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* and a winter variety, *Infinity*,
205 which was on the recommended list of barley varieties in Ireland (where the study was
206 conducted) at the time of the initial field trial whose results were taken into account for
207 varietal selection (Byrne *et al.*, 2022). Selected varieties for further characterization,
208 including transcriptome profiling, were *Golden Promise* (spring variety, 2-row), *Regina* and
209 *Infinity* (winter varieties, 2-row), *Passport* and *Pilastro* (winter varieties, 6-row).

210

211 **Plant growth conditions**

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

216

217 **Soil preparation and seed germination**

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

223

224 **Waterlogging**

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

235

236 **Height measurements**

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

239

240 **Total RNA extraction**

241 Roots of plants grown under control conditions or subjected to waterlogging for were rinsed
242 under running tap water, and frozen in liquid nitrogen. The tissue was ground in liquid
243 nitrogen and total RNA was extracted using Spectrum™ Plant Total RNA kit (Sigma-Aldrich).

244

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

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

258

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

260 For RNA-seq analysis, waterlogging treatment was applied as outlined above for 24 h. For
261 each independent replicate and each condition (waterlogged or untreated), the root
262 systems of 3 plants were pooled prior to grinding for total RNA extraction. Following RNA
263 extraction, RNA integrity was assessed using an Agilent 2100 Bioanalyzer (Agilent). All RNA
264 samples had RIN values > 7.0. Library preparation and single ended 50 bp next-generation
265 sequencing was performed by BGI Genomics (Hong Kong) using the DNBseq sequencing
266 platform.

267

268 **Analysis of RNA-seq data**

269 The third release (Morex V3) of the Morex barley genome was downloaded from e!DAL -
270 Plant Genomics and Phenomics Research Data Repository (PGP) (https://edal-pgp.ipk-
271 gatersleben.de/) (Mascher, 2021). Raw RNA-sequencing reads were aligned to Morex V3
272 using *bowtie2* (v2.4.5) (Langmead and Salzberg, 2012). Files were converted from .sam to
273 .bam files and indexed using *samtools* (v1.15.1) (Danecek *et al.*, 2021). Gene abundance was
274 estimated using *stringtie* (v2.1.7) (Pertea *et al.*, 2015) (Supp. Table S3). Differential gene
275 expression analysis was performed using the Bioconductor package *DeSeq2* (Love *et al.*,
276 2014) in *R* (Team, 2022) using a design in which the factors Variety and Treatment were
277 combined into a single factor to model multiple condition effects. The results of multiple
278 comparisons were extracted and filtered by adjusted *p*-value < 0.05 (Supp. Table S4).
279 Principal component analysis (PCA) was performed using *pcaExplorer* (v2.20.2) (Marini and
280 Binder, 2019) in *RStudio* (v2022.02.0+443). Gene ontology (GO) analysis was performed
281 using *ShinyGO* (v0.75c) (Ge *et al.*, 2020). Read count values were transformed by variance
282 stabilising transformations (VST) to normalise for library size and composition (Supp. Table
283 S5). Venn diagrams were generated using InteractiVenn (Heberle *et al.*, 2015).

284 The means of VST read counts from 3 independent replicates generated from
285 *DeSeq2* were filtered for *k*-means clustering as follows. *Deseq2* DEG analysis was performed
286 and results for the 25 comparisons shown in Supp. Fig S6A were extracted. A list of all
287 11,613 DEGs filtered by adjusted *p*-value < 0.05 was generated by combining DEGs from
288 waterlogged to control samples from the same variety, DEGs from each control to every
289 other control sample and DEGs from each waterlogged to every other waterlogged sample.
290 Next, the DEGs with a mean of <10 normalised reads were removed leaving 10,882 genes
291 for clustering analysis. Clustering analysis was performed using the *k*-means function in *R*
292 (v3.6.2) with the arguments centers=25, nstart = 1000, iter.max = 300 and algorithm =
293 "Lloyd" (gene cluster assignment annotated in (Supp. Table S4)). Clustering heatmaps were
294 generated using *ComplexHeatmap* (v2.10.0) (Gu *et al.*, 2016) in *R*. Plots were generated
295 using *ggplot2* (v3.3.6) (Wickam, 2016) and modified for style in Adobe Illustrator 2022.

296 To compare the datasets obtained to a previously published RNA-seq dataset, raw sets were
297 downloaded from NCBI's Gene Expression Omnibus (GSE144077) (Borrego-Benjumea *et al.*,
298 2020) and Sequence Read Archive (PRJNA889532) (Luan *et al.*, 2022). Raw RNA-sequencing
299 reads were aligned to Morex V3 as described above. Differential gene expression analysis of

300 waterlogging vs control for each variety was performed on 0 h control and 24 h
301 waterlogging datasets for the varieties Franklin and TX9425 from Luan *et al.* as described
302 above. The same differential gene expression analysis was also performed on 72 h control
303 and 72 h waterlogging datasets for the varieties Deder 2 and Yerong from Borrego-
304 Benjumea *et al.*.

305

306 ## Results

307 **Characterization of the transcriptional response of different barley varieties using hypoxia**
308 **response marker genes**

