The metatranscriptomic response of the wheat rhizosphere to drought varies with growth stages.

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Abstract

Microbes can help plant sustain abiotic stresses, such as drought. Plant-microbe interactions are, however, dynamic and the timing of the stress will affect both partners, directly and indirectly. Here, we hypothesize that the effect of drought stress on the wheat rhizosphere microbiome would change between key growth stages. We grew wheat in pots and reduced soil water content for two weeks at stem elongation, booting, or heading. We then sampled the rhizosphere soil and sequenced its metatranscriptome. The timing of the drought strongly affected the transcriptional response of the microbes, but few differentially abundant transcripts were shared across all stages. Some common patterns were however observed at higher taxonomical or functional levels. Drought also affected the normal succession across wheat growth stages. Many of the differentially abundant transcripts, taxa and functions between growth stages of the control plants were not significant anymore for plants that experienced drought. Our results suggest that the timing of the drought event is paramount to the microbial rhizosphere communities, and that it could explain the heightened sensitivity of younger plants to stresses.

Keywords: metatranscriptomics, drought, rhizosphere, microbial communities, wheat.

Introduction

Drought can – depending on the growth stages – kill crops, severely reduce yields and grain quality [1], or have no effect. However, it is not clear if this is due to direct effects on the plant or indirectly through changes in beneficial plant-microbe interactions. A recent metatranscriptomic study from our group supports the indirect route. First, the microbiota – and not the plant – was the main responder to decreasing soil water content [2]. Second, this decrease in water content resulted in higher abundance of microbial transcripts that might be beneficial to the plant and microbes [2]. For instance, one microbial strategy to adapt to the decreased matric potential in dry soil is to accumulate osmolytes [3], and accordingly, we observed more microbial transcripts related to osmolytes in soils with low water content [2]. This could also be beneficial for the plant as microbes can exude osmolytes in the plant environment [4, 5]. Plant tolerance to drought- or salinity-related stresses can indeed be improved by different strains of *Actinobacteria* and *Proteobacteria* [6-9] and by fungal endophytes [10-12]. These beneficial microbial taxa and associated functions will, however, vary with plant growth stages, which could partly explain the varying sensitivity of crops to drought. This interaction between drought and growth stage is not fully understood, as most of the above-mentioned studies focused on a single sampling.

Time and water stress directly, indirectly, and interactively affects microbial communities. First, microorganisms respond directly to soil water availability [13-15], which contributes to seasonal changes in microbial communities [16, 17]. Second, water-stress induced shifts in root exudation [18, 19] can result in indirect effect of water stress on plant-associated microorganisms. Third, time can indirectly affect plant-associated microbial communities as root exudation change with plant growth stage [20, 21], shifting the microbial communities Finally,

56 indirect and direct effects of time and water stress interact to influence the microbial
57 communities. For instance, wheat associated microbial communities were altered by historical
58 and current water stress in interaction with plant growth stage, plant genotype, and plant
59 compartment [13-15]. As most studies look at single factors in isolation, it is difficult to predict
60 the impact of the timing of drought on the microbial response and its feedback to crops, even
61 though this knowledge would be crucial to devise microbial approaches to help crops adapt to
62 water stress.

63 Global change will result in decreased soil moisture and increased drought events [22,
64 23]. Drought can reduce wheat yield by up to 50% [24, 25]. We think that if their response to
65 drought was better understood, microorganisms could help abate these losses. As a first step, we
66 need to understand how microbial communities will respond to water stress at different wheat
67 growth stages. To answer this question, we grew wheat plants in pots without watering them for
68 two weeks at three distinct growth stages (stem elongation, booting, and heading). At tillering,
69 booting, stem elongation, heading and flowering, we uprooted plants, sampled their rhizosphere
70 soil, extracted the RNA and sequenced it. We hypothesized that the effect of drought stress on
71 the wheat rhizosphere microbiome would vary between key growth stages, with the microbiome
72 of younger plants being more affected.
73

Material and methods

Experimental design and sampling

To test our hypothesis, we set-up a greenhouse pot experiment (Fig. 1). Each 1L pot was filled with 1.5 kg of dry soil. The soil was collected from our experimental field at the Institut national de la recherche scientifique (Laval, QC, Canada). This soil is naturally subjected to dry-rewet cycles during the summer [17]. All the soil was air dried, sieved with a 2 mm mesh sieve and homogenized. We then sowed in each pot 8 seeds of *Triticum aestivum* cv. AC Nass (Semences Nicolet, Nicolet, QC, Canada), a drought-sensitive cultivar. After germination, the plants were thinned to 5 per pot. Until the stem elongation stage, all the pots were maintained at 50% SWHC (soil water holding capacity). Drought was imposed for two weeks by dropping to 8% SWHC at the beginning of stem elongation, booting and heading growth stages, resulting in 3 treatments plus a control that was kept at 50% SWHC throughout the experiment (Fig. 1). We wanted to impose drought during the tillering stage as well, but when we tried that during the pre-experiment, all plants died. The treatments were replicated six times in a randomized complete block design, and since our sampling was destructive, this resulted in 120 pots. After their 2-week exposure to drought, the plants were brought back to normal water content and not further exposed to drought. It was observed that during drought plants were weak but continued growth, that there was no delay in the occurrence of the next growth stage and that they showed resilience after rewetting. We sampled at tillering, stem elongation, booting, heading and flowering (Fig. 1). At stem elongation, booting and heading, we waited after the soil water content reached its target value (within a few days) before sampling all treatments and controls, to make sure we sampled during the drought event. Only the pots from the corresponding treatment were under drought at the time of sampling, while all other pots were well watered. All

97 the plants from a pot were uprooted and firmly shaken, after which the soil remaining attached to
98 the roots – the rhizosphere – was sampled, mixed thoroughly and snap frozen in liquid nitrogen.
99 This resulted in 120 rhizosphere soil samples (3 treatments + 1 control x 5 growth stages x 6
100 blocks). Rhizosphere soils were transported to the lab on dry ice and then kept at -80°C until
101 extracted.
102
103 *RNA extraction and sequencing*
104 Total RNA was extracted from 2 g of rhizosphere soil using the RNeasy PowerSoil Total RNA
105 Kit (QIAGEN, Canada). Extracted RNA was treated with DNAse (ThermoFisher, Canada) to
106 remove the DNA prior to sequencing. The absence of DNA was confirmed by the lack of PCR
107 amplification using 16S rRNA gene specific primers. Total RNA was sent for two lanes of
108 NovaSeq6000 S4 PE150 sequencing at the Centre d'Expertise et de Services Génome Québec
109 (Montréal, Québec). Libraries were created using a microbial ribosome subtraction approach.
110 The raw sequencing data produced in this study was deposited in the NCBI under Bioproject
111 accession PRJNA978601.
112
113 *Bioinformatics analyses*
114 The metatranscriptome sequencing of the 120 rhizosphere samples resulted in 12,000M reads for
115 a total of 1,800 giga bases which were processed together through our metatranscriptomics
116 bioinformatics pipeline [26], as detailed in Pande et al. [2]. For gene functional assignment, we
117 used the JGI's guidelines [27] including the assignment of KEGG orthologs (KO). For
118 taxonomic assignment, each contig was blasted (BLASTn v2.6.0+) against NCBI's nt database
119 (version downloaded from NCBI's server on January 9th 2019) and the best hit's taxonomic

identifier was used to assign a taxonomic lineage to the contig. Taxonomic summaries were performed using MicrobiomeUtils v0.9 (github.com/microbiomeutils).

