~~Regulation~~ of nitrogen topdressing and paclobutrazol at different stages on spike differentiation and yield of winter wheat

Dongxiao Li [1] , **Shaojing Mo** [1] , **William D. Batchelor** [2] , **Ruiting Cheng** [1] , **Hongguang Wang** [1] , **Ruiqi** [Corresp. 1]

[1] Hebei Agricultrual University, Baoding, China

[2] Auburn University, Auburn, United States of America

Corresponding Author: Ruiqi
Email address: wheatlrq@163.com

Background. Optimal nitrogen (N) application and plant growth regulators can improve wheat productivity. This can help to improve yield level and ensure food security with limited resources in the Huang-Huai-Hai Plain of China (HPC). **Methods.** A 2-year field experiments were conducted using a randomized block design with four treatments (TS–nitrogen topdressing at pseudostem erection stage ; TPS–TS combined with paclobutrazol application; TJ–nitrogen topdressing at jointing stage; TPJ–TJ combined with paclobutrazol application) in 2011-2013. **Results.** The grain number per ear, thousand kernel weight and yield for the TJ and TPJ treatments were higher than those of the TS and TPS treatments. Grain number per ear, yield, and thousands kernel weigh for the TPJ treatment were significantly higher than for the TS and TPS in 2011-2012 (9.82% and 7.27%, 10.23% and 8.99%, 6.12% and 5.58%) and in 2012-2013 (10.21% and 11.55%, 8.00% and 6.58%, 0.00 and 0.00), respectively. And thousands kernel weight under TJ were significantly higher than those under TS and TPS by13.21% and 14.03%, respectively in 2012-2013. The floret number, significantly correlated with cytokinin content, was also significantly increased under TJ and TPJ at connectivum differentiation stage. For TPJ treatment, the floret number was significantly higher than for the TS, TPS, and TJ by 19.92%, 10.21%, 6.10% in 2011-2012; it was higher than for the TS and TPS by 28.06% and 29.61% in 2012-2013, respectively. The relative expression level of cytokinin oxidase/ dehydrogenase gene (*TaCKX2.2*) in response to cytokinin increasing was improved to regulate hormonal balance. **Conclusions**. Therefore, nitrogen topdressing at jointing stage had increased grain number per ear, thousand kernel weight, and grain yield of wheat. Paclobutrazol could delay spike differentiation and promote cytokinin accumulation that induced expression of *TaCKX2.2*, maintaining hormonal balance and affecting wheat spike morphogenesis.

1   **~~Regulation~~ of nitrogen topdressing and paclobutrazol at different stages on**

2   **spike differentiation and yield of winter wheat**

3   Dongxiao Li[1], Shaojing Mo[1*], William D. Batchelor[2], Ruiting Cheng[1], Hongguang Wang[1] and Rui-Qi Li[1]

4   [1] State Key Laboratory of North China Crop Improvement and Regulation / Key Laboratory of Crop Growth

5   Regulation of Hebei Province / College of Agronomy, Hebei Agricultural University, Baoding, Hebei Province,

6   071000, China.

7   [2]Biosystems Engineering Department, Auburn University, Auburn, Alabama, 36849, United States of America

8   * These authors contributed equally to this work.

9   Corresponding author. Ruiqi Li, wheatlrq@163.com

10   **Abstract.**

11   **Background.** Optimal nitrogen (N) application and plant growth regulators can improve wheat

12   productivity. This can help to improve yield level and ensure food security with limited resources

13   in the Huang-Huai-Hai Plain of China (HPC).

14   **Methods.** A 2-year field ~~experiments were~~ conducted using a randomized block design with four

15   treatments (TS–nitrogen topdressing at pseudostem erection stage; TPS–TS combined with

16   paclobutrazol application; TJ–nitrogen topdressing at jointing stage; TPJ–TJ combined with

17   paclobutrazol application) in 2011-2013.

18   **Results.** The grain number per ear, thousand kernel weight and yield for the TJ and TPJ treatments

19   were higher than those of the TS and TPS treatments. Grain number per ear, yield, and thousands

20 kernel weigh for the TPJ treatment were significantly higher than for the TS and TPS in 2011-

21 2012 (9.82% and 7.27%, 10.23% and 8.99%, 6.12% and 5.58%) and in 2012-2013 (10.21% and

22 11.55%, 8.00% and 6.58%, 0.00 and 0.00), respectively. And thousands kernel weight under TJ

23 were significantly higher than those under TS and TPS by13.21% and 14.03%, respectively in

24 2012-2013. The floret number, significantly correlated with cytokinin content, was also

25 significantly increased under TJ and TPJ at connectivum differentiation stage. For TPJ treatment,

26 the floret number was significantly higher than for the TS, TPS, and TJ by 19.92%, 10.21%, 6.10%

27 in 2011-2012; it was higher than for the TS and TPS by 28.06% and 29.61% in 2012-2013,

28 respectively. The relative expression level of cytokinin oxidase/ dehydrogenase gene (*TaCKX2.2*)

29 in response to cytokinin increasing was improved to regulate hormonal balance. **Conclusions**.

30 Therefore, nitrogen topdressing at jointing stage had increased grain number per ear, thousand

31 kernel weight, and grain yield of wheat. Paclobutrazol could delay spike differentiation and

32 promote cytokinin accumulation that induced expression of *TaCKX2.2*, maintaining hormonal

33 balance and affecting wheat spike morphogenesis.