309 The previous characterization of 403 varieties from the AGOUEB collection under
310 waterlogged and control conditions in the field (Byrne *et al.*, 2022) provided some
311 information on the differential physiological response of these varieties to waterlogging,
312 while also highlighting the difficulties associated with the study of waterlogging tolerance
313 under field conditions. Based on this initial study, we selected a subset of 20 varieties that
314 behaved differently under waterlogged conditions, with the aim to assess their
315 transcriptional response to waterlogging under controlled conditions. The variety *Golden*
316 *Promise* was also included as a model (spring) barley variety that is widely used to generate
317 targeted mutations through *Agrobacterium*-mediated transformation. After identifying
318 Hv*ACTIN* as a suitable reference gene in waterlogged roots (Supp. Figure S1A), we first
319 carried out a time course experiment with four selected varieties and determined the
320 relative expression of *HEMOGLOBIN1 (HB)*, *ALCOHOL DEHYDROGENASE 1 (ADH1)* and
321 *PYRUVATE DECARBOXYLASE 1 (PDC1)*. These genes are hypoxia response markers commonly
322 used to monitor waterlogging response (Loreti *et al.*, 2020; Mendiondo *et al.*, 2016;
323 Mustroph *et al.*, 2010). This initial analysis indicated that (i) the expression of these three
324 hypoxia response genes peaked around 24 h after the onset of the waterlogging treatment;
325 and (ii) there were differences between the four varieties in terms of the amplitude of the
326 transcriptional up-regulation (Supp. Figure S1B). For example, at 24 h after the beginning of
327 the waterlogging treatment, Hv*HB* was expressed at higher levels in *Pilastro* compared to
328 *Arma*, *Tapir* and *Masquerade*. In addition, the up-regulation of Hv*HB* and Hv*ADH1* was
329 stronger than that of Hv*PDC1*, possibly making these first two genes more suitable to

330 identify varieties with differential transcriptional regulation of the hypoxia response
331 program.

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

349 Based on these results, *Pilastro* and *Regina* were chosen as representative 6-row and
350 2-row varieties, respectively, that potentially exhibit a stronger transcriptional response to
351 waterlogging, while *Passport* was selected as a 6-row variety that had a more dampened
352 transcriptional response. We also included *Infinity* as a 2-row variety for further
353 characterization because it is on the recommended list in Ireland (where this study was
354 carried out) and has an average transcriptional response to waterlogging. As highlighted
355 above, *Golden Promis*e was included as a reference variety.

356

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

358 The growth of the five selected varieties was characterized under controlled conditions
359 following a 2-week waterlogging treatment, and a 6-week recovery period with normal

360 watering. Height measurement showed that the growth of *Infinity* and *Passport* was more
361 affected by waterlogging than that of *Pilastro*, *Regina* and *Golden Promise*, whose height
362 was mostly unaffected by the treatment (Figure 2A). In addition, *Golden Promise*, *Infinity*
363 and *Pilastro* produced fewer tillers, whereas *Regina*'s tiller number was largely unaffected
364 (Figure 2B). Because root traits have been shown to be important for waterlogging
365 tolerance (Zhang *et al.*, 2015), we determined the length of the primary root, as well as the
366 number of seminal roots after 2 weeks of waterlogging. The length of the primary root was
367 reduced upon waterlogging stress for all varieties except *Regina* (Figure 2C), while the
368 number of seminal roots increased in all varieties and the difference was statistically
369 significant for all except *Infinity* (Figure 2D). This phenotypic characterization suggests that a
370 variety such as *Regina*, which has a stronger transcriptional response for Hv*ADH1* and Hv*HB*,
371 appears to be less impacted by waterlogging than other varieties.

372

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

374 To determine the transcriptional responses of *Pilastro*, *Regina*, *Passport*, *Infinity* and *Golden*
375 *Promise* to waterlogging, we subjected these varieties to waterlogging for 24 h. This time
376 point was chosen based on the time course experiment described above that showed peak
377 expression of Hv*HB*, Hv*ADH1* and Hv*PDC1* at 24 h of waterlogging. Total RNA was extracted
378 from roots for RNA-seq analysis. Reads obtained were aligned to the Morex barley genome
379 (version 3; Supp. Table S3 and Supp. Figure S3A-C) and differential gene expression
380 (adjusted *p*-value < 0.05) was determined for each of the waterlogged varieties relative to
381 the corresponding untreated variety (Supp. Table S4). A Principal Component Analysis (PCA)
382 indicated that the samples separated based on (i) the treatment (PC1: 35.82% variance)
383 (Figure 3A); (ii) whether they were winter or spring varieties (PC2: 15.64% variance; Supp.
384 Figure S3D); or (iii) whether they were 2-row or 6-row varieties (PC3: 11.34% variance; Supp.
385 Figure S3E). In this analysis, the varieties *Golden Promise*, *Infinity* and *Passport* could also be
386 separated from *Pilastro* and *Regina* (Figure S3F; PC4: 9.92% variance). This suggests that
387 there are underlying differences between these two groups of varieties that are not
388 explained by a known variable.

389 The number of differentially expressed genes (DEGs) identified in the different
390 varieties varied considerably: for *Golden Promise* and *Passport* we identified 220 and 592

391 DEGs (respectively), while *Infinity*, *Pilastro* and *Regina* had a substantially higher number of
392 DEGs (1,307; 1,512; and 1,233, respectively). In all varieties tested, the proportion of up-
393 regulated genes was higher than that of down-regulated genes (Figure 3B). We verified in
394 our datasets the expression of the Hv*HB*, Hv*ADH1* and Hv*PDC1* genes whose up-regulation
395 was initially used to monitor the transcriptional response of the different varieties to
396 waterlogging by RT-qPCR (Figure 1). Induction of all three genes upon waterlogging was
397 found (Figure 3C). We further identified additional homologs of Hv*ADH1* and Hv*HB* and
398 confirmed that these homologs were also up-regulated in response to waterlogging (Supp.
399 Figure S4).

400

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

402 Our RNA-seq experiment identified 2,865 genes that were differentially expressed in at least
403 one variety under waterlogged conditions compared to control plants (Supp. Table S4). The
404 variety with the highest proportion of unique DEGs was *Pilastro* (>50% unique DEGs), while
405 the majority of DEGs identified for *Golden Promise* and *Passport* were shared with other
406 varieties (Figure 4A) (<25% unique DEGs in these 2 varieties). Among the 2,865 DEGs, 1,078
407 genes were called as differentially expressed in more than one variety. Except for 7 genes,
408 the directionality of gene expression change was the same in all varieties, thus further
409 highlighting the similarities in the response of these varieties to waterlogging. As expected
410 based on the transcriptional response of Arabidopsis to waterlogging, Gene Ontology (GO)
411 analysis of the 1,811 DEGs that were up-regulated in at least one variety identified over-
412 represented terms related to hormone and stress responses, oxidoreductase activity, kinase
413 activity, as well as carbohydrate metabolic processes (Figure 4B and D). GO analysis of the
414 1,061 DEGs that were down-regulated in response to waterlogging identified terms related
415 to lipid and carbohydrate metabolism, transmembrane transporter activities and cell
416 periphery, as well as oxidoreductase activity and proteolysis (Figure 4C and E). All of these
417 terms are consistent with functional GO categories known to be central to the
418 transcriptional response of plants to waterlogging.

