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Low temperature exposure influences nitrogen metabolism resulting in decreased Cry1Ac insecticidal endotoxin content in cotton seeds

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Abstract

Background Sudden temperature drops, resulting from extreme weather events, often occur during the boll-setting period of cotton in Xinjiang, China, causing decreased expression of *Bacillus thuringiensis* (Bt) insecticidal proteins in cotton bolls. The precise threshold temperatures and durations that lead to significant changes in Cry1Ac endotoxin levels under low temperatures remain unclear. To address this, we investigated the effects of different temperatures and stress durations on Cry1Ac endotoxin levels in cotton bolls. In 2020–2021, two Bt transgenic cotton varieties, conventional Sikang1 and hybrid Sikang3, were selected as experimental materials. Various low temperatures (ranging from 16 to 20 °C) with different durations (12 h, 24 h and 48 h) were applied during the peak boll-setting period.

Results As the temperature decreased, the Cry1Ac endotoxin content in the boll shell, fiber, and seed exhibited a declining trend. Moreover, the threshold temperature which caused a significant reduction in Cry1Ac endotoxin content increased with the prolonged duration of low-temperature stress. Among the components of cotton bolls, seeds were most affected by low-temperature stress, with the threshold temperature for a significant reduction in Cry1Ac endotoxin content ranging from 17 °C to 19 °C. Correlation analysis indicated that low temperatures led to a decrease in protein synthesis capacity and an increase in degradation ability, resulting in reduced Cry1Ac endotoxin content. Pathway analysis revealed that both free amino acid and peptidase had significant negative effects on Cry1Ac endotoxin content.

Conclusion In summary, when the daily average temperature was ≤ 19 °C, implementing cultural practices to reduce free amino acid content and peptidase activity could serve as effective cold defense strategies for Bt cotton production.

Keywords Low-temperature, Cotton seed, Cry1Ac endotoxin, Protein metabolism

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Introduction

Cotton yield loss increased significantly with the enhanced cotton boll worm population. The number of bollworm larvae and the number of infested squares showed high positive correlation, and one second generation larvae could damage 7.4 squares in average [1]. *Bacillus thuringiensis* (Bt) transgenic cotton, featuring the expression of the Cry1Ac endotoxin (Bt protein), represents a revolutionary cotton variety achieved by introducing the Bt gene into cotton tissue cells. It stands as one of the most extensively cultivated and widely distributed genetically modified crops globally [2]. Bacillus thuringiensis produces a parasporal crystal named δ-endotoxin during its metabolic process, exhibiting toxic effects on various pests, particularly lepidopteran pests like cotton bollworm [3–5]. Fibre quality of transgenic lines was not affected when compared with no transgenic lines [6, 7]. The introduction of the first genetically modified cotton variety with Bt traits in Xinjiang at the end of the last century brought about significant economic and ecological benefits through large-scale cultivation. The expression of insect-resistant proteins notably inhibits the growth of lepidopteran pests, such as cotton bollworm, reducing their impact on cotton and consequently increasing yield. Simultaneously, it curtails the use of chemical pesticides, alleviating environmental pressures [8–11].

Despite the widespread cultivation of Bt cotton in China, the instability in the expression of Bt cotton's insecticidal protein has emerged as a pressing concern in the current landscape of transgenic cotton in China [12, 13]. Cry1Ac endotoxin content gradually decreases throughout the growing season, reaching its lowest concentration during the boll-setting stage [14–16]. Furthermore, Cry1Ac endotoxin content varies across different cotton organs, with higher concentrations in the leaves and lower levels in reproductive organs like squares, flowers, and bolls [17–19]. Adverse environmental conditions, including extreme temperatures, especially low temperatures, can significantly decrease the expression of insecticidal proteins [20–26]. Among these adverse environments, prolonged exposure to low temperatures has been shown to decrease the Cry1Ac endotoxin content in leaves throughout the growth period, with a more pronounced decrease during the boll-setting period [27]. Field experiments have demonstrated that continuous low temperatures lead to a decrease in Cry1Ac endotoxin content, exacerbating cotton bollworm damage [28]. Additionally, under low-temperature conditions, the insecticidal protein content in leaves changes early and significantly decreases throughout the entire stress period [29]. Low temperature also showed a negative impact on cotton bollworm. Gu et al. (2018) showed that less than 60% of eggs and 70% of larvae survived if the daily average temperature was below 10 °C [30]. The relative contribution to the decreased population abundance by the effects on bollworm reproduction was greater than that on the developmental/survival rate (adult longevity), with adult longevity only reduced the population size up to 10%.

With the shift of China's cotton production regions to Xinjiang, over 80% of cotton production in China now originates from Xinjiang, constituting nearly one-fifth of global cotton production [31]. Specifically, in 2020, Xinjiang's cotton planting area and output accounted for 79.0% and 87.3% of China's respective values [32]. Consequently, low-temperature stress occurs more frequently in Chinese cotton production due to the high latitude of Xinjiang, with an increasing trend in the frequency and intensity of extreme low temperatures [33]. Temperatures below 15 °C can impede the growth and development of cotton, and temperatures below 20 °C in later stages are detrimental to cotton maturation [34]. In July and August, the critical boll development stage, Xinjiang often experiences nightly temperatures around 15 °C, even consecutive days with average temperatures below 20 °C [35]. On July 9, 2022, Kashgar experienced temperatures as low as 9 °C, while in mid-August 2022, Altay encountered a week-long period with average temperatures below 15 °C.

Due to the occurrence of low temperatures in the reproductive growth stage and the relatively lower expression of insecticidal protein in reproductive organs, it is crucial to explore the changes in insect resistance of Bt cotton in reproductive organs under low-temperature stress. However, prior studies have predominantly focused on leaves under low-temperature stress, and the impact of low temperatures on different components of cotton bolls, such as boll shells, fibers, and seeds, has been scarcely explored. Therefore, this study aimed to simulate low-temperature conditions that may occur in Xinjiang, compare the changes in the expression of Cry1Ac endotoxin in Bt cotton boll components under different degrees and durations of low temperatures, and investigate the associated physiological mechanisms.

