Barley shows reduced Fusarium Head Blight under drought and modular expression of differential expressed genes under combined stress

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Highlight

Co-expression network analysis reveals association of physiological stress markers and gene expression modules under biotic and abiotic stress

Abstract

Plants often face simultaneous abiotic and biotic stress conditions. However, physiological and transcriptional responses of plants under combined stress situations are little understood. Spring barley is susceptible to Fusarium Head Blight (FHB), which is strongly affected by weather conditions. We therefore studied the potential influence of drought on FHB severity and responses in three differently susceptible spring barley varieties and found strongly reduced FHB severity in susceptible varieties under drought. Quantity of differentially expressed genes (DEGs) and strength of transcriptomic regulation reflected the concentration of physiological stress markers such as abscisic acid or fungal DNA contents. Infection-related gene expression associated rather with susceptibility than resistance. Weighted gene correlation network analysis uncovered 18 modules of co-expressed genes, which reflect the pathogen or drought response in the used varieties. A generally infection-related module contained co-expressed genes for defence, programmed cell death and mycotoxin-detoxification indicating that diverse genotypes use a similar defence strategy towards FHB albeit with different success. Further DEGs showed co-expression in drought or genotype-associated modules correlating with measured phytohormones or the osmolyte proline. The combination of drought stress with infection lead to highest numbers of DEGs and provoked a modular composition of single stress responses rather than a specific transcriptional readout.

Keywords and Abbreviations

barley, drought stress, Fusarium head blight, 3'-RNAseq, transcriptome, weighted gene co-expression network analysis

Abbreviations:

ABA	= abscisic acid
Ba	= Barke
DFc	= drought stress, infected with *Fusarium culmorum*
DEG	= differently expressed gene
DM	= drought stress, mock-inoculated
DPA	= dihydrophaseic acid
F.	= *Fusarium*
Fc	= *Fusarium culmorum*
FHB	= Fusarium Head Blight
IAA	= indol 3-acetic acid
Mo	= Morex
PA	= phaseic acid
Pb	= Palmella Blue
SA	= salicylic acid
SOTA	= self-organizing tree algorithm
WFc	= watered, infected with *Fusarium culmorum*
WGCNA	= weighted gene correlation network analysis
WM	= watered, mock-inoculated
WRKY	= WRKY transcription factor

Introduction

Under natural conditions, plants are facing multiple abiotic and biotic stress factors affecting growth, crop yield and pathogen defence. The simultaneous occurrence of two or more stress factors triggers complex regulatory responses resulting in synergistic, antagonistic or neutral effects on expression and balancing of stress responses (Choi *et al.* 2013; Zhang and Sonnewald 2017; Pandey *et al.* 2015; Ramegowda and Senthil-Kumar 2015; Pandey and Senthil-Kumar 2019). Many stress responses share common factors of plant signalling and metabolism, and co-occurrence of stressors is often fatal for crop productivity (Farooq *et al.* 2009; Suzuki *et al.* 2014; Zandalinas *et al.* 2021). Transcriptional analyses in *Arabidopsis thaliana* indicate that plants respond to combined abiotic and biotic stress with tailored regulatory responses differing from responses towards single stressors (Gupta *et al.* 2016). Stress-stress interactions are often complex and not well understood (Atkinson and Urwin 2012). One of the most limiting factors of plant growth, performance and crop productivity is water availability (Kang *et al.* 2009). In conjunction with global warming, strong and frequent drought periods are predicted to increase in near future and represent serious threats to food security and supply (Meza *et al.* 2020) and especially spring barley will strongly be affected by droughts (Olesen *et al.* 2011; Xie *et al.* 2018). A recent study further predicts that on a global scale proportions of fungal soil borne pathogens will increase with global warming (Delgado-Baquerizo *et al.* 2020). Hence, vulnerability of agriculture may rise with a simultaneously predicted increase in global food demands leading to increasing gaps in food security. In this context, understanding plant physiology under complex stress combinations could unfold regulatory pathways and marker genes important for advanced genotype selection and breeding of disease resistant and stress tolerant crops (Pandey and Senthil-Kumar 2019).

Fusarium head blight (FHB) is one of the most destructive diseases of small grain cereal crops worldwide and its epidemiology is strongly associated with weather conditions. The disease is caused by a pathogen complex of several *Fusarium* species such as *F. graminearum* and *F. culmorum*. Infections with *Fusarium* pathogens can cause yield loss but also quality losses and contamination with hazardous mycotoxins like deoxynivalenol (DON) or zearalenone (Wegulo *et al.* 2015). The occurrence and strength of the disease is dependent on multiple environmental factors especially temperature, air humidity and rainfalls around anthesis, which collectively influence disease incidence and severity in the field (Birr *et al.* 2020; Hoheneder *et al.* 2022). FHB resistance of wheat (Buerstmayr *et al.* 2021; Mesterhazy 2020; Buerstmayr and Lemmens 2015) and barley (Buerstmayr and Lemmens 2015; Ogrodowicz *et al.* 2020) is a quantitative trait expressed from various QTLs and hence difficult to exploit. Furthermore, effective chemical control of FHB is limited to a short time window at around anthesis, when primary head infection usually occurs. Additionally, control measurements and pathogenicity of the fungus strongly interfere with weather conditions, both increasing complexity of effective FHB management (Wegulo *et al.* 2015).

In order to obtain physiological markers for FHB responses under complex environmental conditions, understanding gene regulation networks in response to single and combined abiotic and biotic stressors is crucial. The transcriptional response of wheat and barley to FHB is increasingly well understood (Hameed *et al.* 2022; Kazan and Gardiner 2018) but little information is available for complex stress situations. Hormones regulate various stress responses and physiological and developmental processes in plants. Under drought, plant hormones act as notable endogenous plant growth regulators and mediate abiotic stress tolerance (Farooq *et al.* 2009) and pathogen defence (Bari and Jones 2009). Predominantly, abscisic acid (ABA) governs growth, root-shoot ratio and regulates stomatal conductance to reduce transpiration and water loss under drought over relatively long distances from the root to the shoot. However, while ABA improves abiotic stress tolerance during vegetative growth, the downstream effects of ABA during reproductive stage can be contrary (Dolferus *et al.*

2011). There is evidence that reproductive tissue is most sensitive to water deficit in general (Blum 2009) and drought stress strongly delays flowering or even leads to abortion of spikelets in cereal crops (Barnabás *et al.* 2008). When it comes to pathogen infection, the genetic regulatory mechanisms to balance hormonal regulations associated with specific stress responses are not sufficiently investigated, especially when invading microbes are able to manipulate regulatory networks in the host by biosynthesis of phytohormones (Bari and Jones 2009), which was previously described for *Fusarium* species (Dörffling and Petersen 1984; Jaroszuk-Ściseł *et al.* 2014; Luo *et al.* 2016; Qi *et al.* 2016) Salicylic acid (SA), jasmonic acid (JA) and ethylene are mainly involved in diverse pathogen defence responses against various pathogens and expression of pathogenesis related (PR) genes (Bari and Jones 2009). ABA induces several transcription factors, which contribute to specific abiotic stress but also pathogen defence regulations (Yao *et al.* 2021). SA is further associated as signal for systemic acquired resistance mechanisms. However, the function of hormonal defence signalling network is dependent on the pathogen´s trophic lifestyles (Adie *et al.* 2007), which is often not clearly classifiable and changes during pathogenesis of the hemibiotrophic *Fusarium* species from biotrophic to necrotrophic (Brown *et al.* 2010). However, the antagonistically acting SA and JA/ethylene associated pathways are both involved in responses to *Fusarium* infections (Wang *et al.* 2018). In particular, when it comes to combined stress, functions of either synergistically or antagonistically interacting plant hormones provoke complex stress responses. Hence, the host plant may prioritize particular stress responses, which can lead to either resistance or susceptibility (Bari and Jones 2009; Wang *et al.* 2018; Gupta *et al.* 2020). Although, previous studies revealed associations of ethylene (Chen *et al.* 2009; Xiao *et al.* 2013) auxin (Brauer *et al.* 2019) or ABA (Buhrow *et al.* 2021; Qi *et al.* 2016) with susceptibility or SA (Makandar *et al.* 2012), JA (Sun *et al.* 2016) and gibberellic acid (Buhrow *et al.* 2021) with resistance against *Fusarium* spp., roles of phytohormones remain complex and ambiguous. It was further shown, that their role during infection with *F. graminearum* strongly depends on distinct stages of infection demonstrating strong interactions of the pathogen with plant physiology (Ding *et al.* 2011; Ameye *et al.* 2015; Makandar *et al.* 2010; Makandar *et al.* 2012). Although, several biological and physiological mechanisms of *Fusarium* spp.-barley pathosystem were described, understanding of FHB susceptibility and resistance under complex environmental stress conditions poses new challenges but could support future breeding and selection of pathogen resistant and stress tolerant genotypes. For this purpose, the present study aims to investigate genotype-dependent quantitative resistance and regulatory networks of barley under infection with *Fusarium culmorum* in combination with drought stress. This revealed genotype-independent stress response markers, infection success-related gene expression clusters and a modular gene expression network under combined stress.