419 A total of 98 genes were detected as differentially expressed in the five varieties
420 (Figure 4A), and their directionality of expression change was the same across all datasets.
421 The majority of these genes were up-regulated in response to waterlogging (82 genes;

422 Figure 4B and Supp. Table S4) and included homologs of known core hypoxia response
423 genes in Arabidopsis (Mustroph *et al.*, 2009) such as *ADH1* (HORVU.MOREX.r3.4HG0345760,
424 HORVU.MOREX.r3.1HG0082960 and HORVU.MOREX.r3.4HG0345740), *PDC1*
425 (HORVU.MOREX.r3.4HG0384560), *HB1* (HORVU.MOREX.r3.4HG0394960,
426 HORVU.MOREX.r3.7HG0715010) and a respiratory burst oxidase (*RBOH*)
427 (HORVU.MOREX.r3.5HG0444960). Furthermore, this set included homologs of ERF-VII
428 transcription factors, such as *HRE1* (HORVU.MOREX.r3.4HG0405650 and
429 HORVU.MOREX.r3.4HG0405650), *HRE2* (HORVU.MOREX.r3.5HG0497970) and *RAP2.3*,
430 (HORVU.MOREX.r3.1HG0060770). This result is in agreement with the central role of ERF-VII
431 transcription factors as master regulators of hypoxia and waterlogging response. Sixteen
432 DEGs common to all datasets were down-regulated in response to waterlogging (Figure 4C),
433 including three peroxidase genes (HORVU.MOREX.r3.2HG0215440,
434 HORVU.MOREX.r3.2HG0119650, HORVU.MOREX.r3.2HG0119630) a nitrate transporter
435 (HORVU.MOREX.r3.7HG0700030), and an aldehyde dehydrogenase
436 (HORVU.MOREX.r3.2HG0173750). GO analysis of the set of 98 common DEGs (Figure 4F)
437 revealed largely similar terms to those identified for all up-regulated DEGs (Figure 4D),
438 including GO terms such as oxidoreductase activity, carbohydrate metabolic process, and
439 hormone-mediated signaling.

440 We next compared our RNA-seq results to two recently published RNA-seq datasets.
441 Luan et al. analyzed the transcriptomic response of 4-leaf stage Franklin and TX9425 barley
442 roots to waterlogging for 24 or 72 h (Luan *et al.*, 2022). These were two-row spring varieties,
443 with Franklin showing sensitivity and TX9425 showing tolerance to waterlogging (Luan *et al.*,
444 2022). In addition, Borrego-Benjumea *et al*. analyzed the transcriptomic response of
445 waterlogged barley roots in 2-week-old *Yerong* and *Deder2* for 72 or 120 h (Borrego-
446 Benjumea *et al.*, 2020). Both varieties had been chosen because of their tolerance to
447 waterlogging (Li *et al.*, 2008; Takeda, 1989). Raw RNA-seq data were analyzed using the
448 same pipeline as for our datasets. PCA analysis of all datasets showed that most of the
449 variation was due to the origin of the dataset (Figure S5A; PC1: 68.39% variance), likely
450 because waterlogging stress is known to vary considerably based on experimental
451 differences, such as soil type. As expected, the different samples could also be separated
452 based on treatment (Figure S5A; PC2: 17.03% variance). To facilitate the comparison

453 between the different datasets, we selected the most similar timepoints to those of our
454 study (*i.e.*, 0 h control and 24 h waterlogged for Luan *et al.*; and 72 h control and
455 waterlogged for Borrego-Benjumea *et al.*). Despite the differences in barley varieties, age of
456 the plants and duration of the waterlogging treatment, a statistically significant overlap was
457 observed between both the up and down-regulated genes in our datasets and in those
458 recently published datasets DEGs (Supp. Figure S5B and S5C) (Borrego-Benjumea *et al.*,
459 2020; Luan *et al.*, 2022). GO analysis of the 448 DEGs that were up-regulated in all datasets
460 identified terms related to metabolism and oxidoreductase activity (Supp. Figure S5F), again
461 in agreement with known key aspects of plant responses to waterlogging. Genes associated
462 with transporter activity were found among the 309 shared down-regulated genes (Supp.
463 Figure S5G).

464

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

466 To compare the responses to waterlogging between the 5 varieties used in this study, *k*-
467 means clustering was performed on 10,882 genes identified as differentially expressed in: (i)
468 at least one variety when comparing waterlogged to control samples for each variety, or (ii)
469 between the control samples (*i.e.*, these have intrinsic differential expression between at
470 least 2 varieties in the absence of waterlogging), or (iii) between the waterlogged samples
471 from different varieties (*i.e.*, genes that are differentially expressed between at least 2
472 varieties under waterlogging) (Supp. Figure S6A). Clustering analysis revealed distinct gene
473 expression patterns between samples (Figure 5A). A number of clusters contained genes
474 that were up-regulated in multiple varieties in response to waterlogging compared to
475 untreated plants (*i.e.*, clusters 4, 7, 8, 14, and 21). The largest cluster (#14) comprised 802
476 genes (Supp. Table S4) with a centroid expression that was higher in all varieties in response
477 to waterlogging (Figure 5B and Supp. Figure S6). Cluster 14 included homologs of known
478 hypoxia response genes such as those described above in the common set of 82 up-
479 regulated DEGs in barley (Figure 4B), as well as homologs of Arabidopsis *RAP2.12*
480 (HORVU.MOREX.r3.5HG0481240), additional homologs of *RAP2.3*,
481 (HORVU.MOREX.r3.2HG0112960), and homologs of *ADH1* (HORVU.MOREX.r3.2HG0115170,
482 HORVU.MOREX.r3.3HG0252910) (Supp. Figure S4A and B). Furthermore, transcription
483 factors (TFs) from the ERF, bHLH, bZIP (basic leucine zipper), NAC (NAM, ATAF1,2, CUC2) and

