Transcriptional analysis of waterlogging response in barley identifies signatures of waterlogging tolerance/sensitivity

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Highlight

This study identifies 5 barley varieties with different tolerance to waterlogging. We provide the first RNA-seq datasets identifying both conserved and unique transcriptional responses in both waterlogging tolerant and sensitive barley varieties.

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, the molecular mechanisms underlying this trait are mostly unknown. Here, we surveyed 21 barley varieties for the transcriptional activation of conserved hypoxia-response genes under waterlogging. We selected five varieties that showed differential induction of core hypoxia-response genes during waterlogging treatment and further characterized their phenotypic response to waterlogging stress, with the aim of categorizing them as waterlogging sensitive or tolerant varieties. RNA-sequencing to evaluate the transcriptional responses of these selected varieties to waterlogging led to the identification of a set of 98 core waterlogging-response genes in barley. We further identified groups of genes whose expression correlated with the varietal differences in waterlogging tolerance. These datasets identify potential candidates for molecular markers and/or gene targeting to improve waterlogging tolerance in barley.

Keywords

Waterlogging; barley; 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
CAT: cation exchanger
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
GS: glutamine synthetase 1
GST: glutathione-S-transferase
GWAS: genome-wide association studies
HB: hemoglobin
HRE: hypoxia responsive ethylene response factor
HRPE: hypoxia-responsive promoter element
Hv: *Hordeum vulgare*
IBSC: international barley sequencing consortium
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

102 ## Introduction

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

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

133 under hypoxic conditions, ERF-VIIs accumulate in the nucleus where they bind to *cis*-
134 regulatory elements to trigger the hypoxia response program. Notably, the promoters of
135 conserved hypoxia response gene families are not only enriched for *cis*-regulatory elements
136 bound by transcription factors of the ERF-VII family (e.g. HRPE motif (Gasch *et al.*, 2016)),
137 but also for motifs bound by basic helix-loop-helix (bHLH), MYB and WRKY transcription
138 factors (Reynoso *et al.*, 2019), thus suggesting the involvement of other families of
139 transcription factors in the regulation of the hypoxia response program (Lee and Bailey-
140 Serres, 2021). This is in agreement with the up-regulation of genes coding for transcription
141 factors belonging to the different families mentioned above as part of hypoxia response in
142 plants (Mustroph *et al.*, 2010). Cross-comparison of multiple transcriptomic datasets from
143 different plant species also allowed the identification of core genes of the hypoxia response
144 program that are conserved in plants (Mustroph *et al.*, 2010; Mustroph *et al.*, 2009;
145 Reynoso *et al.*, 2019). These include genes involved in (i) the regulation of carbon
146 metabolism in order to facilitate ATP production *via* glycolysis and NAD+ regeneration
147 through fermentation pathways; (ii) the regulation of important signaling pathways (e.g.
148 MAPK signaling) and molecules (e.g. reactive oxygen species (ROS)) to promote plant
149 tolerance and survival upon hypoxia.

150 Numerous transcriptomic studies using either flooding or waterlogging treatments
151 have been conducted using the model plant *Arabidopsis thaliana* (see list in (Brazel and
152 Graciet, 2023)). While experimental conditions vary, core aspects of the transcriptional
153 reprogramming upon waterlogging or flooding overlap with those identified upon hypoxia
154 (Lee *et al.*, 2011; van Veen *et al.*, 2016). Furthermore, the transcriptomic comparison of 8
155 different natural accessions of Arabidopsis, which had been previously identified as being
156 either sensitive or tolerant to flooding (Vashisht *et al.*, 2011), indicated that the
157 transcriptional response of roots and shoots differ (van Veen *et al.*, 2016). This work
158 identified sets of shoot or root-expressed genes that might contribute to the flooding
159 tolerance phenotype of some natural Arabidopsis accessions (van Veen *et al.*, 2016), which
160 could be relevant to improve crop tolerance to waterlogging/flooding. Notably, the
161 transcriptional reprogramming in response to hypoxia and flooding is also accompanied by
162 other genome-wide regulatory mechanisms such as epigenetic changes, chromatin

163 remodelling (Reynoso *et al.*, 2019), and changes in translation (Lee and Bailey-Serres, 2019;
164 Reynoso *et al.*, 2019).

165 Waterlogging (as well as flooding) is an important source of crop losses (de San
166 Celedonio *et al.*, 2014; Kaur *et al.*, 2020), however, not all crop species are equally affected.
167 Barley has been identified as a particularly sensitive crop with up to 20-25% crop losses due
168 to waterlogging (Byrne *et al.*, 2021; de San Celedonio *et al.*, 2016; de San Celedonio *et al.*,
169 2014; Liu *et al.*, 2020; Miricescu *et al.*, 2021; Setter and Waters, 2003). Losses have been
170 shown to be more severe if waterlogging occurs at the heading stage (de San Celedonio *et*
171 *al.*, 2016; de San Celedonio *et al.*, 2014; Liu *et al.*, 2020; Setter and Waters, 2003), but also
172 at early developmental stages (de San Celedonio *et al.*, 2016; de San Celedonio *et al.*, 2014).
173 The multifaceted nature of waterlogging stress, as well as the complexity of plant responses
174 to this stress, have made it difficult to identify marker genes of waterlogging tolerance, as
175 well as target genes that could be modified to improve crop tolerance to waterlogging.
176 Nevertheless, in recent years, genetic approaches, as well as linkage mapping and genome-
177 wide association studies (GWAS) have led to the identification of potential QTLs and target
178 genes to improve barley tolerance to waterlogging (Bertholdsson *et al.*, 2015; Borrego-
179 Benjumea *et al.*, 2020; Broughton *et al.*, 2015; Gill *et al.*, 2019; Li *et al.*, 2008; Manik *et al.*,
180 2022; Zhang *et al.*, 2017; Zhang *et al.*, 2016; Zhou, 2011). In agreement with the complex
181 response to waterlogging, these QTLs have been identified based on a wide range of
182 phenotypic traits, including leaf yellowing, chlorophyll fluorescence, yield traits,
183 adventitious root formation, aerenchyma formation and ROS levels. QTLs relating to root
184 traits, including the ability to form adventitious roots and aerenchyma under waterlogged
185 conditions (Manik *et al.*, 2022; Zhang *et al.*, 2016), may be of particular relevance
186 considering their known roles in facilitating water and nutrient uptake as well as in oxygen
187 distribution during waterlogging (Zhang *et al.*, 2015).

188 Despite the progress made in recent years in the identification of QTLs for
189 waterlogging tolerance and the realization that transcriptomics may be used to identify
190 potential candidate genes relevant to waterlogging tolerance (Lee *et al.*, 2011; Reynoso *et*
191 *al.*, 2019; van Veen *et al.*, 2016), only few such studies have been conducted with barley.
192 One recent study has characterized the transcriptomic response of two waterlogging-
193 tolerant varieties (Borrego-Benjumea *et al.*, 2020). The authors showed a high degree of

194 conservation between the waterlogging response program in barley and that of other plants
195 (e.g., Arabidopsis). Furthermore, they identified a handful of genes that could be of
196 importance for waterlogging tolerance mechanisms in barley (Borrego-Benjumea *et al.*,
197 2020). In another study, a comparative proteomic analysis of one waterlogging-sensitive and
198 one waterlogging-tolerant variety identified proteins that accumulate differently in
199 waterlogging sensitive or tolerant germplasms (Luan *et al.*, 2018a; Luan *et al.*, 2018b). These
200 proteins included pyruvate decarboxylase (PDC) and an 1-aminocyclopropane-1-carboxylate
201 oxidase 1-like (ACO) protein, which accumulated to higher levels in the waterlogging
202 tolerant variety. In addition, correlation between protein levels and gene expression using
203 RT-qPCR analyses indicated that some genes (*e.g. PDC*, glutamine synthetase (*GS1*), *ACO*
204 and some glutathione-S-transferases (*GSTs*)) are more strongly up-regulated in the
205 waterlogging tolerant variety (Luan *et al.*, 2018b). To our knowledge, no transcriptomic
206 studies have attempted to compare the genome-wide gene expression response of
207 waterlogging-sensitive and tolerant varieties in barley, despite the fact that this approach
208 could lead to the identification of transcriptional signatures that correlate with waterlogging
209 tolerance or sensitivity.

