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Title: Spike development inhibition in the *ftin* mutant is associated with multiple phenotypes and regulated by multiple biological pathways

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Summary

Spike development of wheat line 3558M was strongly inhibited by low temperature stress in spring. The *fertile tiller inhibition (ftin)* gene in the wheat line 3558M is associated with multiple phenotypes, including the production of fewer tillers, delayed floral transition, and death of the shoot apical meristem. We systematically investigated the genes and pathways underlying the differences using ITRAQ proteomics and RNA-sequencing technologies and found multiple biological pathways including to the cold acclimation pathway and multiple defence responses (e.g. reactive oxygen species-mediated hypersensitive response, salicylic acid-mediated systemic acquired resistance) are activated and led to tillers death of the wheat line 3558M under cold stress. Meanwhile, the cold acclimation pathway inhibited the SVP-SCO1-LFY flowering pathway and led to delayed floral transition. Particularly, two TaPIN proteins were significantly downregulated, and multiple auxin signalling genes were also differentially expressed. Knocking down the two *TaPIN* genes using RNAi technology significantly reduced the tiller number. The cold stress inhibited the auxin transport to reduce the tillers of 3558M. Taken together, the *ftin* gene might be a cold-sensitive mutation and that is the cause of multiple biological pathways and phenotypic changes.

Key words: Bread wheat, Spike-development inhibition, Cold Response, Proteomics, Indoleacetic Acids, RNA Interference.

31 Introduction

32 Based on the period development theory, spike development in wheat is divided into three main
 33 physiological stages which were tillering stage, the floral transition from vegetative stage to
 34 reproductive stage and inflorescence development stage (Sreenivasulu & Schnurbusch, 2012).
 35 Tillering is a two-step process: The formation of axillary buds and the outgrowth of the buds. The
 36 dormancy of axillary buds is the result of complex interactions between endogenous
 37 developmental signals and environmental factors (Kebrom et al, 2012). Bud outgrowth is inhibited
 38 by apical dominance and regulated by multiple hormone signalling pathways, e.g., cytokinin (CK),
 39 auxin and strigolactones and their interactions (Thimann & Skoog, 1933; Tanaka et al, 2006). The
 40 regulation of these two steps, which is dependent on both genotypes and environment, determines
 41 the number of productive tillers that form, and thus also the final spike number.

42 The transition from vegetative to reproductive stage is a critical stage of development in which
 43 the outgrowth of tillers will complete the floral transition and finally develop into spikes although
 44 not all wheat tillers can develop into spikes (Deng et al., 2011; Qin et al., 2015). In bread wheat,
 45 the initiation of the floral transition is mainly controlled by photoperiod (*Ppd*) response,
 46 vernalization (*Vrn*), and earliness *per se* (*Eps*) genes (Herndl et al., 2008). The photoperiod
 47 response is mainly regulated by *PPD1*, a member of the *PSEUDO-RESPONSE REGULATOR*
 48 (*PRR*) gene family, which upregulate the expression of the *VRN3/FLOWERING LOCUS T1 (FT1)*
 49 as an activator of the floral transition under long days. Three dominant genes, *Ppd-A1*, *Ppd-B1*
 50 and *Ppd-D1*, which are located on wheat chromosomes 2A, 2B and 2D, respectively, determine
 51 photoperiod insensitivity (Law, Sutka, Worland, 1978; Scarth & Law, 1983). The wheat carrying
 52 the photoperiod-insensitive genotype is rapid flowering regardless of whether they are exposed to
 53 short-day or long-day illumination. The vernalization response is mainly regulated by
 54 *Vernalization1 (VRN1)*, *Vernalization2 (VRN2)* and *Vernalization3 (VRN3)*. *VRN1* is positively
 55 involved in promoting the floral transition which encodes an APETALA1/FRUITFULL-like
 56 MADS box transcription factor. In allohexaploid bread wheat, most of the variation in the
 57 vernalization process is controlled by the three homologs *VRN1* locus (*Vrn-A1*, *Vrn-B1* and
 58 *Vrn-D1*) that are located on chromosomes 5A, 5B and 5D, respectively (Danyluk, 2003; Yan et al.,
 59 2003). *VRN2* encodes a flowering repressor which suppresses the expression of *VRN1*. *VRN3*

encodes a mobile protein that is homologous to *Arabidopsis FT* and is transported from the leaves to the SAM to form a protein complex that regulates the expression of the floral meristem identity gene *VRN1* (Li & Dubcovsky, 2008), *Eps* genes are important for the fine-tuning of flowering and the adaptation of wheat to different environments when the photoperiod and vernalization requirements have been fulfilled. Several *Eps* loci have been mapped to different chromosomes. *LUX ARRHYTHMO/PHYTOCLOCK 1 (LUX/PCL1)*, a candidate gene for *Eps-3^{Am}*, has been mapped and cloned (Gawroński & Schnurbusch, 2012; Gawroński et al., 2014).

Low temperature is an important environmental stress factor that affects the spike development of wheat. Especially in the spring, when the vernalization has been completed and the spike begin the transition from vegetative stage to reproductive stage, the ability of response to cold stress will dramatically descend and the spike will suffer frost injury to lead to significant yield loss under cold stress. However, how the low temperature affects the spike development and how the wheat responds to low temperature stress is still unclear. For this report, we characterized a wheat mutant line 3558M, which displays multiple phenotypic differences, including a reduced tiller number, a delayed floral transition, large spikes, significantly increased grain number and grain weight, compared with the near isogenic line (NIL) wheat 3558. These phenotypes of the 3558M were controlled by a single recessive gene *fertile tiller inhibition gene (ftin)*, which was mapped to 1AS and linked closely to markers Xcfa2153 with 1.0 cM genetic distance (Zhang et al., 2013). In the present study, we find that the mutant line 3558M is cold sensitive and cannot response to normal cold stress in spring. To reveal which biological pathways are responsible for the multiple phenotypes of 3558M and might be regulated by the *ftin* gene, we systematically studied the mutant line 3558M using proteomic, transcriptome sequencing and RNAi technologies. Multiple biological pathways were identified that are closely related to the spike development inhibition phenotype. Our results provide the basis for further studies on the regulatory functions of the *ftin* gene and spike development in bread wheat.

88

89 **Results**

90 **Multiple phenotypes were significant different between wheat line 3558M and NIL** 91 **3558 under low temperature environment**

92 The tillers and spike number of 3558M are significant lower than NIL 3558. Statistical analysis
93 showed that the number of tillers produced before winter without low temperature stress was not
94 significantly different between 3558M and NIL3558 (Fig. 1A). However, at the regeneration stage
95 during a cold environment, 3558M produced tillers significantly fewer than NIL 3558; 3558M
96 produced only approximately two tillers, but 3558 produced approximately seven tillers. Almost
97 all of the tillers of 3558 eventually developed into spikes (average of 10.1), but the tillers of
98 3558M, which were only produced before winter, eventually developed into spikes (average of
99 3.2).

