bioRxiv preprint doi: https://doi.org/10.1101/627117; this version posted May 3, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

1 # Comparative analysis of the Accelerated Aged seed transcriptome
2 # profiles of maize CSSLs (I178 and X178)

3

4 Li Li[1], Feng Wang[1], Xuhui Li[1], Yixuan Peng[1], Hongwei Zhang[2], Stefan Hey[3], Guoying Wang[2],
5 Jianhua Wang[1*], Riliang Gu [1*]

6

7 [1] Seed Science and Technology Research Center, Beijing Innovation Center for Seed Technology
8 (MOA), Beijing Key Laboratory for Crop Genetic Improvement, College of Agronomy and
9 Biotechnology, China Agricultural University, Beijing 100193, China.
10 [2] Institute of Crop Sciences, Chinese Academy of Agricultural Sciences, Beijing 100081, China
11 [3] Department of Agronomy, Iowa State University, Ames, Iowa 50011-1085, United States of
12 America.
13 *Correspondence: Jianhua Wang (Email: wangjh63@cau.edu.cn) and Riliang Gu (Email:
14 rilianggu@cau.edu.cn; Tel: 86-10-62733853)

15

16 Running title: RNA-Seq analysis of I178 and X178

17

18 ## Abstract:

19 Seed longevity is one of the most essential characters of seed quality. Two Chromosome segment
20 substitution lines (CSSL) I178 and X178 with significant difference on seed longevity were
21 subjected to transcriptome sequencing before (0d-AA) and after five days of accelerated ageing
22 (5d-AA) treatments. Compared to the non-accelerated ageing treatment (0d-AA), 286 and 220
23 differential expressed genes (DEGs) were identified in I178 and X178, respectively Among those,
24 98 DEGs were detected in both I178 and X178 after 5d-AA, Enriched GO terms included cellular
25 components of cell part, intracellular part, organelle and membrane etc., including carbohydrate
26 derivative catabolic process, carbohydrate synthesis, sugar isomerase (SIS) family protein etc.

bioRxiv preprint doi: https://doi.org/10.1101/627117; this version posted May 3, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

Transcriptome analysis of I178 and X178 showed that Alternative splicing (AS) occurs in 63.6% of the expressed genes in all samples. Only 381 genes specifically occurred AS in I178 and X178 after 5d-AA, mostly enriched in nucleotide and nucleoside binding. Combined with the reported QTL mapping result, the DEG and the AS information, 13 DEGs in the mapping intervals and 7 AS-DEGs were potential candidates may directly or indirectly associated to seed ageing.

Keywords: Seeds longevity, Chromosome Segment Substitution Lines (CSSL), Transcriptome sequencing, Accelerated Ageing, Alternative Splicing, qRT-PCR

1. Introduction

Ageing is an inevitable process affecting seed longevity, the length of time a seed remains viable, which is accompanied with a progressive loss of quality or viability over time, and a crucial issue for germplasm conservation and seed marketing **[1]**. Seed longevity depends greatly on seed moisture, relative humidity, oxygen pressure and temperature of storage **[2, 3].** Seed storability is a complex trait, many studies have illustrated the oxidative and mitochondrial damage occurs during seed storage, which are the reasons for loss of longevity, and. During seed storage, the activity of the ascorbate and glutathione (AsA-GSH) cycle is reduced resulting in ROS accumulation **[4, 5],** and carbonylation happened of the proteins in seeds **[6, 7]**. These play important roles in scavenging reactive oxygen species (ROS). High concentration of H_2O_2 and the malondialdehyde (MDA), end-products of lipid peroxidation, were considered as the critical factor in contributing to seed deterioration and influencing seed longevity and vigor **[8-11]**. Moreover, the activity of mitochondria, the organelles that supply energy for seed germination, is

bioRxiv preprint doi: https://doi.org/10.1101/627117; this version posted May 3, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

49 significantly decreased during storage, thereby inhibiting seed germination **[5, 12]**. Thus, the level
50 of antioxidants and the level of energy supply are key factors in the regulation of seed longevity.
51 The genetic architecture of crop seed longevity is still far behind the model plant Arabidopsis.
52 Recently, various omic approaches have been used to investigate protein expression patterns
53 during seed storage **[13, 3, 14, 15, 7, 16]**. Sano et al performed an RNA-seq experiment on bulked
54 RILs of Est-1 × Col-0 in Arabidopsis and found that brassinosteroid is important for seed
55 longevity by regulating the seed coat permeability by BR signaling pathway **[13]**. Lv et al
56 conducted proteomic analysis on artificially aged wheat seeds; differentially expressed proteins
57 (DEPs) were mainly involved in metabolism, energy supply, and defense/stress responses.
58 Up-regulated proteins were mainly enriched in ribosome, whereas the down-regulated proteins
59 were mainly accumulated in energy supply, starch and sucrose metabolism and stress defense
60 (ascorbate and aldarate metabolism), revealed that the inability to protect against ageing leads to
61 the incremental decomposition of the stored substance, impairment of metabolism and energy
62 supply, and ultimately resulted in seed deterioration **[14]**. Yin et al performed proteomic analysis
63 on rice embryo with different days of ageing, by comparison analysis, they found most of down
64 regulated proteins were related to energy metabolism (29%), defense (21%), glycolytic pathway
65 (8%), protein synthesis (8%), protein destination and storage (6%), transcription (5%), growth or
66 division (4%), secondary metabolism (3%), transporting (1%), signal transduction (1%). While
67 most of the upregulated proteins were related to storage, energy, disease and defense, metabolism,
68 protein synthesis, growth and division **[7]**. Chen et al compared the storage ability of two wheat
69 varieties (storage tolerant vs. storage sensitive), most of the proteins remarkably different from
70 two varieties were mainly associated with disease or defense, protein destination and storage,

bioRxiv preprint doi: https://doi.org/10.1101/627117; this version posted May 3, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

71 energy, also the storage tolerant seeds possessed a stronger ability in activating the defense system
72 against oxidative damage, utilizing storage proteins for germination, and maintaining energy
73 metabolism for ATP supply **[16]**. While seed longevity is different between species, it also differs
74 between genotypes of a species, the genetic information that involving in seed longevity was far
75 beyond the understood. Also the expression of genes and proteins related to disease defense and
76 energy is largely altered during seed storage. However, the effect of such changes on ageing
77 tolerance and sensitivity of seeds is largely unknown.
78
79 In the present study, the simulation of natural seed deterioration by artificial accelerated ageing
80 (AA) treatment by controlled the moisture up to 95% and the relative temperature at 45 ℃. Two
81 chromosome segment substitution lines (CSSL) (X178 and improved 178, I178), with similar
82 genetic background but shows different sensitivity or endurance in terms of ageing process, were
83 subjected the transcriptional expression analysis, differential expressed genes (DEGs) of
84 non-accelerated ageing treated dry seeds (0d-AA) and 5 days accelerated ageing treatment
85 (5d-AA) in two 178 lines were detected and the co-DEGs as well as the genotype-specific DEGs
86 were subjected qRT-PCR validation, followed by alternative splicing analysis to discover genes or
87 pathways that affected during seeds ageing. We were aiming for uncovering ageing related genes
88 by DEG, alternative splicing (AS) and the candidate gene analysis which existed in QTL mapping
89 interval. Consequently, exploring more biology process that been affected by ageing or those
90 processes that influencing the ageing.
91
92 **2. Materials and Methods**Maternal parent X178 was a widely cultivated maize hybrid

bioRxiv preprint doi: https://doi.org/10.1101/627117; this version posted May 3, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

93 Nongda108 (released by China Agricultural University in 2001), an elite line which has better
94 agronomic trait of storability. I178 (Improved X178) was derive from X178 by introgression of
95 chromosome segments for several generations (CSSL), followed by consecutive self-crossing for
96 at least 10 generations.

97 **Accelerated ageing treatment (AA)**

98 The simultaneously fresh harvested (FH) I178 and X178 seeds were surface disinfected with 1%
99 NaClO for 5 min and washed 10 times with sterile-distilled water, balancing the moisture in the
100 room temperature for overnight, followed by accelerated aging treatment of suspending the seeds
101 on metal mesh trays within closed metal boxes (25× 25×14 cm), maintained in an ageing chamber
102 (LHC-150-11, Beijing Luxi Ltd) at the condition of 95% moisture content and 50 °C temperature
103 for 3, 5 and 7 days of different purpose, 3 replications and non-accelerated aged (0d-AA) seeds as
104 the control. The storage condition of harvested seeds was under 10 °C in a cold room.

105 **Protein Quantification and Zein analysis**

106 Samples of I178 and X178 were prepared for zein extraction, including: 1). dry seeds; 2). seeds
107 after 6 hours imbibed water (0d-6h); 3). Seeds germinated 48 hours (0d-48h); 4). seeds after
108 3d-AA; 5). Seeds after 5d-AA; 6). seeds after 7d-AA were prepared as following methods: dry
109 seeds without any treatment, imbibed into water for 6 hours, 48 hours seeds germination was
110 performed with pre-wetted crepe cellulose papers (CCP), and covered with another piece of CCP,
111 rolling into paper rolls and upright in the ziplock bags for 48 hours under 25 ℃. Above samples
112 were powdered in liquid N_2 and 50 mg of powder was used for zein extraction. Removing the
113 lipids with petroleum benzin and dissolving the samples with protein extraction buffer (12.5 mM
114 sodium borate, 2% 2-mercaptoethanol, 1% SDS and pH10), followed by 5 min incubation at

bioRxiv preprint doi: https://doi.org/10.1101/627117; this version posted May 3, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

115 37 °C, centrifuged at 14,800 rpm for 15 minutes, the supernatant contains total protein was
116 incubated with absolute ethyl alcohol for 2 hours. After centrifugation at 14,800 rpm for 15
117 minutes, the supernatant contains zein was dissolved in IPG solution (8 M urea, 220 mM DTT and
118 2% CHAPS) and measured with the BCA protein assay kit (TRANS, Beijing).

