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Research article

Nitrogen deficit decreases seed Cry1Ac endotoxin expression in Bt transgenic cotton

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ABSTRACT

The spatial and temporal expression of insecticidal gene *Cry1Ac* in transgenic *Bacillus thuringiensis* (Bt) cotton varies with plant organs, plant age, as well as environmental conditions. The research was undertaken to study the impact of nitrogen deficit on Cry1Ac endotoxin concentration in seed in Yangtze River valley region in China in 2015 and 2016. To uncover the underlying mechanism, the nitrogen metabolism process including protein synthesis and degradation was characterized. Based on the results, nitrogen deficit decreased the boll weight, boll volume, boll number per plant, seed Cry1Ac endotoxin concentration, glutamate oxaloacetate transaminase (GOT) and glutamic pyruvic transaminase (GPT) activities, soluble protein concentration, but increased peptidase and protease activities, and free amino acid content in seed. Our results suggested that the decline of seed Cry1Ac endotoxin expression associated with decreased nitrogen was due to the enhanced protein decomposition and reduced protein synthesis, especially the altered activities of GOT and peptidase. There was a significant negative correlation between seed Cry1Ac endotoxin concentration and boll shell Cry1Ac endotoxin content under nitrogen deficiency. Therefore, seed Cry1Ac endotoxin concentration and boll shell Cry1Ac endotoxin concentration should be balanced to guarantee the insecticidal efficiency.

1. Introduction

The *Bacillus Thuringiensis* (Bt) cotton plants could encode the Cry1Ac endotoxin to improve the resistance to lepidopteran pests. The production of Bt transgenic cotton is beneficial to the environment and growers by reducing synthetic insecticides, preserving the population of beneficial arthropods, and increasing worker safety and grower profitability. Transgenic Bt cotton is currently cultivated on a large scale across the world, but it behaves variably in insecticidal efficiency under field conditions. The variability of inseciticidal efficacy caused by unstable Cry1Ac endotoxin expression was attributed to the developmental stage, different plant organs, and/or the extreme environmental factors (Benedict et al., 1993; Chen et al., 2012; Wang et al., 2009,XiaGuo, 2004). The insecticidal efficacy of the Cry1Ac endotoxin was not stable across the growing season and decreased as the crop matures in the field, particularly from flowering period onwards, and the boll period recorded the lowest toxin efficacy (Li et al., 2006; Zhang et al., 2011). Higher insecticidal efficacy was detected in leaves, compared to the reproductive organs, especially the bolls (Shen et al., 2010; Stone, 2011). Besides the temporal and spatial variance, environmental factors such as high temperature, low temperature, soil salinity, and water deficit also affected insecticidal protein expression (Chen et al., 2005; Chen et al., 2012; Chen et al., 2012; Chen et al., 2012; Chen et al., 2013).

The changed nitrogen metabolism might be responsible for variation in Cry1Ac endotoxin content (Chen et al., 2017; Zhang et al., 2007). Bt insecticidal efficacy due to the changed nitrogen metabolism was observed in all the processes including environmental stress, developmental stages, and cultural practices (Chen et al., 2005; Chen et al., 2012; Chen et al., 2012; Chen et al., 2012; Chen et al., 2013). However, how nitrogen metabolism itself affect the Bt protein expression in reproductive organs has not been systematically studied. These benefits and challenges of Bt transgenic cotton underscore the importance to better understand the temporal and spatial variation in toxin efficiency and the resulting mechanism. In this study, we altered the nitrogen metabolism by decreasing nitrogen application compared

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to the local recommended dose, and through this way, nitrogen metabolism was affected, and we could further study the change of insecticidal efficiency of Bt cotton in this process. In our previous study, cotton boll shell, the first target of bollworm of cotton bolls was studied. And decreased Bt protein expression was detected with increasing nitrogen application under N deficiency (Chen et al., 2018). In present study, we will focus on Bt protein expression of cotton seeds, which is the main target of the bollworm. In addition, GPT, GOT, peptidase, and protease activities are frequently recorded parameters in nitrogen metabolism research, and they are correlated with Bt protein content according to our previous study (Chen et al., 2005; Chen et al., 2012; Chen et al., 2012; Chen et al., 2012; Chen et al., 2013). However, the contribution of these parameters to the altered Bt protein expression was not clear.

The research reported here characterized the boll development and seed Cry1Ac endotoxin expression under nitrogen deficit. The primary objective was to compare the differential insecticidal protein expression between the shell and seed to study the insecticidal expression difference in different boll parts under nitrogen deficit. A second objective was to uncover the underlying mechanism of Cry1Ac endotoxin variability by investigating the nitrogen metabolism processes, and the path analysis was used to determine factors affecting Bt protein content in our present study.

2. Materials and methods

2.1. Experimental materials and design

Field experiments were conducted at Yangzhou University Farm of Jiangsu Province, China (32°30′ N, 119°25′ E), in the Yangtze River Valley region of China, from 2015 to 2016. The experimental site has a sandy loam soil (Typical fluvaquents, Entisols (U.S. taxonomy)) with 22.3 g kg^{-1} organic matter and 110.5, 21.6, 85.6 mg kg^{-1} available N–P–K in 2015, 21.9 g kg^{-1} organic matter and 113.7, 20.9, 86.8 mg kg^{-1} available N–P–K in 2016. In our present study, two widely grown Bt transgenic cotton, Sikang1 (conventional) and Sikang3 (hybrid), were used. The seeds were planted on April 3rd in 2015 and April 7th in 2016 in a greenhouse, and seedlings were transplanted to field with the planting density of 27000 (Sikang3) and 37500 (Sikang1) plants per hectare on May 15th in 2015 and May 19th in 2016. K (120 kg ha^{-1} as KCl) and P (300 kg ha^{-1}as single superphosphate) were used before planting, followed by K (120 kg ha^{-1} as KCl) and P (300 kg ha^{-1} as single superphosphate) at early flowering. N (urea) was used before transplanting, at early flowering, and at peak flowering with the proportion of 25%, 18%, 57% respectively. Other management practices were conducted according to local recommendations unless otherwise indicated.