100 The development process of wheat 3558M is remarkably slower than NIL 3558 at floral
101 transition stage. Anatomical examination of 3558M tillers indicated that the shoot apical
102 meristem (SAM) of tillers at single-ridge stage before winter is not significantly different
103 between the 3558M and 3558 (Fig. 1B). However, the spike rachis of 3558 tillers had begun to
104 develop to the spikelets at double-ridge stage after winter, and the SAM of 3558M tillers has still
105 stay at the single ridge stage (Fig. 1B). When the florets of the spikelets of 3558 had begun to
106 develop pistils and stamens, the spikelets of 3558M are still stay in the florets differentiation
107 stage without the pistils and stamens (Fig. 1B). It's obvious that the development process of
108 3558M is significantly slowly than those of the 3558 after winter. In particular, the SAMs of
109 tillers produced after winters not only develop slower, but did not complete the floral transition
110 to develop into spikes. Meanwhile, we found at the floral transition stage, if there are temperature
111 drop fluctuations with normal cold stress (under the zero-degree, Fig 1D), the SAM of 3558M
112 tillers will suffer the freezing damage and lead to death of the shoot apical meristem (SAM) of
113 about two-thirds tillers produced before winter (Fig. 1B and C). Finally, the few tillers of 3558M
114 can develop into spikes.

115 Although the spike number of 3558M was significantly less, the grain number per spike was
116 significantly more than NIL 3558 (Fig. 1A and C). Generally, one spikelet of the spike of 3558M

117 can produce six to seven grains, and the spikelet of the spike of 3558 can produce three to four
118 grains. Finally, the grain number per spike of 3558M is significant higher than 3558. Meanwhile,
119 the length and weight of grains of 3558M is also significantly higher than NIL 3558. The spike
120 development inhibition phenotype was clearly associated with multiple phenotypic
121 characteristics, including reduced tiller and spike numbers, delayed spike development, tiller
122 apex death, high grain number and weight.

123 **Determination of hormone and reactive oxygen species contents**

124 To reveal the differences in hormone contents between the SAM of 3558M and 3558 in
125 response to cold stress, we used an LC-ESI-MS/MS system to analyse six types of hormones:
126 auxin, CKs, gibberellins (GAs), abscisic acid (ABA), jasmonates (JAs) and salicylic acid (SA)
127 (Fig. 2). There were no significant differences in the contents of GAs (GA1, GA3, GA4 and GA7)
128 or ABA between the tillers of 3558M and 3558 (Fig. 2A). The JA and
129 N-[-JASMONOYL]-L-ISOLEUCINE (JA-L-ILE) contents of 3558M were significantly
130 higher than those of 3558, especially after cold stress ($P<0.05$, Fig. 2A and B). The IAA content of
131 3558M was slightly lower than that of 3558, and was decreased in both 3558M and 3558 after
132 cold stress (Fig. 2B). The content of the CK *trans*-Zeatin was significantly higher in 3558M than
133 in 3558, and dramatically increased in both 3558 and 3558M after cold stress (Fig. 2B). The SA
134 content was the highest of all of the detected hormones, reaching over 2000 ng/g in 3558M (Fig.
135 2C). After cold stress, the SA content was higher in 3558M, but slightly lower in 3558. In
136 particular, although there was no significant difference in the whole SAM between 3558M and
137 3558, the IAA content of the base of SAM in 3558M is significant lower than in 3558 after cold
138 stress (Fig.2E, $P<0.05$).

139 To further identify the reactive oxygen species (ROS) mediated hypersensitive response and
140 death of the SAM of tillers of 3558M, we further measured the H₂O₂ concentrations of 3558M
141 and 3558 (Fig. 2D). The pattern of ROS accumulation was similar to that of SA accumulation.
142 ROS levels were significantly higher in 3558M than in 3558. It is worth noting that the ROS
143 concentration of 3558M reached its maximum value after cold stress, but the ROS concentration
144 of 3558 did not change much after cold stress.

145 **Identification and functional classification of differentially expressed proteins**

146 To reveal the effects of normal cold stress on protein expression in 3558M, we performed a
147 high-throughput quantitative proteomic analysis of 3558M and 3558 using ITRAQ technology. In
148 total, 13,577 proteins (unique peptides ≥ 2 and spectral counts ≥ 3) and 9179 proteins were
149 identified in both experimental replicates (Repeat 1 and Repeat 2) (Table S1). Proteins were
150 identified as differentially expressed proteins (DEPs) using strict cut-off criteria (fold
151 change >1.35 or <0.7). Before cold stress, the abundance of 364 proteins in 3558M wheat was
152 significantly different compared with that in 3558; among them, 237 proteins were significantly
153 upregulated, and 175 proteins were significantly downregulated. After cold stress, 611 proteins
154 significantly changed in abundance in 3558M; among them, 335 proteins were upregulated, and
155 175 proteins were downregulated (Table S2).

156 To identify more DEPs and upstream regulators, we isolated the nucleus and extracted
157 nuclear proteins from 3558M and 3558 after cold stress to analyse the nuclear proteomics. A total
158 of 9182 nuclear proteins were quantified (unique peptides ≥ 2 and spectral counts ≥ 3) (Table S1).
159 Among them, 2976 proteins were only identified in the nuclear proteome. A total of 290 proteins
160 significantly changed in abundance in response to cold stress. Of the 201 upregulated proteins, all
161 were identified as being upregulated under cold stress in the total proteome. The abundances of 58
162 proteins were significantly different between the total and nuclear proteomes. Of the 89
163 downregulated proteins, 25 proteins were also identified as being downregulated under cold stress
164 in the total proteome (Table S2). Finally, we performed functional classification and gene ontology
165 (GO) analyses of the DEPs from the nuclear proteome and the total proteome (Fig. 3, $P < 0.05$).
166 Before cold stress, almost all of the upregulated DEPs were involved in photosynthesis, and the
167 downregulated biological pathways mainly included the response to hypoxia and toxin catabolic
168 process. After cold stress, the upregulated biological pathways mainly included the innate immune
169 response (systemic acquired resistance, defence response/incompatible interaction and plant-type
170 hypersensitive response), chitin catabolic process, toxin catabolic process, response to toxic
171 substances and photosynthesis; the downregulated biological pathways mainly included the
172 regulation of chromatin silencing, DNA methylation and DNA packaging. It is worth noting that
173 most of the DEPs in the innate immune response pathway were upregulated in 3558M after cold
174 stress, but they did not significantly different between 3558M and 3558 before cold stress.

