Transcriptional analysis in multiple barley varieties identifies signatures of waterlogging response

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Abstract

Waterlogging leads to major crop losses globally, particularly for waterlogging sensitive crops such as barley. Waterlogging reduces oxygen availability and results in additional stresses, leading to the activation of hypoxia and stress response pathways that promote plant survival. Although certain barley varieties have been shown to be more tolerant to waterlogging than others and some tolerance-related QTLs have been identified, the molecular mechanisms underlying this trait are mostly unknown. Transcriptomics approaches can provide very valuable information for our understanding of waterlogging tolerance. Here, we surveyed 21 barley varieties for the differential transcriptional activation of conserved hypoxia-response genes under waterlogging, and selected five varieties with different levels of induction of core hypoxia-response genes. We further characterized their phenotypic response to waterlogging in terms of shoot and root traits. RNA-sequencing to evaluate the genome-wide transcriptional responses to waterlogging of these selected varieties led to the identification of a set of 98 waterlogging-response genes common to the different datasets. Many of these genes are orthologs of the so-called 'core hypoxia response genes', thus highlighting the conservation of plant responses to waterlogging. Hierarchical clustering analysis also identified groups of genes with intrinsic differential expression between varieties prior to waterlogging stress. These genes could constitute interesting candidates to study 'predisposition' to waterlogging tolerance or sensitivity in barley.

Keywords

Waterlogging; barley; *Hordeum vulgare*; transcriptomics; RNA-seq

Abbreviations

ACO: 1-aminocyclopropane-1-carboxylate oxidase 1-like
ADH: alcohol dehydrogenase
AGOUEB: association genetics of UK elite Barley
bHLH: basic helix-loop-helix
DEG: differentially expressed gene
ERF-VII: group VII ethylene response factor
GABA-T: γ-aminobutyric acid transaminase
GO: gene ontology
Golden P. /GP: Golden Promise

GWAS: genome-wide association studies
HB: hemoglobin
Hv: *Hordeum vulgare*
Inf: Infinity
MAPK: mitogen-activated protein kinase
NAC: NAM (no apical meristem), ATAF1 and −2, and CUC2 (cup-shaped cotyledon)
Pas: Passport
PCA: principal component analysis
PCO: plant cysteine oxidase
PDC: pyruvate decarboxylase
Pil: Pilastro
QTL: quantitative trait locus
RBOH: respiratory burst oxidase
Reg: Regina
ROS: reactive oxygen species
VST: variance stabilising transformations

Introduction

Waterlogging (*i.e.*, the saturation of soil with water) and flooding-related stresses are the cause of major crop losses worldwide. They are predicted to worsen in some countries as a consequence of seasonal increases in rainfall resulting from global climate change (Bailey-Serres *et al.*, 2012a; Bailey-Serres *et al.*, 2012b; Kaur *et al.*, 2020; Langan *et al.*, 2022). Waterlogging and flooding alter biological, physical and chemical parameters in a plant's environment. These combined changes affect negatively different aspects of plant growth, but at the same time, they contribute to the onset of a multi-faceted response. One of the most important changes caused by waterlogging is the reduction in oxygen availability (hypoxia) to roots, and also to shoots in the case of flooding (Bailey-Serres *et al.*, 2012a; Bailey-Serres *et al.*, 2012b; Sasidharan *et al.*, 2017). Other parameters that change and may affect plants negatively upon waterlogging and flooding include the release of toxic chemical compounds in the soil (*e.g.,* iron) (Setter and Waters, 2003), reduced availability of important nutrients such as nitrates (Setter and Waters, 2003), changes in the soil microbiome (Hartman and Tringe, 2019; Wang *et al.*, 2017), or a decrease in light availability because of flood water turbidity.

In recent years, the study of waterlogging stress and of the resulting hypoxic stress has led to the discovery of essential and conserved oxygen-sensing mechanisms in plants (reviewed in (Doorly and Graciet, 2021; Hammarlund *et al.*, 2020; Holdsworth and Gibbs, 2020)). An important oxygen-sensing pathway in plants requires a set of oxygen-dependent PLANT CYSTEINE OXIDASE (PCO) enzymes that oxidize the N-terminus of proteins starting with a cysteine residue (Weits *et al.*, 2014; White *et al.*, 2018; White *et al.*, 2017), including a set of group VII ETHYLENE RESPONSE FACTOR (ERF-VII) transcription factors (White *et al.*, 2018; White *et al.*, 2017) that act as master regulators of the hypoxia response program (reviewed in (Giuntoli and Perata, 2018)). Under normal oxygen conditions, these ERF-VII transcription factors are targeted for degradation by the ubiquitin-dependent N-degron pathway, following oxidation of their N-terminal cysteine residue by PCO enzymes (Gibbs *et al.*, 2011; Licausi *et al.*, 2011; Weits *et al.*, 2014). In contrast, under hypoxic conditions, the activity of PCOs is inhibited, thus preventing the oxidation of ERF-VIIs' N-terminal cysteine and their subsequent N-degron-mediated degradation. As a result, under hypoxic conditions, ERF-VIIs accumulate in the nucleus, where they bind to *cis*-regulatory elements

120 to activate the hypoxia response program. Notably, the promoters of conserved hypoxia
121 response gene families are not only enriched for *cis*-regulatory elements bound by
122 transcription factors of the ERF-VII family (*e.g.*, the HRPE motif (Gasch *et al.*, 2016)), but also
123 for motifs bound by basic helix-loop-helix (bHLH), MYB and WRKY transcription factors
124 (Reynoso *et al.*, 2019), thus suggesting the involvement of other families of transcription
125 factors in the regulation of the hypoxia response program (Lee and Bailey-Serres, 2021). This
126 is in agreement with the up-regulation of transcription factor-coding genes belonging to the
127 different families mentioned above as part of hypoxia response in plants (Mustroph *et al.*,
128 2010). Cross-comparisons of transcriptomic datasets from different plant species further
129 allowed the identification of core genes of the hypoxia response program (Mustroph *et al.*,
130 2010; Mustroph *et al.*, 2009; Reynoso *et al.*, 2019). These include genes involved in (i) the
131 regulation of carbon metabolism in order to facilitate ATP production *via* glycolysis and
132 NAD+ regeneration through fermentation pathways; (ii) the regulation of important
133 signaling pathways (*e.g.*, MAPK signaling) and molecules (*e.g.*, reactive oxygen species
134 (ROS)) to promote plant tolerance and survival upon hypoxia.

135 Numerous transcriptomic analyses using either flooding or waterlogging treatments
136 have been conducted with the model plant *Arabidopsis thaliana* (see list in (Brazel and
137 Graciet, 2023)). These studies revealed that core aspects of the transcriptional
138 reprogramming upon waterlogging or flooding are shared with the response to hypoxia (Lee
139 *et al.*, 2011; van Veen *et al.*, 2016). Furthermore, the transcriptomic comparison of 8
140 different natural accessions of Arabidopsis, which had been previously identified as being
141 either sensitive or tolerant to flooding (Vashisht *et al.*, 2011), indicated that the
142 transcriptional response of roots and shoots differ (van Veen *et al.*, 2016). This work
143 identified sets of shoot or root-expressed genes that might contribute to the flooding
144 tolerance phenotype of some natural Arabidopsis accessions (van Veen *et al.*, 2016), which
145 could be relevant to improve crop tolerance to waterlogging/flooding. Notably, the
146 transcriptional reprogramming in response to hypoxia and flooding is also accompanied by
147 other genome-wide regulatory mechanisms such as epigenetic changes, chromatin
148 remodelling (Reynoso *et al.*, 2019), and changes in translation (Lee and Bailey-Serres, 2019;
149 Reynoso *et al.*, 2019).

