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Increasing plant density increases Bt toxin concentration of boll wall in cotton by decreasing boll setting speed

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Abstract

Background: In order to uncover the mechanism of significantly reduced insect resistance at the late developmental stage in cotton (*Gossypium hirsutum* L.), the relationship between boll setting rate under different planting densities and *Bacillus thuringiensis* (Bt) insecticidal concentrations in the boll wall were investigated in the present study. Two studies were arranged at Yangzhou, China during the 2017–2018 cotton growth seasons. Five planting densities (15 000, 25 000, 45 000, 60 000 and 75 000 plants per hectare) and the flower-removal treatment were imposed separately on Bt cotton cultivar Sikang3 to arrange different boll setting rates, and the boll setting rates and Bt toxin content were compared.

Results: Higher boll setting rate together with lower Bt toxin contents in boll wall was observed under low planting density, whereas lower boll setting rate and higher Bt toxin contents were found under high planting density. Also, higher Bt protein concentration was associated with higher soluble protein content, glutamic-pyruvic transaminase (GPT), and glutamic oxaloacetate transaminase (GOT) activities, but lower amino acid content, and protease and peptidase activities. It was further confirmed that a higher boll setting rate with lower Bt protein content under flower-removal.

Conclusions: This study demonstrated that the insecticidal efficacy of boll walls was significantly impacted by boll formation. Reduced protein synthesis and enhanced protein degradation were related to the reduced Bt toxin concentration.

Keywords: Bt cotton, Boll setting rate, Bt toxin, Protein metabolism

Introduction

The lowest insect resistance was found at the boll developmental stage in Bt cotton (Wu 2007; Kristen et al. 2013; Chen et al. 2017), which was detrimental to final yield formation (Huang et al. 2010). The reduction of insect resistance was a result of dropped Bt toxin concentration (Shen et al. 2010; Levine et al. 2016; Tabashnik et al. 2012). These studies suggest that boll-setting at the boll developmental stage may impact the Bt protein concentration in cotton bolls. Therefore, it is important to determine the relationship between boll formation and Bt protein concentration.

The unstable Bt insect resistance has been reported widely. Temporal and spatial variations of Bt insecticidal protein contents were found at different organs and growth stages in cotton plants (Bravo et al. 2019; Chen et al. 2017; Wan et al. 2005). Higher Bt protein content was detected in cotton leaf compared to reproductive organs (Chen et al. 2004; Kranthi et al. 2005), and bolls exhibited the lowest Bt toxin concentration in the reproductive organs (Kranthi et al. 2005;

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Chen et al. 2017, 2018). Cotton plants had higher insect resistance at the seedling stage, but the insecticidal ability declined during the growth season (Xia et al. 2005; Chen et al. 2012a, 2017). Along with temporal and spatial variations of Bt protein expression, abiotic stress, such as extreme temperature, moisture, and salt, all influenced the expression of Bt toxin (Chen et al. 2005, 2012b, 2013, 2019; Jiang et al. 2006; Zhang et al. 2017). The square size and biomass, boll number and boll size also impacted Bt protein concentration (Wang et al. 2009; Chen et al. 2017; Zhou et al. 2019). Agronomic practices, including plant density, fertilizer, growth regulators, also affect the Bt protein content of cotton plants (Pettigrew and Adamczyk 2006; Chen et al. 2017, 2018, 2019). Cotton with low planting density had higher insecticidal protein concentration, the insecticidal protein content was decreased by leaf cut, but increased by GA_3 application (Chen et al. 2017). These studies showed that Bt insecticidal protein concentration was unstable and could be regulated by various growth conditions and agronomic practices. Moreover, previous studies found that the amount of Bt protein in reproduction organs was positively correlated with levels of soluble protein and amino acid, and activities of GS (glutamine synthetase), GOGAT (glutamine oxoglutarate aminotransferase), GOT (glutamate oxaloacetate transaminase), GPT (glutamic pyruvic transaminase), but negatively correlated with peptidase and protease activities, indicated that the change of nitrogen metabolism was the major factor contributing to altered Bt protein expression (Chen et al. 2012a, b, 2019). Therefore, we proposed the hypothesis that the boll-setting rate would affect Bt toxin content in cotton bolls. In addition, as the first defense of cotton bolls to bollworm, the insect resistance of the boll wall is especially important for insecticidal security. In order to get different boll setting rates, different plant densities and flower-removal treatments were imposed on cotton plants to study the relationship between boll formation and insecticidal concentration in the boll wall.

The primary objective of this study was to explore the relationship between boll setting rate under different planting density and Bt insecticidal protein contents in the boll wall. The secondary objective was to find out the physiological mechanism underlying the dynamics of Bt toxin concentration.