Statistical analyses

All statistical analyses were performed in R version 4.2.3. [28]. Transcript differential abundance analyses between the drought treatments and the growth stages were carried out using the EBTest function of the EBSeq library with a false discovery rate (FDR) of 0.05. Anovas were performed using the aov function of the stats package. Permanovas were performed using the adonis2 function of the vegan package. For both Anova and Permanova, the blocks were considered in the formula. All graphs were generated using ggplot2. The R project folder containing the R code used for data manipulation, statistical analyses, and tables and figure generation is available on our lab GitHub repository (https://github.com/le-labo-yergeau/MT_Time_Wheat). The associated transcript abundance and annotation tables, and the metadata files used with the R code are available on Zenodo (https://doi.org/10.5281/zenodo.7995531).

135 **Results**

136 *Growth stages have a strong influence on the wheat rhizosphere metatranscriptome.*

137 We performed permanova and principal coordinate analysis (PCoA) on the entire
138 metatranscriptome (14,709,097 transcripts) to assess the effect of the four drought treatments and
139 the five growth stages. 16.1% of the variation could be explained by growth stage (P=0.001),
140 3.2% by the drought treatments (P=0.008) and 11.1% by the interaction between the two
141 variables (P=0.001). This can be visualized in the PCoA (Fig. 2a), where the clustering of the
142 samples is mostly driven by the wheat growth stage, with some, less visible in the first two
143 dimensions, effects of the drought treatments. We also looked at the treatments, but classified as
144 when the drought was applied (DR), as rewetted (RW, treatments after the drought event) or as
145 not disturbed (ND, controls and treatments before the drought event) (Fig. 2b). These results
146 show that growth stage has a strong influence on the metatranscriptome.The drought treatments
147 also affected the metatranscriptome, but its effect varied according to the wheat growth stage.

148 We then looked at the taxonomical affiliation of all transcripts across growth stages for
149 all treatments (Fig. 2c) and tested the difference between the control and the different treatments
150 during the drought or after rewetting using paired t-tests. Since there was quite some variability
151 between the treatments, we also kept results with 0.05<P<0.10. As compared to the controls, the
152 relative abundance of *Ascomycota* decreased 1.45 times during drought, when applied at stem
153 elongation (t=2.49, P=0.062). When this treatment was rewetted, the *Bacteroidetes* were 1.34
154 times less abundant than in the control (t=2.31, P=0.069). When drought was applied at booting,
155 none of the most abundant phyla changed their relative abundance, both during drying and
156 rewetting. When soils were dried at heading, the *Actinobacteria* were 1.34 times more abundant
157 than in the controls (t=4.95, P=0.004), whereas the *Chloroflexi* were 1.38 times more abundant

158 (t=3.94, P=0.011). For the *Chloroflexi*, this difference was sustained during rewetting, where
159 they were 1.14 times more abundant in the rewetted treatment as compared to the control
160 (t=3.27, P=0.022).

161 We also looked at the COG category of the transcripts (Fig. 3), and tested differences
162 using paired t-tests as above. As above, since there was quite some variability between the
163 treatments, we also kept results with 0.05<P<0.10.When soils were dried at the stem elongation
164 stage, transcripts related to "Carbohydrate transport and metabolism" were decreased by 1.10
165 times during drought as compared to the controls (t=2.49, P=0.055). When this treatment was
166 rewetted, no difference was found with the controls. When drought was applied at booting,
167 transcripts affiliated to "Energy production and conversion" and "Translation, ribosomal
168 structure and biogenesis" decreased 1.12 and 1.16 times, respectively (t=2.23, P=0.077 and
169 t=2.39, P=0.063, respectively), whereas transcripts classified in the "Transcription" category
170 increased 1.15 times (t=2.06, P=0.095). No significant difference remained for this treatment
171 after rewetting. When soils were dried at heading, transcripts classified in the COG categories
172 "Posttranslational modification, protein turnover, chaperones" and "Transcription" increased
173 1.24 and 1.39 times as compared to the controls, respectively (t=2.26, P=0.074 and t=3.44,
174 P=0.019, respectively). This difference was sustained at rewetting for the "Transcription"
175 category that was 1.12 times more abundant than the controls (t=2.24, P= 0.075).

176

177 *The response to drought across growth stages is idiosyncratic.*

178 To analyze the effect of the drought treatments on the metatranscriptome, we did a transcript
179 differential abundance (DA) analysis between the control and each of the three drought
180 treatments across the different growth stages. In terms of number of differentially abundant

181 transcripts, the patterns varied per treatment (Fig. 4a). When drought was applied at stem
182 elongation, there was a six-fold increase in the number of DA transcripts from stem elongation to
183 booting (from drought to rewetting), followed by a four-fold decrease at heading and another
184 four-fold decrease at flowering (Fig. 4a). When drought was applied at booting, however, there
185 was an 11-fold increase in the number of DA transcripts during drought (booting) followed by a
186 sharp decrease at rewetting (heading) (Fig. 4a). Likewise, when drought was applied at heading,
187 there was a four-fold increase in the number of DA transcripts during drought (heading) followed
188 by a two-fold reduction at rewetting (flowering) (Fig. 4a). Taken together, these results show that
189 the drought treatments resulted in large changes in the rhizosphere metatranscriptome. The
190 amount of DA transcripts varied through the plant growth stages depending on the timing of the
191 drought event.

192 We did a co-occurrence analysis of the DA transcripts found at the dry and rewetting
193 stages for the three treatments (Fig. 4c). Most of the transcripts were unique to a single treatment
194 (Fig. 4c). For the drought at stem elongation treatment, 17,166/21,399 (80.2%) and
195 121,052/126,995 (95.3%) of the DA transcripts were unique at the stem elongation and booting
196 stages, respectively. For the drought at booting treatments, 48,402/59,607 (81.2%) and
197 5,896/7,636 (77.2%) of the DA transcripts were unique at the booting and heading stages,
198 respectively. Finally, for the drought at heading treatment, 25545/32141 (79.5%) and
199 14094/15877 (88.8%) of the DA transcripts were unique at the heading and flowering stages.
200 Across the six samples (total of 247,498 DA transcripts), the largest intersection was between the
201 drought samples at booting and heading, but still only 3,512 DA transcripts were shared (Fig.
202 4c). 800 DA transcripts were shared by 3 or more treatment x growth stage combinations, 14 by
203 4 or more treatments x growth stage combinations, and none by 5 or more treatments x growth

204 stage combinations (Fig. 4c). When comparing the samples taken during the drought events, 174
205 DA transcripts were shared by the three treatments, whereas the samples taken during the
206 rewetting shared only 2 DA transcripts. Taken together, these results suggest that the response of
207 the microbial communities to the drought treatment is very different when drought is applied at
208 different plant growth stages, and that only a minority of DA transcripts are shared across the
209 different treatments.

