Title: Various tomato cultivars display contrasted morphological and molecular responses to a heat wave.

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Running head: Heat wave response in 11 cultivars of tomato.

Summary

Climate change is one of the biggest threats that human society currently needs to face. Heat waves associated with global warming negatively affect plant growth and development and will increase in frequency. Tomato is one of the most produced and consumed fruit in the world but remarkable yield losses occur every year due to the sensitivity of many cultivars to heat stress. New insights into how tomato plants are responding to heat waves will contribute to the development of new cultivars with high yields under harsh temperature conditions. In this study, the analysis of microsporogenesis and pollen germination rate of eleven tomato cultivars after exposure to a simulated heat wave revealed differences between genotypes. The transcriptome of floral buds at two developmental stages of five cultivars selected based on their pollen germination tolerance or sensitivity, revealed common and specific molecular responses implemented by tomato cultivars to cope with heat waves. These data provide valuable insights into the underlying molecular adaptation of floral buds to heat stress and will contribute to the development of future climate resilient tomato varieties.

Keywords

Heat Stress (HS), pollen development, HS transcriptional response, tomato

Introduction

Population growth and global warming are two of the major issues that humanity will have to face in the next decades (IPCC, 2014). Based on current climatic models, experts commonly anticipate that rising temperatures will cause substantial yield losses in the future affecting major plant-food sources (Tripathi *et al.*, 2016). Global warming has already a significant impact on plant crop production and agricultural practices. During the past recent years, many regions of the world experienced exceptional extreme heat waves causing severe damages to ecosystems, human society and crop production (Stillman, 2019). The frequency and intensity of these heat waves will increase in many regions of the globe leading to major losses in agricultural yield (Lau and Nath, 2012; Molina *et al*., 2020).

Plant growth and development can be strongly altered by high temperatures, the most heat-susceptible phases being early seedling and reproductive phases. Heat-stress (HS) can impair essential cellular, physiological and developmental processes, including genome integrity, photosynthesis, flower development or pollen development and viability as reported for a number of plant species (Han *et al.*, 2021; Moore *et al.*, 2021). Detrimental effects of heat on pollen production have been described in various plant taxa, including *Arabidopsis thaliana* (Arabidopsis), *Solanum lycopersicum* (tomato) and *Oryza sativa* (rice) and for different developmental stages such as meiosis, microspore development and pollen maturation (Raja *et al.*, 2019). Pollen development is known to be one of the most temperature-sensitive processes in all plant life cycle (Zinn *et al.*, 2010), and impaired pollen development results in poor fertilization and reduced fruit and seed yield (Giorno *et al.*, 2013; Müller and Rieu, 2016).

The cellular responses to HS have been studied in a variety of plant species and various organ and tissues revealing both common and specific responses. HS disturbs various cellular processes, among which the denaturation of biological molecules, such as proteins, lipids, nucleic acids (Bohnert *et al*., 2006; Kotak *et al*., 2007, Essemine *et al*., 2010) and the disintegration of subcellular structures, including membranes and cytoskeleton networks (Savchenko *et al*., 2011; Saidi *et al*., 2011) have been described. HS affects genome integrity by triggering DNA damage through nucleotide modifications and single-strand or double-strand breaks (Kantidze *et al*., 2016) and it also alters chromatin architecture (Pecinka *et al*., 2012), and affects meiotic recombination (Ning *et al*., 2021; De Jaeger-Brat *et al*., 2021). To cope with HS, plants have developed highly complex intracellular signaling systems involving hormones, Ca2+ and reactive oxygen species (ROS) (Wahid *et al*., 2007). For a long time, ROS were considered as a byproduct that impairs plant growth. However, in recent period, ROS gained attention for its function as a signaling molecule involved in response to environmental stresses like HS (Medina *et al*. 2021). In support to this idea, it has been shown that ROS-scavenging enzymes, involved in detoxification processes, are rapidly induced by HS (Suzuki and Mittler, 2006).

70 HS induces rapid transcriptional changes and the elucidation of the complex
71 transcriptional regulatory networks involved in plant responses to HS is now well advanced
72 (Ohama *et al.*, 2017). That is, HS rapidly activates HS-responsive transcription factors, HSFs,
73 which regulate the transcription of a wide range of target genes involved in signaling and
74 metabolic pathways (Koskull-Döring *et al.*, 2007). Among the most notable responses, the
75 expression of Heat Shock Proteins (HSPs) is known to play essential roles in cellular protection
76 through their action on protein misfolding or aggregation but also in protein translocation and
77 degradation (Vierling 1991; Wang et al. 2004; Schleiff & Becker 2011). Transcriptomic profiling
78 of HS responses in plants has identified several molecular pathways induced under elevated
79 temperature conditions including photosynthesis, response to light or rRNA processing
80 (Jayakodi, Lee and Yang, 2019). Interestingly, in addition to the classically induced heat shock
81 proteins, these studies suggested potential roles for several factors involved in epigenetic,
82 post-transcriptional and post-translational regulation as well as in hormonal regulation.
83 Deciphering the effects of HS at the transcriptome level greatly helped defining candidate
84 genes potentially involved in mediating plant tolerance to HS and several attempts aiming to
85 improve thermotolerance by knocking out or overexpressing these candidate genes have been
86 reported in different plant species including Arabidopsis, tomato, rice and *Glycine max*
87 (soybean) (Zhu *et al.*, 2006; Yokotani *et al.*, 2008; Xin *et al.*, 2010; Wu *et al.*, 2012; Li *et al.*,
88 2013; Shen *et al.*, 2015; Xue, Drenth and McIntyre, 2015; Wan *et al.*, 2016). It is however likely
89 that the activation of two or more independent -but mutually complementary- pathways are
90 able to improve more effectively thermotolerance in crops species. In this regard, new
91 pathways need to be discovered in order to pave the way towards the generation of highly
92 tolerant genotypes using the newly identified candidates for gene stacking.
93 HS response has been extensively investigated in plants in the past two decades. Most
94 studies have been carried out applying very high temperatures (45°C to 50°C) for a short period
95 of time (from 30 minutes to 3 hours) on the whole plant, or focused on specific developmental
96 stages or organs (Qu *et al.*, 2013; Driedonks *et al.*, 2016; Janni *et al.*, 2020). Strikingly so far,
97 quite a few studies have been carried out that simulate heat wave conditions as defined by the
98 STAtistical and Regional dynamical Downscaling of EXtremes for European regions (Stardex)
99 project: 5 consecutive days with 5 degrees anomaly with respect to mean temperature in
100 summer (Jagadish *et al*., 2021). Because the effect of short-term heat treatments is unlikely to
101 completely reproduce what happens during heat waves, studying the impact of long periods of
102 heat on plants is essential to eventually select plant cultivars with increased heat tolerance.
103 Tomato is one of the most produced and consumed fruits in the world. However, tomato
104 producers face dramatic yield losses in very warm summers, due to poor fruit setting that leads
105 to lower fruit number and small fruits of low quality (Adams *et al.*, 2001). In recent years, several
106 transcriptomic, metabolomic, proteomic or lipidomic analyses were performed to study the

response of tomato plants to HS (Pressman *et al*., 2002; Spicher *et al*., 2016; Paupière *et al.*, 2017; Almeida *et al.*, 2021). Studies focusing on floral buds revealed that short-term HS induces the expression of *HSF* genes, *HSP* genes, ROS scavenger genes and genes involved in the control of sugar levels (Frank *et al.*, 2009). However, most of these studies are limited to one tomato genotype and, more importantly, the short-term treatment is unlikely to completely reproduce the molecular responses to heat wave.

Here, we aimed at identifying the common and/or specific molecular responses to a simulated heat wave in a greenhouse, in tomato floral buds of various tomato cultivars. Seeds from 11 modern tomato cultivars were obtained from different seed companies, and plants were grown and subjected to a heat wave, in order to identify tolerant and sensitive cultivars based on pollen germination. Among these 11 cultivars, 2 were selected as thermotolerant and 3 as thermosensitive for subsequent genome-wide transcriptomic profiling analysis of their floral buds, to identify the molecular pathways regulated during heat wave. These analyses highlighted the different strategies that tomato cultivars set-up for facing heat waves.

Results

Morphological analyses of flower development under elevated ambient temperatures

To assess phenotypic differences in response to HS of various tomato genotypes, we examined the behavior of eleven tomato cultivars corresponding to ten commercial varieties and the cherry tomato West Virginia 106 (WVA106) cultivar. This set of cultivars was selected in order to cover some of the tomato diversity in terms of i) fruit morphology: small- (WVA106, Sassari, Brioso, M82) medium- (Docet, Moneymaker, Clodano, JAG8810), and large size (Rebelski, Marbonne, DRK7024) (**Figure S1**); ii) growth pattern: determinate (JAG8810, M82) *versus* indeterminate plants (DRK7024, WVA106, Brioso, Marbonne, Sassari, Moneymaker, Rebelski); and **iii)** market suitability: fresh market- (Sassari, Brioso, M82, Clodano, Moneymaker) and processing tomatoes (JAG8810, DOCET). Plants were grown in a greenhouse under non-stress (NS) temperature conditions (24°C during the day and 18°C during the night) until the plants reach the bolting stage. Thereafter, a simulated heat wave was applied, consisting in 3 weeks at 35°C during the day and 25°C during the night. Given that floral development is critical for tomato yield, we focused our analysis on the morphological and cytological changes occurring during this process under elevated ambient temperatures treatment.