Material and methods

Plant material and experimental design

Field studies were conducted at Yangzhou University Farm, Jiangsu Province, China (32 ° 30 ′ N, 119 ° 25 ′ E) in 2017–2018. Bt transgenic cultivar Sikang3 (hybrid) was used as experimental material. The soil (sandy loam) contained about 19.8 $g{\cdot}kg^{-1}$ organic matter, 105.0, 26.0, and 85.0 $mg{\cdot}kg^{-1}$ available N-P-K in the studied duration, respectively. Other cultivation practices, including insecticides application, DPC (1,1-dimethyl piperidinium chloride, $C_7H_{16}ClN$) spraying, and irrigation, were conducted according to local cultivation practices. Each plot consisted of 6 rows, the plot size was 6 m in length with 5.4 m in width, row space was 0.90 m apart. Seeds were sown on April 15th (2017) and April 16th (2018) in a greenhouse. Seedlings were transplanted to the field on May 18th (2017) and May 19th (2018).

In 2017 and 2018, a randomized complete block design with three replications was conducted. Five plant densities (D1–D5: 15 000, 25 000, 45 000, 60 000, and 75 000 plants per hectare) were imposed on cotton plants.

In 2018, a flower-removal study was carried out with three treatments. A quarter (1/4) and a half (1/2), and none (CK) of white flowers were removed during the peak flowering stage, and the untreated plants were used as control (CK). The experiment was arranged according to a randomized complete block design with three replications. The planting density was 30 000 plants per hectare.

Preparation of plant material

Five bolls at 10 days after flowering were sampled and frozen with liquid nitrogen, stored in − 80 °C, and used for the measurements of Cry1Ac protein content, chemicals, and enzyme activities for protein metabolism in the boll wall.

Physiological measurements

Boll setting rate

The boll number was recorded on Jul 31th and Aug 10th (peak boll stage) for 20 plants in the two central rows in each plot, and the boll setting rate per plant was calculated by the difference of boll number within the two recording dates divided by the days.

The CryIAc protein concentration assay

The CryIAc protein concentrations of the boll wall for 10-day boll after anthesis were determined by immunological analysis ELISA (enzyme-linked immunosorbent assay) (Chen et al. 1997). Three subsamples of 0.5 g shell were prepared by homogenizing the frozen tissue in a 2 mL extraction buffer (Na_2CO_3 1.33 g, DTT 0.192 g, NaCl 1.461 g, Vitamin C 0.5 g dissolved in 250 mL distilled water), then transferred to a 10 mL centrifugation tube, the residue remaining on the wall of the mortar was washed again with 3 mL of the extraction buffer and this was also added to the centrifugation tube. The contents of this tube were shaken with hand and stored at 4 °C for 4 h. The supernatants were collected after centrifugation at 10 000×*g* at 4 °C for 20 min, passed through a C^{18} Sep-Pak Cartridge (Waters, Milford, MA), and three subsamples were pooled for CryIAc quantification. Quantification of the CryIAc in the combined samples was conducted using a commercially available kit (Scientific Service, Inc. China Agriculture University, Beijing). Microtitration plates were coated with the

standard CryIAc insecticidal proteins and samples, incubated at 37 °C for 4 h. The antibodies were added to each well and incubated for another 30 min at 37 °C. The antibodies against the CryIAc insecticidal protein were obtained as described by Weiler and Conrad (1981). Then horseradish peroxidase-labeled goat anti-rabbit immunoglobulin was added to each well and incubated for 30 min at 37 °C. Finally, the enzyme-substrate buffer (orthopenylenediamino) was added, and the enzyme reaction was carried out in the dark at 37 °C for 15 min, then terminated using 3 mol·L^{-1} H_2SO_4. The absorbance was recorded at 490 nm. Calculation of the ELISA data was performed as described by Weiler and Conrad (1981).

Assay of free amino acid and soluble protein content

The boll wall samples (0.5 g) from different treatments were used for the extraction and analysis of amino acid concentration and soluble protein content. The sample was homogenized at 4 °C in 5 mL cold water (Milli-Q reagent grade) and centrifuged at 800×*g* for 5 min. The supernatant was stored on ice, and the pellet was resuspended in 3 mL cold water prior to re-centrifugation (800×*g*) for another 5 min, The supernatant from both centrifugations were pooled and stored on ice, the pellet was re-suspended in 2 mL cold water, and centrifuged at 800×*g* again. The supernatant was pooled for analysis. The total free amino acid content was determined by ninhydrin assay (Yemm et al. 1955). The absorbance readings (in amino acid, fresh weight (FW)) were converted to μg·g^{-1} using the glycine standard curve. The total soluble protein content was determined by the Coomassie Blue dye-binding assay of Bradford (1976). The absorbance readings were converted to protein concentration using BSA standard curve.

