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

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Background. Optimal nitrogen application and plant growth regulator is essential for individual 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 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.** We found that grain number per ear, thousand kernel weight and yield under TJ and TPJ were higher than those under TS and TPS. Grain number per ear, yield under TPJ and thousands kernel weight under TJ were significantly higher than those under TS and TPS. The floret number, significantly correlated with cytokinin content, was also significantly increased under TJ and TPJ at connectivum differentiation stage. The relative expression level of cytokinin oxidase 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.

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2 **spike differentiation of winter wheat**

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8 ## Abstract.

9 **Background.** Optimal nitrogen application and plant growth regulator is essential for individual

10 wheat productivity. This can help to improve yield level and ensure food security with limited

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

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

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

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

15 paclobutrazol application) in 2011-2013.

16 **Results.** We found that grain number per ear, thousand kernel weight and yield under TJ and TPJ

17 were higher than those under TS and TPS. Grain number per ear, yield under TPJ and thousands

18 kernel weight under TJ were significantly higher than those under TS and TPS. The floret number,

19 significantly correlated with cytokinin content, was also significantly increased under TJ and TPJ

20 at connectivum differentiation stage. The relative expression level of cytokinin oxidase gene

21 (*TACKX2.2*) in response to cytokinin increasing was improved to regulate hormonal balance.

22 **Conclusions**. Therefore, nitrogen topdressing at jointing stage had increased grain number per ear,

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

24 and promote cytokinin accumulation that induced expression of *TACKX2.2*, maintaining hormonal

25 balance and affecting wheat spike morphogenesis.

26 **Keywords** topdressing stages, floret number, phytohormone, spike differentiation, *TACKX2.2*

27 **INTRODUCTION**

28 The North China Plain, one of the most important grain base, is experiencing prominent conflicts

29 on limited natural resources and crop production. In order to pursue higher grain yield, excessive

30 use of nitrogen fertilizer exists commonly in local wheat-maize planting system. This can also lead

31 to large losses of nitrogen, resulting in serious environmental pollution (*Azam Shah et al. 2009;*

32 *Wang et al. 2010*). Additionally, the planting area of winter wheat is shrinking for the sake of

33 saving over-exploit underground water and improving ecological benefits (*Xu et al. 2005*). The

34 yield pressure and resources cost such as farmland, water and fertilizer have become increasingly

35 growing. Therefore, it is an important way to ensure China's food security by improving grain

36 production capacity. Wang *et al*. (2015) referred that a key factor for increasing wheat yield was

37 increasing the grain number of spike and seed weight, which was often influenced by nitrogen

38 fertilizer in the high-yield wheat region of Hebei Province. Nitrogen fertilizer application cannot

39 only promote allocation of assimilate, but also increase assimilation amount and contribution rate

40   of post-anthesis photosynthate to grain (*Ma et al. 2008a*). There were three nitrogen absorption

41   peaks of wheat at stage before winter, jointing-booting stage, and flowering-filling stage during

42   the whole growth period. The transport of carbon assimilates to grain was more efficient with

43   nitrogen topdressing at jointing stage than at flowering stage (*Zhai and Li, 2006*). But the nitrogen

44   application should be delayed until a later booting stage under the super-high-yield conditions

45   (*Kara and Uysal, 2009; Liu et al. 2019*). Additionally, the optimal nitrogen application time is

46   often related to local water condition and wheat varieties. Under drought conditions or limited

47   irrigation, nitrogen topdressing should be applied early, that would be more beneficial to

48   transportation of carbon photosynthate to grain at jointing stage than at flowering stage (*Gevrek*

49   *and Atasoy, 2012*). But, for spring wheat, it was more appropriate to topdressing nitrogen at

50   flowering time (*Walsh et al. 2018*). Under irrigated conditions, the topdressing time should be

51   delayed for enhancing photosynthesis and increasing wheat yield (*Chen et al. 2008*).