Under non-stress (NS) conditions, the 11 selected cultivars displayed variability in size and morphology of floral buds during development (**Figure 1**). Under HS, the size and shape of floral buds were unevenly affected in different cultivars. In most cultivars, floral bud size distribution was similar under NS and HS conditions (**Figure S2**)**,** but clear morphological alterations were observed (**Figure 1**). In particular, the flowers of the Sassari cultivar exhibited recessed stigmas with complete absence of stigma exertion under NS conditions, whereas many flower buds in this cultivar showed stigma exertion under HS. On the other hand, HS induced the appearance of curled petals in Clodano, Docet, M82, Marbonne and Moneymaker cultivars. In addition, HS caused the sepals and petals to open earlier than in NS conditions in Moneymaker, Brioso and WVA106. In this latter cultivar, sepals displayed burned extremities likely due to HS. The number of floral organs developed by all the cultivars studied was the same in both conditions suggesting that HS did not affect floral architecture. In conclusion, floral development in various tomato cultivars was affected differently under HS, suggesting that the response to HS is at least partially dependent on the genetic background.

Variability in pollen germination of tomato cultivars under elevated ambient temperatures

Flower fertilization, and subsequent fruit set and seed production, can be impaired by poor pollen germination and pollen tube growth. In heat-sensitive tomato cultivars, high

159 temperatures reduce pollen production, viability and germination capacity of pollen grains,
160 leading to impaired fruit set and to lower number of seeds per fruit (Firon *et al.*, 2006). To
161 investigate the tolerance/sensitivity of pollen germination to HS in the eleven selected tomato
162 cultivars, we determined the percentage of pollen germination as a good proxy of pollen
163 potential for fertilization under NS and HS conditions (**Figure 2**). Under NS conditions, pollen
164 germination was highly variable between the cultivars studied, with a maximum percentage of
165 of 80% for WVA106, and a minimum of 18.6% for DRK7024. Such a variability between tomato
166 cultivars has already been reported and can be attributed to differences of pollen germination
167 capacity between genotypes (Gentile *et al*., 1971; Huner and Huystee, 1982; Abdul-Baki and
168 Stommel, 1995). In addition, this variability can also be due to the composition of the
169 germination medium used which is more or less suitable for some cultivars than others
170 (Karapanos *et al.*, 2006). Overall, HS impacted pollen germination negatively in all cultivars,
171 and it prevented even totally any germination for Clodano, DRK7024, Moneymaker, Rebelski
172 and Sassari, indicating the extreme sensitivity to HS of these cultivars (**Figure 2**). In Brioso,
173 Docet, M82 and Marbonne cultivars, the pollen germination rate under HS was reduced
174 respectively by 10-, 21-, 12- and 33-fold, when compared to NS condition, indicating a high
175 sensitivity to HS of these cultivars even though some germination capacity was maintained.
176 Conversely, pollen germination in JAG8810 and WVA106 was only reduced by 1.4- and 3.5-
177 fold respectively, suggesting a better tolerance to HS treatment in these cultivars. Taken
178 together, these results indicated that pollen germination percentage is highly affected under
179 HS conditions although the degree of sensitivity is clearly genotype dependent.
180
181 **Cytological analyses of pollen development under elevated ambient temperatures**
182 To determine if the effect of the heat wave on pollen germination was related to a perturbation
183 of pollen development, we selected 5 cultivars showing contrasting behaviors for pollen
184 germination under HS: namely WVA106 and JAG8810 as tolerant cultivars, and Clodano,
185 DRK7024 and M82 as sensitive cultivars.
186 It has been described that under NS conditions, tomato floral bud size is generally
187 synchronized with the developmental stage of the reproductive organs (Brukhin *et al.*, 2003;
188 **Figure 3 A-D**). Therefore, we determined first, under our NS growth conditions, the
189 developmental stage of the male gametophyte in relation to floral bud size, using a histological
190 approach. Four pollen developmental stages were considered because they were easily
191 recognizable: microspore mother cell (**Figure 3A**), present in 4.5 mm buds in WVA106; Meiosis
192 (**Figure 3B**), in 5.5 mm buds in WVA106; Tetrad (**Figure 3C**) in 6 mm buds in WVA106 and
193 Mature pollen grain (**Figure 3D**) in 7mm buds in WVA106. Most of the time, only one pollen
194 developmental stage was observed for a determined bud size under NS conditions, indicating
195 the synchronization of floral bud and pollen development in the different cultivars studied.

196 Indeed, Clodano, JAG8810, M82 and DRK7024 floral buds at 13mm, 9mm, 6mm, and 18mm
197 respectively only contained mature pollen grains (**Figure 3 E-H**) and 6mm floral buds in
198 WVA106 only contained tetrads (**Figure 3 I**). Under HS conditions, all cultivars, except
199 DRK7024, showed several pollen developmental stages for a determined bud size (**Figure 3**
200 **J-T**). For DRK7024, while only one pollen developmental stage was observed at a time, pollen
201 development was delayed compared to NS condition as indicated by the observed pollen
202 mother cells instead of mature pollen in 18mm floral buds (**Figure 3 H and M**).
203 These results were confirmed when reporting the size of the flower buds in relation to pollen
204 developmental stage. In three cultivars (Clodano, DRK7024 and JAG8810), the stressed
205 flower buds were longer and showed more variability than in NS conditions for most pollen
206 developmental stages, thus suggesting a delay in pollen development (**Figure 4**). Conversely,
207 floral bud size for a defined pollen developmental stage in WVA106 and M82 was not different
208 indicating that pollen development was not delayed but rather desynchronized under HS
209 condition (**Figure 4**). Altogether these results indicate that pollen development is either
210 delayed (Clodano, DRK7024) and/or desynchronized (Clodano, JAG8810, M82, WVA106) by
211 HS, regardless of the observed tolerance to the heat wave in terms of pollen germination
212 capacity as determined in Figure 2.
213
214 **Genome-wide expression profiling in response to elevated ambient temperatures**

215 In an attempt to identify the molecular pathways differentially regulated in the five selected
216 tomato cultivars displaying contrasting behaviors for pollen germination and floral development
217 under elevated temperature, we carried out a genome-wide transcriptomic analysis by
218 RNAseq. RNA was extracted from floral buds of each tomato cultivar grown under NS or HS
219 conditions (3 weeks at 35°C during the day (16h) and 25°C during the night (8h)). Based on
220 the previous histological analysis, floral buds were harvested at the tetrad stage (tetrad floral
221 bud, TFB) and at the pollen mature stage (pollen floral bud, PFB) based on their size **(Table**
222 **S1)**.
223 Following quality check and pre-processing of the sequence data, the reads were
224 mapped against the SLmic1.0 tomato reference genome (gene model version 1.1). Principal
225 Component Analysis (PCA) of the RNA sequencing data showed that the samples clustered
226 together according to both developmental stages and growth conditions factors explaining
227 respectively about 60% and 10% of the whole variance (**Figure S3**). In TFB and PFB, the
228 transcriptomic profiles corresponding to samples subjected to HS were clearly separated from
229 the ones in NS conditions, indicating that the simulated heat wave triggered a transcriptional
230 response in all cultivars.
231

232 **General and common transcriptomic response to HS**

233 Differentially expressed genes (DEGs) in HS versus NS conditions were computed for each
234 condition and in the two developmental stages as described in material and methods. The
235 RNA-seq analysis yielded a total of 26017 expressed genes among which 3647 were
236 significantly differentially expressed (adjusted p-value<0.05, 1<Log2FC< -1) across the five
237 cultivars at the two stages and between the two growth conditions (**Table S2**). To analyze the
238 global response to HS at each developmental stage independently of the cultivar considered,
239 the down- or up-regulated genes from all cultivars were pooled and classified based on their
240 GO annotation focusing on biological processes. In both PFB (1415 up and 1420 down, **Table**
241 **S3**) and TFB (1369 up and 929 down, **Table S3**), most DEGs having enhanced or decreased
242 expression in response to HS belonged to GO classes linked to response to stimulus, response
243 to stress and response to abiotic stimulus (GO:0050896; GO:0006950 and GO:0009628
244 respectively) corresponding to 20 to 40% of the set of genes with an associated GO term in
245 the studied list **(Table S4)**. As expected, the categories "response to heat" (GO:0009408) and
246 "cellular response to heat" (GO:0034605) were found enriched in the upregulated DEGs.
247 Additionally, "response to reactive oxygen species" (ROS, GO:0000302), "response to oxygen-
248 containing compound" (GO:1901700) and "cellular response to oxygen-containing compound"
249 (GO:1901701) were enriched categories, containing around 15% of the PFB and TFB up-
250 regulated genes. Interestingly down-regulated genes corresponding to the GO "sporopollenin
251 biosynthetic process" (GO:0080110) were found enriched in PFB suggesting that pollen
252 development might be affected. This global analysis thus reveals that the floral buds at both
253 tetrad and pollen developmental stages were severely affected by the heat wave resulting in
254 an oxidative stress.

255 We then analyzed the DEGs commonly up- or down-regulated at PFB and TFB stages.
256 820 out of 1964 (42%) and 534 out of 1815 (29%) DEGs were found to be commonly up-
257 regulated and down-regulated, respectively in PFB and TFB samples, highlighting the
258 response similarity to HS independently of the developmental stage (**Figure 5A-B**). Most
259 DEGs with enhanced expression in response to HS belonged to GO classes linked to response
260 to stimulus such as response to heat (GO:0009408, GO:0034605), response to ROS
261 (GO:0000302 and GO:1901700), and cellular processes such as protein folding (GO:0006457
262 and GO:0042026) (**Figure 5C; Table S5)**. For the down-regulated DEGs the GO enrichment
263 analysis revealed mainly classes related to metabolic processes such as photosynthesis
264 (GO:0009765). These results indicated that the global tomato transcriptome response at both
265 TFB and PFB stages is largely common and suggested that both tissues undergo inhibition of
266 photosynthetic processes.