Glutamic-pyruvic transaminase (GPT) and glutamate oxaloacetate transaminase (GOT) assay

The boll wall samples of the different treatments were used as an analysis of GPT and GOT activities. The samples (0.5 g) were homogenized in a buffered medium (0.05 mmol · L_{-1} Tris-HCl, pH 7.2), and the homogenate was centrifuged at 26 100×*g* for 10 min at 0 °C. The supernatant was analyzed for GOT activity. A mixture of 0.5 mL of a 0.8 mol·L^{-1} alanine in 0.1 mol·L Tris-HCl (pH 7.5) together with 0.1 mL of 2 mmol·L^{-1} pyridoxal phosphate solution was used, and to this mixture 0.2 mL of 0.1 mol · L^{-1} 2-oxoglutarate solution and 0.2 mL the prepared enzyme were added. The reaction mixture was incubated at 37 °C for 10 min followed by termination of reaction with 0.1 mL of 0.2 mol · L^{-1} trichloroacetic acid solution, then the pyruvate with chromogen was converted to pyruvate hydrazone. The color intensity of the hydrazone in saturated water toluene was measured at 520 nm. The GOT activity, in terms of pyruvate production, was calculated from authentic pyruvate standards simultaneously. The procedure used for assaying the activity of GPT was identical to the GOT assay, except that in the GPT assay, 0.5 mL of 0.8 mol alanine in 0.1 mol·L^{-1} Tris-HCl (pH 7.5) were substituted for 0.5 mL of 0.1 mol·L^{-1} buffered aspartate solution in the reaction mixture and aniline citrate addition was omitted (Tonhazy et al. 1950).

Assay of protease and peptidase activity

The boll wall samples were also used as an analysis of protease and peptidase activities. The samples (0.8 g) were homogenized at 4 °C in 1 mL of β-mercaptoethanol extraction buffer (a mixture of ethylene glycol, sucrose, and phenylmethylsulfonyl fluoride, pH 6.8). Cell debris was removed by centrifugation, and the supernatant was placed on ice and immediately used to estimate the square protease. Protease activity was determined using azocasein as substrate (Vance and Johnson 1979) and expressed as the change of absorbance (400 nm) in mg (protein) ·g^{-1} (fresh weight).

The boll wall samples (0.5 g) were homogenized at 4 °C in 8 mL of Tris-HCl extraction buffer (a mixture of 4 mmol·L^{-1}DTT, 4 mmol·L^{-1} EDTA, 1% pH 7.5), and then centrifuged at 15 000 ×*g* for 30 min at 0 °C. The supernatant was used to estimate the peptidase activity (Vermon 1979). A mixture of 0.4 mL acetate buffer (pH 4.8), 1% bovine hemoglobin compounded with 0.2 mL acetate buffer (pH 4.8) was incubated at 37 °C for 10 min. Then 0.4 mL of the enzyme sample was added to the mixture and was incubated at 38 °C for 60 min followed by termination of reaction with 1 mL of 10% trichloroacetic acid solution. For the control treatment, the termination solution was added before the reaction. The solution was then centrifuged at 4 000×*g* for 5 min again after incubation at 4 °C for 30 min. The supernatant was pooled for analysis of the amino acid content which was determined by ninhydrin assay (Yemm et al. 1955), and expressed as the change in absorbance 'μmol (amino acid) ·g^{-1} (fresh weight)'.

Statistics analysis

Analysis of variance (ANOVA) was conducted for insecticidal content etc. using Proc ANOVA in SAS (SAS Institute 1989). The differences between treatments were tested for significance using LSD ($P < 0.05$). The correlation study was computed as the Pearson correlation coefficient.

Results

The effect of plant density on boll setting rate and boll wall insecticidal protein concentration

Reduced boll setting rate per plant was detected with increased planting density (Table 1). There were 0.51 bolls set in each plant per day under treatment D1, but only

0.24 bolls formed under D5 treatment in the 2017 study. A greater reduction of boll setting rate was observed under lower planting density. The boll setting rate was reduced by 23.5% from treatment D1 to D2, but the reduction was only 4.0% from treatment D4 to D5. Similar results were found in 2018.

Enhanced insecticidal protein concentrations in the boll wall were observed with increased planting densities (Table 1). The Bt toxin contents in the boll wall were only 126.7 $ng{\cdot}g^{-1}$ FW under treatment D1, but 181.3 $ng{\cdot}g^{-1}$ FW under treatment D5. A greater increase for Bt toxin content was detected under low planting density. The Bt protein was increased by 20.4% from treatment D1 to D2 but only enhanced by 4.1% from treatment D4 to D5. Similar results were also observed in 2018.