A split plot designs with three replications was used. The main plot was cultivars (Sikang3 and Sikang1), while the nitrogen rates (0, 75, 150, 225 and 300 kg/ha) constituted the subplots. Each subplot contained 6 rows, 6 m long spaced 0.9 m apart.

2.2. Data collection

Data collection were performed in the middle two rows of each subplot. The seed Cry1Ac endotoxin contents and nitrogen metabolic parameters were assessed at 10, 20, 30 and 40 days after flowering (DAF). Fifteen bolls locating at the first fruiting position of the fourth to sixth fruiting branches were collected for further analysis in each subplot. For each parameter, the subsamples per each subplot were used.

As described by Chen et al., (Chen et al., 1997), Cry1Ac endotoxin content was analyzed by immunological analysis ELISA. We analyzed the total free amino acid content by ninhydrin assay according to Yemm et al., (Yemm et al., 1955). The total soluble protein concentration was determined by the Coomassie Blue dye-binding assay of Bradford (1976). Seed samples (0.2 g) were homogenized in 5 ml 0.05 mM Tris-HCl butter (pH 7.2) and the homogenate was centrifuged at 26,100 *g* at 4 °C for 10 min. The supernatant was used for analysis of GOT and GPT activities, following the procedure described by Tonhazy (Tonhazy et al., 1950). Seed 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). The supernatant was collected to estimate the square protease activity (VanceJohnson, 1979). For peptidase activity measurement, seed samples (0.5 g) were homogenized in 8 ml Tris-HCl buffer (4 mM DTT, 4 mM EDTA, 1% PVP, pH 7.5) at 4 °C and measured for the peptidase activity based on Setlow (1975).

2.3. Statistical analysis

Analysis of variance was performed using PROC ANOVA in SAS 9.4. Means were separated using LSD test at the 5% significant level.

3. Results

3.1. Seed and the subtending leaf insecticidal protein concentration under decreased nitrogen application

The seed Cry1Ac endotoxin content was lower as the nitrogen rate decreased from 300 to 0 kg/ha (Table 1). Nitrogen treatments 3/4N, 1/2N, 1/4N, and 0N reduced the seed Cry1Ac endotoxin contents by 8.5%, 19.0%, 41.1%, 47.8% in Sikang1 and by 10.6%, 17.8%, 42.5%, 48.8% in Sikang3 compared with the control treatment (300 kg/ha N) at 40 DAF in 2015. The decline caused by nitrogen treatments 3/4N, 1/2N, 1/4N, and 0N on seed Cry1Ac endotoxin content was 5.5%, 12.0%, 21.6%, 26.8% in SK-1 and 8.4%, 16.6%, 23.2%, 29.4% in SK-3 at 40 DAF in 2016. The Cry1Ac endotoxin content in the subtending leaves of corresponding bolls exhibited the same trend as that of seed, which

Table 1
Seed Cry1Ac endotoxin contents (ng g^{-1} FW) of Bt transgenic cotton Sikang1 (SK-1) and Sikang3 (SK-3) under nitrogen treatments in 2015 and 2016.

Cultivar	N rate (kg/ha)	2015				2016			
		10 DAF	20 DAF	30 DAF	40 DAF	10 DAF	20 DAF	30 DAF	40 DAF
SK-1	0	56.68j	100.45g	103.07h	116.98g	94.00g	113.24i	125.98g	157.21h
	75	93.53i	121.96f	125.59f	131.88f	105.95f	128.26g	142.12e	168.24g
	150	140.41f	171.45d	175.12d	181.46d	117.80d	134.24f	162.12d	188.82e
	225	169.14e	186.28c	189.82c	204.92c	127.30b	153.54c	173.90c	202.95c
	300	203.30b	207.40b	215.43b	224.07b	128.98b	180.36a	188.37b	214.67b
SK-3	0	103.64h	114.73f	114.93g	128.84f	112.52e	117.35h	133.93f	168.42g
	75	133.40g	136.03e	144.12e	144.60e	119.84cd	127.06g	146.46e	183.07f
	150	186.39d	191.71c	194.54c	206.70c	124.94bc	139.44e	159.00d	198.79d
	225	196.77c	213.68 ab	214.42b	224.82b	129.41b	149.06d	185.54b	218.35b
	300	212.69a	220.82a	229.27a	251.47a	137.77a	171.83b	199.72a	238.40a

DAF is acronym of "days after flowering". Same letter among treatments within the same sampling date represent non-significant difference ($p < 0.05$).

Table 2
The subtending leaf Cry1Ac endotoxin contents (ng g^{-1} FW) of Bt transgenic cotton Sikang1 (SK-1) and Sikang3 (SK-3) under nitrogen treatments in 2015 and 2016.

