 and 9 weeks were very similar. The fact that
143 time points mostly clustered together before species indicated a common response to cold across
144 species. We also observed a clear effect of the diurnal rhythm, with time points sampled in the
145 morning (W0, W4 and W9) forming one cluster and time points sampled in the afternoon (D0
146 and D1) forming another.

147 *Cold responsive genes were primarily species specific*

148 We next examined similarities in short and long term cold response between species by
149 analysing changes in gene expression from before cold treatment to eight hours and 4-9 weeks
150 after cold treatment (Fig. 1d). For all species pairs, there was a low, but statistically significant,
151 correlation between the expression fold changes of orthologs in HCOGs (Fig. 2b). A similar
152 pattern was observed when investigating the number of orthologs classified as differentially
153 expressed in pairs of species (FDR adjusted p-value < 0.05 and fold change > 2, Table S4, see
154 Methods): these numbers were low compared to the number of differentially expressed genes
155 (DEGs) in individual species, but higher than expected by chance (Fisher´s exact test $p < 0.05$,

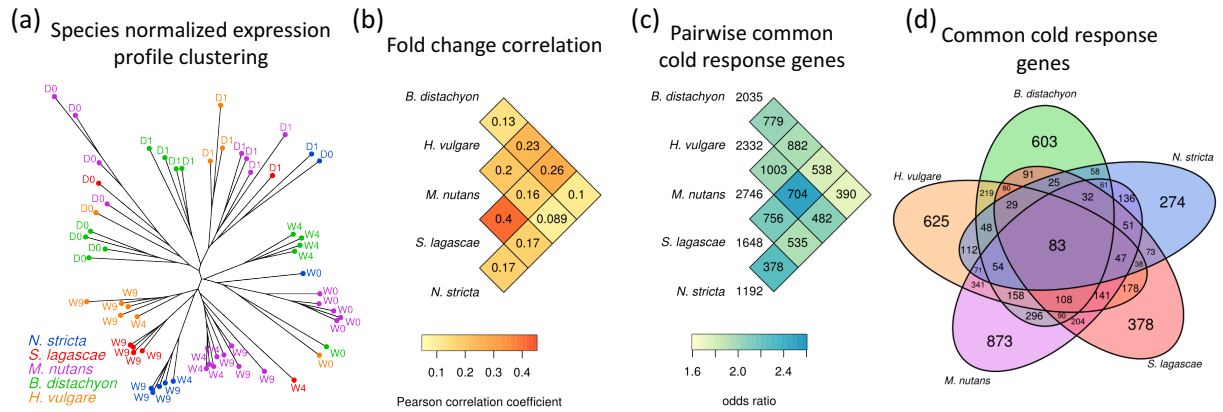

Figure 2. Comparison of cold response across the Pooideae. *(a) Expression clustering of the samples. The tree was generated by neighbor-joining of Manhattan distances given as the sum of log fold changes between all highly expressed genes after subtracting the mean expression per species. Each tip corresponded to one sample. (b) The Pearson correlations of log fold expression changes (only short-term cold response is shown) between pairs of species. The correlations were computed based on the high confidence ortholog groups (HCOG). (c) The number of differentially expressed genes per species and shared between pairs of species. The statistical significance of the overlaps between pairs of species were indicated with odds ratios. (d) The number of differentially expressed genes in each species (FDR adjusted p-value < 0.05 and absolute fold change > 2 in either short- or long-term cold response) and overlap between species.*

Fig. 2c). Finally, the number of orthologs with differential expression in more than two species were very low (Fig. 2d), with only 83 DEGs common to all five species. Taken together, these observations suggest that cold response in Pooideae is primarily lineage specific, with low but significant similarities between pairs of species both with respect to fold change and differential expression. Noticeably, neither the similarities in differential expression nor the fold change correlations reflected the phylogenetic relationship between the species, that is, the cold responses of related species were not more similar than that of distantly related species.

Shared cold response genes included known abiotic stress genes

Sixteen genes shared the same cold response (short- or long-term) in the same direction (up or down) in all five Pooideae species, thus representing a response to cold that might have been

Table 1. High confidence ortholog groups with conserved cold response in all five Pooideae. *S = short-term response, L = long-term response. ↗ = up regulated, ↘ = down regulated. Annotations were inferred from literature using orthologs. These 16 genes were the subset of the 83 genes in Fig. 2d with the same type of cold response (short- or long-term) in the same direction (up- or down-regulation) in all five species.*