Different planting densities influenced protein metabolism in boll wall

Amino acid and soluble protein content in boll wall

Similar results were detected for boll wall amino acid content under different planting densities in 2017 and 2018 (Figs. 1 and 2). Reduced amino acid contents were observed in the boll wall with increased planting densities. The amino acid content was decreased by 36.3 and 40.1% when planting density increased from D1 to D5 in 2017 and 2018, respectively.

In contrast to the trend of amino acid content, soluble protein content in the boll wall raised with increased planting density, with the values increased by 54.7 and 58.9% when planting density increased from D1 to D5 in 2017 and 2018, respectively (Figs. 1 and 2).

GPT and GOT activity in boll wall

Increased GPT and GOT activities in the boll wall were observed with increased planting density in 2017 and 2018 (Figs. 3 and 4). The GPT and GOT activities were enhanced by 32.7 and 36.2% with planting density increased from D1 to D5 in 2017 and increased by 33.6 and 33.9% with planting density increased from D1 to D5 in 2018. Greater increases of GPT and GOT activities were detected under a lower reduction extent of boll setting rate under high planting density.

Protease and peptidase in boll wall

Significantly reduced protease activity in the boll wall was observed with an increased planting density in both studied years (Figs. 5 and 6). The values were decreased by 45.7 and 47.3% with planting density increased from D1 to D5 in 2017 and 2018, respectively. A similar trend was observed in peptidase activity with increased planting density in 2017 and 2018 (Figs. 5 and 6). However, the reduced extent of peptidase activity was smaller with the increased planting density, compared with that of protease activity. The values were only reduced by 6.1 and 4.1% with planting density increased from D1 to D5 in 2017 and 2018, respectively.

The effect of flower-removal on boll setting rate and boll wall insecticidal protein concentration

Compared with the control, significant lower boll setting rates under the flower-removal treatments were found in 2018 (Fig. 7). The values reduced by 21.2 and 45.4% under one-fourth and half of the white flowers removed treatments, respectively. In contrast, the boll wall Bt protein contents under the flower-removal treatments were higher than the control. The boll wall Bt protein content increased by 6.7 and 26.9% under one-fourth and half of the white flowers removed treatments, respectively. The lower boll setting rate had higher boll wall insecticidal protein concentration.

Discussion

Enhanced boll setting rate reduced insect resistance of boll wall by reduced palnting density

It was reported that lower insect resistance was detected at the boll development stage (Benedict et al. 1993; Xia et al. 2005; Chen et al. 2019). Wang et al. (2009) found that cultivar with bigger bolls exhibited lower insect resistance in boll walls and seeds. Higher reproductive organ number was also reported after Bt gene's introduction for Bt cotton cultivars (Abidallha et al. 2017a, b),

Table 1 Effect of plant density on boll setting rate and Bt toxin content in boll wall in 2017 and 2018

Treatment	2017		2018	
	Boll setting rate/ ($boll{\cdot}plant^{-1}{\cdot}d^{-1}$)	Bt protein content/ ($ng{\cdot}g^{-1}FW$)	Boll setting rate/ ($boll{\cdot}plant^{-1}{\cdot}d^{-1}$)	Bt protein content/ ($ng{\cdot}g^{-1}FW$)
D1	0.51a	126.7d	0.50a	129.7d
D2	0.39b	152.5c	0.38b	155.1c
D3	0.29c	165.9b	0.31bc	170.2b
D4	0.25d	174.2ab	0.24cd	181.5ab
D5	0.24d	181.3a	0.22d	192.6a

D1-D5: 15 000, 25 000, 45 000, 60 000, and 75 000 plants per hectare, FW indicated fresh weight; the same letter among treatments are not significantly different (LSD test at 0.05 significance level).