Cultivar	N rate (kg/ha)	2015				2016			
		10 DAF	20 DAF	30 DAF	40 DAF	10 DAF	20 DAF	30 DAF	40 DAF
SK-1	0	196.3i	187.2h	180.2i	159.3i	193.1i	181.3i	173.2i	145.6i
	75	226.5g	203.6 fg	191.6gh	172.6gh	221.7g	215.2g	186.7gh	157.8hi
	150	260.8ef	230.4e	215.4f	187.5 fg	250.1ef	240.5ef	220.6ef	180.3ef
	225	295.6cd	268.9cd	238.2cd	210.2de	283.6c	270.1c	241.6cd	225.4c
	300	310.6 ab	290.3 ab	263.6 ab	236.1 ab	297.8 ab	286.7 ab	267.8 ab	230.6bc
SK-3	0	202.7hi	195.2gh	186.3hi	165.7hi	196.7hi	193.3hi	180.1hi	158.9gh
	75	245.6 fg	231.6e	216.3ef	189.3ef	235.4 fg	226.7 fg	209.8 fg	178.3f
	150	276.3de	251.5d	235.7d	216.5cd	257.3de	245.1de	230.6de	192.4de
	225	306.2bc	287.1bc	254.4bc	232.6bc	290.8bc	276.8bc	251.9bc	236.7 ab
	300	321.6a	298.7a	267.6a	250.2a	311.2a	296.5a	278.2a	246.5a

DAF is acronym of "days after flowering". Same letter among treatments within the same sampling date represent non-significant difference ($p < 0.05$).

decreased with decreasing nitrogen rate. Higher Cry1Ac endotoxin content was detected in leaves, in contrast to seeds. And the leaf Cry1Ac endotoxin content decreased in the growing season (Table 2).

3.2. The relationship between seed Cry1Ac endotoxin content and boll growth parameters under decreased nitrogen rate

Cotton plants exhibit lower insect resistance to boll worm at early stage of boll development, which is around 20 DAF. There were significant positive correlations between seed Cry1Ac endotoxin content and boll number per plant in 2015 (r = 0.927**) and 2016 (r = 0.841**), seed Cry1Ac endotoxin content and boll volume in 2015 (r = 0.918**) and 2016 (r = 0.944**), seed Cry1Ac endotoxin content and individual boll dry weight in 2015 (r = 0.962**) and 2016 (r = 0.953**) (Fig. 1). The highest correlation was observed between Cry1Ac endotoxin content with boll weight, in contrast the lowest correlation was recorded between Cry1Ac endotoxin content and boll number.

3.3. The relationship between boll shell Cry1Ac endotoxin content and seed Cry1Ac endotoxin content under decreased nitrogen rates

Significant negative correlation was detected between seed Cry1Ac endotoxin content and boll shell Cry1Ac endotoxin content in both years. The correlation between seed Cry1Ac endotoxin content and boll shell Cry1Ac endotoxin content was −0.879**, −0.914**, −0.968**, −0.965** at 10, 20, 30, 40 DAF in 2015, and -0.745*, −0.927**, −0.931**, −0.775** at 10, 20, 30, 40 DAF in 2016 (Fig. 2). The correlation was highest at 30 and 40 DAF in 2015, and at 20 and 30 DAF in 2016. Therefore, higher correlation was observed at middle to late boll developing period, and the correlation was higher in 2015.

3.4. Seed nitrogen metabolism under decreased nitrogen rates

Enhanced seed soluble protein and amino acid concentration was detected during the growing season. In contrast, the seed soluble protein content increased and the amino acid content reduced as the nitrogen application dose reduced in both cultivars (Fig. 3 and Fig. 4). Greater reduction of soluble protein with decreasing nitrogen application was recorded at early boll developing period. At 10 DAF, by decreasing nitrogen rates from 300 to 0 kg/ha, the soluble protein content was declined by 43.2% in Sikang1 and 45.3% in Sikang3, compared to the decrease of 27.7% in Sikang1 and 39.3% in Sikang3 at 40 DAF in 2015. In the year 2016, when nitrogen rates reduced from 300 to 0 kg/ha, the reduction of the soluble protein content was 37.9% in Sikang1 and 36.2% in Sikang3 at 10 DAF, in contrast the decline was 26.0% in Sikang1 and 23.9% in Sikang3 at 40 DAF. This result indicated that soluble protein content in seed was less sensitive to the nitrogen rates at late boll developing stage.