175

176 **Transcriptome sequencing and identification of differentially expressed genes**

177 Although we identified the top 15,000 proteins in our analysis of the total proteome and the
178 nuclear proteome, only a few upstream regulators were identified. For example, only a few DEPs
179 were predicted as transcription factors (TFs), and only 36 DEPs were identified as TFs. To reveal
180 the differences in upstream regulator dynamics between 3558M and 3558, we performed
181 RNA-seq on the SAMs of 3558M and 3558 before and after cold stress using the Illumina HiSeq
182 2500 platform. Twelve RNA-seq libraries (four samples with three biological replicates per
183 sample) were prepared, and total of 997,215,482 clean paired-end 150-bp reads, consisting of
184 149.57 Gb, were obtained with an average GC content of 52.6% (Table S3.1). Among the clean
185 reads, 893,955,370 (89.6%) could be uniquely mapped to the wheat reference genome (Table
186 S3.2). More than 90% of the reads mapped to annotated genes and 88% mapped to protein-coding
187 genes. We detected 48,421 to 50,092 expressed genes with FPKM > 1 in each sample (Table S3.3).
188 Finally, we analysed the differentially expressed genes (DEGs) between 3558M and 3558
189 according to the criteria $|\log_2\text{Fold Change (FC)}| > 1$ and $Q\text{-value} < 0.05$. In total, we identified
190 3780 DEGs between 3558M and 3558 before cold stress and 3426 DEGs after cold stress. Before
191 cold stress, 2692 DEGs were upregulated and 1088 DEGs were downregulated in 3558M. After
192 cold stress, 2965 DEGs were upregulated, and 511 DEGs were downregulated in 3558M (Fig 4A
193 and 4B, Table S4).

194 To identify the major functional profiles of DEGs, GO enrichment analysis was carried out
195 separately for four sets of DEGs (3558M_T1 vs 3558_T1_up, 3558M_T1 vs 3558_T1_down,
196 3558M_T2 vs 3558_T2_up and 3558M_T1 vs 3558_T1_down). For the 3558M_T2 vs
197 3558_T2_up set, 49 GO enriched terms in the biological process category ($P < 0.05$ and $\text{DEGs} \geq 10$)
198 were identified; these terms included metabolic process, oxidation-reduction, photosynthesis, and
199 lipid transport (Fig 4C). In particular, 12 significantly enriched GO terms were associated with the
200 response to cold stress, such as oxidative stress, response to cold stress, and biotic and abiotic
201 stimulus, were identified after cold stress (Fig. 4C). The DEGs associated with these terms had the
202 highest expression levels in 3558M_T2. For example, of the 63 DEGs annotated to the GO term
203 oxidation-reduction, 40 showed highest expression levels in 3558MU_T2 (Fig. 5A). Interestingly,

few significantly enriched GO terms were identified for the other three sets, and no GO terms associated with response to oxidative stress or cold stress were identified (Table S5).

TFs are important upstream regulators that regulate developmental and defence processes in response to physiological signals and different types of stress. A total of 3292 genes were predicted to encode TFs using iTAK software in PlnTFDB. Among them, 1055 TFs were differentially expressed in at least one pairwise combination (Table S6). A total of 511 TFs were identified before cold stress, and 689 TFs were identified after cold stress. Before cold stress, 227 TFs were significantly upregulated and 284 TFs were significantly downregulated in 3558M compared with 3558. After cold stress, 302 TFs were significantly upregulated in 3558M, and 387 TFs were downregulated. Interestingly, TF DEGs with the same functional domain tended to have the same expression patterns, especially after cold stress. Most of the TF DEGs involved in stress and defence responses showed significantly upregulated expression in response to cold. The upregulated TFs mostly belonged to five main domain families: APETALA2/ethylene-responsive element binding protein (AP2-EREBP) (52), bHLH (23), MYB (23), WRKY (32), and NAC (15). All of the WRKY family members were significantly upregulated in 3558M, and 34 WRKY family members were significantly upregulated in 3558M after cold stress (Fig. 5A). Most AP2-EREBP and NAC family members showed the highest expression level in 3558M (Fig. 5A). Most of the 15 C-repeat binding factor (CBF) TFs, which are members of the AP2-EREBP family, were upregulated in response to cold stress (Fig. 5B). These results imply that multiple defence mechanisms were quickly activated in response to cold stress in 3558M. In contrast, some TF DEGs involved in the positive regulation of flowering time and development were downregulated. The downregulated TFs belonged to six main categories of domain families: TCP (20), MADS-box (11), GRF (11), SBP-box (12), GATA (8), and AUXIN RESPONSE FACTOR (ARF) (4). Both before and after cold stress, 17 of the 19 MADS-box family members were downregulated in 3558M (Fig. 5C).

The tillers number is significantly reduced after knocking down the two TaPIN genes in wheat using RNAi technology

In the proteome and transcriptome data, multiple DEPs and DEGs were involved in the AUXIN signal transduction. Among them, there were two TaPIN proteins significantly downregulated in

233 3558M (Table S2). Meanwhile, there was a significant difference in transcription levels between
 234 3558M and 3558 after cold stress ($P < 0.05$, Fig 6A). To further verify the expression patterns of
 235 the two PIN proteins, we used a western blot assay to determine the abundances of these proteins
 236 in 3558M and 3558. The results showed that both PIN proteins were significantly downregulated
 237 in the wheat line 3558M (Fig. 6B and C), and were consistent with our ITRAQ results. To
 238 determine the relevance of the correlation between the spike number and TaPIN protein expression,
 239 we constructed an RNAi vector targeting *PINa*, pBIOS2043-PINa-RNAi, and one targeting *PINb*,
 240 pBIOS2043-PINb-RNAi, separately. The sense and antisense DNA sequences of *TaPINa*
 241 (nucleotides 784 to 1036 bp) and *TaPINb* (1013 to 1312 bp) were synthesized using an ABI
 242 3730x1 DNA Analyser and inserted into the plasmid pBIOS2043. Six and eight putative transgenic
 243 plants were obtained from bombarding 35 and 48 immature cv. Svevo embryos with
 244 pBIOS2043-TaPINa and pBIOS2043-TaPINb (RNAi), respectively. A PCR-based assay confirmed
 245 the presence of the transgene in three pBIOS2043-TaPINa-RNAi plants and two
 246 pBIOS2043-TaPINb-RNAi plants. Homozygous plants in the T₂ generation we identified using
 247 the same PCR-based assay. Finally, two independent homozygous transgenic lines called 1954-1
 248 (RNAi-TaPINa) and 1955-4 (RNAi-TaPINb) that had a reduced tiller phenotype were used for
 249 subsequent phenotype analysis and mRNA/protein expression experiments (Fig. 6D and E).