210 We then looked at the genus and COG function annotation of DA transcripts of Fig. 4a. A
211 large portion (~75%) of the DA transcripts could not be annotated at the genus and the COG
212 function levels and were removed from the figure for clarity. As the results we are presenting are
213 the taxonomic or the functional make-up of a list of DA transcripts generated by comparing all
214 replicates, it is not possible to perform statistical comparisons as we end up with a single DA
215 transcript list per treatment comparison. The taxonomic makeup of the DA transcripts varied
216 across growth stages across all treatments, even before the drying and rewetting events (Fig. 5a).
217 The total fraction of DA transcripts that belonged to the genera listed in Fig. 5a – that made up at
218 least 0.25% of the DA transcripts across all samples – was lower at the stage that showed the
219 largest increases in DA transcripts in Fig. 4a. This could be related to an increase in the
220 unannotated DA transcripts or an increase in other genera that did not cross the inclusion
221 threshold across all samples. Besides, there are no clear trends in the shifts among the genera that
222 made up the DA transcripts. For the COG functions, however, the make-up of the DA transcripts
223 was much less variable, with similar proportions of the most abundant functions across most
224 stages for all treatments (Fig. 5b). There were some shifts visible, mostly at the stage where we
225 had seen the largest number of DA transcripts (Fig. 4a), in many cases for COG functions related

226 to amino acids (Fig. 5b). In any case, the shifts in the COG functions of the DA transcripts are
227 subtler than the shifts observed at the genus level.
228
229 *Shifts in metatranscriptome across growth stages are compounded by drought.*
230 We also did differential transcript abundance analyses between each contiguous growth stage
231 (tillering vs. stem elongation, stem elongation vs. booting, booting vs. heading, heading vs.
232 flowering), for both treatments and control. We did this to disentangle the effects of growth
233 stages on the metatranscriptome vs. the temporal effects of the drought treatments. Here again,
234 since we incorporated all replicates in DA analyses, we end up with a single list of DA
235 transcripts per treatment comparison, and it is therefore not possible to perform statistical
236 comparisons. In the controls, the number of DA transcripts was 2.8 to 4.6 times higher for the
237 heading vs. flowering contrast as compared to the other contrasts (Fig. 4b), suggesting large
238 shifts in the rhizosphere metatranscriptome during flowering. For the drought at stem elongation
239 treatment, there were many DA transcripts (150,083 to 287,091) for the stem elongation vs.
240 booting, booting vs. heading and heading vs. flowering contrasts, which was up to 15.5 times
241 more than the controls (for the booting vs. heading contrast) (Fig. 4b). For the drought at booting
242 treatment, the number of DA transcripts was higher than in the controls only for the stem
243 elongation vs. booting and booting vs. heading contrasts (Fig. 4b), up to a factor of 2.7. For the
244 drought at heading treatment, the number of DA transcripts was higher than the controls for the
245 booting vs. heading and the heading vs. flowering contrasts (Fig. 4b), with a 7.3 time increase for
246 the latter case. These results indicate that the microbial metatranscriptome does change with
247 plant growth stages, but that these changes are compounded by water stress.

248 Most of the DA transcripts were not shared between the treatments, for all contrasts
249 including the ones before drying (Fig. 6). This suggests that at the transcript level, there was a
250 lack of a coherent temporal response of the rhizosphere microbiome to drought and to growth
251 stage. The genus-level affiliation of the DA transcripts varied across the contrasts (Fig. 7a).
252 Although there were some common taxa represented in the DA transcripts, the water-stressed
253 microbial succession differed from the well-watered (control) microbial succession (Fig. 7a). For
254 instance, when comparing the stem elongation vs. the booting stages, the DA transcripts for the
255 drought at stem elongation and booting treatments contained less *Cylindrospermum* and
256 *Pelomonas* genera as compared to the controls (Fig. 7a). When comparing the booting and
257 heading stages, the DA transcripts in all the treatments were enriched in *Sorangium* and depleted
258 in *Cylindrospermum* and *Nitrospira* (Fig. 7a). Additionally, the drought at heading treatments
259 transcripts were enriched in *Entotheonella*, whereas it was the inverse for the two other
260 treatments. (Fig. 7a). For the heading vs. flowering contrast, the DA transcripts of the drought at
261 booting treatments were more often affiliated to *Asticcacaulis*, *Devosia*, *Cylindrospermum* and
262 *Sorangium* than the control DA transcripts (Fig. 7a). In contrast, the two other treatments showed
263 inverse trends.
264 At the functional level – COG functions – the affiliation of the dominant DA transcripts
265 was relatively stable for the control treatment (Fig. 7b). For the drought at stem elongation
266 treatment, there were more DA transcripts identified to "Serine/threonine protein kinase" and
267 "Signal transduction histidine kinase" than in the control for both the stem elongation vs. booting
268 and booting vs. heading contrasts (Fig. 7b). For the stem elongation vs. booting contrast, there
269 were less DA transcripts related to "Chaperonin GroEL" and "DNA-directed RNA polymerase"
270 in the drought at stem elongation and at booting treatments, relative to the control (Fig. 7b). For

271 the booting vs. heading contrast, the DA transcripts identified in the three treatments were
272 depleted in “Chaperonin GroEL” and “Outer membrane membrane protein…” as compared to
273 the control (Fig. 7b). Both the drought at booting and drought at heading treatments had more
274 DA transcripts related to “ABC-type dipeptide transport system” and “ABC-type sugar transport
275 system”. Finally, for the heading vs. flowering contrast, the affiliations of the DA transcripts of
276 the treatments were like the control (Fig. 7b). There was, however, more DA transcripts in the
277 “ABC-type dipeptide transport system” and “ABC-type sugar transport system” and “Outer
278 membrane protein…” for the drought at stem elongation and drought at booting treatments,
279 relative to the control (Fig. 7b). Taken together, these results suggest that at the genus and COG
280 function levels, there are some groups that are consistently showing a difference in their
281 succession when subjected to drying and rewetting.
282

Discussion

We had previously shown that, within the wheat holobiont, the strongest responders were the microbial communities [2]. Here, we looked at how these microbial communities respond to a 2-week dry spell imposed at different growth stages. For all growth stages, drying resulted in many differentially abundant (DA) transcripts. However, only a small percentage of these DA transcripts were shared across growth stages, suggesting idiosyncratic responses. This response altered the microbial succession across wheat growth stages.

When we imposed drought at an early stage (tillering), we killed all our wheat plants. Similarly, many plants resist better to disease as they age [29]. Plant aging also modulates the response to drought of the associated microbial communities. For example, the root microbial community composition of older sorghum plants is less impacted by drought than the one of younger plants [30]. Our youngest plants that survived drought (stem elongation stage) were the ones that showed the largest metatranscriptomic shifts. These shifts were larger, more intense during rewetting and lasted longer, as compared to the ones observed in older plants. For these older plants – at the booting and heading stages – the metatranscriptome showed smaller changes that rapidly returned to unstressed control levels. It therefore appears that, in agreement with our hypothesis, the rhizosphere metatranscriptome of younger wheat plants reacts more to drought.