Bd ortholog	Description and relation to stress response	Response
Bradi2g39230	**HyperOSmolality-gated CA2+ permeable channel (OSCA)** - Stress-activated calcium channels [65] that are highly conserved in eukaryotes [66]. In *Oryza sativa*, OSCA genes are differentially expressed in response to osmotic stress [67].	S↗
Bradi2g06830	**Calcium-binding EF-hand containing calcium exchange channel (EF-CAX)** - Calcium ions are important mediators of abiotic stress in plants [68, 69]. Expression of calcium binding proteins correlates with exposure to cold stress in several plants, e.g. *Arabidopsis thaliana* [30], *Musa × paradisiaca* [70] and *Hordeum vulgare* [38].	S↗
Bradi2g05226	**GIGANTEA** - Promotes flower development in plants [71]. In *Arabidopsis thaliana*, this gene is involved in CBF-independent freezing tolerance [72, 73], and is responsive to cold in *Zea mays* [74]. Also part of the circadian clock.	S↗
Bradi4g24967	**Arabidopsis Pseudo-Response Regulator 3-like (AtPRR3-like)** - AtPRR3 is a member of the circadian clock quintet AtPRR1/TOC1 [75, 76]. No association to stress response found in literature. However, AtPRR3-like might be closer related to AtPRR5/9 than to AtPRR3 (See Bradi4g36077, PRR95).	S↗
Bradi2g09060	**Triacylglycerol lipase, alpha/beta-Hydrolase superfamily** - Studies in *Arabidopsis thaliana* [77] and *Ipomoea batatas* [78] suggest that genes with alpha/beta-Hydrolase domains respond to osmotic stress. In *Triticum monococcum*, atriacylglycerol lipase was induced by pathogen stress [79].	S↗
Bradi2g07480	**Late-Embryogenesis-Abundant protein 14 (LEA-14)** - Responsive to drought, salt and cold stress in *Arabidopsis thaliana* [80, 81], Betula pubescens [82] and *Brachypodium distachyon* [83].	S↗
Bradi1g04150	**SNAC1-like / NAC transcription factor 67** - NAC transcription factors mediate abiotic stress responses. Osmotic stress increases the expression of SNAC1 in *Oryza sativa* [84], NAC68 in *Musa × paradisiaca* [70, 85] and NAC67 *Triticum aestivum* [86].	S↗
Bradi4g36077	**Pseudo-Response Regulator 95 (PRR95)** - Homologous to conserved circadian clock gene AtPRR5/9 [87, 88]. AtPRR5 gene is cold regulated in Arabidopsis thaliana [89] and PRR95 is cold responsive in Zea mays [74].	S↗
Bradi2g43040	**DnaJ chaperon protein** - DnaJ co-chaperons are vital in stress response and has been found to be involved in maintenance of photosystem II under chilling stress and enhances drought tolerance in tomato [90, 91].	S+L ↗
Bradi3g33080	**Glycogenin GlucuronosylTransferase (GGT)** - GGT belongs to the GT8 protein family [92]. In *Oryza sativa*, OsGGT transcripts are induced in submerged plants and respond to various abiotic stresses except cold [93, 94].	L↗
Bradi1g04500	**Major facilitator superfamily transporter** - Association to stress response unknown.	L↗
Bradi3g14080	**Glycosyl transferase** - Association to stress response unknown.	L↗
Bradi1g35357	**Uncharacterized membrane protein** - Association to stress response unknown.	S↗
Bradi2g48850	**Uncharacterized protein** - Association to stress response unknown.	S↗
Bradi1g33690	**Uncharacterized protein** - Association to stress response unknown.	S↗
Bradi1g07120	**Putative S-adenosyl-L-methionine-dependent methyltransferase** - Association to stress response unknown.	L↘

166 conserved throughout the evolution of Pooideae (Table 1). Several of these shared cold
167 responsive genes belonged to families known to be involved in cold stress or other abiotic stress
168 responses in other plant species. The most common type of response was short-term up

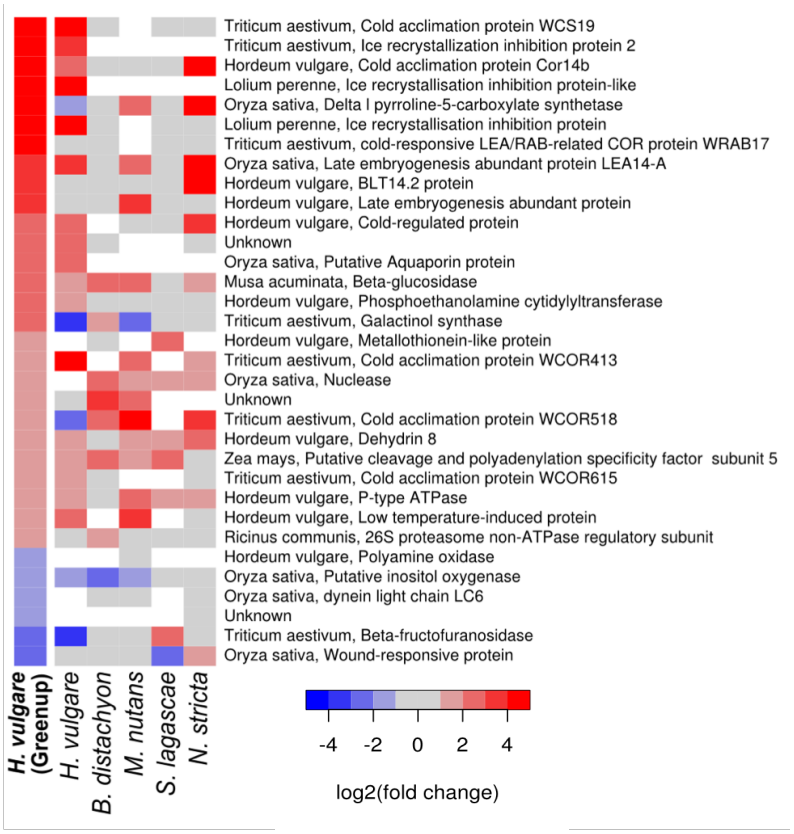

***Figure 3. Comparison of cold response to previous studies**. A reference set of H. vulgare genes independently shown to respond to cold in several studies [38] is compared to our data using short-term log fold change values. White cells represent missing orthologs. Grey cells represent orthologs that were not differentially expressed (not DEGs).*

169 regulation, indicating that stress response, as opposed to long-term acclimation response, is
170 potentially more conserved.