267

268 **Cultivar specific transcriptomic response to HS**

269 To evaluate the common and specific responses to HS between cultivars, we first
270 compared the number of DEGs in the different tomato cultivars under HS condition. We noticed
271 that this number was higher in the pollen sensitive- than in the pollen tolerant cultivars: at the
272 TFB stage, we found 872 and 742 DEGs for JAG8810 and WVA106 respectively, as the two
273 tolerant cultivars, and 1233, 1089 and 1293 DEGs for M82, DRK7024 and Clodano
274 respectively, as the three sensitive cultivars (**Table S3**). At the PFB stage, 587 and 1402 DEGs
275 were found for JAG8810 and WVA106 respectively, and 1327, 1418 and 1388 for M82,
276 DRK7024 and Clodano respectively. These observations suggested that the higher number of
277 responding genes could be indicative of a higher sensitivity of pollen to the HS.
278 We then compared the DEGs found in the five cultivars at each developmental stage. The
279 Venn diagram of the up-regulated genes in TFB showed that 147 genes were common
280 between all cultivars which represent between 17% (for Clodano) and 32% (for WVA106) of
281 the up-regulated DEGs (**Figure 6A, Table S6)**. In PFB, 57 genes were found common between
282 the five cultivars representing between 7% (for M82) and 15% (for JAG8810) of the up-
283 regulated DEGs (**Figure 5B, Table S6**). Interestingly only 13 out of 502 genes were found
284 specifically up-regulated in JAG8810 indicating that the response at this stage in this cultivar
285 is almost completely shared by the others. Concerning the down-regulated DEGs, 97 genes
286 (representing between 20% for DRK7024 and 34% for WVA106) and 69 genes (representing
287 between 9% for DRK7024 and 32% for JAG8810) were commonly down-regulated in TFB and
288 PFB respectively between all cultivars (**Figure 5C and 5D, Table S6**).
289 The 147 commonly up-regulated DEGs identified in TFB were mainly involved in response
290 to stimulus (GO:0050896) with 49% of the set of the genes with an associated GO term in the
291 studied list (**Figure 5E and Table S7**). As expected the GO term “response to heat”
292 (GO:0009408) and “response to oxygen-containing compound” (GO:1901700) were found
293 enriched. Similarly, GO terms corresponding to hormonal responses such as salicylic acid
294 (GO:0009751) and ethylene (GO:0009723), as well as regulation of reproductive process
295 (GO:2000241) were also enriched. At PFB stage, GO terms corresponding to metabolic
296 process (GO:0019222) were found enriched among the 57 commonly up-regulated DEGs. The
297 GO enrichment of the down-regulated DEGs at both TFB and PFB stages revealed mainly
298 photosynthesis related genes (GO:0009765) indicating that both stages are affected in this
299 process during HS. Altogether these results suggested that the common response to HS in the
300 five cultivars mainly corresponds to the upregulation of heat stress response genes, oxidative
301 stress and hormonal pathways and the down-regulation of photosynthesis related genes.
302 To gain some insight into the common and specific responses of the two developmental
303 stages studied, we then compared the DEGs between PFB and TFB in each cultivar.
304 Interestingly, the percentage of commonly up-regulated DEGs between PFB and TFB was
305 higher in sensitive cultivars (36.5% in Clodano; 42.6% in DRK7024 and 43.3% in M82) than in

306 tolerant cultivars (17.5% in WVA106 and 17.0% in JAG8810) (**Figure S4**). A similar result was
307 observed for commonly down-regulated DEGs with 22.7% in Clodano, 32.5% in M82 and 31%
308 in DRK7024 compared with 16.0% and 17.5% in WVA106 and JAG8810 respectively (**Figure**
309 **S4**). This might be the consequence of the observed developmental alterations and
310 desynchronization of pollen development in sensitive cultivars in which PFB still contain non-
311 mature pollen that might share more DEGs with TFB compared to pollen of tolerant cultivars
312 in which the pollen is fully mature in PFB.
313
314 **Flower, pollen and anther development related genes mis-expressed upon HS**
315 The "regulation of reproductive process" (TFB and PFB; GO:2000241; 44 genes) and
316 "developmental process involved in reproduction" (PFB, GO:000300; 130 genes) categories
317 were found enriched in the pool of up-regulated and down-regulated DEGs for all cultivars
318 (**Table S8**), confirming that HS affected flower development. Given the fact that the applied HS
319 affects pollen development, we further examined the transcriptional expression of several
320 genes expressed during tomato pollen development (Chaturvedi *et al*., 2013) under NS and
321 HS conditions. We also included in our dataset genes proposed to be related to tomato pollen-
322 and tapetum development (Jeong *et al.*, 2014; Liu *et al.*, 2019) (**Table S9**). Few genes
323 specifically expressed during anther development and in the microspore were found to be
324 down-regulated under HS in several cultivars, although no clear response pattern was
325 observed (**Table S9**). Interestingly, under NS conditions, genes encoding proteins displaying
326 high expression levels in pollen mother cells such as histones (histone H4, Solyc04g011390 ;
327 histone H2A, Solyc09g010400), proteasome α-subunit (Solyc07g055080) and ribosomal
328 proteins (S17, Solyc11g006690 and L12, Solyc06g075180) displayed higher expression in
329 TFB than in PFB in NS conditions. However, the fact that these genes are not up-regulated
330 under HS in PFB samples suggests that the observed cytological desynchronization of pollen
331 development is not visible at the gene expression level. Conversely, genes encoding proteins
332 specifically expressed in mature pollen (Jeong *et al*., 2014) such as the anther-specific protein
333 TA29 (Solyc02g078370), TomA108 (Solyc01g009590), a gene specifically expressed in
334 anthers and tapetum (Xu, Liu and Chen, 2006), a cysteine protease (Solyc07g053460) or
335 AtAMS-like (Solyc08g062780), encoding a fatty acyl-CoA reductase important for pollen wall
336 development (Chen *et al*., 2011) were specifically expressed in PFB in NS condition. While
337 validating the sampling stages chosen based on cytological observations, these observations
338 did not reveal any striking differences between NS and HS conditions concerning pollen-
339 related genes.
340
341 **Genes encoding HSPs and HSFs are mis-expressed under HS**

342 Among the genes up- and down-regulated, we identified a high number of *HSPs* (heat
343 shock proteins) encoding chaperone proteins and *HSFs* (heat shock factors) encoding DNA-
344 binding proteins, known to be HS related genes. 48 and 44 out of 208 tomato HS-related genes
345 (Keller *et al*., 2018) were found up-regulated in at least one cultivar in TFB and PFB
346 respectively, for a total number of 50 genes at both stages (**Table S10**). Interestingly, more
347 *HSPs* were found upregulated in sensitive (45 for Clodano; 33 for DRK7024; 22 for M82) than
348 in tolerant (20 for WVA106 and 20 for JAG8810) cultivars. Moreover, for the 13 commonly up-
349 regulated *HSPs* in all cultivars, despite a similar absolute expression level under NS
350 conditions, the mean log2-fold change between NS and HS conditions in sensitive cultivars
351 was higher than in tolerant cultivars for the two stages analyzed (2.7-3.8 for Clodano; 2.8-2.7
352 for DRK7024; 2.4-2.8 for M82; 1.4-1.7 for WVA106 and 1.8-1.5 for JAG8810 (values indicated
353 for TFB-PFB)) suggesting that the sensitive cultivars might sense the stress in a stronger
354 manner. Among these 13 commons genes, 11 encode transcription factors belonging to the
355 HSF class, and 2 to the protein folding class HSP40. Several of these genes have been already
356 described as important factors for HS tolerance in male reproductive tissues and particularly
357 at the tetrad stage such as HsfA2, HsfA6b (Fragkostefanakis *et al.*, 2016). These results
358 indicate that depending on the cultivar considered, the level and diversity of HS related proteins
359 induced may vary, suggesting that this differential behavior could lead to the differences
360 observed in the responses to HS.
361
362 **ROS related genes mis-expressed under HS**
363 A total of 259 genes (out of 1860) related to the response to oxygen-containing compound
364 GO (GO:1901700) and 89 genes related to the response to oxidative stress (GO:0006979)
365 were up-regulated at both stages in all cultivars (**Table S11**). Genes encoding ROS
366 scavenging enzymes such as the Ascorbate peroxidase (Solyc09g007270), already reported
367 to be responding to HS in tomato microspores (Frank *et al*., 2009), was found upregulated in
368 HS condition. Antioxidant enzymes such as Catalases (Solyc04g082460 and
369 Solyc12g094620) were upregulated under HS and Solyc12g094620 displayed higher
370 expression levels under NS condition in tolerant cultivars compared to sensible cultivars in
371 both TFB and PFB stages (between 2.1- and 2.6-fold increase in TFB and between 1- and 2-
372 fold in PFB). Moreover, enzymes involved in ROS detoxication such as the iron superoxide
373 dismutase (Solyc06g048410) were found up-regulated.
374
375 **Discussion**
376
377 **Various tomato cultivars respond unequally to HS**