119 **Tetrazolium chloride (TTC) staining**

120 By refer to the Tetrazolium staining method (TZ) on soybean (*Glycine max.*) vigor test **[48]**, corn
121 seeds were imbibed with water for 20 hours at room temperature prior to staining, cut the seeds
122 longitudinally through embryo, then staining with 0.1% Tetrazolium chloride solution (TTC,
123 aqueous solution of 2,3,5-triphenyl tetrazolium chloride) for 1 h and washing 3 times before
124 observation.

125 **RNA-seq and qRT-PCR**

126 For RNA-Seq experiment, 100 artificial accelerated aged seeds (5d-AA) were pooled and grinding
127 promptly in liquid nitrogen, 0.1 g of powder was used for isolating the mRNA with the RNAprep
128 pure Plant Kit (Cat#DP432, TIANGEN, Beijing), RNA was quality check the total RNA with the
129 2% agrose gel, high quality RNA was used for RNA-Seq library preparation and sequenced on a
130 Illumina HiSeq2500 platform (Berry Genmics, Beijing). Two biological replications included and
131 the Non-accelerated aged dry seeds (0d-AA) as the control.

132

133 RNA for qRT-PCR experiment was extracted as above procedure, Quality checking the RNA and
134 performed the reverse transcription with the OneScript cDNA Synthesis Kit (Cat#G234, ABM,
135 Canada), primer of the genes was designed with software Primer Premier5.0. The Fast Sybr Green
136 Master Mix (Applied Biosystems, Foster City, CA, USA) was employed, according to the

bioRxiv preprint doi: https://doi.org/10.1101/627117; this version posted May 3, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

137 manufacturer's instructions, in a reaction volume of 10 μl. qRT-PCR was conducted on a ABI
138 Quantstudio™ DX Real-Time PCR system (Applied Biosystems). PCR conditions included initial
139 denaturation for 2 min at 95 ℃, followed by 40 cycles of denaturation at 95 ℃ for 30 s,
140 hybridization at 60 ℃ for 40 s, and elongation at 68 ℃ for 10 s. The actin2 gene was used as an
141 internal control. The $2^{-\Delta\Delta ct}$ method was used to calculate the relative level of gene expression, and
142 the B73 sample served as a control. A relative level of gene expression greater than 1 was
143 considered to indicate up-regulation, and less than 1 indicated down-regulation. All qRT-PCR
144 reactions were performed with the three biological replicates.

145 **Data analysis**

146 For gene expression level in I178 and X178, transcription with FPKM (Fragments per Kilo bases
147 per Million fragments mapped) >0.1 was considered as expressed genes calculated by htseq-count
148 in HTSeq software. To identify genes involving in seeds ageing, the comparison of genes
149 expressed after 0d-AA and 5d-AA was performed in both I178 and X178, DESeq2 was used for
150 differential expression analysis with the Fold Change of 1.5, with adjusted P-value (*q-value)*
151 <0.05 as the threshold value. Venn diagram was performed with online software
152 (http://bioinfogp.cnb.csic.es/tools/venny/index.html) **[49]**. Agri-Go enrichment was also
153 performed with online (Agri GO v2.0; http://systemsbiology.cau.edu.cn/agriGOv2/#) database
154 **[50].** Gene Organ or tissue specific expression level was compared with online q-teller database
155 ((http://www.qteller.com/qteller4/)).

156

157 ## 3. Results

158 ### 3.1 Comparison of seeds storability for I178 and X178

bioRxiv preprint doi: https://doi.org/10.1101/627117; this version posted May 3, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

159 Few morphological and the physical differences were observed between X178 and I178 because
160 of the similar genetic background. Seed storability can be reflected by color of seed coat, seed
161 viability and vigor after long term storage or AA treatment. Seed coat of I178 was obviously
162 oxidized after 5d-AA as the brown color, and the seed viability was reduced dramatically in I178
163 based on triphenyl tetrazolium chloride (TTC) staining, fresh harvested (FH) seeds have highest
164 viability and dehydrogenase activity as the embryo part stained with bright-color while
165 light-colored after 3d-AA and especially no color after 5d-AA **(Fig 1A, B).** FH seeds of two lines
166 showed slight difference of relative conductivity (RC) before 3d-AA, significant difference
167 observed after 5d-AA. After 1-year storage, the RC of I178 was two times higher than X178, as
168 the continued AA treatment, the storability was reduced significantly in I178 after 5d-AA **(Fig
169 1C)**. Comparative SDS-PAGE analysis of seed storage protein (SSP) zein in dry seeds, imbibed
170 seeds (6 hours imbibition), germinated seeds (48 hours germination) and 3, 5 and 7 days
171 accelerated aged seeds (3d-AA, 5d-AA and 7d-AA), and no significant difference was observed
172 between two 178 lines after AA treatment except the degradation of 40 kD protein happened in
173 I178 after 5d-AA, after 7d-AA, the proteins smaller than 25 kD (including γ27, α22, α19, γ16, β15
174 and δ10) were also dramatically degraded in I178 (**Fig 1D**).
175

176 **3.2 Transcriptome profile of I178 and X178 seeds**

177 The cDNA libraries of the non-accelerated aged dry seeds (0d-AA), 5 days of accelerated aged
178 seeds (5d-AA) for I178 and X178 were prepared and sequenced using an Illumina HiSeq 2500
179 platform, 8 Gb data of two biological replicates for each sample were obtained. Reads of low
180 sequencing quality were filtered out (about 0.12%~0.18%), and totally 34.4~43.4 million 100 bp

bioRxiv preprint doi: https://doi.org/10.1101/627117; this version posted May 3, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

181 paired-end reads were generated in I178 and X178, with the average of 40.8 million reads in each
182 sample. At least 97.22% high quality reads used for analysis. Of these reads, average 68.8%
183 unique mapped reads (289.9 million) were aligned to the B73 reference genome to estimate the
184 transcript levels (ZmB73_RefGen_v3). Expression values were calculated with units of fragments
185 per kilo-base per million reads mapped (FPKM). As expected, 92.92% high quality reads can be
186 mapped to protein-coding genes, 1.36% and 5.72% were mapped to intron and intergenic region,
187 respectively **(Table S1)**.
188

189 **3.3 Identification of the DEGs in I178 and X178**

190 Genes with same expression patterns during seed ageing may related to ageing metabolic
191 processes **[17]**. To identify the function of genes, researchers clustered genes with similar
192 expression patterns as clues to study the function of unknown genes **[18]**. The RNA-Seq reads of 8
193 samples for I178 and X178 after 0d-AA and 5d-AA (2 replications) were aligned to the maize
194 reference genome (ZmB73_RefGen_v3), the reads coverage and the correlation between 2
195 replications was relatively high which reflected by the FPKM distribution and pearson correlation
196 as shown in supplementary **(Fig S1)**. The expressed genes in I178 and X178 were 26,909 and
197 26,514, respectively, among that 25,242 common genes (more than 94%) expressed in both 178
198 seeds (FPKM > 0.1). To identify genes involving in seeds ageing, we focused on genes that
199 differential expressed after 5d-AA (compared to 0d-AA), totally 286 and 220 DEGs were detected
200 in I178 and X178, and 98 common DEGs in both I178 and X178 (log2FC $\geq$ 0.585). Only 2
201 common up-regulated and 86 down-regulated genes identified in two 178 (**Fig 2C; Table S2**).
202

bioRxiv preprint doi: https://doi.org/10.1101/627117; this version posted May 3, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

203 **3.4 GO enrichment of DEGs after 5d-AA**

204 GO is an internationally standardized gene function classification system used to describe the
205 properties of genes and their products in any organism, which contains three ontologies: biological
206 process, cellular component and molecular function **[19].** The 286 I178 differential expressed
207 genes were mainly involving in biology process of abiotic response like temperature, salt stress,
208 osmotic stress, light intensity, heat, ethanol, abiotic stimulus, heat acclimation etc., and the
209 molecular function of nutrient reservoir activity, chitin binding and catalytic activity **(Fig S2A).**
210 While for the 220 X178 DEGs were mainly enriched in cellular component of nuclear part,
211 organelle lumen, intracellular part and heterochromatin etc. (**Fig S2B**).
212 Most of the down-regulated genes in I178 were enriched in stimulus response, including response
213 to biotic stress (organism stress, external biotic etc), abiotic stress (inorganic substance stress,
214 chemical, oxygen-containing compound, acid chemical, organic substance, endogenous stimulus,
215 hormone etc), immune response (defense response, innate immune etc.), and most of DEGs in
216 X178 were enriched in DNA or protein biosynthesis process like peptide biosynthesis, amide
217 biosynthesis and translation **(Fig 2A-B; D-E).** The common 98 DEGs in two 178 were mainly
218 enriched in cellular component of cell part (GO:0005623; GO:0044464), membrane-enclosed
219 lumen (GO:0031974), organelle and intracellular organelle (GO:0043226; GO:0044422;
220 GO:0043233; GO:0043229) and intracellular part (GO:0044424; GO:0070013; GO:0044446;
221 GO:0043231), RNA polymerase (GO:0030880; GO:0000428; GO:0055029; GO:0016591),
222 membrane bounded organelle (GO:0043227), transferase complex (GO:0061695) and the nuclear
223 part (GO:0005634; GO:0044428; GO:000319981; GO:0005654), most of DEGs were
224 down-regulated, and enriched in biology process of carbohydrate derivative catabolic and

bioRxiv preprint doi: https://doi.org/10.1101/627117; this version posted May 3, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