171 *Identified cold response genes confirmed previous findings*

172 We compared the cold response genes from our data to a compilation of *H. vulgare* genes shown
173 to be responsive to low temperature in several previous microarray studies, subsequently referred
174 to as the Greenup genes (table S10 in [38]). We could map 33 of these 55 genes to unique OGs,
175 of which 11 were HCOGs. We observed significant similarity in cold response between the 33
176 Greenup genes and the short-term cold response observed in our data (Fig. 3); for all five species
177 ($p < 0.05$, see Methods). However, this similarity was noticeably larger in *H. vulgare* than in the
178 other four species. This comparison showed that our transcriptome data was consistent with

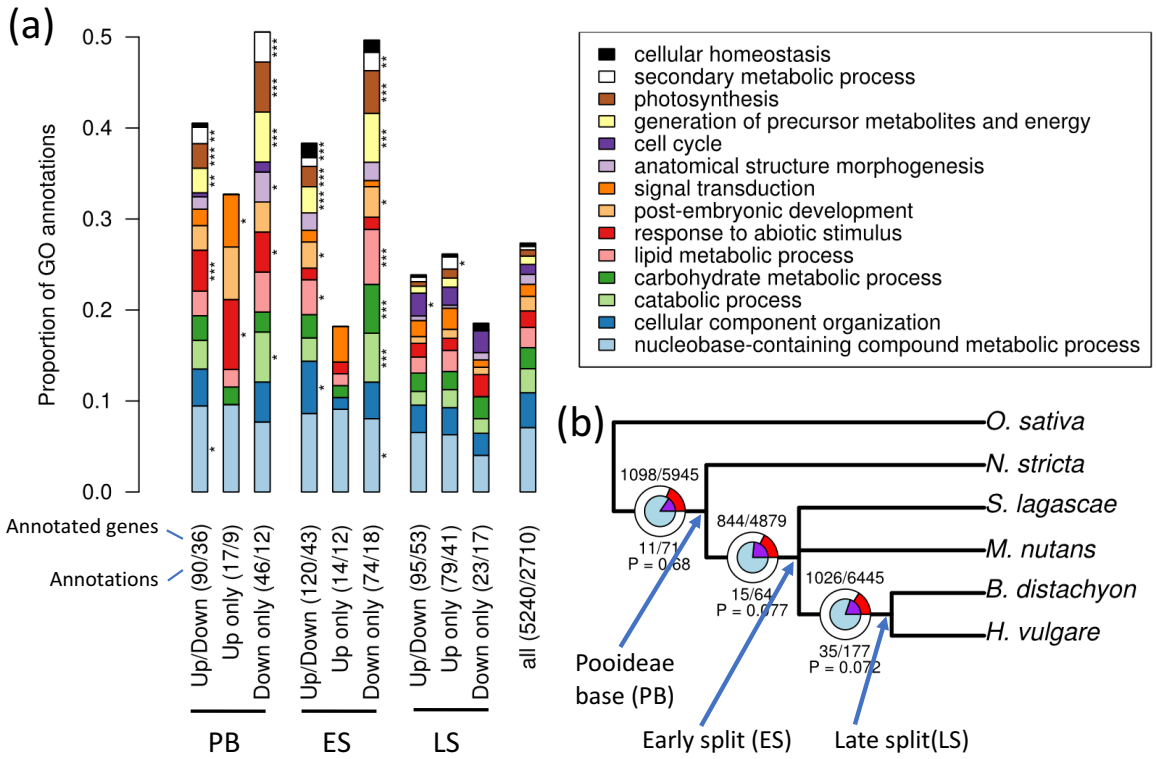

Figure 4. Gene Ontology enrichment and positive selection in branch specific cold responsive genes. *(a) Gene ontology enrichment analysis of high confidence ortholog groups (HCOG) that were differentially expressed (DEGs) in all species (PB), only in species after N. stricta split off (ES) or only in B. distachyon and H. vulgare (LS) (* P < 0.05, ** P < 0.01, *** P < 0.005, Fisher's exact test). Both the number of annotated genes and the number of annotations were indicated for each set of branch specific DEGs. (b) Positive selection at different stages in Pooideae evolution. The circles represent the high confidence ortholog groups that were tested for positive selection at each split (see Methods for the criteria). The inner blue circle represented HCOGs with branch specific differential expression (i.e. with genes that were cold responsive exclusively in the species under the respective branch) while the outer circle represented all other HCOGs. The purple and red pie-slices represented the proportions of HCOGs with positive selection (P < 0.05). The P-value indicated the overrepresentation of positive selection among the branch specific DEGs (Hypergeometric test).*

179 previous findings in *H. vulgare*, and that cold response genes identified in *H. vulgare* exhibits
180 some cold response in other Pooideae.