378 The effects of different types of stresses, including HS, on flower development have
379 been studied for a long time. It is known that the optimal temperature for growing tomato plants
380 is 25°C during the day and 20°C at night (Alsamir *et al.*, 2017). For most tomato cultivars, if
381 the temperatures exceed 26ºC during the day and 20ºC during the night, fruit set is impaired
382 mainly due to floral development alteration, and therefore fruit yield is reduced (Sherzod *et al.*,
383 2020). Because the response to stress varies widely between genotypes (Arena *et al.*, 2020;
384 Gonzalo *et al.*, 2021), gaining deeper knowledge of the developmental and molecular response
385 of various cultivars may provide clues for innovative breeding strategies aiming at improving
386 commercial tomato cultivars for better adaptability to climate change. In the present work, we
387 investigated the responses to heat stress in eleven tomato cultivars producing fruits of different
388 sizes and shapes. The data show that one of the most characteristic effects of HS on floral
389 morphology is stigma exertion, which negatively impacts self-pollination due to the excessive
390 elongation of the styles minimizing pollen access to the stigmas and reducing fertilization, and
391 consequently decreasing fruit yield and fruit quality (Fernandez-Muñoz and Cuartero, 1991;
392 Giorno *et al.*, 2013). A similar response was previously described in the tomato cultivar Micro-
393 Tom (Pan *et al.*, 2019) and the wild species *S. pimpinellifolium*, *S. pennellii* and *S. chillense*
394 (Alsamir *et al.*, 2017) as a consequence of increased temperature. Interestingly, we only
395 observed this effect in the cultivar Sassari. The absence of stigma exertion in the other cultivars
396 could be explained by the milder stress applied in our study compared to the harsh heat
397 condition used in previous studies (35°C/30°C and 16 h/8 h day/night) for 12 days in Pan *et al.*
398 (2019) and two months in a tunnel house with max of 50ºC and min of 30ºC in Alsamir *et al.*
399 (2017). The pistil exertion in the Sassari cultivar could thus indicate that this cultivar is very
400 sensitive to HS. This high sensitivity is also highlighted by the absence of pollen germination
401 under the HS conditions we applied (**Figure 2**). As previously observed (Giorno *et al.*, 2013),
402 HS triggered additional morphological alterations including curled petals and sepals, and early
403 opening of the petals that were more or less pronounced depending on the cultivar. The
404 morphological observations after HS thus reveal that the cultivars respond differently to the
405 stress applied.
406
407 **Under HS, pollen germination was affected in all cultivars but with different ranges**
408 Pollen is easily damaged by exposure to HS (Bita *et al.*, 2011; Paupière *et al.*, 2017; Rieu,
409 Twell and Firon, 2017). The exposure of tomato plants to high temperatures has two effects
410 on pollen grains: a reduction in the number of grains and a reduction in germination and viability
411 (Pressman, Peet and Pharr, 2002). The impact of HS on pollen germination was described
412 previously in many species such as in rice (Coast *et al.*, 2016), sorghum (Sunoj *et al.*, 2017),
413 peanuts (Kakani *et al.*, 2002), spring wheat (Bheemanahalli *et al.*, 2019) and tomato (Sherzod
414 *et al.*, 2020). Thermotolerant tomato cultivars have been defined as plants with higher yield,

415 high quantity of viable pollen grains, and higher germination capacity under HS conditions
416 when compared to thermosensitive plants (Firon *et al.*, 2006). In our study, we found that two
417 cultivars were possibly thermotolerant, namely WVA106 and JAG8810, since they maintain
418 high pollen germination under HS. However, these two cultivars differ greatly with regard to
419 their germination capacity under NS conditions (82.7% and 23.4%, respectively). These
420 variations were also observed in other studies as in spring wheat (*Triticum aestivum*) where
421 pollen germination of 22 cultivars was compared and a germination percentages of 87%
422 maximum and 30% minimum were observed (Bheemanahalli *et al.*, 2019). The decrease in
423 the capacity of pollen to germinate under HS conditions is probably due to damages in the
424 physical structure of pollen, pollen wall composition or lower energy (sugar) status (Jiang *et*
425 *al.*, 2015; Sita *et al.*, 2017; Sunoj *et al.*, 2017; Djanaguiraman *et al.*, 2018; Jagadish *et al.*,
426 2021). JAG8810 might be more thermotolerant than WVA106 since the decrease of pollen
427 germination was less important than for WVA106. In other cultivars, we found that pollen
428 germination was lower than 10%, a very low rate probably leading to reduced fruit set. These
429 findings indicate that these 9 cultivars are sensitive to the stress applied when considering the
430 pollen germination trait. For that trait too, it seems that the genetic background influences
431 differently the response to HS.
432

433 **HS affects pollen development in the sensitive cultivars**

434 Pollen grains are formed inside the anthers through a series of developmental steps from
435 microsporocyte meiosis to pollen release (Gómez *et al.*, 2015). In general, pollen development
436 is synchronized until microsporocyte meiosis I, and sometimes until meiosis II (Brukhin *et al.*,
437 2003). Cell division synchronization is necessary for successful and coordinated pollination
438 and fertilization (Magnard *et al.*, 2001). Specifically, an environmental stress (extreme
439 temperature or drought) induces asynchrony during pollen development and gives rise to
440 meiotic abnormalities (Pécrix *et al.*, 2011; De Storme and Geelen, 2020). When cultivated
441 under continuous mild heat, a simultaneous reduction in pollen viability and appearance of
442 anthers with pistil-like structures was observed in tomato indicating that HS impairs anther
443 development (Müller *et al.*, 2016). In our study we show that pollen development is affected at
444 very early stages by HS, impairing meiosis and leading to a delay or a desynchronization of
445 pollen development depending on the cultivar considered. At the molecular level, few genes
446 related to pollen development were mis-regulated under HS, although no clear response
447 pattern was found between cultivars or time points.

448 **Stress-related genes are commonly induced in the various cultivars**

449 Under a short HS treatment, the *HSP* genes are strongly induced and play important roles to
450 protect the plant. HSP act to maintain the homeostasis of the plant as they have a chaperone
451 activity to limit the misfolding of proteins, but also to protect protein complexes like
452 photosystem II (Vierling, 1991; Tsvetkova *et al.*, 2002; Waters, 2013). Our global transcriptome
453 analysis showed that all cultivars respond to heat wave by maintaining an increased
454 expression of numerous *HSF* and *HSP* genes and a stronger upregulation in sensitive than in
455 tolerant cultivars. As indicated in previous studies (Fragkostefanakis *et al.*, 2016) these results
456 not only strongly suggest that pollen tolerance to HS is linked to the ability of the plant to
457 mitigate the HS effects through a higher expression of heat-response factors, but also by
458 activating a common response for short term extreme HS and heat wave exposure to moderate
459 high temperatures.

460 **Conclusion**

461 Our results showed that heat waves are detrimental for pollen development in all tested tomato
462 cultivars but the tolerance to heat stress widely varies between genotypes. The effects of global
463 warming with the increase in heat waves magnitude and frequency on reproductive organs
464 development need to be investigated using different approaches than those implemented for
465 the study of extreme short high temperature-stress. The transcriptome dataset generated in
466 the present study revealed the complex response of tomato to heat and the common and
467 specific response of each tomato cultivar.

468

469 **Experimental procedures**

470 **Plant material and growth**

471 Plant material consisted in eleven different tomato (*Solanum lycopersicum*) cultivars: Brioso
472 (Rijk Zwaan), Clodano (Syngenta™), Docet (Monsanto™), DRK7024 (De Ruiter™), JAG8810
473 (Bayer™), M82, Marbonne (Gautier Semences), Moneymaker, Rebelski (De Ruiter™), Sassari
474 (Rijk Zwaan) and West Virginia 106 (WVA106). In NS conditions, plants were cultivated in a
475 greenhouse with a photoperiod of 16h/8h with a mean temperature of 24°C during the day and
476 a mean temperature of 18°C during the night. For heat-stress treatments, the plants at bolting
477 stage have been submitted during 3 weeks to 35°C during the day (16h) and 25°C during the
478 night (8h). In both conditions, the hygrometry was around 55% all day and plants had a daily
479 watering.

480

481 **Pollen developmental stage analysis**

482 To analyze pollen development, floral buds of different sizes were dipped in Carnoy's fixative
483 solution (60% (v/v) ethanol, 30% (v/v) chloroform, 10% (v/v) acetic acid). Then, the anthers
484 from the flower buds were dissected in acetocarmine staining solution (1% (p/v) carmine 40,
485 0,5% (p/v) ferric chloride, 45% (v/v) glacial acetic acid) using gauge needles under a
486 stereomicroscope and squashed between a slide glass and a coversleep to release the male
487 reproductive cells as described by (Puchtler *et al*. 1968). Pollen developmental stages were
488 observed using a bright field microscope (Zeiss, Axioplan) and photographed with a CCD
489 camera (Motic 3 megapixels).

490

491 **Pollen germination assays**

492 Pollen grains from tomato flowers at anthesis stage were sprayed on the top of pollen
493 germination medium (18% sucrose, 0.01% Boric acid, 1mM $CaCl_2$, 1mM $Ca(NO_3)_2$, 1mM
494 $MgSO_4$, 0.5% agar; pH=7). After 16 hours of incubation at 25°C in the dark, the preparation
495 was observed using a stereomicroscope Olympus SXZ16 and photographed using a camera
496 (Motic 10 megapixels). The pollen germination rate expressed as a percentage was
497 determined by dividing the number of germinated pollens corresponding to those emitting a
498 pollen tube by the total number of pollen grains.