34 **Keywords** topdressing stages, floret number, phytohormone, spike differentiation, *TaCKX2.2*

35 **INTRODUCTION**

36 The North China Plain is one of the most important grain production regions in China and is

37 experiencing conflicts between limited natural resources and crop production. Excessive use of

38 nitrogen fertilizer is common in order to increase yields in the wheat-maize planting system. This

39 often leads to large losses of nitrogen, resulting in serious environmental pollution (*Azam Shah et*

40   *al. 2009; Wang et al. 2010*). The planting area of winter wheat has been decreasing in order to

41   enhance ecological benefits and due to limited water available for irrigated due to historic over-

42   exploitation of ground water (*Xu et al. 2005*). In order to achieve China's food security goals, it is

43   important to ~~improving~~ wheat yields. Wang *et al*. (2015) found that a key factor for increasing

44   wheat yield was increasing the grain number per spike and seed weight, which is influenced by

45   nitrogen fertilizer in the high-yield wheat region of Hebei Province. Nitrogen fertilizer promotes

46   allocation of assimilate and increases assimilation amount and contribution rate of post-anthesis

47   photosynthate to grain (*Ma et al. 2008a*). There are three nitrogen absorption peaks of winter wheat

48   including fall vegetative growth before winter, the jointing-booting stage, and the flowering-filling

49   stage. The transport of carbon assimilates to grain was found to be more efficient with nitrogen

50   topdressing at the jointing stage than at the flowering stage (*Zhai and Li, 2006*). However, the

51   nitrogen application should be delayed until booting stage under super-high-yield conditions (*Kara*

52   *and Uysal, 2009; Liu et al. 2019*). The time of optimal nitrogen application is often related to local

53   water conditions and wheat varieties. Under drought conditions or limited irrigation, nitrogen

54   topdressing applied at the jointing stage was more beneficial to transport carbon photosynthate to

55   grain compared to the flowering stage for winter wheat (*Gevrek and Atasoy, 2012*). However, for

56   spring wheat, it was more beneficial to apply topdressing nitrogen at the flowering stage(*Walsh et*

57   *al. 2018*). Under irrigated conditions, a later topdressing application enhanced photosynthesis and

58   increased wheat yield (*Chen et al. 2008*).

59   Paclobutrazol (pp333), a plant growth regulator that inhibits synthesis of endogenous

60   gibberellin, is easily absorbed by wheat root, stem, and leaf. The application of paclobutrazol can

61 inhibit crop height, increase stress tolerance, promote tillering, and increase spike number and

62 yield (*Hajihashemi et al. 2007; Gómez et al. 2011; Peng et al. 2014; Dwivedi et al. 2017*).

63 Combining nitrogen topdressing with paclobutrazol can also improve photosynthetic rate, increase

64 grain weight per spike, and achieve further increase in wheat yield when applied at the jointing

65 stage (*Yang et al. 2008*). A combination of nitrogen topdressing at the pseudostem erection stage

66 with a paclobutrazol application can increase dry matter accumulation, grain weight per spike, and

67 grain yield *(Zhang et al. 2017*). The appropriate amount of nitrogen and paclobutrazol can increase

68 stem lodging resistance, nitrogen uptake and maintain high duration of the flag leaf to promote

69 high and stable yield (*Chen et al. 2011; Nouriyani et al. 2012a,b*). Paclobutrazol also regulated

70 root morphological characteristics, maintained physiological function and root activity, increased

71 grain yield by enhancing the levels of osmolytes, endogenous hormone contents, and antioxidant

72 activities under adverse environmental conditions (*Soumya et al. 2017; Kamran et al. 2018*).

73 Additionally, nitrogen can also regulate endogenous hormone content and affect ear and flower

74 development (*Ma et al. 2008a*). Spikelet number, regulated by hormones in spike and roots, was

75 closely related to nitrogen supply (*Chen et al. 2008; Zhang et al. 2009*). At the early grain

76 formation stage, zeatin riboside (ZR) content could effectively regulate panicle flower

77 development and spikelet number (*Ma et al. 2008a*). Decreasing cytokinin and ABA contents in

78 grain and root would be possible resulting in sterile spikelet (*Chen et al. 2008; Zhang et al. 2009*).

79 Paclobutrazol application could influence hormone directly and modify flowering and

80 development of plants (*Zhang et al. 2016*). During ear differentiation, cytokinins

81 oxidase/dehydrogenase (CKX), controlling endogenous cytokinins content, often negatively

82   regulates time of flowering and grain formation of crops (*Zalewski et al. 2010; Yeh et al. 2015;*

83   *Ashikari et al. 2005; Bartrina et al. 2011*). For wheat, *TaCKX2.2* was involved in the formation of

84   the spike grain number and yield (*Zhang et al. 2011*). Inhibiting *TaCKX2* expression by RNAi

85   increased the grain number per spike in bread wheat plants (*Li et al. 2018*). Previous studies have

86   shown that the optimal application amount of a nitrogen and paclobutrazol combination at the stem

87   elongation stage (*Nouriyani et al. 2012a,b*) and nitrogen topdressing at the jointing stage was

88   advantageous for yield and grain quality (*Wu et al., 2014*). It is not clear what the optimum

89   topdressing nitrogen combined paclobutrazol application is for optimal ear differentiation and

90   individual wheat productivity, nor how changing phytohormones and related gene expression

91   occur in plants under different nitrogen topdressing treatments in the North China Plain. Our

92   hypothesis was that nitrogen topdressing and paclobutrazol application at different stages would

93   influence cytokinin (IPA and ZR) content of spike differentiation. Accordingly, the related gene

94   expression varied. Combining molecular biology methods and crop growth analysis, we explored

95   the differential expression law of cytokinin synthesis related genes in individual spike

96   differentiation that directly influence grain yield. The objective of this study was to 1) evaluate the

97   effect of topdressing nitrogen with paclobutrazol application on spike differentiation, and 2)

98   elucidate the possible mechanisms of improving the productivity of wheat. The focus of this study

99   was to determine the impact of N and paclobutrazol on individual spikelet formation, resulting

100   differences in final yield, and to determine how phytohormones and related gene expression

101   changes during spike differentiation. This study will help to provide guidance for optimizing

102   fertilizer and increasing high yield potential of wheat in the North China Region.

103   **Materials and methods**

104   *Experimental site and soil*

105   A field experiment was carried out from 2011 to 2013 on super-high-yield testing farmland in

106   Gaocheng, Shijiazhuang (37°79'N, 115°31'E), China. The climate was classified as subhumid

107   continental monsoon. The mean annual rainfall is typically less than 520 mm, one-third of which

108   typically falls during the wheat-growing season. Winter wheat-summer maize rotation is the

109   typical planting system in this region. The soil type was light-loamy Chao Soil and the soil

110   properties are shown in Table 1.