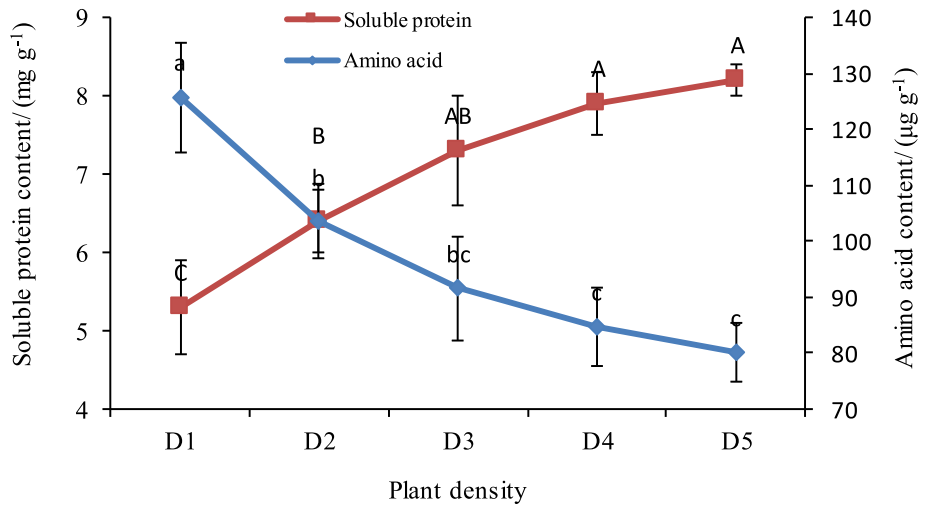

Fig. 1 The amino acid and soluble protein content in boll wall with different planting densities in 2017. D1-D5: 15 000, 25 000, 45 000, 60 000, and 75 000 plants per hectare. The vertical bar represents S.E. of the mean ($n = 3$) when the value exceeds the size of the symbol. Lowercase letters indicate significance analysis among amino acids, and capital letters indicate significance analysis among soluble proteins. The same letter among treatments is not significantly different ($P \geq 0.05$)

and lower Bt protein concentration was detected together with higher boll number and boll volume (Chen et al. 2018). Our studies further extended these findings, indicated that Bt insecticidal concentration in boll wall reduced significantly with enhanced boll setting rate by reduced planting density, and a significantly negative correlation was found between boll setting rate and Bt toxin content in boll wall ($R_{2017} = 0.995$ and $R_{2018} = 0.992$). The results were proved further by the flower-removal study. Higher Bt protein content was observed under a low boll setting rate created by flower removal. The results suggest that it is necessary to regulate the boll setting rate for both high lint yield and Bt toxin concentration for insect resistance. DPC applications increased both boll setting rate and Bt protein content in our study (Fig. 8). It was also confirmed by other studies with plant growth regulators and amino acid application (Abidallha et al. 2017a, b; Chen et al. 2017). Therefore, these agronomic measures could be used to improve both yield formation and insect resistance, which can provide a guarantee for the sustainable application of Bt cotton.

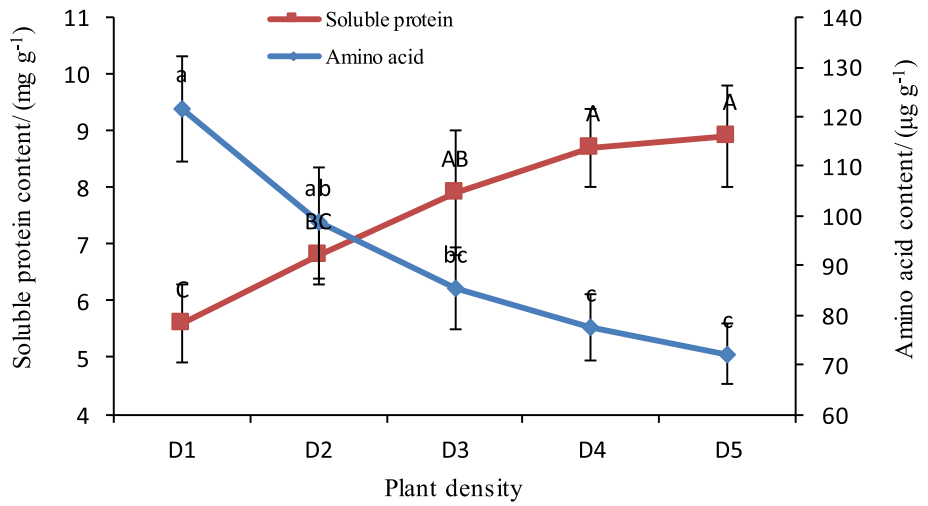

Fig. 2 The amino acid and soluble protein content in boll wall with different planting densities in 2018. D1-D5: 15000, 25 000, 45 000, 60 000, and 75 000 plants per hectare. The vertical bar represents S.E. of the mean ($n = 3$) when the value exceeds the size of the symbol. Minuscule letters indicate significance analysis among amino acids, and capital letters indicate significance analysis among soluble proteins. The same letter among treatments is not significantly different ($P < 0.05$)