499

500 **Genome wide expression profiling**

501 Total RNAs were isolated from 200 and 500 mg of floral buds at different developmental stages
502 using TRIzol Reagent (Life Technologies). Total RNA extract was purified with the Qiagen
503 RNAeasy mini kit RNA. After DNase treatment (DNA-free Kit, Life Technologies), the total RNA
504 quantity and quality (RNA integrity number, RIN) were evaluated using an Agilent 2100
505 Bioanalyzer (Agilent Technologies). Only RNA extracts with a RIN of 10 were used for

506 sequencing. The RNA libraries were constructed as described in the Illumina TruSeq Stranded
507 mRNA guide. mRNAs were sequenced in a HiSeq 3000 sequencing system with 2 × 125 bp
508 paired-end sequences (Illumina HiSeq SBS Kit v4) by the Genotoul bioinformatics platform,
509 Toulouse (http://bioinfo.genotoul.fr/index.php).
510 Three biological replicates per plant and per stage were harvested for NS and heat-stress
511 conditions, resulting in 60 samples (5 cultivars × 2 conditions × 2 developmental stages × 3
512 biological replicates). To obtain the genome-wide expression profiling, RNA samples were
513 subjected to next-generation sequencing.
514 For each RNA sample, more than 20 million of paired-end reads (10 million fragments) were
515 generated, using an Illumina Hiseq 3000 platform. More than 95% of total clean reads were
516 mapped against the SL3.0 version of the reference tomato genome for read mapping
517 (ftp://ftp.solgenomics.net/genomes/Solanum_lycopersicum/Heinz1706/assembly/build_3.00/)
518 and almost 90% of the mapped reads matched a feature on the related gene model.
519
520 **Bioinformatic analyses**
521 Statistical analyses and graphs have been performed with the R software and homemade
522 scripts. The DE analysis has been carried out with the DESeq2 R-package (Love *et al*., 2010).
523 Some analyses, such as the PCA analysis, were carried out with functions contained in the
524 DESeq2 package. Reads were checked using fastQC, cleaned using trimGalore, and mapped
525 using Star, a spliced aware mapper software on the new tomato genome SLmic1.0 generated
526 in the frame of TOMGEM project (http://tomatogenome.gbfwebtools.fr/). The mapping was
527 guided using the gene model annotation version 1.1. FeatureCount was then used to calculate
528 the read counts for each gene from each mapping file. A normalization step was performed in
529 order to obtain comparable expression values between conditions and between genes. For
530 this purpose, our pipeline takes into account the relative size of studied transcriptomes, the
531 library sizes and the gene lengths, as described in Maza *et al.* (2013) and Maza (2016). After
532 data normalization using DESeq2 package, a PCA analysis has been conducted with
533 expression data in all conditions and replicates, in order to check global sample variability.
534 Genome-wide expression data have been analyzed in order to identify genes
535 differentially expressed between different conditions that have been tested. The “Relative Log
536 Expression” normalization (RLE) implemented in the DESeq2 package has been used as
537 normalization method. To highlight DEGs, the genes exhibiting an adjusted p-value < 0.05 and
538 a log2 fold-change < -1 or > +1 have been selected. To eliminate very lowly expressed genes,
539 the up-regulated DEGs with a count value < 10 in HS condition and the down-regulated DEGs
540 with a count value < 10 in NS condition were filtered out.
541 To calculate the intersections of DEGs lists within the different conditions, Venn
542 diagrams corresponding to textual outputs were generated by using the Venn diagram tools at

http://bioinformatics.psb.ugent.be/webtools/Venn/BAR web site at http://bar.utoronto.ca/ or at https://www.biovenn.nl/index.php (Hulsen *et al*., 2008) for area-proportional Venn diagrams. The TomExpress web site, at http://tomexpress.toulouse.inra.fr/query, was used to extract gene expression data obtained from previous genome-wide expression analyses (Zouine et al., 2017).

The GO enrichment analysis has been carried out on PLAZA 5.0 (Van Bel et al., 2022) using the Plaza workbench, with a significance threshold of 0.05 and without any data filter.

Authors' contributions

M.H., C.C., F.D., M.B. and N.G. conceived and supervised the study, obtained funding, and provided resources. M.H., F.D. and N.B. designed the methodology. M.H., F.D., N.B. and A.D. performed the experiments. E.M., M.Z. did the RNA sequencing. M.H., F.D., R.M-P and N.B. analyzed the data. N.G., R.M-P and N.B. wrote the original draft. All authors reviewed and edited the manuscript.

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781

782 **Figure legends**

783 **Figure 1.** Effects of heat stress (HS) and non-stress (NS) conditions on flower bud growth in the 11
784 selected tomato cultivars. For each cultivar, floral buds are ordered according to their size in each
785 condition showing the effect of the heat stress at different developmental stages. Scale bar = 1 cm
786 (applies to all panels).

787 **Figure 2.** Pollen germination rate expressed as the percentage of germinating pollen grains (%) under
788 non-stress (NS) and heat stress (HS) conditions in 11 tomato cultivars.

789 **Figure 3.** Histological analyses of pollen development in relation with bud size under non-stress (NS)
790 and heat stress (HS) conditions in different tomato cultivars.

791 Pollen developmental stages observed are the following: microspore mother cell (A, L, M, N); Meiosis
792 (B, J, R); Tetrad (C, I, K, O, Q, T); Mature pollen grains (D, E, F, G, H, P, S). Bar, 20µm.

793 **Figure 4.** Size of floral buds for a defined pollen developmental stage in Clodano, DRK7024, JAG8810,
794 M82, WVA106,). Pollen Mother Cell (MC); Meiosis (Me) ; Tetrad (Te) ; Mature Pollen (Po). * p-
795 value<0.05 (Kruskal Wallis test).

796 **Figure 5**. Global transcriptome response to HS in all tomato cultivars.

797 (A-B) Venn diagram presenting the overlap of DEGs between upregulated (A) and downregulated (B)
798 genes between TFB and PFB stages in all cultivars. (C) Gene ontology (GO) enrichment analyses of the
799 common DEGs between PFB and TFB. The dot size is representative of the number of DEGs associated
800 with the process and the fold enrichment is according to a heat map (dot color).

801 **Figure 6:** Common transcriptome response to HS between all cultivars

802 (A-D) Venn diagram presenting the overlap of DEGs between all cultivars: upregulated genes in TFB (A)
803 and in PFB (B); downregulated genes in TFB (C) and in PFB (D). (E) Gene ontology (GO) enrichment
804 analyses of the common DEGs between all cultivars.

805 The dot size is representative of the number of DEGs associated with the process and the fold
806 enrichment is according to a heat map (dot color).

807 **Figure S1.** Fruit morphology in the eleven selected cultivars used in the study. Size differences allowed
808 the classification of the 11 cultivars into three main groups: small (WVA106, Sassari, Brioso and M82),
809 medium (Docet, Moneymaker, Clodano and JAG8810), and large fruits (Rebelski, Marbonne and
810 DRK7024). Scale bar = 1 cm (applies to all panels).

811 **Figure S2.** Distribution of floral bud length (A) and width (B) under NS and HS conditions in the 11
812 cultivars.

813 **Figure S3**. Principal component analysis (PCA) of gene expression in TFB and PFB in NS and HS
814 conditions. PC1, explaining 60% of the total variance, differentiates the TFB stage (diamond) from the
815 PFB stage (circle). PC2, explaining 10% of the total variance, separates the heat stress samples (red)
816 from the control samples (blue).

817 **Figure S4**. Common and specific responses between PFB and TFB stages in all cultivars Venn diagram
818 analysis of the upregulated (A) and downregulated (B) DEGs between PFB and TFB stages in pollen
819 sensitives and pollen tolerant cultivars

820

821

822 ## Tables legends

823 **Table S1**. Sizes of the floral buds containing tetrads (TFB) and mature pollen grains (PFB) harvested
824 for the transcriptomic analysis.

825 **Table S2.** List of DEGs under heat-stress conditions in all cultivars in TFB and PFB.

826 **Table S3.** Number of differentially expressed genes under heat-stress conditions based on a log2 fold-
827 change of 1 and a p-value <0.05.

828 **Table S4.** Functional categories overrepresented in the list of all up- and down-regulated DEGs across
829 all the tomato cultivars under heat stress.

830 **Table S5.** Functional categories overrepresented in the list of commonly up- and down-regulated DEGs
831 across all the tomato cultivars in PFB and TFB under heat stress.

832 **Table S6**. List of common DEGs between all cultivars in TFB and PFB.

833 **Table S7.** Functional categories overrepresented for each developmental stage in the common DEGs
834 between all cultivars

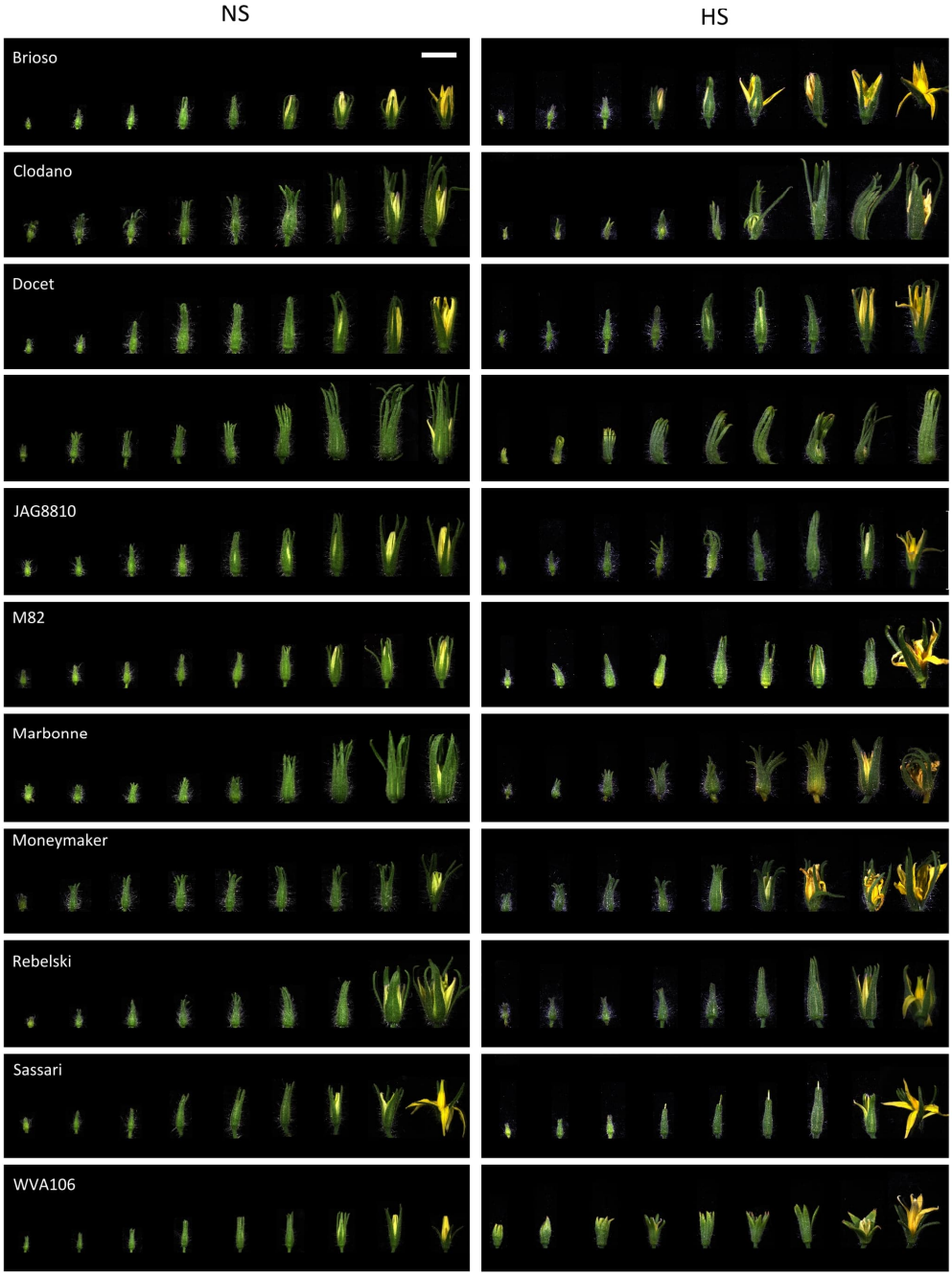
835 **Table S8.** (A) List of DEGs corresponding to the GO "regulation of reproductive process" found
836 enriched in the upregulated DEGs of TFB and PFB. (B) List of DEGs corresponding to the GO
837 "developmental process involved in reproduction" found enriched in the downregulated DEGs PFB.