484 WRKY families were also identified in cluster 14 (Supp. Table S4), all of which belong to
485 families with established and conserved roles in the regulation of hypoxia response genes
486 (Lee and Bailey-Serres, 2021; Mustroph *et al.*, 2010; Reynoso *et al.*, 2019). GO analysis of
487 the 802 genes in cluster 14 identified terms related to hormone and stress responses,
488 oxidoreductase and kinase activity and metabolism (Figure 5C).

489 In contrast, clusters 5, 9, 13, 15, 20 and 24 comprised genes that were down-
490 regulated in response to waterlogging in multiple varieties. Cluster 9 contained 435 genes
491 that were down-regulated in response to waterlogging (Figure 5D and Supp. Figure S6),
492 including 14 of the 16 genes that were down-regulated in waterlogged samples from all
493 varieties (Figure 4C and Supp. Table S4). Among these genes was an aminotransferase
494 (HORVU.MOREX.r3.2HG0190680), whose top-scoring Arabidopsis homolog is γ-
495 aminobutyric acid transaminase (GABA-T; AT3G22200). In Arabidopsis, the expression of this
496 gene is also reduced in response to hypoxia (Branco-Price *et al.*, 2008). Another member of
497 cluster 9 whose expression was downregulated in all varieties in response to waterlogging is
498 a cation exchanger (HORVU.MOREX.r3.3HG0266310). In Arabidopsis, the closely related
499 Ca^{2+}/proton exchanger 11 (CAX11) is important to maintain cytosolic Ca^{2+} homeostasis and
500 was shown to be down-regulated in response to hypoxia and waterlogging (Wang *et al.*,
501 2016). Multiple transcription factors from the heat-shock and MYB transcription factor
502 families were also found in cluster 9. This is consistent with the observation that
503 differentially expressed MYB transcription factors were predominantly found to be down-
504 regulated in response to waterlogging in *Yerong* and *Deder2* (Borrego-Benjumea *et al.*,
505 2020). GO analysis of the 435 genes in cluster 9 showed an enrichment for terms related to
506 lipid and carbohydrate metabolism, transmembrane transporter activities and cell periphery
507 (Figure 5E), similarly to the GO terms identified in all genes down-regulated in response to
508 waterlogging (Figure 4E).

509 Clustering analysis also identified gene expression signatures that were specific to
510 individual varieties: cluster 1 (257 genes) contained genes whose expression was highest in
511 the barley varieties *Golden Promise* and *Infinity* and intermediate in *Passport*. In contrast,
512 the expression of these genes was markedly lower in the varieties *Pilastro* and *Regina*
513 (Figure 5F and Figure S6). GO analysis of genes in cluster 1 identified terms related to
514 catabolic and metabolic processes (Figure 5G), which could be relevant to waterlogging

515 tolerance/sensitivity. In contrast, genes in cluster 16 (359 genes) had an opposite behavior,
516 in that they were more highly expressed in *Pilastro* and *Regina*, but were only expressed at
517 low levels in the other varieties (Figure 5H and Figure S6). GO analysis of genes in cluster 16
518 revealed an enrichment for genes associated with diverse processes found in other clusters
519 or sets of genes, except the term 'nicotianamine metabolic process' which was only found in
520 the GO analysis of cluster 16 genes. This GO term relates to metal (including iron)
521 homeostasis, which could be of relevance to waterlogging stress tolerance because of the
522 increased uptake of metals and their toxicity to plants in waterlogged conditions. Notably,
523 genes in clusters 1 and 16 were not differentially expressed in response to waterlogging.
524 Instead, it is their intrinsic (or varietal-specific) expression level that differs between the
525 samples.

526

527 **Discussion**

528 Maintaining yields in the context of global climate change is a challenge, as crops are likely
529 to experience a wider range of stresses simultaneously or sequentially. Accordingly, efforts
530 are now made to improve crop resilience to multiple abiotic and biotic stresses, as opposed
531 to tolerance to a specific stress. At the same time, the trade-off between the gain of new
532 traits and yield also requires careful consideration (Bailey-Serres *et al.*, 2019). Waterlogging
533 in particular causes important crop losses and will become more prevalent in some regions.
534 Several QTLs have been identified with genes that could be targeted to improve crop
535 tolerance to waterlogging, but the role of candidate genes often needs to be validated, as
536 well as their suitability to improve crop tolerance to waterlogging. Alternatively, targeted
537 mutations in genes coding for key hypoxia response components, such as genes of the N-
538 degron protein degradation pathway, have yielded Arabidopsis and barley lines that are
539 more tolerant to waterlogging and other abiotic stresses (Mendiondo *et al.*, 2016; Vicente *et*
540 *al.*, 2017), albeit with a potential trade-off on their ability to resist infection by some
541 pathogens (de Marchi *et al.*, 2016; Gravot *et al.*, 2016; Vicente *et al.*, 2019).
542 Barley is an essential crop that exhibits varietal differences in its sensitivity to
543 waterlogging. The identification of potential candidate genes that can be targeted or used
544 as markers to identify waterlogging tolerant varieties would be of importance. One
545 approach to identifying candidate targets and markers is the comparison of genome-wide