181 *Photosynthesis was down-regulated under cold stress*

182 To identify biological processes that evolved regulation during different stages of Pooideae
183 evolution, we targeted gene sets that were exclusively differentially expressed in all species
184 within a clade in the phylogentic tree (i.e. branch specific DEGs), and tested these for enrichment
185 of Gene Ontology (GO) biological process annotations (Fig. 4a). For the genes that were
186 differentially expressed in all our species (Pooideae base [PB] in Fig 4b), we found enrichments
187 for annotations related to response to abiotic stimulus, photosynthesis and metabolism. Dividing
188 the branch specific DEGs into up- or down-regulated genes revealed up-regulation of signal
189 transduction (two pseudo response regulators and diacylglycerol kinase 2 (DGK2)) and abiotic
190 stimulus (Gigantea, LEA-14, DnaJ and DGK2), and down-regulation of photosynthesis and
191 metabolism. For the genes that were exclusively differentially expressed in all species except *N.*
192 *stricta* (early split [ES] in Fig. 4b), down-regulated genes were again enriched for GO
193 annotations related to metabolism and photosynthesis.

194 *Cold response genes were associated with positive selection on amino acid content*

195 For each HCOG, we tested for positive selection in coding sequences at each of the internal
196 branches of the species tree. The tests were only performed on the branches where the gene tree
197 topology was compatible with the species tree topology (see Methods). 16-18% of the HCOGs
198 showed significant signs of positive selection ($P < 0.05$) depending on the branch (Fig. 4b). Next,
199 we tested for overrepresentation of positive selection among the branch specific DEGs. There
200 was a tendency that gain of cold response was associated with positive selection at the early split
201 (ES) and late split (LS) branches ($P = 0.077$ and $P = 0.072$, respectively) (Fig. 4b).

202 # Discussion

203 The ecological success of the Pooideae subfamily in the northern temperate regions must have
204 critically relied on adaptation to colder temperatures. However, it is unclear how this adaptation
205 evolved within Pooideae. To test whether molecular responses to cold are conserved in the
206 Pooideae subfamily, we applied RNA-seq to identify short- and long-term cold responsive genes
207 in five Pooideae species ranging from early diverging lineages to core Pooideae species. Since
208 three of the species lacked reference genomes, we employed a *de novo* assembly pipeline to
209 reconstruct the transcriptomes and showed that this pipeline could recover a set of *H. vulgare*
210 genes previously identified as cold responsive (Fig. 3). In order to compare the five
211 transcriptomes, we compiled a set of 8633 high confidence ortholog groups with resolved gene
212 tree topologies. Gene expression clustering based on these ortholog groups arranged samples
213 according to replicates, then time points and finally species, indicating that cold response was the
214 primary signal in the data and confirming the soundness of the approach (Fig. 2a).

215 *Lineage specific adaptations to cold climates*

216 A substantial portion of the individual Pooideae transcriptomes responded to cold (1000-3000
217 genes), however, only a small number of genes responded to cold in all the investigated species
218 (83 genes, Fig. 2d). Even fewer genes responded similarly to cold in all species (e.g. short-term
219 up-regulation, 16 genes, Table 1) and these shared cold response genes primarily included
220 general abiotic stress genes clearly not representative of all the different molecular pathways
221 constituting a fully operational cold response program. We also observed low correlations in
222 expression fold changes between species, a result that was independent of our ability to correctly
223 classify genes as differentially expressed. All these results were based on high confidence
224 ortholog groups that excluded complex families with duplication events shared by two or more
225 species. Since many of the previously described *H. vulgare* cold responsive genes belonged to
226 such complex families, we could have underestimated the number of shared cold responsive
227 genes. However, we specifically investigated the regulation of these previously described genes
228 using all ortholog groups, and again found that few genes displayed shared cold response across
229 all species (Fig. 3), thus confirming our conclusion that cold response in Pooideae is largely
230 species specific. Taken together, our findings indicated that the most recent common ancestor of
231 the Pooideae possessed no, or only a limited, response to cold, and, consequently, that our data

232 appears more consistent with the lineage specific hypothesis of Pooideae cold adaptation than the
233 ancestral hypothesis.

234 The drastic cold stress during the E-O transition was likely an important cause for the evolution
235 of cold adaptation in Pooideae. Previous studies have shown that many temperate plant lineages
236 emerged during the E-O transition [25] and that the expansion of well-known cold responsive
237 gene families in Pooideae coincided with this transition [15, 26]. From the dated phylogeny (Fig.
238 1a) as well as from earlier studies of the Pooideae phylogeny [2, 28], it was clear that all major
239 Pooidaee lineages, including the core Pooideae, had emerged by the late Eocene. Hence, the five
240 lineages studied here experienced the E-O transition as individual lineages (Fig. 1c).
241 Furthermore, we found that closely related species did not share a higher fraction of cold
242 responsive genes than more distantly related species (no phylogenetic pattern, Fig. 2b-d). The
243 observation that the five Pooideae lineages emerged during a relatively warm period before the
244 E-O transition, and the finding that these species harbored high numbers of species specific cold
245 responsive genes with no phylogenetic pattern, together suggested that most of the cold response
246 in Pooideae lineages evolved in parallel during the last 40 M years. During this period,
247 temperatures were constantly decreasing and dramatic cooling events took place, such as the E-O
248 transition and the current Quaternary Ice Age.

