iTRAQ-Based quantitative proteome revealed metabolic changes between a cleistogamous wheat mutant and its wild-type wheat

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Abstract

Background: Wheat is one of the most important staple crops worldwide. However, *Fusarium* head blight severely affects wheat yield and quality. A novel bread wheat mutant, *ZK001*, characterized as cleistogamic was isolated from a non-cleistogamous ~~varieties~~ variety (YM18) through static magnetic field~~s~~ mutagenesis. Cleistogamy should be a new strategy and approach to control *Fusarium* head blight. However, little is known about the mechanism of cleistogamy in wheat.

Methods: *Fusarium* head blight resistance test was performed to identify the *Fusarium* head blight infection rate of *ZK001*. And we measured the agronomic trait of *ZK001* and the contents of starch and total soluble sugar of lodicules in YM18 and *ZK001*. In addition, ~~Comparative~~ comparative studies at the proteome level were employed between YM18 and *ZK001* based on the proteomic technique of isobaric tags for relative and absolute quantification.

Results: The infection rate of *ZK001* is lower than its wild type and AK58. The abnormal lodicules of *ZK001* lost the ability to push the lemma and palea apart during the flowering stage. Proteome analysis showed that the main differentially abundant proteins were related to carbohydrate metabolism, protein transport and calcium ion binding. These differentially abundant proteins may work together to regulate cellular homeostasis, osmotic pressure and the development of lodicules. This theory is supported by the analysis of starch, soluble sugar content in the lodicules as well as the results of qRT-PCR.

Conclusions: In this paper, we demonstrate that the proteome analysis provides comprehensive information for further research on the lodicule development mechanism in wheat. This mutant is optimal for studying flower development in wheat and could be very important for *Fusarium* head blight resistant projects via conventional crossing.

Key words: Bread wheat, Cleistogamous, *Fusarium* head blight, Lodicule, iTRAQ

Introduction

Bread wheat (*Triticum aestivum* L.) is one of the most important staple crops worldwide. The world population continues to grow and arable area is decreasing year by year, therefore higher production in crop plants may prove to be necessary to satisfy the increasing demand in food (FAO, 2015). However, many challenges like biotic and abiotic stress severely affect wheat fields and the product quality. For instance, *Fusarium* head blight (FHB) is a critical factor badly damaging wheat security (Walter, Nicholson & Doohan, 2010), and the application of chemical insecticides and fungicides used for controlling pests and diseases is increasing the amount of residues in wheat and in the environment (Hollingsworth et al., 2008). Therefore, scientists and breeders have to find eco-friendly and cost-effective strategies to guarantee wheat yield and quality. Induced mutation, such as Cobalt-60 (^{60}Co-γ-ray)-mutagenesis, static magnetic fields (SMFs)-mutagenesis, spaceflight-mutagenesis, and ethyl methane sulfonate (EMS), ~~is~~ are ~~an~~ important method~~s~~ employed for crop breeding improvement (Krasileva et al., 2017).

In the last decade, severe epidemics caused by *Fusarium* ~~ssp~~spp. have occurred worldwide and up to 100% yield loss was recorded under optimal disease conditions (Yumurtaci et al., 2017). FHB is a complex disease and the *pore-forming toxin-like* (*PFT*) gene at the quantitative trait locus (QTL) *Fhb1* which so far was the first FHB-resistance gene ~~was~~ isolated and found to confer resistance to FHB in Sumai 3 (SM3) (Rawat et al., 2016). FHB infection usually occurs on the inner surfaces of lemmas and palea~~e~~s after germination of the *Fusarium* ~~ssp~~spp. conidia (Zange, Kang & Buchenauer, 2005). The anther can provide the initial path for FHB infection (Pugh, Johann & Dickson, 1933; Walter, Nicholson & Doohan, 2010); therefore, cleistogamous cultivars, which contain few anthers exposed to glumes, may provide structural barriers for diseases that appear during the flowering stage. In barley (*Hordeum vulgare* L.), cleistogamous cultivars, which self-fertilize within permanently closed flowers (Culley & Klooster, 2007), showed greater resistance to FHB infection than chasmogamous cultivars, which have open flowers (Yoshida, Kawada & Tohnooka, 2005). In wheat, cleistogamous cultivars (U24) have a lower risk of FHB infection than chasmogamous cultivars such as Saikai 165 (Kubo et al., 2010). Therefore, cleistogamy is a new strategy for controlling *Fusarium* head blight. Cleistogamy in barley is genetically determined by the presence of the recessive allele *cly1*, but the dominant allele at the linked locus, *Cly2*, is epistatic over *cly1* (Wang et al., 2013). Loss of the miRNA172 target site causes *cly1* to express a protein, HvAP2, which effectively suppress~~es~~ lodicule swelling (Turuspekov et al., 2004; Nair et al., 2010; Wang et al., 2015). In rice, there are many cleistogamous mutants resulting from abnormal lodicules. *ld(t)* (*lodiculeless spikelet(t)*), a single recessive gene, controls the cleistogamous mutant lacking lodicules (Won & Koh, 1998; Maeng