Material and methods

Description of greenhouse experiments

Three spring barley genotypes (Barke, Morex and Palmella Blue) were grown under controlled conditions in glass house cabins with air conditioning for temperature control. The genotypes were preselected according to different resistance to FHB as assessed in inoculation trials in the field (Hoheneder *et al.* 2022) and preliminary greenhouse.

Six barley grains were sown in each pot containing 3 L peat substrate (Einheitserde C700, Stender, Germany). 12 pots were prepared per barley genotype and stress treatment. We applied daily automatic watering and additional lightning for 16 h per day. Plants were randomized twice a week. Temperature was set to 18 °C (day) and 16 °C (night) with a relative air humidity of 60%.

Drought conditions were set for half of the pots from seven days before expected flowering (growth stage 65) on by a stop of automatic watering on separate flooding tables. To prevent

149 plants from early and premature plant death due to fast desiccation of the substrate, each pot
150 received little amount of water (50 mL) in the first three days after stopping daily irrigation. All
151 plants without irrigation showed reduced turgor pressure and partial loss of green leaf area
152 indicating strong drought stress of the plants. Continuously watered plants showed normal
153 growth and phenology.
154 Seven days after the beginning of drought conditions, spikes of irrigated or drought stressed
155 plants were sprayed with spray flasks till run-off either with *F. culmorum* spore solution or mock
156 solution to obtain four different treatment contrasts (WM: watered-mock, WFc: watered-
157 infected, DM: drought-mock, DFc: drought-infected). To maintain optimal moist conditions for
158 infection (99% relative air humidity), spikes were covered and sealed with transparent
159 polythene bags for two days. Respective mock sprayed plants were similarly treated. Two and
160 four days after inoculation, individual spikes were cut from each pot and flash frozen in liquid
161 nitrogen for further DNA, RNA and metabolite extraction.
162
163 *Preparation of Fusarium culmorum inoculum*
164 Fugal inoculum was cultured and propagated according to (Linkmeyer *et al.* 2013). Therefore,
165 three isolates of *F. culmorum* (Fc002, Fc03, Fc06 – culture collection, Chair of Phytopathology,
166 Technical University of Munich) known to strongly infect barley spikes and to produce DON
167 (Linkmeyer *et al.* 2013; Hofer *et al.* 2016; Hoheneder *et al.* 2022) were combined in equal
168 amounts. Spore solution was adjusted to 50,000 conidia per mL tap water and contained
169 1 mL/L Tween 80 to improve wetting of the spike tissue. The respective mock solution
170 contained the same amount of Tween 80.
171
172 *Sample preparation, DNA and RNA extraction from immature spike tissue*
173 Each individual sample of each biological triplicate per treatment variation was divided into two
174 pieces using one part for RNA extraction. The remaining spike tissues was put together to a
175 pooled sample of three spikes for DNA extraction. After separation, the spike samples were
176 immediately ground in liquid nitrogen and stored at minus 70 °C. DNA extraction was carried
177 out according to the protocol of (Fraaije *et al.* 1999) with minor modifications as described by
178 (Hofer *et al.* 2016). DNA concentration was adjusted to 20 ng total DNA μL^{-1} with nuclease free
179 water. RNA extraction was performed with Direct-zol RNA Miniprep Plus Kit (ZymoResearch,
180 USA) according to manufacturer's protocol.
181
182 *Quantification of fungal DNA*
183 Fungal and barley DNA was determined with qPCR according to (Nicolaisen *et al.* 2009) using
184 species specific primers and 10-fold dilution series of pure target DNA as standards. Non
185 template controls only contained water. Quantitative PCR reactions were carried out using
186 Takyon Low ROX SYBR 2X MasterMix blue dTTP (Eurogentec, Belgium) with a AriaMx real-
187 time PCR system (Agilent Technologies, USA). DNA contents of each sample was determined
188 in duplicates. Finally, fungal DNA was normalized with barley DNA in pg *F. culmorum* DNA
189 ng^{-1} $barley^{-1}$ DNA^{-1}.
190
191 *Library preparation for Illumina HiSeq2500 sequencing*
192 Preparation of libraries for 3'-RNA sequencing was carried out with Lexogen QuantSeq 3'-
193 RNA-Seq Library Prep Kit (FWD) (Lexogen, Austria) for Illumina sequencing according to
194 manufacturer's protocol. Quantification of input RNA was conducted with Qubit Fluorometer
195 2.0 (Invitrogen, USA) and Qubit RNA BR (broad range) Assay Kit (Invitrogen, USA) according
196 to manufacturer's protocol in a range of 10 to 1200 ng total RNA. Quantification of library size
197 was performed with Qubit Fluorometer 2.0 und Qubit DNA High Sensitivity Assay Kit
198 (Invitrogen, USA). A check for distribution of mRNA fragment size of each library was
199 performed with am Agilent 2100 Electrophoresis Bioanalyzer (Agilent Technologies, USA).
200 Final quantification of each library was determined according to Illumina qPCR guide with a
201 KAPA SYBR Fast Mastermix Low ROX (Peqlab, Germany) in a QuantStudio 5 real-time PCR
202 system (Applied Biosystems, USA). Final libraries were normalized with elution buffer (Qiagen,
203 Germany) to a final concentration to 2 nM and pooled with an equal amount of each sample

library. Denaturation and dilution of libraries for HiSeq Clustering was performed according to protocol A of user guide (Illumina) with a concentration for clustering of 10 pM.

Illumina HiSeq2500 sequencing

Sequencing of libraries was carried out on a HiSeq2500 sequencing platform using HiSeq Rapid SR Cluster Kit v2 (Illumina, USA) and HiSeq Rapid SBS Kit v2 (50 Cycle) with run parameters set for multiplexed single-reads (read 1: 100 cycles) and single-indexed reads of 7 cycles. HiSeq Control Software 2.2.70 was used for sequencing. Image analysis and base calling was carried out with Real-Time Analysis (RTA) 1.18.66.4. Fastq-files were generated with CASAVA BCL2FASTQ Conversion Software v2.20. Raw data have been stored on NCBI-Gene Expression Omnibus with the accession number GSE223521.

3'-RNAseq trimming, mapping and read count

The quality of the raw 3'-RNAseq data was analysed with FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). The trimming step was done with Trimmomatic (Bolger *et al.* 2014) using the parameters ILLUMINACLIP:Illumina-SE.fasta:2:30:10 LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:40. We used Hisat2 (Kim *et al.* 2015) with parameters -U --rna-strandness FR to map the trimmed data to the Morex v2 assembly (Monat *et al.* 2019). In order to add an artificial 3'UTR because of the 3'-RNAseq reads, we used a custom script to elongate the last exon either by 3 kb or until the next gene in the gff file of Morex v2. The counts of the generated bam files were assigned to genes using featureCounts (Liao *et al.* 2014) with the parameters -t gene -s 1 -M -O.

CPM calculation

CPM (counts per million) were calculated using the *edgeR*-package (Robinson *et al.* 2010). The CPMs were calculated from the "DGEList" object with log = FALSE, normalized.lib.sizes = TRUE.

Differential gene expression and cluster analysis

The differential gene expression analyses were carried out using the *edgeR*-package (Robinson *et al.* 2010). We kept all genes with a CPM equal or bigger than 15 in at least 10% of all samples. To increase the signal-to-noise ratio for the analyses, we pooled the two time points (48h and 96h post infection) for each sample and calculated the differential expressed genes with the following contrast formula: "((variety_condition_48h + variety_condition_96h)/2) - ((variety_watered-mock_48h + variety_watered-mock_96h)/2)" with variety := Barke, Morex, Palmella Blue and condition := watered-infected, drought-mock, drought-infected.