838 **Table S9.** List of DEGs corresponding to pollen development-related genes and their expression under
839 NS and HS conditions.

840 **Table S10**. List of DEGs corresponding to HSP and HSF genes and their expression under NS and HS
841 conditions.

842 **Table S11.** List of DEGs corresponding to GO:1901700 "response to oxygen-containing compound"
843 and their expression under NS and HS conditions.

Figure 1


NS
HS
Brioso
Clodano
Docet
JAG8810
M82
Marbonne
Moneymaker
Rebelski
Sassari
WVA106

844

Figure 2

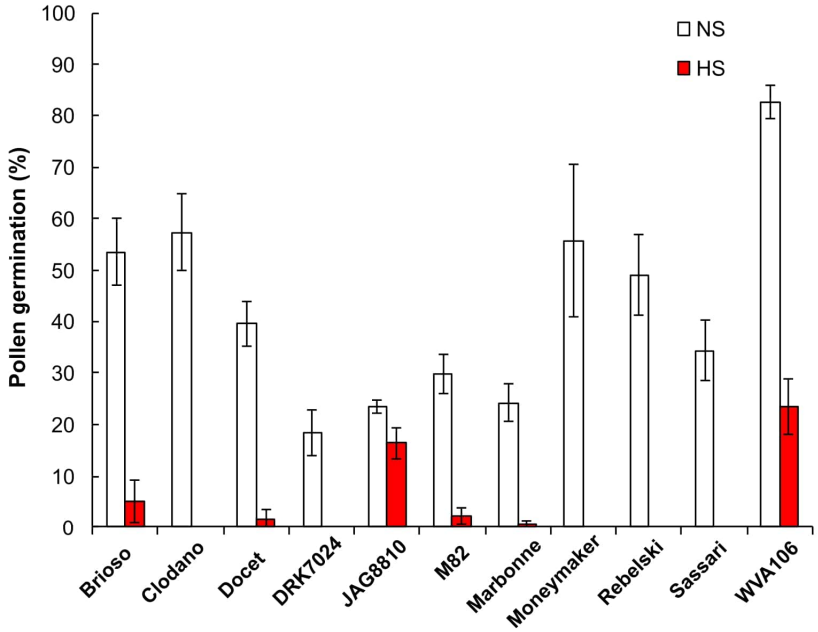


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Figure 3

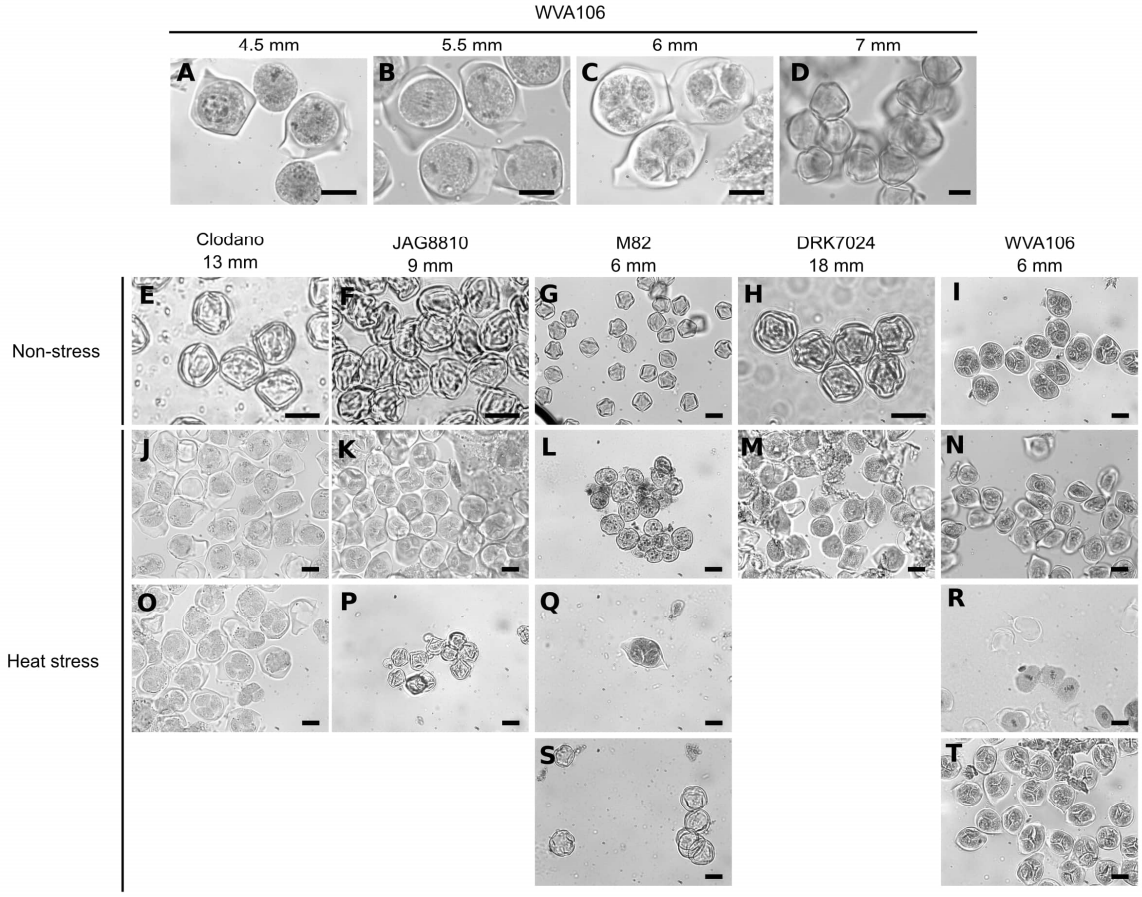

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Figure 4

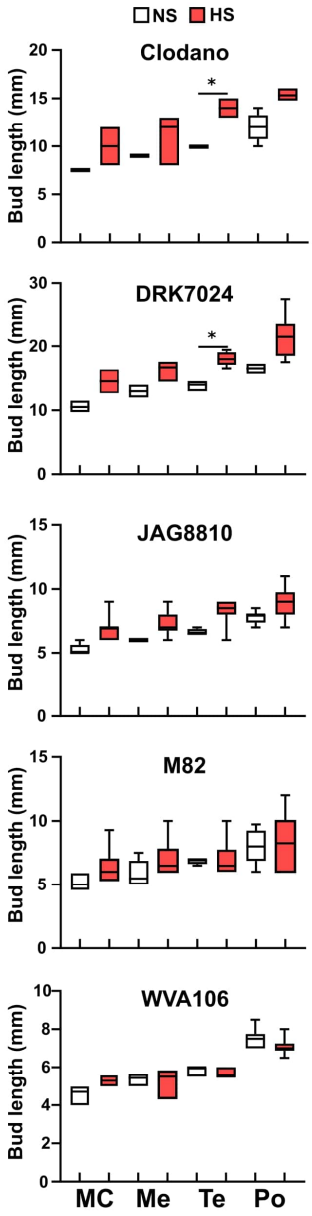

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Figure 5

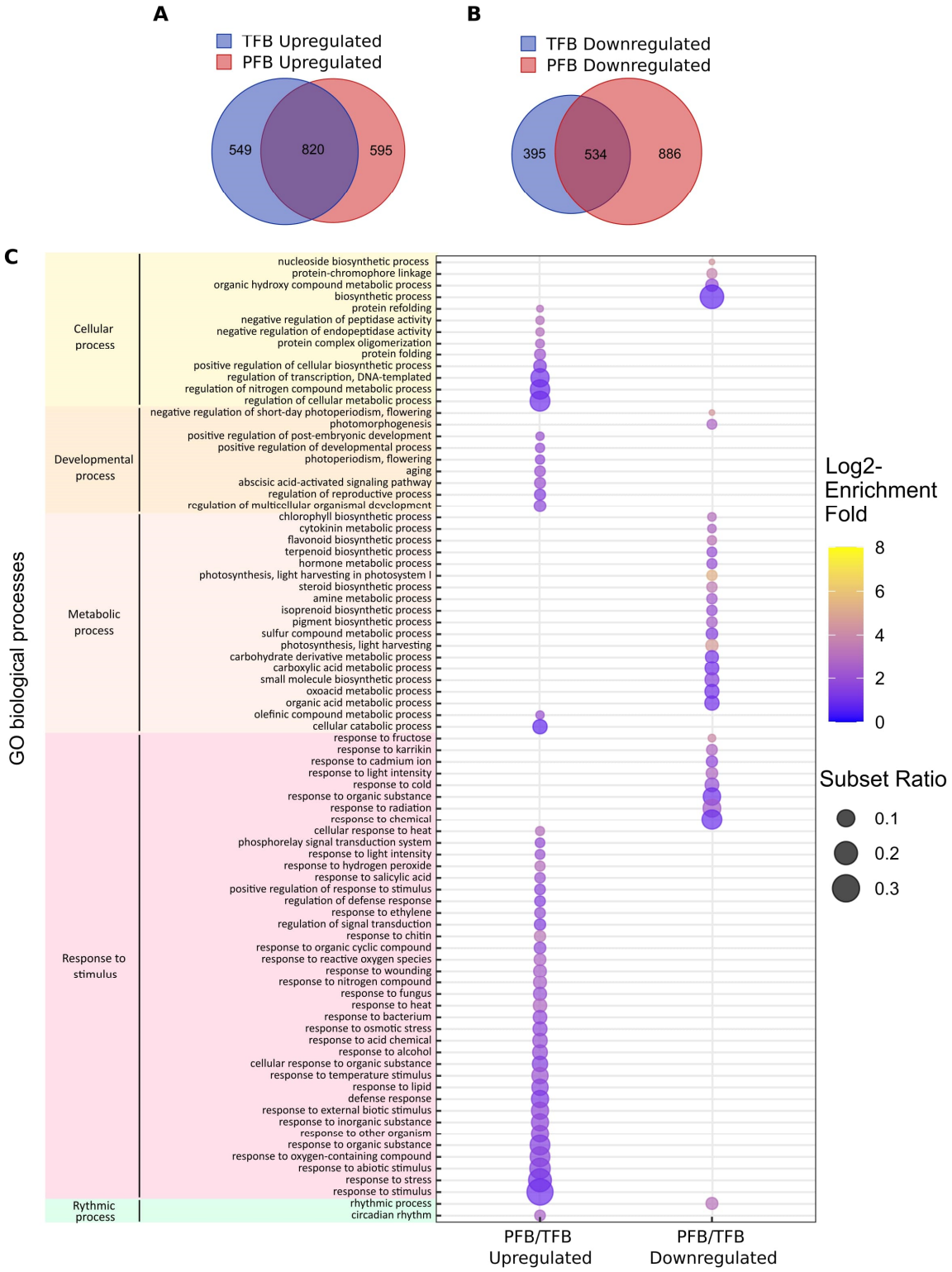

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Figure 6

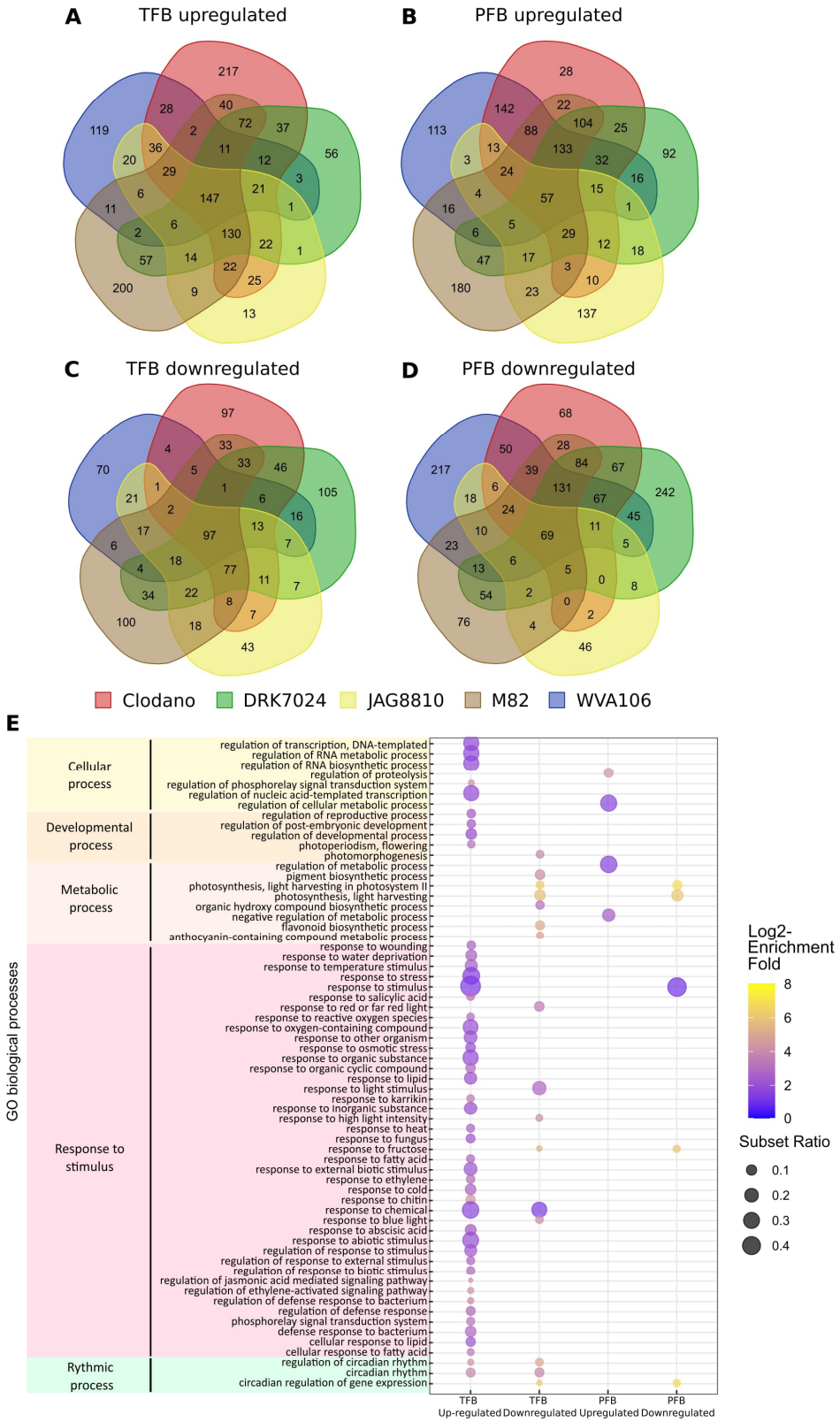
A
TFB upregulated
B
PFB upregulated
C
TFB downregulated
D
PFB downregulated
Clodano
DRK7024
JAG8810
M82
WVA106
E
GO biological processes
Cellular process
Developmental process
Metabolic process
Response to stimulus
Rythmic process
regulation of transcription, DNA-templated
regulation of RNA metabolic process
regulation of RNA biosynthetic process
regulation of proteolysis
regulation of phosphorelay signal transduction system
regulation of nucleic acid-templated transcription
regulation of cellular metabolic process
regulation of reproductive process
regulation of post-embryonic development
regulation of developmental process
photoperiodism, flowering
photomorphogenesis
regulation of metabolic process
pigment biosynthetic process
photosynthesis, light harvesting in photosystem II