80 et al., 2006). Another rice mutant, which has a truncated DEP2 determined by the *cl7(t)* gene, has
81 a cleistogamous phenotype because of weak swelling ability in the lodicules (Ni et al., 2014). A
82 third rice mutant, spw1-cls, has normal stamens, but the lodicules are transformed homeotically
83 into lodicule-glume mosaic organs, thereby engendering cleistogamy with temperature-sensitivity
84 (Yoshida et al., 2007; Ohmori et al., 2012). A novel temperature-stable cleistogamous mutant,
85 *spw1-cls2*, can maintain the cleistogamous phenotype under low temperatures (Lombardo et al.,
86 2017). The glumes open in the flowering stage because the swelling of the lodicule is primarily
87 responsible for pushing the lemma and palea, thereby opening the floret (Nair et al., 2010).
88 However, there is very little information on cleistogamy in wheat.
89   The probability of primary infection is approximately proportional to the number of spores
90 reaching the open florets during the flowering process, and the breeding of varieties of flower that
91 are partially or completely cleistogamous might reduce *Fusarium* susceptibility in wheat (Schuster
92 & Ellner, 2008). In order to probe the mechanism of cleistogamy, Ning et al. (Ning et al., 2013)
93 studied the structure, transcription and post-transcriptional regulation of the cleistogamous gene,
94 *TaAP2*, which is homologous to barley in wheat. *TaAP2* alleles may also generate a cleistogamous
95 wheat and improve the resistance to FHB. Additionally, anther extrusion is a complex trait with
96 significant markers which has either favorable or unfavorable additive effects and impart minor to
97 moderate levels of phenotypic variance in spring and winter wheat (Muqaddasi et al., 2017). Okada
98 et al. (Okada et al., 2018) proposed a novel mechanism where ovary swelling could push the lemma
99 and palea apart to open the florets in wheat flowers.
100   Large-scale transcriptomic analyses have been employed in wheat to better understand the
101 molecular mechanisms of flower development (Winfield et al., 2010; Diallo et al., 2014; Feng et
102 al., 2015; Kumar et al., 2015; Yang et al., 2015; Ma et al., 2017). However, because of post-
103 transcriptional and post-translational modifications, the mRNA levels do not always correlate with
104 the corresponding protein levels (Schweppe et al., 2003; Canovas et al., 2004; Zhao et al., 2013).
105 Proteins are directly correlated with cellular functions (Yan et al., 2005; Zhang et al., 2012);
106 therefore, proteomic analysis is essential for studying global protein expression levels in wheat to
107 further unravel the complex mechanisms of cleistogamy. In particular, isobaric tags for relative
108 and absolute quantitation (iTRAQ) technology, which is a quantitative gel-free proteomic
109 approach, coupled with liquid chromatography/mass spectrometry/mass spectrometry
110 (LC/MS/MS) enables the direct quantification and comparison of protein levels among samples
111 with more efficiency and accuracy than traditional gel-based techniques which fail to identify low-
112 abundance protein species and have difficulties identifying proteins with extreme biochemical
113 properties (Wu et al., 2006).
114   Here, we show that an SMFs-induced wheat mutant, *ZK001*, is a cleistogamous line with
115 lower FHB infection vulnerability than the chasmogamous line, Yumai 18 (YM18). Additionally,
116 we perform a comparative proteomic analysis of different development stages in YM18 and *ZK001*
117 at Bangfei Bioscience (Beijing Bangfei Bioscience Co., Ltd. http://www.bangfeibio.com/) to
118 characterize the protein expression profiles. In the present study, we attempted to provide insight
119 into proteomic changes associated with the cleistogamous phenotype in wheat, specifically

120 exploring lodicule expanding mechanisms at the protein level. The results have the potential to
121 benefit future research efforts in controlling FHB via conventional crossing and studying wheat
122 flower development. It could also be important for better control of genetically modified lines of
123 agriculturally important crops; and it has time-saving and cost-saving benefits in the purification
124 of genotypes.

Materials & Methods

Plant Material

127 The cleistogamy mutant line, *ZK001*, was isolated from a mutagenized population of wheat
128 varieties, YM18, using a SMFs ~~with~~ of 7 Tesla for 5 hours. And it propagated via self-pollination
129 until the cleistogamous phenotype was completely stable. All wheat seeds were stored in Hefei
130 Institutes of Physical Science, Chinese Academy of Sciences (CASHIPS), Anhui, P. R. China.
131 Sumai 3 (SM3), Aikang 58 (AK58), YM18 and *ZK001* were grown in a greenhouse in an
132 experimental field (31°54′ N, 117°10′ E) at CASHIPS. Fertilizer and weed management were
133 similar to the process for wheat breeding (Li et al., 2014). The spikelets and lodicules of YM18
134 and *ZK001* which had three biological replicates were harvested during the white anther stage
135 (WAS), green anther stage (GAS), yellow anther stage (YAS) and anthesis stage (AS), respectively
136 (Zadoks, Chang & Konzak, 1974; Kirby & Appleyard, 1987; Guo & Schnurbusch, 2015). These
137 samples were collected and frozen in liquid nitrogen and preserved at -80 °C.