150 Waterlogging is an important source of crop losses, however, not all crops are
151 equally affected (de San Celedonio *et al.*, 2014; Kaur *et al.*, 2020). Barley is particularly
152 sensitive to waterlogging with reported crop losses of up to 20-25% (Byrne *et al.*, 2022; de
153 San Celedonio *et al.*, 2016; de San Celedonio *et al.*, 2014; Liu *et al.*, 2020; Miricescu *et al.*,
154 2021; Setter and Waters, 2003). These losses are particularly severe if waterlogging occurs
155 at the heading stage (de San Celedonio *et al.*, 2016; de San Celedonio *et al.*, 2014; Liu *et al.*,
156 2020; Setter and Waters, 2003), but also at early developmental stages (de San Celedonio *et*
157 *al.*, 2016; de San Celedonio *et al.*, 2014). The multifaceted nature of waterlogging stress, as
158 well as the complexity of plant responses to this stress, have made it difficult to identify
159 marker genes of waterlogging tolerance, as well as target genes that could be modified to
160 improve crop tolerance to waterlogging. Nevertheless, in recent years, genetic approaches,
161 as well as linkage mapping and genome-wide association studies (GWAS), have led to the
162 identification of potential QTLs and target genes to improve barley tolerance to
163 waterlogging (Bertholdsson *et al.*, 2015; Borrego-Benjumea *et al.*, 2020; Broughton *et al.*,
164 2015; Gill *et al.*, 2019; Li *et al.*, 2008; Manik *et al.*, 2022; Zhang *et al.*, 2017; Zhang *et al.*,
165 2016; Zhou, 2011). In agreement with the complex response to waterlogging, these QTLs
166 have been identified based on a wide range of phenotypic traits, including leaf yellowing,
167 chlorophyll fluorescence, yield traits, adventitious root formation, aerenchyma formation
168 and ROS levels. QTLs relating to root traits, including the ability to form adventitious roots
169 and aerenchyma under waterlogged conditions (Manik *et al.*, 2022; Zhang *et al.*, 2016), may
170 be of particular relevance considering their known roles in facilitating water and nutrient
171 uptake as well as in oxygen distribution during waterlogging (Zhang *et al.*, 2015).

172 Despite the recent progress made in the identification of QTLs for waterlogging
173 tolerance and the realization that transcriptomics may be used to identify potential
174 candidate genes relevant to waterlogging tolerance (Lee *et al.*, 2011; Reynoso *et al.*, 2019;
175 van Veen *et al.*, 2016), only few such studies have been conducted with barley. Recent
176 studies have characterized the transcriptomic response of waterlogging-tolerant and
177 sensitive varieties (Borrego-Benjumea *et al.*, 2020; Luan *et al.*, 2022). From these datasets,
178 the authors identified a handful of genes that could be of importance for waterlogging
179 tolerance mechanisms in barley (Luan *et al.*, 2022). In another study, a comparative
180 proteomic analysis of one waterlogging-sensitive and one waterlogging-tolerant variety

181 identified proteins that accumulate differently in waterlogging sensitive or tolerant
182 germplasms (Luan *et al.*, 2018a; Luan *et al.*, 2018b).

183 Here, to dissect the transcriptomic response of barley to waterlogging, we selected
184 two 2-row and two 6-row winter barley varieties based on the differential expression of
185 known hypoxia response marker genes after waterlogging treatment. These varieties were
186 chosen from the Association Genetics Of UK Elite Barley (AGOUEB) population (Thomas *et*
187 *al.*, 2014) and from the list of recommended barley varieties in Ireland (where the study was
188 conducted; list at the time the experiments were carried out). Our transcriptomic study
189 focused on roots because of their central role in mediating waterlogging tolerance (Zhang *et*
190 *al.*, 2015). We also included the model spring barley variety *Golden Promise* to provide a
191 reference dataset to the community (this variety is widely used to target specific genes
192 using molecular approaches). We identified sets of common genes that are consistently
193 differentially expressed in barley in response to waterlogging. Hierarchical clustering
194 identified groups of genes with intrinsic differential expression between varieties prior to
195 waterlogging stress. Low or elevated expression of some of these genes could 'predispose'
196 some varieties to waterlogging tolerance or sensitivity. In sum, the datasets presented serve
197 as an additional reference for the study of waterlogging response in barley and provide
198 insights into potential avenues of research to improve waterlogging tolerance in this crop.

199

200 Materials and Methods

201 Plant material

202 Cultivars used in this study (Supp. Table S1) included winter varieties that originated from
203 the 'Association Genetics of UK Elite Barley' (AGOUEB) population of barley (Thomas *et al.*,
204 2014), as well as the model spring variety *Golden Promise* (obtained from Teagasc, Oak Park,
205 Ireland) and a winter variety, *Infinity* (obtained from Teagasc, Oak Park, Ireland), which was
206 on the recommended list of barley varieties in Ireland (where the study was conducted) at
207 the time of the initial field trial whose results were taken into account for varietal selection
208 (Byrne *et al.*, 2022). Selected varieties for further characterization, including transcriptome
209 profiling, were *Golden Promise* (spring variety, 2-row), *Regina* and *Infinity* (winter varieties,
210 2-row), *Passport* and *Pilastro* (winter varieties, 6-row).

211

212 **Plant growth conditions**

213 Plants were grown in a plant growth room under long-day conditions (16 h light/8 h dark) at
214 15°C (constant temperature), approximately 45% relative humidity. Light intensity was
215 determined to be ~138 µmol/m^2/s and was provided by LED bulbs (Philipps LED tubes High
216 Output, T8 20W/865).

217

218 **Soil preparation and seed germination**

219 Commercial John Innes N°2 (Vitax; UK) soil was soaked in water after filling round pots of 9-
220 cm diameter and 9-cm height without compressing the soil. Untreated seeds were sown
221 directly in soil at a depth of 2 cm. The sown seeds were stratified in the dark for 14 d at 4°C
222 to ensure homogenous germination. After cold treatment, the pots were transferred to the
223 plant room for germination and growth.

224

225 **Waterlogging**

226 Plants were grown as indicated above for ten days (corresponding to L1/L2 stage) for
227 transcriptomic experiments, and for 10-14 d (corresponding to L1/L2 stage) for phenotypic
228 characterization. The pots were then transferred to a large tub, which was subsequently
229 filled with tap water up to 1 cm above soil level. The water level was kept constant
230 throughout the duration of the experiment (Figure 1A). The plants were kept in the same
231 growth conditions and were treated for 15 d for phenotypic characterization, or for shorter
232 periods of time, as indicated in the text. Control plants were left in the same growth
233 conditions but received normal watering (every two days, avoiding any standing water in
234 the trays). For the recovery period, plants were taken out of the water and kept in the same
235 growth conditions with a normal watering regime.

236

237 **Height measurements**

238 Plant height was taken from the soil surface to the tip of the tallest leaf at the indicated
239 time points.

240

241 **Total RNA extraction**

242 Roots of plants grown under control conditions or subjected to waterlogging for were rinsed
243 under running tap water, and frozen in liquid nitrogen. The tissue was ground in liquid
244 nitrogen and total RNA was extracted using Spectrum™ Plant Total RNA kit (Sigma-Aldrich).
245 For each condition (waterlogged or untreated), the root systems of 3 plants of the same
246 variety were pooled prior to grinding for total RNA extraction. This experiment was
247 conducted independently at least 3 times to obtain samples from at least 3 biological
248 replicates, as indicated in the figure legends.

249

250 **Reverse transcription coupled to quantitative PCR (RT-qPCR)**

251 Total RNA was reverse transcribed using an oligo$(dT)_{18}$ primer and the RevertAid reverse
252 transcriptase (Thermo) according to the manufacturer's instructions. For reverse
253 transcription reactions, 1 µg of total RNA was used. cDNA obtained was used for qPCR with
254 a Lightcycler 480 (Roche). Each PCR reaction mix contained 5 µL of 2x SYBR green master 1
255 (Roche), 1 µL cDNA, 1 µL of 10 µM primers and 3 µL of molecular biology grade water.
256 LightCycler melting curves were obtained to check for single peak melting curves for all
257 amplification products. The second derivative maximum method was used to analyze the
258 amplification data. The resulting Cp values were converted into relative expression values
259 using the comparative Ct method (Livak and Schmittgen, 2001). All primer sequences are
260 provided in Supp. Table S2. One reference gene (Hv*ACTIN*) was used to normalize the RT-
261 qPCR data following the screening of a set of reference genes during waterlogging (Supp.
262 Fig. S1).

263

264 **Next-generation sequencing of RNA samples**

265 For RNA-seq analysis, waterlogging treatment was applied as outlined above for 24 h. For
266 each condition (waterlogged or untreated), the root systems of 3 plants of the same variety
267 were pooled prior to grinding for total RNA extraction. This experiment was conducted
268 independently 3 times to obtain samples from 3 biological replicates (i.e. for each variety, 6

269 RNA samples were sent for sequencing, corresponding to 3 biological replicates for the
270 untreated plants and 3 biological replicates for the waterlogged plants). Following RNA
271 extraction, RNA integrity was assessed using an Agilent 2100 Bioanalyzer (Agilent). All RNA
272 samples had RIN values > 7.0. Library preparation and single ended 50 bp next-generation
273 sequencing was performed by BGI Genomics (Hong Kong) using the DNBseq sequencing
274 platform.