On top of having different magnitude, the shifts observed at the transcript level across the drought treatments were idiosyncratic. Idiosyncrasy is often seen in the response of microbial communities to various factors. For instance, depending on the plant compartment and the water stress history of the soil, drought altered differently the wheat microbial communities [13-15]. The wheat microbial community is also influenced by wheat genotype, but constrained by the plant growth stages, compartment and time [16]. This idiosyncrasy also depends on the

306 resolution of the analyses: at coarse taxonomic or functional levels, some patterns are conserved
307 (e.g. increased relative abundance of *Actinobacteria* following drought) [13, 15, 31-33], but at
308 finer levels, there is no coherent response. For instance, the microbial responses to drought at
309 two different plant growth stages differed at the OTU level but resulted in the same enrichment
310 of monoderms [30]. Here, most of the DA transcripts were unique for a single treatment, but
311 when looking at functional categories (i.e. COG functions), the differences were less important.
312 This supports the idea that shifts at fine taxonomic or at the individual transcript levels might be
313 seemingly random but are coherent at the functional category level or at coarser taxonomical
314 levels [31, 34]. Alternatively, part of the idiosyncratic responses observed could be due to
315 stochastic variation, as even before the drought treatments were applied, many of the DA
316 transcripts were unique and showed distinct taxonomic affiliations across treatments.
317 Some of the idiosyncrasies observed could be explained by the microbial succession
318 through plant developmental stages. As plant develops, its microbial community fluctuates, and
319 drought does not affect similarly microbial communities of different diversity and composition.
320 For instance, Gram-negative bacteria produce osmolytes in response to drought, whereas they are
321 constitutively produced in Gram-positive [35]. At the same time, Gram-positive bacteria
322 generally have a thicker cell wall that Gram-negative bacteria [36]. Accordingly, Gram-positive
323 bacteria (mostly *Actinobacteria* and *Firmicutes*) made up 90% of the wheat microbial isolates
324 that could grow under high osmotic pressure [37]. As mentioned above, *Actinobacteria* generally
325 increase in relative abundance following drought [13, 15, 31-33], suggesting a heightened
326 capacity to resist to that stress. The relative activity of *Actinobacteria* also increased at the
327 flowering stage in our study. As compared to bacteria, the wheat fungal community composition
328 often doesn't respond as much to changes in soil water content [17, 38], but they strongly

329 responded to decreasing soil water content at the metatranscriptomic level [2] and many fungal
330 isolates from wheat could grow at high osmotic pressure [37]. Hence, the magnitude of the
331 metatranscriptomic response of the microbial community to drought will likely be defined by the
332 relative dominance of fungi, Gram-positive bacteria, and Gram-negative bacteria, which all have
333 different life-strategies when facing water stress [31, 38, 39]. As all these groups vary through
334 plant development, this could partly explain the contrasting responses of the microbial
335 communities to drought imposed at different plant growth stages.

336 In addition to its strong idiosyncratic effect on microbial transcripts, drought resulted in a
337 different microbial transcriptomic succession as compared to the well-watered controls.
338 Previous studies had shown that the plant root exudates varies across plant growth stages [20],
339 shifting the microbial community and the metatranscriptome of the rhizosphere [16, 21, 40, 41].
340 The effect of drought on microbial communities was, however, shown to trump the effect of
341 plant development [30]. Indeed, across developmental stages, we found a much larger number of
342 DA transcripts in the treated rhizospheres as compared to the controls. In the controls, the largest
343 number of DA transcripts was found when comparing heading to flowering. The flowering vs.
344 heading comparison was also the one that yielded the largest number of DA transcripts in the
345 treated samples, probably because it compounded the large shifts due to succession with the ones
346 due to drought. Under drought, the microbial transcriptomic succession in the rhizosphere was
347 heightened and had little to do with the well-watered (control) succession, even though the
348 development of the plants was not visibly disrupted by the drought.

349 We worked with a single wheat genotype that was not bred for resistance to water stress.
350 The response of wheat to water stress does vary across genotype, with some being better at
351 tolerating water stress at particular growth stages [42]. The wheat microbiome also varies across

genotypes [13, 15], but this is constrained by plant compartment, growth stages and with environmental conditions [16]. Historical exposure of the soil microbiome to water stress also modulates its interaction with wheat genotypes [13-15]. It would be important to confirm the results presented here for genotypes with a gradient of water stress resistance and across multiple soils, ideally in the field.

We showed here that drought strongly affects the wheat rhizosphere microbial transcriptome, but in a growth stage-dependent fashion. Our results imply that - as for the plant - early drought events impact more the microbial communities. These altered microbial communities might be less beneficial, which could partly explain the vulnerability of young plants to stresses. Microbial communities also responded when drought was applied at other growth stages, but the identity of the transcripts was very different. In all cases, drought altered the microbial transcriptome of the rhizosphere, which could have repercussions on wheat growth and grain quality. Understanding the interaction between crop growth stage and drought events on the rhizosphere microbiome is essential to optimize crop resistance and resilience under the current climate emergency.

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375 **Data availability**

376 The raw data produced in this study was deposited in the NCBI under Bioproject accession
377 PRJNA978601. The R project folder containing the R code used for data manipulation, statistical
378 analyses, and tables and figure generation is available on our lab GitHub repository
379 (https://github.com/le-labo-yergeau/MT_Time_Wheat/). The associated transcript abundance and
380 annotation tables, and the metadata files used with the R code are available in the Zenodo
381 archive (https://doi.org/10.5281/zenodo.7995531).

382

383 **Author contributions**

384 PMP: Designed, set-up and maintained the greenhouse experiment, performed the soil sampling
385 and molecular analyses. JT: Performed the bioinformatic analyses. MSA: Designed the
386 experiment, supervised students and secured funding. EY: Designed the experiment, performed
387 the statistical analyses, secured funding, supervised students and wrote the manuscript with
388 inputs from all authors.

389

390 **Competing interests**

391 The authors declare no competing interests.

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501

502 **Figure legends**

503 **Figure 1. Experimental design.** Water stress was imposed at Stem Elongation (SE), Booting
504 (B) or Heading (H), and we sampled at Tillering, Stem Elongation, Booting, Heading, and
505 Flowering. Created in https://BioRender.com

506 **Figure 2. Growth stage has a strong influence on the wheat rhizosphere**
507 **metatranscriptome.** Principal coordinate analysis based on the relative abundance of all
508 transcripts (A,B) and community composition at the phylum level (C) for the rhizosphere
509 metatranscriptome of wheat plants at tillering, stem elongation, booting, heading and flowering
510 stages that were stressed at stem elongation (SE), booting (B) or heading (H), or not stressed
511 (control).

512 **Figure 3. Growth stage also influences the functional composition of the metatranscriptome**
513 **in the wheat rhizosphere.** Community composition at the COG category level for the
514 rhizosphere metatranscriptome of wheat plants at tillering, stem elongation, booting, heading and
515 flowering stages that were stressed at stem elongation, booting or heading, or not stressed
516 (control).

517 **Figure 4. The response to drought across growth stages is idiosyncratic.** Number of DA
518 transcripts (treatment vs. control) in the rhizosphere metatranscriptome of wheat plants at
519 tillering, stem elongation, booting, heading and flowering stages that were stressed at stem
520 elongation, booting or heading (A). Number of DA transcripts between adjacent growth stages
521 for the rhizosphere metatranscriptome of wheat plants that were stressed at stem elongation,
522 booting or heading, or not stressed (B). Number of shared DA transcripts (treatment vs. control)
523 in the rhizosphere metatranscriptome of wheat plants during drought at stem elongation, booting

524 or heading, or after rewetting. Only intersection with size above 100 are shown (C). SE: stem
525 elongation, B: booting, H: heading, F: flowering.

526 **Figure 5. The taxonomical and functional makeup of the DA transcripts vary without clear**
527 **patterns.** Genus (A) and COG function (B) makeup of the DA transcripts (treatment vs. control)
528 in the rhizosphere metatranscriptome of wheat plants at tillering, stem elongation, booting,
529 heading and flowering stages that were stressed at stem elongation, booting or heading.