225 molecular function of carbohydrate derivative binding **(Fig S2D, E**). Only two common
226 up-regulated genes in I178 and X178, gene GRMZM2G353885, encodes a TATA box binding
227 protein (TBP) associated factor 2, and another gene of no annotation (**Fig S2C**).
228
229 **3.5 Analysis of DEGs by qRT-PCR**
230 According to the RNA-Seq results, 9 genes including 7 randomly selected DEGs and 2 longevity
231 related genes were selected for qRT-PCR validation. The expression patterns of the 7 DEGs were
232 consistent between qRT-PCR and RNA-seq, indicating that the RNA-seq gene expression was
233 reliable, two genes (ZmLOX11 and ZmPIMT1) were not able to detected in RNA-Seq analysis,
234 further qRT-PCR was conducted on I178 and X178 before and after 5d-AA treatment, as
235 expectation, the expression was extremely low, and was consistent to the q-Teller whole
236 transcriptome expression result (http://www.qteller.com/qteller4/) **(Fig 3)**.
237
238 **3.6 Alternative splicing (AS) analysis of ageing related transcriptions and GO enrichment**
239 In I178 and X178, we totally identified 51,388~59,146 AS events, including 12 different types
240 which covered 46,521~ 48,724 transcript isoforms (including 15,984~17,070 genes) **(Table S3)**.
241 Among that, TSS (alternative 5' first exon) and TTS (alternative 3' last exon) account for more
242 than 72% of the total AS events, and IR (Intron retention), AE (Alternative exon) and SKIP
243 (Skipped exon) were also frequently occurred in I178 and X178 (**Fig S3A**). In dry seeds (0d-AA),
244 we detected 56,228 and 53,593 AS events in I178 and X178, respectively, which cover 20,446 and
245 19,623 transcripts in I178 and X178, respectively. After 5d-AA, we detected 55,763 and 56,794
246 AS events in I178 and X178, which cover 20,073 and 19,845 transcripts, respectively **(Fig S3A;**

bioRxiv preprint doi: https://doi.org/10.1101/627117; this version posted May 3, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

247 **Table S3)**. By comparing AS in I178 and X178 before and after 5d-AA, we noticed that 63.6%
248 transcript isoforms (15,606, cover 12,834 genes) occurred AS in all samples. In order to discover
249 that AS genes may involving in seed ageing, we only focus on AS genes that specifically
250 identified in I178 and X178 after 5d-AA: 381 transcript isoforms (including 169 genes) occurred
251 AS in both 178 lines, 849 transcript isoforms (including 415 genes) specifically occurred AS in
252 I178 and 760 transcript isoforms (including 343 genes) specifically occurred AS in X178 after
253 5d-AA (**Fig S3B**). In order to identify the relationship of AS genes and the ageing related
254 procedure, Agri-Go analysis on common AS genes in I178 and X178 showed that the enriched in
255 nucleotide biosynthesis process, function as nucleoside binding **(Fig 4A)**. For those AS genes
256 specifically occurred in I178 and X178 after 5d-AA, beside of the molecular function of
257 ribonucleoside binding, ATP binding etc., some were also enriched in freezing response **(Fig 4B)**.
258 Combine the DEG and the AS gene information in this study, only 6 X178 specific DEGs
259 specifically occurred AS and one I178 specific DEG specifically occurred AS after 5d-AA (**Table
260 1; Fig S3C**).
261

262 ## 4. Discussion

263 ### 4.1 Seed storability was decreased dramatically in I178 after AA treatment

264 I178 was derived from X178 and less polymorphic segments was detected among the
265 chromosomes except chromosomes 7 and 10 by performing 6K maize array chip, the only
266 significant difference between X178 and I178 is the seed vigor which was validated by previous
267 study **[20]**. The electrolytic exudate conductivity of the seed reflects the permeability of the seeds
268 coat, also represent the damage of cellular membrane system, our test of seed vigor of FH seeds

bioRxiv preprint doi: https://doi.org/10.1101/627117; this version posted May 3, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

269 after 3d-AA and 5d-AA was consistent to Liu's result. Obviously, the seeds storability was
270 constantly decreased even stored at relative low temperature, and the ageing level of fresh
271 harvested seeds after 5d-AA was stronger than 1-year storage in cold room **(Fig 1)**. As we know
272 the deterioration exists inevitably, to slow down the loss of longevity, keep the seeds in a lower
273 temperature (possible 4 °C) is a better choice. Seeds storage protein (SSP) have been described as
274 a primary target for oxidation in seeds, zein is the largest component of SSP which account for
275 60% of SSP **[21].** Carbonylation of SSPs during long-term storage was an irreversible form of
276 oxidation leading to deterioration in both after-ripen seeds and aged seeds **[22, 23]**. More studies
277 found that SSPs were degraded during seed germination, which also differential expressed in aged
278 seeds, indicating the role of SSPs in seed longevity **[24, 6, 7, 16]**. In our study, we do observed the
279 degradation of the SSPs (zein) after 48 hours of germination in both I178 and X178. Previous
280 study proved that the oxidative SSPs were more easily degraded into smaller polypeptides or
281 amino acids **[16]**, in our observation, the total amount of zein was decreased significantly after
282 5d-AA in I178 and 7d-AA in both 178 lines, was consistent to above result.

283

284 **4.2 Seed ageing affects numerous biology processes including stress defense and**
285 **carbohydrates metabolisms**

286 Seed ageing is an inevitable procedure occurred on all living things. The consensus molecular
287 mechanism associated to the ageing is including: 1) Peroxidation of plasma membrane and
288 disintegration of membrane system structures. 2) Variation of biomacromolecule, including
289 variation of nucleic acid (RNA and DNA) and enzyme/proteins. 3) The accumulation of toxic
290 substances, i.e., Reactive oxygen species (ROS), malondialdehyde (MDA), and the by-products of

bioRxiv preprint doi: https://doi.org/10.1101/627117; this version posted May 3, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

291 seeds physiological activity, organic substance like alcohols, free fatty acids etc. **[25, 16]**. In our
292 RNA-Seq analysis 5d-AA treated 178 lines, 98 common DEGs were identified in both 178 seeds,
293 while only 88 genes have same expression pattern in I178 and X178, 86 of which were
294 down-regulated and enriched in carbohydrate derivative catabolic process **(Fig S2E)**.
295 Interestingly, Lv et al revealed that the proteins in carbohydrate derivative biological pathway
296 were up-regulated in aged wheat seed especially the up-regulation of genes in amide biosynthesis
297 pathway, and the genes in defense- and stress response were down regulated after ageing **[14]**. In
298 our study, most of the DEGs (including up- and down-regulated genes) were enriched in defense-
299 and stress pathways in I178 after ageing. The inconsistency between two studies can be explained
300 by the different ageing level of the wheat seeds and maize seeds, in Lv's study, the germination
301 rate of aged wheat was lower than 20% while based on Liu's result, the GR of 5d-AA treated I178
302 and X178 was around 20% and 80%, respectively [20]. We do observed a enrichment of
303 up-regulated genes in carbohydrate catabolic process in I178 (GO:0016052), while
304 down-regulated in X178, i.e., GRMZM2G176307, which encodes a glyceraldehyde-3-phosphate
305 dehydrogenase C2. In Xin's proteome analysis on maize, they mentioned that the carbohydrates
306 utilization was important in seed ageing and seed vigor, was been influenced in aged seeds **[4]**,
307 which was also consistent to our results. The X178 down-regulated genes were enriched in amide
308 and peptide biosynthesis after 5d-AA **(Fig 2E)**, which was inconsistent with Lv's result, it was
309 possible that X178 possess a better resistant to ageing, after 5d-AA treatment of, the DNA and
310 protein repair systems was slightly affected which reflected by the down-regulation of ageing
311 affected genes, while as the constant ageing treatment once GR was below 20%, some of the stress
312 response genes will be activated and up-regulated to coping with DNA and protein damage.

bioRxiv preprint doi: https://doi.org/10.1101/627117; this version posted May 3, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

313

314 **4.3 ZmPIMT1 and LOX11 were down-regulated after 5d-AA**

315 To validate the RNAseq data, qRT-PCR was performed on 9 of randomly selected genes may
316 related to ageing and consistent results were obtained on both platforms. Protein-L-isoaspartyl
317 (d-aspartyl) O-methyltransferase (PIMT), a typical protein repair methyltransferase related to seed
318 longevity by recognizes isoAsp residues in proteins or peptides and catalyzes the transfer of a
319 methyl group from S-adenosyl methionine (AdoMet) to the free a-carboxyl group of abnormal
320 L-isoAsp residues (as well as the b-carboxyl group of D-aspartyl residues) **[26, 27].** There are two
321 PIMT orthologues in Arabidopsis and maize, in this study, ZmPIMT2 share 65% sequence
322 identity with the AtPIMT1, was not affected in both I178 and X178, ZmPIMT1 share 71%
323 sequence identity to AtPIMT1, was not been detected in RNA-Seq experiment. qRT-PCR of
324 PIMT1 showed that ZmPIMT1 was almost no expression signal in 178 seeds in all samples (**Fig**
325 **3)**, indicating that the spatio-temporal expression specificity of PIMT1 in dicotyledon and.
326 Monocotyledon plants. Lipoxygenase (LOX) was also a typical longevity related protein which
327 associated to the lipid oxidation in seed or other tissue **[28]**. There are 13 ZmLOX gene have been
328 identified in maize so far, but few of them have been cloned or further studies in molecular
329 biology level **[27]**. In Arabidopsis. LOX2 is essential for formation of green leaf volatiles and
330 five-carbon volatiles **[29]**, the homolog ZmLOX11 in this study was no expression in RNA-Seq as
331 well, which is normal since the gene expression in q-Teller showed that this gene was only highly
332 expressed in the young seeds while little expression observed in mature seed **(Fig S4)**. qRT-PCR
333 showed that ZmLOX11 was down-regulated in I178, while up-regulated in X178 after 5d-AA
334 treatment, a possibility that LOX11 was not specific expressed in seeds, or the spatiotemporal

bioRxiv preprint doi: https://doi.org/10.1101/627117; this version posted May 3, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

335 specificity of the LOX11 in tissue except the seeds **(Fig 3)**.