249 Our results suggested that the Pooideae lineages evolved cold response in parallel using, to a
250 large degree, unrelated genes. This implies that different genes can be co-opted into the
251 functional cold response of the Pooideae. It is worth noticing, however, that although we
252 observed many species specific cold response genes, all species pairs displayed a statistically
253 significant correlation in cold response across all HCOGs (Fig. 2b) and a statistically
254 significantly overlap in cold responsive genes (Fig. 2c). This may reflect that some genes code
255 for proteins with biochemical functions more suited to be recruited for cold response than others
256 [39, 40], and that different species thus have ended up co-opting orthologous genes into their
257 cold response program more often than expected by chance.

258 *An adaptive potential in the Pooideae ancestor*

259 Multiple independent origins of cold adaptation raise the question whether connecting traits
260 exists in the evolutionary history of the Pooideae that can explain why the Pooideae lineages
261 were able to shift to the temperate biome. The transcripts that were cold responsive across all

262 five species (Table 1) represented genes that might have gained cold responsiveness in the
263 Pooideae most recent common ancestor and contributed to increase the potential of Pooideae
264 lineages to adapt to a cold temperate climate. Several of these conserved genes were known to be
265 involved in abiotic stresses in other plants such as drought or other osmotic stress, which share
266 some physiological effects experienced during freezing. Co-option of such genes into a cold-
267 responsive pathway might have been the key to acquire cold tolerance. In fact, other studies have
268 implied that drought tolerance might have facilitated the shift to temperate biome [26, 41, 42].
269 Interestingly, most of the conserved genes were short-term cold responsive (Table 1) and this
270 observation strengthened the hypothesis that existing stress genes might have been the first to be
271 co-opted into the cold response program. Also, three of the conserved cold responsive genes
272 (GIGANTEA, PRR95 and AtPRR3-like) were associated with the circadian clock that is known
273 to be affected by cold [43–45]. This might suggest that clock genes have had an important
274 function in the Pooideae cold adaptation, for example by acting as a signal for initiating the cold
275 defense. More generally, transcripts involved in photosynthesis and response to abiotic stimuli
276 were significantly enriched among the genes with cold response in all species (Fig. 4a). An
277 expanded stress responsiveness towards cold stress and the ability to down-regulate the
278 photosynthetic machinery during cold temperatures to prevent photoinhibition might have
279 existed in the early evolution of Pooideae. In conclusion, the conserved stress response genes
280 discussed here may have represented a fitness advantage for the Pooideae ancestor in the newly
281 emerging environment with incidents of mild frost, allowing time to evolve the more complex
282 physiological adaptations required to endure the temperate climate with strong seasonality and
283 cold winters that emerged following the E-O transition [23]. Consistent with this, Schubert et al.
284 (unpublished) showed that the fructan synthesis and ice recrystallization inhibition protein gene
285 families known to be involved in cold acclimation in core Pooideae species [10] evolved around
286 the E-O split, whereas also earlier evolving Pooideae species show capacity to cold acclimate.

287 *Evolution of coding and regulatory sequences*

288 The molecular mechanisms behind adaptive evolution are still poorly understood, although it is
289 now indisputably established that novel gene regulation plays a crucial role [46]. The evolution
290 of gene regulation proceeds by altering non-coding regulatory sequences in the genome, such as
291 (cis-) regulatory elements [47], and has the potential to evolve faster than protein sequence and

292 function. The high number of species specific cold response genes observed in this study is thus
293 most consistent with the recruitment of genes with existing cold tolerance functions by means of
294 regulatory evolution. However, previous studies have also pointed to the evolution of coding
295 sequences [27] as underlying the acquisition of cold tolerance in Pooideae. To investigate
296 possible coding evolution, we tested for the enrichment of positive selection among branch
297 specific cold responsive genes (Fig. 4b). Although not statistically significant, there was a
298 tendency for positive selection in genes gaining cold response in a period of gradual cooling
299 preceding the E-O event. Thus, we saw evidence of both coding and regulatory evolution playing
300 a role in cold adaptation in Pooideae, and that these processes may have interacted. Finally, gene
301 family expansion has previously been implied in cold adaptation in Pooideae [15, 26]. As
302 previously discussed, the conservative filtering of ortholog groups employed in this study
303 removed complex gene families containing duplication events shared by two or more species.
304 Interestingly, out of the 33 previously described *H. vulgare* cold responsive genes (Fig. 3), as
305 many as 22 were not included in the high confidence ortholog groups, the main reason being that
306 they belonged to gene families with duplications. This observation thus confirms that duplication
307 events are a relatively common feature of cold adaptation. Although *de novo* assembly of
308 transcriptomes from short-read RNA-Seq data is a powerful tool that has vastly expanded the
309 number of target species for conducting transcriptomic analysis, the approach has limited power
310 to distinguish highly similar transcripts such as paralogs. Further insight into the role of
311 duplication events in Pooideae cold adaptation would therefore benefit immensely from
312 additional reference genomes.