52   Paclobutrazol (pp333), a plant growth regulator to inhibit synthesis of endogenous

53   gibberellin, could be absorbed easily by root, stem, and leaf. The application of paclobutrazol

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

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

56   Combing nitrogen topdressing with paclobutrazol could improve photosynthetic rate, increase

57   grain weight per spike, and achieve further increase in wheat yield at jointing stage (*Yang et al.*

58   *2008*). Also, nitrogen topdressing at pseudostem erection stage with paclobutrazol application

59   could increase dry matter accumulation, grain weight per spike, and grain yield *(Zhang et al. 2017)*.

60   On the one hand, appropriate amount of nitrogen and paclobutrazol could increase stem lodging

61   resistance to guarantee high and stable yield (*Chen et al. 2011*). On the other hand, paclobutrazol

62   regulated root morphological characteristics, maintained physiological function and root activities,

63   increased grain yield by enhancing the levels of osmolytes, endogenous hormones contents, and

64   antioxidant activities under disadvantage conditions (*Soumya et al. 2017; Kamran et al. 2018*).

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

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

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

68   stage, zeatin riboside (ZR) content could effectively regulate panicle flower development and

69   spikelet number (*Ma et al. 2008a*). Decreasing cytokinin and ABA contents in grain and root would

70   be possible resulting in sterile spikelet (*Chen et al. 2008; Zhang et al. 2009*). Paclobutrazol

71   application could influence hormone directly and modify flowering and development of plants

72   (*Zhang et al. 2016*). During ear differentiation, cytokinins oxidation dehydrogenase (CKX),

73   controlling endogenous cytokinins content, often negatively regulates flowering time and grain

74   formation of crop (*Zalewski et al. 2010; Yeh et al. 2015; Ashikari et al. 2005; Bartrina et al. 2011*).

75   For wheat, *TaCKX2.2* was involved in the formation of the spike grain number and yield (*Zhang*

76   *et al. 2011*). Inhibiting *TaCKX2* expression by RNAi, the grain number per spike would be

77   increased in bread wheat plants(*Li et al. 2018*). So far, it is still not clear when topdressing nitrogen

78   combined paclobutrazol application is optimal for ear differentiation and individual wheat

79   productivity; and how changing phytohormones and related gene expression in plant under

80   different nitrogen topdressing treatments in the North China Plain. The objective of the present

81   study was thus to evaluate the effect of topdressing nitrogen with paclobutrazol application on

82   spike differentiation and to elucidate the possible mechanism of improving productivity of wheat.

83   This study will help to provide guidance for optimizing irrigation and fertilizer and both

84   combination measures in drought and semi-drought regions.

85   **Materials and methods**

86   *Experimental site and soil*

87   The field experiment was carried out from 2011 to 2013 on the super-high-yield testing farmland,

88   Gaocheng, Shijiazhuang, China. The climate was classified as a subhumid continental monsoon.

89   The mean annual rainfall is less than 520 mm, one-third of which would be allocated to wheat-

90   growing season. Winter wheat-summer maize rotation is a typical planting system. The soil type

91   was light-loamy Chao Soil. Soil properties are shown in Table 1.

92   *Experimental design and crop management*

93   The cultivar Shimai18 was provided by Shijiazhuang Academy of Agricultural Sciences.

94   During the whole growth period, fertilizers were based applied with the rates of 112 kg ha^{-1}

95   nitrogen, 60 kg ha^{-1} phosphorus, 87 kg ha^{-1} potassium and 9 kg ha^{-1} zinc. Another 112 kg ha^{-1}

96   nitrogen topdressing was applied in next April. Fore-rotating corn straw crushed 2 times was

97   returned to the field. Then rotary tillage operations were conducted 2 times with a 15 cm depth.

98   Seeding rate was 150 kg ha^{-1}. Paclobutrazol was applied by a foliar spray of aqueous solution

99   containing 10% wettable powder at effective concentration of 200 mg ha^{-1} and spraying amount

100   of 675 kg ha^{-1}. Wheat was sown on October 7 in 2011 and October 9 in 2012. The equal row

101   spacing was 0.15 m. Planting density was approximately 270◊10^4 plants ha^{-1}.