Results

Low temperature occurrence during boll development stage in Xinjiang

Summarizing the daily average temperatures from July 1 to August 31 in the past decade (2014–2023), the East Xinjiang Cotton Region generally experienced higher temperatures with a lower threat of low temperatures. In recent years, both the North Xinjiang Cotton Region and the South Xinjiang Cotton Region have faced greater challenges from low temperatures during the boll-setting period, with particular attention needed in the North Xinjiang Cotton Region (Fig. 1). Compared to the previous five years (2014–2018), in the last

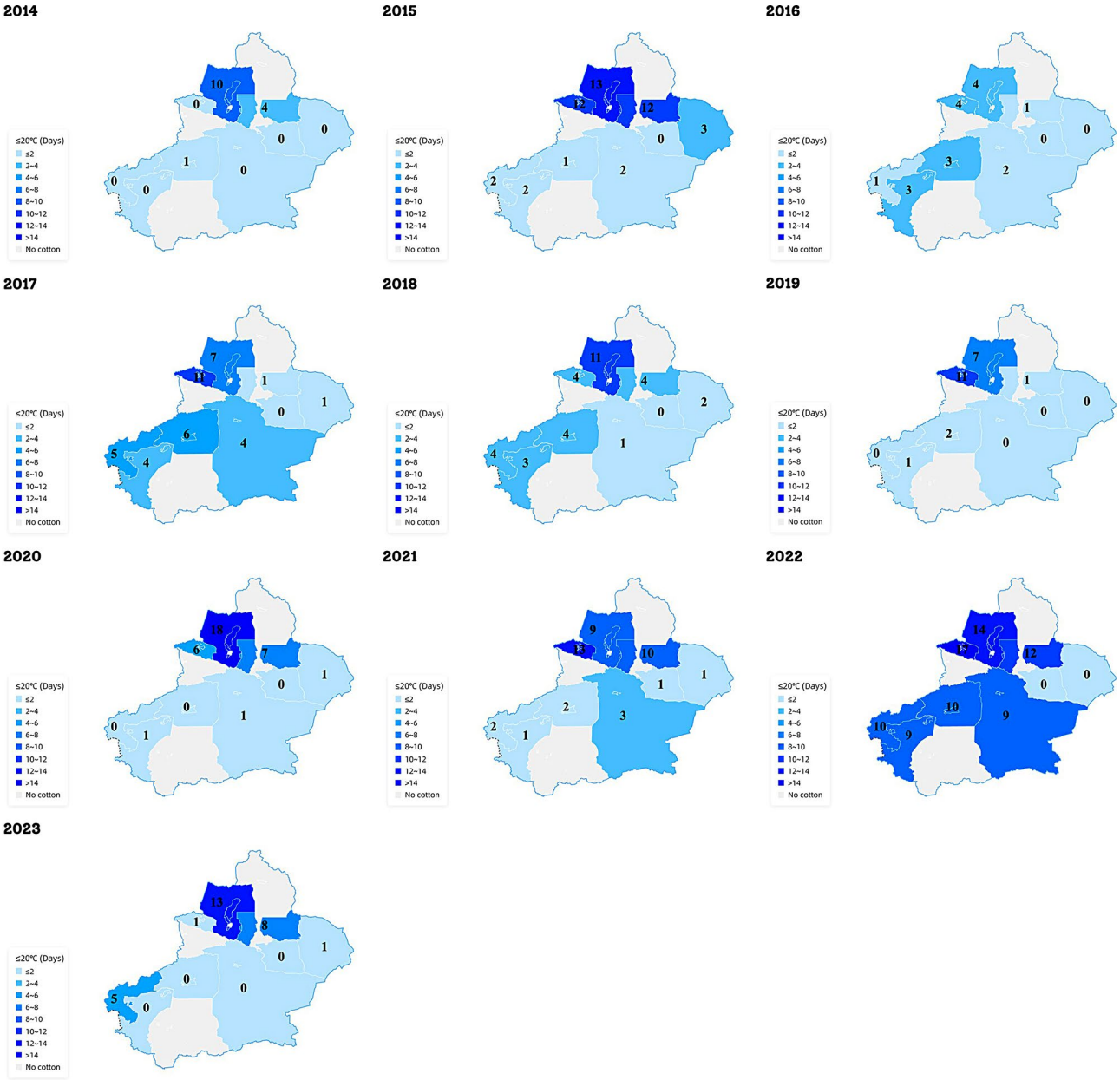

Fig. 1 Statistics on the number of days with daily mean temperature less than or equal to 20℃ in the main cotton areas of Xinjiang, China from 2014 to 2023

five years (2019–2023), the total number of days with a daily average temperature lower than 20 °C increased by 72.73% in Changji Hui Autonomous Prefecture, by 35.56% in Tacheng Prefecture, and by 54.84% in Bortala Mongol Autonomous Prefecture. It is evident that, in recent years, with climate change, the cotton-growing areas of Xinjiang, especially in Northern Xinjiang, have experienced more frequent occurrences of cold weather during the peak boll-setting period of Bt cotton. Consequently, investigating the impact of low temperatures on the Cry1Ac endotoxin content in cotton bolls holds significant importance for cotton security in China and globally.

Cry1Ac endotoxin content

According to the low-temperature levels and frequency in Xinjiang, we set the low-temperature range from 16 to 20 °C and tested the effect of different levels and durations of low temperature on Cry1Ac endotoxin contents in cotton bolls. Under low temperatures, the Cry1Ac endotoxin content in boll shell, fiber, and seed decreased, and with the decrease in temperature, their content showed a decreasing trend (Table 1). In 2020, compared

Table 1 Effects of varying temperatures and stress durations on the Cry1Ac endotoxin content in cotton boll shell, fiber, and seed (ng g^{-1} FW)