The cluster-analyses of the DEGs was done with the Multiple Expression Viewer (MeV, (Howe *et al.* 2011) using the implemented self-organizing tree algorithm (SOTA) with the default parameters. As input we used all differentially expressed genes with an FDR < 0.05 in at least one variety for one certain stress condition. For each analyses the $\log_2$ fold-change values over all samples for the respective gene sets were used.

GO enrichment analysis

GO-enrichment analyses were performed on the differentially expressed genes using the *topGO*-R-package (Alexa and Rahnenfuehrer 2022). The GO-terms for each barley gene were downloaded from https://biit.cs.ut.ee/gprofiler/gost. As gene universe we have used all genes found in our RNAseq analyses that passed the threshold of having a CPM equal or bigger than 15 in at least 10% of all samples (35657 genes). The enrichment analyses for "molecular function" and "biological process" was done using the following settings: algorithm="weight01", statistic="fisher". Shown are the graphs for the 5 most significant nodes.

Weighted gene correlation network analysis (WGCNA)

Co-expression analyses were performed using the R-package WGCNA (Langfelder and Horvath 2008, 2012). First we calculated the mean of the CPM values for the three replicates for each time point, variety, treatment and stress condition. We then selected the 12818 genes which showed in the differential gene expression analysis a sig. regulation with an FDR < 0.05

260 in at least one variety. The mean CPM values of those genes were normalized using variance
261 stabilizing transformation from the *vsn* R-package (Huber *et al.* 2002). For the WGCNA
262 analysis we have used the default settings with a soft power of 6 for the calculation of network
263 adjacency of gene counts and the topological overlap matrix, clustering the genes into 18
264 modules plus 42 unassigned genes collected in the grey module.
265 For all the external traits (phytohormones, plant metabolites and fungal DNA) that we have
266 correlated with our modules, we previously calculated the mean of the three replicates for each
267 time point, variety, treatment and stress condition. The mean value was than subjected into
268 the network analysis.
269 For the network analysis, the 25 edges between any two genes with the highest edge weight
270 in each module have been selected and the corresponding network of those selected edges
271 was drawn using the *igraph* R-package (Csardi and Nepusz 2006). For better visualization the
272 networks were manually adjusted.
273
274 *Measurement of phytohormones and plant metabolites*
275 Contents of phytohormones and secondary plant metabolites (abscisic acid, abscisic acid
276 glucoside, phaseic acid, dihydrophaseic acid, auxin, salicylic acid, salicylic acid glucoside and
277 proline) was carried out with mass spectrometry according to methods described in
278 (Chaudhary *et al.* 2020) and (Abramov *et al.* 2021). For this purpose, 150 mg of grinded barley
279 spike tissue as used for RNA extraction was used to determine contents of phytohormones
280 and plant metabolites in relation to fresh weight.

281

282 **Results**

283 *Global transcription analysis reflects variety-dependent susceptibility to FHB and*
284 *enhanced resistance under drought*

285 To study variety-dependent and -independent stress responses in barley and the effect of
286 drought stress on FHB, we grew three diverse spring barley varieties in pots under controlled
287 conditions in the greenhouse. The varieties Barke, Morex and Palmella Blue were selected
288 based on differences in their resistance against FHB in preliminary greenhouse experiments
289 and in inoculation trials in the field (Hoheneder *et al.* 2022) with Barke showing the most
290 resistant phenotype and Palmella Blue being the most susceptible variety. Morex showed an
291 intermediate resistance phenotype and represents the variety with the sequenced reference
292 genome for the later analysis. Further genotype characteristics and agronomic traits for all
293 three varieties can be found in the supplemental table T1.

294 Plants were either continuously irrigated (watered samples) or irrigation was stopped (drought
295 samples) seven days before flowering (GS 57-59), a time period in which weather conditions
296 are of pivotal importance for FHB pathogenesis in the field (Hoheneder *et al*, 2022). Inoculation
297 or mock-treatment of the spikes was carried out with *Fusarium culmorum* (*Fc*) spore
298 suspension on watered samples (watered-Fc, WFc or watered-mock, WM) or drought samples
299 (drought-Fc, DFc or drought-mock, DM) at mid of flowering (GS 65) and samples were taken
300 48 and 96 hours post-infection (hpi) (Fig. 1A).

301 In order to better understand the underlying differences in response to infection, drought stress
302 or the combination of drought with infection, we analysed the gene expression of barley spikes
303 using 3'-RNA-sequencing. Mapping of 3'-RNA reads on the reference genome, identified
304 transcripts from 41746 (Barke), 42653 (Morex) or 41363 (Palmella Blue) barley genes in our
305 samples. For 34969 genes, we obtained reliable reads from all three varieties. We compared
306 for each variety all treatments against the watered, not-infected (watered-mock) samples and
307 counted all significant (false discovery rate (FDR) corrected $p < 0.05$) differentially expressed
308 genes (DEGs) (Fig. 1B). There were large variety-dependent differences in the amount of

309 DEGs and in the distribution of up- and downregulated genes, ranging for example from 229
310 DEGs in Palmella Blue under drought stress over 953 genes in Morex up to 7447 genes in
311 Barke. Under infection, by contrast, susceptible Palmella Blue showed most DEGs. In total and
312 over all genotypes, we found 2949 DEGs after *F. culmorum* infection, 7879 DEGs under
313 drought, and 10188 DEGs under drought plus infection. Because the gene sets overlap, this
314 corresponds to a sum of 12818 DEGs (Supplemental data D1.1).

315 We used quantification of fungal DNA to assess the severity of infection in the same
316 experiment. Under irrigation we observed strong differences in the amount of fungal DNA from
317 Barke (Ba, 6 pg *Fc* DNA ng^{-1} $barley^{-1}$ DNA^{-1}) over Morex (Mo, 45 pg *Fc* DNA ng^{-1} $barley^{-1}$
318 DNA $^{-1}$) to Palmella Blue (Pb, 70 pg *Fc* DNA ng^{-1} $barley^{-1}$ DNA^{-1}; averages of 48 and 96 h post
319 inoculation) (Fig. 1C), corresponding well to the previously observed differences in basal
320 resistance. When compared to this, all three varieties showed a reduced amount of fungal
321 DNA when the plants were exposed to drought before infection (Ba 5 pg *Fc* DNA ng^{-1} $barley^{-1}$
322 DNA^{-1}; Mo 3 pg *Fc* DNA ng^{-1} $barley^{-1}$ DNA^{-1}; Pb 13 pg *Fc* DNA ng^{-1} $barley^{-1}$ DNA^{-1}).

323 We measured the accumulation of abscisic acid in all spike samples as one of the major
324 responses to drought stress. We observed low values of abscisic acid in most of the irrigated
325 samples, whereas the samples from drought-stressed barley showed clearly elevated levels
326 of ABA, with strong genotype-dependent differences (Ba 4260 ng ABA g^{-1} FW^{-1}; Mo 2057 ng
327 ABA g^{-1} FW^{-1}; Pb 1578 ng ABA g^{-1} FW^{-1}; averages of 48 and 96 h post inoculation, 9 or 11
328 days after stop of irrigation) (Fig. 1C).

329 Despite strong differences in susceptibility to *F. culmorum* infection and in drought responses
330 as measured by accumulation of ABA and the stress-associated amino acid proline
331 (Supplemental data D2), our data also revealed DEGs that were regulated in all three
332 genotypes under either infection-related stress (146 DEGs), drought stress (64 DEGs) or the
333 combination of both (167 DEGs) (Supplemental data D1.2 - D1.4). Because our genotypes are
334 diverse in geographic origin and pedigree (Supplemental table T1), those genes may serve as
335 general variety-independent markers for the core response of barley to the respective stresses.
336 In support of this, several of the DEGs in those lists are identical or homologous to previously
337 reported DEGs in other barley or wheat genotypes infected by *Fusarium graminearum* or
338 suffering from drought. Examples for such generally *Fusarium*-responsive genes are Fusarium
339 resistant-orphan protein, tryptophan decarboxylases, anthranilate synthase, laccases,
340 HvWRKY23 and DMR6-like 2-oxoglutarate and Fe(II)-dependent oxygenase genes
341 (Buerstmayr *et al.* 2021; Low *et al.* 2020; Soni *et al.* 2020; Boddu *et al.* 2006; Boddu *et al.*
342 2007; Karre *et al.* 2019; Perochon *et al.* 2019; Tucker *et al.* 2021). Additionally, many enzyme
343 genes that are potentially involved in detoxifying DON are among the commonly infection-
344 regulated DEGs such as glycosyltransferases and Glutathione-S-transferase genes and a
345 cysteine synthase (compare (Gardiner *et al.* 2010)). Prominent examples in that respect are
346 HvUGT13248 (HORVU.MOREX.r2.5HG0384710) and HvUGT6
347 (HORVU.MOREX.r2.5HG0430540) (He *et al.* 2020; Michlmayr *et al.* 2018; Schweiger *et al.*
348 2010). In the list of genotype-independent drought-associated DEGs, we find previously
349 reported dehydrins and late-embryogenesis-abundant protein genes, potential ABA-receptor
350 complex PP2c protein phosphatases and a downregulated SAUR auxin response protein
351 gene. 46 of the combined stress-associated variety-independent DEGs are also variety-
352 independently regulated in one of the single stress situations. Those genes hence reliably
353 showed the infection-related stress response even under drought and the drought-related
354 response in additionally infected situations over all barley varieties (Supplemental data D1.5).