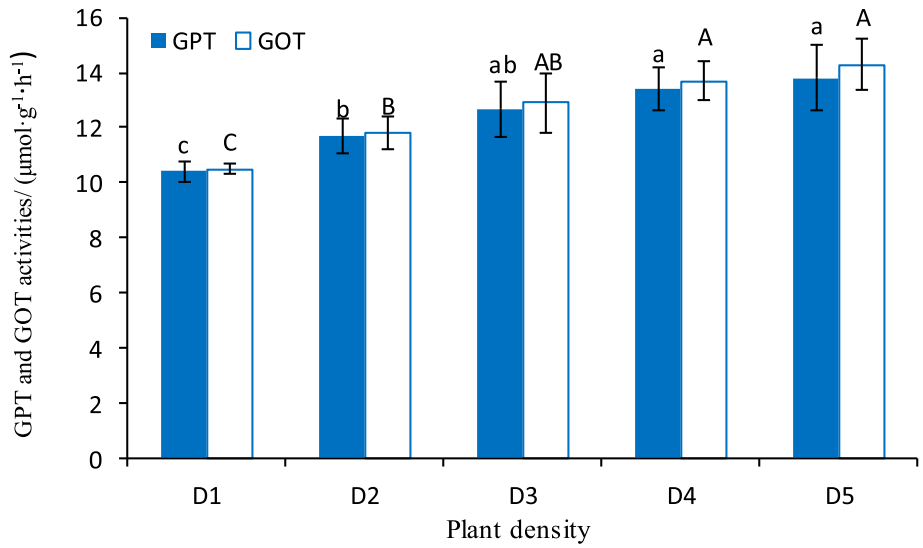

Fig. 3 The GPT and GOT activity in boll wall under different planting densities in 2017. D1-D5: 15 000, 25 000, 45 000, 60 000, and 75 000 plants per hectare. The vertical bar represents S.E. of the mean ($n = 3$) when the value exceeds the size of the symbol. Lowercase letters indicate significance analysis among GPT activity, and capital letters indicate significance analysis among GOT activity. The same letter among treatments is not significantly different ($P \geq 0.05$)

Reduced protein synthesis and increased protein degradation resulted in the decrease of Bt toxin concentration in the boll wall

It was found that lower protein synthesis and higher protein degradation in boll wall was related to high boll setting rate under lower planting density in our study. In contrast, when the rate of the boll setting reduced under high planting density, protein synthesis in the boll wall enhanced, and protein degradation reduced. These results indicated that Bt cultivars with enhanced protein synthesis at the peak boll stage were unfavorable for boll formation. The conclusions were in line with previous research in cotton (Wang et al. 2009; Zhang et al. 2014). Moreover, enhanced Bt insecticidal protein concentration was observed together with high soluble protein contents, GPT and GOT activities, but low amino acid

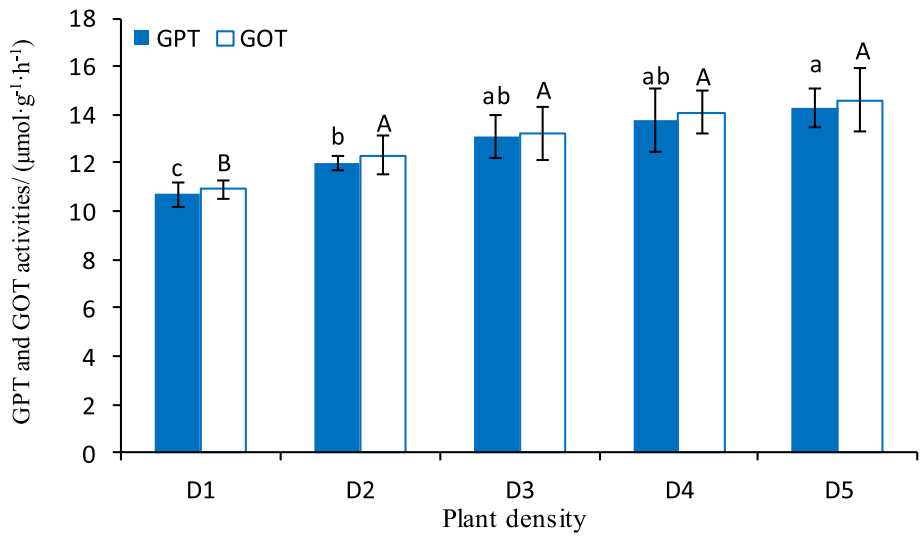

Fig. 4 The GPT and GOT activity in boll wall under different planting densities in 2018. D1-D5: 15 000, 25 000, 45 000, 60 000, and 75 000 plants per hectare. The vertical bar represents S.E. of the mean ($n = 3$) when the value exceeds the size of the symbol. Lowercase letters indicate significance analysis among GPT activity, and capital letters indicate significance analysis among GOT activity. The same letter among treatments is not significantly different ($P \geq 0.05$)