313 Conclusion

314 Here we investigated the cold response of five Pooideae species, ranging from early diverging
315 lineages to core Pooideae species, to elucidate evolution of adaptation to cold temperate regions.
316 We primarily observed species specific cold response that seems to have evolved chiefly after
317 the *B. distachyon* lineage and the core Pooideae diverged, possible initiated by the drastic
318 temperature drop during the E-O transition. However, we do also see signs of conserved
319 response that potentially represents a shared potential for cold adaptation that explain the success
320 of Pooideae in temperate regions. This included several general stress genes with conserved
321 short-term response to cold as well as the conserved ability to down-regulate the photosynthetic

322 machinery during cold temperatures. Taken together, our observations are consistent with a
323 scenario where many of the biochemical functions needed for cold response were present in the
324 Pooideae common ancestor, and where different Pooideae lineages have assembled, in parallel,
325 different overlapping subsets of these genes into fully functional cold response programs through
326 the relatively rapid process of regulatory evolution.

327 Methods

328 *Plant material, sampling and sequencing*

329 To address our hypothesis, we selected five species to cover the phylogenetic spread of
330 Pooideae. The selected species also represent major, species rich lineages or clades in the
331 Pooideae subfamily, or belong to very early diverging lineages [5]. Seeds were collected either in
332 nature: *Nardus stricta* (collected in Romania, [46.69098, 22.58302], July 2012) and *Melica*
333 *nutans* (collected in Germany, [50.70708, 11.23838], June 2012); or acquired from germplasm
334 collections: *Stipa lagascae* (PI 250751, U.S. National Plant Germplasm System (U.S.-NPGS) via
335 Germplasm Resources Information Network [GRIN]), *Brachypodium distachyon* (line 'Bd1-1',
336 W6 46201, U.S.-NPGS via GRIN) and *Hordeum vulgare* (line 'Igri', provided by Prof. Åsmund
337 Bjørnstad, Department of Plant Sciences, Norwegian University of Life Sciences, Norway).
338 Seeds were germinated and initially grown in a greenhouse at a neutral day length (12 hours of
339 light), 17°C and a minimum artificial light intensity of 150 µmol/m2s. Because the seedlings of
340 the phylogenetically diverse species grew at different rates, the sampling was based on
341 developmental stages rather than time. Plants were grown until three to four leaves had emerged
342 for *M. nutans*, *S. lagascae*, *B. distachyon* and *H. vulgare*, or six to seven leaves for *N. stricta*
343 (which is a cushion forming grass that produces many small leaves compared to its overall plant
344 size). Depending on the species, this process took one (*H. vulgare*), three (*B. distachyon* and *S.*
345 *lagascae*), six (*M. nutans)* or eight (*N. stricta*) weeks from the time of sowing. Subsequently,
346 plants were randomized and distributed to two cold chambers with short day (8 hours of light),
347 6°C and a light intensity of 50 µmol/m2s. Leaf material for RNA isolation was collected i) in the
348 afternoon (8 hours of light) before cold treatment (D0) and 8 hours after cold treatment (D1) and
349 ii) in the morning (at lights on) before cold treatment (W0), 4 weeks after cold treatment (W4)
350 and 9 weeks after cold treatment (W9) (Fig. 1d). Flash frozen leaves were individually

351 homogenized using a TissueLyser (Qiagen Retsch) and total RNA was isolated (from each leaf)
352 using RNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions. The purity and
353 integrity of total RNA extracts was determined using a NanoDrop 8000 UV-Vis
354 Spectrophotometer (Thermo Scientific) and 2100 Bioanalyzer (Agilent), respectively. For each
355 time point, RNA extracts from five leaves sampled from five different plants were pooled and
356 sequenced as a single sample. In addition, replicates from single individual leaves were
357 sequenced for selected timepoints (see Table S1 and "Differential expression" below). Two time
358 points lacked expression values: W9 in B. distachyon (RNA integrity was insufficient for RNA
359 sequencing) and W0 in S. lagascae (insufficient supply of plant material). Samples were sent to
360 the Norwegian Sequencing Centre, where strand-specific cDNA libraries were prepared and
361 sequenced (paired-end) on an Illumina HiSeq 2000 system. The raw reads are available in the
362 ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-5300.

363 *Transcriptome assembly and ortholog inference*

364 Using Trimmomatic v0.32 [48], all reads were trimmed to a length of 120 bp, Illumina TruSeq
365 adapters were removed from the raw reads, low quality bases trimmed using a sliding window of
366 40 bp and an average quality cut-off of 15 and reads below a minimum length of 36 bp were
367 discarded. Read quality was controlled using fastqc v0.11.2. For each species, transcripts were
368 assembled *de novo* with Trinity v2.0.6 [49] (strand specific option, otherwise default parameters)
369 using reads from all samples. Coding sequences (CDS) were identified using TransDecoder
370 rel16JAN2014 [50]. Where Trinity reported multiple isoforms, only the longest CDS was
371 retained. Ortholog groups (OGs) were constructed from the five *de novo* transcriptomes and
372 public reference transcriptomes of *H. vulgare* (barley_HighConf_genes_MIPS_23Mar12), *B.*
373 *distachyon* (brachypodium v1.2), *O. sativa* (rap2), *Z. mays* (ZmB73_5a_WGS), *S. bicolor*
374 (sorghum 1.4) and *L. perenne* (GenBank TSA accession GAYX01000000) using OrthoMCL
375 v2.0.9 [51]. All reference sequences except *L. perenne* were downloaded from
376 http://pgsb.helmholtz-muenchen.de/plant/plantsdb.jsp. A summary of the results is provided in
377 Table S1.