355 Interestingly, there are only two DEGs (HORVU.MOREX.r2.5HG0401150;
356 HORVU.MOREX.r2.5HG0372030) that show significant regulation in all genotypes under

357 combined stress but not after one of the single stresses in at least one genotype. Hence, barley
358 seems to express no genotype-independent specific response to the applied combined stress.

359 The questions arose whether we find genotype-dependent stress markers in our data sets. We
360 found many Barke-specific DEGs under drought and many Palmella Blue-specific DEGs after
361 infection, because those genotypes showed the strongest gene expression responses to the
362 respective stresses. We therefore more specifically asked whether there are Barke-specific
363 DEGs under *Fusarium* stress that may explain higher quantitative resistance. This generated
364 a list of 20 upregulated and 47 downregulated genes, which are neither differentially expressed
365 in Morex nor in Palmella Blue (Supplemental data D1.6). Vice versa, we found 148 genes that
366 were upregulated in both susceptible Morex and Palmella Blue but not in Barke (Supplemental
367 data D1.7). Those lists possibly contain new factors for quantitative resistance or susceptibility
368 to FHB.

369 With a self-organizing tree algorithm (SOTA) analysis, DEGs clustered according to their
370 differential expression pattern over all varieties and treatments (Fig. 1C). Under infection with
371 *F. culmorum,* the expression strength of infection-related cluster I11 (2283 genes; 77.4% of all
372 infection-related DEGs) reflects the amount of fungal DNA (Fig. 1C). Cluster I9 and I10 (223
373 and 243 genes) show downregulation only in the most susceptible variety Palmella Blue. Taken
374 together, 93.2% of all infection-related DEGs are in those clusters (I9-I11) and their expression
375 strength rather reflects disease progression than resistance to FHB.

376 Under drought stress, the quantity of DEGs and the strength of expression in most of the
377 clusters reflected the amount of ABA that we measured in individual varieties with Barke
378 showing the strongest ABA accumulation (all drought-related clusters except D3, Fig. 1B, C).

379 The combination of drought stress with infection lead to the highest number of DEGs (10188
380 genes). Most of the clusters (DI1, 2, 4, 5, 9, 10, 11; 6394 genes, 62.8%) mirror very well the
381 expression pattern under drought stress alone, indicating that those genes are mainly
382 responsive to the drought stress. Cluster DI3 contains rather *Fusarium*-responsive genes, as
383 they are not upregulated under drought stress alone, but show a strong infection-strength
384 dependent regulation, similar to the before mentioned cluster I11. In particular, Palmella Blue,
385 which is still strongly infected under drought, shows upregulation of genes in cluster DI3.
386 Additionally, there are 3 clusters with variety-dependent expression that is similar in all stress
387 situations (DI6 for Palmella Blue, DI7 for Morex and DI8 for Barke).

388 Taken together, we observed variety-dependent differences both in infection severity and
389 strength of drought responses and all plants were less infected when plants were exposed to
390 drought before infection compared to their respective controls. Each single stress provokes a
391 mostly stress-specific gene expression response and the combination of drought stress and
392 infection was mainly dominated by the firstly applied drought stress response in our
393 experiment. However, in highly susceptible Palmella Blue many of combined stress-regulated
394 DEGs are also infection-related DEGs. In many cases, number of DEGs and strength of gene
395 regulation reflect an increase of a stress marker such as content of ABA or fungal DNA in the
396 same samples.

397

398 *Expression network analysis supports global similarities in the FHB-responses, but*
399 *also differences in drought and combined stress responses*
400

401 For further analysis of the DEGs we performed a weighted gene co-expression network
402 analysis (Zhang and Horvath 2005) to find DEGs that cluster into modules according to their
403 expression pattern over all samples, time points and treatments (Fig. 2A), resulting in 19

404 modules of DEGs. 42 DEGs did not match any co-expression pattern (grey module) (Fig. 2A).
405 The rest of the DEGs clustered into one of the remaining 18 modules, range in size from 63
406 genes up to 5287 genes in the turquoise module.

407 We checked for each barley variety and stress the relative contribution of modules to the
408 overall stress response and displayed the five modules with the highest contribution to the sum
409 of DEGs (Fig. 2B, supplementary Fig. S1). The blue module contains more than 60% of the
410 infection-related largely upregulated DEGs for all three varieties. This may indicate that all
411 three varieties show a similar response to infection with *F. culmorum* despite the fact that p-
412 value based numbers of DEGs strongly differed. Gene ontology analyses ranked protein
413 phosphorylation and response to biotic stress as most significantly enriched biological process
414 (supplementary data D3 and D4A) and heme binding, DNA-binding transcription factor activity
415 and protein serine/threonine kinase activity as most significantly enriched molecular functions
416 in the blue module (supplementary data D3 and D4) possibly reflecting a general pathogen
417 response in the blue module.

418 The drought stress response in Barke and Morex is dominated by the turquoise co-expression
419 module but also the green module contributes to the drought response in all genotypes. In
420 Palmella Blue the salmon module contains most DEGs but the turquoise module is also
421 represented. Gene ontology analyses identified photosynthesis related functions as most
422 significantly enriched in the turquoise module, and most of the genes are downregulated under
423 drought (supplementary data D1.3, D3 and D4). The green module contains many similarly
424 up-regulated genes and is most significantly enriched in gene ontology terms photorespiration,
425 response to stress ending in defence response and embryo development ending in seed
426 dormancy, and the salmon module with protein dephosphorylation and response to water
427 (supplementary data D3 and D4).

428 After the combination of drought stress and infection most of the DEGs in Palmella Blue cluster
429 in the blue module as seen for infection alone. In Barke and Morex the turquoise module is still
430 the biggest one. Interestingly, none of the modules showed a significant association with the
431 trait “combined stress” (see “DFc against all” in supplemental Figure S2A).

432 Additionally, we can also see genotype-related modules such as the yellow module being
433 regulated in Barke under all three stress conditions but contributing less to the sum of DEGs
434 in Morex and Palmella Blue DEGs (Fig. 2B, supplemental Figure S1).

435 The contribution pattern of individual modules to the overall stress response supports the
436 previous observation that the combination of drought stress with infection is mainly dominated
437 by the drought stress in Barke, because the distribution of the biggest modules is almost not
438 altered in Barke under drought stress alone and under drought plus infection. In comparison,
439 the distribution pattern of DEGs under drought plus infection matches better to infection alone
440 in Palmella Blue (Fig. 2B, supplemental Figure S1). Morex shows a more complex response,
441 but the drought stress pattern is largely recovered under combined stress.

442

443 *Co-expression modules correlate with FHB-severity, phytohormones or stress marker*
444

445 For plant stress responses, little information exists on association of gene co-expression
446 clusters with quantitative physiological traits of stress responses. We analysed module-trait
447 associations by correlating WGCNA-module-sample eigengenes with determined contents of
448 phytohormones and their derivates, abundance of the stress marker proline and the amount
449 of fungal DNA to identify significant associations (Fig. 3).