210 Here, to identify barley varieties that are waterlogging sensitive or tolerant, we used
211 varieties from the Association Genetics Of UK Elite Barley (AGOUEB) population, which
212 consists of barley varieties that have been commercialized on the UK National and
213 Recommended List trials between 1988 and 2006 (Thomas *et al.*, 2014). Next, we
214 characterized selected varieties, using a previously established controlled-condition
215 waterlogging protocol (Miricescu *et al.*, 2021). We then dissected the transcriptomic
216 response of two 2-row and two 6-row winter barley varieties that were moderately sensitive
217 and tolerant to waterlogging in order to identify genes whose expression may be associated
218 with these traits. Our study focused on the transcriptomic response of roots, because of
219 their central role in mediating waterlogging tolerance (Zhang *et al.*, 2015). We identified
220 core waterlogging response genes in barley, as well as sets of genes whose expression is
221 specific to either waterlogging-sensitive or waterlogging-tolerant varieties. The datasets
222 presented therefore serve as a reference for the study of waterlogging stress response in
223 barley and provide insights into potential target genes and new avenues of research to
224 understand tolerance to waterlogging in this crop.

225

226 ## Materials and Methods

227 **Plant material**

228 Cultivars used in this study included winter varieties that originated from the 'Association
229 Genetics of UK Elite Barley' (AGOUEB) population of barley (Thomas *et al.*, 2014), as well as
230 the model spring variety *Golden Promise* and a winter variety, *Infinity*, which has been on
231 the recommended list at the time of an initial field trial whose results were taken into
232 account for varietal selection (Byrne *et al.*, 2021). Both 2-row and 6-row varieties were
233 included (Supp. Table S1). Selected varieties for further characterization, including
234 transcriptome profiling, were *Golden Promise* (spring variety, 2-row), *Regina* and *Infinity*
235 (winter varieties, 2-row), *Passport* and *Pilastro* (winter varieties, 6-row).

236

237 **Plant growth conditions**

238 Plants were grown in a plant growth room under long-day conditions (16 h light/8 h dark) at
239 15°C (constant temperature), approximately 45% relative humidity. Light intensity was
240 determined to be ~138 µmol/m^2/s and was provided by LED bulbs (Philipps LED tubes High
241 Output, T8 20W/865).

242

243 **Soil preparation and seed germination**

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

249

250 **Waterlogging**

251 Plants were grown as indicated above for ten days (corresponding to L1/L2 stage) for
252 transcriptomic experiments, and for 10-14 d (corresponding to L1/L2 stage) for phenotypic

253 characterization. The pots were then transferred to a large tub, which was subsequently
254 filled with tap water up to 1 cm above soil level. The water level was kept constant
255 throughout the duration of the experiment (Figure 1A). The plants were kept in the same
256 growth conditions and were treated for 15 d for phenotypic characterization, or for shorter
257 periods of time, as indicated in the text. Control plants were left in the same growth
258 conditions but received normal watering (every two days, avoiding any standing water in
259 the trays). For the recovery period, plants were taken out of the water and kept in the same
260 growth conditions with a normal watering regime.

261

262 **Height measurements**

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

265

266 **Total RNA extraction**

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

270

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

272 Total RNA was reverse transcribed using an oligo$(dT)_{18}$ primer and the RevertAid reverse
273 transcriptase (Thermo) according to the manufacturer's instructions. For reverse
274 transcription reactions, 1 µg of total RNA was used. cDNA obtained was used for qPCR with
275 a Lightcycler 480 (Roche). Each PCR reaction mix contained 5 µL of 2x SYBR green master 1
276 (Roche), 1 µL cDNA, 1 µL of 10 µM primers and 3 µL of molecular biology grade water.
277 LightCycler melting curves were obtained to check for single peak melting curves for all
278 amplification products. The second derivative maximum method was used to analyze the
279 amplification data. The resulting Cp values were converted into relative expression values
280 using the comparative Ct method (Livak and Schmittgen, 2001). All primer sequences are
281 provided in Supp. Table S2. One reference gene (Hv*ACTIN*) was used to normalize the RT-

282 qPCR data following the screening of a set of reference genes during waterlogging (Supp.
283 Fig. S1).

284

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

286 For RNA-seq analysis, waterlogging treatment was applied as outlined above for 24 h. For
287 each independent replicate and each condition (waterlogged or untreated), the root
288 systems of 3 plants were pooled prior to grinding for RNA extraction. Following RNA
289 extraction, RNA integrity was assessed using an Agilent 2100 Bioanalyzer (Agilent). All RNA
290 samples had RIN values > 7.0. Library preparation and next-generation sequencing were
291 performed by BGI Genomics (Hong Kong) using the DNBseq sequencing platform.

292

293 **Analysis of RNA-seq data**

294 The third release (Morex V3) of the Morex barley genome was downloaded from e!DAL -
295 Plant Genomics and Phenomics Research Data Repository (PGP) (https://edal-pgp.ipk-
296 gatersleben.de/) (Mascher, 2021). Raw RNA-sequencing reads were aligned to Morex V3
297 using *bowtie2* (v2.4.5) (Langmead and Salzberg, 2012). Files were converted from .sam to
298 .bam files and indexed using *samtools* (v1.15.1) (Danecek *et al.*, 2021). Gene abundance was
299 estimated using *stringtie* (v2.1.7) (Pertea *et al.*, 2015) (Supp. Table S3). Differential gene
300 expression analysis was performed using the Bioconductor package *DeSeq2* (Love *et al.*,
301 2014) in *R* (Team, 2022) using a design in which the factors Variety and Treatment were
302 combined into a single factor to model multiple condition effects. The results of multiple
303 comparisons were extracted and filtered by adjusted *p*-value < 0.05 (Supp. Table S4).
304 Principal component analysis (PCA) was performed using *pcaExplorer* (v2.20.2) (Marini and
305 Binder, 2019) in *RStudio* (v2022.02.0+443). Gene ontology (GO) analysis was performed
306 using *ShinyGO* (v0.75c) (Ge *et al.*, 2020). Read count values were transformed by variance
307 stabilising transformations (VST) to normalise for library size and composition (Supp. Table
308 S5). Venn diagrams were generated using InteractiVenn (Heberle *et al.*, 2015).

309 The means of VST read counts from 3 independent replicates generated from
310 *DeSeq2* were filtered for *k*-means clustering as follows. *Deseq2* DEG analysis was performed
311 and results for the 25 comparisons shown in Supp. Fig S6A were extracted. A list of all

312 11,613 DEGs filtered by adjusted *p*-value < 0.05 was generated by combining DEGs from
313 waterlogged to control samples from the same variety, DEGs from each control to every
314 other control sample and DEGs from each waterlogged to every other waterlogged sample.
315 Next, the DEGs with a mean of <10 normalised reads were removed leaving 10,882 genes
316 for clustering analysis. Clustering analysis was performed using the *k*-means function in *R*
317 (v3.6.2) with the arguments centers=25, nstart = 1000, iter.max = 300 and algorithm =
318 "Lloyd" (gene cluster assignment annotated in (Supp. Table S4)). Clustering heatmaps were
319 generated using *ComplexHeatmap* (v2.10.0) (Gu *et al.*, 2016) in *R*. Plots were generated
320 using *ggplot2* (v3.3.6) (Wickam, 2016) and modified for style in Adobe Illustrator 2022.

