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Identification of heat responsive genes in pea stipules and anthers through transcriptional profiling

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Author Contribution:

TW and KG conceptualized the study. SH, KG, RB, BT and TW designed the experiments; SH conducted the experiment, analysis and wrote the manuscript; RL, NC and KG contributed to sequencing data alignment and DEG analysis. All authors reviewed the manuscript.

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Abstract

Field pea (*Pisum sativum* L.), a cool-season legume crop, is known for poor heat tolerance. Our previous work identified PR11-2 and PR11-90 as heat tolerant and susceptible lines in a recombinant inbred population. CDC Amarillo, a Canadian elite pea variety, was considered as another heat tolerant variety based on its similar field performance as PR11-2. This study aimed to characterize the differential transcription. Plants of these three varieties were stressed for 3h at 38°C prior to self-pollination, and RNAs from heat stressed anthers and stipules on the same flowering node were extracted and sequenced via the Illumina NovaSeq platform for the characterization of heat responsive genes. *In silico* results were further validated by qPCR assay. Differentially expressed genes (DEGs) were identified at log2 fold change, the three varieties shared 588 DEGs which were up-regulated and 220 genes which were down-regulated in anthers when subjected to heat treatment. In stipules, 879 DEGs (463/416 upregulation/downregulation) were consistent among varieties. The above heat-induced genes of the two plant organs were related to several biological processes i.e., response to heat, protein folding and DNA templated transcription. Ten gene ontology (GO) terms were over-represented in the consistently down-regulated DEGs of the two organs, and these terms were mainly related to cell wall macromolecule metabolism, lipid transport, lipid localization, and lipid metabolic processes. GO enrichment analysis on distinct DEGs of individual pea varieties suggested that heat affected biological processes were dynamic, and variety distinct responses provide insight into molecular mechanisms of heat-tolerance response. Several biological processes, e.g., cellular response to DNA damage stimulus in stipule, electron transport chain in anther that were only observed in heat induced PR11-2 and CDC Amarillo, and their relevance to field pea heat tolerance is worth further validation.

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24 # Introduction

25 Human activities have contributed approximately 1°C temperature increase globally since the
26 Industrial Age, and are predicted to cause another 0.5-1°C increase in the period between 2030
27 and 2052 according to current greenhouse gas emission rates [1]. The evidence of the rising
28 temperature causing lowered grain production was reported in the three major crops, maize,
29 wheat, and rice [2]. Heat stress (HS) also limits the production on legume crops including pea. In
30 Canada, where its pea production accounts for one third of the global production, lowered grain
31 yield was observed in summers when the maximum temperature exceeded 28 °C during
32 flowering, or the seasonal temperature was over 17.5 °C [3, 4]. Because of the concern about a
33 warming summer in North America, physiological studies on HS related damage on field pea,
34 particularly the reproductive plant parts, have been conducted in the last decade. When pea
35 plants at anthesis were exposed to 36/18°C day/night for 7 days in a growth chamber, the pollen
36 germination percentage, pollen tube length, pod length, seed number per pod, and the seed–ovule
37 ratio dropped dramatically compared to pea exposed to normal conditions of 24/18°C [5]. In
38 addition, HS reduced both pollen and ovule viability, but pollen appeared to be more heat
39 susceptible [6]. In terms of pea breeding, progress has also been made in the characterization of
40 heat tolerance based on field trials. A longer duration from sowing to flowering termination, and
41 greater pod production per plant contributed to increased grain yield potential at both hot and
42 normal conditions, and several stable quantitative trait loci were characterized related to
43 flowering and yield component traits [4]. Lodging resistance and the semi-leafless leaf type
44 resulted in a cooler pea canopy and greater yield potential [7]. Additionally, the authors further
45 characterized putative genomic loci of heat responsive traits, e.g., canopy temperature, pod
46 number and chlorophyll concentration, via a pea genome wide association mapping study [8].

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47 The discovery of heat responsive genes started with the characterization of heat shock protein
48 (HSP) genes and their transcription factors (HSFs). Findings in this aspect were firstly well
49 documented in *Arabidopsis thaliana*. In addition to the 21 known HSFs [9], the Arabidopsis heat
50 response is partly mediated by 13 HSP20s [10], 18 HSP70s [11], seven HSP90s [12], and up to
51 eight members of the HSP100s [13]. The gene family of HSP20 was most highly expressed
52 under HS, followed by the gene family of HSP70 and HSP90, and the gene family of HSP100
53 was not responsive to heat stress [14]. Subsequent studies on the global transcriptome profiling
54 under HS revealed that heat responsive genes could expand to those other genes involved in
55 plant hormone biosynthesis and signaling, calcium and sugar signaling, primary and secondary
56 metabolism [15-17]. Cell wall and secondary metabolite pathways were also highly affected
57 under HS in lentil [18]. However, both the number of up- and down-regulated genes and the ratio
58 of up- and down-regulated genes under HS varied among the above mentioned studies depending
59 on HS treatments, plant species, genotypes and different plant organs used for RNA isolation.

60 Research on heat responsive gene discovery in pea is limited to the findings of HSP genes.
61 Among the reported pea HSP genes, the expression of *PsHSP 18.1* and *PsHSP71.2* genes
62 appeared to be heat inducible [19, 20]. The relation of HSPs to heat tolerance was subsequently
63 confirmed as the induction of these HSP genes improved survival rate of pea seedlings and
64 mature plants at high temperature [21]. Moreover, several HSP genes had greater heat-induced
65 expression in one of the heat tolerant cultivars, Acc.623, than in one susceptible variety Acc.476.
66 The conclusion that the heat tolerant variety, in general, outperformed the susceptible variety in
67 terms of HSP heat induction threshold is still in question because this study did not conduct a full
68 comparison of HSP heat induced expression patterns among the pair of pea heat tolerant and
69 susceptible varieties.

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70 Although lacking the reference genome previously, transcriptome profiling via RNA-seq studies
71 were carried out in pea over the last decade, mainly focusing on the mining of genetic markers.
72 The first pea transcriptome reference was developed using next generation sequencing with the
73 Roche/454 platform [22]. Later Illumina high-throughput sequencing was applied to sequence 23
74 cDNA libraries from multiple tissues of the Australian field pea cultivars Kaspa and Parafield
75 [23]. A large proportion of the assembled contigs were expressed in both cultivars. To date, no
76 transcriptome-wide mapping of pea response to HS has been conducted, but this method was
77 utilized in the discovery of responsive genes in field pea seed aging [24], root nodulation [25]
78 and most recently in water-logging stress studies [26, 27]. The utilization of RNA-seq technique
79 in pea HS research allows for the genome-wide mining of heat responsive genes and the global
80 description of the complex regulatory pathway in the protection against HS at the cellular level,
81 as well as comparative analysis of genes responsive to HS among different pea varieties, or
82 between pea and other crop species. Thus, the objectives of this research included 1)
83 characterization of additional gene response toward high temperature besides previously
84 characterized pea HSP genes; 2) comparative analysis of heat responsive gene expression
85 differences between anthers and stipules, as well as between heat tolerant and heat susceptible
86 varieties.

87 Methods

88 Plant materials

89 Three pea varieties were used as plant material for this experiment, that is, PR11-2 (heat tolerant
90 variety), PR11-90 (heat susceptible variety) and CDC Amarillo (check variety). PR11-2 and
91 PR11-90 are recombinant inbred lines from the population PR11, which was derived from the

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92 cross CDC Centennial/CDC Sage made in 2008 at the Crop Development Centre (CDC),
93 University of Saskatchewan [4]. CDC Centennial was developed at CDC. It is a high yielding
94 yellow pea cultivar with semi-leafless leaf type with moderately large seeds [28]. CDC Sage is a
95 high yielding cultivar from the CDC with green cotyledons and medium-small seeds [29]. PR11-
96 2 and PR11-90 have white flowers and green cotyledons, but PR11-2 has greater pod number per
97 plant, longer flowering duration and greater grain yield than PR11-90 based on field trials at both
98 normal and hot conditions, thus PR11-2 is considered to have better heat tolerance than PR11-90.
99 CDC Amarillo [30], a yellow pea variety and one of the best yielding varieties in western
100 Canada, was included as a check. Because CDC Amarillo has similar field performance as PR11-
101 2 in our field test at normal and heat stressful conditions (Table 1), it is also considered as heat
102 tolerant compared with PR11-90.

103

104 **Table 1. Characteristics of flowering and yield-related traits of PR11-2, PR11-90 and CDC**
105 **Amarillo at normal and late seeding trials in 2017-2019 at Saskatoon, Canada.**

variety	seeding date	DTF	DOF	RNN	PN	SNPP	TSW(g)	plot yield(kg/ha)
PR11-2	normal	56.9	15.6	5.4	7.7	4.1	215.4	2827.7
	late	51.9	12.7	4.8	7.4	3.5	216.6	2665.2
PR11-90	normal	48.0	17.7	4.5	7.1	5.8	200.9	2288.9
	late	47.7	13.0	3.3	5.2	4.8	177.6	1144.9
CDC Amarillo	normal	56.5	14.5	5.0	7.6	4.6	236.4	3064.5
	late	51.4	13.6	4.4	7.1	3.9	200.9	2734.2

106 Note: DTF, days to flowering; DOF, duration of flowering; RNN, reproductive node number on
107 main-stem; PN, pod number on main-stem; SNPP, seed number per pod; TSW, thousand seed
108 weight (g). Late seeding trail is more heat stressful trial.