546 transcriptional responses of different varieties to waterlogging. Here, after identifying a
547 time point (24 h) at which the expression of well-known hypoxia response marker genes
548 peaked, we screened a subset of 20 winter varieties to identify some with a differential
549 transcriptional response to waterlogging. Based on this analysis, we determined the
550 genome-wide transcriptional response to waterlogging of 4 winter barley varieties and of
551 the model spring variety *Golden Promise*. In agreement with previous transcriptional studies
552 with a range of plants and crops (Borrego-Benjumea *et al.*, 2020; Lee *et al.*, 2011; Lee and
553 Bailey-Serres, 2021; Luan *et al.*, 2022; Luan *et al.*, 2023; Mustroph *et al.*, 2010; Reynoso *et*
554 *al.*, 2019; van Veen *et al.*, 2016), our RNA-seq datasets indicate that the transcriptional
555 reprogramming that accompanies the onset of waterlogging response in barley involves
556 cellular processes such as carbohydrate metabolic processes, regulation of oxidative stress,
557 and metal homeostasis. Many of the up and down-regulated genes identified in this study
558 had also been shown to be differentially expressed in two other datasets generated using
559 distinct barley varieties and different experimental conditions (Borrego-Benjumea *et al.*,
560 2020; Luan *et al.*, 2022; Luan *et al.*, 2023). This overlap, together with the results of GO and
561 RT-qPCR analyses validate our datasets. We also identified a set of 98 waterlogging
562 response genes that were common to the five datasets generated in this study. Many of
563 these common DEGs are homologs of 'core hypoxia response genes' that were identified
564 across a range of plant species (Mustroph *et al.*, 2009) and that are involved in processes
565 such as cell wall metabolism, ethylene biosynthesis and signalling, carbohydrate metabolism
566 and ROS regulation. Multiple transcription factors belonging to the ERF, bHLH, and WRKY
567 families, which are known to be important for the onset of the waterlogging response
568 program, are also among these 98 common DEGs. Hence, our data indicate that, as
569 expected, the gene regulatory network that controls waterlogging response is conserved
570 amongst monocots and dicots (Mustroph *et al.*, 2010; Tamura and Bono, 2022).
571 Clustering analysis of the different DEGs identified groups of genes that behave
572 similarly between the 5 varieties in response to waterlogging (*e.g.*, all genes in clusters 14
573 and 9 are up or down-regulated, respectively). This analysis also revealed genes that are
574 differentially expressed between the varieties in the absence of treatment (see clusters 1
575 and 16 (Figures 5F and 5H)). While their expression does not typically change in response to
576 waterlogging, it is possible that either the low or high intrinsic expression of some of these
577 genes may 'predispose' certain varieties to either sensitivity or tolerance to waterlogging. In

578 other words, these genes could constitute variety-specific waterlogging susceptibility or
579 tolerance factors. Validating this possibility would require (i) an in-depth characterization of
580 the physiological responses and yield losses of a large number of varieties that would be
581 first grouped based on the intrinsic expression level of genes of interest; (ii) dissecting the
582 function of these genes, many of which have paralogs in barley; and (iii) generating barley
583 plants that are mutated or that constitutively express these different genes, followed by a
584 detailed characterization of the physiological response and yield under waterlogged
585 conditions. Potential trade-offs on other traits of agronomic relevance and on resilience to
586 other abiotic and biotic stresses would also need to be assessed. Considering the variety of
587 gene functions present in clusters 1 and 16, it is however difficult to predict which genes (or
588 gene families) are most likely to be of potential relevance to crop improvement. Their
589 association with GWAS and QTL identification would most likely help to pinpoint the best
590 candidates.
591
592

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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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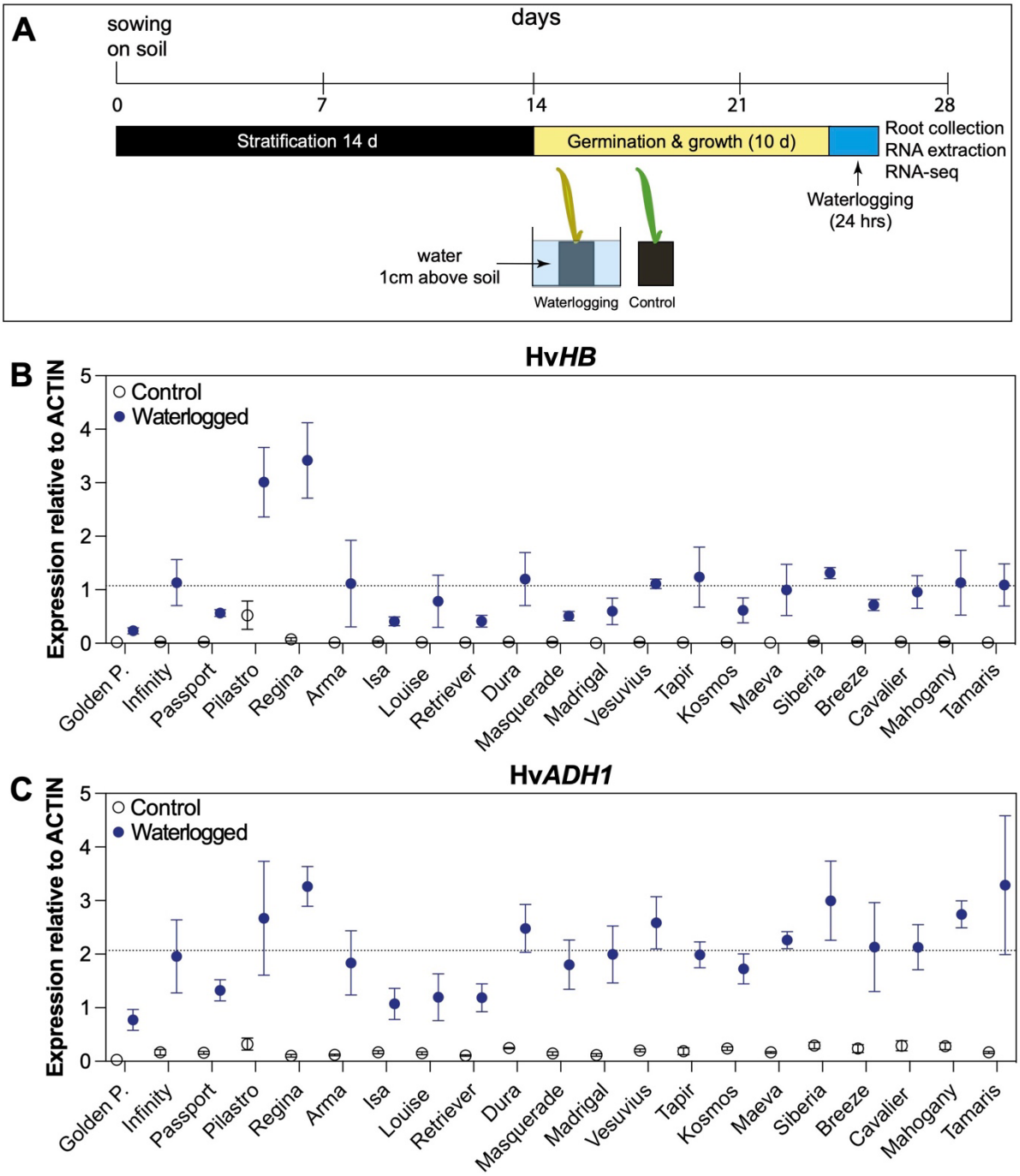
825 **Figures**