336

337 **4.4 Identify genes potentially associated to seed longevity**

338 Numerous studies reported that the seed longevity genes may involve in switching off metabolic
339 activity in seeds, repair systems during seed imbibition and DNA, RNA or protein repair systems
340 **[25]**. In previous study, QTL mapping of seeds ageing traits on RILs and $F_{2:3}$ populations of I178
341 × X178, 17 QTL were identified on 5 chromosomes **[20]**. In order to excavating genes that
342 involving in seed longevity, DEGs in QTL mapping intervals for both I178 and X178 were
343 selected for analysis, 13 DEGs located in the mapped QTL of chromosome 3 (11 genes) and
344 chromosome 5 (2 genes), for the 10 DEGs with explicated annotations, DEG4 encodes a
345 peroxisomal ABC transporter 1, previous study showed that the peroxisomal ABC transporter in
346 plant was essential for transporting hydrophobic fatty acids and large cofactor molecules (carrier
347 for ATP, NAD and CoA), and play an indispensable role in pathways like fatty acid β-oxidation,
348 photorespiration, and degradation of reactive oxygen species **[30]**, it was possible that during seed
349 ageing, the accumulation of reactive oxygen species in seed resulted the down-regulation of
350 DEG4. DEG7 encodes a proteases 6, In Arabidopsis, the aspartic protease 1 (ASPG1) was
351 affected the seed longevity and germination by the process of proteolysis **[31]**, proteases 6 was the
352 major cellular machinery of proteolysis in eukaryotic organisms, it was possible that DEG7 was
353 also regulated by seed ageing. DEG10 encodes a BURP domain-containing protein, a newly
354 identified protein that is unique to plants and plays an important role in plant abiotic stresses,
355 development and metabolism via regulating the level of diverse proteins **[32, 33].** DEG12 encodes
356 a phenylalanine ammonia lyase homolog1 (PAL1), PAL genes was been reported involving in

bioRxiv preprint doi: https://doi.org/10.1101/627117; this version posted May 3, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

357 multiple biology process including response to environmental stress **[35].** Based on the annotation
358 of above genes, it was possible that those genes may function in ageing induced defense response,
359 energy metabolism and the DNA/RNA and protein repair systems **(Table 1)**.
360
361 Alternative splicing is a process whereby multiple functionally distinct transcripts are encoded
362 from a single gene by the selective removal or retention of exons and/or introns from the maturing
363 RNA **[36, 37],** which is common in many eukaryote lineages, including metazoans, fungi, plants
364 and showing over 95% of multi-exon genes in human genome produce at least one alternatively
365 spliced isoform **[38-42]**. In this study we identified 7 AS-DEGs in I178 and X178, DEG14
366 encodes a HSP20-like chaperones superfamily protein. DEG15 encodes a NADH dehydrogenase
367 subunit 4, an important enzyme in the respiratory chain of all organisms having an aerobic or
368 anaerobic electron-transport system in mitochondria **[43]**. DEG16 encodes an embryo defective
369 3012, was down-regulated and affected by ageing. DEG17 is an auxin transport protein (BIG) that
370 in charge of the auxin polar transportation and distribution, gibberellin status in seed **[44, 45].**
371 DEG18 encodes a sucrose synthase 3, which was participate in respiration and related to plant
372 growth **[46].** DEG19 encodes a 27-kDa zein protein (zp27), specifically expressed in maize and
373 may functions like a protease inhibitor **[47].** Up-regulation of DEG14, 15, 17**,** 18 and 19 indicated
374 those genes were potential ageing related genes that involved in stress response, energy
375 metabolism, development regulation etc.
376
377
378 ## Reference

bioRxiv preprint doi: https://doi.org/10.1101/627117; this version posted May 3, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

379 1. Agacka-Moldoch M, Nagel M, Doroszewska T, Lewis RS, Börne A. Mapping quantitative
380 trait loci determining seed longevity in tobacco (Nicotiana tabacum L.). Euphytica. 2015;
381 202(3): 479-486.
382 2. Walters C. Understanding the mechanisms and kinetics of seed ageing. Seed Sci. Res. 1998;
383 8: 223-244.
384 3. Groot S, Surki A, Vos R and Kodde J. Seed storage at elevated partial pressure of oxygen, a
385 fast method for analysing seed ageing under dry conditions. Ann. Bot. 2012; 110: 1149-1159.
386 4. Xin X, Lin X, Zhou Y, Chen X, Liu X, Lu X. Proteome analysis of maize seeds: the effect of
387 artificial ageing. Physiol. Plantarum. 2011; 143, 126-138.
388 5. Xin X, Tian Q, Yin G, Chen X, Zhang J, Ng S. Reduced mitochondrial and
389 ascorbate-glutathione activity after artificial ageing in soybean seed. J. Plant Physiol. 2014;
390 171, 140-147.
391 6. Nguyen TP, Cueff G, Hegedus DD, Rajjou L, Bentsink L. A role for seed storage proteins in
392 Arabidopsis seed longevity. J. Exp. Bot. 2015; 66, 6399-6413.
393 7. Yin G, Xin X, Fu S, An M, Wu S, Chen X, Zhang J, He J, Whelan J, Lu X. Proteomic and
394 carbonylation profile analysis at the critical node of seed ageing in oryza sativa. Sci. Rep.
395 2017; 7, 1-12.
396 8. He Y, Cheng J, Li X. Acquisition of desiccation tolerance during seed development is
397 associated with oxidative processes in rice. Botanique. 2015; 94(2): 91-101.
398 9. Debeaujon I, Leon-Kloosterziel KM, Koornneef M. Influence of the testa on seed dormancy,
399 germination, and longevity in Arabidopsis. Plant Physiol. 2000; 122(2): 403-414
400 10. Murthy UMN, Kumar PP, Sun WQ. Mechanisms of seed ageing under different storage

bioRxiv preprint doi: https://doi.org/10.1101/627117; this version posted May 3, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

401 conditions for Vigna radiata (L.) Wilczek: lipid peroxidation, sugar hydrolysis, Maillard
402 reactions and their relationship to glass state transition. J Exp Bot. 2003; 54(384): 1057-1067.
403 11. Zhan J, Li W, He HY, Li CZ, He LF. Mitochondrial alterations during Al-induced PCD in
404 peanut root tips. Plant Physiol Biochem. 2014; 75:105-113.
405 12. Yin G, Whelan J, Wu S, Zhou J, Chen B, Chen X, Zhang J, He J, Xin X, Lu X.
406 Comprehensive mitochondrial metabolic shift during the critical node of seed ageing in rice.
407 PLoS One. 2016; 11, e0148013.
408 13. Sano N, Kim JS, Onda Y, Nomura T, Mochida K. Okamoto M, Seo M. RNA-Seq using
409 bulked recombinant inbred line populations uncovers the importance of brassinosteroid for
410 seed longevity after priming treatments. Scientific report. 2017; 7: 8095
411 https://doi:10.1038/s41598-017-08116-5
412 14. Lv Y, Zhang S, Wang J, Hu Y. Quantitative Proteomic Analysis of Wheat Seeds during
413 Artificial Ageing and Priming Using the Isobaric Tandem Mass Tag Labeling. PLOS ONE.
414 2016; DOI:10.1371/journal.pone.0162851 September 15, 2016
415 15. Wang W, Liu S, Song S, Møller IM. Proteomics of seed development, desiccation tolerance,
416 germination and vigor. Plant Physiol. Biochem. 2015; 86, 1-15.
417 16. Chen X, Yin G, Börnerb A, Xin X, He J, Nage M, Liu X, Lu X. Comparative physiology and
418 proteomics of two wheat genotypes differing in seed storage tolerance. Plant Physiology and
419 Biochemistry. 2018; 130:455-463. https://doi.org/10.1016/j.plaphy.2018.07.022
420 17. Mao X, Cai T, Olyarchuk JG, Wei L. Automated genome annotation and pathway
421 identification using the KEGG Orthology (KO) as a controlled vocabulary. Bioinformatics.
422 2005; 21, 3787±93. https://doi. org/10.1093/bioinformatics/bti430 PMID: 15817693.

bioRxiv preprint doi: https://doi.org/10.1101/627117; this version posted May 3, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