321 To compare the datasets obtained to a previously published RNA-seq dataset, data
322 presented in the supplementary materials of Borrego-Benjumea *et al*. were collated
323 (Borrego-Benjumea *et al.*, 2020). This list included a subsection of DEGs of interest filtered
324 by adjusted *p*-value < 0.05. To directly compare the data presented here to the results from
325 Borrego-Benjumea *et al*. (Borrego-Benjumea *et al.*, 2020), raw RNA-sequencing reads were
326 aligned to the same version of the barley genome used in this previous study as described
327 above (Supp. Table S6, Supp. Table S7). The second release (Morex IBSC 2 V1 2016) of the
328 Morex Barley genome was downloaded from the IPK Galaxy Blast Suite.

329

330 Results

331 Identification of barley varieties sensitive or tolerant to waterlogging

332 It has been shown that plants constitutively expressing hypoxia response genes are more
333 tolerant to waterlogging than wild-type plants (*e.g.* N-degron pathway mutant plants that
334 constitutively accumulate ERF-VII transcription factors (Gibbs *et al.*, 2011; Licausi *et al.*,
335 2011; Mendiondo *et al.*, 2016)). Plants ectopically expressing specific hypoxia response
336 genes such as *HEMOGLOBIN1 (HB1)* (De Castro *et al.*, 2022; Thiel *et al.*, 2011)) are also more
337 tolerant to waterlogging, and in the case of *HB1*, there also appears to be a positive
338 correlation between the level of gene expression and waterlogging tolerance. Other genes
339 of interest, whose expression is important for hypoxia tolerance, include *ALCOHOL*
340 *DEHYDROGENASE 1 (ADH1)* and *PYRUVATE DECARBOXYLASE 1 (PDC1)*, both of which are
341 involved in ethanol fermentation (Banti *et al.*, 2013) and the plants' ability to continue

342 producing ATP *via* glycolysis during waterlogging. Prior to monitoring the expression of
343 these selected hypoxia response genes with the aim of screening for barley varieties that
344 show differential up-regulation of these genes (*i.e.* that are potentially either sensitive or
345 tolerant to waterlogging), we tested 5 different reference genes previously used in barley
346 (Mendiondo *et al.*, 2016; Walling *et al.*, 2018) to identify those whose expression was stable
347 in waterlogged roots. To this end, barley plants of the *Golden Promise* variety were
348 subjected to waterlogging stress for 8 h, 1 d or 4 d, and roots were collected for RNA
349 extraction and RT-qPCR analysis. This analysis identified Hv*ACTIN* as a suitable reference
350 gene in waterlogged roots (Supp. Figure S1).

351 Next, we monitored the expression of Hv*HB*, Hv*ADH1* and Hv*PDC1* in root tissue at
352 different time points after the beginning of waterlogging treatment. Four different varieties
353 were used in order to determine the suitability of these genes to screen for differential
354 expression between varieties. This analysis indicated that (i) the expression of these 3
355 hypoxia response genes peaked around 1 d after the onset of waterlogging treatment; (ii)
356 there were differences between the 4 varieties in terms of the amplitude of the
357 transcriptional up-regulation (Supp. Figure S2). For example, at 1 d after the beginning of
358 the waterlogging treatment, Hv*HB* was expressed at higher levels in *Pilastro* compared to
359 *Arma*, *Tapir* and *Masquerade*. In addition, the up-regulation of Hv*HB* and Hv*ADH1* was
360 stronger than that of Hv*PDC1*, possibly making these first two genes more suitable to
361 identify varieties with differential regulation of the hypoxia response program and
362 potentially revealing differences between varieties for their tolerance/sensitivity to
363 waterlogging.

364 We next tested the expression of Hv*HB*, Hv*ADH1* and Hv*PDC1* in 20 different winter
365 barley varieties from the AGOUEB collection, which were selected because of their
366 differential response to waterlogging in field experiments (Byrne *et al.*, 2021). The variety
367 *Golden Promise* was also included, as a model (spring) barley variety that is widely used in
368 the community to generate targeted mutations using *Agrobacterium*-mediated
369 transformation. Plants were subjected to waterlogging for 24 h, at which time point, the
370 roots were harvested for RNA extraction and gene expression analysis (Figure 1A). Under
371 normal watering conditions (mock treatment), most varieties had low levels of expression
372 for each of the hypoxia-response genes tested (Figures 1B, 1C and Supp. Figure S3). One

373 exception was *Pilastro*, which had a higher expression level of Hv*HB* under normal watering.
374 This may be relevant to *Pilastro*'s ability to tolerate waterlogging stress, as plants having
375 constitutively higher expression of hypoxia-response genes or ectopically expressing *HB*
376 have been shown to be more tolerant to waterlogging (De Castro *et al.*, 2022; Mendiondo *et*
377 *al.*, 2016; Thiel *et al.*, 2011). Waterlogging stress for 24 h triggered the up-regulation of the
378 three hypoxia response genes tested, with some varieties showing a stronger induction. As
379 previously (Supp. Figure S2), differences were more marked with Hv*HB* and Hv*ADH1* (Figure
380 1B, C) compared to Hv*PDC1* (Supp. Figure S3). The latter gene was therefore not further
381 considered to distinguish between varieties that could be more sensitive or tolerant to
382 waterlogging. After 1 d of waterlogging, some varieties reached higher expression levels
383 than the average expression observed for these genes in the population of 21 varieties.
384 These included *Pilastro* and *Regina* for both Hv*HB* and Hv*ADH1*, as well as *Dura*, *Vesuvius*,
385 *Siberia*, *Mahogany* and *Tamaris* for Hv*ADH1*. These varieties could potentially have better
386 waterlogging tolerance. In contrast, some varieties had a reduced up-regulation of hypoxia
387 response genes. For example, varieties such as *Golden Promise*, *Passport*, *Isa*, *Louise* and
388 *Retriever* had lower expression levels of both Hv*HB* and Hv*ADH1* compared to the average
389 of the population. These could be potential waterlogging sensitive barley varieties. Other
390 varieties, such as *Infinity*, had average expression levels of Hv*HB* and Hv*ADH1* after 1 d of
391 waterlogging treatment.

392 Based on these results, *Pilastro* and *Regina* were chosen as 6-row and 2-row
393 varieties, respectively, for the further characterization of possible waterlogging tolerant
394 varieties, while *Passport* was selected as a 6-row variety that might be more sensitive to
395 waterlogging. To select a 2-row variety that could be potentially more sensitive to
396 waterlogging, we also took into account previously published phenotypic data that had
397 identified *Infinity* as being waterlogging sensitive (Miricescu *et al.*, 2021). *Golden Promise*
398 was also included as a reference variety.

399

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

401 The selected varieties were subjected to waterlogging for 2 weeks and were then returned
402 to normal watering for an additional 6-week period. We have previously shown that under
403 these conditions, waterlogging tolerant and sensitive varieties can be distinguished

404 (Miricescu *et al.*, 2021). Height measurements showed that the growth of *Infinity* and
405 *Passport* was more affected by waterlogging than that of *Pilastro*, *Regina* and *Golden*
406 *Promise*, whose height was mostly unaffected relative to control plants (Figure 2A). Tiller
407 count after a 6-week recovery period indicated that *Golden Promise*, *Infinity* and *Pilastro*
408 produced fewer tillers, whereas *Regina* was largely unaffected (Figure 2B). The effect
409 observed for *Passport* was not statistically significant. Because root traits have been shown
410 to be important for waterlogging tolerance (Zhang *et al.*, 2015), we determined the length
411 of the primary root, as well as the number of seminal roots after 2 weeks of waterlogging.
412 We found that the length of the primary root was reduced upon waterlogging stress for all
413 varieties except *Regina* (Supp. Figure S3B). In contrast, in response to waterlogging, the
414 number of seminal roots increased in all varieties and the difference was statistically
415 significant for all except *Infinity* (Figure 2C). Hence, this phenotypic characterization largely
416 confirmed the initial variety selection for RNA-seq analysis: (i) *Pilastro* and *Regina* as 6-row
417 and 2-row varieties (respectively) that are waterlogging tolerant; and (ii) *Passport* and
418 *Infinity* as 6-row and 2-row (respectively) waterlogging-sensitive varieties. Finally, based on
419 the data obtained, *Golden Promise* can be considered as a putative waterlogging-sensitive
420 variety (Figure 2D).