102   The experiment was a randomized block design with four experimental treatments including

103   nitrogen topdressing at pseudostem erection stage (TS), TS with paclobutrazol application (TPS),

104   nitrogen topdressing at jointing stage (TJ), TJ with paclobutrazol application (TPJ). Each treatment

105   was replicated three times. Each plot area was 60 m^2.

106   *Variable measurements*

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

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

109   young ears marked on the same flowering date would be sampled at 0, 3, 6, 9 and 12 days after

110   flowering. From regreening stage, representative wheat plants were selected at 3 days intervals.

111   The numbers of spikelets and florets were recorded in a constant state (on about 21st April) using

112   anatomical lens (Stemi 2000-c, Germany).

113   Total ribonucleic acid (RNA) samples were extracted. RNA concentration and purity were

114   detected by spectrophotometer (Nanodrop, USA); the RNA integrity was detected by agarose gel

115   electrophoresis. And cDNA obtained by reverse transcription of RNA was used for subsequent

116   fluorescence quantitative expression. Priming sequence: GGGAGAAGAAGCACTTTGGTC

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

118   gene *GAPDH* was selected for mRNA transcription studies using quantitative real-time

119   polymerase chain reaction (PCR).

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

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

122   by enzyme linked immunosorbent assay (ELISA). The test kit was provided by College of

123   Agriculture and Biotechnology, China Agricultural University. Young ear samples were cut into

124   0.5 g pieces and were ground to a fine powder in liquid nitrogen with a pre-cooled mortar. The

125   powder diluted with extracting solution was extracted 4 h at 4 ℃ in cold 80% methanol based on

126   Oliver *et al.* (2007) protocol. The homogenate was then centrifuged at 3500 r min^{-1} for 20 min,

127   and the supernatant was collected to be washed by 80% methanol in C-18 solid phase extraction

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

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

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

131   Plant height was measured at pseudostem erection, jointing, booting, and ripening stage,

132   respectively. Yield and yield components were measured by aconventional method. The harvest

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

134   1000-kernel weight was determined by counting two 500-kernel samples and weighing them with

135   the relative error was less than 5%. If not, the third 500-kernel sample would be weighted. The

136   number of spikes was recorded by counting the samples in one-meter-length at mature stage. The

137   number of kernels per spike was counted in a set of 20 plants per plot.

138   *Statistical analysis*

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

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

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

142   differences were labelled based on DMR.

143   **RESULTS**

Plant height

144

145   Table 2 showed that plant height increased gradually with the growth and development of

146   seedlings in all treatments. At pseudostem erection stage, there were no significant differences

147   among all untreated plots. At jointing stage, plant height in TS increased rapidly and was

148   significantly higher than that in TPS, TJ (2011-2012) and TPJ (2011-2012). No obvious changes

149   were observed between TJ and TPJ. At booting stage, the plant height in TS was higher

150   significantly than other treatments; and TPJ was significantly lower than others in 2012-2013. No

151   significant differences were existed between TPS and TJ. At mature stage, plant height reached to

152   a peak value under all treatments. Also, the same changing trend and arranged order of plant height

153   were observed at booting stage. These findings suggested that paclobutrazol application could

154   inhibit plant growth effectively at different stages.

Wheat yield and yield components

155

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

157   TJ and TPJ were higher than those in TS and TPS (Table 3). The yield was observed in a decreasing

158   order as TPJ>TJ>TPS>TS, in which TPJ was significantly higher than that in TPS and TS.

159   Whether or not spraying paclobutrazol, grain yield and yield components of wheat were not

160   affected obviously comparing two treatments of nitrogen topdressing at the same time. The

161   thousand grain weight has shown that TJ and TPJ were significantly higher than TS, and TPJ was

162   significantly higher than TPS in 2011-2012; TJ was significantly higher than TS and TPS in 2012-

163   2013. This result suggested that nitrogen topdressing at jointing stage was more beneficial to grain

164   weight than at pseudostem erection stage. The grains per spike in TJ and TPJ were significantly

165   higher than in TS and TPS, and that in TPJ was significantly higher than in TS and TPS in 2011-

166   2012. This value of TJ was significantly higher than that of TS and TPS in 2012-2013. The results

167   also showed that grains per spike could be increased by 1.81-3.81 under nitrogen topdressing with

168   paclobutrazol application at jointing stage. There were no significant differences in spikes per hm^2

169   among four treatments.