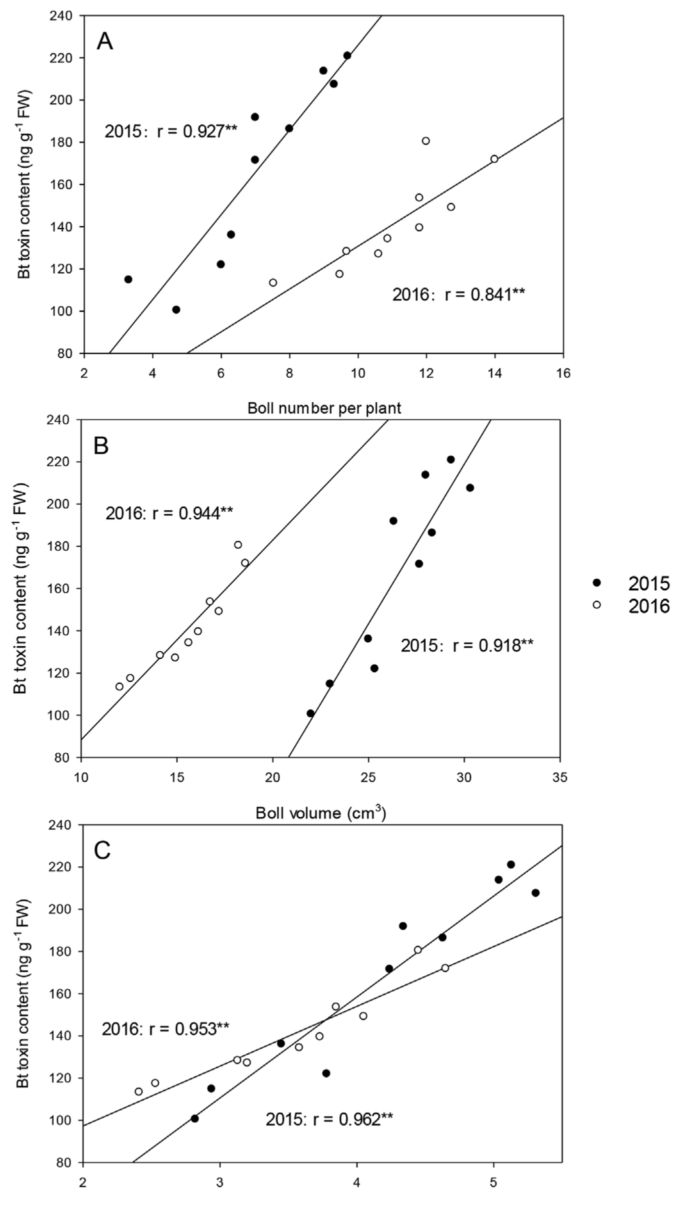

Fig. 1. The correlations between seed Cry1Ac endotoxin content and boll number per plant (A), seed Cry1Ac endotoxin content and boll volume (B), and seed Cry1Ac endotoxin content and boll dry weight (C) at 20 DAF (days after flowering) in 2015 and 2016.

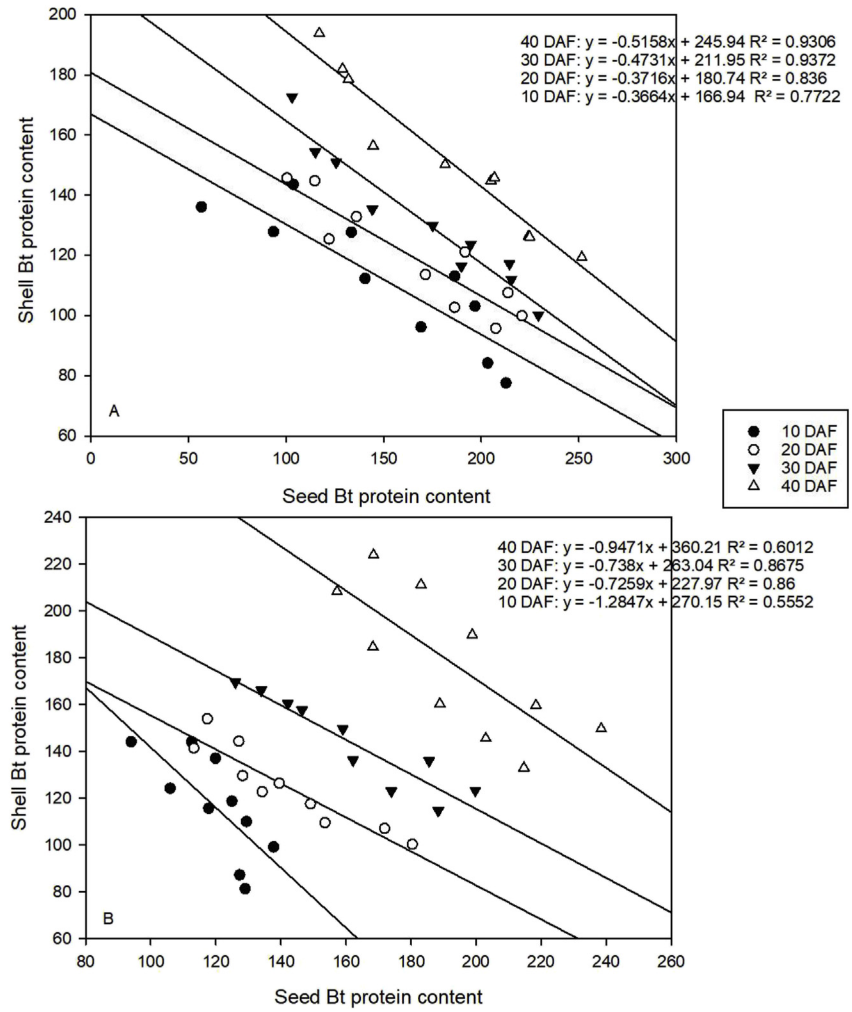

Fig. 2. The Correlations between boll shell Cry1Ac endotoxin content and seed Cry1Ac endotoxin content at 10, 20, 30, 40 DAF (days after flowering) in 2015 (A) and 2016 (B).

The activities of GPT and GOT tended towards lower values as the nitrogen application dose decreased (Table 3 and Table 4). GPT and GOT activity decreased greater at early boll developing period with the decreasing nitrogen rates, compared with late boll developing period. By reducing nitrogen rates from 300 to 0 kg/ha, the decline in GPT activity was 31.1% in Sikang1 and 35.8% in Sikang3 at 10 DAF, compared to the reduction of 24.1% in Sikang1 and 23.9% in Sikang3 at 40 DAF in 2015. Similar trend was observed in 2016. The activity of GS was lower with the decreasing nitrogen rate. By reducing nitrogen rates from 300 to 0 kg/ha, the decline in GS activity was 59.1% in Sikang1 and 50.5% in Sikang3 in 2015, compared with the reduction of 37.1% in Sikang1 and 57.2% in Sikang3 in 2016 (Table 5).