421

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

423 To determine the transcriptional response of *Pilastro*, *Regina*, *Passport*, *Infinity* and *Golden*
424 *Promise* to waterlogging, we subjected these varieties to waterlogging for 24 h. We then
425 extracted RNA from roots and performed RNA-seq analysis. Reads obtained were aligned to
426 the Morex barley genome (version 3; Supp. Table S3) and differential gene expression
427 (adjusted p-value < 0.05) was determined for each of the waterlogged varieties relative to
428 the corresponding untreated variety (Supp. Table S4). A Principal Component Analysis (PCA)
429 on sample expression profiles (Figure 3 and Supp. Figure S4) indicated that the samples can
430 be separated based on (i) the treatment itself (PC1: 35.82% variance) (Figure 3A); (ii)
431 whether they are winter or spring varieties (PC2: 15.64% variance; Supp. Figure S4D); or (iii)
432 whether they are 2-row or 6-row varieties (PC3: 11.34% variance; Supp. Figure S4E).
433 Notably, the different samples could also be separated based on whether the varieties had

434 been categorized as either sensitive or tolerant (Figure 3B; PC4: 9.92% variance), thus
435 providing a validation for the selection and categorization of the varieties.

436 The number of DEGs we detected under waterlogging in the different varieties
437 varied considerably: for *Golden Promise* and *Passport* we identified 220 and 592 DEGs
438 (respectively), while *Infinity*, *Pilastro* and *Regina* had a substantially higher number of DEGs
439 (1,307, 1,512, and 1,233, respectively). In all varieties tested, the proportion of up-regulated
440 genes was higher than that of down-regulated genes (Figure 3C). We verified in our datasets
441 the expression of the Hv*HB*, Hv*ADH1* and Hv*PDC1* genes whose up-regulation was used to
442 monitor the response of the different varieties to waterlogging, and compared the RNA-seq
443 results to those of the RT-qPCR experiments (Figure 1). Induction of all three genes upon
444 waterlogging was found (Figure 3D). Although relative differences between varieties were
445 not always identified for Hv*ADH1*, the relative behaviour of the different varieties for Hv*HB*
446 expression was conserved, including the higher expression of Hv*HB* in *Pilastro* in the
447 absence of waterlogging. We further identified additional homologs of Hv*ADH1* and Hv*HB*
448 and confirmed that these homologs were also up-regulated in response to waterlogging
449 (Supp. Figure S5A and B).

450

451 **Both conserved and divergent transcriptional reprogramming occurs in response to**
452 **waterlogging in barley**

453 Our RNA-seq experiment identified 2,865 genes that were differentially expressed (adjusted
454 *p*-value < 0.05) in at least one variety under waterlogged conditions compared to control
455 plants (Supp. Table S4). The variety with the highest proportion of unique DEGs was *Pilastro*
456 (>50% unique DEGs), while the majority of DEGs identified for *Golden Promise* and *Passport*
457 were shared with other varieties (Figure 4A) (<25% unique DEGs in these 2 varieties).
458 Among these 2,865 DEGs, 1,078 genes were called as differentially expressed in more than
459 one variety. Except for 7 genes, the directionality of gene expression change was the same
460 in all the different varieties. As expected based on the transcriptional response of
461 Arabidopsis to waterlogging, Gene Ontology (GO) analysis on the 1,811 DEGs that were up-
462 regulated under waterlogging in at least one variety identified over-represented terms
463 related to hormone and stress responses, oxidoreductase activity, kinase activity, as well as
464 carbohydrate metabolic processes (Figure 4B and D). GO analysis of the 1,061 DEGs that

465 were down-regulated in response to waterlogging identified terms related to lipid and
466 carbohydrate metabolism, transmembrane transporter activities and cell periphery, as well
467 as oxidoreductase activity and proteolysis (Figure 4C and E). All of these terms are
468 consistent with functional GO categories known to be central to the transcriptional
469 response of plants to waterlogging.

470 A total of 98 genes were detected as differentially expressed in all varieties tested
471 (Figure 4A). In addition, the directionality of the observed expression changes was the same
472 across all datasets. Hence, these genes may be used to define a set of core waterlogging
473 response genes in barley. The majority of these genes were up-regulated in response to
474 waterlogging (82 genes; Figure 4B and Supp. Table S4) and included homologs of known
475 core hypoxia response genes in Arabidopsis (Mustroph *et al.*, 2009) such as *ADH1*
476 (HORVU.MOREX.r3.4HG0345760, HORVU.MOREX.r3.1HG0082960 and
477 HORVU.MOREX.r3.4HG0345740), *PDC1* (HORVU.MOREX.r3.4HG0384560), *HB1*
478 (HORVU.MOREX.r3.4HG0394960, HORVU.MOREX.r3.7HG0715010) and a respiratory burst
479 oxidase (*RBOH*) (HORVU.MOREX.r3.5HG0444960). Furthermore, this set included homologs
480 of ERF-VII transcription factors, such as *HRE1* (HORVU.MOREX.r3.4HG0405650 and
481 HORVU.MOREX.r3.4HG0405650), *HRE2* (HORVU.MOREX.r3.5HG0497970) and *RAP2.3*,
482 (HORVU.MOREX.r3.1HG0060770). This result is in agreement with the central role of ERF-VII
483 transcription factors as master regulators of hypoxia and waterlogging response. Sixteen
484 DEGs common to all datasets were down-regulated in response to waterlogging (Figure 4C),
485 including three peroxidase genes (HORVU.MOREX.r3.2HG0215440,
486 HORVU.MOREX.r3.2HG0119650, HORVU.MOREX.r3.2HG0119630) a nitrate transporter
487 (HORVU.MOREX.r3.7HG0700030), and an aldehyde dehydrogenase
488 (HORVU.MOREX.r3.2HG0173750). GO analysis of the core set of 98 DEGs (Figure 4F)
489 revealed largely similar terms to those identified for all up-regulated DEGs (Figure 4D),
490 including GO terms such as oxidoreductase activity, carbohydrate metabolic process, and
491 hormone-mediated signaling.

492 We next compared our RNA-seq results to a previously published RNA-seq dataset
493 and to QTLs identified based on waterlogging-related root and yield traits. In a recent
494 publication, Borrego-Benjumea *et al*. analyzed the transcriptomic response of 2-week-old
495 *Yerong* and *Deder2* barley roots waterlogged for 72 or 120 h (Borrego-Benjumea *et al.*,