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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

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

830 watering) and waterlogged (blue symbols) plants after 24 h of treatment. The dashed line
831 corresponds to the average relative expression of Hv*HB* for waterlogged samples of all
832 varieties and replicates. (C) Hv*ADH1* expression relative to *ACTIN* in untreated (open
833 symbols) and waterlogged (blue symbols) plants after 24 h of treatment. The dashed line
834 corresponds to the average relative expression of Hv*ADH1* for waterlogged samples of all
835 varieties and replicates. (D) *p*-value results of two-way ANOVA analysis of the data
836 presented in (B) and (C), and in Supp. Figure S2. Data shown in (B) and (C) are from three
837 independent replicates, except for *Golden Promise* for which 4 independent replicates were
838 used. Errors bars indicate SEM. Each independent replicate was obtained by pooling the
839 root systems from 3 plants prior to grinding for RNA extraction. Note: expression values for
840 *Infinity* shown in (B) and (C) were published in (Miricescu *et al.*, 2021).
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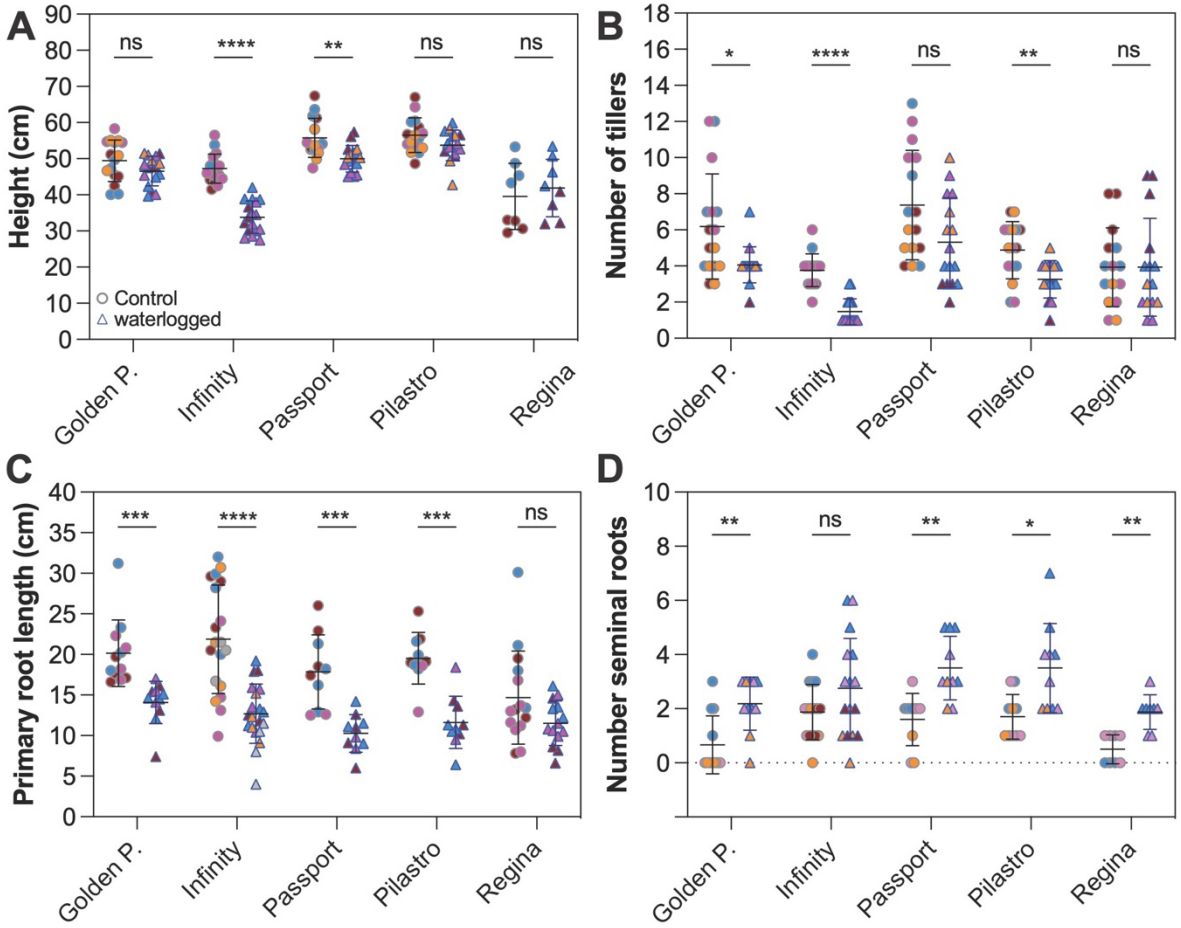