109

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110 ## Experimental design

111 A randomized complete block design experiment utilizing the three varieties with three
112 biological reps and two temperature treatments was carried out in a phytotron chamber in the
113 Agriculture Building, University of Saskatchewan. Temperature treatments consisted of control
114 temperature treatment 24/16°C, 16/8h day/night, and high temperature treatment 38/16°C, 16/8h
115 day/night. Three seeds of each variety were planted in individual 3.8 L pots containing Sunshine
116 mix #4 (Sun Gro, Seba Beach, AB, Canada). The three plants in one pot were bulked later as one
117 biological replication. Starting from 1 week after crop emergence, the plants were watered every
118 2-3 days based on the growth stage and water use. Once a week, a quick release fertilizer (20
119 N:20 P_2O_5:20 K_2O) prepared at a concentration of 3 g L^{-1} was applied at a rate of 100 ml per pot
120 starting 1 week after emergence. At the stage when plants developed the first flower bud, but
121 before anther dehiscence, pots of all varieties in the heat treatment group were transferred to a
122 38°C chamber for 3h. Then all the anthers and stipules on the first flowering node of the three
123 plants within one pot were sampled from both normal and high temperature treatments, and then
124 were freshly frozen in liquid nitrogen and kept at -80°C for storage.

125 ## RNA extraction and RNA integrity check

126 The whole experiment constituted a library of 36 samples from three varieties, two plant organs,
127 two temperature treatments and three biological reps, as detailed in the previous section. For
128 each organ sample, the extraction of total RNA was conducted using QIAGEN RNeasy plant
129 mini kit from QIAGEN Inc, and then a further clean-up step by digesting any remaining DNA
130 contaminant was carried out using QIAGEN RNase-free DNase set. The quantity of extracted
131 RNA sample was then determined by evaluating optical density at 260 nm and the

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132 OD260/OD280 absorption ratio using NanoDrop 8000 UV spectrophotometer. The integrity of
133 all 36 RNA samples were profiled for integrity via Bioanalyzer 2100 according to the
134 manufacturer's manual, and all RNA samples had integrity scores in the range of 9-10 on the
135 scale of 0-10, which passed the integrity standard for sequencing.

136 RNA-Seq protocol

137 Construction of cDNA libraries and subsequent sequencing was done at MedGenome Inc
138 (https://www.medgenome.com, Foster City, CA, USA).

139 Raw data processing and sequencing read alignment

140 In the pre-processing step of the raw reads, the adapter sequences and low-quality bases were
141 trimmed using AdpaterRemoval-V2 [31]. From the preprocessed reads, ribosomal RNA
142 sequences were removed by aligning the reads with SILVA database [32] using Bowtie2_v2.2.9
143 [33]. The remaining reads were aligned to the pea reference genome (Pisum_sativum_v1a.fa)
144 and gene model (Pisum_sativum_v1a_genes.gff3) [34]. The alignment was preformed using
145 STAR_v2.5.3a [35].

146 Differential gene expression analysis and annotation

147 Firstly, a homology search was executed for all 44,756 gene sequences against UniProt plant
148 [36] using Diamond_v0.9.3.104 [37]. Out of 44,756 genes, 33,669 genes were annotated based
149 on tophit. Then for each variety, the differential expression analysis between heat-treatment (3H
150 38°C) and control (22°C) was conducted via cuffdiff program in cufflinks package_v 2.2.1 [38].
151 Log2 fold change (FC) cutoff 2/-2 and p-value cutoffs 0.01 were used separately as cutoffs for
152 up and down regulated genes to characterize differentially expressed genes (DEGs). The unit of

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153 measurement used by Cufflinks to estimate transcript abundance is fragments per kilobase of
154 transcript per million mapped reads.

155 Quantitative real-time PCR validation

156 To validate the correctness of above DEG results analysed *in silico*, qPCR bench assay was
157 conducted to test the result consistency between the two methods. Eleven random genes were
158 originally selected from the pea genome and primers were designed for each gene via IDT
159 Primer quest tool (Integrated DNA Technologies Inc) according to the following criteria, i.e., Tm
160 of 62 ± 1°C, PCR amplicon lengths of 90-120 bp, primer length of 20-22 bp, and GC content of
161 45-55%. A series of 10-time cDNA dilutions on PR11-90_control leaf cDNA library was made
162 for primer efficiency test. And primer efficiency (%) of each gene was equaled to $(10^{-1/slope}$
163 $-1) * 100$, and all primers had their efficiency rates between 90-110% and qualified for assay
164 use (S1 Fig).

165 Subsequently, the relative expression of the 11 genes was separately quantified among 18 stipule
166 samples and the 18 anther samples, which were used for RNA sequencing. RT-qPCR data were
167 analyzed according to the comparative $2^{-\Delta\Delta Ct}$ method [39], where ΔCt = (Ct of gene of interest –
168 Ct of reference gene). The relative gene expression change was compared between qPCR bench
169 assay and RNA-Seq on stipules and anthers separately.

170 Gene ontology (GO) enrichment analysis on DEGs

171 Further comparative analyses on DEGs were conducted among the three pea varieties with
172 different heat tolerances, and between anther (reproductive plant organ) and stipule (vegetative
173 plant organ). The results were output in Venn diagram via online software
174 (http://bioinformatics.psb.ugent.be/webtools/Venn/). Subsequently, GO terms of heat responsive

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175 genes were tested against the pea reference transcriptome (Pisum_sativum_v1a_GO, database
176 was retrieved in November, 2020) via agriGO v2.0 [40], and significant GO terms in biological
177 processes were filtered using hypergeometric test method at FDR adjusted p value<0.01.

178 Results

179 Sequencing quality assessment

180 To understand transcriptional reprogramming of field pea in response to heat stress, we
181 performed deep RNA sequencing of stipule and anther organs subjected to 38°C for 3h among
182 three varieties using the NovoSeq sequencing platform. The sequencing platform produced a
183 high confidence sequencing output with a <2% maximum read error rate among the 36 libraries.
184 After removing the error reads, the anther libraries had an average of 84 million 100 bp paired-
185 end reads across the three varieties (Table 2). Stipule libraries resulted in a similar average of 88
186 million reads (Table 3). Both types of libraries outputted high sequencing depths for the global
187 transcriptome analysis as compared with previous pea transcriptome studies [23, 25].
188 Subsequently the reads were mapped to the pea reference genome [34], and nearly all reads were
189 successfully aligned to the pea genome, which also implies good quality of the deep sequencing.

190 **Table 2. Summary of sequencing depth and percentage of sequencing reads aligning to pea**
191 **genome on anther samples.**

Genotype	Rep	Treatment	No. of million reads	Alignment (%)
CDC Amarillo	1	22°C control	65.2	99.6
CDC Amarillo	2	22°C control	100.1	99.7
CDC Amarillo	3	22°C control	88.3	99.7
CDC Amarillo	1	38°C stressed	65.0	99.2
CDC Amarillo	2	38°C stressed	88.1	99.2
CDC Amarillo	3	38°C stressed	87.4	99.6

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PR11-2	1	22°C control	74.4	99.7
PR11-2	2	22°C control	75.9	99.7
PR11-2	3	22°C control	94.6	99.8
PR11-2	1	38°C stressed	83.8	99.1
PR11-2	2	38°C stressed	82.1	99.0
PR11-2	3	38°C stressed	80.8	99.2
PR11-90	1	22°C control	88.6	99.6
PR11-90	2	22°C control	80.2	99.7
PR11-90	3	22°C control	98.0	99.7
PR11-90	1	38°C stressed	85.0	99.6
PR11-90	2	38°C stressed	85.7	99.4
PR11-90	3	38°C stressed	90.9	99.4

192

193 **Table 3. Summary of sequencing depth and percentage of sequencing reads aligning with**

194 **pea genome on stipule samples.**

Genotype	Rep	Treatment	No. of million reads	Alignment (%)
CDC Amarillo	1	22°C control	101.7	99.3
CDC Amarillo	2	22°C control	92.1	99.5
CDC Amarillo	3	22°C control	100.1	99.5
CDC Amarillo	1	38°C stressed	82.6	97.3
CDC Amarillo	2	38°C stressed	78.3	98.4
CDC Amarillo	3	38°C stressed	80.4	99.1
PR11-2	1	22°C control	90.3	99.6
PR11-2	2	22°C control	90.4	99.3
PR11-2	3	22°C control	87.9	99.6
PR11-2	1	38°C stressed	85.2	98.8
PR11-2	2	38°C stressed	84.7	99.3
PR11-2	3	38°C stressed	106.4	99.3
PR11-90	1	22°C control	77.5	99.0
PR11-90	2	22°C control	109.8	99.6
PR11-90	3	22°C control	80.4	99.5
PR11-90	1	38°C stressed	74.7	98.9
PR11-90	2	38°C stressed	66.5	99.0
PR11-90	3	38°C stressed	107.7	99.3

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195
DEG analysis validation

196 The stipule expression response (log2 FC) of the 11 randomly selected genes between heat
197 treatment and control temperature were characterized via cuffdiff program and qPCR
198 respectively, and the results are shown in a heat map. Nine genes out of the eleven, had
199 consistent heat responses between qPCR and RNA-Seq result *in silico* (Fig 1), implying a good
200 quality of RNA-Seq analysis. And the significant correlation ($R^2 = 0.97$) between bench result
201 and *in silico* result further confirmed a correct analysis via cuffdiff program (Fig 2a). Two genes
202 had some unmatched results between the two methods. 0s3930g0040 displayed a consistently
203 up-regulated expression via qPCR among the three pea varieties when subjected to heat
204 treatment (Fig 1), whereas for the *in silico* result only CDC Amarillo had the same trend. From
205 *in silico* result, 5g006560 demonstrated a consistent downregulation towards HS in all varieties;
206 whereas in qPCR result, only PR11-90 had the similar trend. Likewise, among anther samples, a
207 high consistency was found between bench results and cuffdiff result (Fig 1 and 2b). Unmatched
208 results were mainly observed on gene 5g006560. The significantly high correlation ($R^2 = 0.93$)
209 between the two methods confirmed the correctness of the analyses. The qPCR results
210 successfully validated the correctness of RNA-Seq analysis on both anther and stipule samples.

211

212 **Fig 1. Transcriptional heat response heatmap of 11 randomly selected genes in the pea**
213 **genome via qPCR and cuffdiff *in silico* methods. FC values are the average log2 (FC)**
214 **across three biological reps; red color is for upregulation and blue color is for**
215 **downregulation**.