496 2020). Both varieties had been chosen because of their tolerance to waterlogging (Li *et al.*,
497 2008; Takeda, 1989). To facilitate the comparison between the different datasets, we
498 repeated the analysis of our RNA-seq data using the same version of the barley genome that
499 was used by Borrego-Benjumea *et al* (*i.e*. Morex version 1). This resulted in the identification
500 of 1,726 up-regulated genes and 1,236 down-regulated genes in at least one of the varieties
501 in our experiment (adjusted *p*-value <0.05). Despite the differences in the barley varieties, in
502 the age of the plants and in the duration of the waterlogging treatment, a statistically
503 significant overlap was observed between both the up and down-regulated genes in our
504 datasets and in the list of DEGs presented by Borrego-Benjumea *et al*. (Supp. Figure S5C and
505 Supp. Table S7) (Borrego-Benjumea *et al.*, 2020). GO analysis of the 52 DEGs that were up-
506 regulated in all datasets identified terms related to alcohol dehydrogenase activity,
507 metabolism and detoxification (Supp. Figure S5D), again in agreement with known key
508 aspects of plant responses to waterlogging. Genes associated with nitrate transport and
509 oxygen response were found among the 11 shared down-regulated genes. We also
510 compared our data to a list of 28 genes located in QTLs for waterlogging tolerance that were
511 identified following a screen of nearly 700 barley varieties with a specific focus on root traits
512 known to be relevant (*i.e.* the formation of adventitious roots and of root cortical
513 aerenchyma) (Manik *et al.*, 2022). Two potassium transporter genes that are part of these
514 28 genes were down-regulated in *Regina* after waterlogging (Supp. Table S7), which may be
515 of relevance to the previously shown importance of potassium flux during waterlogging (Gill
516 *et al.*, 2018). Comparison with another list of 48 candidate genes located in QTLs identified
517 in a waterlogging field trial focused on growth and yield parameters with nearly 250 barley
518 varieties identified a single gene with unknown function (HORVU2Hr1G011650) that was
519 significantly down-regulated in *Pilastro* under waterlogging (Supp. Table S7). This particular
520 gene was also identified as a candidate that contained significant SNPs associated with
521 waterlogging tolerance under control conditions (Borrego-Benjumea *et al.*, 2021).

522

523 **Identification of unique expression signatures in putative waterlogging-tolerant and**
524 **sensitive varieties**

525 To compare the responses to waterlogging between varieties, *k*-means clustering was
526 performed on 10,882 genes identified as differentially expressed (adjusted *p*-value <0.05) in:

527 (i) at least one variety when comparing waterlogged to control samples for each variety, or
528 (ii) between the control samples (*i.e*. these have intrinsic differential expression between at
529 least 2 varieties in the absence of waterlogging), or (iii) between the waterlogged samples
530 from different varieties (*i.e*., genes that are differentially expressed between at least 2
531 varieties under waterlogging) (Supp. Figure S6). Clustering analysis revealed distinct gene
532 expression patterns between samples (Figure 5A). A number of clusters contained genes
533 that were up-regulated in multiple varieties in response to waterlogging compared to
534 untreated plants (*i.e.* clusters 4, 7, 8, 14, and 21). Cluster 14 comprised 802 genes, the
535 largest in any of these clusters (Supp. Table S4). The centroid expression of genes in cluster
536 14 was consistently higher in response to waterlogging in all varieties (Figure 5B and Supp.
537 Figure S7). Cluster 14 included homologs of known hypoxia response genes such as those
538 described above in the core set of up-regulated DEGs in barley, as well as homologs of
539 Arabidopsis *RAP2.12* (HORVU.MOREX.r3.5HG0481240), additional homologs of *RAP2.3*,
540 (HORVU.MOREX.r3.2HG0112960), and homologs of *ADH1* (HORVU.MOREX.r3.2HG0115170,
541 HORVU.MOREX.r3.3HG0252910) (Supp. Figure S5A and B). Furthermore, a number of
542 transcription factors (TFs) from the ERF, bHLH, bZIP (basic leucine zipper), NAC (NAM,
543 ATAF1,2, CUC2) and WRKY families were also identified in cluster 14 (Supp. Table S4).
544 Notably transcription factors belonging to these families have established and conserved
545 roles in the regulation of hypoxia response (Lee and Bailey-Serres, 2021; Mustroph *et al.*,
546 2010; Reynoso *et al.*, 2019). GO analysis of the 802 genes in cluster 14 identified terms
547 related to hormone and stress responses, oxidoreductase and kinase activity and
548 metabolism (Figure 5C).

549 Clusters comprising genes that are down-regulated in response to waterlogging in
550 multiple varieties include clusters 5, 9, 13, 15, 20 and 24. Cluster 9 contained 435 genes that
551 were down-regulated in response to waterlogging (Figure 5D and Supp. Figure S7), including
552 14 of the 16 genes that were down-regulated in waterlogged samples from all varieties
553 (Supp. Table S4). Among these genes was an aminotransferase
554 (HORVU.MOREX.r3.2HG0190680), whose top-scoring Arabidopsis homolog is γ-
555 aminobutyric acid transaminase (GABA-T; AT3G22200). In Arabidopsis, the expression of this
556 gene is reduced in response to hypoxia, leading to an accumulation of GABA (Branco-Price
557 *et al.*, 2008). Another member of cluster 9 whose expression was downregulated in all

558 varieties in response to waterlogging is a cation exchanger
559 (HORVU.MOREX.r3.3HG0266310). In Arabidopsis, the closely related Ca^{2+}/proton exchanger
560 11 (CAX11) is important to maintain cytosolic Ca^{2+} homeostasis and was shown to be down-
561 regulated in response to hypoxia and waterlogging (Wang *et al.*, 2016). Multiple
562 transcription factors from the heat-shock and MYB transcription factor families were also
563 found in cluster 9. This is consistent with the observation that differentially expressed MYB
564 transcription factors were predominantly found to be down-regulated in response to
565 waterlogging in *Yerong* and *Deder2* (Borrego-Benjumea *et al.*, 2020). GO analysis of the 435
566 genes in cluster 9 showed an enrichment for terms related to lipid and carbohydrate
567 metabolism, transmembrane transporter activities and cell periphery (Figure 5E), similarly
568 to the GO terms identified in all genes down-regulated in response to waterlogging (Figure
569 4E).

570 Clustering analysis also identified gene expression signatures that were specific to
571 either the waterlogging-tolerant or sensitive varieties. Specifically, cluster 1 (257 genes)
572 contained genes whose expression was highest in the waterlogging-sensitive barley varieties
573 *Golden Promise* and *Infinity*, and intermediate in *Passport* (considered as a sensitive variety).
574 In contrast, the expression of these genes was markedly lower in the two waterlogging-
575 tolerant varieties *Pilastro* and *Regina* (Figure 5F and Figure S7). GO analysis of genes in
576 cluster 1 identified terms related to catabolic and metabolic processes (Figure 5G), which
577 could be relevant to waterlogging tolerance/sensitivity. In contrast, genes in cluster 16 (359
578 genes) had an opposite behavior, in that they were more highly expressed in waterlogging-
579 tolerant varieties, but were only expressed at low levels in the 3 waterlogging-sensitive
580 varieties (Figure 5H and Figure S7). GO analysis of genes in cluster 16 revealed an
581 enrichment for genes associated with diverse processes found in other clusters or sets of
582 genes, except the term 'nicotianamine metabolic process' which was only found in the GO
583 analysis of cluster 16 genes. This GO term relates to metal (including iron) homeostasis,
584 which could be of relevance to waterlogging stress tolerance because of the increased
585 uptake of metals and their toxicity to plants in waterlogged conditions. Notably, genes in
586 clusters 1 and 16 were not differentially expressed in response to waterlogging. Instead, it is
587 their intrinsic (or varietal-specific) expression level that may contribute to waterlogging
588 tolerance or sensitivity.

589

590 **Discussion**

591 Maintaining yields in the context of global climate change is a challenge, as crops are likely
592 to experience a wider range of stresses simultaneously or sequentially. Accordingly, efforts
593 are now made to improve crop resilience to multiple abiotic and biotic stresses, as opposed
594 to tolerance to a specific stress. At the same time, the trade-off between the gain of new
595 traits and yield also requires careful consideration (Bailey-Serres *et al.*, 2019). Waterlogging
596 in particular causes important crop losses and will become more prevalent in some regions.
597 Several QTLs have been identified with genes that could be targeted to improve crop
598 tolerance to waterlogging. However, the role of these candidate genes often needs to be
599 validated, and their suitability to improve crop waterlogging tolerance also needs to be
600 demonstrated in many cases. Alternatively, targeted mutations in genes coding for key
601 hypoxia response components, such as genes of the N-degron protein degradation pathway,
602 have yielded Arabidopsis and barley lines that are more tolerant to waterlogging and other
603 abiotic stresses (Mendiondo *et al.*, 2016; Vicente *et al.*, 2017), albeit with a potential trade-
604 off on their ability to resist infection by some pathogens (de Marchi *et al.*, 2016; Gravot *et*
605 *al.*, 2016; Vicente *et al.*, 2019).