423 18. Rajandeep S, Sekhon RB, Candice N, Hirsch CL, Natalia L, Shawn M, et al. Maize gene
424 atlas developed by RNA sequencing and comparative evaluation of transcriptomes based on
425 RNA sequencing and microarrays. PLoS ONE. 2013; 8:E61005.
426 https://doi.org/10.1371/journal.pone.0061005. PMID:23637782.
427 19. Young MD, Wakefield MJ, Smyth GK, Oshlack A. Gene ontology analysis for RNA-seq:
428 Accounting for selection bias. Genome Biol. 2010; 11, R14.
429 https://doi.org/10.1186/gb-2010-11-2-r14 PMID: 20132535.
430 20. Liu Y, Zhang H, Li X, Wang F, Lyle D, Sun L, Wang G, Wang J, Li L, Gu R. Quantitative
431 trait locus mapping for seed artificial aging traits using an $F_{2:3}$ population and a recombinant
432 inbred line population crossed from two highly related maize inbreds. Plant Breeding. 2018;
433 (138):29-37. https://doi.org/10.1111/pbr.12663.
434 21. Shewry PR, Halford NG. Cereal seed storage proteins: Structures, properties and role in grain
435 utilization. J Exp Bot. 2002; 53:947-958.
436 22. Rajjou L, Miche L, Huguet R, Job C, Job D. The use of proteome and transcriptome profiling
437 in the understanding of seed germination and identification of intrinsic markers determining
438 seed quality, germination efficiency and early seedling vigour. In: Navie SC, Adkins SW,
439 Ashmore S, eds. Seeds: Biology, Development and Ecology . Oxfordshire, CAB
440 International, 2007; 149-158.
441 23. Rajjou J, Lovigny Y, et al. Proteome-Wide Characterization of Seed Aging in Arabidopsis: A
442 Comparison between Artificial and Natural Aging Protocols. Plant Physiology. 2008; 148:
443 620-641. https://doi.org/10.1104/pp.108.123141
444 24. Bewley JD. Seed germination and dormancy. Plant Cell. 1997; 9: 1055-1066.

bioRxiv preprint doi: https://doi.org/10.1101/627117; this version posted May 3, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

445 25. Sano N, Rajjou L, North HM, Debeaujon I, Marion-Poll A, Seo M. Staying Alive: Molecular
446 Aspects of Seed Longevity. Plant Cell Physiol. 2016; 57(4):660-74. https://doi:
447 10.1093/pcp/pcv186.
448 26. Petla BP, Kamble NU, Kumar M, Verma P, Ghosh S, Singh A, Rao V, Salvi P, Kaur H,
449 Saxena SC, Majee M. Rice PROTEIN l-ISOASPARTYL METHYLTRANSFERASE
450 isoforms differentially accumulate during seed maturation to restrict deleterious isoAsp and
451 reactive oxygen species accumulation and are implicated in seed vigor and longevity. New
452 Phytol. 2016; 211(2):627-45. https://doi.org/ 10.1111/nph.13923.
453 27. Ogunola OF, Hawkins LK, Mylroie E, Kolomiets MV, Borrego E, Tang JD, Williams WP,
454 Warburton ML. Characterization of the maize lipoxygenase gene family in relation to
455 aflatoxin accumulation resistance. PLoS One. 2017; 12(7):e0181265. https://doi:
456 10.1371/journal.pone.0181265. eCollection 2017.
457 28. Li Z, Gao Y, Lin C, Pan R, Ma W, Zheng Y, Guan Y, Hu J. Suppression of LOX activity
458 enhanced seed vigour and longevity of tobacco (Nicotiana tabacum L.) seeds during storage.
459 Conserv Physiol. 2018; 6(00): coy047; https://doi.org/10.1093/conphys/coy047.
460 29. Mochizuki S, Sugimoto K, Koeduka T, Matsui K. Arabidopsis lipoxygenase 2 is essential for
461 formation of green leaf volatiles and five-carbon volatiles. FEBS Lett. 2016; 590(7):1017-27.
462 https://doi:10.1002/1873-3468.12133.
463 30. Charton L, Plett A, Linka N. Plant peroxisomal solute transporter proteins. J Integr Plant
464 Biol. 2019; Accepted. https://doi.org/10.1111/jipb.12790
465 31. Yashwanti Mudgil, Shin-Han Shiu, Sophia L. Stone, Jennifer N. Salt, and Daphne R. Goring.
466 A Large Complement of the Predicted Arabidopsis ARM Repeat Proteins Are Members of

bioRxiv preprint doi: https://doi.org/10.1101/627117; this version posted May 3, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

467 the U-Box E3 Ubiquitin Ligase Family Plant Physiol. 2004; (134):59-66

468 32. Shen W, Yao X, Ye T, Ma S, Liu X, Yin X, Wu Y. Arabidopsis Aspartic Protease ASPG1
469 Affects Seed Dormancy, Seed Longevity and Seed Germination. Plant Cell Physiol. 2018;
470 59(7):1415-1431. https://doi: 10.1093/pcp/pcy070.

471 33. Dinh SN and Kang H. An endoplasmic reticulum-localized Coffea arabica BURP
472 domain-containing protein affects the response of transgenic Arabidopsis plants to diverse
473 abiotic stresses. Plant Cell Rep. 2017; 36(11):1829-1839. https://doi:
474 10.1007/s00299-017-2197-x.

475 34. Li Y, Chen X, Chen Z, Cai R, Zhang H, Xiang Y. Identification and Expression Analysis of
476 BURP Domain-Containing Genes in Medicago truncatula. Front Plant Sci. 2016; 7:485.
477 https://doi: 10.3389/fpls.2016.00485. eCollection 2016.

478 35. Huang J, Gu M, Lai Z, Fan B, Shi K, Zhou YH, Yu JQ, Chen Z. Functional analysis of the
479 Arabidopsis PAL gene family in plant growth, development, and response to environmental
480 stress. Plant Physiol. 2010; 153(4):1526-38. https://doi: 10.1104/pp.110.157370.

481 36. Chow LT, Gelinas RE, Broker TR, Roberts RJ. An amazing sequence arrangement at the
482 5'ends of adenovirus 2 messenger RNA. Cell. 1977; 12, 1-8. https://doi:
483 10.1016/0092-8674(77)90180-5

484 37. Bush SJ, Chen L, Tovar-Corona JM, Urrutia AO. Alternative splicing and the evolution of
485 phenotypic novelty. Philos Trans R Soc Lond B Biol Sci. 2017; 372(1713): 20150474.
486 https://doi: 10.1098/rstb.2015.0474

487 38. Kim N, Alekseyenko AV, Roy M, Lee C. The ASAP II database: analysis and comparative
488 genomics of alternative splicing in 15 animal species. Nucleic Acids Res. 2007; 35,

bioRxiv preprint doi: https://doi.org/10.1101/627117; this version posted May 3, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

489 D93-D98. https://doi: 10.1093/nar/gkl884

490 39. Grutzmann K, Szafranski K, Pohl M, Voigt K, Petzold A, Schuster S. Fungal alternative
491 splicing is associated with multicellular complexity and virulence: a genome-wide
492 multi-species study. DNA Res. 2014; 21, 27-39. https://doi: 10.1093/dnares/dst038

493 40. Zhang C, Yang H, Yang H. Evolutionary character of alternative splicing in plants.
494 Bioinform. Biol. Insights. 2016; 9(Suppl 1), 47-52. https://doi: 10.4137/BBI.S33716.
495 eCollection 2015.

496 41. Pan Q, Shai O, Lee LJ, Frey BJ, Blencowe BJ. Deep surveying of alternative splicing
497 complexity in the human transcriptome by high-throughput sequencing. Nat. Genet. 2008;
498 40, 1413-1415. https://doi: 10.1038/ng.259

499 42. Wang ET, Sandberg R, Luo SJ, Khrebtukova I, Zhang L, Mayr C, Kingsmore SF, Schroth
500 GP, Burge CB. Alternative isoform regulation in human tissue transcriptomes. Nature. 2008;
501 456, 470-476. https://doi: 10.1038/nature07509

502 43. Matsushita K, Otofuji A, Iwahashi M, Toyama H, Adachi O. NADH dehydrogenase of
503 Corynebacterium glutamicum. Purification of an NADH dehydrogenase II homolog able to
504 oxidize NADPH. FEMS Microbiol Lett. 2001; 204 (2):271-6.
505 https://doi.org/10.1111/j.1574-6968.2001.tb10896.x. PMID: 11731134

506 44. Gil P, Dewey E, Friml J, Zhao Y, Snowden KC, Putterill J, Palme K, Estelle M, Chory J.
507 BIG: a calossin-like protein required for polar auxin transport in Arabidopsis. Genes Dev.
508 2001; 15(15):1985-97.

509 45. Desgagné-Penix I, Eakanunkul S, Coles JP, Phillips AL, Hedden P, Sponsel VM. The auxin
510 transport inhibitor response 3 (tir3) allele of BIG and auxin transport inhibitors affect the

bioRxiv preprint doi: https://doi.org/10.1101/627117; this version posted May 3, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

511 gibberellin status of Arabidopsis. Plant J. 2005; 41(2):231-42.

512 46. Daloso DM, Williams TC, Antunes WC, Pinheiro DP, Müller C, Loureiro ME, Fernie AR.
513 Guard cell-specific upregulation of sucrose synthase 3 reveals that the role of sucrose in
514 stomatal function is primarily energetic. New Phytol. 2016; 209(4):1470-83. https://doi:
515 10.1111/nph.13704.