216

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217 **Fig 2. Gene expression result correlation on stipule samples (panel a) and anther samples**
218 **(panel b) between qPCR and cuffdiff program.**

219

220 Global comparisons of HS related transcriptomes between stipules 221 and anthers among three pea varieties

222 To gain the knowledge on gene response to heat treatment, genes whose expression differed
223 between HS and control temperature at log2|FC|≥2 were characterized as heat responsive genes.
224 A total of 3565 responsive genes were identified in anthers, among which 2322 genes had greater
225 expression and 1243 had lower expression in heat treatment compared to control temperature.
226 Stipules on the same flowering node had 4381 responsive genes, with 1886 up-regulated genes
227 and 2495 down-regulated genes. Among anther transcriptomes of the three varieties, the number
228 of genes that were up-regulated under HS was almost twice the number of down-regulated genes
229 (Fig 3). The three varieties shared 588 genes with up-regulated expression under HS, which
230 comprised of 25% up-regulated genes in total. The overlap between PR11-2 and PR11-90, where
231 the two varieties were derived from the same recombinant inbred population, accounted for a
232 higher proportion (~70% in PR11-2 and ~60% in PR11-90). CDC Amarillo, which has a
233 different genetic background, contributed a major group of DEGs that were distinctly up-
234 regulated. Among the 1343 genes whose expression was inhibited, 220 genes were found
235 common among the three varieties.

236 Whereas among the surrounding stipule leaf transcriptomes, the pattern was opposite compared
237 to the anther transcriptome, i.e., a greater number of genes were down-regulated in the heat
238 treatment. The result revealed a different heat response in stipules compared to anthers. Still,

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239 there were common DEGs between anthers and stipules, 220 common DEGs with their up-
240 regulated expression and 25 DEGs with down-regulated expression. Respective gene ontology
241 (GO) enrichment analysis of the two groups of DEGs was conducted to cluster their functions in
242 plant biological processes, and results were elucidated in the section below on GO analysis.

243 Among the three varieties, PR11-2, considered to be best heat tolerant, had the lowest number of
244 its DEGs in both anthers and stipules, indicating that PR11-2 might be able to maintain a
245 relatively steady transcriptome when subjected to short term HS. In anthers, PR11-90 had a
246 similar number of total DEGs as that of CDC Amarillo, but CDC Amarillo had a greater number
247 of up-regulated genes and a less number of down-regulated genes than PR11-90. Whereas in
248 stipules, CDC Amarillo had both higher number of up-regulated and down-regulated genes than
249 PR11-90. It is worth noting that CDC Amarillo appeared to have more unique DEGs in heat
250 response compared with the other two varieties whose genetic backgrounds were more similar.
251 This finding implied that heat response could depend on genetic variability.

252

253 **Fig 3. Venn diagram showing the number of common and specific differentially expressed**
254 **genes (log2 |FC| ≥ 2; false discovery rate < 0.05) at 3h 38°C heat treatment among three pea**
255 **varieties, and between anther and stipule on the same node.** Panel a-c are for up-regulated
256 genes (from the left to right are anther, stipule and comparison between the two. Panel d-f are for
257 down-regulated genes in the same order mentioned above.

258

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GO grouping on common DEGs among varieties

With the purpose of characterizing a general pea plant heat response, GO enrichment analysis was conducted on the common DEGs among the three pea varieties in this study. In anthers, GO terms relating to the 588 common up-regulated genes and 220 down-regulated genes were tested separately against the pea reference transcriptome (Pisum_sativum_v1a_GO, database was retrieved in November, 2020) to identify the significantly over-represented GO terms in biological processes under HS. All significant GO terms were filtered via hypergeometric test method at FDR adjusted p value≤0.01. Respective analysis was similarly conducted on the common genes in stipules as well, i.e., 463 DEGs with upregulation and 416 DEGs with downregulation. Up-regulated genes were enriched with 31 and 13 GO terms in biological processes for anthers and stipules, respectively (Fig 4, S1 Table). The top 10 most significant GO terms in anthers were protein folding (GO:0006457, 21 enriched terms), embryo development (GO:0009790, 9), multicellular organismal process (GO:0032501, 17), response to heat (GO:0009408, 5), multicellular organism development (GO:0007275, 15), galactose metabolic process (GO:0006012, 4), regulation of transcription, DNA-templated (GO:0045449, 43), regulation of cellular metabolic process (GO:0031323, 44), regulation of RNA metabolic process (GO:0051252, 26), and regulation of gene expression (GO:0010468, 44). In the stipules located on the same anther bearing node, the ten most over-represented GO terms were protein folding (GO:0006457, 13), response to heat (GO:0009408, 4), cellular protein modification process (GO:0006464, 12), carbohydrate metabolic process (GO:0005975, 80), post-translational protein modification (GO:0043687, 12), transcription, DNA-templated (GO:0006351, 21), RNA biosynthetic process (GO:0032774, 19), phosphate-containing compound metabolic process (GO:0006796, 11), phosphorus metabolic process (GO:0006793, 11) and regulation of RNA

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282 metabolic process (GO:0051252, 18). Four GO terms were common between anthers and
283 stipules, which were GO:0006457 (protein folding), GO:0009408 (response to heat),
284 GO:0006351 (transcription, DNA-templated) and GO:0051252 (regulation of RNA metabolic
285 process). GO:0051252 is one of the ancestor terms of GO:0006351 in the cluster. Other enriched
286 GO terms of consistently up-regulated genes in anthers were involved with primary metabolic
287 processes, cellular respiration and reproductive structure development and the regulations of
288 several biosynthetic and metabolic clusters including cellular metabolic and biosynthetic process,
289 RNA metabolic process, macromolecule metabolic and biosynthetic process (S1 Table).

290

291 **Fig 4. Top ten over-representative GO terms with up-regulation at log2 FC >2 in biological**
292 **process in anthers (blue column) and stipules (orange column).**

293

294 To further compare the most heat responsive genes among different varieties, we arbitrarily
295 filtered the DEGs of each variety within the top 20% fold threshold range and found the most
296 heat inducible genes were quite similar, though the greatest gene expression fold threshold varied
297 slightly among the three varieties. The gene group relating to heat shock transcription factor
298 (HSF) and heat shock protein (HSP) accounted for a large proportion.

299 Many of these HSF and HSP genes were reported here for the first time; HSF genes in particular,
300 which expanded the previously limited findings. Putative HSF family A and B genes appeared to
301 heat inducible, these genes included three HSF A genes and two HSF B genes (corresponding
302 gene locus refers to Table 4). In addition to the two pea HSP genes that were previously
303 documented, 11 other small HSP genes on chromosomes II, IV, V, VI and VII were highly heat

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304 inducible among all varieties regardless of plant organs. Increased expression of six HSP70
305 genes, two HSP90 family genes and three other HSP genes were also identified among all three
306 varieties. Several heat shock cognate genes (HSCs), whose expression was previously considered
307 as constitutive during normal plant development, appeared to be heat responsive as well (e.g.
308 PsHSC 71.0, HSC 70-2 like etc). The response of several other genes whose functions closely
309 interacted with HSP were also detected in this study.

310 Interestingly, several HSP genes only responded in one organ. Anthers had unique HSFs
311 (Psat0s3914g0040, putative HSF; Psat4g086800, HSF24-like), three HSP genes which were
312 Psat1g222760 (Stromal HSP70), Psat3g104360 (HSP83-like fragment), Psat5g229840 (class IV
313 HSP). Stipules had unique HSF (Psat6g078240, HSF A3-like) and two HSP genes
314 (Psat7g255520, HSP26.5; Psat3g180040, HSC70 2-like). Several other HSF and HSP genes
315 were specific to variety, e.g., two HSP relating genes (Psat4g003160 and Psat4g035840) were
316 only induced in PR11-90.

Table 4. List of pea HSP and HSF related genes that were induced in response to 3 h 38°C heat treatment; numbers in the table are averaged log2 FC across three biological replicates.

Locus ID	PR11-2		PR11-90		CDC Amarillo		Gene annotation
	anther	stipule	anther	stipule	anther	stipule	
Psat0s1635g0080	8.8	8.1	9.0	8.7	8.2	8.0	PsHSP18.1
Psat2g036480	10.8	10.4	12.5	9.3	11.4	9.1	PsHSP17.9 fragment
Psat0s3930g0040	10.9		12.1		11.7	8.2	PsHSP71.2
Psat3g049640	2.8		2.9	2.3	2.9	2.3	PsHSC71.0
Psat0s3914g0040			2.5		2.4		putative HSF
Psat1g102600	2.0	3.9	2.1	4.0		5.4	HSF B-2A-like
Psat2g021040	inf		inf	2.4			putative HSF
Psat3g061600	9.3	7.8	9.7	7.8		7.5	HSF A3
Psat4g086800	inf		inf		inf		HSF24-like
Psat5g036400	7.7	7.4	9.2	8.1	7.1	7.5	HSF A fragment
Psat6g059040	10.1	6.1	9.8	6.8	8.7	6.2	putative HSF A3
Psat6g078240		5.7		5.8		6.9	HSF A3-like protein
Psat6g200480	5.8	7.1	7.2	7.9	5.6	8.1	HSF B2A-like isoform X2
Psat6g204120				3.2		2.5	putative HSF A3
Psat7g004560		2.0				2.7	putative HSF
Psat7g131680						2.4	putative HSF
Psat7g170680						3.6	HSF A1B-like isoform X2
Psat0s529g0040	10.1	10.0	12.5	9.0	12.6	9.0	putative class I HSP20
Psat2g046440	6.3	6.6	8.2	8.2	6.2	7.8	HSP15.7 peroxisomal-like
Psat4g136720	7.3	7.4	8.1	8.5	7.0	7.4	small HSP
Psat5g035320	10.5	10.3	11.9	9.3	12.2	9.3	class II HSP17.1
Psat4g166400	10.3	9.9	12.3	8.9	13.0	9.5	cytosolic class II HSP
Psat5g073280	11.7	10.5	13.1	9.8	11.1	8.9	small HSP
Psat5g174800	9.1	7.4	11.0	6.9	8.5	7.1	putative HSP20
Psat6g112800	10.8	10.0	13.0	9.4	11.8	9.0	class IV HSP22.7
Psat7g114760	7.5	9.9	11.3	9.0	11.5	11.9	class I HSP17.6