Starch and total soluble sugar content

139 Twenty pairs of lodicules with three biological replications from YM18 and *ZK001* were sampled
140 and snap frozen in liquid nitrogen at four flower development stages. The samples were ~~grinded~~
141 ground using Tissuelyser-24 (Shanghai Jingxin Industrial Development Co., Ltd.) for 45 seconds
142 at 50 Hz.

143 Starch and total soluble sugars were extracted following the instructions included with the Starch
144 content kit and Plant soluble sugar content test kit (Nanjing Jiancheng Bioengineering Institute),
145 respectively. The starch and total soluble sugars in the supernatant were determined using a UV-
146 VIS Spectrophotometer (Lambda 365, PerkinElmer) with a wave length of 620 nm. The formula
147 of calculating starch and total soluble sugars as follow:

$$\text{Starch content} \left(\frac{\text{mg}}{\text{g}}\right) = \frac{\text{OD}_{\text{sample}} - \text{OD}_{\text{blank}}}{\text{OD}_{\text{standard}} - \text{OD}_{\text{blank}}} \times \text{Standard solution concentration} \left(0.2\frac{\text{mg}}{\text{ml}}\right) \times$$
$$\text{V}_{\text{pretreatment solution}} \text{ (ml)} \times \text{Dilution ratio} \div \text{Weight of sample (g)}$$

$$\text{Total soluble sugar content (µg/g)}$$
$$= \frac{\text{OD}_{\text{sample}} - \text{OD}_{\text{blank}}}{\text{OD}_{\text{standard}} - \text{OD}_{\text{blank}}} \times \text{Standard solution concentration} \left(100\frac{\text{µg}}{\text{ml}}\right)$$
$$\div \frac{\text{Weight of sample (g)}}{10 \times \text{V}_{\text{distilled water used for homogenating}}\text{(ml)}} \times \text{Dilution ratio}$$

Observation of spikes and lodicules

157 Spike images of YM18 and *ZK001* were captured with a camera (Nikon) at the AS. After 90
158 minutes, the lodicules of YM18 and *ZK001*, which were separated from the central young spikes

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159 in triplicate during GAS and cultured on Murashige and Skoog (MS) medium, were observed with
160 an upright fluorescence stereomicroscope (Olympus) and captured by a camera (Olympus).

161 **FHB resistance testing**

162 FHB resistance testing was performed during the flowering stage of SM3, AK58, YM18 and
163 *ZK001* in the greenhouse by spraying the FHB spore F0601 (*Fusarium graminearum* Schw. cv.
164 F0601) in both 2013-2014 and 2014-2015. The inoculum (50 μL at 105 spores per mL) was
165 deposited by spraying on both sides of the ears. AK58 and SM3 were used as the susceptible and
166 resistant controls, respectively. The diseased spikelet rate was calculated using the following
167 formula:

168 $?? =$ infected spikelets/total number of spikelets×100.

169 **Protein extraction and iTRAQ labelling**

170 Total soluble proteins were extracted according to a published procedure (Yang et al., 2013) with
171 slight modifications. Protein concentrations of the samples were estimated using the Bradford
172 method (Bradford, 1976) (Table S1) and the samples were stored at -80 °C for further use. All
173 protein samples were checked via sodium dodecyl sulphate polyacrylamide gel electrophoresis
174 (SDS-PAGE) according to the Schägger protocol (Schägger, 2006). SDS-PAGE gels (Figure S1)
175 were stained with Coomassie Brilliant Blue (CBB) staining solution (Commassie Blue Fast
176 Staining Solution, Beijing Dingguo Changsheng Biotechnology Co., LTD) (Kang et al., 2002).

177 After determining the protein concentration, Trypsin (Promega, V5113) was used to digest
178 and incubate for 12-16 h at 37 °C. About 100 μg peptides of different samples were labelled with
179 iTRAQ based on the Unwin et al. protocol (Unwin, Griffiths & Whetton, 2010). The peptides of
180 different samples were labelled with iTRAQ reagents (isobaric tags 113, 114, 115, 116, 117, 118,
181 119 and 121 for group YM18-WAS, *ZK001*-WAS, YM18-GAS, *ZK001*-GAS, YM18-YAS,
182 *ZK001*-YAS, YM18-AS and *ZK001*-AS, respectively) according to the manufacturer's
183 instructions (Applied Biosystems).