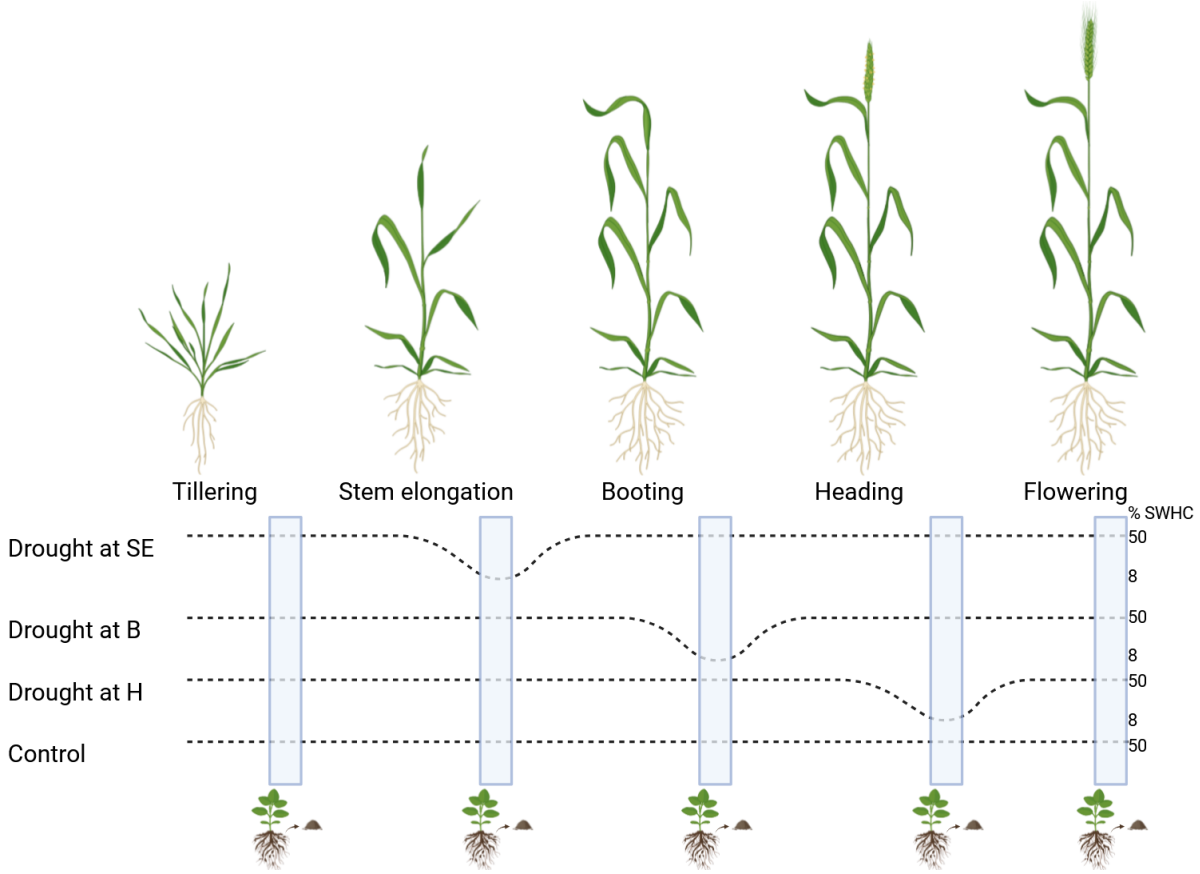
530 **Figure 6. Drought treatments affect the normal microbial succession across growth stages.**
531 Number of shared DA transcripts between tillering and stem elongation (A), stem elongation and
532 booting (B), booting and heading (C), and heading and flowering (D) for the rhizosphere
533 metatranscriptome of wheat plants that were stressed at stem elongation, booting or heading, or
534 not stressed. T: tillering, SE: stem elongation, B: booting, H: heading, F: flowering.

535 **Figure 7. Drought treatments affect the normal microbial taxonomical and functional**
536 **succession across growth stages.** Genus (A) and COG function (B) makeup of the DA
537 transcripts between adjacent growth stages for the rhizosphere metatranscriptome of wheat plants
538 that were stressed at stem elongation, booting or heading, or not stressed**.** T: tillering, SE: stem
539 elongation, B: booting, H: heading, F: flowering.

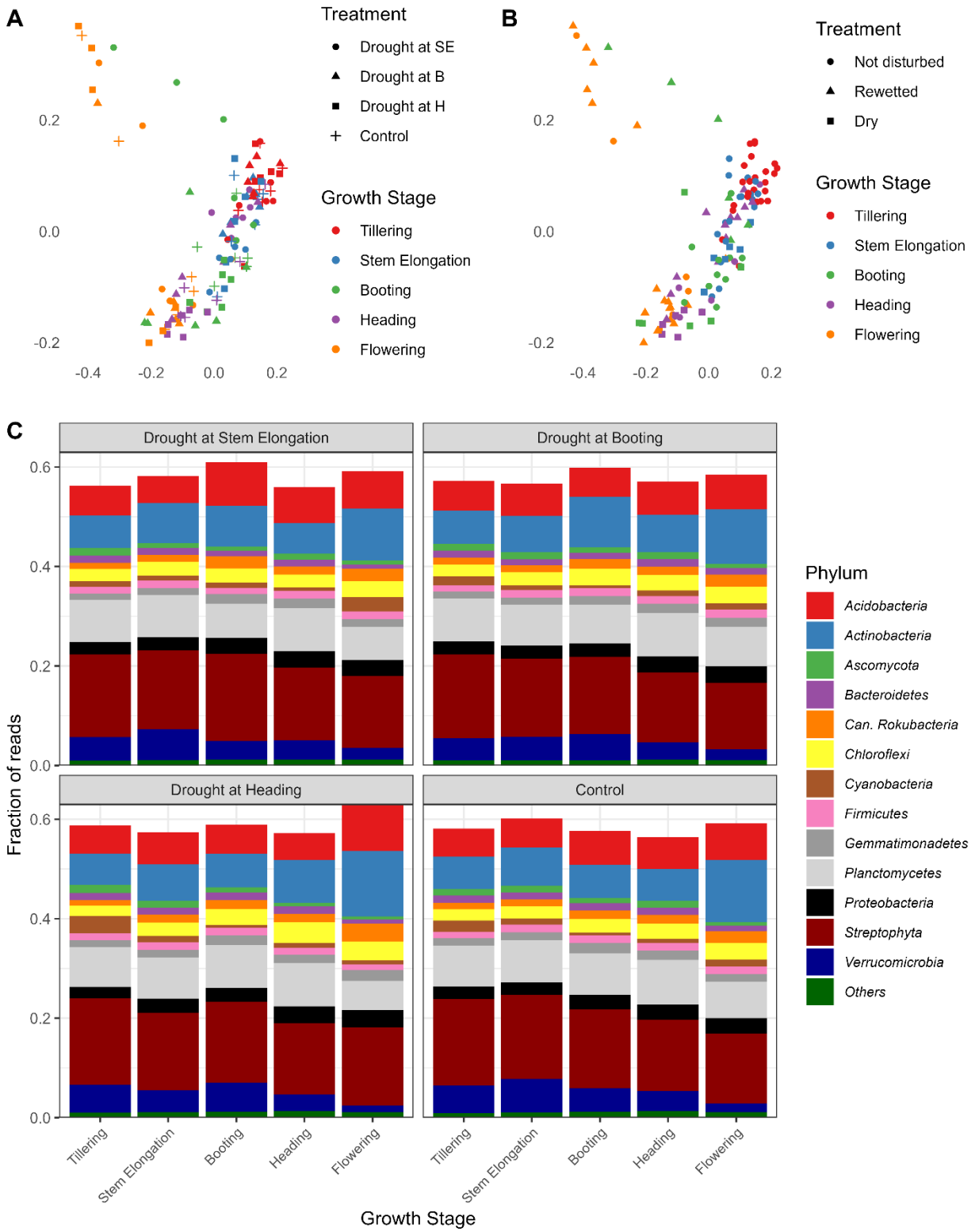
540

541

Figure 1.