Psat7g115480	7.1	9.0	7.6	9.3	6.7	8.4	HSP18.1
Psat7g211720	5.4	6.4	6.0	6.9	4.5	6.2	class I HSP17.5
Psat7g255520		2.8		3.2		3.6	HSP26.5
Psat6g238960					2.3		DnaJ/HSP40 cysteine-rich domain protein
Psat1g212880	7.1	4.9	8.1	5.7	7.1	6.5	HSP70, mitochondrial
Psat1g222760	4.9		5.2		4.5		Stromal HSP70
Psat2g051360	8.9	6.5	10.4	6.6	8.4	7.2	HSP70
Psat3g143400		2.5	2.1	2.6		3.5	HSP70-interacting protein
Psat3g180040		2.0		2.4		2.2	HSC70 2-like
Psat3g183720	5.1	5.9	5.4	6.5	4.6	6.1	putative HSP70 family
Psat4g003160			2.1				HSP70-interacting protein
Psat4g035840				2.2			HSP70 8-like
Psat4g210520	inf	3.9	6.5	4.2	5.4	5.0	HSP70
Psat5g299000	3.8	2.9	4.3	3.1	3.6	3.8	putative HSP70 family
Psat7g023360	4.5	4.3	5.2	4.9	4.5	4.8	HSP70
Psat7g218840	8.9	7.7	10.0	8.3	8.6	7.8	HSC70 2-like
Psat7g237280	4.7	4.4	5.1	4.9	4.7	5.3	HSP70 nucleotide exchange factor FES1-like
Psat2g006440	5.4	4.6	5.7	5.4	5.2	5.4	HSP81-2
Psat0ss29864g0040	10.8	10.2	12.1	9.1	13.0	9.2	HSP83-like fragment
Psat3g104360	8.5		9.9		8.3		HSP83-like fragment
Psat5g164840	2.6	2.0	3.0	2.8	2.5	2.8	HSP80 cognate protein
Psat6g123080	4.2	3.8	4.7	4.2	3.9	4.9	activator of HSP90 ATPase homolog 1-like
Psat2g178800	5.0	3.8	5.5	4.0	4.9	4.6	HSP70-HSP90 organizing protein 3-like
Psat3g067000	5.2	3.9	5.7	4.4	4.9	5.2	activator of HSP90 ATPase homolog 1
Psat0s3618g0080	2.4	3.4	3.0	4.0	2.2	5.2	HSP DnaJ; putative transcription factor C2H2 family
Psat1g204360	10.3	10.2	11.7	9.2	12.5	9.4	HSP DnaJ
Psat2g037160	3.3						HSP
Psat5g035280	10.7	10.4	12.0	9.4	10.9	9.2	Class II HSP
Psat5g229840	3.8		3.7		2.3		Class IV HSP

Psat6g021840	9.5	8.2	11.1	8.8	10.4	7.5	Class II HSP
Psat7g114360	10.6	10.2	12.5	9.4	10.9	9.0	HSP

319 Note: for the cells denoting 'inf' as FC, the reason was that their transcript at control temperature was too low to quantify. Because
320 their transcript at HS were significant, they were still considered as heat responsive.

321

322 A total of 220 commonly down-regulated genes in anthers among the three varieties were enriched in 18 GO terms in biological
323 process category and 416 consistently down-regulated genes in stipules had 16 GO terms significantly over-represented (Fig 5). Ten
324 GO terms overlapped between the two organ types, that is, GO:0006629 (lipid metabolic process), GO:0006869 (lipid transport),
325 GO:0010876 (lipid localization), GO:0044036 (cell wall macromolecule metabolic process), GO:0071554 (cell wall organization or
326 biogenesis), GO:0006979 (response to oxidative stress), GO:0005975 (carbohydrate metabolic process), GO:0006022 (aminoglycan
327 metabolic process), GO:0043086 (negative regulation of catalytic activity), and GO:0044092 (negative regulation of molecular
328 function). GO:0006508 (proteolysis), GO:0006468 (protein phosphorylation), GO:0015833 (peptide transport) and GO:0006857
329 (oligopeptide transport) were distinctly enriched in stipule down-regulated genes, whereas GO:0005976 (polysaccharide metabolic
330 process), GO:0010383 (cell wall polysaccharide metabolic process), GO:0042545 (cell wall medication) and GO:0071555 (cell wall
331 organization) were only enriched in anthers. Although more than half of the over-represented GO terms overlapped between heat
332 stressed pea anthers and stipules at the same flowering node, surprisingly, the gene composition relating to these biological processes
333 varied between the two organs. For example, three GO terms related to lipid biological processes were both down-regulated in anthers
334 and stipules. However, only two genes (*PsLTP1* and *PsLTP2*) for lipid transport/localization were common, and seven genes

335 (Psat1g060840, Psat1g082320, Psat1g085080, Psat2g027880, Psat3g005680, Psat5g104040, and Psat5g295040) for lipid metabolic

336 processes were common between the two organ types (Table 5).

337

338 **Fig 5. Significant GO terms (FDR adjusted p value at 0.01) in biological process of down-regulated genes at log2 FC <-2 in**

339 **anther (blue column) and stipule (orange column)**

340

341 **Table 5. Gene locus and function list of commonly down-regulated genes that are associated with lipid transport, localization**

342 **and metabolic process among the three pea varieties in anthers and stipules.**

GO:0006869 lipid transport/ GO:0010876 lipid localization			
anther		stipule	
locus ID	gene function	locus ID	gene function
Psat0s1251g0040	Non-specific lipid-transfer protein	Psat0s2857g0040	Lipid transfer protein
Psat0s4118g0160	Non-specific lipid-transfer protein	Psat1g217760	Non-specific lipid-transfer protein
Psat3g119520	Non-specific lipid-transfer protein	Psat3g097600	Putative non-specific lipid-transfer protein AKCS9-like protein
Psat3g119560	Non-specific lipid-transfer protein	Psat5g029400	lipid transfer protein EARLI 1-like
Psat7g233960	Non-specific lipid-transfer protein	Psat5g112720	Lipid transfer protein
Psat7g234520	Non-specific lipid-transfer protein 2 (PsLTP2)	Psat6g027760	14 k Da proline-rich protein DC2.15-like
Psat7g234640	Non-specific lipid-transfer protein 2 (PsLTP2)	Psat7g226840	Non-specific lipid-transfer protein
Psat7g234720	Non-specific lipid-transfer protein 3 (PsLTP1)	Psat7g228160	Non-specific lipid-transfer protein 1 (LTP1)
		Psat7g234680	Non-specific lipid-transfer protein 2 (PsLTP2)
		Psat7g234720	Non-specific lipid-transfer protein 3 (PsLTP1)
GO:0006629 lipid metabolic process			

anther		stipule	
locus ID	gene function	locus ID	gene function
Psat0s1560g0040	GDSL esterase/lipase	Psat0s1401g0160	GDSL-like lipase/acyl hydrolase (EC 3.1.1.3)
Psat1g060840	Pathogen-inducible alpha-dioxygenase	Psat0s1926g0240	Auxilin-like protein (Fragment)
Psat1g081400	uncharacterized protein LOC101505667 isoform	Psat0s2010g0040	GDSL-like lipase/acyl hydrolase (EC 3.1.1.3)
Psat1g082320	GDSL esterase/lipase	Psat0s3211g0160	GDSL esterase/lipase (Fragment)
Psat1g085080	GDSL esterase/lipase LTL1-like	Psat1g017360	Fungal proteinase A aspartic proteinase superfamily protein
Psat1g086280	Lipase	Psat1g060840	Pathogen-inducible alpha-dioxygenase
Psat1g096440	3-ketoacyl-CoA synthase-like protein	Psat1g082320	GDSL esterase/lipase
Psat1g200800	GDSL-like lipase/acyl hydrolase (EC 3.1.1.3)	Psat1g085080	GDSL esterase/lipase LTL1-like
Psat2g027880	Uncharacterized protein	Psat1g193000	GDSL-like lipase/acyl hydrolase (EC 3.1.1.3)
Psat3g005680	GDSL-like lipase/acyl hydrolase (EC 3.1.1.3)	Psat2g027800	GDSL-like lipase/acyl hydrolase (EC 3.1.1.3)
Psat3g006280	GDSL esterase/lipase	Psat2g027880	Uncharacterized protein
Psat5g104040	GDSL esterase/lipase At2g04570-like	Psat2g083600	3-ketoacyl-CoA synthase (EC 2.3.1.-)
Psat5g284160	3-ketoacyl-CoA synthase (EC 2.3.1.-)	Psat2g132440	PI-PLC X domain-containing protein At5g67130
Psat5g295040	GDSL esterase/lipase apg-like protein	Psat3g000920	3-ketoacyl-CoA synthase (EC 2.3.1.-)
Psat6g041080	GDSL-like lipase/acyl hydrolase	Psat3g005640	GDSL-like lipase/acyl hydrolase (EC 3.1.1.3)
Psat6g184320	Patatin (EC 3.1.1.-)	Psat3g005680	GDSL-like lipase/acyl hydrolase (EC 3.1.1.3)
Psat7g066400	GDSL esterase/lipase	Psat3g010160	Uncharacterized protein
Psat7g066440	GDSL-like lipase/acyl hydrolase	Psat4g010320	Fatty acid hydroxylase protein (EC 4.1.99.5)
Psat7g066520	GDSL-like lipase/acyl hydrolase	Psat4g020480	cyprosin-like
Psat7g125680	PLC-like phosphodiesterase superfamily protein	Psat4g190160	Phospholipase D alpha
		Psat4g196720	GDSL esterase/lipase apg-like protein
		Psat5g104040	GDSL esterase/lipase At2g04570-like
		Psat5g177200	GDSL-like lipase/acyl hydrolase (EC 3.1.1.3)
		Psat5g295040	GDSL esterase/lipase apg-like protein
		Psat6g002160	GDSL-like lipase/acyl hydrolase
		Psat7g059400	GDSL-like lipase/acyl hydrolase (EC 3.2.1.51)

343 **Note:** for the genes in the list, their transcription level between heat treatment and control temperature was at log2 FC <-2.