Years	Varieties	T	Boll shell			Fiber			Seed		
			12 h	24 h	48 h	12 h	24 h	48 h	12 h	24 h	48 h
2020	SK-1	27℃	129.68a	130.08a	130.01a	66.30a	66.89a	66.04a	233.35a	233.2a	232.74a
		20℃	128.53a	124.68a	119.97b	65.26a	63.47ab	63.91a	230.75a	220.96a	213.51a
		19℃	128.76a	124.21a	118.88b	65.18a	62.57ab	63.75a	228.99a	212.33ab	177.17b
		18℃	128.25a	111.41b	107.16c	65.49a	61.11b	54.05b	228.99a	197.89b	161.16bc
		17℃	115.18b	109.90b	95.30d	64.03a	55.77c	53.44b	228.03a	176.70c	143.99 cd
		16℃	116.08b	99.08c	94.05d	57.26b	50.10d	47.70c	225.49a	154.64d	124.66d
	SK-3	27℃	134.60a	134.10a	133.87a	69.58a	70.03a	69.69a	322.85a	302.19a	305.34a
		20℃	133.96a	129.25a	122.73b	69.16a	67.99a	68.21a	321.58a	291.91a	287.77a
		19℃	133.54a	126.55a	122.87b	69.45a	67.76a	67.28a	321.30a	283.91ab	274.91ab
		18℃	129.79ab	112.71b	107.30c	68.91a	67.33a	61.46ab	320.53a	267.08b	246.54bc
		17℃	125.12b	111.46bc	97.42d	67.41ab	62.09b	55.53bc	318.74a	244.17c	228.19 cd
		16℃	125.37b	102.46c	96.14d	62.68b	56.51c	49.00c	319.39a	221.26d	211.74d
2021	SK-1	27℃	120.43a	120.82a	117.93a	62.12a	61.52a	60.63a	282.68a	281.96a	277.95a
		19℃	118.88a	116.66a	107.88b	61.23a	58.34ab	57.18a	281.67a	270.76a	250.43b
		18℃	118.00a	106.90b	95.74c	61.65a	57.08b	52.00b	278.04a	263.36a	233.06c
		17℃	111.76b	102.46b	84.20d	60.11a	51.99c	50.49b	276.90a	216.74b	200.99d
		16℃	111.35b	90.72c	83.55d	55.99b	47.23d	46.26c	273.06a	202.09b	180.42e
	SK-3	27℃	133.10a	132.82a	132.12a	65.12a	65.95a	64.76a	413.65a	404.90a	386.83a
		19℃	132.52a	128.69a	121.17a	64.33a	63.88a	62.42ab	405.66a	385.21ab	370.04a
		18℃	130.73ab	119.00b	107.83b	63.61a	62.99a	56.40bc	406.19a	359.75b	330.47b
		17℃	124.68b	113.90b	95.31c	63.34ab	58.85b	55.44bc	403.82a	320.57c	294.26c
		16℃	124.44b	101.08c	94.73c	59.50b	55.07c	50.57c	400.2a	310.14c	269.08d

Note FW represents fresh weight. Different lowercase letters indicate significant differences between treatments of each variety at $P<0.05$ in the same year. SK-1 and SK-3 represent cultivars Sikang 1 and Sikang 3, respectively

with control check (CK: 27 °C), after 12 h of stress, the Cry1Ac endotoxin content in SK1 and SK3 boll shell significantly decreased at 16–17 °C, fiber at 16 °C, while the seeds showed no significant difference from CK. After 24 h of stress, the Cry1Ac endotoxin content in SK1 boll shell, fiber, and seeds significantly decreased at 16–18 °C, and the Cry1Ac endotoxin content in SK3 boll shell significantly decreased at 16–18 °C, fiber at 16–17 °C, and seeds at 16–18 °C. After 48 h of stress, the Cry1Ac endotoxin content in SK1 boll shell significantly decreased at 16–20 °C, fiber at 16–18 °C, seeds at 16–19 °C, the Cry1Ac endotoxin content in SK3 boll shell significantly decreased at 16–20 °C, fiber at 16–17 °C, and seeds at 16–18 °C. From the above results, it can be concluded that the temperatures cause a significant decrease in Cry1Ac endotoxin content in boll shell, fiber, and seed. Such decrease in Cry1Ac endotoxin increased with the prolonged duration of low-temperature stress. Additionally, with the prolonged duration of stress, the Cry1Ac endotoxin content in boll shell, fiber, and seeds under each low-temperature treatment showed a gradual decrease.

In 2020, after 12–48 h of stress at 20 °C, Cry1Ac endotoxin content decreased by 0.89-7.72% in the SK1 boll shell, 1.57-3.23% in fiber, and 1.11-8.26% in seeds (Fig. 2). Similarly, after 12–48 h of stress at 19 °C, Cry1Ac endotoxin content in the SK1 boll shell, fiber and seeds decreased by 0.71-8.56%, 1.69-6.46%, and 1.87-23.88%, respectively. This trend continued with 12–48 h of stress at 18 °C, where Cry1Ac endotoxin content in the SK1 boll shell, fiber and seeds decreased by 1.10-17.58%, 1.22-18.16%, and 1.87-30.76%, respectively. The pattern persisted with further decreases in temperature; after 12–48 h of stress at 17 °C, Cry1Ac endotoxin content in the SK1 boll shell, fiber and seeds decreased by 11.18-26.70%, 3.42-19.08%, 2.28-38.13%, respectively. Similarly, after 12–48 h of stress at 16 °C, Cry1Ac endotoxin content in the SK1 boll shell, fiber and seeds decreased by 10.49-27.66%, 13.63-27.77%, and 3.37-46.44%, respectively.

The comparison of the decrease in Cry1Ac endotoxin content in the cotton boll, fiber, and seeds after the same duration of stress revealed that in 2020, Cry1Ac endotoxin content in SK1 boll shell, fiber, seeds decreased by 0.89 −10.49%, 1.57 −13.63%, and 1.11 −3.67% after 12 h of stress at 16–20℃. After 24 h of stress, Cry1Ac endotoxin content decreased by 4.15 −23.83%, 5.11 −25.10%, and 5.25 −33.69% in the cotton boll shell, fiber and seeds, respectively. Similar trend was observed with longer stress duration, after 48 h of stress, Cry1Ac endotoxin content in the boll shell, fiber and seeds decreased by 7.22 −27.66%, 3.23 −27.77%, and 8.26 −46.44%,