378 *High confidence ortholog groups*

379 To compare gene expression across Pooideae, we identified ortholog groups containing one gene
380 from each species that all descended from a single gene in the Pooideae ancestor. As the ortholog

381 groups (OGs) inferred using orthoMCL sometimes cluster more distantly related homologs as
382 well as include both paraphyletic and monophyletic paralogs, we further refined the OGs by
383 phylogenetic analysis. Several approaches to phylogenetic refinement has been proposed
384 previously (see e.g. [52]). Here we first aligned protein sequences within each OG using mafft
385 v7.130 [53] and converted to codon alignments using pal2nal v14 [54]. Gene trees were then
386 constructed from the codon alignments using Phangorn v1.99.14 [55] (maximum likelihood
387 GTR+I+G). Trees with apparent duplication events before the most recent common ancestor of
388 the included species were split into several trees. This was accomplished by identifying in-group
389 (Pooideae) and out-group (*Z. mays*, *S. bicolor* and *O. sativa*) clades in each tree, and then
390 splitting the trees so that each resulting sub-tree contained a single out-group and a single in-
391 group clade. Finally, we only retained the trees were all species in the tree formed one clade each
392 (i.e. only monophyletic paralogs), *B. distachyon* and *H. vulgare* formed a clade and at least three
393 of the five studied species were included. These trees constituted the high confidence ortholog
394 groups (HCOGs).

395 *Species tree*

396 Ortholog groups with a single ortholog from each of the five *de novo* Pooideae species and *O.*
397 *sativa* (after splitting the trees, see "High confidence ortholog groups") were used to infer dated
398 gene trees. To this end, BEAST v1.7.5 [56] was run with an HKY + Γ nucleotide substitution
399 model using an uncorrelated lognormal relaxed clock model. A Yule process (birth only) was
400 used as prior for the tree and the monophyly of the Pooideae was constrained. Prior estimates for
401 the *Oryza*-Pooideae (53 Mya [SD 3.6 My], [37]) and *Brachypodium-Hordeum* (44.4 Mya [SD
402 3.53 My], [28]) divergence times were used to define normally distributed age priors for the
403 respective nodes in the topology. MCMC analyses were run for 10 million generations and
404 parameters were sampled every 10.000 generation. For each gene tree analysis, the first 10
405 percent of the estimated trees were discarded and the remaining trees were summarized to a
406 maximum clade credibility (MCC) tree using TreeAnnotator v1.7.5. The topology of the species
407 tree was equal to the most common topology among the 3914 MCC trees, with internal node
408 ages set equal to the mean of the corresponding node age distributions of the MCC gene trees.

409 *Differential expression*

410 Reads were mapped to the *de novo* transcriptomes using bowtie v1.1.2 [57], and read counts
411 were calculated with RSEM v1.2.9 [58]. In HCOGs, read counts of paralogs were summed
412 (analogous to so called monophyly masking [59]) and missing orthologs were assumed to not be
413 expressed (i.e. read counts equal to zero). To identify conserved and diverged cold response
414 across species, we probed each HCOG for differentially expressed genes (DEGs). Specifically,
415 DEGs were identified using DESeq2 v1.6.3 [60] with a model that combined the species factor
416 and the timepoint factor (with timepoints W4/9 as a single level). Pooled samples provided
417 robust estimates of the mean expression in each time point. To also obtain robust estimates of the
418 variance, the model assumed common variance across all timepoints and species within each
419 HCOG, thus taking advantage of both biological replicates available for individual time points
420 within species and the replication provided by analysing several species. For each species, we
421 tested the expression difference between D0 and D1 (short-term response) and the difference
422 between W0 and W4/9 (long-term response) (Fig. 1d). *B. distachyon* lacked the W9 samples and
423 long-term response was therefore based on W4 only. *S. lagascae* lacked the W0 sample and
424 long-term response was therefore calculated based on D0. As a result, the observed diurnal effect
425 (Fig. 2a) might have resulted in more unreliable estimates of the long term cold response in *S.*
426 *lagascae* since for this species the afternoon sample (D0) was used to replace the missing
427 morning sample (W0). Genes with a false discovery rate (FDR) adjusted p-value < 0.05 and a
428 fold change > 2 were classified as differentially expressed.

429 *Sample clustering*

430 Sample clustering was based on read counts normalized using the variance-stabilizing
431 transformation (VST) implemented in DESeq2 (these VST-values are essentially log
432 transformed). HCOGs that lacked orthologs from any of the five species, or that contained
433 orthologs with low expression (VST < 3), were removed, resulting in 4981 HCOGs used for the
434 clustering. To highlight the effect of the cold treatment over the effect of absolute expression
435 differences between species, the expression values were normalised per gene and species: First,
436 one expression value was obtained per timepoint per gene by taking the mean of the replicates.
437 Then, these expression values were centered by subtracting the mean expression of all timepoint.
438 Distances between all pairs of samples were calculated as the sum of absolute expression

439 difference between orthologs in the 4981 HCOGs (i.e. manhattan distance). The tree was
440 generated using neighbor-joining [61].