111   *Experimental design and crop management*

112   The experiment was a randomized complete block design with four experimental treatments:

113   1) nitrogen topdressing at pseudostem erection stage (TS), 2) TS with paclobutrazol application at

114   pseudostem erection stage (TPS), 3) nitrogen topdressing at the jointing stage (TJ), and 4) TJ with

115   paclobutrazol application at the jointing stage (TPJ). Each treatment was replicated three times.

116   Each plot area was 60 m^2.

117   The cultivar Shimai18 was provided by Shijiazhuang Academy of Agricultural Sciences,

118   Shijiazhuang, Hebei. This variety was planted on October 7, 2011 and October 9, 2012 with a row

119   spacing of 0.15 m and plant density of 270◊10^4 plants ha^{-1}. Fertilizers were applied before planting

120   at rates of 112 kg ha^{-1} nitrogen, 60 kg ha^{-1} phosphorus, 87 kg ha^{-1} potassium and 9 kg ha^{-1} zinc.

121   Topdressed N was applied at either pseudostem erection stage or jointing at a rate of 112 kg ha^{-1}.

122   Fore-rotating corn straw from the previous crop was crushed 2 times and returned to the field at a

123   15 cm depth. Paclobutrazol (provided by Shenggong Bioengineering (Shanghai) Co., LTD) was

124 applied by a foliar spray of aqueous solution containing 10% wettable powder at an effective

125 concentration of 200 mg kg^{-1} which equated to a rate of 675 kg ha^{-1} at either pseudostem erection

126 or jointing stage.

127 *Variable measurements*

128   Young ears were sampled and then quickly-frozen at −80°C in the differentiation stages of

129 stamen and pistils, early connectivum, late connectivum, and tetrad formation, separately. Partial

130 young ears marked on the same flowering date were sampled at 0, 3, 6, 9 and 12 days after

131 flowering. Beginning with the regreening stage, representative wheat plants were selected at 3

132 days intervals. The numbers of spikelets and florets were recorded in a constant state (on about

133 21st April) using an anatomical lens (Stemi 2000-c, Germany).

134   Total ribonucleic acid (RNA) was extracted from wheat samples with a PlantRNA Kit

135 (Tiangen, Beijing, China) according to the manufacturer's protocol. RNA concentration and purity

136 were detected by spectrophotometer (Nanodrop, USA); the RNA integrity was detected by agarose

137 gel electrophoresis. cDNA obtained by reverse transcription of RNA was used for subsequent

138 fluorescence quantitative expression. First-strand cDNA was generated from 2 μg of total RNA

139 using a PrimeScript II First Strand cDNA Synthesis Kit (TaKaRa, Dalian, China). The primer used

140 for qRT-PCR were designed according to the published sequence *TaCKX2* (geneID: GU084177.1)

141 by software Primer 5 (Zhang et al., 2011). Priming sequence: GGGAGAAGAAGCACTTTGGTC

142 (*TaCKX2.2*-F); CCTGCAGTAAACTCAAACCATATC (*TaCKX2.2*-R). The optimal reference

143 gene *GAPDH* (GAPDH-F: TGTCCATGCCATGACTGCAA; GAPDH-R:

144 CCAGTGCTGCTTGGAATGATG) was selected for mRNA transcription studies using

145   quantitative real-time polymerase chain reaction (PCR). All PCR reactions were repeated three

146   times and the data were normalized to constitutively expressed TaGAPDH according to the 2–

147   ΔΔCt method described by Livak and Schmittgen (2001). Thermal-cycling conditions included an

148   initial denaturation at 95°C for 5 s, followed by 45 cycles at 95°C for 5 s, 60°C for 10 s, and 72°C

149   for 15 s, then a final melt step from 60°C to 95°C.

150   Endogenous phytohormone content (IAA, auxin; ABA, abscisic acid; GA, gibberellin;

151   cytokinin, CTK including isopentenyl adenosine-IPA and trans zein nucleoside-ZR) was measured

152   by enzyme linked immunosorbent assay (ELISA) (Li et al., 2017). The test kit was provided by

153   College of Agriculture and Biotechnology, China Agricultural University, Beijing, China. Young

154   ear samples were collected from each treatment and cut into 0.5 g pieces and were ground to a fine

155   powder in liquid nitrogen with a pre-cooled mortar. The powder was diluted with extracting

156   solution and was extracted over 4 h at 4 ℃ in cold 80% methanol based on the protocol outlined

157   by Oliver *et al.* (2007). The homogenate was then centrifuged at 3500 rpm min^{-1} for 20 min, and

158   the supernatant was collected and washed by 80% methanol in a C-18 solid phase extraction

159   column. Next, the filtered fluid of all the samples was pooled and dried in a vacuum chamber.

160   Sample diluent was added to a final volume of 1 mL, which was diluted in tris-buffered saline and

161   GA, CTK, ABA, IAA concentrations were measured according to Jin *et al.* (2011).

162   Plant height was measured at pseudostem erection, jointing, booting, and ripening stages,

163   respectively. Yield and yield components were measured by conventional methods. The harvest

164   area was 3 m^2 for each plot. All plants were harvested, threshed, and dried to weigh seed yield.

165   And 1000-kernel weight was determined using the sum of two 500-kernel sample weights

166 according with absolute value of the difference between two 500-kernel weights divided the

167 average of both was less than 5%. If not, the third 500-kernel sample was weighted. The number

168 of spikes was measured by counting the samples in one-meter-length of row in each replication at

169 maturity. The number of kernels per spike was measured by counting the number of kernels per

170 spike in 20 plants per plot.

171 *Statistical analysis*

172   All data were run using analysis of variance (ANOVA) with three replicates according to

173 Excel 2003, SPSS17.0 (SPSS Inc., Chicago, USA). The Duncan's new Multiple Range (DMR)

174 test at 5% probability level was used to test the differences among the mean values. Significant

175 differences were labelled based on DMR.

176 **RESULTS**

177 **Plant height**

178 Plant height increased gradually with the growth and development of seedlings in all treatments

179 (Table 2). At pseudostem erection stage, there were no significant differences in plant height across

180 all treatments. At the jointing stage, plant height in the TS treatment was significantly higher than

181 in the TPS, TJ (2011-2012) and TPJ treatments (2011-2012). Plant height in the TJ and TPJ

182 treatments were not significantly different. At booting stage, the plant height in the TS treatment

183 was significantly higher than other treatments, while the TPJ treatment had significantly lower

184 plant height than other treatments (2012-2013). No significant differences were existed between

185 TPS and TJ. At mature stage, plant height reached to a peak value for all treatments. The

186 paclobutrazol treatments had a significantly lower plant height than the non-paclobutrazol

187 treatments.