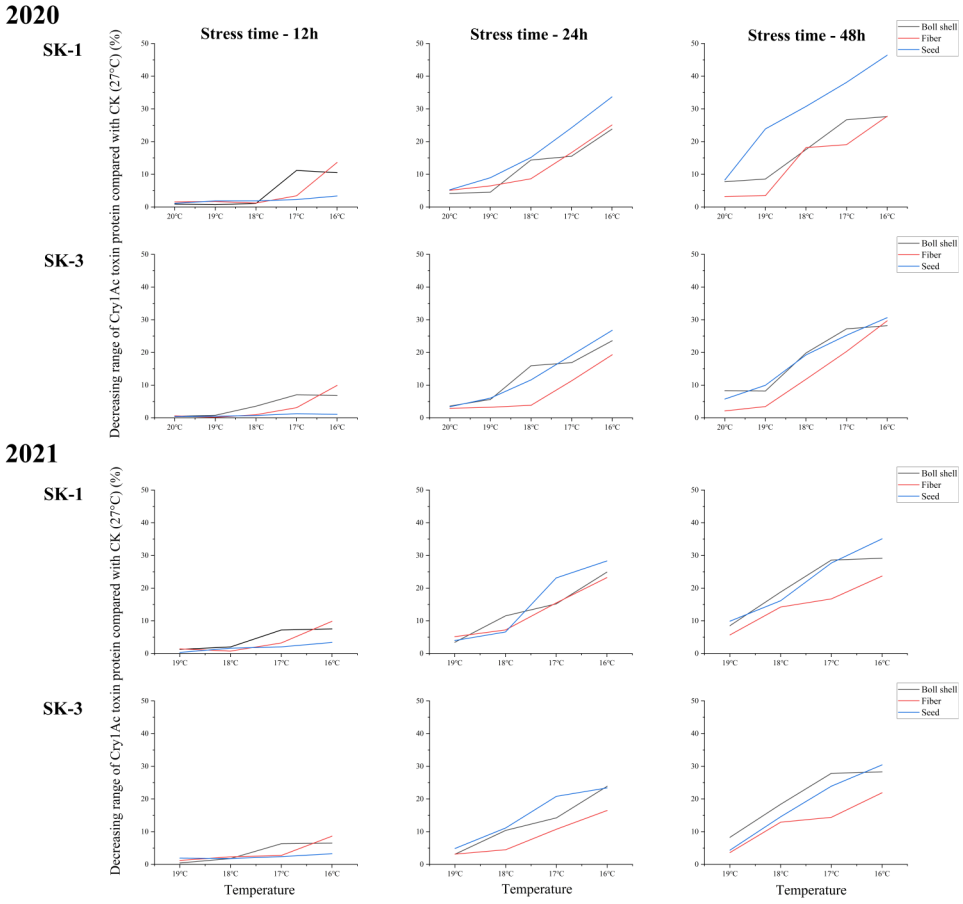

Fig. 2 The decreasing range in Cry1Ac endotoxin protein content in boll shell, fiber, and seed under varying temperatures and stress durations treatment compared with CK (27 ℃). SK-1 and SK-3 represent cultivars Sikang 1 and Sikang 3, respectively

respectively. The trend of change in SK3 was similar to SK1. The experimental results in 2021 were similar to those in 2020. Consequently, with decreasing temperature and prolonged stress duration, the Cry1Ac endotoxin content in seeds within cotton bolls exhibited the greatest decline, rendering it the most unstable compared to boll shell and fiber, particularly susceptible to low temperatures. Therefore, this study focused on the seed insecticidal protein level under low temperatures and the nitrogen metabolism to uncover the underlying mechanism.

In summary, compared to bolls and fibers, low temperature exhibited a greater impact on the Cry1Ac endotoxin content in cotton seed. After 24 h and 48 h of stress, the temperatures causing significant changes in Cry1Ac endotoxin content in SK1 seed were 18℃ and 19℃, and for SK3 were 18℃ and 18℃ in 2020; in 2021, for SK1, they were 17℃ and 19℃ respectively, and for SK3 were 18℃ and 18℃ respectively. Therefore, extending the duration of low-temperature stress not only increased the temperature at which Cry1Ac endotoxin content significantly decreased but also further reduced the Cry1Ac endotoxin content.

Nitrogen metabolism

Soluble protein and amino acid content

Under low-temperature treatment, the soluble protein content in seeds decreased, and with the decrease in temperature and extension of stress duration, its content showed a declining trend (Fig. 3A and B). In 2020, after 12 h of stress, the soluble protein content in seeds under low-temperature showed no significant difference compared to CK; after 24 h of stress, low-temperature significantly decreased the soluble protein content in seeds at 16℃-17℃, with reductions of 23.10-30.69% and 26.48-29.60%, respectively, compared to CK; after 48 h of stress, low-temperature significantly decreased the SK1 soluble protein content in seeds at 16℃-18℃, and the SK3 soluble protein content in seeds at 16℃-19℃, with reductions of 16.32-33.68% and 14.78-37.11%, respectively, compared to CK. The experimental results in 2021 were similar to those in 2020. In conclusion, the temperature leading to a significant decrease in soluble protein content in seeds increased with the prolongation of low-temperature stress duration.

Under low-temperature treatment, the content of free amino acids in seeds increased, and with the decrease