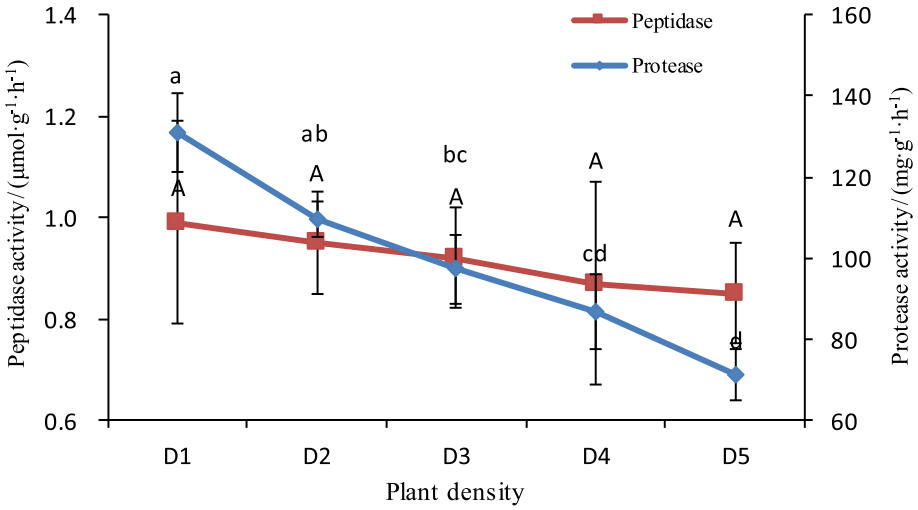

Fig. 5 The protease and peptidase activity in boll wall under different planting densities in 2017. D1-D5: 15 000, 25 000, 45 000, 60 000, and 75 000 plants per hectare. The vertical bar represents S.E. of the mean ($n = 3$) when the value exceeds the size of the symbol. Lowercase letters indicate significance analysis among protease activity, and capital letters indicate significance analysis among peptidase activity. The same letter among treatments is not significantly different ($P \geq 0.05$)

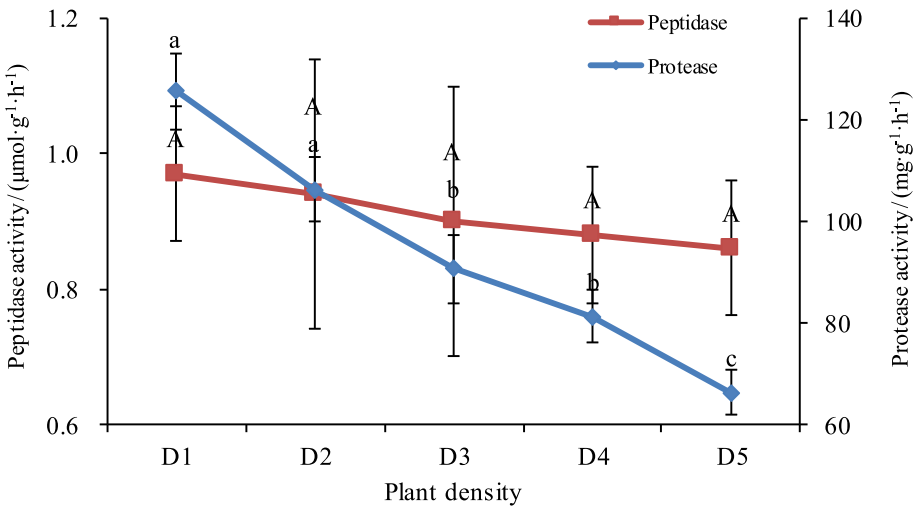

Fig. 6 The protease and peptidase activity in boll wall under different planting densities in 2018. D1-D5: 15 000, 25 000, 45 000, 60 000, and 75 000 plants per hectare. The vertical bar represents S.E. of the mean ($n = 3$) when the value exceeds the size of the symbol. Lowercase letters indicate significance analysis among protease activity, and capital letters indicate significance analysis among peptidase activity. The same letter among treatments is not significantly different ($P \geq 0.05$)