606 Barley is an essential crop that exhibits considerable difference in the sensitivity to
607 waterlogging depending on the variety. The identification of potential candidate genes that
608 can be targeted or used as markers to identify waterlogging tolerant varieties would be of
609 major importance. One approach to identifying candidate targets and markers is the
610 comparison of genome-wide transcriptional responses of varieties that are either tolerant or
611 sensitive to waterlogging. To date, few transcriptomic datasets have been generated using
612 barley waterlogged samples, and to the best of our knowledge there has not been a
613 transcriptional comparison between waterlogging sensitive and tolerant varieties. Here, we
614 identified winter barley varieties with different levels of sensitivity/tolerance to
615 waterlogging based on the expression of known core hypoxia response genes and the effect
616 of waterlogging treatment on their growth. We then performed RNA-seq on the roots of
617 these selected varieties under waterlogging and normal watering conditions. In agreement
618 with transcriptional studies done on other plants and crops (Lee *et al.*, 2011; Lee and Bailey-
619 Serres, 2021; Mustroph *et al.*, 2010; Reynoso *et al.*, 2019; van Veen *et al.*, 2016), our RNA-

620 seq datasets indicate that the transcriptional reprogramming that accompanies the onset of
621 waterlogging response in barley involves cellular processes such as carbohydrate metabolic
622 processes, regulation of oxidative stress, and metal homeostasis.
623 By identifying those genes whose transcriptional regulation in response to
624 waterlogging is conserved amongst the 5 barley varieties used, a set of 98 core waterlogging
625 response genes in barley was defined. Many of these core waterlogging response genes are
626 homologs of core hypoxia response genes defined in Arabidopsis (Mustroph *et al.*, 2009)
627 and are involved in processes known to be important for the waterlogging response,
628 including cell wall metabolism, ethylene biosynthesis and signalling, carbohydrate
629 metabolism and ROS regulation. Multiple transcription factors belonging to the ERF, bHLH,
630 and WRKY families, which are known to be important for the onset of the waterlogging
631 response program, are also among these core barley waterlogging response genes. Hence,
632 our data confirms that the gene regulatory network that controls waterlogging response is
633 conserved amongst monocots and dicots, as suggested based on previous comparisons
634 which did not include barley data (Mustroph *et al.*, 2010; Tamura and Bono, 2022). In
635 addition, it is possible that some of these core waterlogging response genes in barley could
636 serve as markers for waterlogging response or tolerance, if the amplitude of their gene
637 expression change correlates with waterlogging tolerance.
638 Clustering analysis of the different DEGs further identified groups of genes that are
639 intrinsically differentially expressed between waterlogging tolerant and sensitive varieties.
640 These genes could constitute variety-specific waterlogging susceptibility or tolerance factors
641 in barley, so that either their inactivation *via* mutagenesis, or their constitutive expression in
642 plants may allow the development of barley varieties with increased tolerance to
643 waterlogging. Validating this idea would require (i) studying the function of these genes; and
644 (ii) generating barley plants that are mutated or that constitutively express these different
645 genes. Potential trade-offs on other traits of agronomic relevance and on resilience to other
646 abiotic and biotic stresses would also need to be assessed. Considering the variety of gene
647 functions present in these clusters, it is however difficult to predict which genes (or gene
648 families) are most likely to be of potential relevance to crop improvement. Their association
649 with GWAS and QTL identification would most likely help to pinpoint the best candidates.
650

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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 GSExxxxx. *Note to reviewers*: the data has been submitted and the authors are waiting for reference number.

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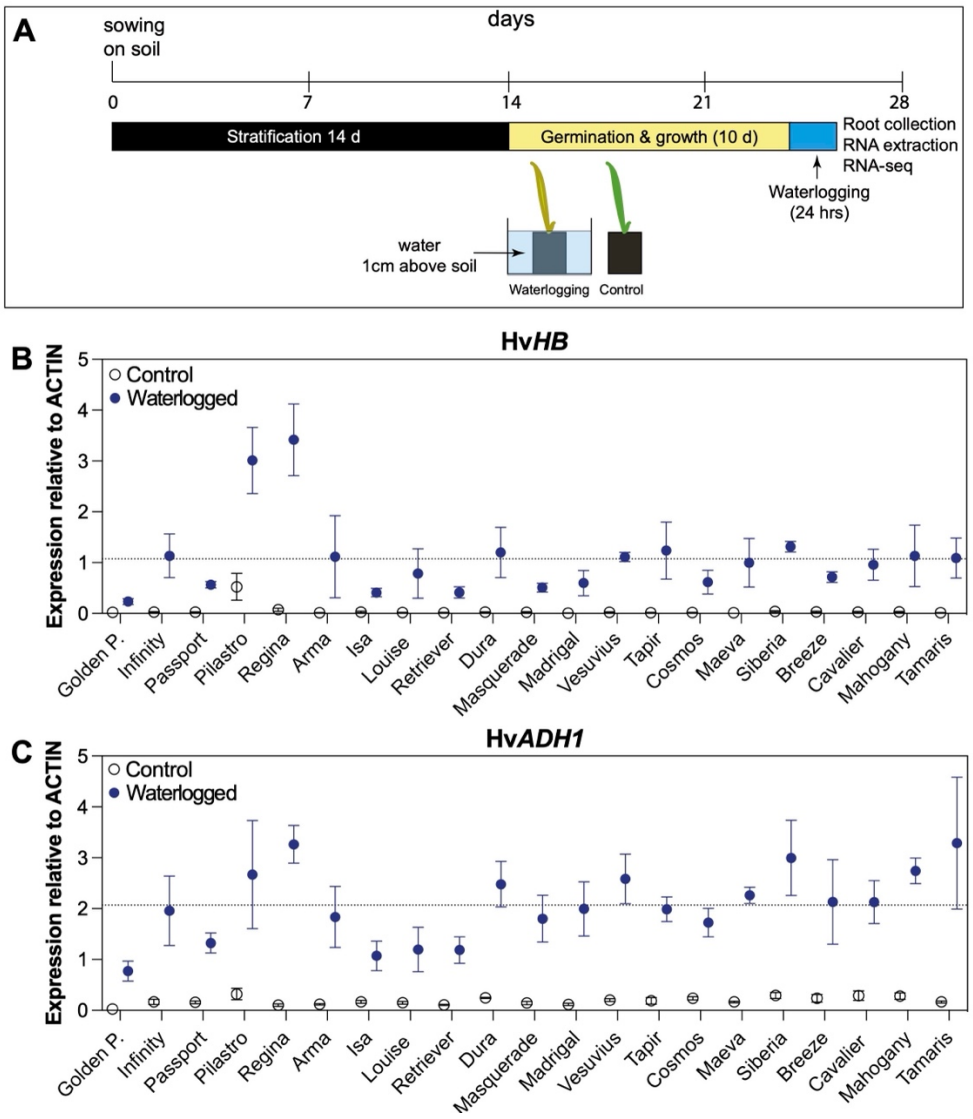
897 **Figures**



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899 **Figure 1: Differential expression of selected hypoxia response genes in 21 different barley**
900 **varieties.** (A) Experimental design of waterlogging experiments (see Materials and Methods
901 for details). (B) Hv*HB* expression relative to *ACTIN* in mock-treated (open symbols) and
902 waterlogged (blue symbols) plants after 1 d of treatment. The dashed line corresponds to
903 the average relative expression of Hv*HB* for waterlogged samples of all varieties and
904 replicates. (C) Hv*ADH1* expression relative to *ACTIN* in mock-treated (open symbols) and
905 waterlogged (blue symbols) plants after 1 d of treatment. The dashed line corresponds to
906 the average relative expression of Hv*ADH1* for waterlogged samples of all varieties and
907 replicates. Data shown in (B) and (C) are from three independent replicates, except for
908 *Golden Promise* for which 4 independent replicates were used. Each independent replicate

909 was obtained by pooling the root systems from 3 plants prior to grinding for RNA extraction.