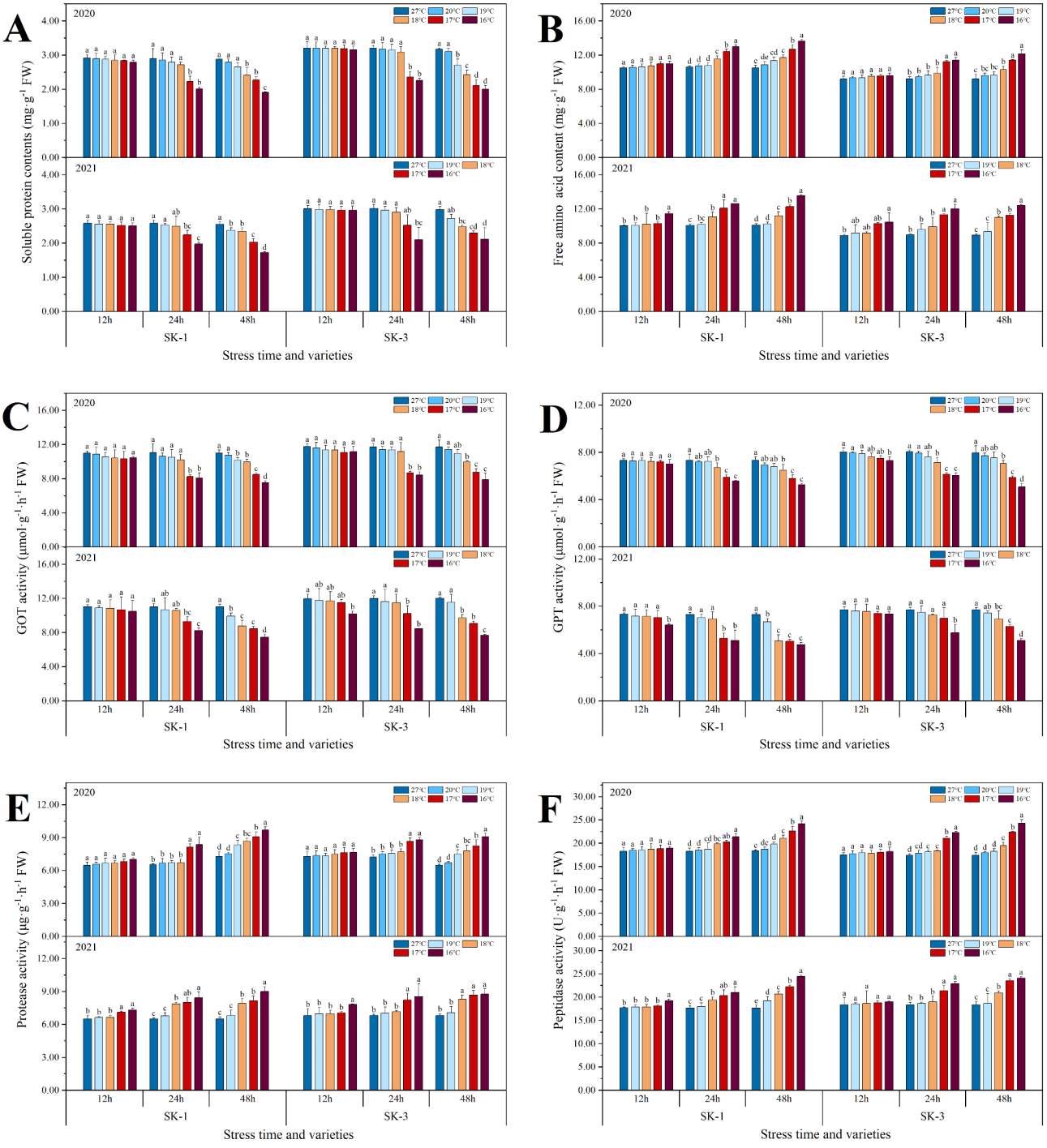

Fig. 3 Effects of low temperature stress on protein turnover in cotton seed in 2020 and 2021. FW represents fresh weight. **A**: soluble protein contents. **B**: free amino acid contents. **C**: GOT activities. **D**: GPT activities. **E**: protease activities. **F**: peptidase activities. Different lowercase letters indicate significant differences between different temperature treatments under the same stress time and variety ($P < 0.05$). SK-1 and SK-3 represent cultivars Sikang 1 and Sikang 3, respectively

in temperature and prolongation of stress duration, its content showed an upward trend. In 2020, after 12 h of stress, the free amino acid content in seeds for SK1 and SK3 under 16℃-20℃ treatment showed no significant difference compared to CK; after 24 h of stress, low-temperature significantly increased the free amino acid content in SK1 seeds at 16℃-18℃, and significantly increased the free amino acid content in SK3 seeds at 16℃-17℃, with increases of 9.25-22.45% and 21.54-23.70%, respectively, compared to CK; after 48 h of stress,

low-temperature significantly increased the free amino acid content in SK1 seeds at 16℃-19℃, and significantly increased the free amino acid content in SK3 seeds at 16℃-18℃, with increases of 8.08-29.66% and 11.73-31.92%, respectively, compared to CK. The experimental results in 2021 were similar to those in 2020. In conclusion, the temperature leading to a significant increase in free amino acid content in seeds increased with the prolongation of low-temperature stress duration.

Glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) activity

Under low-temperature treatment, the activities of seed GOT and GPT decreased, and with decreasing temperature and prolongation of stress duration, their activities showed a declining trend (Fig. 3C and D). In 2020, after 12 h of stress, the seed GOT activity of SK1 and SK3 under treatment at 16–20 °C showed no significant difference compared to CK; after 24 h of stress, low-temperature significantly decreased seed GOT activity at 16–17 °C, with decreases of 25.50-26.85% and 25.83-28.13% compared to CK; after 48 h of stress, low-temperature significantly decreased the SK1 seed GOT activity at 16–19 °C, and significantly decreased the SK3 seed GOT activity at 16–18 °C, with decreases of 7.45-31.45% and 14.75-32.65% compared to CK. In 2020, after 12 h of stress, under treatment at 16–20 °C, the seed GPT activity of SK1 showed no significant difference compared to CK, while low-temperature significantly decreased the SK3 seed GPT activity by 8.96% at 16 °C; after 24 h of stress, low-temperature significantly decreased seed GPT activity at 16–18 °C, with decreases of 8.31-23.71% and 11.17-24.69% in SK1 and SK3 respectively compared to CK; after 48 h of stress, low-temperature significantly decreased seed GPT activity at 16–18 °C, with decreases of 11.56-28.44% and 11.28-36.34% in SK1 and SK3 compared to CK. The results of the 2021 experiment were similar to those of 2020. In summary, the temperatures leading to a significant decrease in seed GOT and GPT activities increased with the prolonged duration of low-temperature stress.

Protease and peptidase activity

Under low-temperature treatment, the activities of seed protease and peptidase increased, and with decreasing temperature and prolonged stress duration, their activities showed an upward trend (Fig. 3E and F). In 2020, after 12 h of stress, the seed protease activity of SK1 and SK3 at 16–20 °C showed no significant difference compared to CK; after 24 h of stress, low-temperature significantly increased seed protease activity at 16–17 °C, with increases of 24.43-27.79% and 19.92-21.85% respectively in SK1 and SK3, compared to CK; after 48 h of stress, low-temperature significantly increased seed protease activity at 16–19 °C, with increases of 15.54-40.00% and 14.40-32.78% compared to CK.

In 2020, after 12 h of stress, there was no significant difference in cotton seed peptidase activity at 16–20 °C compared to the control (CK); after 24 h of stress, low-temperature significantly increased the SK1 seed peptidase activity at 16–18 °C, and significantly increased the SK3 seed peptidase activity at 16–19 °C, with increases of 8.63-16.88% and 4.13-28.00%, respectively, compared to CK; after 48 h of stress, low-temperature significantly increased the SK1 seed peptidase activity at 16–19 °C, and significantly increased the SK3 seed peptidase activity at 16–18 °C, with increases of 7.61-31.52% and 11.68-39.21%, respectively. The results of the 2021 experiment were similar to those of 2020. In summary, the temperatures leading to a significant increase in cotton seed protease and peptidase activity increased with the prolonged duration of low-temperature stress.