275

276 **Analysis of RNA-seq data**

277 The third release (Morex V3) of the Morex barley genome was downloaded from e!DAL -
278 Plant Genomics and Phenomics Research Data Repository (PGP) (https://edal-pgp.ipk-
279 gatersleben.de/) (Mascher, 2021). Raw RNA-sequencing reads were aligned to Morex V3
280 using *bowtie2* (v2.4.5) (Langmead and Salzberg, 2012). Files were converted from .sam to
281 .bam files and indexed using *samtools* (v1.15.1) (Danecek *et al.*, 2021). Gene abundance was
282 estimated using *stringtie* (v2.1.7) (Pertea *et al.*, 2015) (Supp. Table S3). Differential gene
283 expression analysis was performed using the Bioconductor package *DeSeq2* (Love *et al.*,
284 2014) in *R* (Team, 2022) using a design in which the factors Variety and Treatment were
285 combined into a single factor to model multiple condition effects. The results of multiple
286 comparisons were extracted and filtered by adjusted *p*-value < 0.05 (Supp. Table S4).
287 Principal component analysis (PCA) was performed using *pcaExplorer* (v2.20.2) (Marini and
288 Binder, 2019) in *RStudio* (v2022.02.0+443). Gene ontology (GO) analysis was performed
289 using *ShinyGO* (v0.75c) (Ge *et al.*, 2020). Read count values were transformed by variance
290 stabilising transformations (VST) to normalise for library size and composition (Supp. Table
291 S5). Venn diagrams were generated using InteractiVenn (Heberle *et al.*, 2015).

292 The means of VST read counts from 3 biological replicates generated from *DeSeq2*
293 were filtered for *k*-means clustering as follows. *Deseq2* DEG analysis was performed and
294 results for the 25 comparisons shown in Supp. Fig S6A were extracted. A list of all 11,613
295 DEGs filtered by adjusted *p*-value < 0.05 was generated by combining DEGs from
296 waterlogged to control samples from the same variety, DEGs from each control to every
297 other control sample and DEGs from each waterlogged to every other waterlogged sample.
298 Next, the DEGs with a mean of <10 normalised reads were removed leaving 10,882 genes
299 for clustering analysis. Clustering analysis was performed using the *k*-means function in *R*

300 (v3.6.2) with the arguments centers=25, nstart = 1000, iter.max = 300 and algorithm =
301 "Lloyd" (gene cluster assignment annotated in (Supp. Table S4)). Clustering heatmaps were
302 generated using *ComplexHeatmap* (v2.10.0) (Gu *et al.*, 2016) in *R*. Plots were generated
303 using *ggplot2* (v3.3.6) (Wickam, 2016) and modified for style in Adobe Illustrator 2022.

304 To compare the datasets obtained to a previously published RNA-seq dataset, raw sets were
305 downloaded from NCBI's Gene Expression Omnibus (GSE144077) (Borrego-Benjumea *et al.*,
306 2020) and Sequence Read Archive (PRJNA889532) (Luan *et al.*, 2022). Raw RNA-sequencing
307 reads were aligned to Morex V3 as described above. Differential gene expression analysis of
308 waterlogging vs control for each variety was performed on 0 h control and 24 h
309 waterlogging datasets for the varieties Franklin and TX9425 from Luan *et al.* (2022) as
310 described above. The same differential gene expression analysis was also performed on 72 h
311 control and 72 h waterlogging datasets for the varieties Deder 2 and Yerong from Borrego-
312 Benjumea *et al.* (2020).

313 To compare the datasets to a previously published list of 28 genes found in QTLs for
314 waterlogging tolerance, we downloaded the gene list from Supp. Table 3 in (Manik *et al.*,
315 2022). This gene list used Morex V1 (r1) gene IDs. To compare this list to our data, we
316 performed a BLASTN search of the CDS of each of these genes to the Morex V3 genome
317 using *Phytozome 13* (Goodstein *et al.*, 2012) and used the transcript with the highest
318 percentage identity to a Morex V3 transcript for the comparison (Supp. Table S4).

319

320 Results

321 Characterization of the transcriptional response of different barley varieties using hypoxia 322 response marker genes

323 The previous characterization of 403 varieties from the AGOUEB collection under
324 waterlogged and control conditions in the field (Byrne *et al.*, 2022) provided some
325 information on the differential physiological response of these varieties to waterlogging,
326 while also highlighting the difficulties associated with the study of waterlogging tolerance
327 under field conditions. Based on this initial study, we selected a subset of 20 varieties that
328 behaved differently under waterlogged conditions, with the aim to assess their
329 transcriptional response to waterlogging under controlled conditions. The variety *Golden*

330 *Promise* was also included as a model (spring) barley variety that is widely used to generate
331 targeted mutations through *Agrobacterium*-mediated transformation. After identifying
332 Hv*ACTIN* as a suitable reference gene in waterlogged roots (Supp. Figure S1A), we first
333 carried out a time course experiment with four selected varieties and determined the
334 relative expression of *HEMOGLOBIN1 (HB)*, *ALCOHOL DEHYDROGENASE 1 (ADH1)* and
335 *PYRUVATE DECARBOXYLASE 1 (PDC1)*. These genes are hypoxia response markers commonly
336 used to monitor waterlogging response (Loreti *et al.*, 2020; Mendiondo *et al.*, 2016;
337 Mustroph *et al.*, 2010). This initial analysis indicated that (i) the expression of these three
338 hypoxia response genes peaked around 24 h after the onset of the waterlogging treatment;
339 and (ii) there were differences between the four varieties in terms of the amplitude of the
340 transcriptional up-regulation (Supp. Figure S1B). For example, at 24 h after the beginning of
341 the waterlogging treatment, Hv*HB* was expressed at higher levels in *Pilastro* compared to
342 *Arma*, *Tapir* and *Masquerade*. In addition, the up-regulation of Hv*HB* and Hv*ADH1* was
343 stronger than that of Hv*PDC1*, possibly making these first two genes more suitable to
344 identify varieties with differential transcriptional regulation of the hypoxia response
345 program.

346 We next tested the expression of Hv*HB*, Hv*ADH1* and Hv*PDC1* at 24 h of
347 waterlogging in the set of 21 barley varieties we selected based on (Byrne *et al.*, 2022)
348 (Figure 1A). As expected, under normal watering conditions, most varieties had low levels of
349 expression for each of the hypoxia response genes, with the exception of *Pilastro*, which
350 exhibited higher Hv*HB* expression (Figures 1B, 1C and Supp. Figure S2). Waterlogging stress
351 triggered the up-regulation of the hypoxia response genes in all varieties tested, but some
352 differences in their response were observed. As previously, differences were more marked
353 with Hv*HB* and Hv*ADH1* (Figure 1B-D) than with Hv*PDC1* (Supp. Figure S2), making these
354 first two genes more suitable to identify varieties with a differential transcriptional response
355 to waterlogging. After 24 h of waterlogging, some varieties reached higher expression levels
356 than the average relative expression observed for these genes in the population of 21
357 varieties. These included *Pilastro* and *Regina* for both Hv*HB* and Hv*ADH1*, as well as *Dura*,
358 *Vesuvius*, *Siberia*, *Mahogany* and *Tamaris* for Hv*ADH1*. In contrast, some varieties showed
359 reduced up-regulation of hypoxia response genes compared to the population average. For
360 example, varieties such as *Golden Promise*, *Passport*, *Isa*, *Louise* and *Retriever* had lower

361 expression levels of both Hv*HB* and Hv*ADH1* compared to the average of the population.
362 Other varieties, such as *Infinity*, had average expression levels of Hv*HB* and Hv*ADH1*.

363 Based on these results, *Pilastro* and *Regina* were chosen as representative 6-row and
364 2-row varieties, respectively, that potentially exhibit a stronger transcriptional response to
365 waterlogging, while *Passport* was selected as a 6-row variety that had a more dampened
366 transcriptional response. We also included *Infinity* as a 2-row variety for further
367 characterization because it is on the recommended list in Ireland (where this study was
368 carried out) and has an average transcriptional response to waterlogging. As highlighted
369 above, *Golden Promis*e was included as a reference variety.

370

371 **Effect of waterlogging on the growth of selected varieties**

372 The growth of the five selected varieties was characterized under controlled conditions
373 following a 2-week waterlogging treatment, and a 6-week recovery period with normal
374 watering. Height measurement showed that the growth of *Infinity* and *Passport* was more
375 affected by waterlogging than that of *Pilastro*, *Regina* and *Golden Promise*, whose height
376 was mostly unaffected by the treatment (Figure 2A). In addition, *Golden Promise*, *Infinity*
377 and *Pilastro* produced fewer tillers, whereas *Regina*'s tiller number was largely unaffected
378 (Figure 2B). Because root traits have been shown to be important for waterlogging
379 tolerance (Zhang *et al.*, 2015), we determined the length of the primary root, as well as the
380 number of seminal roots after 2 weeks of waterlogging. The length of the primary root was
381 reduced upon waterlogging stress for all varieties except *Regina* (Figure 2C), while the
382 number of seminal roots increased in all varieties and the difference was statistically
383 significant for all except *Infinity* (Figure 2D). This phenotypic characterization suggests that a
384 variety such as *Regina*, which has a stronger transcriptional response for Hv*ADH1* and Hv*HB*,
385 appears to be less impacted by waterlogging than other varieties.