184 **HPLC grading of C18 columns at high pH and LC-electrospray ionization-MS/MS analysis**

185 The lyophilized peptide mixture was reconstituted with 100 μL of solution A (2% acetonitrile
186 (ACN) and 20 mM ammonium formate, pH 10). Then, the samples were loaded onto a reverse-
187 phase column (C18 column, 1.9 μm 150 μm × 120 mm, Thermo Scientific) and eluted using a step
188 linear elution program (Table S2). The samples were collected every 1.5 min and centrifuged at
189 14,000 × g for 5-90 min. The 60 fractions collected were dried and re-dissolved with 5 μL 0.5%
190 formic acid (FA). The collected fractions were finally combined into 10 pools and centrifuged at
191 14,000 × g for 10 min.

192 The reconstituted peptides were analysed with the Q-Exactive HF mass spectrometer
193 (Thermo Fisher Scientific) coupled with a nano high-performance liquid chromatography system
194 (1260 Infinity II, Agilent) using 320 degrees centigrade temperature conditions (Scheltema et al.,
195 2014). The peptides were loaded onto a C18-reversed phase column (C18 column, 3 μm 100 μm
196 × 200 mm, Thermo Scientific) and separated on an analytical column (xBridge peptide BEH 130
197 C18 column, Waters) using mobile phases A (0.1% FA/H2O) and B (0.08% FA, 80% ACN) (Table

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198 S3). The HPLC effluent was directly electrosprayed into the mass spectrometer and analysed based
199 on pre-set parameters (Figure S2).
200 **Data analysis**
201 The raw mass data were processed for peptide identification using Proteome Discoverer 1.4 (ver.
202 1.4.0.288, Thermo Fisher Scientific) with specific parameters (Table S4) for searching the Uniprot
203 Triticum database. False discovery rate (FDR) ≤ 0.01 was estimated for protein identification using
204 a target-decoy search strategy (Elias & Gygi, 2007). The mass spectrometry proteomics data have
205 been deposited to the ProteomeXchange Consortium
206 (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository (Vizcaino et al.,
207 2016) with the dataset identifier < PXD010188 >. Up-regulated and down-regulated proteins were
208 determined based on 1.5-fold-changes and peptides spectral matches (PSMs) ≥ 2 (Sharma et al.,
209 2017) between *ZK001*-WAS vs YM18-WAS (Group 1), *ZK001*-GAS vs YM18-GAS (Group 2),
210 *ZK001*-YAS vs YM18-YAS (Group 3), *ZK001*-AS vs YM18-AS (Group 4), YM18-WAS vs
211 YM18-GAS (Group 5), YM18-WAS vs YM18-YAS (Group 6), YM18-WAS vs YM18-AS
212 (Group 7), *ZK001*-WAS vs *ZK001*-GAS (Group 8), *ZK001*-WAS vs *ZK001*-YAS (Group 9), and
213 *ZK001*-WAS vs *ZK001*-AS (Group 10), respectively.
214   Protein annotation was conducted by a BLAST search against NCBI and Uniprot databases.
215 Protein function was classified based on the following databases: Gene Ontology
216 (http://www.geneontology.org/, GO), and Kyoto Encyclopedia of Genes and Genomes
217 (http://www.genome.jp/kegg/, KEGG). For analysis of DAPs, significant GO enrichment and
218 KEGG enrichment were defined as a corrected FDR with a p–value (Benjamini & Hochberg, 1995)
219 less than 0.01. Proteins containing at least two peptides spectral matches (PSMs) per protein and
220 fold change ratios ≥ 1.5 or ≤ 0.67 were considered as more abundant or less abundant proteins,
221 respectively. In order to validate the DEP profile, the EnsemblPlants database
222 (http://plants.ensembl.org/index.html) was searched for corresponding DNA sequences. A total of
223 10 DAPs, which were carbohydarte, calcium ion binding and transport, were selected for qRT-
224 PCR validation.
225 **Quantitative Real-time PCR validation**
226 Total RNA was isolated using a Plant RNA Kit (Omega, R6827) according to the manufacturer's
227 instructions. The quality of each RNA sample was checked on 1% agarose gels. Measurement of
228 the concentration of RNA samples was performed using the NanoDrop 2000 spectrophotometer
229 bioanalyzer (Thermo Fisher Scientific). cDNAs were synthesized using TransScript One-Step
230 gDNA Removal and cDNA Synthesis SuperMix (Transgen Biotech) according to the
231 manufacturer's protocol. qRT-PCR was used to measure the transcript levels of the proteins of
232 interest. Each experiments wasere performed in three technical replicates with three biological
233 replicates. Target gene-specific primers (Table S5) were designed using the online software Primer
234 3 version 0.4.0 (http://bioinfo.ut.ee/primer3-0.4.0/primer3/) (Untergasser et al., 2012).
235 Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed according
236 to the manufacturer's instructions for the FastStart Essential DNA Green Master (Roche), run in
237 the Roche LightCycler® 96 Instrument. The *glyceraldehyde-3-phosphate dehydrogenase* gene