Correlation analysis and pathway analysis

According to Fig. 4, it can be observed that after 12 h of low-temperature treatment, Cry1Ac endotoxin content in seeds was not correlated with the soluble protein content and peptidase activity. However, it showed a significant positive correlation with GOT, GPT, and protease

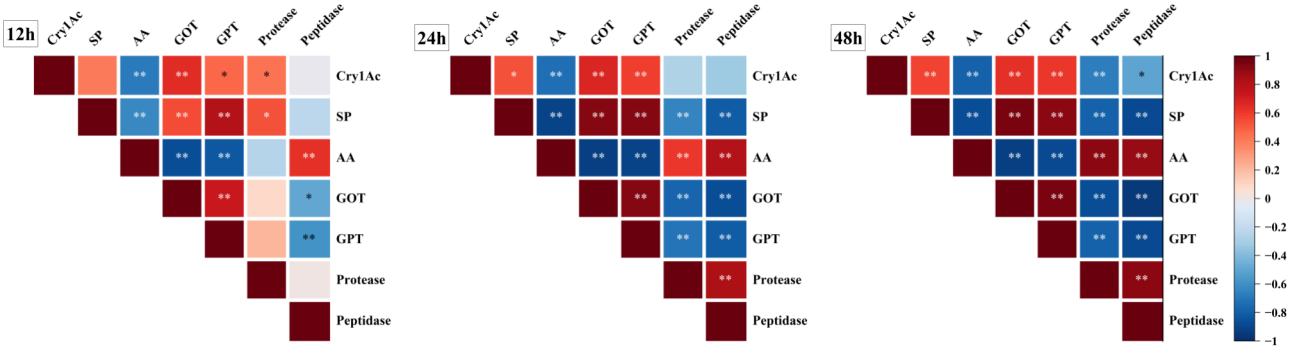

Fig. 4 Correlations between Cry1Ac endotoxin content and nitrogen metabolism related parameters in 2020 and 2021. Cry1Ac: Cry1Ac endotoxin content. SP: soluble protein. AA: free amino acid. * and ** represent the significance levels of 5% and 1%, respectively

Table 2 Results of stepwise regression analysis

Dependent variable	Significant level	The order of stepwise	Regression equation
Cry1Ac endotoxin content	0.05	X_2, X_6	$Y = 655.956 - 75.095 \times_2 + 21.308 \times_6$

Note X2 represents AA content. X6 represents peptidase activity. Y represents seed Cry1Ac endotoxin content

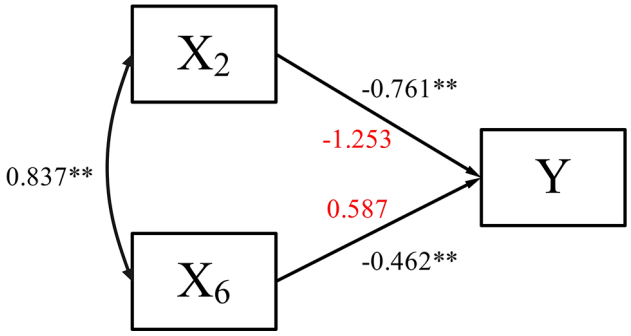

Fig. 5 Path coefficients of key physiological index X_2 represents AA content. X_6 represents peptidase activity. Y represents seed Cry1Ac endotoxin content. Black numbers indicate correlation (combined effect). Red numbers indicate direct effect. The indirect effect of X_2 on Y through X_6 is 0.491, and the indirect effect of X_6 on Y through X_2 is -1.049

activity, and a highly significant negative correlation with free amino acid content. After 24 h of low-temperature treatment, the Cry1Ac endotoxin content in seeds showed a significant positive correlation with soluble protein content, GOT, and GPT activity, a highly significant negative correlation with free amino acid content, and a negative correlation with protease and peptidase activity, although not significant. After 48 h of low-temperature treatment, the Cry1Ac endotoxin content in seeds exhibited a highly significant positive correlation with soluble protein content, GOT, and GPT activity, and a significant negative correlation with free amino acid content, as well as protease and peptidase activity. The above results indicated that low temperature primarily reduced protein synthesis capacity, and enhanced protein degradation capability, thereby affecting the Cry1Ac endotoxin content in seeds. Moreover, with the prolonged duration of stress, a more significant correlation was found between Cry1Ac endotoxin content in seeds and the content of key nitrogen metabolism substances and key enzyme activities.

Using Cry1Ac endotoxin content (Y) as the dependent variable and SP (X1), AA (X2), GOT (X3), GPT (X4), protease (X5), and peptidase activity (X6) as independent variables, stepwise regression analysis was conducted, and the coefficient of determination of the regression equation was R2=0.682 (Table 2). This indicated that the selected indicators reflected the main factors influencing the content of Cry1Ac endotoxin in seeds. To further clarify the effects of the indicators determined by stepwise regression on Cry1Ac endotoxin content, pathway analysis was performed on the selected indicators with Cry1Ac endotoxin content (Y) as the dependent variable. The results showed that AA (X2) and peptidase (X6) both had significant negative effects on Cry1Ac endotoxin content. Although peptidase had a direct positive effect on Cry1Ac endotoxin content, its indirect effect was −1.049 (Fig. 5). In summary, the increase in amino acid content and peptidase activity in cotton seeds under low-temperature stress mainly contributed to the decrease in Cry1Ac endotoxin content.

Discussion

The increasingly frequent low-temperature stress reduced the Cry1Ac endotoxin content in cotton bolls, with the greatest impact observed on cotton seeds

Due to the distribution of soil and topographical influences in China, regions like Xinjiang and Inner Mongolia, characterized by vast land and sparse population, have formed fertile and expansive plains suitable for mechanized cultivation of crops. However, the higher latitudes in regions like Xinjiang result in lower temperatures throughout the year. Previous studies have shown that temperatures below 15℃ seriously affect the growth and development of cotton [36]. Maho et al. found that the survival rate of cotton bollworm larvae was higher under low temperatures (14～22℃) [37]. Zhou et al. studied the impact of temperature on the insecticidal activity of expressed insecticidal proteins in cotton plants, finding that at 16℃, the insecticidal activity of Bt cotton was significantly reduced [38]. Zhang et al. demonstrated that the entire growth period of Bt cotton was affected by low-temperature and high-humidity conditions. The decline in insecticidal protein content in Bt cotton leaves varied with the degree and duration of stress, with a faster decline during the boll development period compared to the flowering period [27]. Chen et al. demonstrated that low-temperature stress reduced the insecticidal protein content in cotton bolls, and prolonging the duration of low temperatures increased the temperature at which significant changes occur [39]. Therefore, it can be concluded that low-temperature stress during the cotton growth stages, especially during the boll development period, leads to a decrease in insecticidal protein content of Bt cotton.