Tillering
Stem elongation
Booting
Heading
Flowering
% SWHC
Drought at SE
Drought at B
Drought at H
Control
50
8
50
8
50
8
50

546 **Figure 2.**



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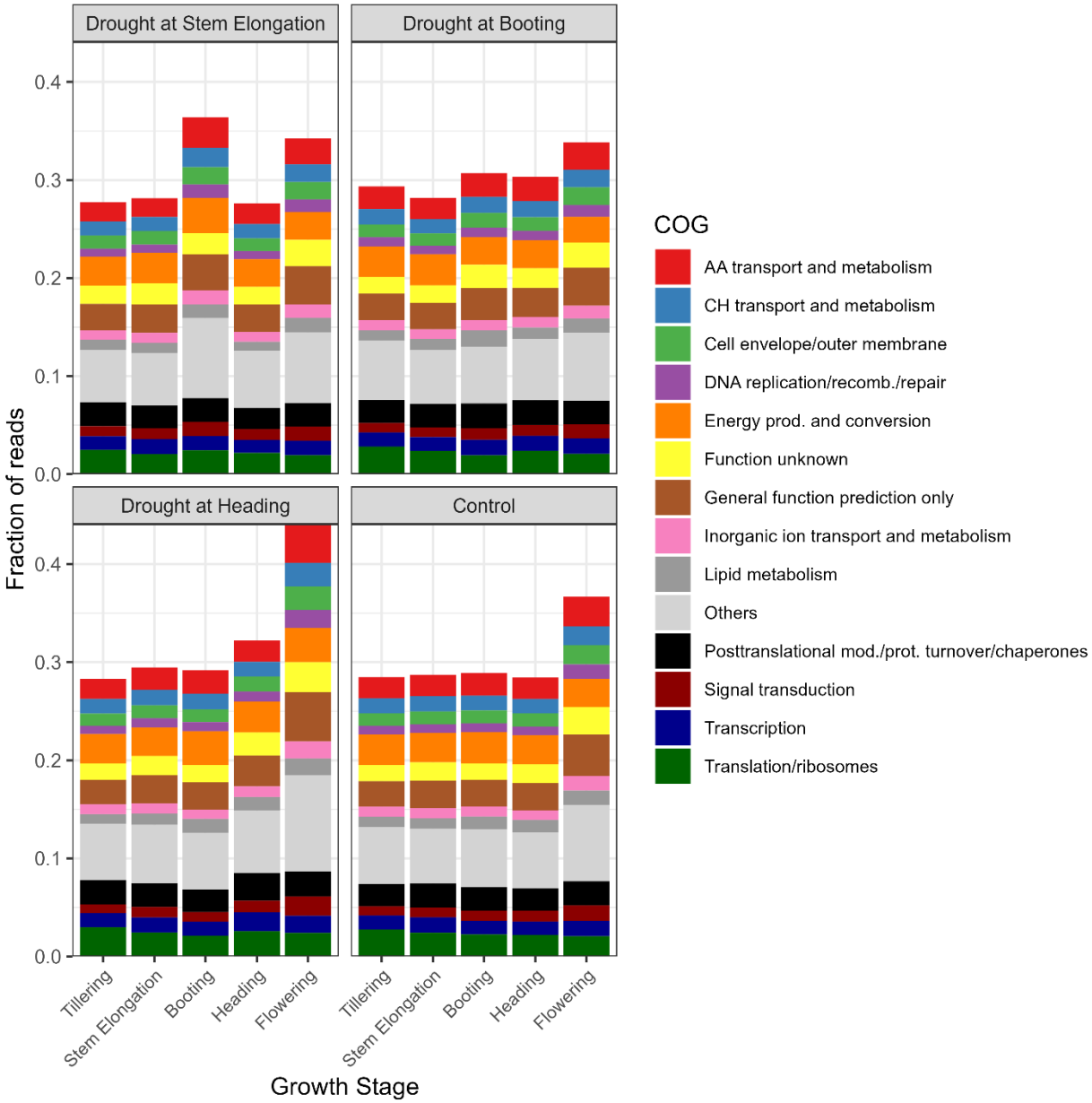
549 **Figure 3.**



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552 **Figure 4.**

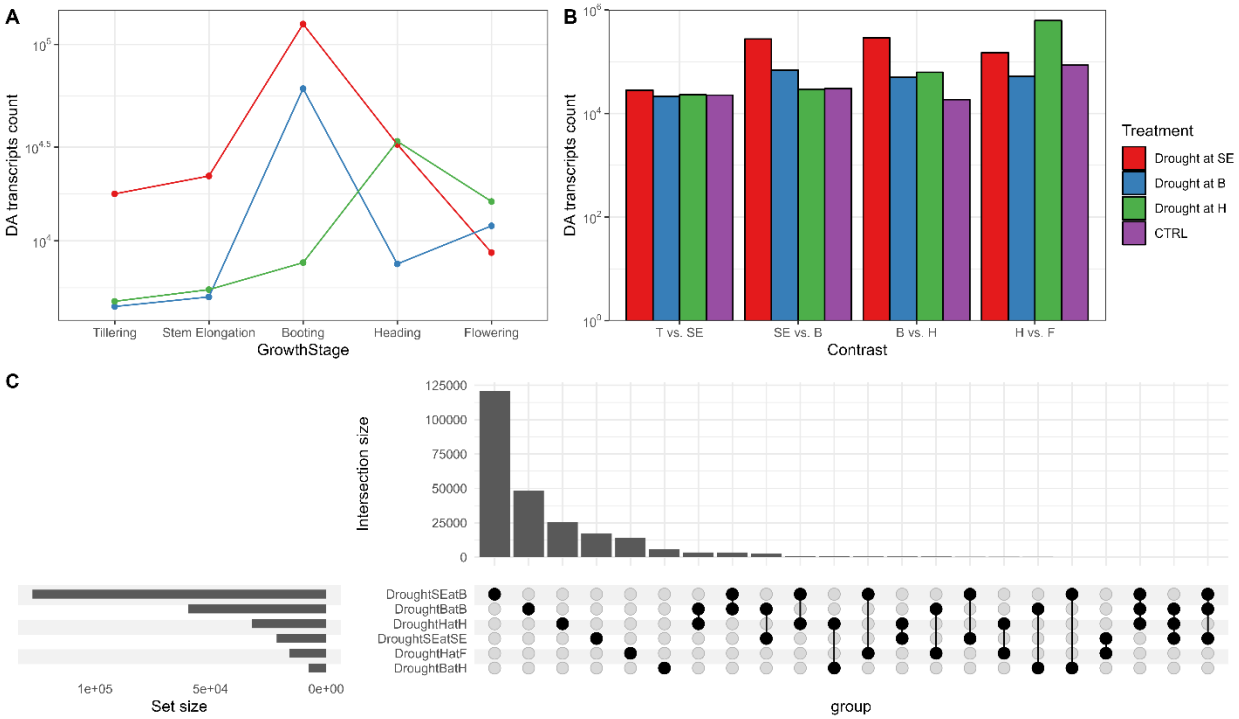

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Figure 5.