450 The blue module is the only module which has a significant positive correlation with the amount
451 of fungal DNA and the phytohormone auxin (IAA). The turquoise module shows the strongest
452 negative correlation with ABA; most genes in this module are downregulated under drought.
453 The green module has the highest positive correlation with the content of ABA. Similarly, to
454 the turquoise module, the tan module shows a significant negative correlation with ABA
455 contents. The salmon module, containing most of the DEGs in Palmella Blue under drought,
456 shows a significant positive correlation with proline, ABA and the ABA derivates phaseic acid,
457 dihydrophaseic acid and ABA glucoside. Hence, the module indicates a specific set of DEGs
458 for stress regulation under drought. Additionally, many modules show by-trend contrasting
459 correlations with salicylic acid or auxin versus ABA or derivates of ABA. The purple module is
460 even significantly positively associated with phaseic acid and dihydrophaseic acid but
461 negatively with salicylic acid. Hence, module-trait associations added functional value in
462 showing significant linkages between stress physiology and complex gene expression patterns
463 (Fig. 3).

464

465 *Co-expression levels reveal potential hub genes in selected modules*

466 For a better understanding of the inter-connection and functionality-related linkage between
467 the most co-regulated genes within selected modules we took the 25 edges with the highest
468 edge weight from each of those modules. The edge weight represents the connection strength
469 between two nodes, corresponding to the co-expression of those two genes.

470 The blue module is the only one with a significant positive correlation with the amount of fungal
471 DNA within barley spikes (Fig. 3) and most of the heavy edges are directly connected with the
472 DON-detoxifying Glycosyltransferase HvUGT13248 (HORVU.MOREX.r2.5HG0384710)
473 (Mandalà *et al.* 2019; Schweiger *et al.* 2010) (Fig. 4A, B, supplemental table D5). All of the
474 selected genes are infection-specifically expressed and the expression strength partially
475 reflects the amount of fungal DNA detected in each variety, with Palmella Blue having the
476 highest amount of fungal DNA detected by qPCR (see Fig. 1C). Most of the genes show a
477 positive fold change after infection of watered and drought stressed plants, but no significant
478 regulation under drought stress alone (Fig. 4B). Manual gene reannotations suggested a
479 possible function of genes in DON-toxin response (HvUGT13248 and a GSTU6-like
480 Glutathione S-transferase, HORVU.MOREX.r2.7HG0532730), plant defence and regulation of
481 cell death (e.g. heat shock transcription factor similar to rice lesion mimic gene *spl7*,
482 HORVU.MOREX.r2.1HG0067240; a NAC transcription factor, similar to OsNAC4 involved in
483 cell death responses, HORVU.MOREX.r2.3HG0247070; disease resistance protein a, which
484 represents a Toll-interleukin receptor 1 domain only protein,
485 HORVU.MOREX.r2.2HG0110590) (Kaneda *et al.* 2009; Yamanouchi *et al.* 2002) and of plant
486 immunity (homologs of Arabidopsis PUB21 and PUB23 plant U-box domain-containing family
487 proteins, HORVU.MOREX.r2.6HG0521570, HORVU.MOREX.r2.7HG0555030) (Trujillo *et al.*
488 2008).

489 The salmon module shows positive correlation with drought stress associated ABA and ABA-
490 derivates and the drought stress marker proline but no association with fungal infection
491 success (Fig. 3). The genes corresponding to the 25 highest co-expression values are in all
492 three varieties upregulated under drought stress, both alone and in combination with infection
493 (Fig. 4D, supplemental table D5). Typical drought and dehydration stress-associated genes
494 are among the strongly co-expressed genes. These are for instance dehydrins
495 (HORVU.MOREX.r2.6HG0516710, HORVU.MOREX.r2.6HG0516720) or a seed maturation
496 protein (HORVU.MOREX.r2.7HG0529950). Additionally, we find a phytoene synthase
497 potentially involved in ABA biosynthesis (HORVU.MOREX.r2.5HG0419050) and
498 hexosyltransferase similar to galactinol synthase 2, a drought stress tolerance protein that

499 catalyses raffinose synthesis for osmoregulation (HORVU.MOREX.r2.2HG0090050) (Gu *et al.*
500 2016; Li *et al.* 2020; Selvaraj *et al.* 2017) and an ABA-related Arabidopsis AtHB-7-like
501 homeobox transcription factor (HORVU.MOREX.r2.6HG0496860) (Söderman *et al.* 1996).

502 The green module is significantly correlated with ABA and proline but not fungal infection
503 success (Fig. 3). The genes corresponding to the 25 highest co-expression values are, similar
504 to what we found for the salmon module, genes associated with responses to dehydration
505 stress, like late-embryogenesis abundant proteins (HORVU.MOREX.r2.1HG0049080,
506 HORVU.MOREX.r2.1HG0049090, HORVU.MOREX.r2.3HG0213440,
507 HORVU.MOREX.r2.5HG0352360), potential osmoregulatory sodium/hydrogen_exchanger
508 (HORVU.MOREX.r2.7HG0560260) (Brini *et al.* 2007) or desiccation-induced 1VOC proteins
509 (HORVU.MOREX.r2.4HG0322480) (Mulako *et al.* 2008) (Fig. 4E, supplemental table D5). A
510 lot of the genes show a stronger regulation in Barke compared to the two others varieties
511 potentially reflecting higher ABA contents in Barke under drought (Fig. 4F).

512 The turquoise module is by far the biggest module (5287 genes) (Fig. 2A) and is negatively
513 correlated with ABA (Fig.3). Most of the strongest co-expressed genes are either associated
514 with photosynthesis or are associated with translation (Suppl. Fig. S3A+B, supplemental table
515 D5), two process being already known to be downregulated under stress.

516 The midnightblue module is not associated with any measured trait (Fig. 3), but is among the
517 modules dominating the drought stress response of especially Palmella Blue (Fig. 2B).
518 Interestingly, 8 out of the highly connected 11 genes with strongest co-expression are
519 annotated as heat-shock proteins (Suppl. Fig. S3C+D, supplemental table D5).

520 Taken together the correlation of the modules and phytohormones or stress-markers is further
521 strongly supported by the predicted functions of most strongly co-expressed genes in each
522 module. Corresponding gene annotations support the hypothesis that the blue module may
523 partly link to DON-responses and cell death during differentially progressing pathogenesis in
524 the different varieties. The salmon and green module showed a significant correlation with
525 several typical drought stress markers, like ABA or proline, and also the genes showing the
526 strongest co-expression in these modules strongly support a key function in the response to
527 drought stress and ABA.

528

529 **Discussion**

530 Comparably little is known about the response of plants to a combination of different stresses
531 and there is a high demand for understanding complex stress responses in crop plants (Rivero
532 *et al.* 2022). Severity, frequency and combination of stresses collectively define the likelihood
533 that stress results in tissue damage or complete organ or plant death (Buchanan 2000).
534 Literature provides examples for plant responses to diverse simultaneous stresses that act
535 additive or mutually inhibitory or synergistic (Ben Alaya *et al.* 2021; Loo *et al.* 2022; Rivero *et*
536 *al.* 2022). We wanted to better understand how drought, which increasingly often occurs before
537 flowering and seed set in mid European barley growing areas, may influence FHB infection.
538 We used diverse spring barley genotypes that have different degrees of quantitative resistance
539 to FHB under field and glasshouse conditions (Hoheneder *et al.* 2022) to survey a broad
540 spectrum of possible responses in barley. We observed strong differences in the amount of
541 the drought stress hormone ABA (Fig. 1C; Supplemental data D2) whereas the drought stress
542 metabolite and osmolyte proline accumulated more uniformly among the three genotypes
543 (Supplemental data D2). This might indicate that the genotypes experienced a similar change
544 of water potential but Barke particularly strongly responded to this by accumulation of ABA and
545 the strongest drought-related gene expression response. In contrast, Barke reacted least to

546 *F. culmorum* infection, although it showed the highest degree of quantitative resistance as
547 assessed by fungal DNA in infected ears (Fig. 1). By contrast, highly susceptible Palmella Blue
548 showed most DEGs and strongest upregulation under infection. Morex showed an
549 intermediate response in both aspects. Therefore, the global gene expression rather reflects a
550 transcriptional response associated with successful FHB pathogenesis. However, many genes
551 that are significantly regulated in susceptible barley are also infection-responsive in more
552 resistant Barke albeit at a lower level and not significantly different from controls (Fig. 1;
553 Supplemental data D1.1). We speculate that in less susceptible Barke less tissue gets in direct
554 contact to the developing fungus and our low numbers of DEGs in most resistant Barke may
555 be biased due to a dilution effect from healthy parts of the tissue. Nevertheless, high numbers
556 and strong regulation of DEGs in susceptible barley do not reflect efficient defence, an
557 observation that has been made before for FHB in wheat (Biselli *et al.* 2018; Buerstmayr *et al.*
558 2021; Wang *et al.* 2018).