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344 We arbitrarily filtered the DEGs of each variety within the top 20% fold threshold range to
345 further characterize a list of genes whose functions were most inhibited in heat stress. In anthers,
346 35 genes were shared among the three varieties, among which seven genes were involved in
347 pectin metabolism, and three with lipid metabolism. Pectin, a polysaccharide polymer of
348 galacturonic acid with different degrees of esterification via an a-1, 4-glycosidic bond, is a
349 primary composition in the plant cell wall and cell interlayer. In stipules, 51 genes were common
350 among the three varieties. The functions of these genes seemed various, including four lipase
351 genes. Only one gene at locus Psat3g196040 was present in both lists.

352 GO analysis on variety-dependant DEGs

353 To compare heat response among the three pea varieties, individual GO enrichment analyses
354 were performed on the distinct DEGs of each variety, which were exclusive DEGs from the
355 varieties' common DEGs in individual variety DEG list. Among the three varieties, PR11-2 had
356 the lowest number of enriched GO terms in down-regulated genes and the highest number of
357 over-representative GO terms in up-regulated genes of both anthers and stipules, implying that
358 PR11-2 is likely to have a superior heat tolerance compared to the other two varieties (Fig 6). In
359 the anther transcriptome of PR11-2, no GO term was significantly enriched for down-regulated
360 DEGs but 13 terms were up-regulated. These terms corresponded to four biological pathways,
361 i.e., cell respiratory electron transport chain (GO:0022904), cell wall lignin metabolic and
362 catabolic process (GO:0009808, GO:0046274), oxidation-reduction process (GO:0055114), and
363 cellular modified amino acid catabolic process (GO:0042219). In contrast, the up-regulated GO
364 terms in its stipule were related to regulation of transcription (GO:0006355), DNA repair
365 (GO:0006281), and response to hormone (GO:0009725).

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366 PR11-90 (heat susceptible) had none and two GOs significantly up-regulated in anthers and
367 stipules, respectively; whereas four and 39 GO terms were down-regulated in anther and stipule.
368 The two up-regulated terms corresponded with response to water (GO:0009415). The four anther
369 down-regulated terms were associated with amide transport (GO:0042886), oligopeptide
370 transport (GO:0006857) and oxidation-reduction process. And the 39 GOs with down-regulation
371 in stipule included 19 terms in the cluster of nucleosome assembly (GO:0006334), two terms in
372 microtubule-based movement (GO:0007018) and response to auxin (GO:0009733) that were
373 only over-representative in PR11-90. These distinctly heat prohibited processes in PR11-90 were
374 predicted to link with its heat susceptible property.

375 In both anther and stipule transcriptomes of CDC Amarillo, the number of down-regulated GO
376 terms was also greater than that of GO terms with up-regulation (29/12
377 downregulation/upregulation in anther; 46/9 downregulation/upregulation in stipule) and had the
378 highest total number of GOs in both anthers and stipules among the three pea varieties. This
379 differential of heat responsive GO among the three varieties demonstrated the genetic variation
380 of field pea in heat response and shed a light in deciphering molecular mechanism involved in
381 pea heat response and heat tolerance. In anthers, the significantly enriched GO terms in
382 transcriptionally inhibited genes consisted of many GOs in the cluster ATP biosynthetic
383 (GO:0006754) and metabolic (GO:0046034) process, which was uniquely observed in CDC
384 Amarillo. The 12 enriched terms of genes, whose expression was induced, were associated with
385 rRNA processing (GO:0006364), response to zinc ion (GO:0010043), electron transport chain
386 (GO:0022900) and carbon utilization (GO:0015976). rRNA processing was also up-regulated in
387 stipule in addition to cellular response to DNA damage stimulus (GO:0006974) and protein
388 folding (GO:0006457). The stipule down-regulated GO terms were mainly linked with amino

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389 acid transport (GO:00068650), cell wall polysaccharide metabolic process (GO:0010383), lignin
390 metabolic and catabolic process, lipid transport (GO:0006869), lipid localization (GO:0010876),
391 lipid metabolic process (GO:0006629) and protein phosphorylation (GO:0006468).

392 In stipules, 7 GO terms were down-regulated in all three varieties and were involved in apoptotic
393 process (GO:0006915), defense response (GO:0006952) cell wall macromolecule metabolic
394 process (GO:0044036) and polysaccharide metabolic process (GO:0005976). Cell wall
395 polysaccharide metabolic process, plant-type cell wall organization (GO:0009664),
396 photosynthesis, light harvesting (GO:0009765), amine transport (GO:0015837) and aminoglycan
397 metabolic process (GO:0006022) were down-regulated in PR11-90 and CDC Amarillo. Lipid
398 transport and localization were down-regulated in PR11-2 and CDC Amarillo. It is noted that
399 cellular response to stress (GO:0033554) and cellular response to DNA damage stimulus
400 (GO:0006974) was only up-regulated in the stipules of two heat-tolerant varieties, PR11-2 and
401 CDC Amarillo. Comparing the between the two varieties, transcripts of four genome loci were
402 common, which were Psat2g148040, Psat5g135640, Psat6g105320 and Psat6g199840. In
403 anthers, generation of precursor metabolites and energy (GO:0006091) and electron transport
404 chain (GO:0022900) were only up-regulated in PR11-2 and CDC Amarillo as well, and the
405 transcriptional level of three relating genes (Psat1g132320, Psat1g132440, and Psat6g041400)
406 was induced in both varieties. Interestingly, contrasting response was observed in oxidation-
407 reduction process (GO:0055114) among the anthers' transcriptomes of the three varieties. This
408 biological process was enriched in the up-regulated genes in PR11-2, but significant in the down-
409 regulated genes in PR11-90 and were over-representative in both down/up-regulated genes in
410 CDC Amarillo. Among these genes, 8 genes were commonly up-regulated in PR11-2 and CDC
411 Amarillo, 28 genes were down-regulated in CDC Amarillo and PR11-90 (S2 Table).

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412 **Fig 6. Enriched GO terms in biological processes of variety distinct DEGs in anthers and**
413 **stipules. Red color are up-regulated processes, and blue color is down-regulated processes.**
414 **More intense color means greater significance.** Up-regulated biological process is colored in
415 red, red scale for significance p value is shown as follows, 0.01≤p<0.001
416 0.001≤p<0.0001 ≤0.0001. Down-regulated biological process is colored in blue, blue
417 scale for significance p value is shown as follows, 0.01≤p<0.001,
418 0.001≤p<0.0001, ≤0.0001.

419 Discussion

420 General and genotype specific heat response at cellular level

421 Separate heat responsive genes of individual variety were identified at log2 FC >2 for anther and
422 stipules, and two heat tolerant varieties in our study demonstrated different transcriptomic
423 response, i.e., PR11-2 had the lowest number of DEGs among the three varieties, contrastingly
424 DEG number in CDC Amarillo was the greatest. This was also seen in maize, where tolerant
425 cultivar S058 and L043 had the most and least abundant DEGs among four tolerant and four
426 susceptible varieties, respectively [16]. Collectively, it is suggested that plant heat tolerance
427 could be achieve in different mechanism.

428 Individual GO enrichment analysis was carried out on common DEGs among the three varieties
429 and variety unique DEGs, aiming to characterize the general heat response in biological
430 processes of pea plant as well as unique responses relating to heat tolerance. Response to heat
431 (GO:0009408), protein folding (GO: 0006457) and transcription, DNA-templated (GO:0006351
432 and GO:0051252) were commonly upregulated between stipules and anthers (Fig 4). The

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433 transcriptome re-program and chaperone function of HSPs are considered to contribute to plant's
434 basal thermo-tolerance [41]. Regressed biological processes were mainly related to lipid
435 transport, lipid metabolic process and cell wall macromolecule metabolic process, and their
436 relevance to HS and relating genes are further discussed in latter section.

437 Regarding variety unique heat response, anther of PR11-2 had only up-regulated processes,
438 belonging to three biological process clusters, i.e., respiratory electron transport chain, lignin
439 catabolic process and cellular modified amino acid catabolic process (Fig 6). PR11-90 had none
440 induced biological process in anther. This could partly explain heat tolerance of PR11-2 over
441 PR11-90. Intriguingly, electron transport chain (ETC) was also upregulated in CDC Amarillo. In
442 electron transport chain, Psat1g132320 and 6g041400 encoding mitochondrial cytochrome b and
443 Psat1g132440 encoding uncharacterized protein were upregulated. Cytochrome b-c1 complex is
444 an essential component of the mitochondrial ETC. Chilling induced accumulation of reactive
445 oxygen species resulting from over-reduction of ETC led to oxidative stress [42].

446 In stipules, cellular response to DNA damage stimulus was only induced in two heat tolerant
447 varieties. Four genes were common between gene lists of the two varieties, which were
448 2g148040 (DNA mismatch repair protein MLH3), 5g135640 (DNA excision repair protein),
449 6g105320 (cryptochrome 2b), and 6g199840 (DNA mismatch repair protein MSH3). The
450 putative functions of the four genes were involved with three DNA repair pathways, but these
451 pathways were well studied in UV light induced stress [43]. Elucidation on the connection of the
452 plant DNA repair to abiotic stress responses remains scarce, plant's ability to maintain its
453 genome integrity is likely to play a role in stress tolerance [44].