Figure 2: Effect of waterlogging on shoot and root growth. (A) Plant height after 14 d of waterlogging and a 6-week recovery period. For *Golden Promise*, *Passport* and *Pilastro*, three independent replicates (4 plants per replicate) were carried out. For *Regina* and *Infinity*, two independent replicates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

858 (Holm-Sidak method). Asterisks: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$; ns: not
859 statistically significant.
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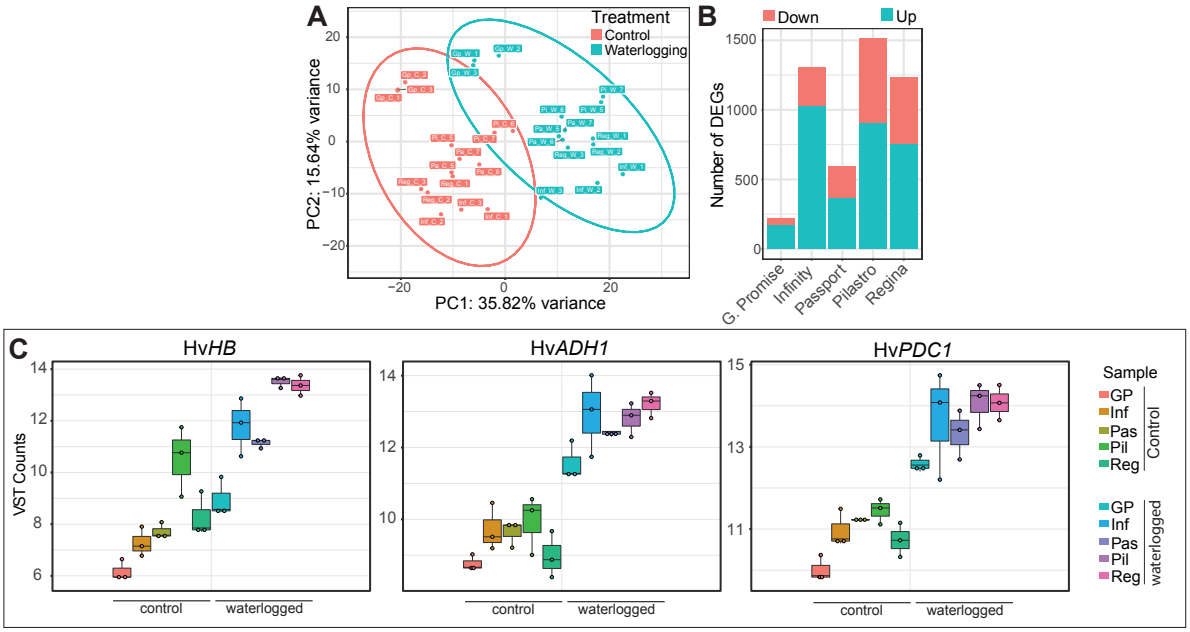

Figure 3: Results of RNA-seq experiments. (A) PCA plot showing Principal Component 2 (PC2) *versus* PC1 with ellipses showing sample grouping by treatment. (B) Absolute number of DEGs (filtered by adjusted *p*-value<0.05) in waterlogged *versus* control samples for each variety. (C) Box plots showing 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)).