170   **Spike differentiation**

171   The numbers of spikelet and floret of wheat in TPS were both less than those in other treatments,

172   which suggested that nitrogen topdressing with paclobutrazol application at pseudostem erection

173   stage had evident inhibitory effect on spikelet differentiation (Table 4). Spikelet of wheat in TS,

174   the most one, was significantly higher than in TPS, but had no obvious differences with that in TJ

175   and TPJ. Floret of wheat in TPJ, the most one in 2011-2012, was significantly higher than that in

176   TPS and TS; and this value in TPS was significantly lower than that in other treatments in 2012-

177   2013. The florets differentiated from stamen and pistil of wheat in TPJ was significantly higher

178   than in TJ (2011-2012), TS, and TPS, respectively; this value in TJ was significantly higher than

179   in TPS and TS (2012-2013). The floret number at connectivum differentiation stage in TPJ, the

180   most one, was significantly higher than other treatments in 2011-2012 and in 2012-2013(TJ

181   exception), respectively. These results illustrated that nitrogen topdressing at jointing stage with

182   paclobutrazol application was more helpful in improving development of florets, inhibiting its

183   degeneration in wheat, and increasing grains number per spike.

184   **Phytohormone content**

185   The variation of endogenous hormone levels in wheat spike were similar among all treatments

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

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

188   GA content showed a single-peak curve with peaks of CTK and IAA appearing at stamen and pistil

189   differentiated stage and with peak of GA appearing at floret primordia differentiation stage. The

190   contents of CTK, IAA, GA in spike under different treatments exhibited a decreasing order of

191   TS>TPS>TJ>TPJ during peak periods. This result showed that it was easier to promote cumulation

192   of growth acceleration hormone under nitrogen topdressing at pseudostem erection stage than at

193   jointing stage. IAA content of wheat spike with paclobutrazol application was obviously decreased

194   comparing with that without paclobutrazol treatment at pseudostem erection stage. But this value

195   was significantly higher under nitrogen topdressing with paclobutrazol application than that

196   without paclobutrazol treatment at jointing stage. The differences between two paclobutrazol

197   treatments were increasingly smaller during the panicle differentiation. ABA content in spike

198   showed an increasing trend throughout the growth period. At floret primordium formation stage,

199   this value showed a descending order as TJ>TPJ>TS>TPS. At booting stage, it reached the

200   maximum value and showed a descending order as TS>TPS>TJ>TPJ. These results suggested that

201   ABA content could be decreased under nitrogen topdressing with paclobutrazol application at

202   jointing stage.

203   **IPA and ZR content**

204   Further research on CTK had been conducted after tetrad stage. Fig. 2 showed an "up-down"

205   single-peak changing trend of IPA, ZR, (IPA+ZR) content in wheat grains under all treatments

206   from tetrad stage to flowering stage. The peak values appeared mainly at 0–6 days after flowering.

207   There were no significant differences in IPA content among all treatments at tetrad stage. IPA

208   content, which was the highest in TPJ at 0, 3, 12, and 15 days after flowers, showed significantly

209   higher in TJ and TPJ than in TS and TPS at 6 days after flowers. ZR contents in grains under TPS

210   and TPJ were both significantly higher than that under TS and TJ at booting stage. And this value

211   was higher in TPJ and TJ than in TS and TPS at 0, 12, 15 days after flowering. Additionally, the

212   highest ZR content appeared in TPJ and TJ at 3 and 9 days after flowering, respectively. The

213   IPA+ZR content, showing similar changing trend to ZR content, was higher in TJ and TPJ than in

214   TS and TPS. This value in TPJ was significantly higher than others at 3 and 15 days after flowering.

215   **_TACKX2.2_ Expression**

216   _TACKX2.2_ expression trending was shown in Fig. 3 under different treatments during spike

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

218   level of _TACKX2.2_ was very low in all treatments. Thereafter, it rose sharply and reached a peak