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454 **Regulatory importance of HSFA3 and HSFB2 in heat response**

455 Although HSFs are believed to play a central regulation role in the transcriptional induction of
456 downstream HS responsive genes, HSFs display their variation in HS response in terms of
457 induction fold threshold and regulation, and thereby could affect various gene expression
458 induction. Structurally plant HSFs are classified into three classes, namely, HSF A, B, and C,
459 based on their structural peculiarities. The best characterized HSF gene family in plants has been
460 firstly reported in Arabidopsis (21 HSF genes) [9]. Wheat (56 HSF genes) [45]; and soybean (52
461 HSF genes) [46] were reported to have the largest families in monocot and dicot crops,
462 respectively. Among the three classes, the function of HSFAs was more clearly elucidated, and
463 here is broad agreement that their role most directly leads to heat-induced activation of heat
464 shock genes. HSFA1s are predicted to be the "master regulators" that have the direct role in the
465 activation of transcriptional networks. Knockdown of HSFA1 genes in Arabidopsis led to a
466 reduced induction of many HS-responsive genes, as a result plants demonstrated HS susceptible
467 phenotypes [47, 48]. The thermo-tolerance conferred by Arabidopsis HSFA1d was further
468 confirmed in a recent study in pea [49], where transformant pea plants with this Arabidopsis HSF
469 was more heat tolerant than its wild type due to the increased antioxidant enzyme activity and
470 reduced hydrogen per oxide. Another study in Arabidopsis concluded that HSFA3 was also an
471 important HS-responsive TF, because knockout or knockdown mutation of HSFA3 resulted in
472 reduced expression of putative target HSP genes during HS [50]. OsHSFA3 and A2s were
473 identified to responsive in rice panicle when exposed to multiple hours of HS [51]. In
474 comparison, in common wheat (*Triticum aestivum* L.), HSFA2 and A6 had the highest
475 transcriptional induction among 56 TaHSF members when subjected to HS, which revealed the
476 regulatory importance of these two subclasses during HS [45]. Among legume plants, over-

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477 expression of soybean *GmHSFA1* could enhance the thermotolerance of transgenic soybeans via
478 the activation of various HSP gene expression [52]. In the other study, the induction of GmHSFs
479 at HS was found to variate at different plant stages, including HSFA1 [53]. In *Lotus japonicus*,
480 HSFA1 did not dominantly express in heat-stressed seedlings, A2, A3, A6, A7, B2 and B5 were
481 exclusively heat induced and other hsf subclasses could also be involved in other abiotic stress
482 responses [54]. Q-PCR expression analysis of chickpea HSFs under heat stress at pod
483 development and at 15 days old seedling stage showed that CarHSFA2, A6, and B2 were
484 constitutively up-regulated at both plant development stages indicating their importance in the
485 regulatory network relative to HS [55]. In the present study, various transcripts of putative pea
486 HSFs were characterized that were responsive to 3h heat treatment, among which putative HSFA
487 stood out in its amount abundance, the A3 subclass in particular. Three HSFA transcripts
488 (Psat3g061600, Psat5g036400 and Psat6g059040) were highlighted because their transcriptional
489 levels were dominantly increased in both anthers and stipules in all three varieties (Table 4),
490 suggesting they are essential transcriptional regulators in pea HS response. Further analysis on
491 knock-out mutants of these HSF genes will validate their exact role, whether directly or not, in
492 heat regulation. Interesting, individual HSF were identified for anthers and stipules, indicating
493 different regulatory networks may exist between vegetative and reproductive organs.

494 Functions and molecular mechanism of HSFBs were less elucidated, but they were found to
495 interact closely with HSFA in plant's HS response. The role of HSFBs were reported either as a
496 repressor or activator in the transcription of HSFA depending on plant species, as a result, they
497 participated in different mechanisms in HS regulation. In *A. thaliana*, HSFB suppressed the
498 transcriptional activities of HS-inducible HSFs, including HSFA2, A7a, at both normal
499 temperature environment and HS condition [56]. On the contrary, the function of tomato's

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500 HSFB1 seemed more complex, it could work either as a co-activator of some HSFs e.g.,
501 HSFA1a or as a transcription repressor of other HSFs such as HSFA1b and HSFA2([57-59]. In
502 our result, transcription levels of two putative HSFB2 genes (Psat1g102600 and Psat6g200480)
503 were highly heat induced along with HSFA genes independent of organ types and genotypes,
504 implying their positive role in transcriptional regulation of field pea in HS, which was in
505 agreement with the finding in chickpea [55]. It seemed that the role of HSFB in legume crops
506 was similar to the coactivator characteristics of tomato HSFB.

507 Transcriptional induction of various pea sHSPs and HSP70 at HS

508 In plant cellular defense against HS, the induction of HSP is one of the major responses. HSPs
509 act as molecular chaperones which are proteins that facilitate folding of other functional proteins
510 especially at the secondary and tertiary structure and prevent them from denaturation and
511 aggregation during exposure to HS. Depending on the molecular size, HSPs are divided into five
512 conserved classes: small HSPs (sHSPs), HSP60, HSP70, HSP90 and HSP100.

513 sHSPs range in size from 10 to 42 kDa and share a conserved C-terminal domain that is common
514 to all eukaryotic organisms. Generally, sHSP functions as a molecular chaperone and protects the
515 substrate proteins against thermal aggregation or denaturation. In six legume species, more than
516 5 different sHSPs were detected from plant tissues exposed to HS [60]. In pea, several sHSPs
517 belonging to two classes based on their sequence alignment and immunological cross-reactivity
518 were isolated. *PsHSP 17.7, 17.9, 18.1* were located in the cytoplasm, whereas *PsHSP 21 and*
519 *PsHSP 22* were located in chloroplasts and mitochondria, respectively ([19, 20, 61]. From these
520 reports, we could conclude that they were all involved in establishing cellular thermotolerance to
521 some degree, though the induction of their expression was triggered at different temperatures.

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522 Transcriptome profiling in our experiment revealed that the transcriptional levels of cytoplasmic
523 sHSPs were drastically increased at HS among the three pea varieties (Table 4), which was in
524 agreement with the above-mentioned result on other pea genotypes, suggesting the function of
525 these sHSPs is general in field pea plant. Beyond that, transcriptional response of other sHSPs in
526 relation to HS were also characterized, which provides a more comprehensive picture of sHSP
527 heat response in pea.

528 HSP70 proteins have also been extensively studied; they are ATP-driven molecular chaperones
529 with an N-terminal ATPase domain and a C-terminal peptide binding domain. Similar to the
530 gene family encoding sHSPs, HSP70 genes also encode proteins targeted to different cellular
531 compartments, including mitochondria, chloroplast, endoplasmic reticulum, and the cytoplasm.
532 Similarly, HSPs isolated in pea differed in their expression under different temperature
533 environments, inferring functional differences between heat-induced and constitutively
534 expressed HSP 70 homologues. In our study we confirmed the significance of various HSP70
535 genes in field pea heat response.

536 HS response in pea cell wall

537 Various biological processes relating to cell wall were significantly down-regulated when
538 exposed to HS in our study, which helped decipher the molecular mechanism of heat damage on
539 pea cell wall (Fig 5 & 6). Similar in heat stressed lentil, a major group of heat responsive genes
540 were involved in plasma membrane and cell wall [18].

541 Plant cell walls have multiple layers and are made up of three sections, i.e., the middle lamella,
542 primary cell wall, and secondary cell wall. The primary wall surrounds growing cells or cells
543 capable of cell growth and its plasticity is essential for cell expansion and growth; whereas the

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544 secondary wall is a highly specialized and thickened structure to provide the sufficient rigidity,
545 which undergoes irreversible changes in many fully developed cells. The middle lamella is a
546 pectin layer to provide necessary adhesive between two adjoining cells [62]. Pectin, a mixture of
547 polysaccharides, is also a major composition in primary cell wall, especially in dicotyledonous
548 plants [63]. In addition to its adhesive property, adjustment of its content in cell wall is proposed
549 to link with various physiological function during plant life cycle as well as contribute to signal
550 transduction to various conditions. Reproductive tissues are particularly rich in pectin compared
551 with other tissues, for example pectin constituted ~40% and 15% in rice pistil and anther cell
552 wall, respectively, whereas the proportion of pectin was only 5% in the cell wall of mature leaf
553 [64]. Transcriptome comparison of this study between HS and normal temperature characterized
554 a cluster of genes encoding pectin esterase (enzymes for pectin metabolism), only heat
555 responsive in anthers of all three varieties, not in stipule, and it is proposed to be associated with
556 contrasting pectin composition between reproductive organ and vegetative plant organ. The
557 reduced expression of pectin methyl esterase (PME; EC 3.1.1.11) genes under HS was consistent
558 with the finding in canola [17]. Intriguingly, recent studies in pea aluminum stress and cold
559 stress suggested that the degree of pectin methyl-esterification and PME activity could also play
560 a role in both abiotic stresses [65, 66]. Still, the stress effect on the architecture of cell wall
561 remodeling by PME activity may depend on the plant species, genotype, and growth stage, and
562 also rely on the intensity and timing of the stress [62].

563 Lignin is a major composition in secondary cell wall and provides cell structural rigidity. Its
564 biosynthesis consists of a very complicated network, where cinnamyl alcohol dehydrogenase
565 (CAD), laccase (LAC) and peroxidase are involved. In *A.thaliana*, CAD function defective
566 mutant displayed inhibited plant and male sterile compared with wild type, likely attributed to

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567 the abnormally reduced lignin biosynthesis in the anther [67]. Likewise, CAD1 mutant of *M.*
568 *truncatula* had a much lower lignin content than the wild type, though causing no growth
569 difference between two materials at normal temperature environment (22°C), the growth of this
570 *MtCAD1* mutant was significantly suppressed at 30°C [68]. In our study, lignin metabolic and
571 catabolic process was identified to be uniquely up-regulated in the anther's transcriptome of heat
572 tolerant variety, PR11-2, when exposed to HS (Fig 6). The genes in this process were identified
573 to be LAC encoding genes on pea chromosome II, III, V and VII, which are predicted to be
574 associated with heat tolerance. In Anadiplosis, functions of LAC 1, 4 and 17 were linked with
575 anther dehiscence success [69]. A QTL was identified for HS susceptibility index of percent
576 spikelet sterility in rice on chromosome XII, and one LAC gene was included in this QTL
577 interval [70].