250 Southern blot analysis suggested that the TaPINa-RNAi and TaPINb-RNAi sequences were
 251 integrated into the wheat genome. The 1954-1 line contains three copies of TaPINa-RNAi, and the
 252 1955-4 line contains one copy of TaPINb-RNAi (Fig. 6F). The mRNA and protein abundances of
 253 TaPINa and TaPINb were estimated by qRT-PCR and western blot assay, respectively. The
 254 qRT-PCR results showed that the abundance of *TaPINa* and *TaPINb* transcripts was reduced
 255 by >80% in the TaPINa-RNAi and TaPINb-RNAi lines, respectively (Fig. 6G). The western blot
 256 assay results showed that the TaPINa and TaPINb proteins were significantly downregulated in the
 257 TaPINa-RNAi and TaPINb-RNAi lines, respectively (Fig. 6A and B). The effect of TaPINa and
 258 TaPINb downregulation on the spike number was assessed by comparing the transgenic lines with
 259 the wild-type plants. Statistical analysis showed that the axillary buds/tillers of TaPINa-RNAi and
 260 TaPINb-RNAi were significantly reduced by >50% (Fig. 6H), but all of the produced tillers
 261 eventually developed into spikes. As the spring wheat, the Fielder and transgenic-Fielder did not

262 experience a low temperature stress during their life cycle. So the death of meristem in the TaPIN
263 RNAi lines and delayed flowering time was not observed. On the contrary, the flowering time is
264 earlier in the TaPINb-RNAi line, than in wild type. Finally, the number of tillers is congruent with
265 the number of spikes without aborted phenotype. The results showed that the downregulation of
266 TaPIN proteins results in a reduced spike number phenotype.

267 **DISCUSSION**

268 **The wheat line 3558M is cold sensitive at the regeneration stage and activates multiple** 269 **defence responses**

270 Previous studies of inhibition of spike development in wheat line 5660M have shown that
271 multiple cold-regulated (COR) proteins and CBF genes are significantly upregulated and that
272 high expression levels are closely related to the 5660M inhibition phenotype (Zheng et al., 2013).
273 The wheat line 3558M contains a recessive mutation in the same gene mutated in 5660M, *fin*,
274 and has a similar spike development inhibition phenotype as 5660M. To further verify the
275 cold-sensitive phenotype of the mutant 3558M, we compared the shoot apices of tillers of
276 3558M and 3558 before and after cold stress (Fig. 2E and 2F). Eight COR proteins were
277 identified, and all were upregulated in 3558M proteome as determined using ITRAQ in
278 combination with nano LC-MS/MS analysis (Table S2). By analysing the transcriptome of
279 3558M, most of the CBF/DREB TFs were significantly upregulated after cold stress (Fig 5B).
280 The changes in CBF/DREB TFs expression in 3558M were consistent with those in 5660M
281 according to qRT-PCR analysis (Zheng et al., 2013). Although the *fin* gene has not been cloned,
282 the fact that the mutant 3558M has the same tiller inhibition phenotype and the same expression
283 trends of CBFs and COR proteins as the mutant 5660M indicates that the recessive mutation of
284 *fin* in these lines causes cold sensitivity.

285 GO analysis of DEPs showed that 18 DEPs potentially involved in plant innate immunity were
286 upregulated in 3558M after cold stress. Among these, ten DEPs were involved in the
287 ROS-mediated plant-type HR (Fig. 4). Meanwhile, GO analysis showed that 63 DEGs associated
288 with oxidative stress were significantly upregulated after cold stress (Fig. 5A). ROS are signalling
289 agents that drive the hypersensitive response and are correlated with disease resistance (Asai &

Yoshioka, 2010). We found that the relative H₂O₂ concentration in 3558M was consistently higher than that in 3558 and reached a maximum value after cold stress (Fig. 2D). Consistent with the higher concentrations of ROS, two NADPH oxidase (RBOH) proteins involved in the generation of ROS were also found to be significantly upregulated after cold stress. RBOH proteins can generate ROS, which antagonize SA-dependent HR signals and suppress the spread of cell death (Torres et al., 2005). FLAVIN-DEPENDENT MONOOXYGENASE1 (FMO1) protein which is involved in defence activation and the HR in *Arabidopsis* (Bartsch et al., 2007) was upregulated after cold stress. VASCULAR ASSOCIATED DEATH1 (VAD1), a regulator of cell death and defence responses, was significantly upregulated after cold stress (Lorrain et al., 2004). Most of the proteins in the hypersensitive response pathway were not significantly different before cold stress but were significantly upregulated after cold stress. In addition, other DEPs involved in the hypersensitive response, such as EP3, CHIN, Calreticulin 3 (CRT3), HCHIB and RPM1, were upregulated after cold stress. At the transcription level, four RBOH and twenty CDPK genes were also significantly upregulated and had the same expression trends as those DEPs after cold stress. Taken together, these results indicate that 3558M experienced a strong ROS-mediated HR under cold stress. The activation of this response may explain why tiller damage and cell death in the SAM occur in 3558M after cold stress.

The results of the hormone content analysis showed that the content of the defence response-related hormone SA in 3558M was significantly higher than that in 3558 (Fig. 2C). Five DEPs involved in SA-mediated SAR were identified, namely FMO1, BGL2, DIR1, SABP2, and PR4. SABP2 is required to convert methyl salicylate to SA as part of signal transduction pathways that activate SAR in systemic tissue (Forouhar et al., 2005). The SABP2 protein was identified in both the nuclear proteome and the total proteome and was significantly upregulated after cold stress. The ankyrin-repeat protein NPR1 and WRKY TFs are the central positive regulators of SAR, transducing the SA signal to activate PR gene expression (Li et al., 1999). Five of six NPR1 genes were upregulated after cold stress at the transcriptional level. Three homeologs of NPR1-type protein 2 were significantly upregulated after cold stress. Multiple WRKY TFs have been shown to be involved in the SA-mediated SAR pathway. WRKY71 functions as a regulatory factor upstream of NPR1 and PR1b (Liu et al., 2007). Although NPR1 and WRKY TF proteins

were not detected by ITRAQ, seven pathogenesis-related (PR) proteins downstream of the SAR pathway were identified and found to be significantly upregulated: four PR1 proteins, one PRB1-3, one PRB1.2, and one PR10. In summary, the ROS-mediated HR and SA-mediated SAR were strongly activated after cold stress in 3558M, indicating that the normal cold stress induced multiple forms of plant innate immunity in 3558M.