441 *Comparison with known cold responsive genes*

442 A set of *H. vulgare* genes independently identified as cold responsive were acquired from
443 supplementary table S10 in [38]. These genes were found to be responsive to cold in three
444 independent experiments with Plexdb accessions BB64 [62], BB81 (no publication) and BB94
445 [38]. The probesets of the Affymetrix Barley1 GeneChip microarray used in these studies were
446 blasted (blastx) against all protein sequences in our OGs. Each probe was assigned to the OG
447 with the best match in the *H. vulgare* reference. If several probes were assigned to the same OG,
448 only the probe with the best hit was retained. Correspondingly, if a probe matched several
449 paralogs within the same OG, only the best match was retained. DESeq2 was used to identify
450 short-term response DEGs for all transcripts in all OGs (i.e. this analysis was not restricted to the
451 HCOGs), and these were compared to DEGs from [38]. The statistical significance of the overlap
452 between our results and those reported in [38] was assessed for each species by counting the
453 number of genes that had the same response (up- or down-regulated DEGs) and comparing that
454 to a null distribution. The null distribution was obtained from equivalent counts obtained from
455 100 000 trials where genes were randomly selected from all expressed genes (mean read count >
456 10) with an ortholog in *H. vulgare*.

457 *Gene ontology enrichment tests*

458 Gene Ontology (GO) annotations for *B. distachyon* were downloaded from Ensembl Plants
459 Biomart and assigned to the HCOGs. The TopGO v2.18.0 R package [63] was used to calculate
460 statistically significant enrichments (Fisher's exact test, $p < 0.05$) of GO biological process
461 annotations restricted to GO plant slim in each set of branch specific DEGs using all annotated
462 HCOGs as the background. Branch specific DEGs were those genes that were exclusively
463 differentially expressed in all species within a clade in the phylogenetic tree.

464 *Positive selection tests*

465 Each of the HCOGs were tested for positive selection using the branch-site model in codeml,
466 which is part of PAML v4.7 [64]. We only tested branches for positive selection in HCOGs
467 meeting the following criteria: (i) The tested branch had to be an internal branch also in the gene

468 tree (i.e. there was at least two species below the branch). (ii) The species below and above the
469 tested branch in the gene tree had to be the same as in the species tree or a subset thereof. (iii)
470 The first species to split off under the branch had to be the same as in the species tree (for the
471 early split, either *S. lagascae* or *M. nutans* was allowed). We then used the Hypergeometric test
472 to identify statistically significant overrepresentation of positive selection among branch specific
473 DEGs (see "Gene ontology enrichment tests") at the Pooideae base (PB), the early split (ES) and
474 the late split (LS) branches.

475 Declarations

476 *Ethics approval and consent to participate*

477 Not applicable.

478 *Consent for publication*

479 Not applicable.

480 *Availability of data and material*

481 The raw reads, the assembled transcripts and the raw read counts are available in the
482 ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-5300.

483 *Competing interests*

484 The authors declare that they have no competing interests.

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489 *Authors´ contributions*

490 All authors designed the experiment. M.S. performed the growth experiments, sampled and
491 prepared RNA for sequencing, helped designing the data analysis pipeline, contributed to the
492 positive selection analysis and performed the phylogenetic analyses. L.G. developed,
493 implemented and conducted the data analysis. All authors interpreted the results. M.S. and L.G.
494 wrote the manuscript with input from S.F., T.R.H., and S.R.S.

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741

742 # Supporting Information

743 **Table S1**. **Summary statistics for the sampling, the transcriptome assembly, coding**
744 **sequence detection and ortholog group inference**. Isoforms were not included in the counts.
745 Only ortholog groups with at least one coding transcript from the five studied species were
746 included.

747 **Table S2. High confidence ortholog groups (HCOGs**). HCOGs generated by filtering and
748 splitting ortholog groups (see Methods). Ortholog groups were stored as a table with the ortholog
749 group IDs as rows and species as columns. Each cell contains sequence IDs separated by ",".
750 Groups that were the result of splitting larger ortholog groups were marked by a number suffix in
751 the group ID.

752 **Table S3. A cross species expression matrix.** Combined read counts for high confidence
753 ortholog groups. Column represents samples and rows represents HCOGs. The sample IDs in the
754 column header consists of the species ID, the time point, indication of whether the sample is
755 pooled from five individual plants ("mix") or just a single individual plant ("ind") and the
756 replicate number.

757 **Table S4. Differential expression results for the high confidence ortholog groups (HCOGs).**
758 A table with rows representing HCOGs and columns representing differential expression results
759 including log2 fold changes, P-values and FDR adjusted p-values for short- and long-term
760 responses.

761 **Figure S1: The four most common gene tree topologies.** We generated gene trees from a
762 selected set of 3914 ortholog groups (see Methods). This figure depicts the four most common
763 topologies.