578 Effects of HS on Lipid Transport and Metabolism

579 HS in our study adversely affected lipid transport and localization in both pea anther and stipule
580 independent of genotypes (Fig 5). The lipid process was inhibited mainly via the down-
581 regulation of various transcripts encoding non-specific lipid transfer proteins (LTPs; Table 5).
582 Plant LTPs are broadly categorized into LTP1 and LTP2 groups based on the molecular weighs.
583 LTP1s generally consist of 90 amino acids, whereas LTP2s have around 70 amino acids.
584 Although the biological functions of LTP have not been clear yet, previous studies suggested that
585 LTPs genes can be divided into three groups depending on expression patterns of the related
586 genes, that is, 1) genes only expressed in aerial plant parts; 2) genes only expressed in root; and
587 3) genes whose expression was restricted in reproductive tissues [71]. Our results added another
588 piece of evidence to support tissue-specific expression of LTP genes, because different
589 transcripts of LTP genes were characterized between field pea anther and stipule at normal

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590 development as well as at HS condition. Except that the two genes encoding *PsLTP1* & *2*,
591 previously isolated in pea seeds [72], were heat responsive in both plant samples, other
592 corresponding genes variated. To the authors' knowledge, our work is the first to report the link
593 between LTP genes with pea normal plant development and heat response, and their biological
594 functions are worth being validated via mutation experiment. In wheat, *LTP3* accumulation was
595 detected in cell membrane after HS at plant seedling and grain-filling stages, what's more, in
596 transgenic Arabidopsis seedling with the overexpression of *TaLTP3* was better tolerant to HS
597 than control plants, possibly because of a less membrane injury [73].

598 In addition, the lipid metabolic process was negatively damaged by HS in both anther and stipule
599 among all three pea varieties (Fig 5), which was also seen in rice heat stressed anther [15]. The
600 damage was mostly due to that the transcriptional activity of multiple genes associated with
601 GDSL lipase were adversely affected, although GDSL gene family was differentially expressed
602 between anther and stipule (Table 5). Studies in this aspect are scarce in legume including pea. In
603 the model plant *A. thaliana*, GDSL lipase gene has a family of 108 gene members, which are
604 distributed across plant genome [74, 75]. Among them, 20 members were expressed in all
605 tissues, and the other 16 and five members were exclusively expressed in flower and root,
606 respectively. Mayfield et al. (2001) reported one GDSL lipase to be involved in the formation of
607 pollen coat [76]. With the advance in omics technology, the integration of lipidome and
608 transcriptome provides a new perspective of studying HS as shown in [77]Higashi et al. (2015).

609 Coincidence of Heat Responsive Genes among Field Pea Studies

610 Attempts in genomic understanding of pea HS and selecting for heat tolerant varieties have
611 started since last decade ago, benefiting from the rapid advancement in sequencing technology.

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612 However, results from individual research can not all be compared because the types of genetic
613 markers applied were various. Our characterized heat responsive genes can be compared with a
614 recent association mapping study[8] by Tafesse et al. (2020), as pea genome locus markers were
615 used in their work. Twelve DEGs in our study coincided with putative candidate genes for heat
616 responsive trait characterized in the field condition from their work (S3 Table). The response of
617 these 12 genes fell into three patterns: 1) responsive in all tissue types among the three varieties
618 (e.g. Psat5g303760 encoding uncharacterized protein); 2) specifically responsive to tissue type
619 (e.g. Psat2g144160 encoding pectin acetylesterase); 3) only responsive in certain genotype (e.g.
620 Psat2g166520 encoding putative rapid alkalinization factor). Further functional annotation of
621 individual gene would benefit to explicit its role in HS response.

622 Conclusions

623 Our research profiles a global transcriptome response to short term HS among different field pea
624 varieties. Common effects of HS in biological processes are shared between anthers
625 (reproductive organ) and stipules on the same flowering node (vegetative organ), though the
626 involved genes in certain processes differed between the two organs (e.g., lipid transport and
627 metabolic process). Distinct heat responses were characterized on individual pea varieties, which
628 provides insight into molecular mechanisms of heat-tolerance response. This research supports
629 the utilization of RNA-Seq for the identification of heat responsive genes, provides preliminary
630 result for marker assisted selection, and is proposed to be applicable in other abiotic stress
631 studies of pea.

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844

845 # Supporting information

846 **S1 Table. Over-representative GO terms with significance p value in the anther's**

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847 **consistently up-regulated genes among three pea varieties.**

848

849 **S2 Table. Genes in GO:0055114, oxidation-reduction process was upregulated in PR11-2**
850 **and CDC Amarillo or down-regulated in PR11-90 and CDC Amarillo.**

851

852 **S3 Table. Overlapping of heat responsive genes between our study and Tafesse et al. (2020).**

853 Note: Trait names, SPAD: Soil plant analysis development meter for the estimation of leaf
854 chlorophyll concentration, CT: canopy temperature, RSL: reproductive, PN: pod number. Red
855 cell represents up-regulated gene expression at HS in our study, whereas blue cell represents a
856 down-regulation.

857 **S1 Fig. qPCR primer efficiency standard curves.**

858

Locus ID	gene function	stipule						anther					
		PR11-2		PR11-90		CDC Amarillo		PR11-2		PR11-90		CDC Amarillo	
		RNA-Seq	qPCR	RNA-Seq	qPCR	RNA-Seq	qPCR	RNA-Seq	qPCR	RNA-Seq	qPCR	RNA-Seq	qPCR
Psat0s1067g0080	protein dimerization activity												
Psat0s3930g0040	HSP70-like_chaperone												
Psat2g036360	HSP20-like_chaperone												
Psat3g186840	protein/chaperone binding												
Psat4g021840	negative regulation of catalytic activity												
Psat4g047480	protein metabolic process/ ATP binding												
Psat4g175760	macromolecule metabolic process												
Psat4g206200	protein binding/signal transduction												
Psat5g006560	organ specific protein												
Psat6g021800	HSP20-like_chaperone												
Psat6g175400	carbohydrate binding												