Seed protease and peptidase activities tended toward higher values as the nitrogen application dose decreased (Table 6 and Table 7). We obtained the greater increase of peptidase activity at early boll developing stage than late boll developing stage in both years. Similar trend for protease activity was recorded in both cultivars in the year 2016.

Path analysis was applied to determine the direct and indirect effects of the nitrogen metabolism parameters on seed Bt protein content. In path analysis, Bt protein content was the response variable and the explanatory variables were the four nitrogen metabolism parameters GPT, GOT, peptidase and protease activity. According to the path analysis results, only GOT and peptidase activity had significant effect in the model for the explanatory variable Bt protein content (Fig. 5). The direct effect of GOT on seed protein was positive and significant (0.86**), and the correlation coefficient between GOT and Bt protein content in the present study was high ($r = 0.816^{**}$). The direct effect of peptidase on seed protein was negative (−0.373**), and the correlation coefficient between peptidase and Bt protein content was lower than that of GOT ($r = -0.271^{**}$). Along with the direct effect, there was also indirect effect of GOT (−0.044) and peptidase (0.102) on Bt protein content.

4. Discussions

4.1. Nitrogen deficit decreased seed Cry1Ac endotoxin concentration

Our research reported here showed that nitrogen deficit significantly reduced Cry1Ac endotoxin content, and our previous research revealed that boll weight, boll volume, and boll number all decreased under nitrogen deficit. The positive correlation between Cry1Ac endotoxin content and boll development parameters (boll weight, boll volume, and boll number) was further confirmed by correlation analysis. To guarantee the requirement of both boll development and Cry1Ac endotoxin content, sufficient nitrogen supply is needed (Guinn, 1986). Since the recommended nitrogen rates for research conducted area was 300 kg/ha, nitrogen was not enough to meet the requirement of both fast developing bolls and insecticidal protein synthesis in our study. Thus, both reduced boll development and Bt protein contents were detected with the decreasing dose of nitrogen application.

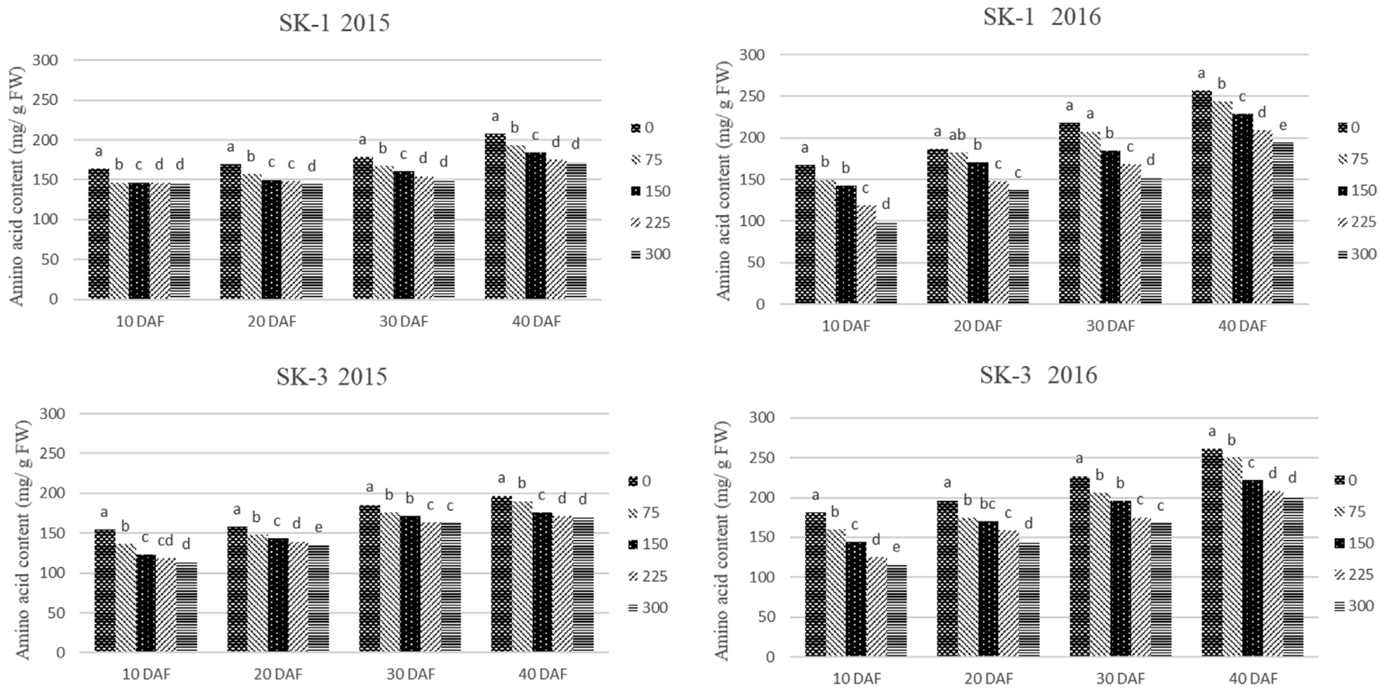

Fig. 3. Seed amino acid content of Bt transgenic cotton Sikang1 (SK-1) and Sikang3 (SK-3) under nitrogen treatments at 10, 20, 30, 40 DAF (days after flowering) in 2015 and 2016. Same letter among treatments within the same sampling date represent non-significant difference ($p < 0.05$).