Our current study indicated that in recent years, both Southern Xinjiang and Northern Xinjiang cotton regions frequently experience low temperatures during the Bt cotton boll development period. Moreover, low temperatures reduced the Cry1Ac endotoxin content in boll shell, fiber, and seeds of cotton bolls. As the temperature decreased, the Cry1Ac endotoxin content in boll shell, fiber, and seeds all showed a declining trend. Additionally, the temperature at which the Cry1Ac endotoxin content

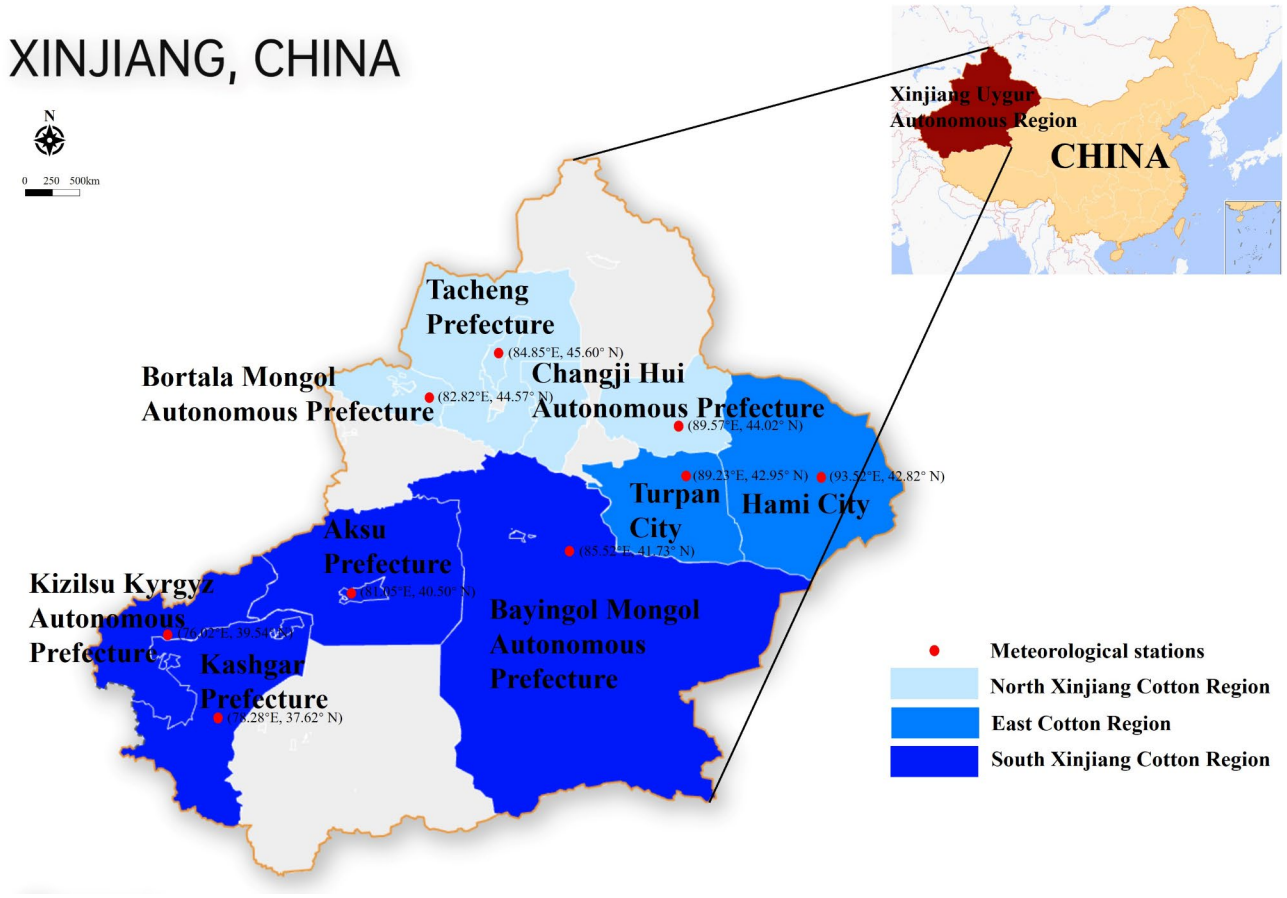

Fig. 6 Location and information map of meteorological stations in the main cotton areas of Xinjiang for this study

underwent significant changes increased with the prolonged duration of low-temperature stress. Moreover, as the most affected part of bolls by low-temperature stress, after 24 h and 48 h of low-temperature stress, the Cry1Ac endotoxin content in cotton seeds experienced the greatest decline, reaching up to 33.69% and 46.44% in SK1 and SK3, respectively, with a significant decrease occurring at temperatures between 17℃ and 19℃. The decrease in Cry1Ac endotoxin content would endanger the safety of cotton production in Xinjiang, and thus special attention should be given to low-temperature hazards, and appropriate practices should be applied when the temperature drops below 19℃ during the boll development period. The hybrid variety SK-3 had a smaller decrease compared to the conventional variety SK-1, possibly due to the better stress resistance of the hybrid variety. However, this conclusion needs further verification.

Low-temperature stress reduced the protein synthesis capacity and enhanced the protein degradation capability, leading to decreased Cry1Ac endotoxin content in cotton seeds

Protein metabolism in living organisms is in dynamic balance. While new proteins are synthesized, there is also degradation of old proteins, and the amino acids produced from degradation can be utilized for protein synthesis [40]. Multiple studies have shown that the concentration of Cry1Ac endotoxin protein content is closely related to protein metabolism in plants [41–43]. Chen et al. [44] proved the altered square nitrogen metabolic intensity was main physiological cause of changed square growth and Cry1Ac endotoxin content. Moreover, the changes in seed Cry1Ac endotoxin content under excessive use of nitrogen or nitrogen deficient conditions have also been shown to be the result of protein turnover [16, 45].

In terms of environment, the protein synthesis in the alternating high temperature cotton boll shell decreases and degradation increases, thereby reducing the Cry1Ac endotoxin content [46]. Under high-temperature conditions, the insecticidal protein content in Bt cotton leaves decreased, possibly due to the greater degradation of soluble proteins in leaves by high temperatures [21]. When the temperature was above 38℃, and diurnal temperature variations persisted for more than 7 days, the ability of protein degradation in squares and bolls exceeded the synthetic capacity, resulting in a significant decrease in insecticidal protein content [47]. Therefore,

high-temperature stress accelerates protein degradation, slows down protein synthesis, and ultimately leads to a decrease in insecticidal protein content.