219   on 6 days after flowering under TS and TPS. During 0–9 days after flowering, the _TACKX2.2_

220   expression in spike under TJ and TPJ was higher than that under TS and TPS obviously. And there

221   were the first peak appeared on 0 day and the second higher peak appeared on 6 days after

222   flowering. On 9 days after flowering, _TACKX2.2_ expression was substantially decreased in all

223   treatments. Thereafter, it rose modestly under TJ and TPJ with a higher rate of increase than under

224   TS and TPS at 12 days after flowering. These results suggested that *TACKX2.2* expression could

225   be induced by paclobutrazol at jointing stage and its expression in TPJ reached maximum value

226   during the flowering stage.

227   **Correlation Analysis**

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

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

230   from stamen and gynoecium and from connectivum were all very significantly correlated to

231   cytokinin (IPA+ZR) content. At 3 days, 9 days, and 15 days after flowering, there were a

232   significant correlation between cytokinin (IPA+ZR) content and numbers of floret differentiated

233   from stamen and gynoecium (15 days exception) and from connectivum. These results showed

234   that both IPA and ZR could control and regulate floret number and development during full-

235   blossom period.

236   **DISCUSSION**

237   It was reported that nitrogen topdressing at jointing stage could increase the number of grains per

238   ear and wheat yield (*Li et al. 2010*). The application of paclobutrazol combined with nitrogen

239   fertilizer can improve wheat yield mainly due to increase in nitrogen use efficiency and reduction

240   in nitrogen loss (*Nouriyani et al. 2012b*). In this study, the number of grains per ear, thousand

241   kernel weight, and yield of wheat in TPJ were significantly higher than those in TS and TPS

242   treatment, which was in agreement with the report of Wu *et al*. (2014). These results showed that

243   nitrogen topdressing combined with paclobutrazol at jointing stage promoted more photosynthate

244   accumulation in grain and improved nitrogen use efficiency. Paclobutrazol application decreased

245   plant height effectively from booting stage to maturity stage; and that would be further decreased

246   when the application time was delayed from pseudostem erection stage to jointing stage. The

247   inhibitory effect of paclobutrazol on overgrowth of wheat plants could avoid excessive

248   consumption of nutrient substance. Therefore, it is an effective measure to increase wheat yield by

249   combing nitrogen topdressing and paclobutrazol application at jointing stage. Although a single

250   application of paclobutrazol had no significant effect on yield and yield components, the increment

251   of topdressing nitrogen with paclobutrazol on yield indexes was higher than those without

252   paclobutrazol application. It was reported that the best effect was achieved at the combination of

253   150 mg L^{-1} paclobutrazol and 160 kg ha^{-1} nitrogen, for which paclobutrazol increased the

254   absorption and transportation of nitrogen in plant and significantly affected photosynthetic

255   pigments to keep high duration of flag leaf area and increase the grain yield (*Nouriyani et al.*

256   *2012a*). Paclobutrazol could also reduce plant height and flag-leaf area of black rice, but increase

257   sucrose and amylopectin content in the grain at a concentration of 50 ppm (*Dewi and Darussalam,*

258   *2018*). Previous report indicated that paclobutrazol was prone to regulation on physiological

259   balance at early growth stage, not on final yield of winter wheat directly (*Yang et al. 2008*).

260   Therefore, the regulatory effect of paclobutrazol on plant would be different duo to different

261   developmental phase of plant and concentration variation from paclobutrazol or nitrogen or both.