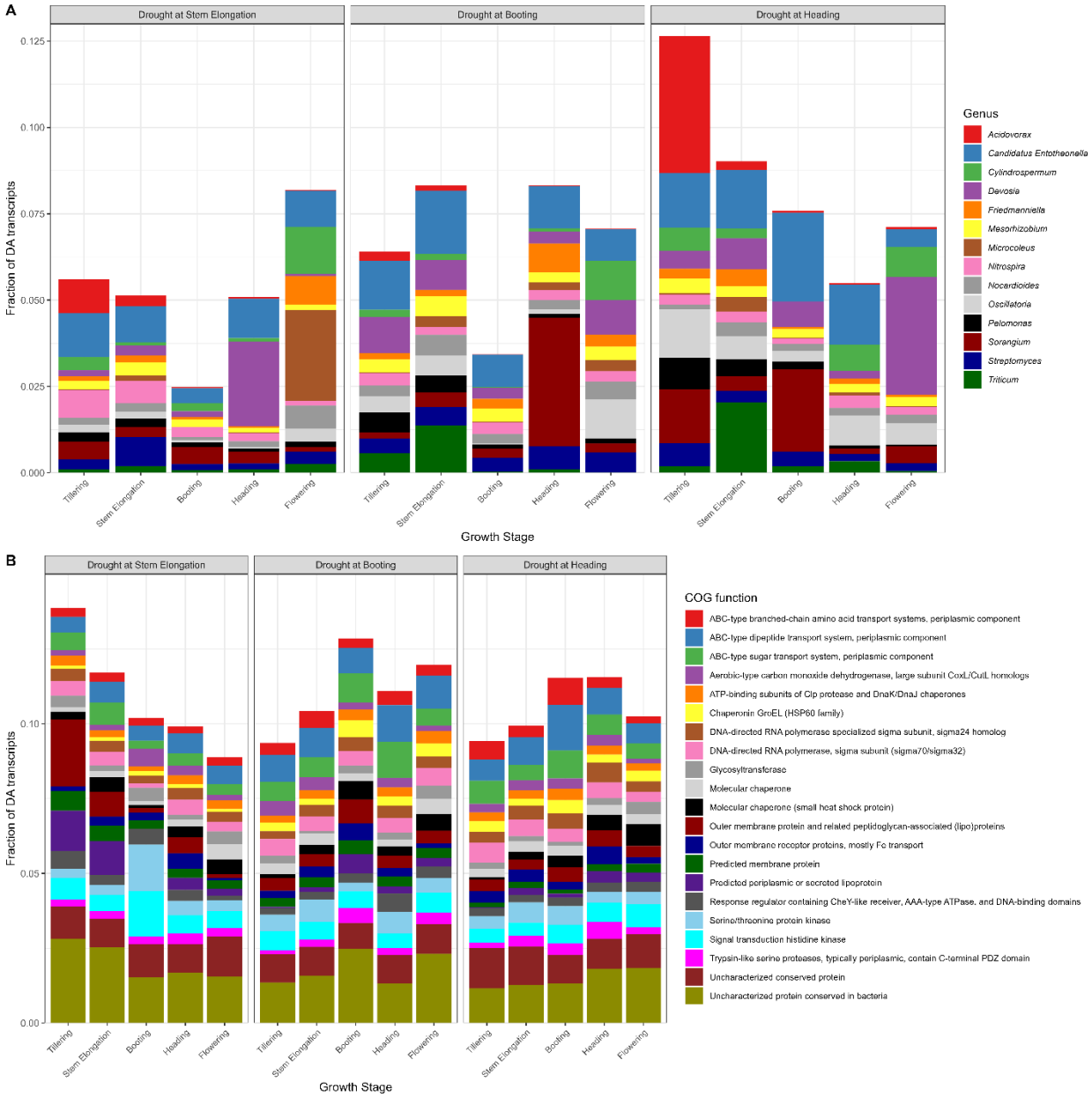

A
Drought at Stem Elongation
Drought at Booting
Drought at Heading
Fraction of DA transcripts
0.125
0.100
0.075
0.050
0.025
0.000
Tillering
Stem Elongation
Booting
Heading
Flowering
Growth Stage
Genus
Acidovorax
Candidatus Entotheonella
Cylindrospermum
Devosia
Friedmanniella
Mesorhizobium
Microcoleus
Nitrospira
Nocardioides
Oscillatoria
Pelomonas
Sorangium
Streptomyces
Triticum
B
Drought at Stem Elongation
Drought at Booting
Drought at Heading
Fraction of DA transcripts
0.10
0.05
0.00
Tillering
Stem Elongation
Booting
Heading
Flowering
Growth Stage
COG function
ABC-type branched-chain amino acid transport systems, periplasmic component
ABC-type dipeptide transport system, periplasmic component
ABC-type sugar transport system, periplasmic component
Aerobic-type carbon monoxide dehydrogenase, large subunit CoxL/CutL homologs
ATP-binding subunits of Clp protease and DnaK/DnaJ chaperones
Chaperonin GroEL (HSP60 family)
DNA-directed RNA polymerase specialized sigma subunit, sigma24 homolog
DNA-directed RNA polymerase, sigma subunit (sigma70/sigma32)
Glycosyltransferase
Molecular chaperone
Molecular chaperone (small heat shock protein)
Outer membrane protein and related peptidoglycan-associated (lipo)proteins
Outer membrane receptor proteins, mostly Fe transport
Predicted membrane protein
Predicted periplasmic or secreted lipoprotein
Response regulator containing CheY-like receiver, AAA-type ATPase, and DNA-binding domains
Serine/threonine protein kinase
Signal transduction histidine kinase
Trypsin-like serine proteases, typically periplasmic, contain C-terminal PDZ domain
Uncharacterized conserved protein
Uncharacterized protein conserved in bacteria

Figure 6.