386

387 **Overview of transcriptional responses to waterlogging in root tissue of selected varieties**

388 To determine the transcriptional responses of *Pilastro*, *Regina*, *Passport*, *Infinity* and *Golden*
389 *Promise* to waterlogging, we subjected these varieties to waterlogging for 24 h. This time
390 point was chosen based on the time course experiment described above that showed peak

391 expression of Hv*HB*, Hv*ADH1* and Hv*PDC1* at 24 h of waterlogging. Total RNA was extracted
392 from roots for RNA-seq analysis. Reads obtained were aligned to the Morex barley genome
393 (version 3; Supp. Table S3 and Supp. Figure S3A-C) and differential gene expression
394 (adjusted *p*-value < 0.05) was determined for each of the waterlogged varieties relative to
395 the corresponding untreated variety (Supp. Table S4). A Principal Component Analysis (PCA)
396 indicated that the samples separated based on (i) the treatment (PC1: 35.82% variance)
397 (Figure 3A); (ii) whether they were winter or spring varieties (PC2: 15.64% variance; Supp.
398 Figure S3D); or (iii) whether they were 2-row or 6-row varieties (PC3: 11.34% variance; Supp.
399 Figure S3E). In this analysis, the varieties *Golden Promise*, *Infinity* and *Passport* could also be
400 separated from *Pilastro* and *Regina* (Figure S3F; PC4: 9.92% variance). This suggests that
401 there are underlying differences between these two groups of varieties that are not
402 explained by a known variable.

403 The number of differentially expressed genes (DEGs) identified in the different
404 varieties varied considerably: for *Golden Promise* and *Passport* we identified 220 and 592
405 DEGs (respectively), while *Infinity*, *Pilastro* and *Regina* had a substantially higher number of
406 DEGs (1,307; 1,512; and 1,233, respectively). In all varieties tested, the proportion of up-
407 regulated genes was higher than that of down-regulated genes (Figure 3B). We verified in
408 our datasets the expression of the Hv*HB*, Hv*ADH1* and Hv*PDC1* genes whose up-regulation
409 was initially used to monitor the transcriptional response of the different varieties to
410 waterlogging by RT-qPCR (Figure 1). Induction of all three genes upon waterlogging was
411 found (Figure 3C). We further identified additional homologs of Hv*ADH1* and Hv*HB* and
412 confirmed that these homologs were also up-regulated in response to waterlogging (Supp.
413 Figure S4).

414

415 **Genome-wide transcriptional reprogramming of barley in response to waterlogging**

416 Our RNA-seq experiment identified 2,865 genes that were differentially expressed in at least
417 one variety under waterlogged conditions compared to control plants (Supp. Table S4). The
418 variety with the highest proportion of unique DEGs was *Pilastro* (>50% unique DEGs), while
419 the majority of DEGs identified for *Golden Promise* and *Passport* were shared with other
420 varieties (Figure 4A) (<25% unique DEGs in these 2 varieties). Among the 2,865 DEGs, 1,078
421 genes were called as differentially expressed in more than one variety. Except for 7 genes,

422 the directionality of gene expression change was the same in all varieties, thus further
423 highlighting the similarities in the response of these varieties to waterlogging. As expected
424 based on the transcriptional response of Arabidopsis to waterlogging, Gene Ontology (GO)
425 analysis of the 1,811 DEGs that were up-regulated in at least one variety identified over-
426 represented terms related to hormone and stress responses, oxidoreductase activity, kinase
427 activity, as well as carbohydrate metabolic processes (Figure 4B and D). GO analysis of the
428 1,061 DEGs that were down-regulated in response to waterlogging identified terms related
429 to lipid and carbohydrate metabolism, transmembrane transporter activities and cell
430 periphery, as well as oxidoreductase activity and proteolysis (Figure 4C and E). All of these
431 terms are consistent with functional GO categories known to be central to the
432 transcriptional response of plants to waterlogging.

433 A total of 98 genes were detected as differentially expressed in the five varieties
434 (Figure 4A), and their directionality of expression change was the same across all datasets.
435 The majority of these genes were up-regulated in response to waterlogging (82 genes;
436 Figure 4B and Supp. Table S4) and included homologs of known core hypoxia response
437 genes in Arabidopsis (Mustroph *et al.*, 2009) such as *ADH1* (HORVU.MOREX.r3.4HG0345760,
438 HORVU.MOREX.r3.1HG0082960 and HORVU.MOREX.r3.4HG0345740), *PDC1*
439 (HORVU.MOREX.r3.4HG0384560), *HB1* (HORVU.MOREX.r3.4HG0394960,
440 HORVU.MOREX.r3.7HG0715010) and a respiratory burst oxidase (*RBOH*)
441 (HORVU.MOREX.r3.5HG0444960). Furthermore, this set included homologs of ERF-VII
442 transcription factors, such as *HRE1* (HORVU.MOREX.r3.4HG0405650 and
443 HORVU.MOREX.r3.4HG0405650), *HRE2* (HORVU.MOREX.r3.5HG0497970) and *RAP2.3*,
444 (HORVU.MOREX.r3.1HG0060770). This result is in agreement with the central role of ERF-VII
445 transcription factors as master regulators of hypoxia and waterlogging response. Sixteen
446 DEGs common to all datasets were down-regulated in response to waterlogging (Figure 4C),
447 including three peroxidase genes (HORVU.MOREX.r3.2HG0215440,
448 HORVU.MOREX.r3.2HG0119650, HORVU.MOREX.r3.2HG0119630) a nitrate transporter
449 (HORVU.MOREX.r3.7HG0700030), and an aldehyde dehydrogenase
450 (HORVU.MOREX.r3.2HG0173750). GO analysis of the set of 98 common DEGs (Figure 4F)
451 revealed largely similar terms to those identified for all up-regulated DEGs (Figure 4D),

452 including GO terms such as oxidoreductase activity, carbohydrate metabolic process, and
453 hormone-mediated signaling.

454 We next compared our RNA-seq results to two recently published RNA-seq datasets.
455 Luan et al. analyzed the transcriptomic response of 4-leaf stage Franklin and TX9425 barley
456 roots to waterlogging for 24 or 72 h (Luan *et al.*, 2022). These were two-row spring varieties,
457 with Franklin showing sensitivity and TX9425 showing tolerance to waterlogging (Luan *et al.*,
458 2022). In addition, Borrego-Benjumea *et al*. analyzed the transcriptomic response of
459 waterlogged barley roots in 2-week-old *Yerong* and *Deder2* for 72 or 120 h (Borrego-
460 Benjumea *et al.*, 2020). Both varieties had been chosen because of their tolerance to
461 waterlogging (Li *et al.*, 2008; Takeda, 1989). Raw RNA-seq data were analyzed using the
462 same pipeline as for our datasets. PCA analysis of all datasets showed that most of the
463 variation was due to the origin of the dataset (Figure S5A; PC1: 68.39% variance), likely
464 because waterlogging stress is known to vary considerably based on experimental
465 differences, such as soil type. As expected, the different samples could also be separated
466 based on treatment (Figure S5A; PC2: 17.03% variance). To facilitate the comparison
467 between the different datasets, we selected the most similar timepoints to those of our
468 study (*i.e*., 0 h control and 24 h waterlogged for Luan *et al.*; and 72 h control and
469 waterlogged for Borrego-Benjumea *et al.*). Despite the differences in barley varieties, age of
470 the plants and duration of the waterlogging treatment, a statistically significant overlap was
471 observed between both the up and down-regulated genes in our datasets and in those
472 recently published datasets DEGs (Supp. Figure S5B and S5C) (Borrego-Benjumea *et al.*,
473 2020; Luan *et al.*, 2022). GO analysis of the 448 DEGs that were up-regulated in all datasets
474 identified terms related to metabolism and oxidoreductase activity (Supp. Figure S5F), again
475 in agreement with known key aspects of plant responses to waterlogging. Genes associated
476 with transporter activity were found among the 309 shared down-regulated genes (Supp.
477 Figure S5G).