262   The panicle primordium differentiation stage is a critical phase inreproductive organ

263   construction and the formation of final yield of wheat. Nitrogen could increase floret survival rate

264   and grain number in wheat (*Ferrante et al. 2010*). And paclobutrazol could promote fertile florets

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

266 Further observation showed that the differentiation of young panicle could be obviously inhibited

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

268 erection stage comparing with at jointing stage. That may explain why grain number per ear and

269 yield of wheat with nitrogen topdressing at pseudostem erection stage was lower than at jointing

270 stage. It is known that the growth of young ears and development of spikelet was closely related

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

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

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

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

275 wheat (*Aly et al. 2011*). This study indicated that paclobutrazol application took a decreasing effect

276 on the number of spikelet and floret at pseudostem erection stage, but took an increasing effect on

277 floret number at jointing stage according to two-years data. The application of paclobutrazol

278 inhibited not only GA content during spike differentiation but also CTK, IAA, and ABA content

279 before tetrad stage. Maybe this is possible due to dynamic phytohormone levels and an inconstant

280 inhibitory effect of paclobutrazol on spike differentiation. The mutual antagonism and interactions

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

282 IAA, CTK, and GA contents in spike were lower or not different under nitrogen topdressing with

283 or without paclobutrazol at jointing stage comparing with that at pseudostem erection stage. This

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

285 not determined mainly by the phytohormone levels at this stage. At tetrad stage, ABA content in

286   spikelet under TPS was higher than that under TPJ, suggesting that other promoting hormone

287   would take possible regulatory effect on panicle and floret development after tetrad stage.

288   Cytokinin, which was degraded by cytokinin oxidizes dehydrogenase to IPA and ZR, has

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

290   Jameson *et al*. 2015). From tetrad stage to anthesis stage, cytokinin content in spikelet was further

291   researched to reveal the possible regulatory mechanism. At tetrad stage, ZR content in spikelet

292   under nitrogen topdressing with paclobutrazol treatment was significantly higher than that without

293   paclobutrazol treatment, which help to increase yield sink capacity (*Dewi and Darussalam, 2018*).

294   From this stage onwards, the contents of ZR and IPA and the expression level of *TACKX2.2* were

295   increased obviously with nitrogen topdressing during 0–6 days after flowering; and the increment

296   was higher at jointing stage than at pseudostem erection stage. It may be suggested that cytokinin

297   oxidation dehydrogenase would perform stronger degradation to maintain hormone balance when

298   accumulation of cytokinin in ear exceeded demand, which was often resulted from the rapid

299   development of wheat ears with nitrogen topdressing during reproductive growth (*Werner et al.*

300   *2006; Zhang et al. 2011*). Also, panicle development was faster under nitrogen topdressing at

301   jointing stage, when *TACKX2.2* expression was higher than that at standing stage. Meantime,

302   paclobutrazol application would increase *TACKX2.2* expression to inhibit cytokinin content. Our

303   study revealed that the contents of IPA and ZR in TPJ, ZR and (IPA+ZR) in TPS at 9days and 3

304   days after flowering, respectively, were suppressed by paclobutrazol, which was partly consistent

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

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

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

308 would regulate increases in leaf water potential and carbon-nitrogen ratio of mango (*Upreti et al.*

309 *2013*). This study also showed that cytokinin (IPA+ZR) content was significantly correlated with

310 the florets number at stamen and gynoecium differentiation and at connectivum differentiation

311 stage. It was established that paclobutrazol had regulated floret differentiation and development

312 indirectly by regulating cytokinin content and influenced the final yield. There were also lots of

313 environmental factors, such as water status, paclobutrazol concentrations, growth stage, etc, should

314 be involved in the process of understanding the regulatory process. Additionally, the differences

315 of gene expression in spike among treatments was not only influenced by cytokinin content but

316 also by multiple hormone interactions and other hormone levels changing at crucial stage. Further

317 research still required to elucidating the main molecular mechanism by which assimilate of grain

318 increased by nitrogen and paclobutrazol treatment

319 **Conclusion**

320 In this paper, it could be concluded that nitrogen topdressing at jointing stage significantly

321 increased floret number, kernels number per spike, and yield. And this result was partly related to

322 cytokinin (especial ZR) content increasing after tetrad stage. Application of paclobutrazol could

323 delay spike development to some extent and promote cytokinin content elevation, along with

324 *TACKX2.2* relative expression increasing to keep hormonal equilibrium and regulate spikelet and

325 floret number. It is crucial to focus future research on the deep mechanism of interaction between

326 paclobutrazol and phytohormone. Therefore, our study shows still an effective measure to promote

327   wheat productivity by nitrogen topdressing with paclobutrazol at jointing stage.