516 47. Krishnan HB, Jang S, Kim WS, Kerley MS, Oliver MJ, Trick HN. Biofortification of
517 soybean meal: immunological properties of the 27 kDa γ-zein. J Agric Food Chem. 2011;
518 59(4):1223-8. https://doi: 10.1021/jf103613s.

519 48. International rules for seed test. International seed testing association (ISTA). Switzerland:
520 Zurich. 2018; Chapter 15-6.

521 49. Oliveros JC. VENNY. An interactive tool for comparing lists with Venn Diagrams. 2007;
522 http://bioinfogp.cnb.csic.es/tools/venny/index.html.

523 50. Tian T, Liu Y, Yan H, You Q, Yi X, Du Z, Xu W, Su Z. AgriGO v2.0: a GO analysis toolkit
524 for the agricultural community. Nucleic Acids Res. 2017; 45(Web Server issue):
525 W122-W129. https://doi: 10.1093/nar/gkx382.

526

527 **Acknowledgements**

528 We acknowledge the financial support from National Key R&D Program of China
529 (2017YFD0102001-3 & 2018YFD0100900-3), National Natural Science Foundation of China
530 (31701437 & 31771891) and the China Agriculture Research System (CARS-02-10).

531

532 **Supporting Information**

bioRxiv preprint doi: https://doi.org/10.1101/627117; this version posted May 3, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

533 **Fig 1. Storage characterization of I178 and X178 seed. A).** Observation of 3 and 5d-AA seeds;
534 **B).** TTC staining of embryo at 0d-AA, after 3 and 5d-AA; **C).** The relative conductivity of fresh
535 harvest (FH) seeds, 1-year storage seeds treated by 3 and 5d-AA; the dash lines indicate the vigor
536 of FH seeds after 5d-AA was lost more than that of 1-year storage seeds. **D).** SDS-PAGE analysis
537 of seeds zein, lane 1-12: Dry seeds of X178 and I178 (X178-Dry, I178-Dry), X178 seeds after 6
538 hours imbibed water (X178-0d-6h), 48-hours germinated X178 seeds (X178-0d-48h), I178 seeds
539 after 6 hours imbibed water (I178-0d-6h), 48 hours-germinated I178 seeds (I178-0d-48h), X178
540 seeds after 3d-AA (X178-3d), I178 seeds after 3d-AA (I178-3d), X178 seeds after 5d-AA
541 (X178-5d), I178 seeds after 5d-AA (I178-5d), X178 seeds after 7d-AA (X178-7d), I178 seeds
542 after 7d-AA (I178-7d). The degradation of 40 kD protein was denoted as arrow.
543 **Fig 2. RNA-Seq of I178 and X178 and gene expression analysis before and after 5d-AA. A).**
544 Heatmap of differential expressed genes in I178 after 5d-AA compared with 0d-AA. **B).** Most of
545 the significantly differential expressed genes in I178 were enriched in 5 categories of Immune
546 system (red), biotic stress response (green), abiotic stress response (blue), carbohydrate catabolic
547 process (light blue), nutrient reservoir activity (purple) and extracellular region (pink). **C).**
548 Compared to the 0d-AA treatment, DEGs after 5d-AA identified in two materials with the
549 adjusted *P*-value $\leq$0.05 and the FoldChange $\geq$1.5, there are 188 specific I178 DEGs and 122 X178
550 specific DEGs, among that, 98 common DEGs identified in I178 and X178, with 2 common
551 up-regulated and 86 common down-regulated genes. 10 genes were up-regulated in I178 while
552 down-regulated in X178 was labeled in the triangle. **D).** Heat map of DEGs in X178. **E).** GO
553 enrichment of the most significantly differential expressed genes in X178.
554 **Fig 3. qRT-PCR validation of the RNA-Seq result. A).** Nine genes including 7 differential

bioRxiv preprint doi: https://doi.org/10.1101/627117; this version posted May 3, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

555 expressed genes and 2 ageing related genes validated by qRT-PCR. RNA-Seq gene expression
556 calculated by fold-change in I178 **(B)** and X178 **(C)**.
557 **Fig 4. GO enrichment of AA induced alternative splicing genes (ASG) in I178 and X178. A).**
558 Biology process, cellular component and the molecular function of common ASGs in I178 and
559 X178. **B).** 381 alternative splicing genes specifically expressed after 5d-AA in I178 and X178.
560 **Fig S1. Data quality demonstration of RNA-Seq. A).** FPKM distribution of 2 replications of
561 I178 and X178. **B).** Pearson correlation analysis between samples of I178 and X178.
562 **Fig S2. GO enrichment analysis of the DEGs.** Biology process, cellular component and the
563 molecular function of 286 I178 DEGs **(A)** and 220 X178 DEGs **(B); C).** Only 2 co-up regulated
564 genes detected in both I178 and X178, one of gene encodes a TBP associated factor; **D).** Total 98
565 common DEGs identified in both I178 and X178; **E).** 86 I178 and X178 commonly down
566 regulated genes identified in this study, most of the genes involving in carbohydrate catabolic
567 process and carbohydrate derivative binding.
568 **Fig S3. AS, ASG and AS-DEGs identified in 178. A).** 12 types of AS identified in I178 and
569 X178 after 0d-AA and 5d-AA. **B).** Transcript isoforms (and the covered genes in brackets) that
570 occurred AS in I178 and X178 after0d-AA and 5d-AA, the red colored are genes specifically
571 spliced in two 178 after 5d-AA. **C).** Alternative spliced DEGs in I178 and X178 after 5d-AA. Six
572 and one AS-DEGs were identified specifically in X178 and I178, respectively.
573 **Table 1. Potential seed ageing related genes.**
574 **Table S1. Number of reads sequenced and mapped to the maize genome.**
575 **Table S2. The expression of common 98 DEGs and the related biology process classification.**
576 **Table S3. Alternative splicing events and the genes involved in I178 and X178.**

bioRxiv preprint doi: https://doi.org/10.1101/627117; this version posted May 3, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

577

578 **Author Contributions**

579 Conceptualization: Li Li

580 Data curation: Li Li.

581 Formal analysis: Li Li, Li Xuhui.

582 Funding acquisition: Wang Guoying and Li Li.

583 Investigation: Li Li, Wangfeng and Peng Yixuan

584 Methodology: Li Li, Wang Feng and Peng Yixuan.

585 Project administration: Li Li.

586 Resources: Wang Guoying, Wang Jianhua and Zhang Hongwei.

587 Software: Li Li, Li Xuhui

588 Supervision: Gu Riliang and Wang Jianhua.

589 Validation: Li Li.

590 Visualization: Li Li.

591 Writing -original draft: Li Li.

592 Writing- review & editing: Li Li and Gu Riliang

Table 1. Potential seed ageing related genes.

DEG ID	Gene ID	I178_ log2FC	X178_ log2FC	Up/ Down	Source.	Homologs	Annotation
DEG1	GRMZM2G058970	0.87	0.86	down	**QTL(Chr3:8-205 Mb)**	At5G49930.1	Zinc knuckle (CCHC-type) family protein
DEG2	GRMZM2G302913	1.00	0.67	down		At1G22060.1	-
DEG3	GRMZM2G358618	0.74	0.84	down		At4G03200.1	Catalytics
DEG4	GRMZM2G375807	1.03	0.88	down		At4G39850.3	Peroxisomal ABC transporter 1
DEG5	GRMZM2G379913	0.98	0.80	down		At3G06530.1	ARM repeat superfamily protein
DEG6	GRMZM2G379929	0.96	0.88	down		At3G06530.2	ARM repeat superfamily protein
DEG7	GRMZM2G438938	0.86	0.86	down		At5G02310.1	Proteolysis 6
DEG8	GRMZM5G867767	0.97	0.70	down		At3G10650.1	-
DEG9	GRMZM2G181135	1.86	0.89	down		At1G34060.1	Pyridoxal phosphate (PLP)-dependent Transferases superfamily protein
DEG10	GRMZM5G800586	1.27	0.77	down		At5G25610.1	BURP domain-containing protein
DEG11	GRMZM5G877838	0.97	0.89	down		Os01g56780.1	Plus-3 domain containing protein
DEG12	GRMZM2G074604	0.80	0.86	down	**QTL(Chr5:185-205 Mb)**	AT2G37040.1	PHE ammonia lyase 1
DEG13	GRMZM5G824439	0.98	0.99	down		AT1G23230.2	-
DEG14	GRMZM2G158232	-0.98	-0.03	up	**I178_AS-DEG**	At1G53540.1	HSP20-like chaperones superfamily protein
DEG15	GRMZM5G804358	-0.13	-1.02	up	**X178_AS-DEG**	AtMG00580.1	NADH dehydrogenase subunit 4
DEG16	GRMZM2G476810	0.52	0.64	down		At5G40480.1	Embryo defective 3012
DEG17	GRMZM2G461586	-0.31	-0.78	up		At3G02260.1	Auxin transport protein (BIG)
DEG18	GRMZM2G311182	-0.39	-0.71	up		At4G02280.1	Sucrose synthase 3
DEG19	GRMZM2G138727	-0.54	-0.63	up		-	27-kDa zein protein
DEG20	GRMZM2G417682	0.69	0.61	down		-	-

Note: 13 DEGs of two 178 were located in QTL interval derived from RILs and $F_{2:3}$ populations of I178 × X178 (DEG1-13); 7 AS-DEGs were identified in I178 (DEG14) and X178 (DEG15-20) after 5d-AA, were possible the Ageing related candidates.