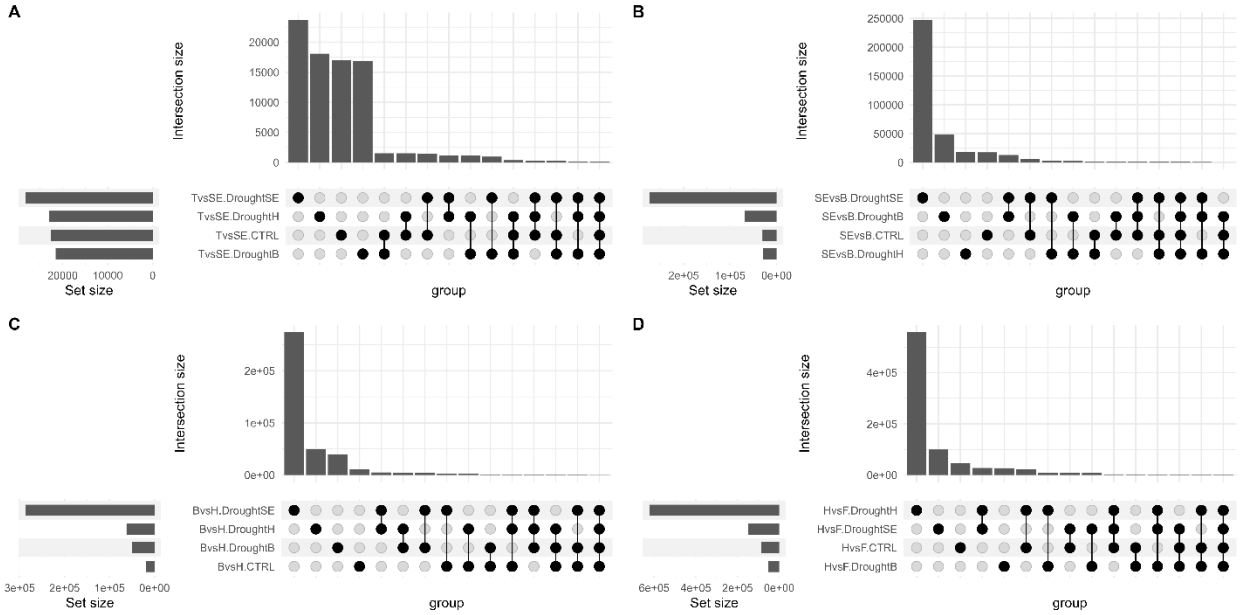
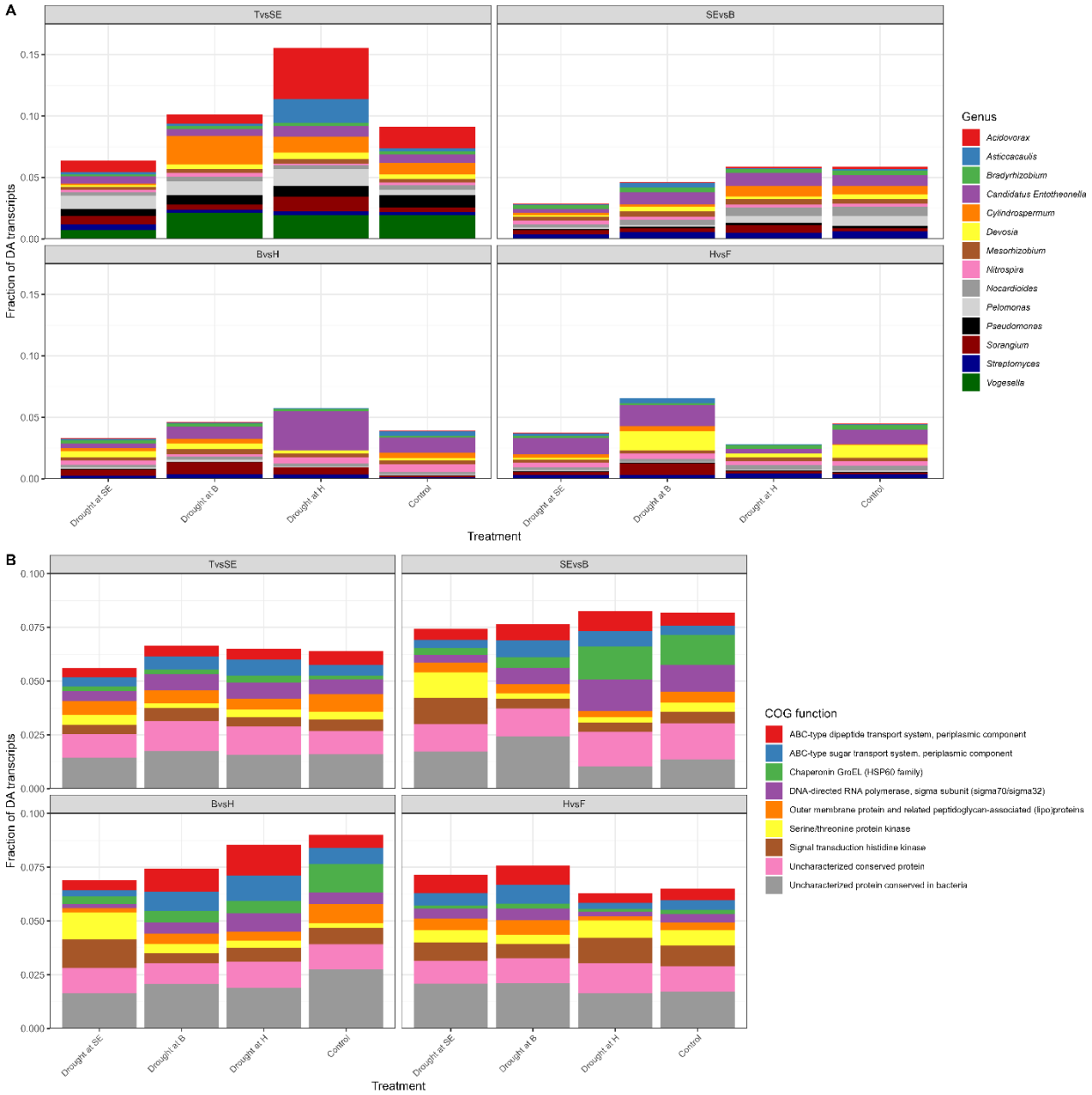

A
Intersection size
20000
15000
10000
5000
0
TvsSE.DroughtSE
TvsSE.DroughtH
TvsSE.CTRL
TvsSE.DroughtB
20000 10000 0
Set size
group
B
Intersection size
250000
200000
150000
100000
50000
0
SEvsB.DroughtSE
SEvsB.DroughtB
SEvsB.CTRL
SEvsB.DroughtH
2e+05 1e+05 0e+00
Set size
group
C
Intersection size
2e+05
1e+05
0e+00
BvsH.DroughtSE
BvsH.DroughtH
BvsH.DroughtB
BvsH.CTRL
3e+05 2e+05 1e+05 0e+00
Set size
group
D
Intersection size
4e+05
2e+05
0e+00
HvsF.DroughtH
HvsF.DroughtSE
HvsF.CTRL
HvsF.DroughtB
6e+05 4e+05 2e+05 0e+00
Set size
group

Figure 7.


A
TvsSE
SEvsB
BvsH
HvsF
Fraction of DA transcripts
0.15
0.10
0.05
0.00
Drought at SE
Drought at B
Drought at H
Control
Treatment
Genus
Acidovorax
Asticcacaulis
Bradyrhizobium
Candidatus Entotheonella
Cylindrospermum
Devosia
Mesorhizobium
Nitrospira
Nocardioides
Pelomonas
Pseudomonas
Sorangium
Streptomyces
Vogesella
B
0.100
0.075
0.050
0.025
0.000
COG function
ABC-type dipeptide transport system, periplasmic component
ABC-type sugar transport system, periplasmic component
Chaperonin GroEL (HSP60 family)
DNA-directed RNA polymerase, sigma subunit (sigma70/sigma32)
Outer membrane protein and related peptidoglycan-associated (lipo)proteins
Serine/threonine protein kinase
Signal transduction histidine kinase
Uncharacterized conserved protein
Uncharacterized protein conserved in bacteria