4.2. Nitrogen metabolism affected seed Cry1Ac endotoxin concentration

In contrast to the seed results, our previous study on boll shell showed that the insecticidal protein expression increased as nitrogen rates dropped from 300 to 0 kg/ha (Chen et al., 2018). Correlation analysis further confirmed a significant negative correlation of Cry1Ac endotoxin concentration in boll shell with Cry1Ac endotoxin concentration in seed. The possible explanation for the different trend of Cry1Ac endotoxin concentration under decreased nitrogen application is the uneven nitrogen distribution among seed and shell in cotton bolls. When the nitrogen rates raised from 0 to 300 ka/ha, most of the nitrogen was assimilated in the seed but not the boll shell. Therefore, nitrogen supply was enough for seed to produce more Cry1Ac endotoxin and feed the development, but in boll shell nitrogen supply was limited and Cry1Ac endotoxin synthesis was sacrificed. The nitrogen content in different parts further confirmed our hypothesis. At 20 DAF in cultivar Sikang1, the nitrogen contents were 1.67%, 2.37%, and 2.51% at 0, 150, and 225 kg/ha nitrogen application in boll shell; the nitrogen contents were 2.12%, 3.21%, and 3.92% at 0, 150, and 225 kg/ha nitrogen application in seed; and the nitrogen contents were

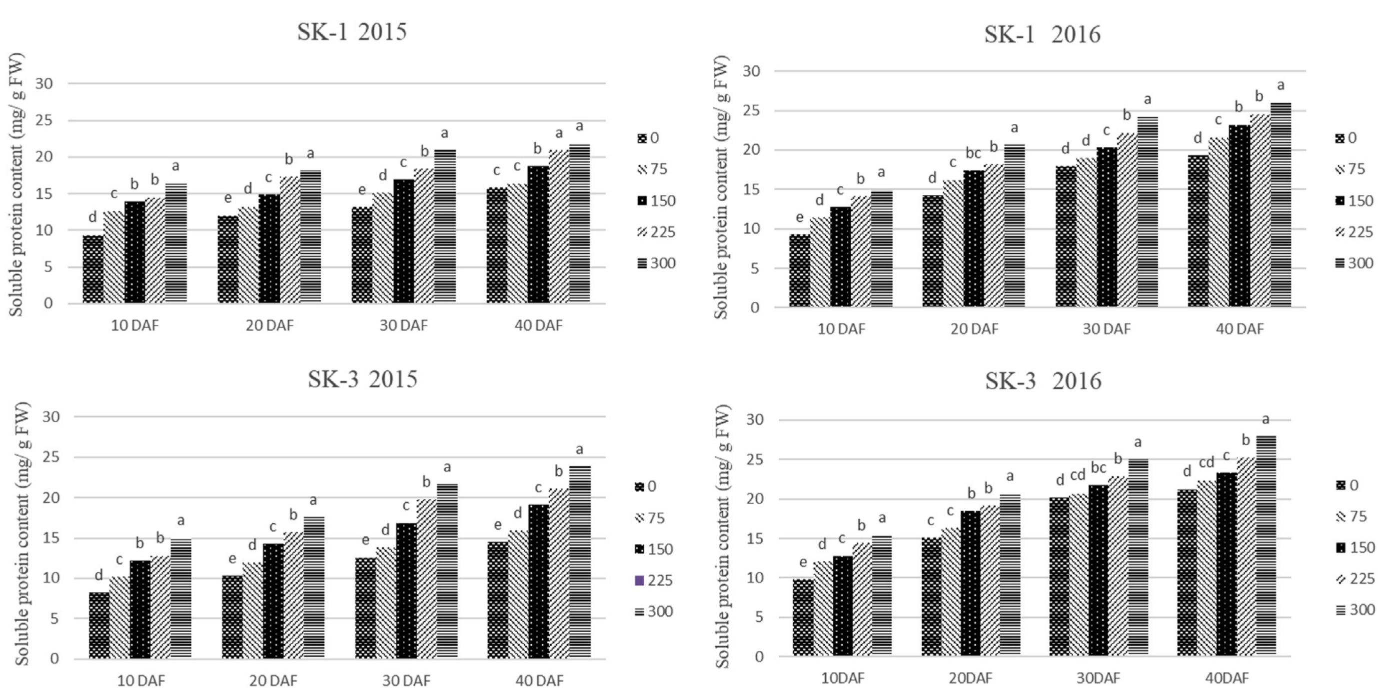

Fig. 4. Seed soluble protein content of Bt transgenic cotton Sikang1 (SK-1) and Sikang3 (SK-3) under nitrogen treatments at 10, 20, 30, 40 DAF (days after flowering) in 2015 and 2016. Same letter among treatments within the same sampling date represent non-significant difference ($p < 0.05$).

Table 3
Boll seed glutamic-oxalacetic transaminase (GOT) and glutamic-pyruvic transaminase (GPT) activities of Bt transgenic cotton Sikang1 (SK-1) and Sikang3 (SK-3) under nitrogen treatments in 2015.