log2 FC
-6 - 10

Fig 1

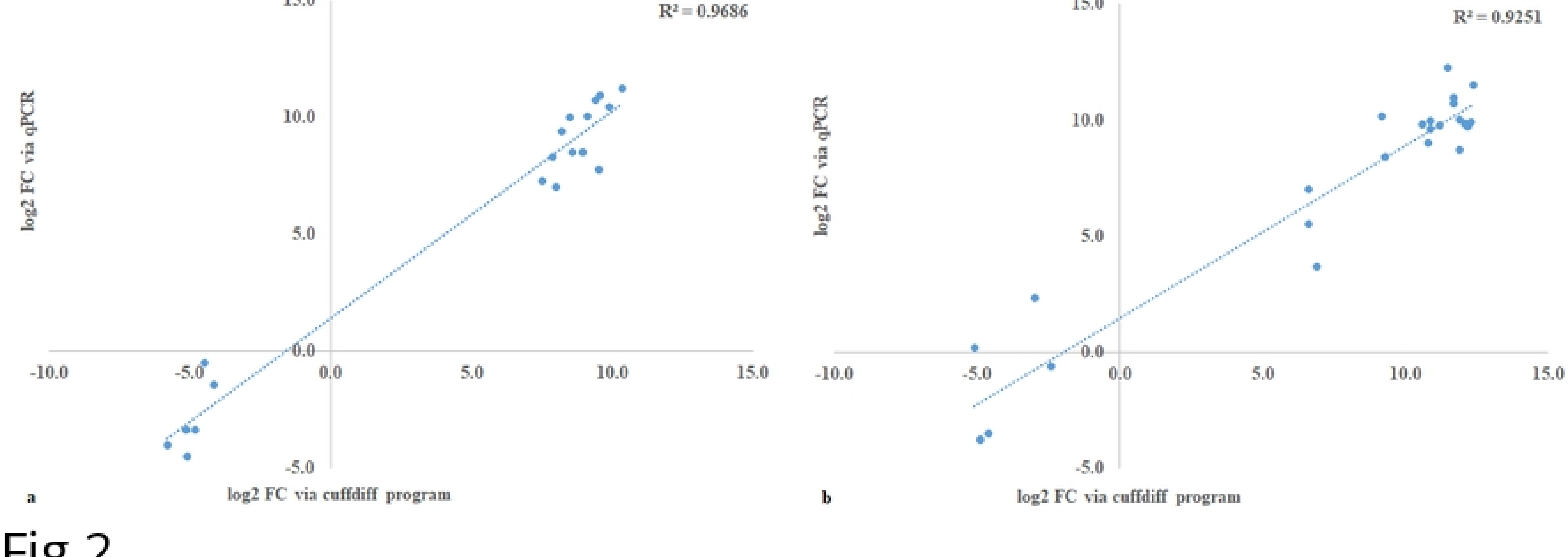

Fig 2

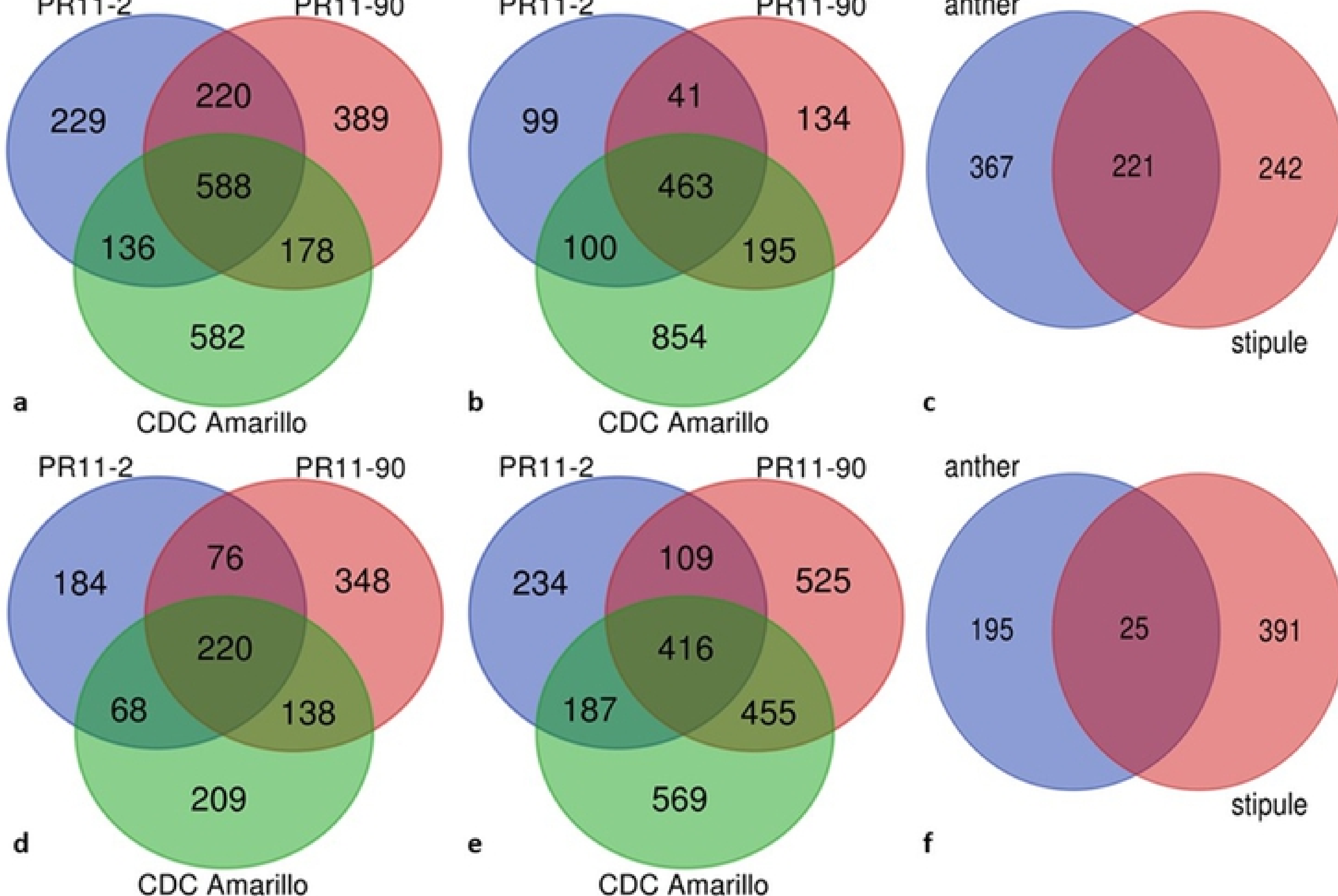

Fig 3

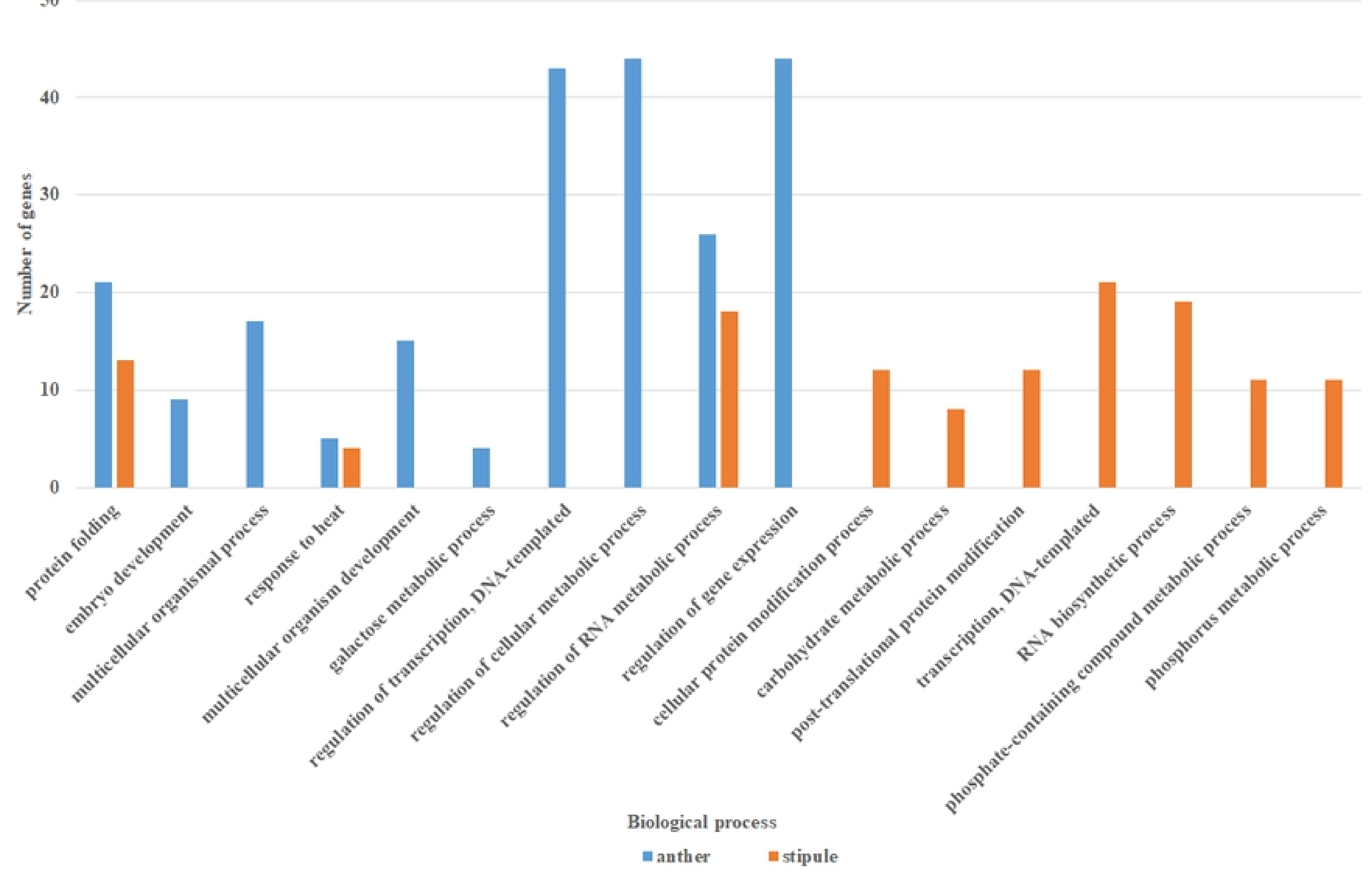

Fig 4

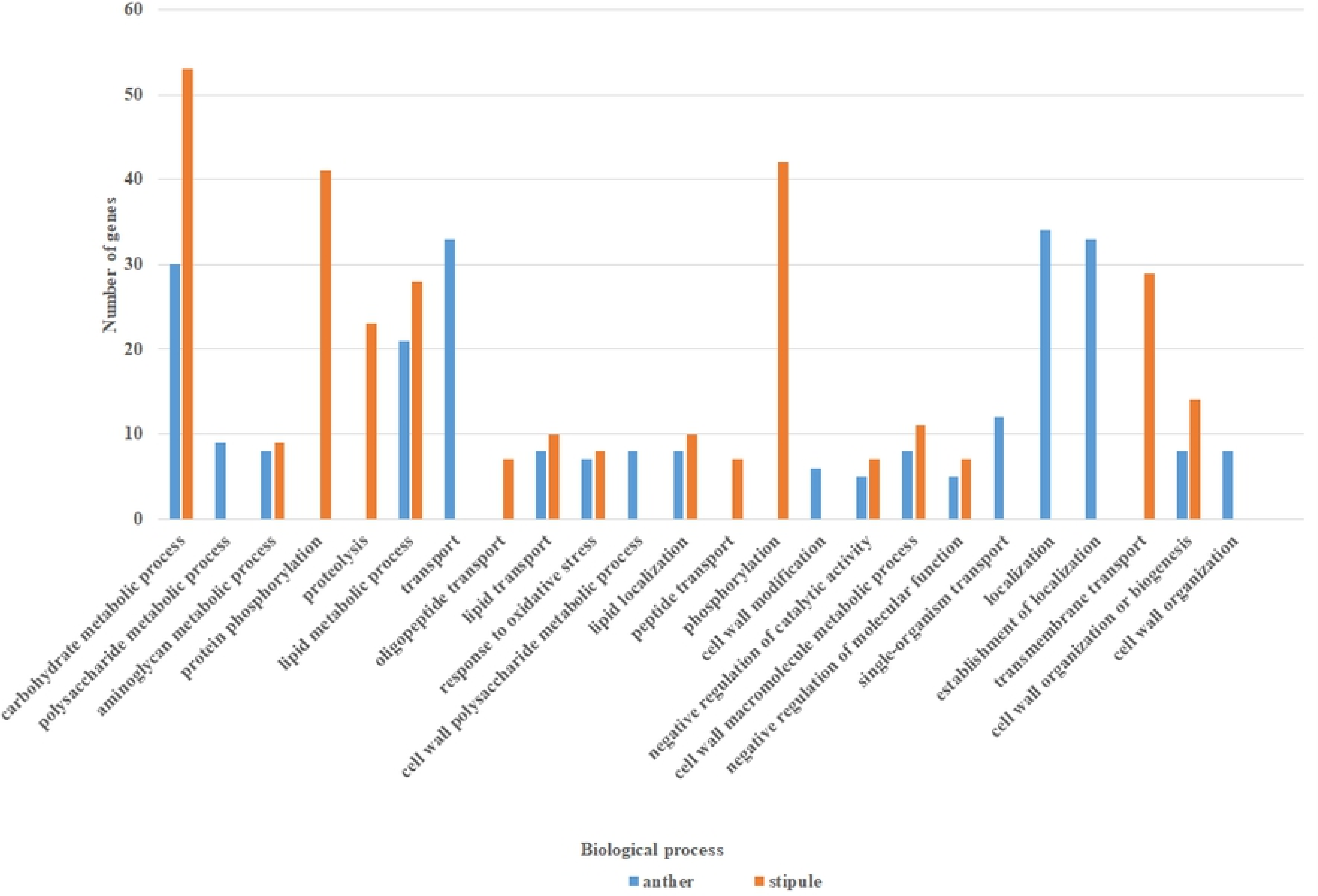

Fig 5

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Fig 6