478

479 **Identification of unique expression signatures between varieties**

480 To compare the responses to waterlogging between the 5 varieties used in this study, *k*-
481 means clustering was performed on 10,882 genes identified as differentially expressed in: (i)
482 at least one variety when comparing waterlogged to control samples for each variety, or (ii)

483 between the control samples (*i.e.*, these have intrinsic differential expression between at
484 least 2 varieties in the absence of waterlogging), or (iii) between the waterlogged samples
485 from different varieties (*i.e.*, genes that are differentially expressed between at least 2
486 varieties under waterlogging) (Supp. Figure S6A). Clustering analysis revealed distinct gene
487 expression patterns between samples (Figure 5A). A number of clusters contained genes
488 that were up-regulated in multiple varieties in response to waterlogging compared to
489 untreated plants (*i.e.*, clusters 4, 7, 8, 14, and 21). The largest cluster (#14) comprised 802
490 genes (Supp. Table S4) with a centroid expression that was higher in all varieties in response
491 to waterlogging (Figure 5B and Supp. Figure S6). Cluster 14 included homologs of known
492 hypoxia response genes such as those described above in the common set of 82 up-
493 regulated DEGs in barley (Figure 4B), as well as homologs of Arabidopsis *RAP2.12*
494 (HORVU.MOREX.r3.5HG0481240), additional homologs of *RAP2.3*,
495 (HORVU.MOREX.r3.2HG0112960), and homologs of *ADH1* (HORVU.MOREX.r3.2HG0115170,
496 HORVU.MOREX.r3.3HG0252910) (Supp. Figure S4A and B). Furthermore, transcription
497 factors (TFs) from the ERF, bHLH, bZIP (basic leucine zipper), NAC (NAM, ATAF1,2, CUC2) and
498 WRKY families were also identified in cluster 14 (Supp. Table S4), all of which belong to
499 families with established and conserved roles in the regulation of hypoxia response genes
500 (Lee and Bailey-Serres, 2021; Mustroph *et al.*, 2010; Reynoso *et al.*, 2019). GO analysis of
501 the 802 genes in cluster 14 identified terms related to hormone and stress responses,
502 oxidoreductase and kinase activity and metabolism (Figure 5C).

503 In contrast, clusters 5, 9, 13, 15, 20 and 24 comprised genes that were down-
504 regulated in response to waterlogging in multiple varieties. Cluster 9 contained 435 genes
505 that were down-regulated in response to waterlogging (Figure 5D and Supp. Figure S6),
506 including 14 of the 16 genes that were down-regulated in waterlogged samples from all
507 varieties (Figure 4C and Supp. Table S4). Among these genes was an aminotransferase
508 (HORVU.MOREX.r3.2HG0190680), whose top-scoring Arabidopsis homolog is γ-
509 aminobutyric acid transaminase (GABA-T; AT3G22200). In Arabidopsis, the expression of this
510 gene is also reduced in response to hypoxia (Branco-Price *et al.*, 2008). Another member of
511 cluster 9 whose expression was downregulated in all varieties in response to waterlogging is
512 a cation exchanger (HORVU.MOREX.r3.3HG0266310). In Arabidopsis, the closely related
513 Ca^{2+}/proton exchanger 11 (CAX11) is important to maintain cytosolic Ca^{2+} homeostasis and

514 was shown to be down-regulated in response to hypoxia and waterlogging (Wang *et al.*,
515 2016). Multiple transcription factors from the heat-shock and MYB transcription factor
516 families were also found in cluster 9. This is consistent with the observation that
517 differentially expressed MYB transcription factors were predominantly found to be down-
518 regulated in response to waterlogging in *Yerong* and *Deder2* (Borrego-Benjumea *et al.*,
519 2020). GO analysis of the 435 genes in cluster 9 showed an enrichment for terms related to
520 lipid and carbohydrate metabolism, transmembrane transporter activities and cell periphery
521 (Figure 5E), similarly to the GO terms identified in all genes down-regulated in response to
522 waterlogging (Figure 4E).

523 Clustering analysis also identified gene expression signatures that were specific to
524 individual varieties: cluster 1 (257 genes) contained genes whose expression was highest in
525 the barley varieties *Golden Promise* and *Infinity* and intermediate in *Passport*. In contrast,
526 the expression of these genes was markedly lower in the varieties *Pilastro* and *Regina*
527 (Figure 5F and Figure S6). GO analysis of genes in cluster 1 identified terms related to
528 catabolic and metabolic processes (Figure 5G), which could be relevant to waterlogging
529 tolerance/sensitivity. In contrast, genes in cluster 16 (359 genes) had an opposite behavior,
530 in that they were more highly expressed in *Pilastro* and *Regina*, but were only expressed at
531 low levels in the other varieties (Figure 5H and Figure S6). GO analysis of genes in cluster 16
532 revealed an enrichment for genes associated with diverse processes found in other clusters
533 or sets of genes, except the term 'nicotianamine metabolic process' which was only found in
534 the GO analysis of cluster 16 genes. This GO term relates to metal (including iron)
535 homeostasis, which could be of relevance to waterlogging stress tolerance because of the
536 increased uptake of metals and their toxicity to plants in waterlogged conditions. Notably,
537 genes in clusters 1 and 16 were not differentially expressed in response to waterlogging.
538 Instead, it is their intrinsic (or varietal-specific) expression level that differs between the
539 samples.

540

541 **Discussion**

542 Maintaining yields in the context of global climate change is a challenge, as crops are likely
543 to experience a wider range of stresses simultaneously or sequentially. Accordingly, efforts

544 are now made to improve crop resilience to multiple abiotic and biotic stresses, as opposed
545 to tolerance to a specific stress. At the same time, the trade-off between the gain of new
546 traits and yield also requires careful consideration (Bailey-Serres *et al.*, 2019). Waterlogging
547 in particular causes important crop losses and will become more prevalent in some regions.
548 Several QTLs have been identified with genes that could be targeted to improve crop
549 tolerance to waterlogging, but the role of candidate genes often needs to be validated, as
550 well as their suitability to improve crop tolerance to waterlogging. Alternatively, targeted
551 mutations in genes coding for key hypoxia response components, such as genes of the N-
552 degron protein degradation pathway, have yielded Arabidopsis and barley lines that are
553 more tolerant to waterlogging and other abiotic stresses (Mendiondo *et al.*, 2016; Vicente *et*
554 *al.*, 2017), albeit with a potential trade-off on their ability to resist infection by some
555 pathogens (de Marchi *et al.*, 2016; Gravot *et al.*, 2016; Vicente *et al.*, 2019).
556 Barley is an essential crop that exhibits varietal differences in its sensitivity to
557 waterlogging. The identification of potential candidate genes that can be targeted or used
558 as markers to identify waterlogging tolerant varieties would be of importance. One
559 approach to identifying candidate targets and markers is the comparison of genome-wide
560 transcriptional responses of different varieties to waterlogging. Here, after identifying a
561 time point (24 h) at which the expression of well-known hypoxia response marker genes
562 peaked, we screened a subset of 20 winter varieties to identify some with a differential
563 transcriptional response to waterlogging. Based on this analysis, we determined the
564 genome-wide transcriptional response to waterlogging of 4 winter barley varieties and of
565 the model spring variety *Golden Promise*. In agreement with previous transcriptional studies
566 with a range of plants and crops (Borrego-Benjumea *et al.*, 2020; Lee *et al.*, 2011; Lee and
567 Bailey-Serres, 2021; Luan *et al.*, 2022; Luan *et al.*, 2023; Mustroph *et al.*, 2010; Reynoso *et*
568 *al.*, 2019; van Veen *et al.*, 2016), our RNA-seq datasets indicate that the transcriptional
569 reprogramming that accompanies the onset of waterlogging response in barley involves
570 cellular processes such as carbohydrate metabolic processes, regulation of oxidative stress,
571 and metal homeostasis. Many of the up and down-regulated genes identified in this study
572 had also been shown to be differentially expressed in two other datasets generated using
573 distinct barley varieties and different experimental conditions (Borrego-Benjumea *et al.*,
574 2020; Luan *et al.*, 2022; Luan *et al.*, 2023). This overlap, together with the results of GO and
575 RT-qPCR analyses validate our datasets. We also identified a set of 98 waterlogging