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335   **Competing Interests**

336   The authors declare there are no competing interests.

337   **Author Contributions**

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

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

340   final draft.

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

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

343   paper, and approved the final draft

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

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

346

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Soil conditions of the experimental field

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

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

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.

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 ($\times10^4\cdot ha^{-1}$)	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.

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.

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.

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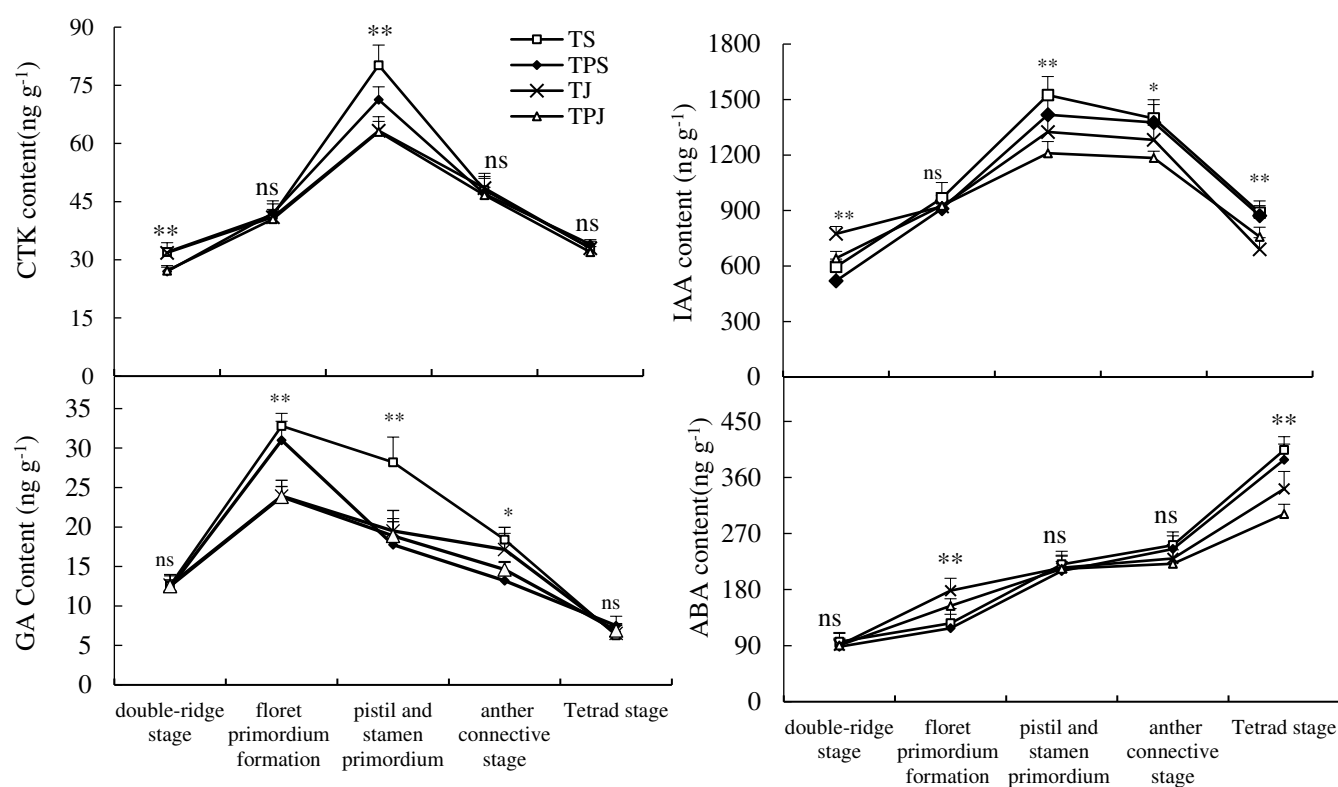
Figure 1   Dynamic of endogenous hormone levels in ears during spike differentiation. *, ** significant at the 0.05 and 0.01 probability levels, respectively, ns, no significant. Each data point represents the average of the measured data.