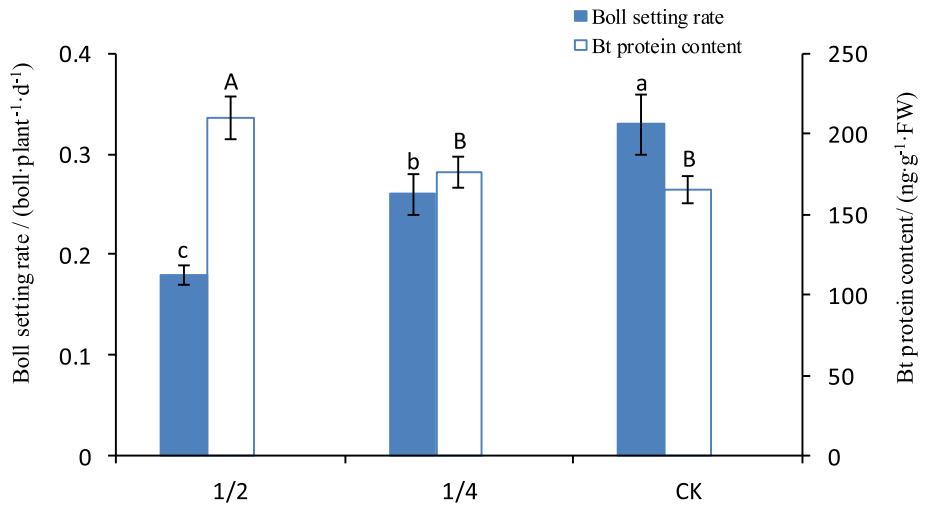

Fig. 7 The boll setting rate per plant and boll wall Bt toxin content under different flower-removal treatments in 2018. Sympol 1/4 and 1/2 represented one-fourth and half of the white flowers was removed during peak flowering stage respectively, CK was the control. The vertical bar represents S.E. of the mean ($n = 3$) when the value exceeds the size of the symbol. Lowercase letters indicate significance analysis among boll setting rate per plant, and capital letters indicate significance analysis among Bt protein content. The same letter among treatments is not significantly different ($P \geq 0.05$)

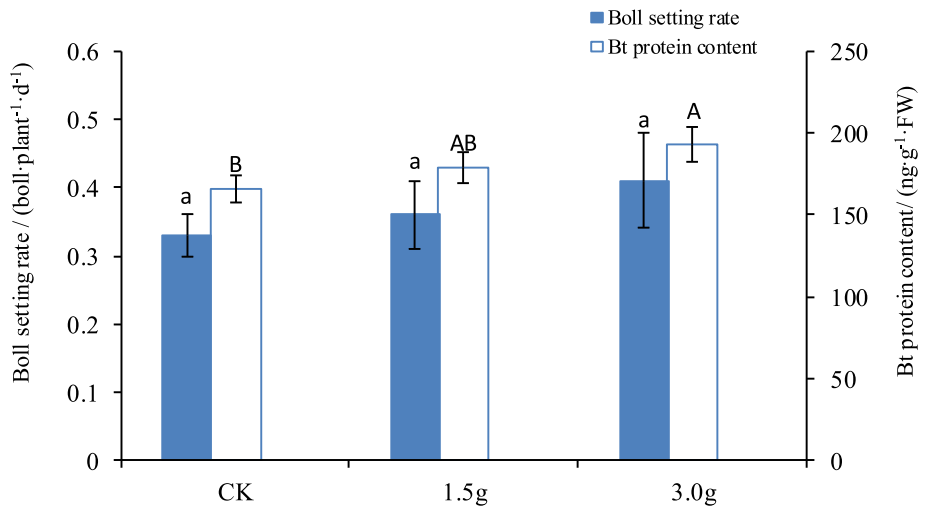

Fig. 8 The boll setting rate per plant and boll wall Bt toxin content under different DPC applications treatments in 2018. Sympol 1.5 g and 3.0 g represented the application rate of DPC during the peak flowering stage respectively, CK was the control. The vertical bar represents S.E. of the mean ($n = 3$) when the value exceeds the size of the symbol. Lowercase letters indicate significance analysis among boll setting rate per plant, and capital letters indicate significance analysis among Bt protein content. The same letter among treatments is not significantly different ($P \geq 0.05$)

content, protease and peptidase activities in our study, which was consistent with previous reports (Chen et al. 2017, 2019). These results suggested that enhanced ability of protein synthesis benefit insect resistance in the boll wall but reduced boll formation. Therefore, a tissue culture method should be applied to increase both boll setting and insect resistance in the boll wall. In our previous study, both boll setting rate and protein synthesis in boll wall could bolster remarkably with DPC application (Chen et al. 2012b), further exploration is necessary for the enhancement of both boll formation and insect resistance.

Abbreviations

Bt: *Bacillus thuringiensis*; GPT: Glutamic-pyruvic transaminase; GOT: Glutamic oxaloacetate transaminase; DPC: Dimethyl piperidinium chloride; FW: Fresh weight; ELISA: enzyme-linkedimmunosorbent assay; GOGAT: glutamine oxoglutarate aminotransferase; GS: glutamine synthetase

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Authors' contributions

Zhou MY and Chen C wrote the paper; Zhou MY, Tambel LIM and Chen C performed the experiments and analyzed the data; Chen Y and Chen DH conceived and designed the research; Chen Y and Zhang X revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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