Flowering genes and their gene regulatory networks were downregulated overall, resulting in delayed spike development in 3558M

Phenotypic analysis of 3558M revealed that spike development was inhibited and delayed in spring. Analysis of proteome and transcription data revealed many spike development genes, most of which were downregulated in 3558M, consistent with the inhibited spike development phenotype of 3558M. MADS-box TFs, such as *OsMADS14*, *OsMADS22*, *OsMADS34*, *OsMADS4*, *OsMADS50*, *OsMADS55*, and *OsMADS58*, are involved in the regulation of flowering time and the transition from the inflorescence meristem to the spikelet meristem phase. In the present study, 19 MADS-box TFs showed downregulated expression after cold stress ($P < 0.05$, Fig. 5C). The *SOC1* gene can repress the expression of *FRIGIDA* (*FRI*) and integrate signals from the photoperiod, vernalization and autonomous floral induction pathways in *Arabidopsis* (Liu et al., 2008; Lee et al., 2000). The three *TaSOC1* genes, which are homeologous MADS-box TFs located in the wheat A, B and D subgenomes, encode proteins sharing 86% and 62% amino acid identity with *SOC1* in rice and *Arabidopsis*, respectively. All three genes were significantly downregulated in 3558M compared with 3558 both before and after cold stress. The *LEAFY* (*LFY*) protein promotes early establishment of floral meristem identity by directly activating the *APETALA1*, *CAULIFLOWER* and *AGAMOUS* genes. Interestingly, all three *LFY* homeologs, which encode proteins sharing 83% and 64.8% identity with *LFY* in rice and *Arabidopsis*, respectively, were significantly downregulated in 3558M after cold stress. Three homeologs of *VRN-1* were also downregulated in 3558M. In particular, *TaAGL10* and *TaAGL11AP1* TFs, were downregulated at the protein level. Sixteen SBP-box TFs were differentially expressed between 3558 and 3558M, 11 of which were downregulated in 3558M at the transcription level after cold stress. One SBP transcription factor, Squamosa promoter-binding-like protein 13 (*SPL13*), was found to be downregulated at the protein level after cold stress. *SPL* proteins can specifically bind to the

consensus nucleotide sequence 5'-TNCGTACAA-3' in MADS-box TF promoters to activate expression and promote both vegetative phase change and flowering. Previous studies of 5660M have shown that, as in 3558M, genes involved in spike development and the floral transition (e.g., *VRN1* and *LFY*) are downregulated in 5660M (Zheng et al., 2013). The results indicate that the block or delay in spike development in 3558M and 5660M after exposure cold temperatures and is closely related to the later flowering of 3558M and 5660M tillers.

The reduced tillers phenotype is closely associated with the downregulation of PIN proteins

In the present study, we found that the contents of auxin in both 3558 and 3558M were significantly reduced after cold stress. Although the total contents of auxin in the whole SAMs organization of 3558M and 3558 were not significant differently, the auxin content in the top SAM organization of 3558M was significantly higher than that in the base SAM organization in 3558 (Fig. 2E). We speculate that auxin transport is inhibited and maintained at a low level at the base organization of the tillers where the lateral buds/tillers grow out. This may be an important cause of the production of fewer tillers. Multiple DEPs and DEGs were annotated as being involved in auxin signal transduction and significant downregulated in 3558M after cold stress. Two PIN proteins were found to be downregulated in the proteome of 3558M and western blot assays of the TaPIN proteins TaPIN1a and TaPIN1b showed that both proteins were significantly downregulated in 3558M compared with 3558, which was consistent with the results of the ITRAQ analysis. One aminopeptidase M1 protein (APM1), which can bind to the auxin transport inhibitor N-1-naphthylphthalamic acid and plays a negative role in the regulation of PIN auxin transport proteins (Murphy et al., 2006), was significantly upregulated in 3558M compared with 3558. Significant upregulation under cold stress was observed for one TOPLESS (TPL) protein, which functions as a co-repressor with AUXIN/INDOLE-3-ACETIC ACID (AUX/IAA) transcriptional repressors to bind ARF proteins and repress the expression of auxin response genes (Krogan et al., 2012; Szemenyei et al., 2008). There were 64 DEGs related to auxin signalling: 10 AUX1 genes, 22 AUX/IAA genes, 7 ARF genes, 19 SAUR genes and 6 GH3 genes ($P < 0.05$). Particularly, all of the seven ARF genes were significantly downregulated after cold stress. Taken together, these results show that the auxin signal transduction pathway might be influenced by

377 cold stress and also suggest that the reduced tillers phenotype of 3558M could be related to auxin
378 transport. When we used RNAi technology to knock down the *TaPINa* and *TaPINb* genes, spike
379 number was significantly reduced. However, the heading and maturation dates of the
380 *TaPINb*-RNAi (1954) lines were significantly earlier than those in the wild type, and there were
381 no significant differences in heading between *TaPINa*-RNAi (1955) lines and wild type. It is thus
382 clear that downregulation of *TaPINa* and *TaPINb* did not delay spike development. Therefore, the
383 delayed spike development and reduced spike number phenotypes were associated with a different
384 biological pathway(s).

385 **The *fin* recessive gene in 3558M influences multiple biological pathways to produce** 386 **multiple phenotypes changes**

387 Tiller formation has been intensely studied in rice and wheat. For example, *OsMOC1*, which
388 controls tillering and tiller formation, has been mapped to the long arm of chromosome 6 and
389 encodes a plant-specific GRAS transcription factor (Li et al., 2003). In bread wheat, the recessive
390 gene *tin* inhibits tiller formation and has been mapped to the short arm of chromosome 1AS
391 (Hyles et al., 2017). The candidate gene for *tin* has been cloned and encodes a cellulose
392 synthase-like protein with homology with members of the CsIA clade (Hyles et al., 2017). We
393 have performed related research on tiller formation and spike development in our lab. In a
394 previous study, we mapped the *fin* gene from a wheat-*Agropyron cristatum* (L.) Gaertn.-derived
395 line 3558M to 1AS and found that it was closely linked (1.0 cM distance) to marker Xcfa2153
396 (Zhang et al., 2013). The *tin* gene is also closely linked to marker Xcfa2153 (Hyles et al., 2017).
397 To further determine whether the two genes are the same locus, we analysed variation of the *tin*
398 gene and found that the *tin* gene sequence was the same between 3558M and 3558 (unpublished
399 data). This indicates that *fin* and *tin* are likely not the same gene, although they cause a similar
400 low tiller number phenotype. In this report, we found that the *fin* gene led to multiple phenotypic
401 differences between 3558M and 3558 wheat. The *fin* gene not only altered the tiller number but
402 also delayed spike development and caused sensitivity to normal cold stress. There were multiple
403 other phenotypes (e.g., large spikes with high grain no. and weight) at maturity. Systematically
404 investigation of the differences between 3558M and 3558 using proteomic, RNA-seq and RNAi
405 technology provided insight into which metabolic pathways are potentially regulated by the *fin*

406 gene.