Wheat yield and yield components

189 Data from both years showed that grains per spike, thousand grain weight, and yield of wheat in

190 the TJ and TPJ treatments were higher than those in the TS and TPS treatments (Table 3). The

191 decreasing order of observed yield was TPJ>TJ>TPS>TS, and the TPJ yield was significantly

192 higher than the yield for the TPS (10.22% and 8.88% higher) and TS (8.99% and 6.58% higher)

193 treatments in 2011-2012 and 2012-2013, respectively. The topdressing N + paclobutrazol

194 treatment yield components were not significantly different than the N treatments alone for the

195 different paclobutrazol application dates. The thousand grain weight for the TJ and TPJ treatments

196 were significantly higher than for the TS by 3.57% and 6.12%, respectively, and TPJ was

197 significantly higher than TPS by 5.58% in 2011-2012. Thousand grain weight in the TJ treatment

198 was significantly higher than in the TS and TPS treatments by 13.21% and 14.03%, respectively,

199 in 2012-2013. This result suggested that nitrogen topdressing at the jointing stage was more

200 beneficial to grain weight than at the pseudostem erection stage. The grains per spike in TJ and

201 TPJ were significantly higher than in TS and TPS in 2011-2012, while grains per spike in TPJ was

202 significantly higher than in TS and TPS in 2012-2013. Grains per spike for the TJ treatment was

203 significantly higher than that of TPS in 2012-2013. There were no significant differences in spikes

204 per hm^2 among four treatments.

Spike differentiation

206   The numbers of spikelets and florets in the TPS treatment were less than those in other treatments

207   (Table 4). Spikelet number in the TS treatment, the most one, was significantly higher than in the

208   TPS treatment by 8.33% and 5.43% in 2011-2012 and 2012-2013, respectively. But spikelet

209   number in the TS had no obvious differences with that in the TJ and TPJ treatments. Florets number

210   in the TPJ was significantly higher than in the TPS and TS treatments by 8.68% and 6.68% in 2011-

211   2012; and florets number in the TPS treatment was significantly lower than in the other treatments

212   in 2012-2013. The number of florets differentiated from stamen and pistil of wheat in the TPJ

213   treatment was significantly higher than in the TJ (2011-2012, 7.11% higher), TS (4.39% and

214   14.49% higher), and TPS (20.94% and 18.05% higher) treatments, respectively; this number in the

215   TJ was significantly higher than in the TPS and TS treatments by 13.28% and 16.81% in 2012-

216   2013, respectively. The floret number at the connectivum differentiation stage in the TPJ treatment

217   was significantly higher than in the TS, TPS, and TJ treatments by 19.92%, 10.21%, and 6.10%,

218   respectively, in 2011-2012; and the numbers in the TJ and TPJ were significantly higher than in

219   the TS (30.75% and 28.06%) and TPS (32.33% and 29.61%) treatments in 2012-2013,

220   respectively. These results suggested that N topdressing and paclobutrazol application at jointing

221   stage was more helpful in improving development of florets, inhibiting its degeneration in wheat,

222   and increasing grains number per spike.

223   **Phytohormone content**

224   The variation of endogenous hormone levels in wheat spikes were similar among all treatments

225   during differentiation of the main stem spike (Fig.1). However, the content of endogenous

226 hormones was different at various phases of spike differentiation. The changes in CTK, IAA, and

227 GA content showed a single-peak curve. CTK contents in the TS and TJ treatments was

228 significantly higher than in the TPS (18.52% and 17.78%) and TPJ (17.65% and 16.91%),

229 respectively, at the double-ridge stage. The peak value of CTK content in the TS treatments was

230 significantly higher than in the TPS treatment by 12.48% at the stamen and pistil differentiated

231 stage, when CTK content in the TPS was significantly higher than in the TJ and TPJ by 12.64%

232 and 13.17%, respectively. The peak values of GA in the TS and TPS were significantly higher than

233 in the TJ (37.12% and 29.60%) and TPJ (37.64% and 30.09%) treatments, respectively, at the

234 floret primordia differentiation stage. IAA contents in the TS and TPS treatments were

235 significantly higher than in the TJ (15.04% and 7.02%) and TPJ (25.98% and 17.19%) treatments,

236 respectively, at the pistil and stamen differentiated stage. These results showed that the nitrogen

237 topdressing at the pseudostem stage promoted accumulation of growth acceleration hormones

238 more than at the jointing stage. The differences between TS and TPS and between TJ and TPJ

239 were increasingly smaller during the panicle differentiation stage. ABA content in spikes showed

240 an increasing trend throughout the growth period. At the tetrad stage, ABA reached the maximum

241 value and showed a descending order of TS>TPS>TJ>TPJ; ABA contents in the TS and TPS

242 treatments were significantly higher than in the TJ (18.22% and 13.69%) and TPJ (34.07% and

243 28.93%) treatments, respectively. These results suggested that ABA content could be decreased

244 under nitrogen topdressing with paclobutrazol application at the jointing stage.

245 **IPA and ZR content**

246   Figure 2 shows single-peak trend in IPA, ZR, and (IPA+ZR) content in wheat grains under

247   all treatments from tetrad stage to flowering stage. The peak values appeared mainly at 0–6 days

248   after flowering. The IPA content, which was the highest in the TPJ treatment at 0, 3, 12, and 15

249   days after flowering, was significantly higher in the TJ and TPJ treatments than in the TS and TPS

250   treatments by 2.71% and 8.51%, 11.54% and 17.83%, respectively, at 6 days after flowers. The

251   ZR contents in grains for the TPS and TPJ treatments were both significantly higher than for the

252   TS (14.15% and 15.41%) and TJ (24.54% and 25.91%) treatments, respectively, at tetrad stage.

253   The ZR contents were significantly higher in the TPJ and TJ treatments than in the TS (20.43%

254   and 16.62%) and TPS (29.75% and 25.64%) treatments, respectively, at 12 days after flowering.