576 response genes that were common to the five datasets generated in this study. Many of
577 these common DEGs are homologs of 'core hypoxia response genes' that were identified
578 across a range of plant species (Mustroph *et al.*, 2009) and that are involved in processes
579 such as cell wall metabolism, ethylene biosynthesis and signalling, carbohydrate metabolism
580 and ROS regulation. Multiple transcription factors belonging to the ERF, bHLH, and WRKY
581 families, which are known to be important for the onset of the waterlogging response
582 program, are also among these 98 common DEGs. Hence, our data indicate that, as
583 expected, the gene regulatory network that controls waterlogging response is conserved
584 amongst monocots and dicots (Mustroph *et al.*, 2010; Tamura and Bono, 2022).
585 Clustering analysis of the different DEGs identified groups of genes that behave
586 similarly between the 5 varieties in response to waterlogging (*e.g.*, all genes in clusters 14
587 and 9 are up or down-regulated, respectively). This analysis also revealed genes that are
588 differentially expressed between the varieties in the absence of treatment (see clusters 1
589 and 16 (Figures 5F and 5H)). While their expression does not typically change in response to
590 waterlogging, it is possible that either the low or high intrinsic expression of some of these
591 genes may 'predispose' certain varieties to either sensitivity or tolerance to waterlogging. In
592 other words, these genes could constitute variety-specific waterlogging susceptibility or
593 tolerance factors. Validating this possibility would require (i) an in-depth characterization of
594 the physiological responses and yield losses of a large number of varieties that would be
595 first grouped based on the intrinsic expression level of genes of interest; (ii) dissecting the
596 function of these genes, many of which have paralogs in barley; and (iii) generating barley
597 plants that are mutated or that constitutively express these different genes, followed by a
598 detailed characterization of the physiological response and yield under waterlogged
599 conditions. Potential trade-offs on other traits of agronomic relevance and on resilience to
600 other abiotic and biotic stresses would also need to be assessed. Considering the variety of
601 gene functions present in clusters 1 and 16, it is however difficult to predict which genes (or
602 gene families) are most likely to be of potential relevance to crop improvement. Their
603 association with GWAS and QTL identification would most likely help to pinpoint the best
604 candidates. For example, we compared our data to a list of 28 genes located in QTLs for
605 waterlogging tolerance (Supp. Table S4) that were identified following a screen of nearly
606 700 barley varieties with a specific focus on root traits (i.e. the formation of adventitious
607 roots and of root cortical aerenchyma) (Manik *et al.*, 2022). A number of candidate genes

608 that are a part of this list are differentially regulated between the control samples or the
609 waterlogged samples of the different varieties we tested. These genes include the homolog
610 of HRE2 (HORVU.MOREX.r3.6HG0621670) – one of the ERF-VII transcription factors that
611 regulates the hypoxia response program, as well as a potassium transporter
612 (HORVU.MOREX.r3.7HG0736590). This gene may be relevant because regulation of
613 potassium flux during waterlogging has been shown to be important (Gill *et al.*, 2018).
614 Although the expression does not necessarily change in response to waterlogging, the
615 intrinsic expression differences between varieties may be of interest to breeding
616 waterlogging tolerant varieties in barley.
617
618

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Author contributions

AM, AJB and EG conducted experiments; AJB, AM, JB, FW and EG analyzed RNA-seq datasets and wrote the manuscript.

Conflict of interest

The authors declare that they have no conflicts of interest.

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Data availability

The datasets supporting the results of this article are included within the article (and its supplemental files). RNA-seq data have been deposited with the Gene Expression Omnibus (GEO) repository (at http://www.ncbi.nlm.nih.gov/) under GSE220532. *Note to reviewers*: the data has been submitted and the authors are waiting for publication before release.

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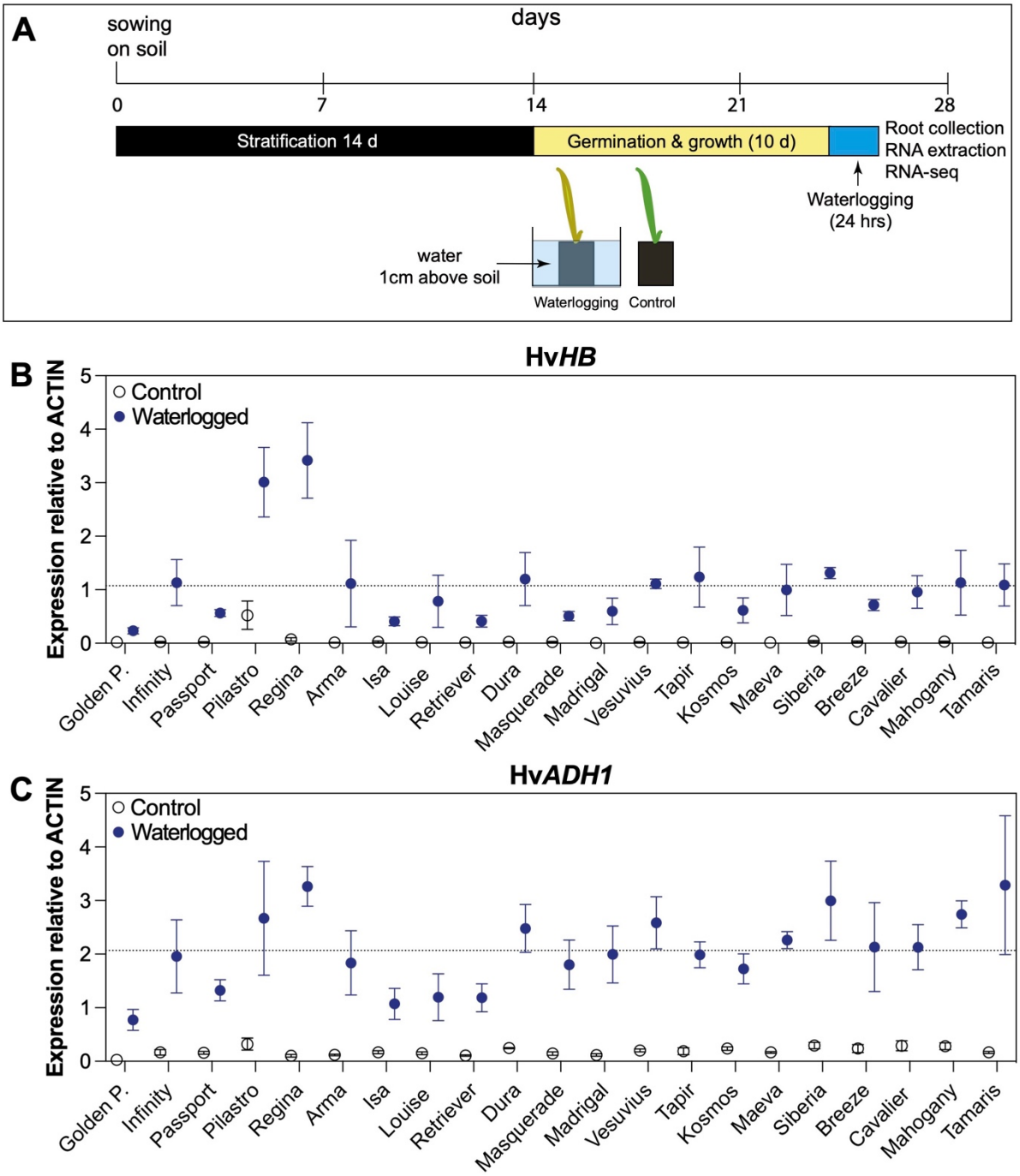
862 **Figures**



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	HvHB1	HvADH1	HvPDC1
Variety factor	<0.0001	0.0245	0.23
Treatment factor	<0.0001	<0.0001	<0.0001
Interaction	0.0002	0.0994	0.6043

863

864 **Figure 1: Differential expression of selected hypoxia response genes in 21 different barley**
865 **varieties.** (A) Experimental design of waterlogging experiments (see Materials and Methods
866 for details). (B) Hv*HB* expression relative to *ACTIN* in untreated (open symbols; normal