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.
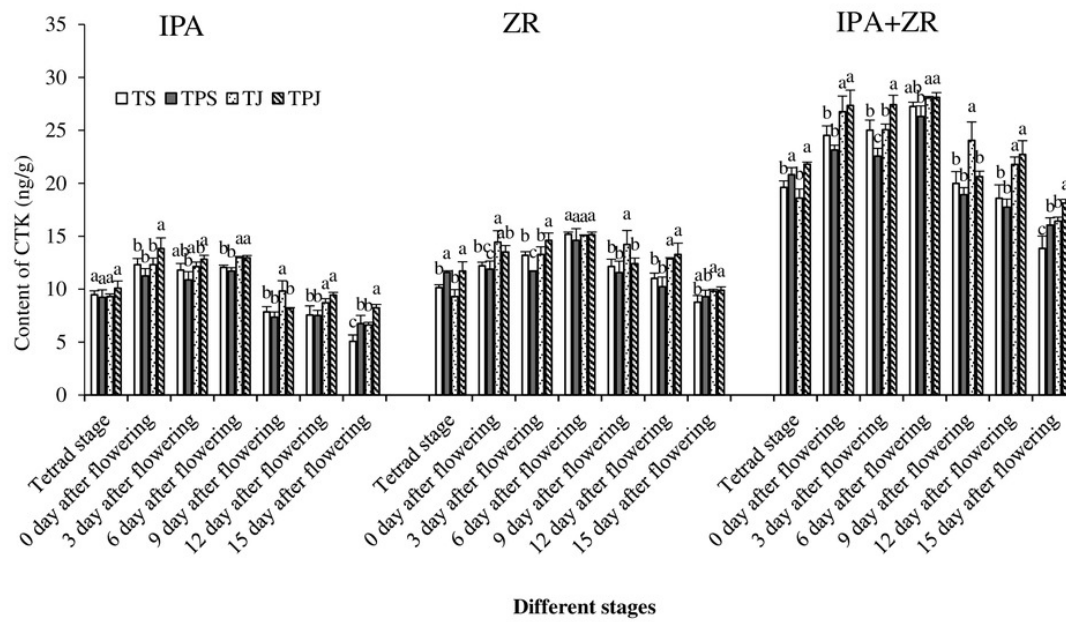
Fig.2. IPA and ZR content of spikes under all treatments at different stages. Different letters indicate significant differences among four treatments, assessed by ANOVA ($P \leqslant 0.05$).

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
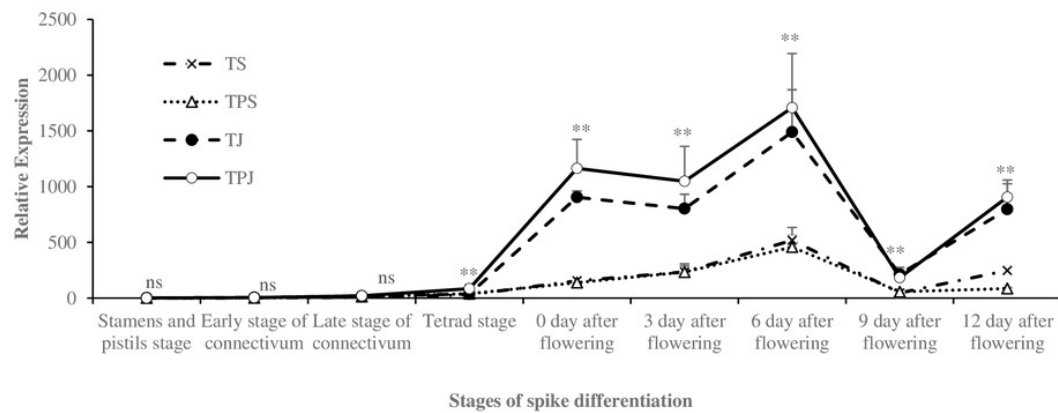
Figure 3 The changing trend of relative expression of *TACKX2.2* at different stages. *, ** significant at the 0.05 and 0.01 probability levels, respectively, ns, no significant. Each data point represents the average of the measured data.