238 from *T. aestivum* (*TaGAPDH*, GI: 7579063) was served as an internal control and the relative
239 expression of target genes was calculated using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001).
240 **Statistical data analysis**
241 The experimental data values represented the average of the measurements conducted from three
242 independent assays and were expressed as the mean ± standard error of the mean (SEM). The data
243 were further analyzed using an ANOVA at a 95% confidence level followed by Duncan's's test
244 (SPSS 18.0, IBM, Somers, NY, USA). The level of significance was set at $p \leq 0.05$.
245

246 # Results
247 **Comparison of resistance to *Fusarium* head blight**
248 Since the cleistogamous wheat mutant was isolated, we identified this cleistogamous mutant with
249 a low FHB infection comparing to chasmoigamous varieties. Kubo et al. (Kubo et al., 2010)
250 showed that cleistogamous wheat cultivars have a lower FHB infection rate than chasmogamous
251 cultivars. Therefore, we tested the FHB resistance of YM18 and *ZK001*. The results of FHB
252 resistance testing showed that the infection rate in Sumai 3 (SM3) and *ZK001* were 7.03% and
253 9.39% in 2013-2014 and 8.61% and 17.60% in 2014-2015, respectively (Table 1). Compared to
254 SM3 and *ZK001*, YM18 and Aikang (AK58) were highly susceptible, 35.16% and 38.12% in
255 2014-2015, respectively. However, the diseased spikelet rates for YM18 and AK58 were 15.20%
256 and 20.41% in 2013-2014, respectively, about half the rate in 2014-2015 (Table 1). It indicates
257 that the FHB infection rate is greatly influenced by environmental factors. These results suggested
258 that cleistogamous cultivars had a lower FHB infection rate than chasmoigamous cultivars. In
259 addition, this mutant should be capable of generating excellent varieties with the phenotype of
260 cleistogamy via conventional hybridization.
261

262 **Comparison of flower between YM18 and *ZK001***
263 Exserted anthers increased the incidence of FHB (Sage & De Isturiz, 1974; PARRY, JENKINSON
264 & McLEOD, 1995). Furthermore, the anthers of cleistogamy wheat were detained in glums during
265 the flowering stage. The individual lines of YM18 and *ZK001* were grown under the same growth
266 and environmental conditions but the morphological differences were obvious. In YM18, the
267 anthers extrude from the palea and lemma at the anthesis stage (AS), whereas no anthers were
268 observed in *ZK001* at all flower development stages (Figure 1A). The morphology of the lodicules
269 of YM18 and *ZK001* was also obviously different. In order to investigate the morphology of the
270 lodicules, the lodicules of YM18 and *ZK001* at the green anther stage (GAS) were harvested and
271 cultured for 90 minutes on MS medium. The results showed that the width of YM18 lodicules
272 (Figure 1B) was larger than that of *ZK001* lodicules (Figure 1C and 1D).
273

274 **Physiological characters of lodicules in YM18 and *ZK001***
275 To reveal the cause of the lodicules difference between YM18 and *ZK001*, the starch and total
276 soluble sugar contents were measured in the lodicules of YM18 and *ZK001* at four flower
277 development stages. Lodicule starch and total soluble sugar contents showed an overall increase

278   from white anther stage (WAS) to AS for YM18 and *ZK001* (Figure 2, Table S6). No significant
279   differences in the starch and soluble sugar contents in the lodicule were observed in YM18 or
280   *ZK001* during WAS and GAS. Additionally, the starch and total soluble sugar contents in *ZK001*
281   decreased by 2.54, 2.40, 2.30, and 1.75-fold compared with YM18 during the yellow anther stage
282   (YAS), detected in the quality fraction and per pair of lodicules, respectively (Figure 2, Table S6).
283   In contrast, the starch and total soluble sugar contents in *ZK001* increased by 4.50, 3.57, 1.87, and
284   1.52-fold compared with YM18 during the AS, detected in the quality fraction and per pair of
285   lodicules, respectively (Figure 2, Table S6).
286

287 **Overview of quantitative proteome analysis**
288   In order to study the protein expression patterns in YM18 and *ZK001*, the proteomes of YM18 and
289   *ZK001* in the four flower development stages were examined and quantitatively catalogued using
290   iTRAQ technology, respectively. In this study, 19,422 peptides were matched to 4,497 proteins in
291   the samples (Table S7); in addition, 11,603 unique peptides were found, and 2,172 proteins were
292   identified with more than two unique peptide sequences excluding PTMs. Figure 3A shows that
293   more than 99% of the peptides covered proteins within the 36 peptides, and protein quantity
294   decreased as the number of matching peptides increased. In terms of protein mass distribution,
295   good coverage (average of 10–18% of total proteins in each protein-mass group) was obtained for
296   proteins > 10 kDa and < 60 kDa (Figure 3B). The length of the identified peptides was between
297   10 and 13 aas at the peak and about 93% of the peptide length was within 24 aas (Figure 3C). Over
298   77% of the proteins had > 5% sequence coverage. Additionally, sequence coverage distribution
299   was high in most of identified proteins: more than 58% had over > 10% coverage and more than
300   37% had over 20% coverage (Figure 3D). These results indicate that the identified peptides were
301   sufficient for protein identification.
302