407 First, the wheat line 3558M is cold sensitive and not response to normal cold stress at the
408 regeneration stage. The ICE-CBF-COR signal cascade system was intensely active to response to
409 normal cold stress in both 3558M and 5660M wheat. After extreme cold stress, the
410 ROS-mediated HR and defence response in 3558M wheat were activated and led to the death of
411 the apexes of 3558M tillers. As a result of the apex death, sections of tillers did not develop into
412 spikes. In response to normal cold stress, multiple innate immune responses (e.g., the HR-, SAR-
413 and FLS2-induced pathogen-associated defence systems) were activated in 3558M wheat.

414 Second, consistent with the known role of *OsPIN* genes in the regulation of tiller development
415 in (Chen et al., 2011), two downregulated TaPIN proteins and multiple DEGs involved in the
416 auxin signalling pathway were identified. Moreover, all of the ARFs, which positively regulate
417 the auxin signalling pathway, were downregulated after cold stress, and all of the *TaGH3* genes,
418 which negatively regulate the auxin signalling pathway, were significantly upregulated (Staswick
419 et al., 2005). The auxin content was also reduced after cold stress, especially in the SAM of
420 3558M. These findings suggest that mutant *ftin* gene influence the auxin signalling pathway and
421 that cause the reduced tiller phenotype of 3558M. This notion is further supported by the finding
422 that knockdown of *PIN* gene expression causes a reduced tiller phenotype. A previous study
423 showed that the regulator OsNPR1, which regulates the SAR pathway, can affect rice growth and
424 development by disrupting the auxin pathway at least partially through indirectly upregulating
425 OsGH3.8 expression (Li et al., 2016). In the present study, we identified six *TaNPR1* genes at the
426 transcription level, five of which were significantly upregulated after cold stress. Furthermore,
427 the death of SAM caused by ROS-mediated HR affects the synthesis and distribution of auxin.
428 Taken together, our results suggest that the reduced tillering phenotype of 3558M is caused by a
429 disrupted auxin signalling pathway that might result from the activation of the SAR pathway and
430 multiple active defence responses.

431 Third, the delayed floral transition is associated with the downregulation of flowering network
432 genes. Using our data, we attempted to elucidate the mechanism by which the *ftin* gene interferes
433 with/regulates the flowering pathway to delay the floral transition of 3558M. The floral transition
434 in wheat is regulated by the three types of genes vernalization (*Vrn*), photoperiod (*Ppd*) response,

435 and earliness *per se* (*EPS*) and their interactions with temperature. In the present study, we found
 436 that the expression of the clock regulators *PRR5* and *PRR7*, and flowering genes that play key
 437 roles in photoperiodic flowering responses, *GI*, *CRY*, and *FT*, was slightly upregulated in 3558M
 438 (Table S7.1). We also compared the expression of the *Vrn* genes, including repressors and
 439 activators. In our unpublished data, we identified 18 MADS-box TFs as repressors that were
 440 silenced by vernalization and 9 MADS-box TFs as activators induced when vernalization is
 441 complete and the floral transition is initiated. Among the 18 repressors, 11 MADS-box TFs were
 442 completely silenced in 3558M; there was no significant change in the expression of the other 7
 443 MADS-box TFs (Table S7.2). We also compared the expression of the *VRN2* (*ZCCT1* and *ZCCT2*)
 444 genes and found they were completely silenced in both 3558M and 3558. These results show that
 445 delayed floral transition is likely not caused by alteration of the photoperiodic or vernalization
 446 pathway. We identified three *TaSOC1* gene homeologs and three *LFY* homeologs, which are
 447 downstream targets of *SOC1*. Both sets of homeologs, which positively regulate spike
 448 development, were significantly upregulated. In *Arabidopsis*, crosstalk between the cold response
 449 and flowering pathways is mediated by *SOC1* (Seo et al, 2009). It has been reported that *SOC1*
 450 can directly bind to the promoters of *CBF* genes and repress their expression. In *Arabidopsis*, *SVP*,
 451 another flowering repressor, plays an important role in the response of plants to ambient
 452 temperature changes (Lee et al., 2007) and associates with the promoter region of *SOC1* to
 453 directly repress its transcription in the shoot apex. In the present study, we found that both alleles
 454 of *TaSVP* were significantly upregulated in 3558M, especially after cold stress. The expression
 455 patterns of *TaSVP*, *TaSOC1* and *TaLFY* genes in 3558M under cold stress are consistent with its
 456 delayed spike-development phenotype. Therefore, we speculate that after cold stress, *TaSVP*
 457 senses the ambient temperature change and represses the expression of *TaSOC1* and *TaLFY*. The
 458 *ftin* gene in 3558M delays spike development through the SVP-SOC1-LFY thermosensory
 459 pathway.

460 To sum up, although we have not cloned the *ftin* mutant gene, according to the current data, the
 461 *ftin* mutant gene might be a cold-sensitive mutation and that is the cause of multiple metabolic
 462 pathways and phenotypic changes.

463

464

465 **Materials and methods**

466 **Plant materials**

467 The wheat mutant line 3558M (*T. aestivum* L., $2n = 6x = 42$, AABBDD) and the NIL 3558 (*T.*
468 *aestivum* L., $2n = 6x = 42$, AABBDD) are near isogenic lines obtained from the seventh
469 generation derived from the cross Fukuhokomugi (*T. aestivum* L., $2n = 6x = 42$, AABBDD) /A.
470 *cristatum* Z559 (*A. cristatum* (L.) Gaertn., $2n = 4x = 28$, PPPP; National Gene Bank accession
471 number Z559)/Youla (*T. aestivum* L., $2n = 6x = 42$, AABBDD) (Zhang et al., 2013). Both of
472 them are winter wheat and planted on the three plots in Jinan on October7, 2014 and October10,
473 2016 in autumn at above 20 °C. Each plot contained 24 rows, 2 m in length, and separated by
474 0.25 m. Crop management was according to the local practice. Both of them would go through a
475 long winter and the tillers will be stop to product as the temperature usually falls below zero. In
476 the spring of next year, the tillers will be going on tillering as the temperature rises. Based on the
477 period development theory, the spike differentiation of winter wheat is divided into several
478 important physiological stages: single ridge stage (vegetative growth), double ridge stage (floral
479 transition), spikelets differentiation stage, florets differentiation (pistils and stamens). The SAM of
480 3558M and 3558 were harvested at the double ridge stage before and after a normal cold stress
481 when the temperature has a zero degree fluctuations (Fig 1.D). Finally, approximately 3 g SAM of
482 3558M and 3558 were collected and stored at -80°C in a cryogenic refrigerator to extract protein,
483 hormone and RNA. The common spring wheat cultivar Fielder is used to transgenic experiments.
484 The seeds of Fielder were grown in a greenhouse at 25 °C with a photoperiod of 16 h light and 8 h
485 dark for 7–10 d. The leafs of Fielder (Wild type) and transgenic Fielder (1954-1 and 1955-4) at the
486 double ridge stage were harvested to quantitative RT-PCR and Southern blotting analysis. As the
487 spring wheat, the Fielder and transgenic Fielder did not experience a low temperature stress during
488 their life cycle.