867 watering) and waterlogged (blue symbols) plants after 24 h of treatment. The dashed line
868 corresponds to the average relative expression of Hv*HB* for waterlogged samples of all
869 varieties and biological replicates. (C) Hv*ADH1* expression relative to *ACTIN* in untreated
870 (open symbols) and waterlogged (blue symbols) plants after 24 h of treatment. The dashed
871 line corresponds to the average relative expression of Hv*ADH1* for waterlogged samples of
872 all varieties and biological replicates. (D) *p*-value results of two-way ANOVA analysis of the
873 data presented in (B) and (C), and in Supp. Figure S2. Data shown in (B) and (C) are from
874 three biological replicates, except for *Golden Promise* for which 4 biological replicates were
875 used. Errors bars indicate SEM. Each biological replicate was obtained by pooling the root
876 systems from 3 plants prior to grinding for RNA extraction. Note: expression values for
877 *Infinity* shown in (B) and (C) were published in (Miricescu *et al.*, 2021).
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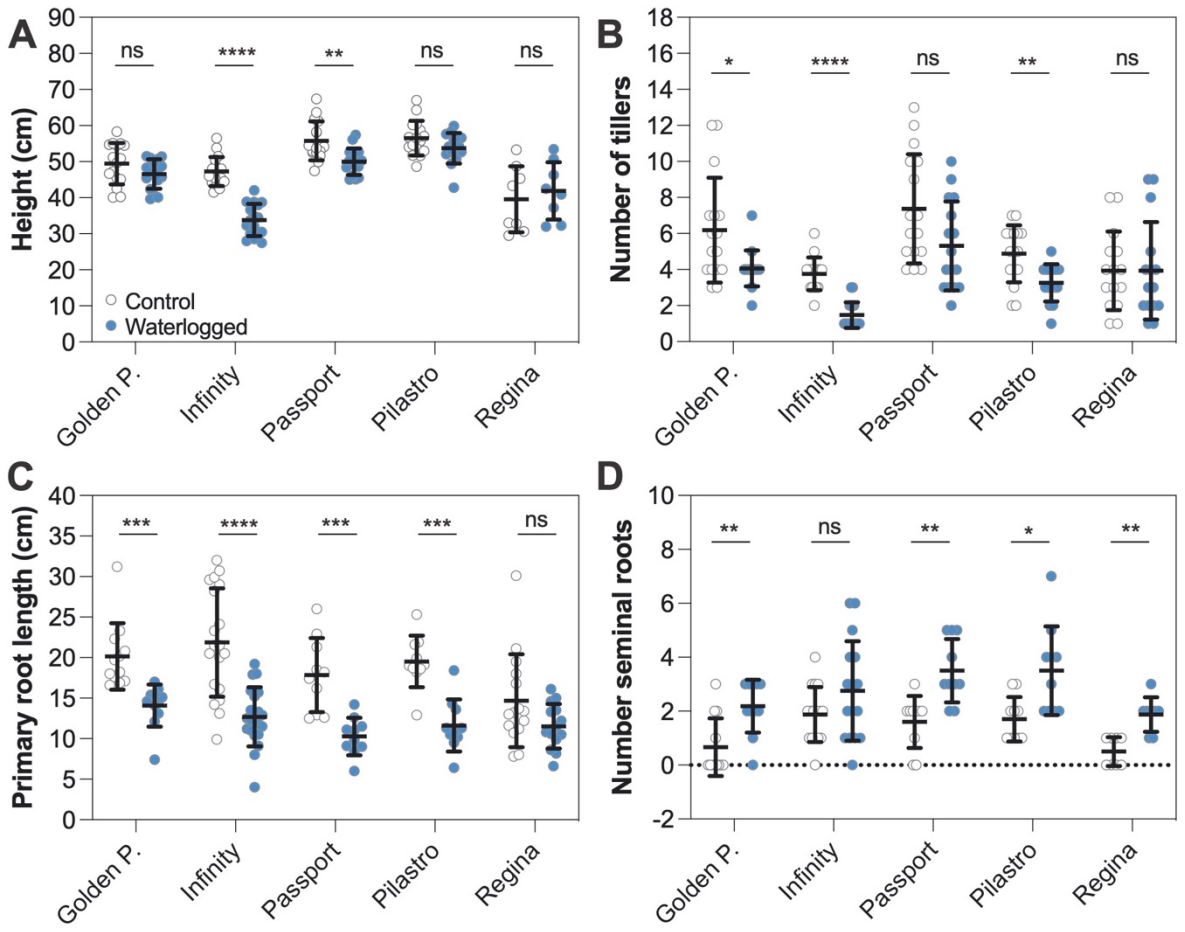

Figure 2: Effect of waterlogging on shoot and root growth. (A) Plant height after 14 d of waterlogging and a 6-week recovery period. For *Golden Promise*, *Passport* and *Pilastro*, three biological replicates (4 plants per biological replicate) were carried out. For *Regina* and *Infinity*, two biological replicates (4 plants per replicate) and 3 biological replicates (4 to 9 plants per replicate) were carried out, respectively. (B) Number of tillers after 14 d of waterlogging and a 6-week recovery period. Three biological replicates were carried out with at least 4 plants per replicate. (C) Length of the primary root after 14 d of waterlogging. For *Golden Promise*, *Passport*, *Pilastro* and *Regina*, three biological replicates with 2 to 7 plants each were carried out. For *Infinity*, 5 biological replicates were carried out with 3 to 4 plants per replicate. (D) Number of seminal roots after 14 d of waterlogging. For *Golden Promise*, *Passport*, and *Pilastro*, three biological replicates (2 to 4 plants per replicate) were carried out. For *Regina*, 2 biological replicates were conducted (4 plants per replicate). For *Infinity*, 4 biological replicates were carried out (4 plants per replicate). In (A-D), data from different biological replicates is color coded. Error bars correspond to standard deviations. Statistical analysis: multiple unpaired t-test with multiple comparison correction (Holm-

895 Sidak method). Asterisks: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$; ns: not
896 statistically significant.
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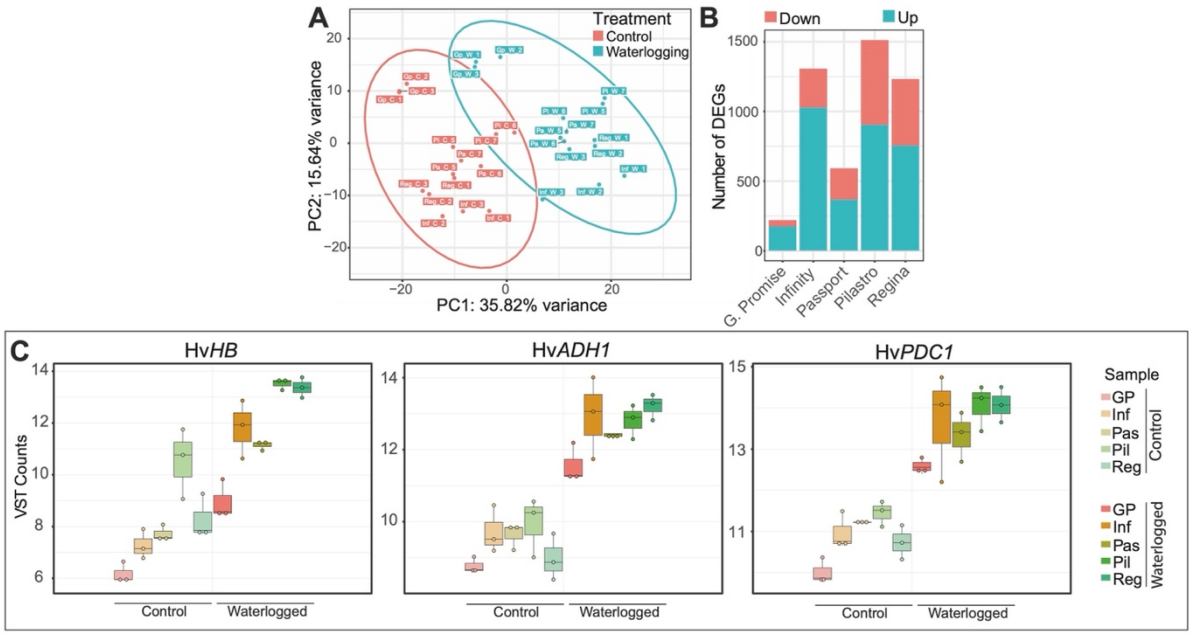

Figure 3: Results of RNA-seq experiments. (A) PCA plot showing Principal Component 2 (PC2) *versus* PC1 with ellipses showing sample grouping by treatment. (B) Absolute number of DEGs (filtered by adjusted *p*-value<0.05) in waterlogged *versus* control samples for each variety. (C) Box plots showing variance stabilised transformed (VST) read counts for Hv*HB* (HORVU.MOREX.r3.4HG0394960), Hv*ADH1* (HORVU.MOREX.r3.4HG0345760.1) and Hv*PDC1* (HORVU.MOREX.r3.4HG0384560) in control and waterlogged samples (*Golden Promise* (GP), *Infinity* (Inf), *Passport* (Pas), *Pilastro* (Pil) and *Regina* (Reg)).