255   Additionally, the ZR contents in the TPJ and TJ treatments were significantly higher than the others

256   treatments, respectively, at 3 and 9 days after flowering. The IPA+ZR content showed a similar

257   changing trend to ZR content and was higher in the TJ and TPJ treatments than in the TS and TPS

258   treatments. The IPA+ZR content in the TPJ treatment was significantly higher than others at 3 and

259   15 days after flowering.

260   *TaCKX2.2* **Expression**

261   The trend of *TaCKX2.2* expression is shown in Fig. 3 under different treatments during spike

262   development. From the stamen and pistil differentiation to tetrad differentiation stage, the

263   expression level of *TaCKX2.2* was very low in all treatments. The level rose sharply and reached

264   a peak on 6 days after flowering under the TS and TPS treatments, which were significantly lower

265   than the TJ (65.22% and 69.27%) and TPJ (69.67% and 73.21%) treatments, respectively. From

266 0–12 days after flowering, the *TaCKX2.2* expression in spikes under the TJ and TPJ treatments

267 was significantly higher than that under the TS and TPS treatments, respectively. The first peak

268 for the TJ and TPJ treatments occurred on 0 days and the second higher peak occurred on 6 days

269 after flowering. At 9 days after flowering, *TaCKX2.2* expression was decreased substantially in all

270 treatments. These results suggested that *TaCKX2.2* expression could be induced by paclobutrazol

271 at the jointing stage and its expression in TPJ reached a maximum value during the flowering

272 stage.

273 **Correlation Analysis**

274 The floret number had a significant linear correlation with IPA and ZR content from 0-12

275 days after flowering (Table 5). At 0, 6, and 12 days after flowering, the florets numbers from the

276 stamen and gynoecium and from connectivum were all very significantly correlated to cytokinin

277 (IPA+ZR) content. At 3, 9, and 15 days after flowering, there was a significant correlation between

278 cytokinin (IPA+ZR) content and numbers of florets differentiated from stamen and gynoecium (15

279 days exception) and from connectivum. These results showed that both IPA and ZR could control

280 and regulate floret number and development during full-blossom period.

281 **DISCUSSION**

282 It has been reported that nitrogen topdressing at the jointing stage can increase the number of grains

283 per ear and wheat yield (*Li et al. 2010*). The application of paclobutrazol at the stem elongation

284 stage can promote nitrogen uptake and reduce nitrogen leaching to improve nitrogen use efficiency

285 and wheat yield (*Nouriyani et al. 2012b*). In this study, the number of grains per ear, thousand

286   kernel weight, and yield of wheat in the TPJ treatment were significantly higher than those in the

287   TS and TPS treatments, which was in agreement with the report of Wu *et al*. (2014). Paclobutrazol

288   application decreased plant height from booting stage to maturity stage, and further reduced plant

289   height when the application time was delayed from the pseudostem erection stage to the jointing

290   stage. The inhibitory effect of paclobutrazol on overgrowth of wheat plants likely reduced excess

291   consumption of nutrients and reserved photosynthate accumulation to support grain growth (Ghosh

292   et al., 2010). Combing nitrogen topdressing and paclobutrazol application at jointing stage

293   increased wheat yield. Although a single application of paclobutrazol had no significant effect on

294   yield and yield components, using topdressing nitrogen with paclobutrazol gave higher yield

295   component values than treatments without the paclobutrazol application. It was reported that the

296   best effect was achieved using a combination of 150 mg L^{-1} of paclobutrazol and 160 kg ha^{-1}

297   nitrogen, for which paclobutrazol increased the absorption and transportation of nitrogen in plant

298   and significantly affected photosynthetic pigments to maintain a high duration of flag leaf area

299   which increased the grain yield (*Nouriyani et al. 2012a*). Paclobutrazol was found to also reduce

300   plant height and flag-leaf area of black rice, but increased sucrose and amylopectin content in the

301   grain at a concentration of 50 ppm (*Dewi and Darussalam, 2018*). Previous reports indicated that

302   paclobutrazol was prone to regulation on physiological balance at early growth stages, but not on

303   final yield of winter wheat directly (*Yang et al. 2008*). Therefore, the regulatory effect of

304   paclobutrazol is different for different developmental phase of plants and concentration variation

305   from paclobutrazol or nitrogen or both.

306   The panicle primordium differentiation stage is a critical phase in reproductive organ

307   construction and the formation of final yield of wheat. Nitrogen can increase floret survival rate

308   and grain number in wheat (*Ferrante et al. 2010*). Paclobutrazol can promote fertile florets

309   differentiation and coordinate the vegetative and reproductive growth (*Hampton et al. 2006*).

310   Further observations have shown that the differentiation of young panicles was inhibited and the

311   floret number was decreased under nitrogen topdressing with paclobutrazol at the pseudostem

312   erection stage compared with the jointing stage. This may explain why grain number per ear and

313   yield of wheat with nitrogen topdressing at the pseudostem erection stage was lower than at

314   jointing stage. It is known that the growth of young ears and development of spikelets is closely

315   related to hormone content (*Zhang et al. 2009*). A recent study reported that paclobutrazol elevated

316   endogenous auxin and abscisic acid levels, suppressed gibberellins (GA4) and trans-zeatin

317   concentrations of plant (*Opio et al. 2020*). However, another report showed that paclobutrazol

318   significantly decreased the content of GA3 and IAA, and increased ABA contents in leaves of

319   wheat (*Aly et al. 2011*). This study indicated that the paclobutrazol application decreased the

320   number of spikelets and florets at the pseudostem erection stage, but increased floret number at

321   the jointing stage based on two- years data. The application of paclobutrazol inhibited not only

322   GA content during spike differentiation but also CTK, IAA, and ABA content before the tetrad

323   stage. This may be possible due to dynamic phytohormone levels and an unconstant inhibitory

324   effect of paclobutrazol on spike differentiation. The mutual antagonism and interactions between

325   those hormones varied in different growth stages of crops (*Wu et al. 2019*). Additionally, IAA,

326   CTK, and GA contents in spikes were lower or not different under nitrogen topdressing with or

327   without paclobutrazol at the jointing stage compared with the pseudostem erection stage. This

328   result suggested that the differences in number of spikelets and florets among all treatments were

329   not primarily determined by the phytohormone levels at this stage. At the tetrad stage, ABA

330   content in spikelets under the TPS treatment was higher than that under the TPJ stage, suggesting

331   that other promoting hormones likely regulated panicle and floret development after the tetrad

332   stage. These results have also verified the hypothesis that individual spikelets number was affected

333   by both nitrogen topdressing and paclobutrazol application, which played a promoting role mainly

334   during the jointing stage.