489 **Methods**

490 Determination of plant hormone content: The SAMs of twelve samples (Table S8, two varieties
491 × three biological replicates × two time points, ~100 mg each) were harvested from the tillers of

492 3558MU and 3558 at T1 (on Mar. 11, 2017) and T2 (on Mar. 15, 2017), immediately frozen in
493 liquid nitrogen, and ground into powder. Hormone extraction and ESI-Q TRAP-MS/MS analysis
494 were performed according to the protocol of Wuhan Metware Biotechnology Co., Ltd.

495 Proteomic analysis

496 protein extraction and labeled with iTRAQ 4plex: About the total proteome, the shoot apical
497 meristem of eight wheat samples (Table S8, two varieties ×2 biological replicates × two time
498 points ~100mg each) were harvested from the tillers of the 3558MU and 3558 at the T1 (on Mar. 7,
499 2015) and T2 (on Mar. 14, 2015).

500 The SAM of tillers of wheat 3558M and 3558 were ground into powders in liquid nitrogen, and
501 the powders were each suspended overnight in 30 mL of a 10% w/v TCA/acetone solution that
502 contained 0.07% v/v mercaptoethanol at 20°C. After centrifugation at 40 000 × g for 1 h at 4°C, the
503 pellets were each washed with 30 mL of ice-cold acetone. After vacuum drying, pellets were
504 reconstituted in 900ul 8M guanidine and pH was adjusted to >7.5 with 1M Tris HCl pH8.1.
505 Disulfide bond was reduced by addition of 20 mM DTT and incubated at 60°C for 30 min. After
506 cooling to room temperature, 40 mM iodoacetamide was added and the samples were kept in the
507 dark for 1 hour to block free cysteine. Protein assay was done at this step with Bradford method.
508 The samples were dialyzed against 2 M urea and 50 mM NH₄HCO₃ for 2 hours and 50 mM
509 NH₄HCO₃ for 2 hours twice. 200ug of protein was digested by Trypsin at 1:50 (w:w,
510 trypsin:sample) and incubated at 37°C overnight. The digested peptides were further filtered with
511 Amicon ultra centrifugal filters (10kD MWCO, Merck Millipore, MA), dried under vacuum
512 extensively to remove residual primary amine from reagents. ½ of the digested samples were
513 labeled with iTRAQ 4plex reagent (ABSciex, MA, US) according manufacturer's instructions and
514 combined after labeling and dried. About the nuclear proteome, four wheat samples (two varieties
515 ×2 biological replicates ~500mg each) were harvested from the tillers of the 3558MU and 3558 at
516 the T2 (on Mar. 14, 2015). The proteins were extracted and then labeled with iTRAQ 4plex
517 reagent according to above methods.

518 MS identification: First dimensional separation of TMT labeled peptides High-pH Reverse
519 phase HPLC were used for peptide fractionation using Gilson 300 series. The iTRAQ4plex

520 labeled and combined samples were solubilized in 200 μ l of 20 mM ammonium formate (pH10),
 521 and injected onto an Xbridge column (Waters, C18 3.5 μ m 2.1X150 mm) using a linear gradient
 522 of 1%B/min from 2-45% of B (buffer A: 20 mM ammonium formate, pH 10, B: 20 mM
 523 ammonium formate in 90% acetonitrile, pH10). 1 min fractions were collected and speed vac
 524 dried. 2.2.2 Nano LC-MSMS Selected fractions were analyzed by nanoLC-MS/MS using a RSLC
 525 system interfaced with a Q Exactive™ Hybrid Quadrupole-Orbitrap Mass Spectrometer
 526 (ThermoFisher, San Jose, CA). Samples were loaded onto a self-packed 100 μ m x 2cm trap
 527 packed with Magic C18AQ, 5 μ m 200 A (Michrom Bioresources Inc, Auburn, CA) and washed
 528 with Buffer A(0.2% formic acid) for 5 min with flow rate of 10 μ l/min. The trap was brought
 529 in-line with the homemade analytical column (Magic C18AQ, 3 μ m 200 A, 75 μ m x 50cm) and
 530 peptides fractionated at 300 nL/min with a multi-stepped gradient (4 to 15% Buffer B (0.16%
 531 formic acid 80% acetonitrile) in 35 min and 15-25%B in 65 min and 25-50%B in 55 min). Mass
 532 spectrometry data was acquired using a data-dependent acquisition procedure with a cyclic series
 533 of a full scan acquired in Orbitrap with a resolution of 120,000 followed by MSMS scans (30% of
 534 collision energy in the HCD cell) of 20 most intense ions with a repeat count of one and the
 535 dynamic exclusion duration of 30 sec.

536 Databases Searching: The LC-MSMS peak list of 13 fractions from each experiment were
 537 searched in MUDPIT style against the corresponding Uniprot database (Uniprot_Triticum
 538 aestivum (Wheat)_20150401 , 94854 sequence) using an in house version of X!tandem
 539 (SLEDGEHAMMER (2013.09.01), the gpm.org) with carbamidomethylation on cysteine and
 540 iTRAQ label on lysine and N-terminus of peptides as fixed modification and oxidation of
 541 methionine as variable modification. A +/-5 ppm and 20 ppm were used as tolerance for precursor
 542 and product ions respectively. Intensity of iTRAQ4plex reporter ions of each spectrum was
 543 extracted using an in-house perl script and corrected for isotope cross-over using values supplied
 544 by the manufacturer. The False Discovery Rate was estimated for all samples by using a reverse
 545 database (FDR<0.01). The treatment/blank control ratio of each spectrum was calculated using
 546 reporter ion intensity and normalized to the median ratio of all identified spectra that fit certain
 547 criteria: peptide belongs to homo sapiens database, peptide log (E)<=-2 (E value<=0.01), total

reporter ion intensity >40,000 for each pair. Spectra that have ratio as “Divide 0” were replaced with an arbitrary number “10”. Pairwise ratios (115/114 and 117/116) of individual protein were calculated using median ratio of all peptides belonging to the protein that has at least two data points that fit the criteria of peptide log₂ < -2 and sum of reporter ions of both channel >20,000.