559 Application of drought stress before FHB infection resulted in less successful pathogenesis.
560 This effect was strong in Morex and moderate in Palmella Blue. In little susceptible Barke,
561 drought did not have a major impact on FHB development. Accordingly, global gene
562 expression responses to the fungus were reduced in susceptible barley under drought.
563 However, in Palmella Blue, the amount of DEGs that appear to be primarily infection-
564 responsive under combined stress was still high, and genes showed strong upregulation.
565 Again, strong gene expression responses to fungal infection associate with fungal success,
566 even if the success is limited under drought.

567 SOTA analysis allowed for a quick overview about global gene expression patterns. Generally,
568 SOTA-resolved gene expression suggested that most combined stress regulated clusters
569 showed a modular gene expression trend. This means that they often reflect gene expression
570 that similarly appeared under drought or infection alone. We did not find clear indications for a
571 specific response of barley to the combined stress but data rather support a combination of
572 the drought and the infection response. If we consider the 2949 infection-related DEGs, we
573 see that most clusters show a lower responsiveness under combined stress, when compared
574 to infection stress alone. However, data do not resolve whether this reflects an inhibitory effect
575 of drought stress on the pathogen response or rather less fungal infection success, although
576 we maintained optimal infection conditions by covering barley spikes with polythene bags for
577 two days after inoculation. Morex profited most strongly from drought when considering
578 reduced fungal infection success. Interestingly, quite some clusters from the combined stress
579 situation, show also mild pathogen- and drought-responsiveness in Morex (Fig. 1C, clusters
580 DI5, 7, 9-11). It is tempting to speculate, that in this case the drought response positively added
581 to the pathogen response and supported effective pathogen defence.

582 We used the full complexity of our data set and all DEGs to calculate gene co-expression
583 networks by WGCNA. This uncovered modules of co-expressed genes, which largely reflect
584 the pathogen response (blue module) or drought response (turquoise, green, salmon modules)
585 in the used varieties. Similar information was extracted from the SOTA and as such not a
586 surprise, e.g. infection related clusters of the SOTA analysis were rediscovered in the blue
587 module of the WGCNA (supplementary dataset D1.1). Added value of the WGCNA derived
588 from physiological trait association and diving into most strongly co-expressed gene sets
589 (arbitrarily extracted 25 heaviest edges of big modules). Association of modules with
590 physiological data of fungal development and of plant stress markers supported that the blue
591 module is positively correlated with fungal DNA and auxin contents (Fig. 3). Previous reports
592 from wheat FHB had already reported a connection between FHB susceptibility and auxin
593 contents or responses (Brauer *et al.* 2019; Luo *et al.* 2016; Wang *et al.* 2018). This could hence
594 reflect a general association in FHB of *Triticeae*. The blue module subnetwork of 25 most
595 strongly co-regulated genes centered around the DON-detoxifying glycosyltransferase

596 HvUGT13248 and more genes with predicted functions in toxin-response, dampening of
597 immune-responses and programmed cell death regulation (Kaneda *et al.* 2009; Mandalà *et al.*
598 2019; Buerstmayr *et al.* 2021) (Fig. 4A). In this context it is interesting, that overexpression of
599 the barley cell death suppressor BAX Inhibitor-1 enhances resistance to *F. graminearum* ear
600 and crown infection in barley (Babaeizad *et al.* 2009). Since gene selection was based solely
601 on edge weight in the network, and several discovered genes have partially described function
602 in *Fusarium* spp. or general immune response, outcome suggests that our pipeline was
603 suitable for finding potentially pivotal gene functions in big co-expression modules. This is
604 supported by the fact that also the green and salmon module subnetworks of heavy edges
605 reflect gene annotations known from drought responses and seed maturation (Fig. 4C, E).
606 Self-pollinating barley is fertilized already before open flowering and seed set is initiated. We
607 applied drought stress before open flowering and our data suggest that seed maturation was
608 accelerated under drought, storage proteins and dehydrins are upregulated and also sugar
609 osmolytes may enrich. This change in plant resource allocation could have also influenced
610 fungal infection success because quick ripening might have limited the hemibiotrophic fungus
611 in access to plant metabolites.

612 The interplay of biotic and abiotic stress responses is complex and difficult to study. Literature
613 provides examples of mutually inhibitory or supportive stress responses, such that combined
614 or consecutive stresses may result in enhanced or reduced stress resistance. Often plant
615 hormone-triggered pathways act antagonistically in complex interactions with the environment,
616 which may have evolved as a measure of checks and balances to fine tune responses for
617 optimal fitness. Our data also show multiple tendencies of opposite correlation of gene
618 expression in WGCNA modules and salicylic acid versus ABA or auxin versus ABA and related
619 metabolites (compare grey-blue versus green squares in vertical columns of Fig. 3). Perhaps,
620 ABA responses and early ripening limited pathogenesis-associated auxin responses and
621 thereby reduced susceptibility to fungal infection.

622 The question arises, whether we can generalize the observations we made in terms of drought
623 associated resistance to fungal infections in barley. Indeed, a recent study found many drought
624 stress related genes to be differentially expressed during Fusarium crown rot development in
625 barley and wheat (Su *et al.* 2021). In a similar context, a NAC transcription factor (HvSNAC1),
626 involved in drought tolerance, was found to mediate resistance towards Ramularia leaf spot
627 disease of barley (McGrann *et al.* 2015). We observed earlier, that Barke and fourteen other
628 spring barley genotypes showed enhanced resistance to Ramularia leaf spots when artificially
629 exposed to long lasting drought stress in the field. However, in open fields, severe Ramularia
630 leaf spots occurred even in hot and dry summers if single short rain events provided sufficient
631 leaf wetness (Hoheneder *et al.* 2021). It is hence difficult to generalize that drought would
632 mitigate severity of fungal infections. The timing of drought stress application may further be
633 pivotal for its influence on fungal infection success. In preliminary experiments, we found that
634 drought can also enhance development of FHB disease, when applied simultaneously instead
635 of before infection. It remains speculative, whether and how altered gene regulation is
636 depending on the order of occurring abiotic and biotic stress situations. We found several NAC
637 and WRKY transcription factors regulated under combined stress and clustering in the blue
638 module (Supplemental data D1.1), which reveals importance of single and versatile acting
639 genes on multiple stress responses. This and future studies may provide an increasing amount
640 of data that allows for a deep understanding of how stress responses interact depending on
641 plant genotype and phenology and timing and combination of stresses.

642 The complexity of gene expression data is a boon and a bane of combined stress response
643 analyses. In front of thousands of stress-regulated genes in our study, using diverse genotypes
644 allowed for identification of genotype-independent stress markers that are reliable both under
645 single and combined stresses (Supplemental data D1). Computational association of gene

expression modules with physiological or agronomic traits appears helpful for interpretation of complex data. Enhancing the complexity of experimental setups and data points also supports co-expression analyses and identification of subnetworks that may function in stress resistance or susceptibility and can identify trait-associated candidate genes for functional analysis.

Supplementary data

Table T1. Detailed information about the three used barley varieties

Figure S1. Full list of co-expression modules in each variety for each stress treatment

Figure S2. Relationships of consensus module eigengenes with binary comparisons

Figure S3. Co-expression levels reveal potential hub genes in selected modules

Supplemental data D1. List of DEGs

Supplemental data D2. Data table with measured phytohormones, stress markers and fungal DNA

Supplemental data D3. Statistics of GO term enrichment for molecular function and biological process in selected modules

Supplemental data D4. Hierarchical trees of GO term enrichment for molecular function and biological process in selected modules

Supplemental data D5. Gene numbers, abbreviations and CPM values for genes with the highest co-expression values in selected module

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Author contributions

FH, CES, MH and RH: conceptualization.
FH, CES and RH wrote the manuscript
FH and conducted the greenhouse experiments.
CW performed the RNA sequencing.
MG and CD measured the phytohormones and stress markers.
MM and KM mapped the RNAseq reads to Barley genome.
CES, NK, RS and RH analysed the obtained sequencing results.