335   Cytokinin, which ~~was~~ degraded by cytokinin oxidizes/dehydrogenase to IPA and ZR, plays

336   an important role in early spikelet development and panicle differentiation (*Ma et al. 2008b;*

337   *Jameson et al. 2015*). From the tetrad to anthesis stage, cytokinin content in spikelets may be a

338   regulatory mechanism. At the tetrad stage, ZR content in spikelets under the nitrogen topdressing

339   with paclobutrazol treatment was significantly higher than that without the paclobutrazol

340   application, which help to increase yield sink capacity (*Dewi and Darussalam, 2018*). From this

341   stage onwards, the contents of ZR and IPA and the expression level of *TaCKX2.2* were higher for

342   the nitrogen topdressing during 0–6 days after flowering. The increment was higher at the jointing

343   stage than at the pseudostem erection stage. It is likely that cytokinin oxidation/dehydrogenase

344   would perform stronger degradation to maintain hormone balance when accumulation of cytokinin

345   in ear exceeded demand, which often results from the rapid development of wheat ears with

346   nitrogen topdressing during reproductive growth (*Werner et al. 2006; Zhang et al. 2011*). Panicle

347   development was faster under nitrogen topdressing at the jointing stage, when *TaCKX2.2*

348   expression was higher than that at the pseudostem erection stage. Meantime, paclobutrazol

349   application increased *TaCKX2.2* expression to inhibit cytokinin content. Our study showed that

350   the contents of IPA and ZR in the TPJ treatment, and ZR and (IPA+ZR) in the TPS treatment at 3

351   and 9 days after flowering, respectively, were suppressed by paclobutrazol, which was partly

352   consistent with Opio *et al*. (2020). However, the contents of ZR and ZR + IPA in TPJ were elevated

353   significantly by paclobutrazol at 3 days after flowering. These results can be interpreted that

354   paclobutrazol induced early flowering by increasing ABA and cytokinins contents in buds, which

355   regulated increases in leaf water potential and carbon-nitrogen ratio of mango (*Upreti et al. 2013*).

356   This study also showed that cytokinin (IPA+ZR) content was significantly correlated with the

357   floret number at the stamen and gynoecium and the connectivum differentiation stage. It was

358   established that paclobutrazol regulates floret differentiation and development indirectly by

359   regulating cytokinin content and influencing the final yield. These results further verified the

360   hypothesis that spikelet number and differentiation was regulated by phytohormone, in which

361   cytokinin (IPA and ZR) content and *TaCKX2.2* expression could keep homeostasis mainly after

362   the tetrad stage. Other factors such as water status, paclobutrazol concentrations, and growth stage

363   should also be considered in understanding the regulatory process. Additionally, the differences in

364   gene expression in spikes among treatments was not only influenced by cytokinin content but also

365   by multiple hormone interactions and other hormone levels changing at crucial stages. Further

366   research is needed to determine the primary molecular mechanisms by which assimilate of grain

367   increased for the nitrogen and paclobutrazol treatment.

368   **Conclusion**

369   In conclusion, nitrogen topdressing at jointing stage was optimal for increasing floret number,

370   kernels number per spike, and yield. And this result was partly related to cytokinin (especial ZR)

371   content increasing after tetrad stage. Application of paclobutrazol could delay spike development

372   to some extent and promote cytokinin content elevation, along with *TaCKX2.2* relative expression

373   increasing to keep hormonal equilibrium and regulate spikelet and floret number. This work has

374   systematically combined the molecular biology, phytohormone physiology and crop growth to

375   analyze seed yield increasing approach. Future research on the deep mechanism of interaction

376   between paclobutrazol and phytohormone is needed. Therefore, our study shows still an effective

377   measure to promote wheat productivity by regulating individual spikelet differentiation with

378   nitrogen topdressing and paclobutrazol application at jointing stage. And for conventional

379   production wheat variety, there also need adequate planting population to achieve high yield and

380   ensure food security in the North China Plain.

381   **ADDITIONAL INFORMATION AND DECLARATIONS**

382   **Funding**

389   **Competing Interests**

390   The authors declare there are no competing interests.

391   **Author Contributions**

392   • Dongxiao Li conceived and designed the experiments, performed the experiments, analyzed the

393   data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the

394   final draft.

395   • Shaojing Mo analyzed the data, authored or reviewed drafts of the paper.

396   • William D. Batchelor improved English language and reviewed the manuscript.

397   • Ruiting Cheng and Hongguang Wang   analyzed the data, authored or reviewed drafts of the

398   paper, and approved the final draft

399   • Ruiqi Li conceived and designed the experiments, authored or reviewed drafts of

400   the paper, funding, and approved the final draft.

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507

508

Table 1(on next page)

Soil conditions of the experimental field

Each data indicates the nutritious elements contents in different soil layers.

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1   **Table 1. Soil conditions of the experimental field.**

Year	Soil layers (cm)	Organic Matter (g·kg⁻¹)	Total N (g·kg⁻¹)	Available N (mg·kg⁻¹)	Available P (mg·kg⁻¹)	Available K (mg·kg⁻¹)
2011-2012	0-20	13.2	1.1	148.8	33.0	128.6
	20-40	5.6	0.6	60.5	7.3	50.0
2012-2013	0-20	20.3	0.9	124.5	21.4	133.3
	20-40	9.6	0.4	42.0	7.5	52.6

3

Table 2(on next page)

Plant height of wheat under different treatments

Each data point indicates the average plant height of 5 repeats plants at different treatments.

1

Table 2. Plant height of wheat under different treatments.