Cultivar	N rate (kg/ha)	GPT activity (μmol g^{-1} FW h^{-1})				GOT activity (μmol g^{-1} FW h^{-1})			
		10DAF	20DAF	30 DAF	40 DAF	10DAF	20DAF	30DAF	40DAF
SK-1	0	8.56e	10.55c	10.50e	12.15e	9.69f	12.15g	12.43e	14.50d
	75	9.24d	11.05c	11.08d	12.92d	11.44e	14.50d	13.99d	16.45c
	150	10.71c	13.00b	12.92c	14.65c	13.67d	16.01b	16.78b	18.52 ab
	225	11.73b	13.69a	13.93b	15.40b	14.41c	17.07a	18.90a	19.41a
	300	12.43a	13.88a	14.60a	16.00a	14.92b	17.30a	19.12a	19.89a
SK-3	0	8.03e	9.17e	9.95d	11.86e	9.92f	11.09h	11.69f	14.69d
	75	8.69d	10.20d	10.44d	12.65d	11.67e	12.93f	13.59d	16.43c
	150	9.72c	12.29c	11.98c	13.87c	13.24d	14.00e	14.91c	17.97b
	225	11.72b	13.23b	12.81b	15.14b	14.37c	14.75cd	16.55b	19.65a
	300	12.50a	13.98a	13.56a	15.59a	15.47a	15.21c	16.99b	19.93a

DAF is acronym of "days after flowering". Same letter among treatments within the same sampling date represent non-significant difference ($p < 0.05$).

Table 4
Boll seed glutamic-oxalacetic transaminase (GOT) and glutamic-pyruvic transaminase (GPT) activities of Bt transgenic cotton Sikang1 (SK-1) and Sikang3 (SK-3) under nitrogen treatments in 2016.

Cultivar	N rate (kg/ha)	GPT activity (μmol g^{-1} FW h^{-1})				GOT activity (μmol g^{-1} FW h^{-1})			
		10DAF	20DAF	30 DAF	40 DAF	10DAF	20DAF	30DAF	40DAF
SK-1	0	8.24d	9.18e	12.32e	15.77e	8.65d	9.64e	12.93e	16.56e
	75	9.18c	9.61d	13.50d	16.42d	9.64c	10.09d	14.18d	17.24d
	150	9.86b	10.45c	14.17c	17.29c	10.35b	10.97c	14.88c	18.15c
	225	10.10b	11.26b	14.55b	17.84b	10.60b	11.83b	15.28b	18.73b
	300	10.71a	11.61a	15.09a	18.23a	11.25a	12.19a	15.85a	19.14a
SK-3	0	8.30e	9.49e	12.90e	15.59e	8.72e	9.96e	13.55e	16.37e
	75	9.07d	10.45d	13.53d	16.37d	9.53d	10.97d	14.21d	17.19d
	150	9.48c	10.64c	14.31c	17.30c	9.96c	11.17c	15.02c	18.17c
	225	10.50b	11.12b	14.76b	17.76b	11.02b	11.68b	15.5b	18.65b
	300	10.87a	11.66a	15.43a	18.29a	11.41a	12.24a	16.2a	19.2a

DAF is acronym of "days after flowering". Same letter among treatments within the same sampling date represent non-significant difference ($p < 0.05$).

Table 5
Boll seed (GS) activities of Bt transgenic cotton Sikang1 (SK-1) and Sikang3 (SK-3) under nitrogen treatments at 10 days after flowering (DAF) in 2015 and 2016.

Cultivar	N rate (kg/ha)	GS activity (μmol g-1 FW h-1)	
		2015	2016
SK-1	0	2.08d	2.99d
	75	2.32cd	3.70c
	150	3.12bc	4.46b
	225	3.76b	4.70 ab
	300	5.08a	4.75a
SK-3	0	1.80d	1.66e
	75	1.80d	3.10d
	150	2.28d	3.63c
	225	3.56b	3.80c
	300	3.64b	3.88c

Same letter among treatments within the same sampling date represent non-significant difference ($p < 0.05$).

0.76%, 1.02%, and 1.11% at 0, 150, and 225 kg/ha nitrogen application in boll fiber. Thus, the increase in nitrogen content was 22.1% in seed as the nitrogen rates raised from 150 to 225 kg/ha, compared to 5.9% increase in boll shell. When the nitrogen supply was enhanced from 0 to 225 kg/ha, the increase recorded in seed and boll shell was 84.9% and 50%, respectively. Therefore, the uneven distribution of nitrogen caused the different trend of Bt protein expression under different nitrogen doses.

Boll shell and seed, as the first target and main target of boll worm respectively, are important aims of insect protection. Maintaining the proper insecticidal efficiency in these both parts are required for insect resistance. Since the Cry1Ac endotoxin concentration in boll shell and seed was negatively correlated, it is important to balance the Cry1Ac endotoxin concentration in seed and boll shell to guarantee proper insecticidal efficiency.