Conflict of interest

No conflict of interest declared

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Figure legends

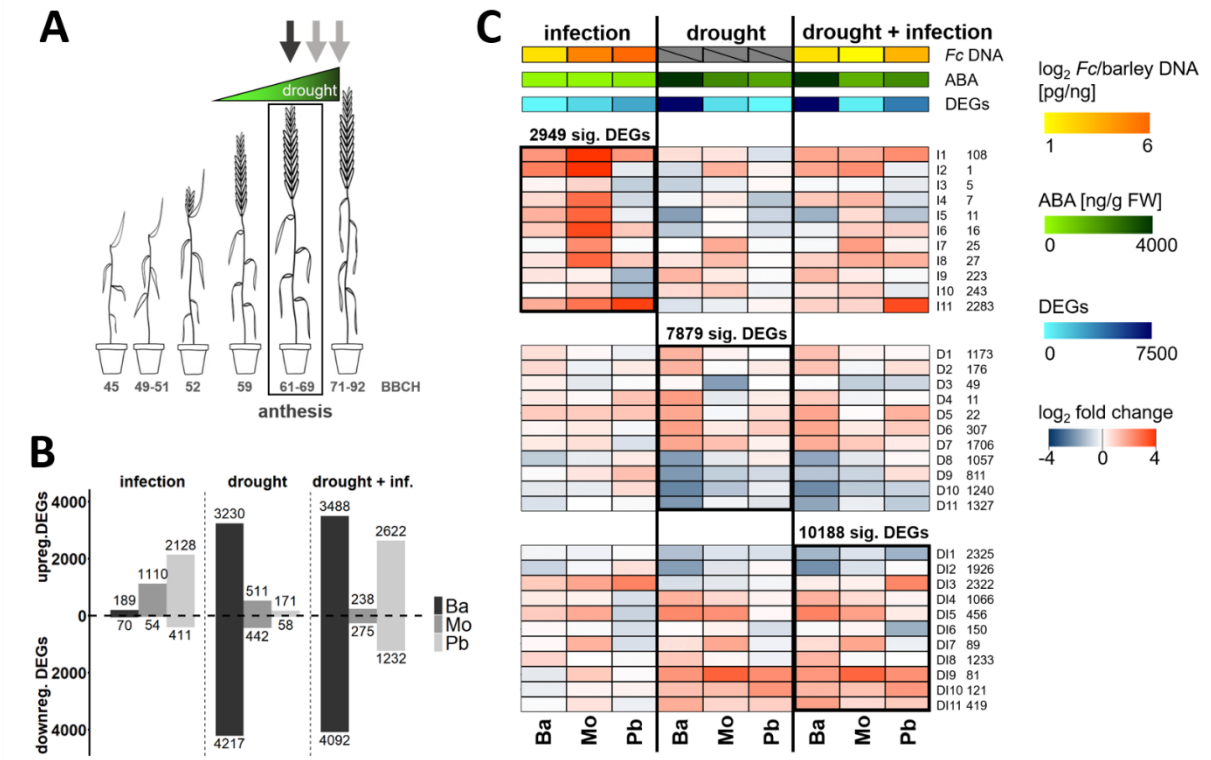
Fig. 1. Global transcription analysis of variety-dependent, reduced susceptibility against FHB under drought stress in the greenhouse. (A) Experimental setup: Three different barley varieties (Barke, Ba; Morex, Mo; and Palmella Blue, Pb) were grown in pots under controlled conditions in the greenhouse. Drought stress was induced by stopped irrigation 7 days before anthesis (growth stage 61-69; dark grey arrow). Irrigated and drought-stressed plants were inoculated with conidia of *Fusarium culmorum* or mock (dark grey arrow) and samples were harvested 2 and 4 days post-inoculation (light grey arrows). (B) Number of DEGs. For each variety all treatments were compared against the watered, mock-infected samples. Differentially expressed genes were counted (FDR $p<0.05$) and number of DEGs were plotted. Upwards directed columns show the numbers of upregulated genes and downwards directed columns show downregulated genes. (C) Heat maps of key experimental outcomes and expression $\log_2$ fold changes of clusters of genes that behave similarly according to self-organizing tree algorithm. Amount of *Fusarium culmorum* DNA per ng barley DNA was determined by qPCR. Values were colour-coded with grey (0), yellow ($\log_2$ 1 pg *Fc* DNA ng^{-1} $barley^{-1}$ DNA^{-1}) to orange ($\log_2$ 6 pg *Fc* DNA ng^{-1} $barley^{-1}$ DNA^{-1}). The average DNA content from 2 and 4 dpi was calculated and the $\log_2$ used for data representation. Abscisic acid content [in pg ng^{-1} $fresh^{-1}$ $weight^{-1}$] in all spike samples was quantified using mass spectrometry. Values were colour-coded with light-green (0 ng ABA ng^{-1} $fresh^{-1}$ $weight^{-1}$) to dark-green (4000 ng ABA ng^{-1} $fresh^{-1}$ $weight^{-1}$). For each variety DEGs (relative to watered, not infected samples) with a FDR-corrected p-value < 0.05 were counted under each stress treatment and the quantity was colour-coded with light-blue (0) to dark-blue (7500). All genes which were significantly regulated ($p < 0.05$) in at least one variety in one particular stress treatment (infection: 2949 genes; drought: 7879 genes; drought + infection: 10188 genes) were subjected to a cluster analysis using a self-organizing tree algorithm (SOTA) using the expression pattern over all varieties and stress treatments. The respective stress treatment for which the DEGs were selected is highlighted by a black frame. All DEGs were split into 11 clusters, labelled with numbers 1-11 and a capital letter for the stress (I=infection, D=drought, DI=drought + infection) next to it and the corresponding number of genes in each cluster. The mean $\log_2$ fold change for each cluster was colour-coded ranging from blue (> -4) over white (0) to red (< 4).

Fig. 2. Modules of DEGs after weighted gene co-expression network analysis. (A) Co-expression modules. Based on the expression pattern over all time points, varieties and

treatments all DEGs clustered into 18 modules (color-coded) with 63 to 5287 genes after WGCNA-analysis. 42 genes didn't follow any co-expression pattern and were clustered in the grey module. The numbers reflect the amount of DEGs in each module. (B) Top 5 biggest co-expression modules in each variety for each stress treatment. We counted the DEGs in each module for each variety under each stress treatment. We show the 5 modules with most DEGs in each stress scenario in percentage relative to the total number of DEGs per variety (Barke, Ba; Morex, Mo; and Palmella Blue, Pb).

Fig. 3. Relationships of consensus module eigengenes with FHB-severity, different phytohormones or stress markers. Each column in the table corresponds to a module and each row to one of the physiological traits: FHB-severity, a phytohormone, its derivate or proline as a stress marker. Module names are shown on top. Square colours in the figure represent the correlations of corresponding module eigengenes and measured stress parameters. The FDR-corrected p-values are coded by size (the bigger the square, the more significant). Highly significant correlations ($p < 0.01$) are highlighted with bold frames. DNA, *F. culmorum* DNA relative to plant DNA; ABA, abscisic acid; PA, phaseic acid; DPA, dihydrophaseic acid; IAA, indol 3-acetic acid; SA, salicylic acid.

Fig. 4. Co-expression levels reveal potential hub genes in selected modules. For three co-expression modules (blue, salmon and green), the 25 edges with the highest edge weight have been selected and their corresponding network was schematically drawn (A blue module, C salmon module, E green module). Each node represents one gene, and in the heat maps on the left the log fold changes of the gene expression values are shown with the respective colour codes and individual scales for each module (B blue module, D salmon module, F green module). The size of the square represents the false discovery rate-corrected p-values (FDR), with bigger squares indicating higher confidence levels. The bold squares indicate a significant FDR p-value smaller than 0.05. For gene identifiers, see supplemental dataset D5.