Year	Treatment	Plant height (cm)			
		Pseudostem erection stage	Jointing stage	Booting stage	Maturity stage
2011-2012	TS	17.8a	29.7a	60.3a	69.2a
	TPS	17.5a	28.1b	58.6b	67.6b
	TJ	19.0a	27.6c	57.1bc	66.4b
	TPJ	18.3a	27.6c	56.3c	64.1c
2012-2013	TS	19.1a	27.1a	54.4a	68.7a
	TPS	18.7a	23.6b	51.3b	67.1b
	TJ	19.0a	26.0a	50.7b	66.0b
	TPJ	19.4a	26.0a	48.1c	61.7c

2 Note: Different letters in each column indicate significant differences among four treatments, assessed by
3 ANOVA ($P \leqslant 0.05$). TS, nitrogen topdressing at erecting stage; TPS, TS combined with paclobutrazol
4 application; TJ, nitrogen topdressing at jointing stage; TPJ, TJ combined with paclobutrazol application.
5

Table 3(on next page)

Wheat yield and yield components under different treatments

Each data point indicates the wheat yield and yield components in three repeats testing plots with different treatments.

1

Table 3. Wheat yield and yield components under different treatments.

Year	Treatments	Spikes ($\times 10^4 \cdot ha^{-1}$)	Grains Per spike	Thousand grain weight (g)	Yield ($t \cdot ha^{-1}$)
2011-2012	TS	796.7a	33.6b	39.2c	8.8b
	TPS	752.3a	34.4b	39.4bc	8.9b
	TJ	798.9a	36.2a	40.6ab	9.4ab
	TPJ	807.8a	36.9a	41.6a	9.7a
2012-2013	TS	829.4a	33.3bc	28.0b	7.5b
	TPS	834.9a	32.9c	27.8b	7.6b
	TJ	806.0a	35.8ab	31.7a	7.8ab
	TPJ	823.4a	36.7a	30.5ab	8.1a

2 Note: Different letters in each column indicate significant differences among four treatments, assessed by

3 ANOVA ($P \leqslant 0.05$). TS, nitrogen topdressing at erecting stage; TPS, TS combined with paclobutrazol

4 application; TJ, nitrogen topdressing at jointing stage; TPJ, TJ combined with paclobutrazol application.

5

Table 4(on next page)

Spike differentiation of wheat under different treatments

Each data point indicates the average performance of 5 repeats in spike differentiation indexes under different treatments.

1 **Table 4. Spike differentiation of wheat under different treatments.**

Year	Treatments	Differentiation Spikelet number	Differentiation Floret number	Floret number differentiated from stamen and gynoecium	Floret number differentiated from connectivum
2011-2012	TS	20.8a	140.8b	118.4b	52.2c
	TPS	19.2b	138.2b	102.2c	56.8b
	TJ	20.8a	145.4ab	115.4b	59.0b
	TPJ	20.4ab	150.2a	123.6a	62.6a
2012-2013	TS	19.4a	148.8a	99.4b	67.0b
	TPS	18.4b	141.8b	96.4b	66.2b
	TJ	18.8ab	150.4a	112.6 a	87.6a
	TPJ	19.0ab	149.6a	113.8a	85.8a

2   Note: Different letters in each column indicate significant differences among four treatments, assessed by

3   ANOVA ($P \leqslant 0.05$). TS, nitrogen topdressing at erecting stage; TPS, TS combined with paclobutrazol

4   application; TJ, nitrogen topdressing at jointing stage; TPJ, TJ combined with paclobutrazol application.

5

Table 5(on next page)

The correlation between (IPA+ZR) content and floret number

Each data point indicates the correlation coefficient of 12 runs between (IPA+ZR) content and floret number differentiated from connectivum, stamens and pistils.

1   **Table 5. The correlation between (IPA+ZR) content and floret number. **P < 0.01 and *P<0.05.**

content of (IPA+ZR)	florets differentiated from connectivum	florets differentiated from stamen and gynoecium
0 day after flowering	0.825**	0.855**
3 days after flowering	0.685*	0.63*
6 days after flowering	0.766**	0.725**
9 days after flowering	0.600*	0.683*
12 days after flowering	0.913**	0.875**
15 days after flowering	0.695*	0.549

2   Notes. *, ** significant at the 0.05 and 0.01 probability levels, respectively, ns, no significant. Each data point

3   represents the average of the measured data.

4

Figure 1(on next page)

Dynamic of endogenous hormone levels in ears during spike differentiation

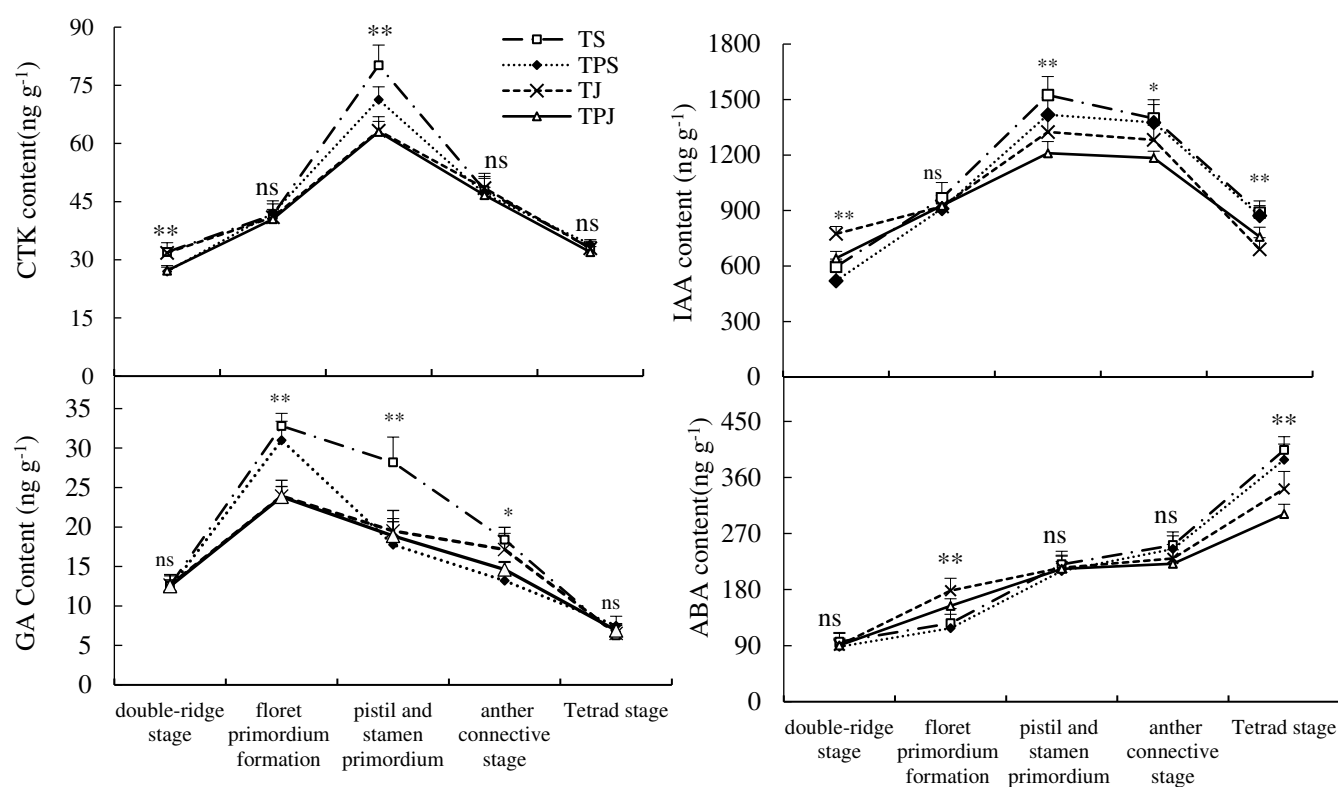E Each data point indicates the average performance of 5 repeats in phytohormone contents with different stages.