Nitrogen deficiency reduced boll weight, boll volume, and boll number per plant, seed Cry1Ac endotoxin concentration, GOT and GPT activities, soluble protein concentration, but increased peptidase and protease activities, and amino acid concentration in seed. It is evident that nitrogen deficit decreased protein synthesis, but increased protein degradation. Therefore, Cry1Ac endotoxin in seed, as a part of total protein, reduced under nitrogen deficit. GPT, GOT, peptidase, and protease activities are frequently recorded parameters in nitrogen metabolism research, and they are correlated with Cry1Ac endotoxin concentration according to our previous study (Chen et al., 2005; Chen et al., 2012; Chen et al., 2012; Chen et al., 2012; Chen et al., 2013). Thus, the decline of Cry1Ac endotoxin concentration under nitrogen deficit was due to altered nitrogen metabolism. The path analysis was used to determine factors affecting Cry1Ac endotoxin concentration in our present study. According to our path analysis results, GOT and peptidase both contributed to the Cry1Ac endotoxin concentration model, whereas GPT and protease did not. The high correlation was noted between GOT and Cry1Ac endotoxin concentration ($r = 0.816^{**}$), while the lower correlation was detected between peptidase and Cry1Ac endotoxin concentration ($r = -0.271^{**}$). Compared to peptidase, GOT had the greater effect on Cry1Ac endotoxin concentration under nitrogen deficit conditions. Therefore, GOT and peptidase activity can be used for management decisions, and as a selection criteria for Cry1Ac endotoxin expression.

In sum, nitrogen deficit reduced boll weight, boll volume, and boll number, together with seed Cry1Ac endotoxin concentration. At the same time, decreased GOT and GPT activities, soluble protein

Table 6
Boll seed protease and peptidase activities of Bt transgenic cotton Sikang1 (SK-1) and Sikang3 (SK-3) under nitrogen treatments in 2015.

Cultivar	N rate (kg/ha)	Protease activity (mg g^{-1} FW h^{-1})				Peptidase activity (μmol g^{-1} FW h^{-1})			
		10 DAF	20 DAF	30 DAF	40 DAF	10DAF	20DAF	30DAF	40DAF
SK-1	0	0.97a	1.20a	1.29a	1.42a	1.10a	1.20a	1.27a	1.34a
	75	0.91b	1.13b	1.23b	1.36b	1.02b	1.14b	1.23b	1.29b
	150	0.81c	1.02c	1.10c	1.23c	0.90c	1.04c	1.14c	1.18c
	225	0.75d	0.93d	1.01d	1.09d	0.85d	0.97d	1.07d	1.09d
	300	0.68e	0.87e	0.94e	1.02e	0.80e	0.92e	1.02e	1.04e
SK-3	0	0.98a	1.14a	1.26a	1.39a	1.12a	1.17a	1.28a	1.33a
	75	0.93b	1.08b	1.20b	1.31b	1.04b	1.13b	1.22b	1.29b
	150	0.85c	0.95c	1.08c	1.16c	0.96c	1.02c	1.11c	1.18d
	225	0.82c	0.86d	0.98d	1.06d	0.88d	0.95d	1.05d	1.11d
	300	0.76d	0.82e	0.91e	0.98e	0.83e	0.91e	1.00e	1.07e

DAF is acronym of "days after flowering". Same letter among treatments within the same sampling date represent non-significant difference ($p < 0.05$).

Table 7
Boll seed protease and peptidase activities of Bt transgenic cotton Sikang1 (SK-1) and Sikang3 (SK-3) under nitrogen treatments in 2016.

Cultivar	N rate (kg/ha)	Protease activity (mg g^{-1} FW h^{-1})				Peptidase activity (μmol g^{-1} FW h^{-1})			
		10 DAF	20 DAF	30 DAF	40 DAF	10DAF	20DAF	30DAF	40DAF
SK-1	0	3.37a	3.40a	3.96a	4.18a	1.16a	1.16a	1.39a	1.39a
	75	2.73b	2.83 ab	3.50b	3.68b	0.98b	1.00 ab	1.19b	1.24b
	150	2.58b	2.56bc	2.97c	3.48b	0.94b	0.92bc	1.05c	1.19b
	225	2.09c	2.02c	2.43d	2.82c	0.79c	0.77c	0.89d	1.00c
	300	1.60d	1.94c	2.07d	2.10d	0.65d	0.75c	0.79d	0.79d
SK-3	0	3.80a	3.71a	3.90a	4.06a	1.28a	1.25a	1.30a	1.35a
	75	3.04b	3.03b	3.24b	3.83a	1.06b	1.05b	1.11b	1.29a
	150	2.31c	2.93b	2.87bc	3.23b	0.85c	1.04b	1.01bc	1.12b
	225	1.69d	2.03c	2.78c	2.91c	0.67d	0.78c	0.99c	1.03c
	300	1.64d	1.75c	1.86d	2.09d	0.66d	0.70c	0.72d	0.79d

DAF is acronym of "days after flowering". Same letter among treatments within the same sampling date represent non-significant difference ($p < 0.05$).

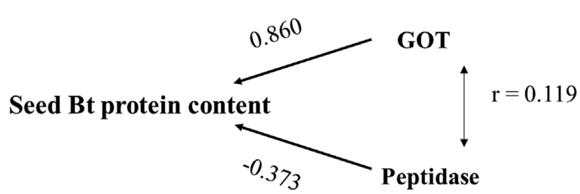

Fig. 5. The contribution of nitrogen metabolism parameters on seed Cry1Ac endotoxin content by path analysis.

concentration, enhanced peptidase and protease activities, and free amino acid were detected with decreasing nitrogen rates. Our results suggested that the decline of seed Cry1Ac endotoxin expression associated with decreased nitrogen was due to the enhanced protein decomposition and reduced protein synthesis, especially the altered activities of GOT and peptidase. There was a significant negative correlation between seed Cry1Ac endotoxin concentration and boll shell Cry1Ac endotoxin concentration under nitrogen deficiency. Therefore, seed Cry1Ac endotoxin concentration and boll shell Cry1Ac endotoxin concentration should be balanced to guarantee the insecticidal efficiency.

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