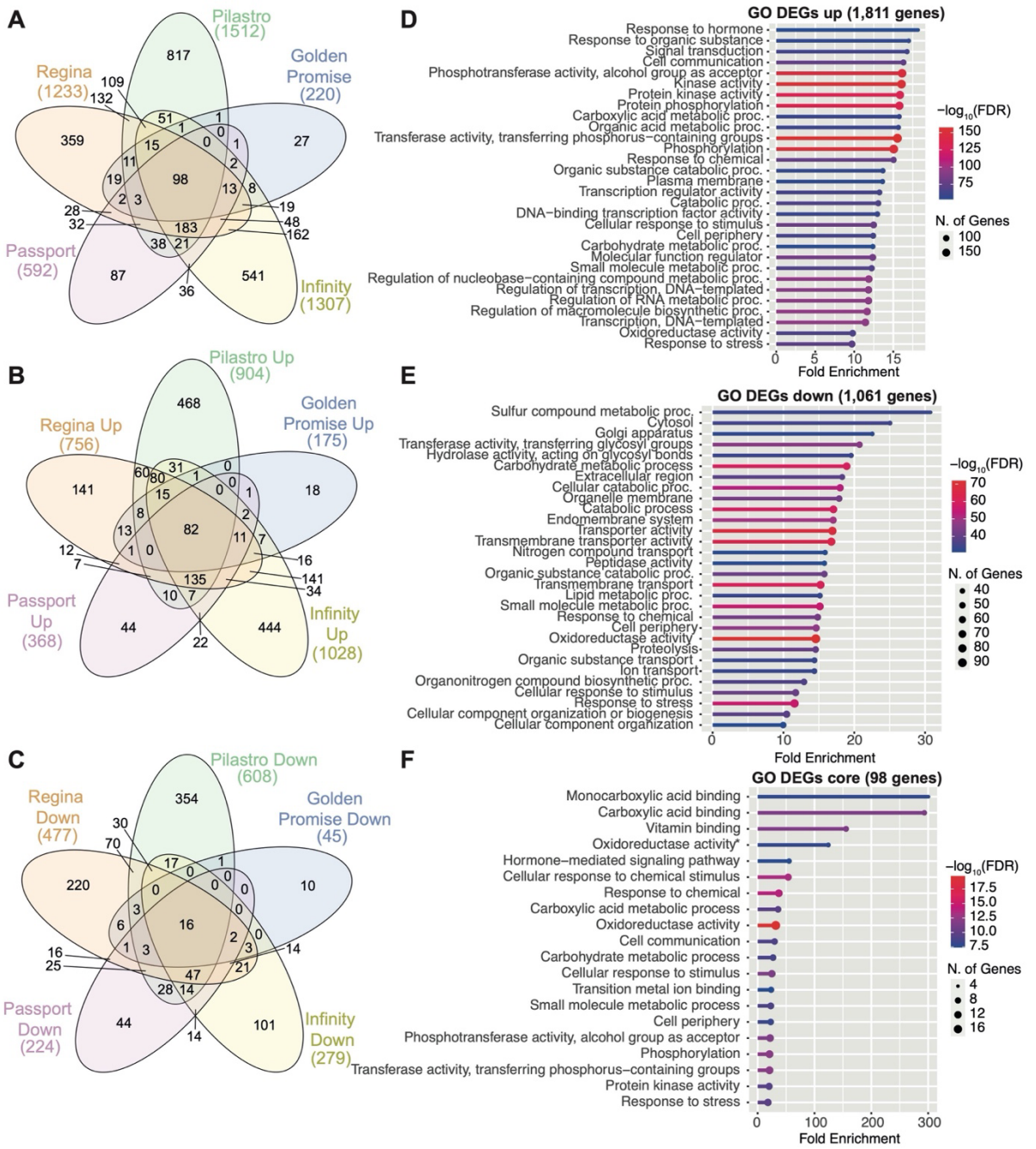

Figure 4: Comparison of *waterlogging* response genes in barley. (A) Overlap between the DEGs identified in the different varieties. (B) Overlap analysis of genes up-regulated in waterlogged samples compared to control samples for each variety. (C) Overlap between datasets for genes that are down-regulated 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

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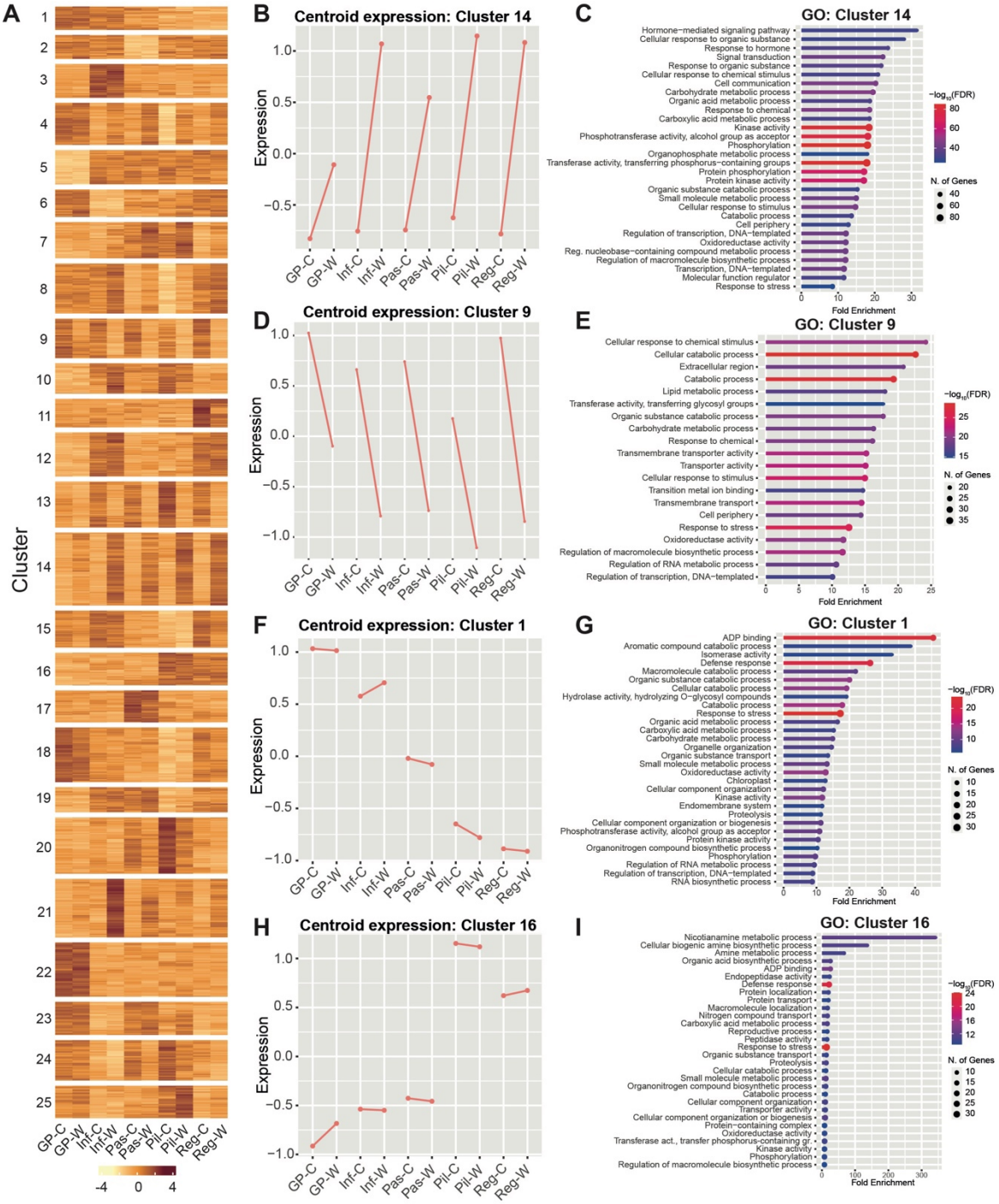

Figure 5: Clustering analysis of DEGs in waterlogged compared to control samples. (A) Heatmap showing the results of *k*-means clustering (*k*=25) of 10,882 DEGs (see Supp. Figure S6A for selection criteria). Image generated using *ComplexHeatmap* in *R*. (B) Plot of centroid expression for genes in 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

891 the 257 genes from cluster 1. (H) Plot of centroid expression for genes in cluster 16. (I) The
892 20 most highly enriched GO terms among the 359 genes from cluster 16.
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