303 **Formatting of Mathematical Components**
304   In order to identify more differentially abundant proteins, we compared the differentially abundant
305   proteins between YM18 and *ZK001* in the flowering development process, *ZK001*-WAS vs YM18-
306   WAS (Group 1), *ZK001*-GAS vs YM18-GAS (Group 2), *ZK001*-YAS vs YM18-YAS (Group 3),
307   *ZK001*-AS vs YM18-AS (Group 4), YM18-WAS vs YM18-GAS (Group 5), YM18-WAS vs
308   YM18-YAS (Group 6), YM18-WAS vs YM18-AS (Group 7), *ZK001*-WAS vs *ZK001*-GAS
309   (Group 8), *ZK001*-WAS vs *ZK001*-YAS (Group 9), and *ZK001*-WAS vs *ZK001*-AS (Group 10),
310   respectively. Increasing abundance and decreasing abundance proteins were determined based on
311   fold-changes (FC) of > 1.5 or < 0.667 for expression difference comparison. For further screening,
312   about 16, 47, 2, 0, 11, 124, 105, 15, 298 and 188 differentially abundant proteins (DAPs) were
313   identified with a corrected P–value for GO KEGG enrichment less than 0.01 in group 1 to 10
314   (Table 2). A Venn diagram of the DAPs and their overlap between Group 1 and Group 2 showed
315   that 2 and 1 common DAPs were increased-abundance and decreased- abundance, respectively
316   (Figure S3). Group 3 and Group 4 showed no overlap with Group 1 or Group 2 (Figure S3). Venn

317 diagrams indicated that 6, 10, 32, 206, 57 and 140 DAPs were specific DAPs of Groups 5, 8, 6, 9,
318 7 and 10, respectively (Figure S3).
319   Furthermore, there were more DAPs during WAS (Group 1) / GAS (Group 2) than during
320 YAS (Group 3) and AS (Group 4). With the growth of the plant, the mass of DAPs continued
321 increasing from WAS to YAS in both YM18 and *ZK001* (Groups 5 to 6 and Groups 8 to 9,
322 respectively). However, there was a disparity between YM18 and *ZK001* from YAS to AS. The
323 number of DAPs decreased by about 39% in *ZK001* from YAS to AS (Group 10), but showed a
324 slight decrease in YM18 (Group 7) (Table 2).
325
326 **Functional classification and subcellular localization of proteins**
327 GO analysis showed that all of the identified proteins in YM18 and *ZK001* were involved in 11
328 subgroups of MF, 19 subgroups of BP, and 14 subgroups of CC (Figure S4). Significant GO
329 enrichment was employed to analyse the DAPs with a corrected FDR P–value less than 0.01, and
330 a ratio FC of more than 1.5. Based on GO annotations and enrichments, the DAPs of Group 1 were
331 enriched in molecular function terms for lipid binding (100%) (Figure 4A), as well as biological
332 process terms for lipid transport (14.06%), lipid localization (14.06%), macromolecule localization
333 (20.31%), organic substance transport (20.31%), single-organism transport (15.63%) and single-
334 organism localization (15.63%) (Figure 4B). GO classification of Group 2 revealed that the DAPs
335 were enriched in the biological process, cellular component and molecular function (Figure 4C).
336 No protein was enriched in Group 3 or Group 4. The DAPs of Groups 5 to 10 were also classified
337 into biological process (Figure S5A), cellular component (Figure S5B) and molecular function
338 (Figure S5C). DAPs involved in carbohydrate metabolism and transport, calcium ion binding and
339 protein transport, fatty acid biosynthesis and signal transduction-related proteins were further used
340 in cluster analyses.
341
342 **Accumulation patterns of DAPs and verification of DAPs interest**
343 Based on the above analyses, 11 genes which corresponded to DAPs of interest were chosen for
344 qRT-PCR analyses using gene-specific primers (Table S5) to explore the expression profile at the
345 transcription level.
346   The morphology of lodicules showed significant differences between YM18 and *ZK001*
347 (Figure 1). qRT-PCR was carried out using the RNA of YM18 and *ZK001* lodicules to study the
348 transcript profiles of the above genes corresponding to the selected DAPs (Figure 5). The results
349 of qRT-PCR indicated that the expression level of The gene of *A0A1D6CCI3* (bidirectional sugar
350 transporter SWEET) was expressed in the lodicules at WAS and GAS of both YM18 and ZK001,
351 while almost no expression at YAS and AS (Figure 3, Table S7); (Figure 5A). Additionally, the
352 expression trend of this gene revealed down-regulation from WAS to GAS and YAS, and then up-
353 regulation to the initial expression level during AS in *ZK001* (Figure 5A). Furthermore, the
354 expression trend in YM18 showed a certain similarity to that in *ZK001* (Figure 5A). The expression
355 level of the gene *A0A1D5WGA3* was extremely down-regulated from WAS to GAS in YM18 and
356 *ZK001*, respectively (Figure 5B). Though the relative expression level in *ZK001* was higher than