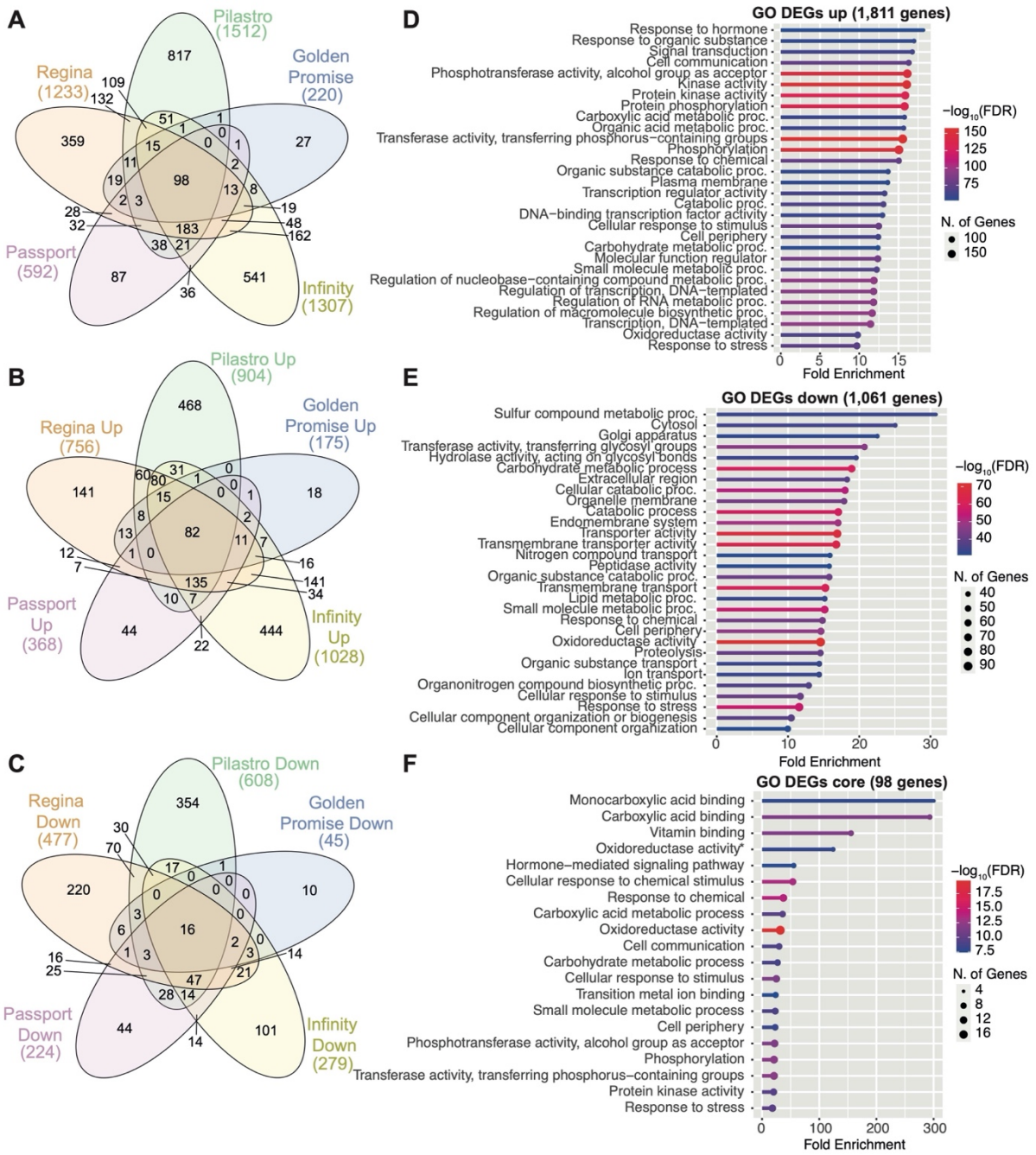

Figure 4: Comparison of *waterlogging* response genes in barley. (A) Overlap between the DEGs identified in the different varieties. (B) Overlap analysis of genes up-regulated in waterlogged samples compared to control samples for each variety. (C) Overlap between datasets for genes that are down-regulated in waterlogged samples compared to the controls in each variety. (D) The 30 most highly enriched GO terms among the 1,811 DEGs that are up-regulated in at least one variety in response to waterlogging. (E) The 30 most highly enriched GO terms enriched among the 1,061 DEGs that are down-regulated in any

916 variety in response to waterlogging. (F) The 20 most enriched GO terms among the 98 DEGs
917 common to all varieties when comparing waterlogged to control samples for each variety.
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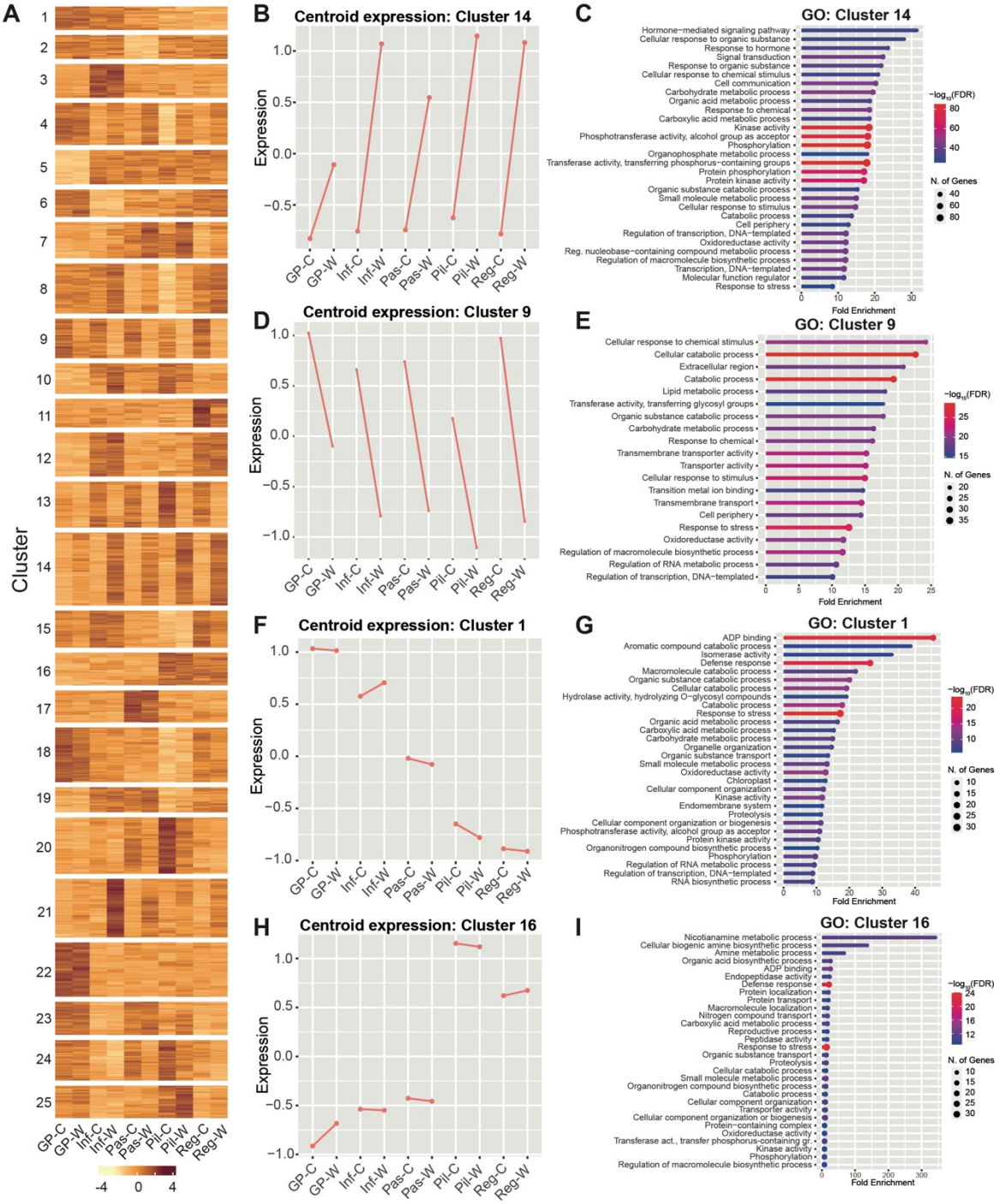

Figure 5: Clustering analysis of DEGs in waterlogged compared to control samples. (A) Heatmap showing the results of *k*-means clustering (*k*=25) of 10,882 DEGs (see Supp. Figure S6A for selection criteria). Image generated using *ComplexHeatmap* in *R*. (B) Plot of centroid expression for genes in cluster 14. (C) The 20 most highly enriched GO terms among the 802 genes identified in cluster 14. (D) Plot of centroid expression for DEGs in cluster 9. (E) The 20 most highly enriched GO terms among the 435 genes identified in cluster 9. (F) Plot of centroid expression for genes in cluster 1. (G) The 20 most highly enriched GO terms among

928 the 257 genes from cluster 1. (H) Plot of centroid expression for genes in cluster 16. (I) The
929 20 most highly enriched GO terms among the 359 genes from cluster 16.
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