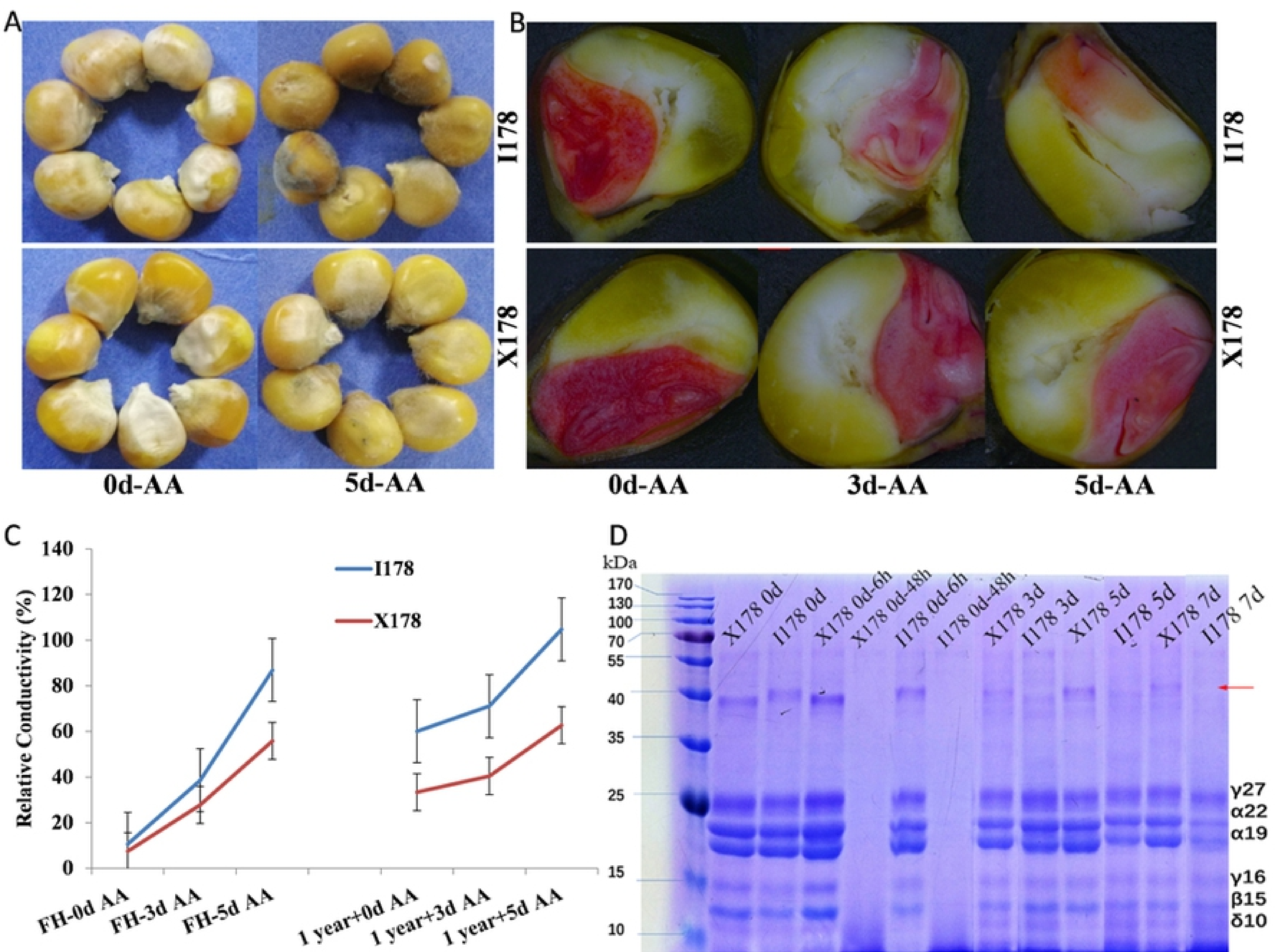
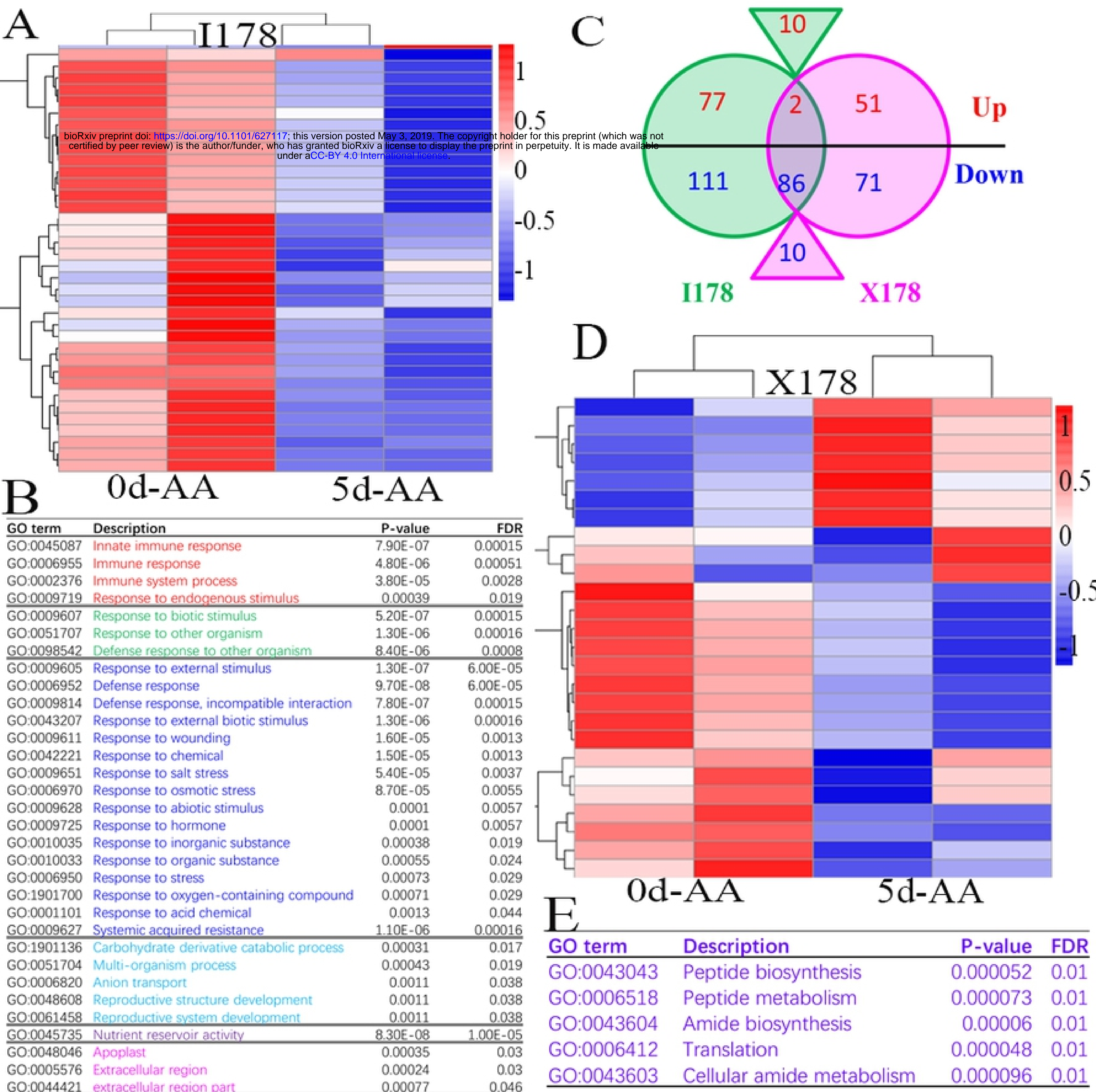


Fig 1

bioRxiv preprint doi: https://doi.org/10.1101/627117; this version posted May 3, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.



GO term	Description	P-value	FDR
GO:0045087	Innate immune response	7.90E-07	0.00015
GO:0006955	Immune response	4.80E-06	0.00051
GO:0002376	Immune system process	3.80E-05	0.0028
GO:0009719	Response to endogenous stimulus	0.00039	0.019
GO:0009607	Response to biotic stimulus	5.20E-07	0.00015
GO:0051707	Response to other organism	1.30E-06	0.00016
GO:0098542	Defense response to other organism	8.40E-06	0.0008
GO:0009605	Response to external stimulus	1.30E-07	6.00E-05
GO:0006952	Defense response	9.70E-08	6.00E-05
GO:0009814	Defense response, incompatible interaction	7.80E-07	0.00015
GO:0043207	Response to external biotic stimulus	1.30E-06	0.00016
GO:0009611	Response to wounding	1.60E-05	0.0013
GO:0042221	Response to chemical	1.50E-05	0.0013
GO:0009651	Response to salt stress	5.40E-05	0.0037
GO:0006970	Response to osmotic stress	8.70E-05	0.0055
GO:0009628	Response to abiotic stimulus	0.0001	0.0057
GO:0009725	Response to hormone	0.0001	0.0057
GO:0010035	Response to inorganic substance	0.00038	0.019
GO:0010033	Response to organic substance	0.00055	0.024
GO:0006950	Response to stress	0.00073	0.029
GO:1901700	Response to oxygen-containing compound	0.00071	0.029
GO:0001101	Response to acid chemical	0.0013	0.044
GO:0009627	Systemic acquired resistance	1.10E-06	0.00016
GO:1901136	Carbohydrate derivative catabolic process	0.00031	0.017
GO:0051704	Multi-organism process	0.00043	0.019
GO:0006820	Anion transport	0.0011	0.038
GO:0048608	Reproductive structure development	0.0011	0.038
GO:0061458	Reproductive system development	0.0011	0.038
GO:0045735	Nutrient reservoir activity	8.30E-08	1.00E-05
GO:0048046	Apoplast	0.00035	0.03
GO:0005576	Extracellular region	0.00024	0.03
GO:0044421	extracellular region part	0.00077	0.046