357 that in YM18, there were almost no significant differences between YM18 and *ZK001* in GAS,
358 YAS or AS (Figure 5B). Compared with the relative expression levels of the gene encoding
359 A0A1D5RR02 in YM18, the gene in *ZK001* was down-regulated during WAS and GAS,
360 respectively (Figure 5C). There was no significant difference during YAS or AS (Figure 5C).
361 Additionally, compared with YM18, P93594-encoding mRNA was all up-regulated in the
362 lodicules of *ZK001* (Figure 5D). Interestingly, the mRNA level of W5B5R3 in *ZK001* indicated
363 up-regulation compared with YM18 at YAS and AS, respectively, while there was no significant
364 difference during WAS or GAS (Figure 5E). The mRNA levels of *A0A1D5SYC3* and
365 *A0A1D5VEI9* showed almost no significant difference between YM18 and *ZK001* at the four
366 flower development stages, except that of A0A1D5SYC3 (Figure 5F) and A0A1D5VEI9 (Figure
367 5G). The relative expression level for *A0A1D5RVB4* was up-regulated in *ZK001* compared with
368 that in YM18 at GAS, YAS and AS (Figure 5H). Comparing with YM18, the gene expression of
369 A0A077S2F2 in *ZK001* was down-regulated during YAS (Figure 5I).
370 Calcium ion plays a key role in the development of plants. Therefore, the relative expression
371 of the genes encoding A0A1D5TN57 and A0A1D5RRV7 was also evaluated to determine the
372 profile during flower development. Compared with YM18, the relative gene expression levels of
373 A0A1D5TN57 in *ZK001* were up-regulated during WAS, YAS and AS, respectively (Figure 5K),
374 while those of A0A1D5RRV7 were up-regulated during WAS, GAS and AS (Figure 5J).

375 **Discussion**

376 **Cleistogamy provides structural barriers for diseases of *Fusarium* head blight**

377 From the physiological point of view, the flowering stage is regarded as the most susceptible period
378 for primary infection of wheat spikes by FHB because of the opening of wheat florets and the
379 extension of anthers (Pugh, Johann & Dickson, 1933; Schroeder & Christensen, 1963; Gilsinger
380 et al., 2005; Schuster & Ellner, 2008). Barley is the plant that self-fertilizes with permanently
381 closed flowers, but it is easy to be infected with *Fusarium* in chasmogamous barley varieties
382 (Yoshida, Kawada & Tohnooka, 2005; Culley & Klooster, 2007). Table 1 shows that the diseased
383 spikelet rate in 2014-2015 was more severe than that in 2013-2014 except in SM3, possibly
384 because of the resistance gene *Fhb1* (Rawat et al., 2016). Compared with YM18, which is a wild
385 type and chasmogamous cultivar, the diseased spikelet rate for *ZK001*, which is a mutant and
386 cleistogamous cultivar, decreased by 38.22% and 50.00% in 2013-2014 and 2014-2015,
387 respectively (Table 1). This indicates that although the diseased spikelet rate is greatly influenced
388 by environmental factors, cleistogamous cultivars that flower partially or completely may have a
389 lower risk of FHB infection than chasmogamous cultivars (Kubo et al., 2010; Wang et al., 2013).
390 Therefore, we further verified the hypothesis that cleistogamous cultivars might have lower
391 *Fusarium* susceptibility in wheat. From the practical point of view, as a new strategy for
392 controlling FHB, it will be very meaningful to introduce the cleistogamous function into other
393 varieties that are suitable for production and promotion but sensitive to FHB through hybridization.

394 **Lodicules play an important role in glume opening/closing in wheat**

395 The molecular mechanism for cleistogamy had been intensively studied in rice (Maeng et al., 2006;
396 Yoshida et al., 2007; Ohmori et al., 2012; Ni et al., 2014; Lombardo et al., 2017) and barley

397 (Turuspekov et al., 2004; Hori et al., 2005; Nair et al., 2010; Wang et al., 2013, 2015; Zhang et al.,
398 2016). However, the molecular mechanism for cleistogamy remains unclear beyond the knowledge
399 that lodicule is a key factor in glume opening/closing in the monocotyledon. The abnormal
400 lodicules may lack the ability to push the lemma and palea apart during the flowering stage in the
401 cleistogamous mutant *ZK001* (Figure 1). This phenomenon is similar to that in barley (Nair et al.,
402 2010).