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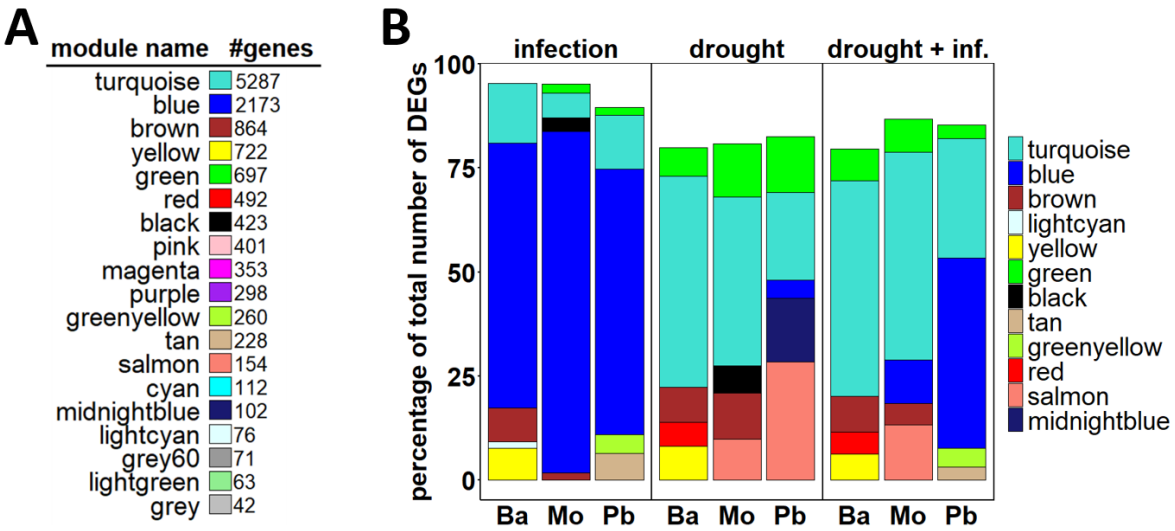

Fig. 2. Modules of DEGs after weighted gene co-expression network analysis.

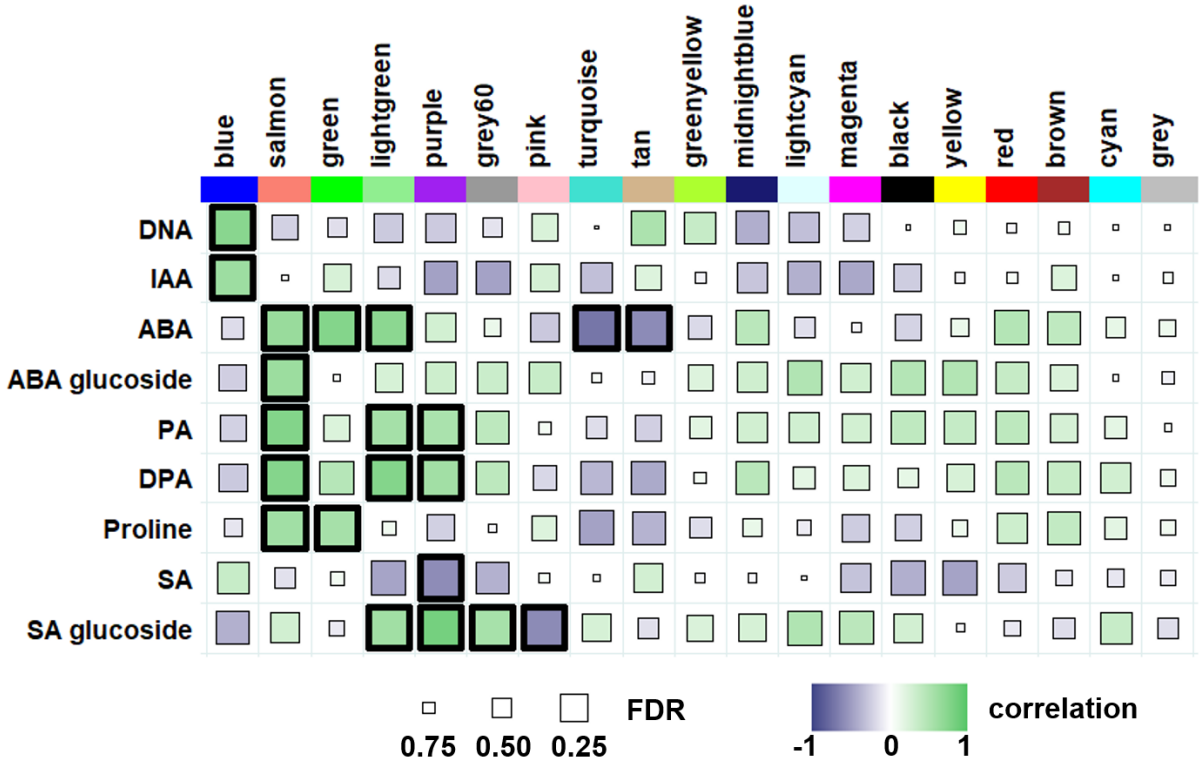

Fig. 3. Relationships of consensus module eigengenes with FHB-severity, different phytohormones or stress markers.

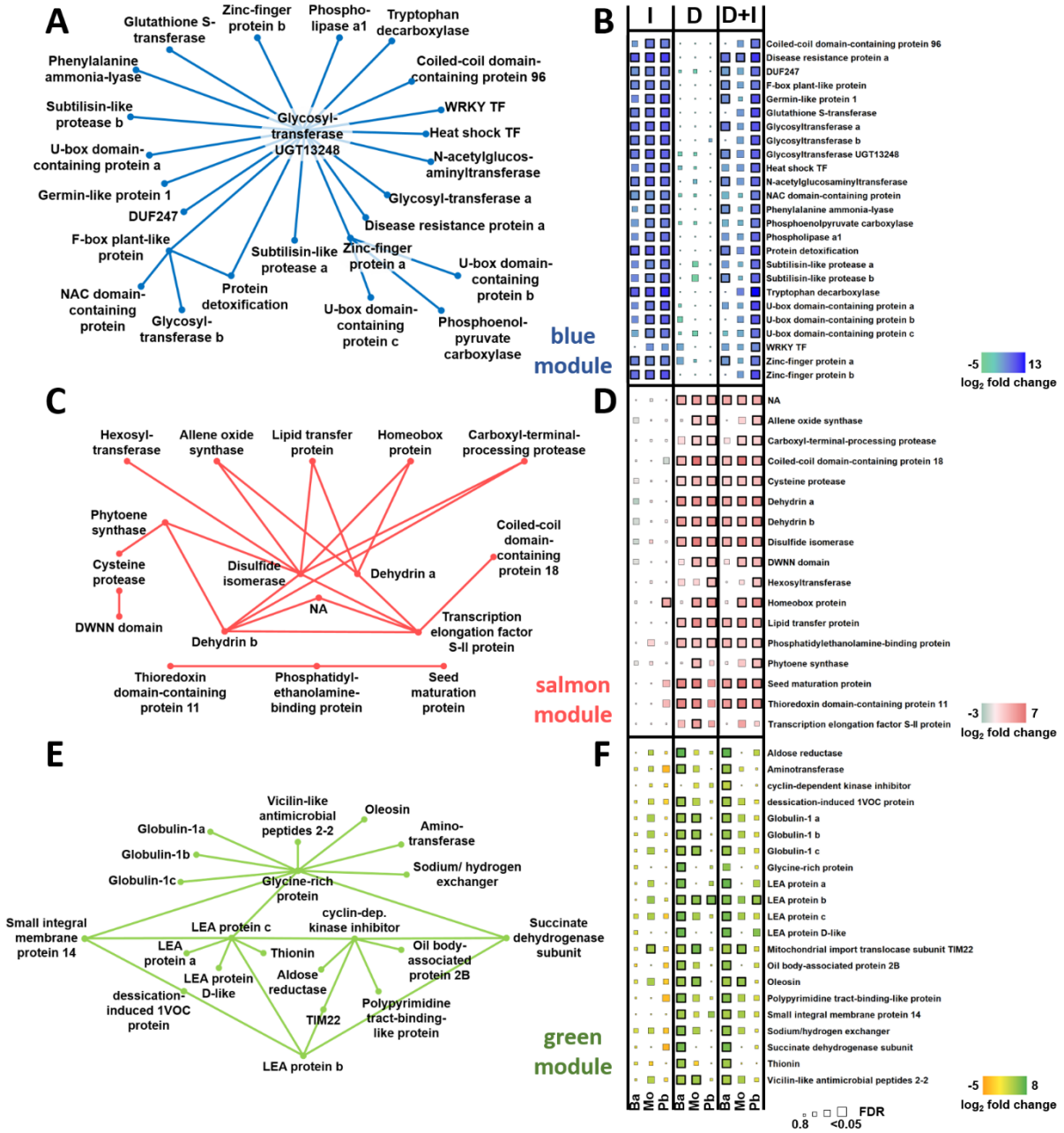

Fig. 4. Co-expression levels reveal potential hub genes in selected modules.