Figure 1 Dynamic of endogenous hormone levels in ears during spike differentiation. *, ** significant at the 0.05 and 0.01 probability levels, respectively, ns, no significant. Each data point represents the average of the measured data.

Figure 2

IPA and ZR content of spikes under all treatments at different stages

Each data point indicates the average performance of 5 repeats in spike with different treatments during all flowering stage.
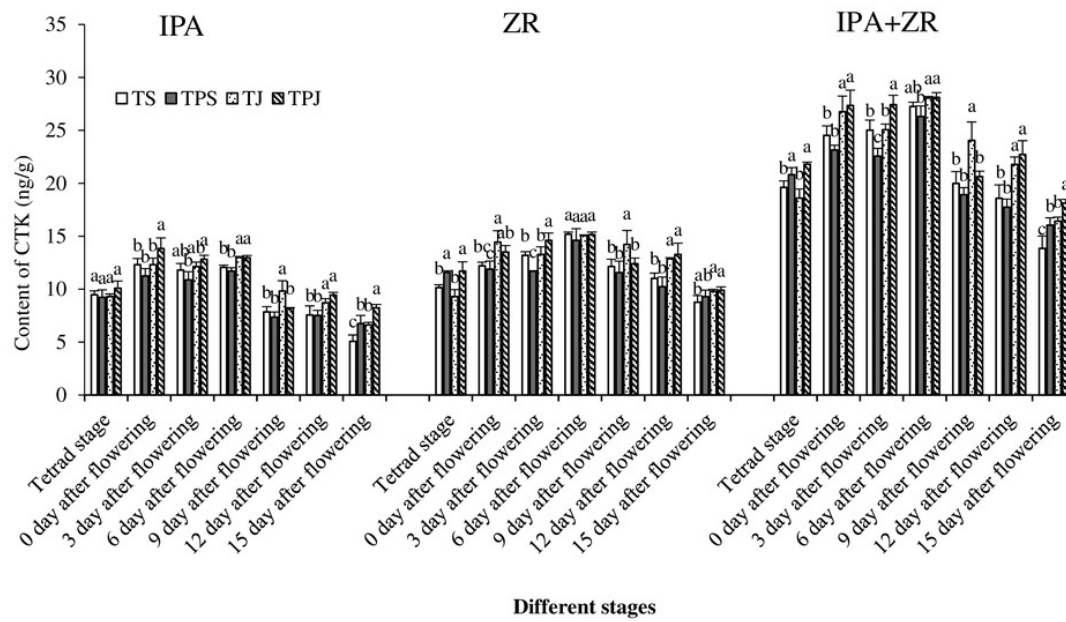
Fig.2. IPA and ZR content of spikes under all treatments at different stages. Different letters indicate significant differences among four treatments, assessed by ANOVA ($P \leqslant 0.05$).

Figure 3

The changing trend of relative expression of TACKX2.2 at different stages

Each data point indicates the average performance of 3 repeats samples after randomly choosing with all treatments at different spike differentiational stages
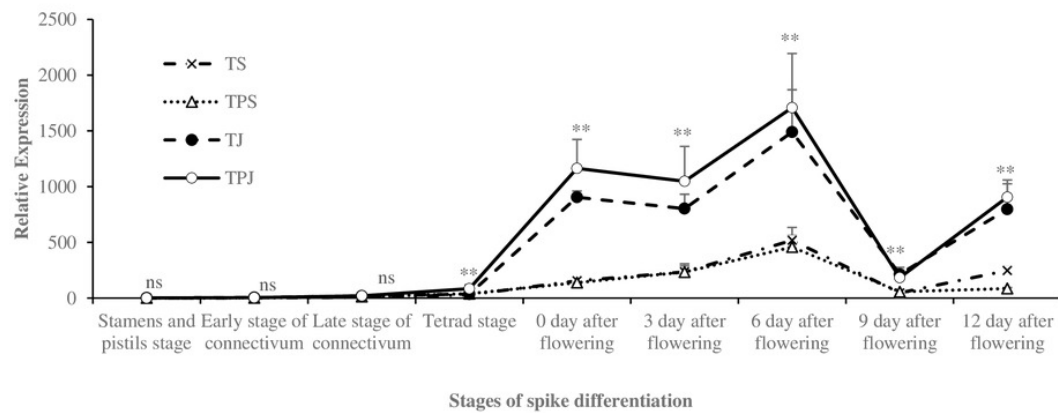
Figure 3 The changing trend of relative expression of *TACKX2.2* at different stages. *, ** significant at the 0.05 and 0.01 probability levels, respectively, ns, no significant. Each data point represents the average of the measured data.