403 **Carbohydrates and calcium are the main factors regulating lodicule osmotic pressure**
404 Sucrose is the primary form by which sugar is transported for photosynthetic carbon assimilation
405 (Chen et al., 2012). The gene of *A0A1D6CCI3* was expressed in the lodicules of both YM18 and
406 *ZK001* (Figure 5A, Table S8). This indicates that carbohydrates can be transferred to the lodicules
407 of both YM18 and *ZK001* normally. Nevertheless, the starch and soluble sugar content in the
408 lodicules of YM18 increased dramatically from GAS to YAS (Figure 2A-D, Table S6). Liu et al.
409 (Liu et al., 2017) suggested that retarded lodicule expansion in ZS97A was caused by reduced
410 water accumulation because of retarded accumulation of osmotic regulation substances. In
411 contrast, the lower soluble sugar content in the lodicules of *ZK001* prevented the accumulation of
412 water during YAS (Figure 2C and 2D, Table S6). The lodicule mass of YM18 was higher than that
413 of *ZK001* because the starch and soluble sugar contents in the lodicules of *ZK001* decreased from
414 GAS to YAS, leading to little water transfer to the lodicules. The lodicules of wheat swell
415 extensively and subsequently contract after rapid autolyzation of the tissues (Craig & O'Brien,
416 1975). Therefore, the starch and soluble sugar contents in the lodicule of YM18 were lower than
417 those in *ZK001* at AS (Figure 2A-D, Table S6).

418 Cytosolic calcium is an important secondary messenger in plants and plays important roles in
419 the response to both environmental and internal signals (Poovaiah & Reddy, 1993; Liao, Zheng &
420 Guo, 2017). Plant annexins are calcium-dependent phospholipid binding proteins with many
421 biological functions; for instance, they participate in calcium ion channel formation, membrane
422 dynamics, plant growth and the stress response (Mortimer et al., 2008; Laohavisit & Davies, 2011).
423 In this study, the gene relative expression levels of annexin (A0A1D5RRV7) and the calcium ion
424 binding-related protein (A0A1D5TN57) in the lodicules of *ZK001* were up-regulated during WAS
425 compared to those of YM18, respectively (Figure 5J and 5K). Therefore, we inferred that WAS
426 was a critical period for the lodicules.

427 **An overview of the pathways for proteome metabolic changes in lodicules**
428 Many factors regulate the osmotic pressure of lodicules, such as soluble sugar (Zee & O'Brien,
429 1971; Wang, Gu & Gao, 1991; Yan et al., 2017), starch (Pissarek, 1971), calcium (Qin, Yang &
430 Zhao, 2005; Chen et al., 2016) and potassium (Heslop-Harrison & Heslop-Harrison, 1996; Chen
431 et al., 2016; Liu et al., 2017). In this paper, we propose an overview of the metabolic pathways
432 involving the carbohydrates that regulate the osmotic pressure of the lodicules. Our results and
433 previous studies provide an overview of the osmotic mechanisms. As shown in Figure 6, sucrose
434 is transferred into the lodicules from the extra-cellular environment through a bidirectional sugar
435 transporter, and is converted into d-fructose-6p by hexokinase after being broken down into d-
436 fructose. d-fructose-6p converted to α-d-flucose-1p, which can be converted to d-flucose by β-

Commented [RB7]: I presume the authors are referring to the D-form not the d-form?
What is flucose?
I presume the authors mean D-glucose?

437 glucosidase and synthetized amylose. The starch formed by amylose can be broken down into d-
438 glucose under the action of β-amylase. UDP-glucose formed from amino sugar and nucleotide
439 sugar can also be converted into d-glucose under the action of β-glucosidase. The accumulation of
440 d-glucose leads to a change in osmotic pressure in the lodicules. Additionally, α-d-glucose-1p can
441 enter the pentose phosphate pathway and the fatty acid biosynthesis pathway through the formation
442 of α-d-glucose-6P and β-d-glucose-6P. Soluble sugar can also enter and exit cells through the
443 bidirectional sugar transporter. Furthermore, annexin can trigger calcium ion influx, increasing the
444 osmotic pressure. Once the osmotic pressure changes, water accumulates in / is excreted from the
445 cells of the lodicules and induces the expansion / shrinkage of the lodicules.

> **Commented [RB8]:** D and d mean different things. Use the correct notation.
>
> e.g. α-D-glucose

Conclusions

448 The wheat mutant, *ZK001*, with the atrophied, thin and ineffective lodicules lost the ability to push
449 the lemma and palea apart in the flower development process. Besides, compared with YM18,
450 *ZK001* showed lower *Fusarium* infection because of the cleistogamous phenotype. Furthemore,
451 we speculated that the thin lodicule of *ZK001* resulted from the lower soluble sugar, calcium ion
452 and potassium ion contents in lodicule which were regulated by carbohydrate metabolic, protein
453 transport, and calcium ion binding-related proteins. Though, little is known about the mechanism
454 of cleistogamy in wheat, we propose an overview metabolic pathway about the carbohydrate which
455 regulates the osmotic pressure of the lodicules. This study provides foundations for researchers to
456 explore the mechanism of cleistogamy. Furthermore, it should be possible to generate a
457 cleistogamous wheat via conventional crossing, which would improve the FHB resistance of wheat
458 and control pollen-mediated gene flow of genetically modified wheat.

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686   Why does Figure 2 B and D have different units? 10^{-3} mg/PL versus ug/PL ?

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