910 Note: expression values for *Infinity* in (B) and (C) were published in (Miricescu *et al.*, 2021).

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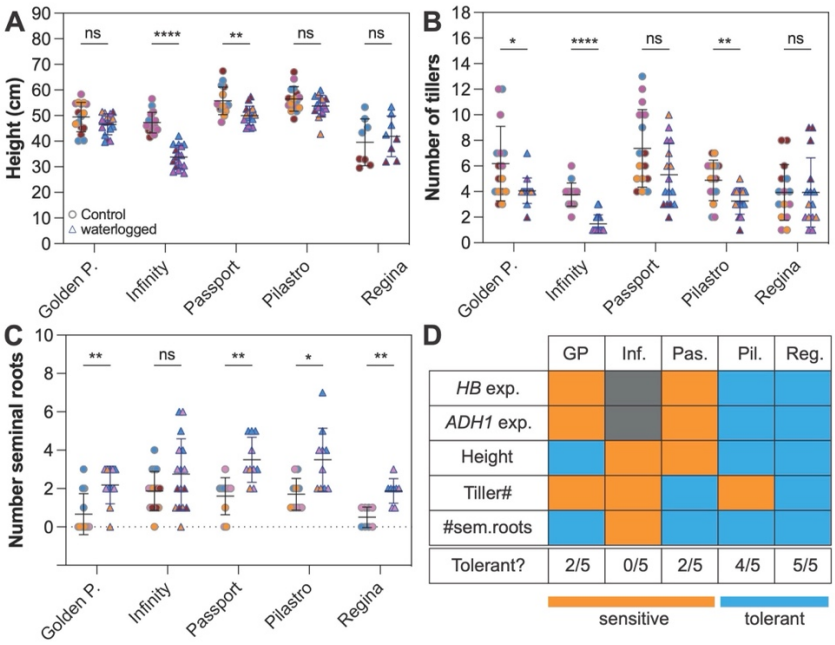
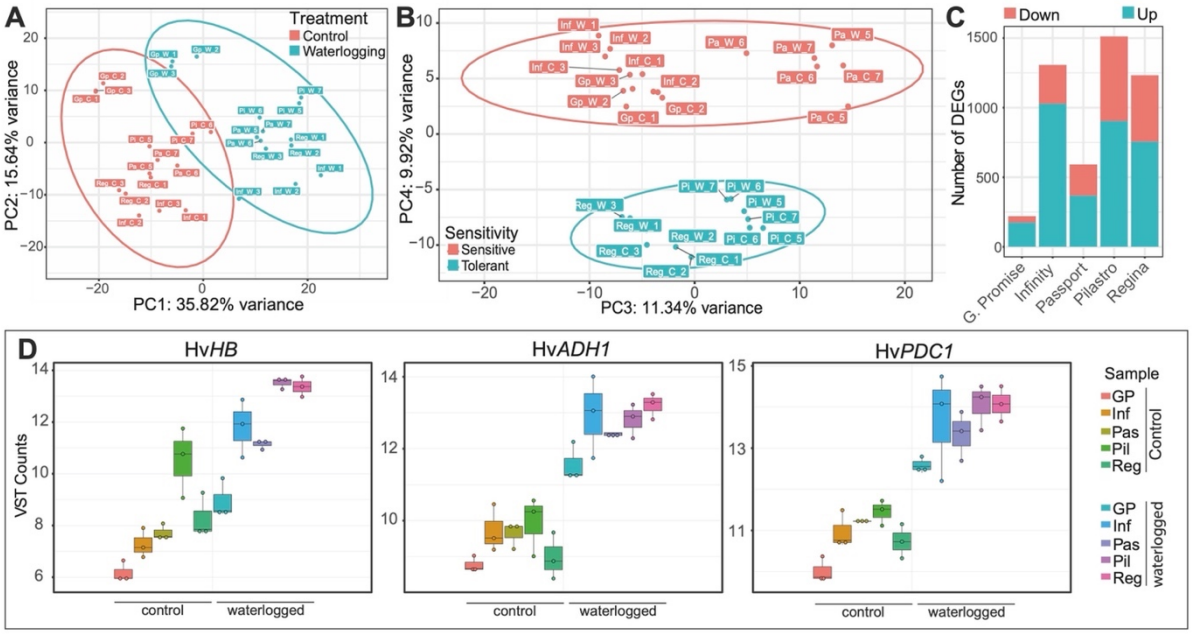


Figure 2: Effect of waterlogging on shoot and root growth. (A) Plant height after 14 d of waterlogging and a 6-week recovery period. For *Golden Promise*, *Passport* and *Pilastro*, three independent replicates (4 plants per replicate) were carried out. For *Regina* and *Infinity*, two independent replicates (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. For *Golden Promise*, *Passport* and *Pilastro*, three independent replicates (4 plants per replicate) were carried out. For *Regina* and *Infinity*, data from three independent replicates are presented (4 to 7 plants per replicate for *Regina*, and 4 to 9 plants per replicate for *Infinity*). (C) 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). (D) Summary of variety category based on at least 3 out of 5 criteria being consistent with the plants being either sensitive or tolerant. Orange: sensitive to waterlogging; blue: tolerant to waterlogging; grey: inconclusive. In (A-C), data from different replicates is color coded. Statistical analysis: multiple unpaired t-test with multiple comparison correction (Holm-Sidak method). Asterisks: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$; ns: not statistically significant.



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933 **Figure 3: Results of RNA-seq experiments .** (A) PCA plot showing Principal Component 2
934 (PC2) *versus* PC1 with ellipses showing sample grouping by treatment. (B) PCA plot showing
935 PC4 versus PC3. Ellipses show sample grouping based on sensitivity/tolerance to
936 waterlogging. (C) Absolute number of DEGs (filtered by adjusted *p*-value<0.05) in
937 waterlogged *versus* control samples for each variety. (D) Boxplots showing variance
938 stabilised transformed (VST) read counts for Hv*HB* (HORVU.MOREX.r3.4HG0394960),
939 Hv*ADH1* (HORVU.MOREX.r3.4HG0345760.1) and Hv*PDC1* (HORVU.MOREX.r3.4HG0384560)
940 in control and waterlogged samples (*Golden Promise* (GP), *Infinity* (Inf), *Passport* (Pas),
941 *Pilastro* (Pil) and *Regina* (Reg)).