photosynthesis, light harvesting
organic hydroxy compound biosynthetic process
negative regulation of metabolic process
flavonoid biosynthetic process
anthocyanin-containing compound metabolic process
response to wounding
response to water deprivation
response to temperature stimulus
response to stress
response to stimulus
response to salicylic acid
response to red or far red light
response to reactive oxygen species
response to oxygen-containing compound
response to other organism
response to osmotic stress
response to organic substance
response to organic cyclic compound
response to lipid
response to light stimulus
response to karrikin
response to inorganic substance
response to high light intensity
response to heat
response to fungus
response to fructose
response to fatty acid
response to external biotic stimulus
response to ethylene
response to cold
response to chitin
response to chemical
response to blue light
response to abscisic acid
response to abiotic stimulus
regulation of response to stimulus
regulation of response to external stimulus
regulation of response to biotic stimulus
regulation of jasmonic acid mediated signaling pathway
regulation of ethylene-activated signaling pathway
regulation of defense response to bacterium
regulation of defense response
phosphorelay signal transduction system
defense response to bacterium
cellular response to lipid
cellular response to fatty acid
regulation of circadian rhythm
circadian rhythm
circadian regulation of gene expression
TFB Up-regulated
TFB Downregulated
PFB Upregulated
PFB Downregulated
Log2-Enrichment Fold
8
6
4
2
0
Subset Ratio
0.1
0.2
0.3
0.4

Figure S1

WVA106
Sassari
Brioso
M82
Docet
Moneymaker
Clodano
JAG8810
Rebelski
Marbonne
DRK7024