GO term	Description	P-value	FDR
GO:0043043	Peptide biosynthesis	0.000052	0.01
GO:0006518	Peptide metabolism	0.000073	0.01
GO:0043604	Amide biosynthesis	0.00006	0.01
GO:0006412	Translation	0.000048	0.01
GO:0043603	Cellular amide metabolism	0.000096	0.01

Fig 2

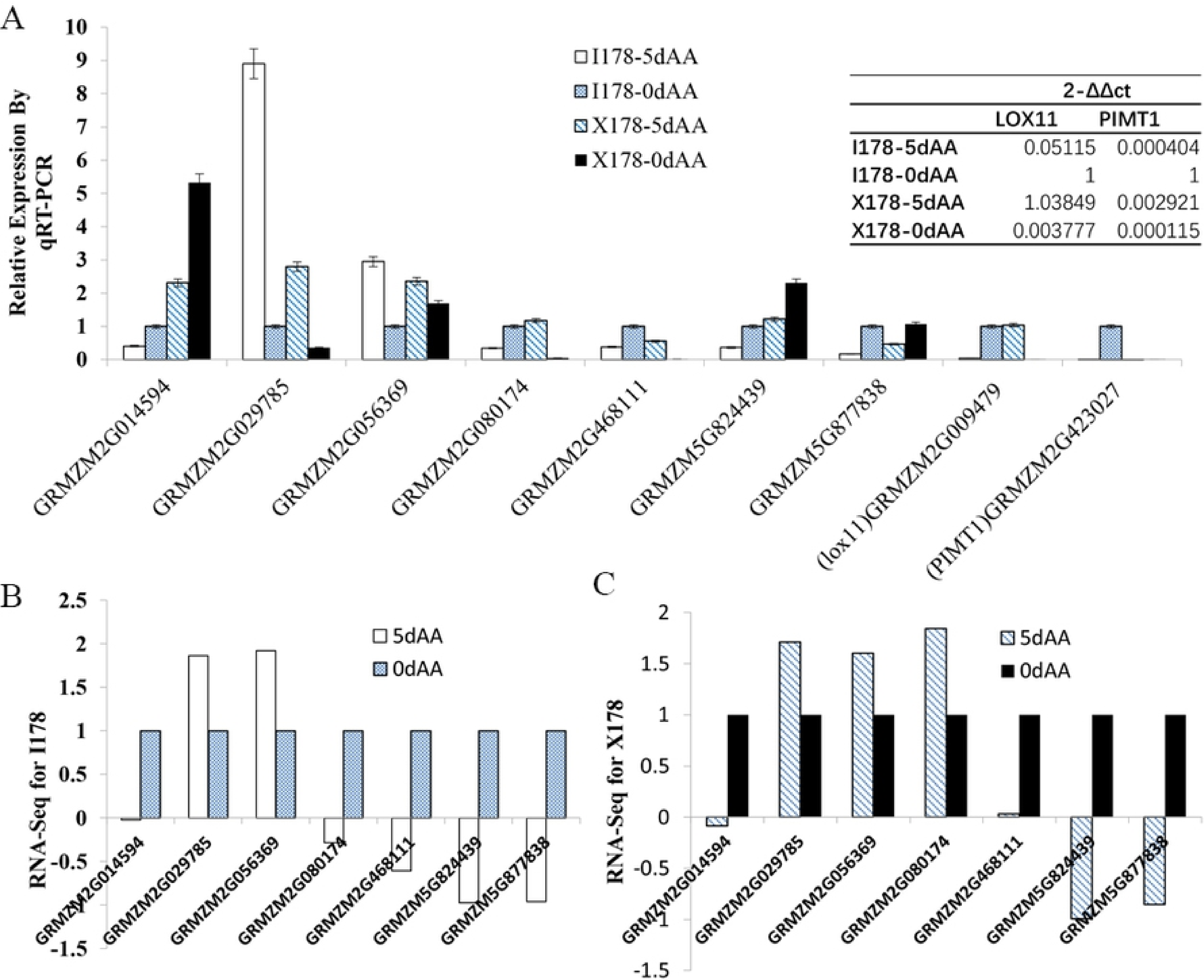


Fig 3

A

$-\log_{10}$(P.value) 20 40 60 80

Gene number 500 1000 1500

ribonucleoside triphosphate biosynthetic process
ribonucleoside monophosphate biosynthetic process
purine ribonucleoside triphosphate biosynthetic process
purine ribonucleoside monophosphate biosynthetic process
purine ribonucleoside biosynthetic process
purine nucleoside triphosphate biosynthetic process
purine nucleoside monophosphate biosynthetic process
purine nucleoside biosynthetic process
nucleoside triphosphate biosynthetic process
ATP biosynthetic process
protein serine/threonine phosphatase complex
phosphatase complex
structural molecule activity
ribonucleotide binding
ribonucleoside binding
purine ribonucleotide binding
purine ribonucleoside triphosphate binding
purine ribonucleoside binding
purine nucleotide binding
purine nucleoside binding
nucleoside binding
ATP binding

BP
CC
MF

2.0 2.5 3.0 3.5

Fold enrichment

bioRxiv preprint doi: https://doi.org/10.1101/627117; this version posted May 3, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

B

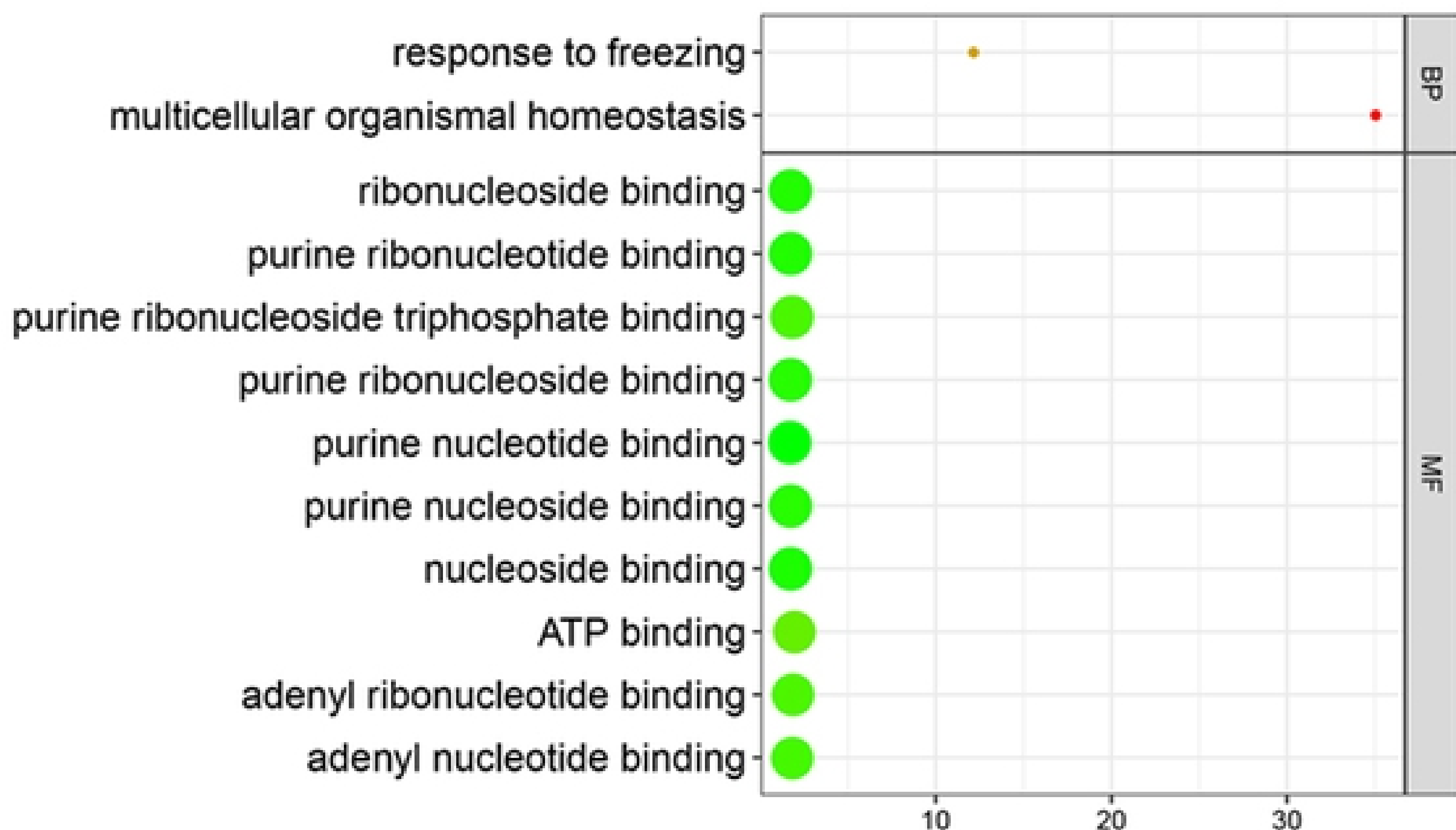


Fig 4