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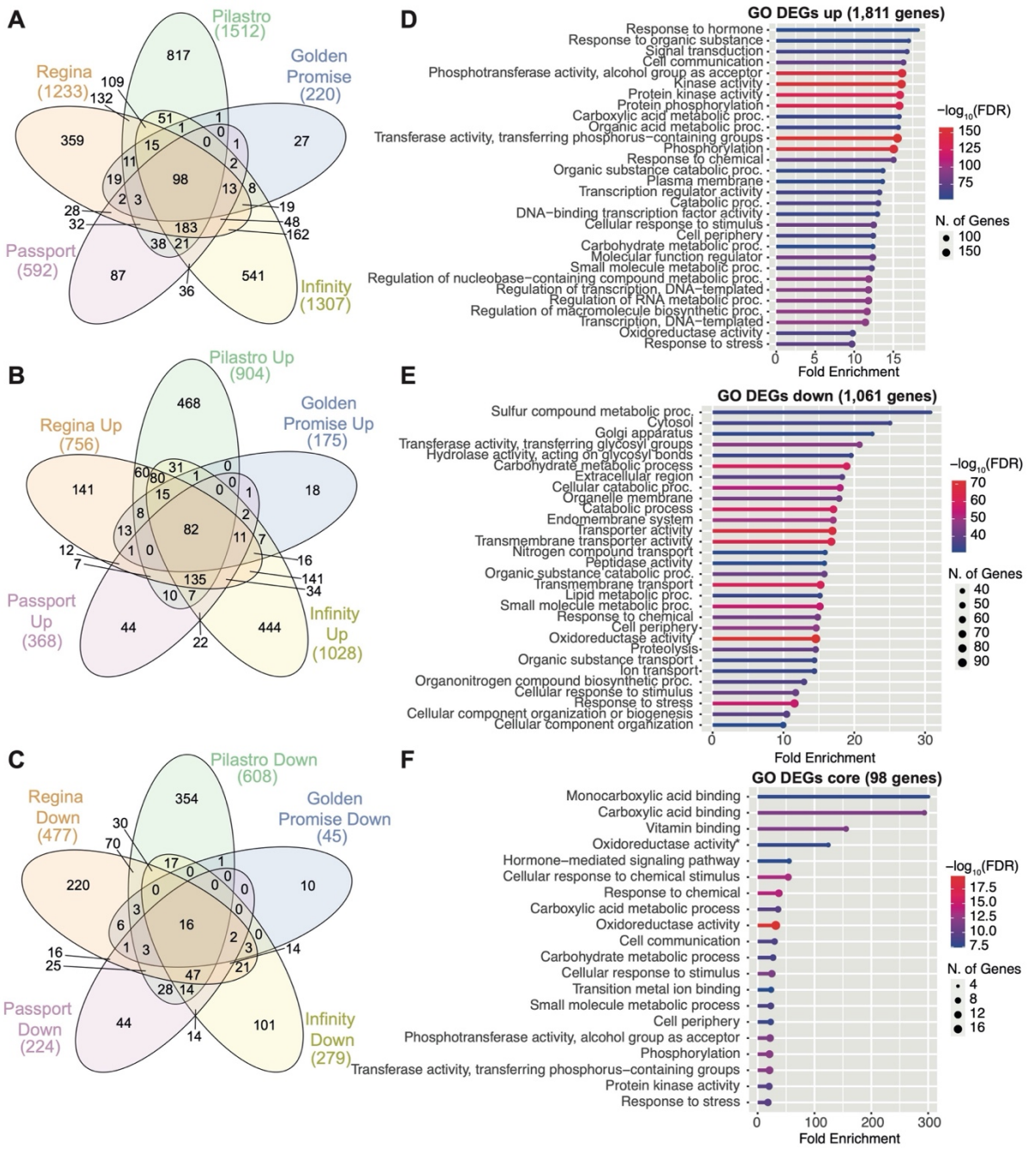

Figure 4: Identification of core waterlogging response genes in barley. (A) Overlap between the DEGs identified in the different varieties. (B) Overlap analysis of genes up-regulated in waterlogged samples compared to control samples for each variety. (C) Overlap between datasets for genes that are down-regulated 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 variety in response to waterlogging. (F) The 20 most enriched GO terms among the 98 core

953 DEGs common to all varieties when comparing waterlogged to control samples for each
954 variety.
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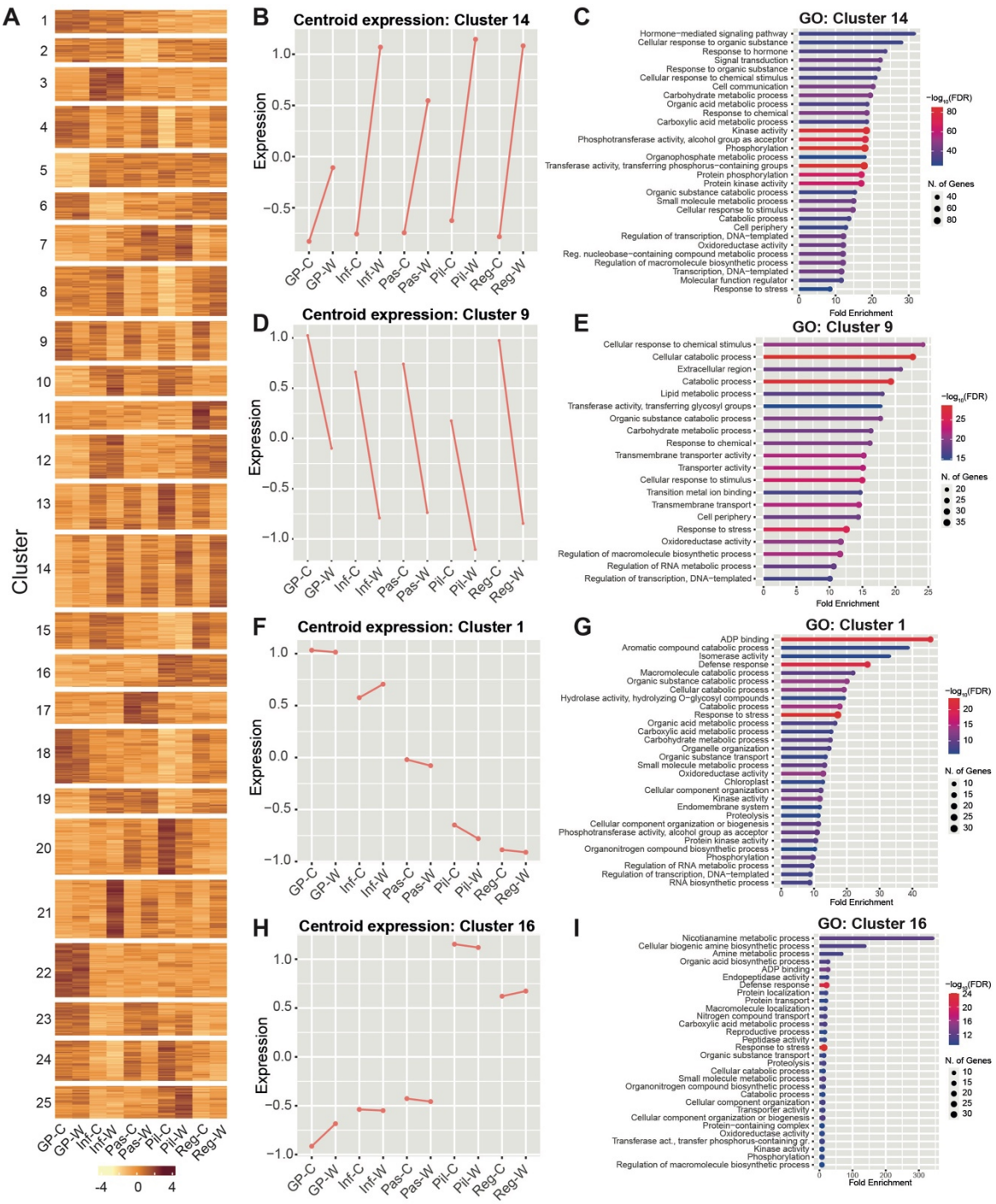

Figure 5: Clustering analysis of DEGs in waterlogged compared to control samples reveals gene expression signatures in waterlogging tolerant and sensitive varieties. (A) Heatmap showing the results of *k*-means clustering (*k*=25) of 10,882 DEGs (see Supp. Figure S6A for selection criteria). Image generated 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

965 centroid expression for genes in cluster 1. (G) The 20 most highly enriched GO terms among
966 the 257 genes from cluster 1. (H) Plot of centroid expression for genes in cluster 16. (I) The
967 20 most highly enriched GO terms among the 359 genes from cluster 16.
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