Figure S2

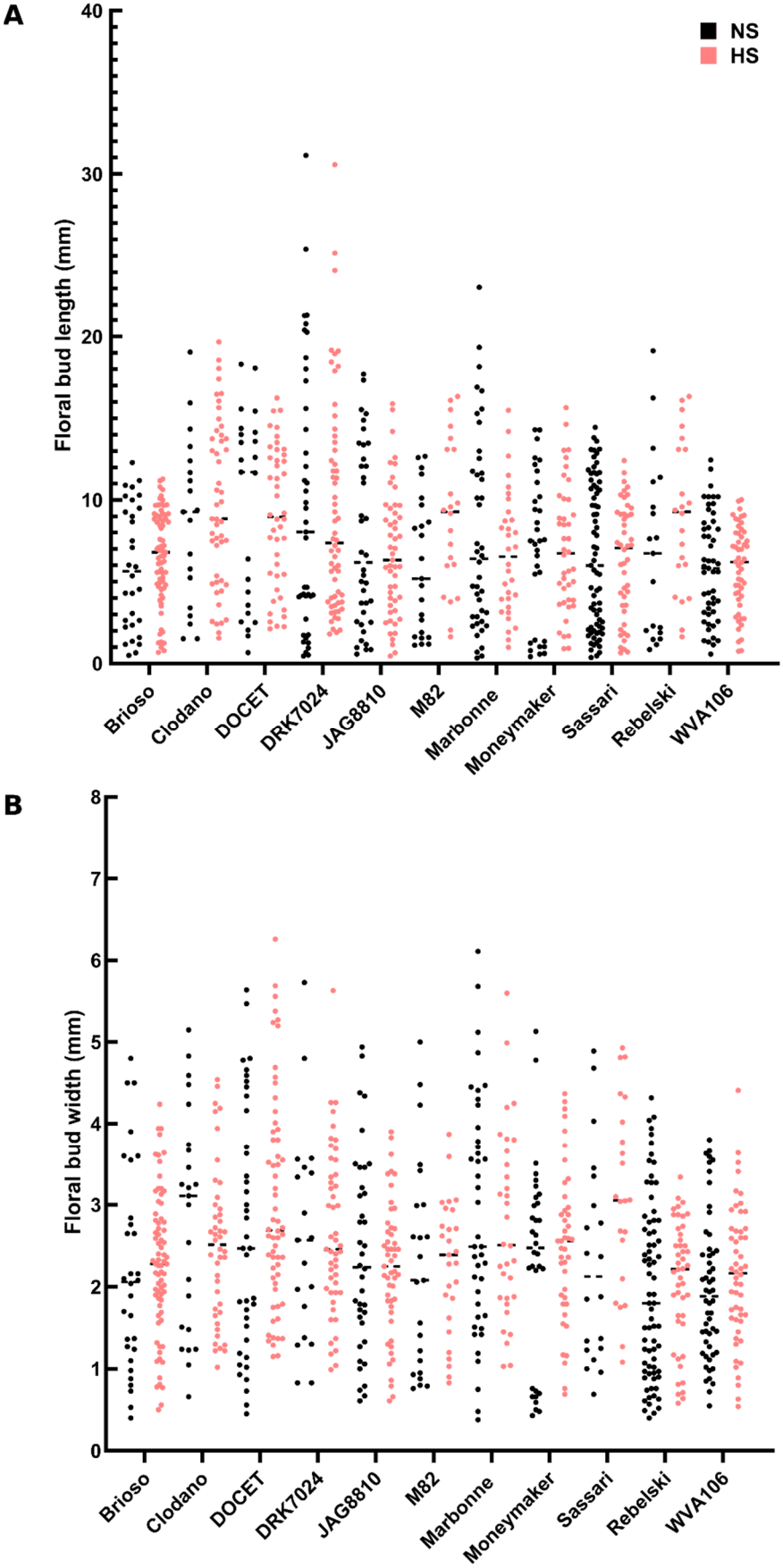

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Figure S3

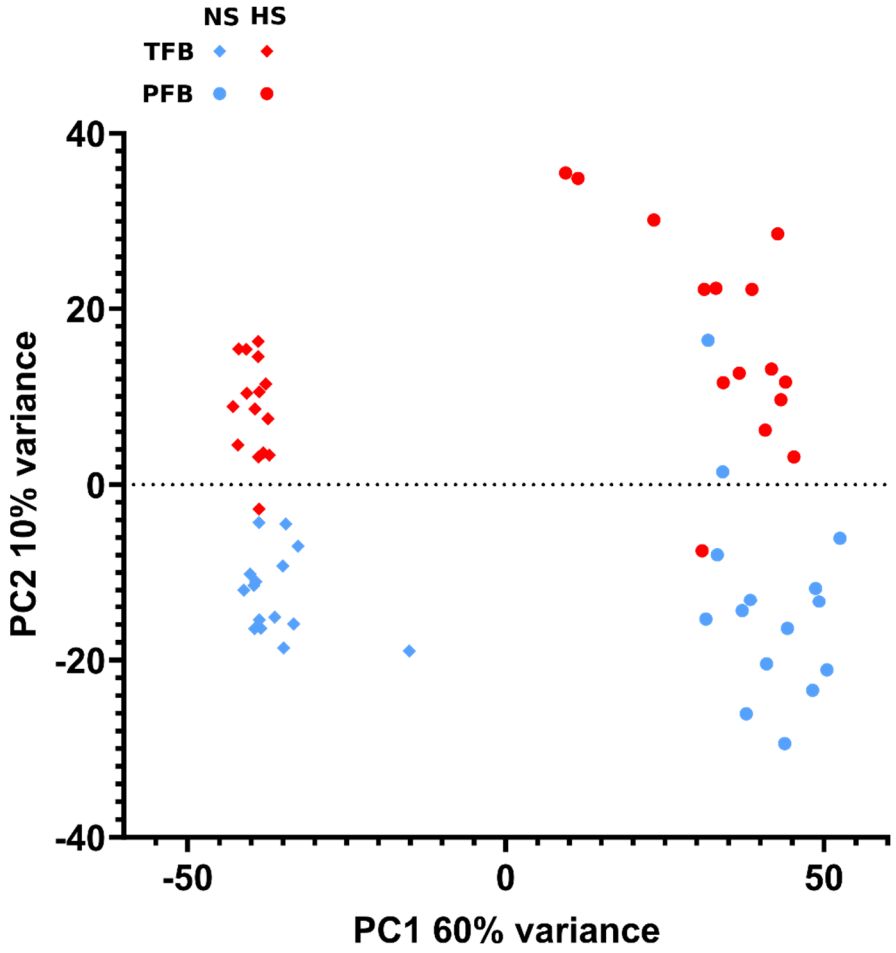
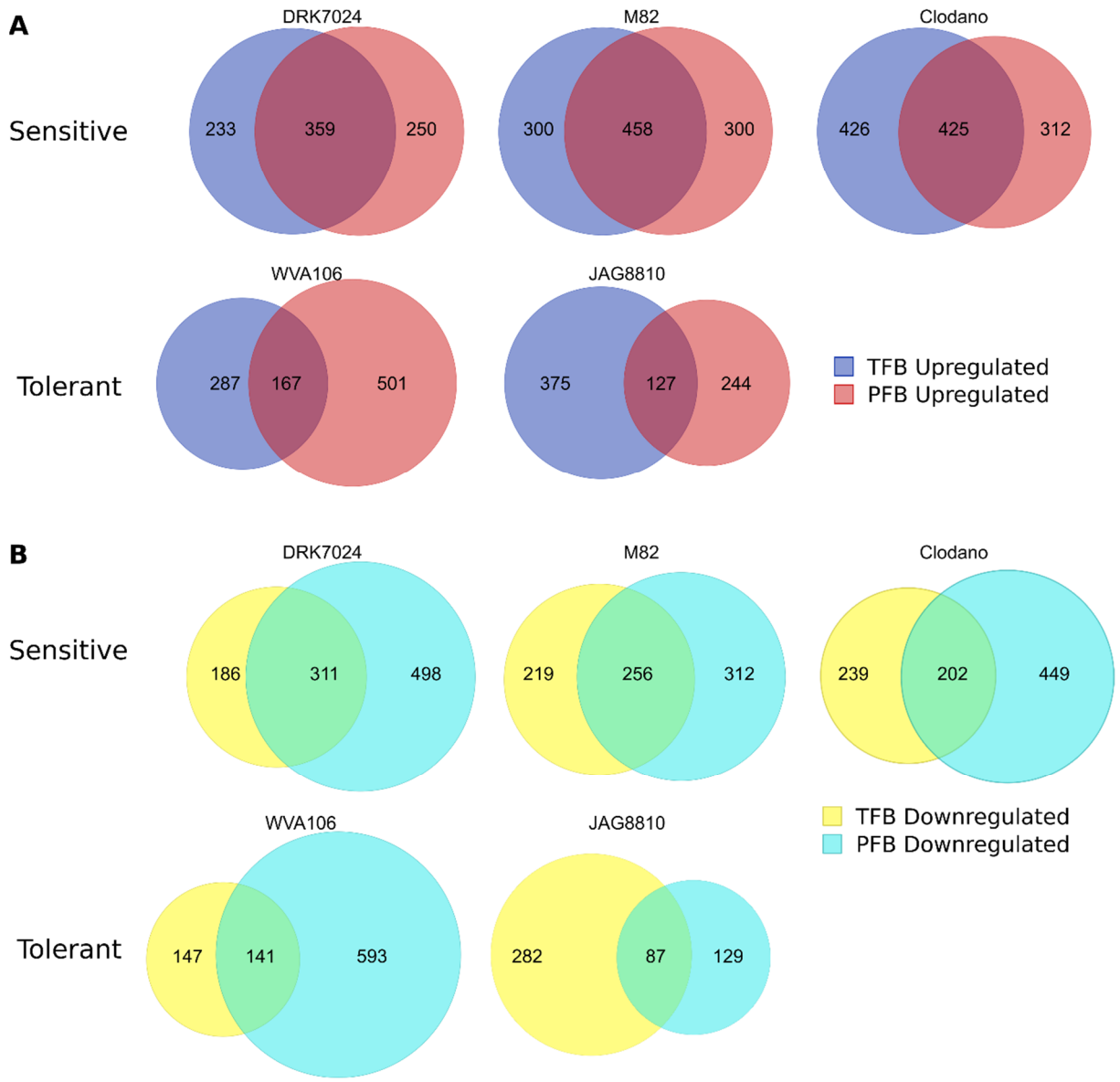

NS HS
TFB
PFB
40
20
0
-20
-40
PC2 10% variance
-50
0
50
PC1 60% variance

Figure S4


A
DRK7024
M82
Clodano
Sensitive
233
359
250
300
458
300
426
425
312
WVA106
JAG8810
Tolerant
287
167
501
375
127
244
TFB Upregulated
PFB Upregulated
B
DRK7024
M82
Clodano
Sensitive
186
311
498
219
256
312
239
202
449
WVA106
JAG8810
TFB Downregulated
PFB Downregulated
Tolerant
147
141
593
282
87
129