Transcriptome analysis

RNA isolation, cDNA library construction and sequencing: the shoot apical meristem of 12 wheat samples (Table S8, two varieties × three biological replicates × two time points ~100mg each) were harvested from the tillers of the 3558MU and 3558 at the T1 (on Mar. 11, 2017) and T2 (on Mar. 15, 2017). Total RNA was extracted using RNeasy Plant Mini Kit (TIANGEN, DP432) according to the manufacturer’s protocol. RNA purity was checked using the NanoPhotometer® spectrophotometer (IMPLEN, CA, USA). RNA concentration was measured using Qubit® RNA Assay Kit in Qubit® 2.0 Fluorometer (Life Technologies, CA, USA). RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA). The sequencing libraries preparation and high-throughput sequencing were carried out by Novogene Bioinformatics Technology CO., Ltd. (Beijing, China, <http://www.novogene.com>).

Western blot assay and quantitative RT-PCR: The protein extracts were from the SAMs of wheat 3558MU_T2, 3558_T2, Fielder wheat and their transgenic lines 1954-1 and 1955-4 (Table S8). The western blot assays and quantitative RT-PCR were performed as described by Zheng et al. (2013 & 2017). Specific primers for TaPINa and TaPINb (TaPINa-F: GTGGACCATCACGCTCTTCT, TaPINa-R: GAGCATGAGCGTGACCAGA; TaPINb-F: CTCTCTCGGACAAGGAAACG, TaPINb-R: AGCAGGCTTGGTGAAGAAGA) were used to detect expression levels. The relative expression levels were calculated using the relative 2^{-ΔΔCt} method.

RNA interference and Southern blotting analysis: The conserved coding sequence of *TaPINa* (784 to 1036 bp) and *TaPINb* (1013 to 1312 bp) were selected separately as the RNAi target sequence and used to design a RNAi hairpin structure that was inserted into pBIOS2043. After sequencing the target sites, the binary vector was transformed into the wheat cultivar Fielder by

576 *Agrobacterium tumefaciens*-mediated transformation. The gene-specific primers
577 5'-ATTCTTATTTCTTTCCAGTAGC and 5'-AGAAGCGGCATAATGTGAGA were used to
578 amplify a 478-bp fragment of the FAD2 intron, a fragment of the interference vector. Genomic
579 DNA was extracted from the leaves of T3 transgenic plants (Table S8, lines 1954-1 and 1955-3)
580 using a plant genomic DNA Extraction Kit (Tiangen Biotech, Beijing, China). About 20 µg of
581 DNA was successfully digested with 5 U of EcoRV and incubated at 37 °C for 24 h. The digested
582 genomic DNA fragments were separated on a 0.8% (w/v) agarose gel, and transferred onto
583 Zeta-Probe GT nylon membrane (Bio-Rad, Hercules, CA, USA). The DNA fragments were fixed
584 to the membrane by UV cross linking. The 1000-bp PCR fragment of the FAD2 intron was labeled
585 with digoxin. Probe labeling, hybridization, washing and detection were performed according to
586 instructions of the DIG-High Prime DNA Labeling and Detection Starter Kit II (Roche, Mannheim,
587 Germany).

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Fig.1 Multiple phenotypic differences between wheat 3558M and NIL line 3558. A. Statistical analysis of the differences in number of spikes, tillers, and grains per spike between 3558MU and 3558 (**stands for $p < 0.01$). B. Anatomical analysis of the differences of SAM in several important physiological stages of single ridge (b1 and b2), double ridge (b3 and b4), spikelets differentiation (b5 and b6). b-7, the death of 3558M meristem. C. At heading time, 3558MU had significantly fewer spikes and more grains per spike compared with 3558. D The time points at which samples were taken for RNA sequencing and protein identification (between the two time points T1 and T2, there was a significant cooling change about zero degrees centigrade).

Fig. 2 Measurement of hormone and H₂O₂ (a reactive oxygen species) contents of 3558 and 3558M (**stands for $p < 0.01$). A and B. The contents of hormones present at concentrations below 10 ng/g (A) and between 10 and 100 ng/g (D). C and D. The contents of salicylic acid (C) and H₂O₂ (D). E. The contents of IAA in top and base SAMs of 3558 and 3558M after cold stress.

Fig. 3 Enriched GO Terms ($P < 0.05$) for differentially expressed proteins of wheat 3558M and 3558WT before and after cold stress. The red stand for upregulated DEPs and the blue stand for downregulated DEPs

Fig. 4 Volcano plot of DEGs and enriched GO Terms ($P < 0.05$) for wheat 3558MU and 3558WT. A and B. The volcano plots showing the number of DEGs before cold stress (A) and after cold stress (B). The red points stand for upregulated DEGs, and green points stand for downregulated. C. Enriched GO biological process terms for wheat 3558M_T2 and 3558_T2 after cold stress.

Fig. 5 Heatmaps and the gene expression levels of DEPs with different functions. A. Heatmap of the DEPs involved in the biological processes of response to oxidative stress, cold stress and DEPs annotated as TFs containing different functional domains (WRKY, NAC and AP2-EREBP). The red stand for upregulated DEGs, and blue stand for downregulated DEGs. B. The gene expression levels of TaCBF TFs, which are involved in cold stress response. C. The gene expression levels of TF DEPs that contain the MADS-box domain, which are involved in flowering development.

Fig. 6 Analysis of TaPINa and TaPINb mRNA and protein accumulation, phenotypic characteristics, and insert number in transgenic RNAi-TaPINa and RNAi-TaPINb plants. A. the genes expression levels of TaPINa and TaPINb in wheat 3558M and 3558; B and C. the proteins expression levels of TaPINa and TaPINb in wheat 3558M and 3558; RNAi-TaPINa, RNAi-TaPINb lines and the wild-type (WT) control, as determined by western blot analysis; D and E. The tiller number phenotype in RNAi-TaPINa, RNAi-TaPINb and WT plants. F. Southern blot analysis revealed three gene copies in RNAi-TaPINa (1954) and one gene copy in RNAi-TaPINb (1955). WT was included as a negative control. G. qRT-PCR analysis of the expression levels of *TaPINa* and *TaPINb* in the RNAi-TaPINa and RNAi-TaPINb lines, respectively, and the WT control (**stands for $p < 0.01$). H. Statistical analysis of the number of tillers and spikes (**stands for $p < 0.01$)