This study yielded consistent findings in low-temperature conditions. Specifically, we observed peptidase and protease activities increased under low temperatures, leading to increased protein degradation and a subsequent rise in free amino acids. Concurrently, diminished activities of enzymes such as GOT and GPT directly contributed to reduced protein synthesis and a decline in soluble protein content, consequently leading to a decrease in Cry1Ac endotoxin content. Cry1Ac endotoxin content exhibited a significant positive correlation with soluble protein content, GOT activity, and GPT activity, while displaying a significant negative correlation with free amino acid content, protease activity, and peptidase activity.

In summary, low temperatures impaired protein synthesis capacity while augmenting protein degradation capacity, ultimately resulting in a reduction in Cry1Ac endotoxin content.

The increase of free amino acid content and peptidase activity in cotton seeds mainly contributed to the deduction in Cry1Ac endotoxin content

Cry1Ac toxin was a part of soluble protein [48]. Previous studies show that activities of protease and peptidase both negatively affected the Cry1Ac toxin content. Protease and peptidase were both involved in protein degradation process, which would affect soluble protein content, and further influence Cry1Ac protein in cotton [42, 43, 45]. While previous studies have highlighted the relationship between Cry1Ac endotoxin content and protein metabolism, few have delved into a detailed analysis. Our study utilized stepwise regression and pathway analysis to elucidate this relationship further. We found that the rise in free amino acid content exerted a significant direct negative effect on Cry1Ac endotoxin content, while the indirect negative effect of peptidase activity was even more pronounced. In summary, the elevation of free amino acid content and the increase in of peptidase activity in seeds under low-temperature stress primarily contributed to the decline in Cry1Ac endotoxin content. These findings offer novel insights and a theoretical foundation for mitigating the detrimental effects of low-temperature stress on the insect resistance of Bt cotton.

Conclusion

In recent years, both the Northern and Southern Xinjiang cotton regions in China have experienced a growing incidence of low-temperature stress during the boll development period. These low temperatures have had a significant impact on the Cry1Ac endotoxin content in cotton bolls. Moreover, as the duration of stress persisted, the threshold temperature leading to a significant decrease in Cry1Ac endotoxin content had risen. Notably, cotton seeds were susceptible to low-temperatures, highlighting the need for practices to bolster the resistance of Bt cotton to temperatures $\leq 19℃$. Protein metabolism analysis revealed that under low-temperature conditions, the decrease in Cry1Ac endotoxin content was primarily related to enhanced protein degradation and diminished synthesis ability. Notably, the levels of free amino acids and peptidase activity had played a key role in this process, underscoring their significant contribution to decreased Cry1Ac endotoxin content.

Materials and methods

Plant materials and experimental design

The study was conducted using two Bt transgenic cotton varieties, 'Sikang1' (conventional, SK1) and 'Sikang3' (hybrid, SK3), at Yangzhou University, Yangzhou, China (32°30'N, 119°25'E) during the 2020–2021 cotton growing season. Seeds were sowed in the greenhouse on April 15th, 2020, and April 18th, 2021, and the seedlings were transplanted to pots (50 cm height, 40 cm diameter, 62.8 L volume) at 35 days after sowing. The pots were filled with 20 kg sandy loam soil (Typic fluvaquents, Entisols), containing 18.8 g kg^{-1} organic matter and available N-P-K at 135.2, 22.8, and 80.9 mg kg^{-1}, respectively. Plants were watered thoroughly on a daily basis.

On the day of transplanting (May 17th), 1.5 g N as urea, 0.7 g P as single superphosphate, and 2.6 g as KCl were mixed into the soil of each pot, and one seedling was transplanted in each pot. At 46 days after transplanting, 1.6 g N as urea, 0.7 g P as single superphosphate, and 2.6 g as KCl were top-dressed into each pot. At 68 days after transplanting, 2.0 g N as urea was top-dressed into each pot.

The experiments were performed using a completely randomized design with six replications, consisting of six temperature regimes (optimum temperature: 27 °C and low temperatures: 16 °C, 17 °C, 18 °C, 19 °C, 20 °C) in 2020 and five temperature regimes (optimum temperature: 27 °C and low temperatures: 16 °C, 17 °C, 18 °C, 19 °C) in 2021. The temperature was set based on low-temperature variations in Xinjiang and the previous study by Chen et al. [29]. At the peak flowering stage (July 18th), flowers on the seventh to eighth fruiting branches were tagged, and temperature treatments were initiated fifteen days after flower appearance by transporting pots to environmentally controlled rooms with different temperature settings (14 h d^{-1} photoperiod at a photon flux density of 200 mmol $m^{-2}s^{-1}$; and 70% relative humidity). Five labeled bolls of each treatment were collected at 12 h, 24 h, and 48 h after treatments and stored at -80 °C for later measurements. Each cotton boll was separated into three parts: shell, fiber, and seed. The boll shell, fiber,

and seed were thoroughly mixed respectively before subsampling.

Meteorological data sources

Xinjiang (73.66°-96.38°E, 34.42°-49.17°N) is located in the border area of Northwest China. Xinjiang is the largest arid and semiarid region in China, characterized by a typical continental climate with a dry climate, little rainfall, sufficient sunshine time, and a large diurnal temperature range (12.9–15℃) [49]. Xinjiang cotton holds a significant position in global cotton production. Currently, based on different climate characteristics and vegetation types, Xinjiang's cotton-growing areas are divided into three cotton regions: East Xinjiang Cotton Region (Hami City, Turpan City), North Xinjiang Cotton Region (Changji Hui Autonomous Prefecture, Tacheng Prefecture, Bortala Mongol Autonomous Prefecture), and South Xinjiang Region (Aksu Prefecture, Kizilsu Kyrgyz Autonomous Prefecture, Kashgar Prefecture, Bayingol Mongol Autonomous Prefecture) (Fig. 6). This data is sourced from the Xinjiang Statistical Yearbook [50]. Due to the cotton boll formation period being concentrated from July to August each year, we selected the daily average temperature data (T) from July 1st to August 31st in the past decade. The meteorological data is sourced from the National Oceanic and Atmospheric Administration (NOAA) of the United States (www.noaa.gov) and the National Center for Environmental Information (NCEI) (ncei.noaa.gov). We used the basic GAODE map data compiled from the DISHU Platform (https://dycharts.com), which includes the boundary of Xinjiang, provincial boundaries, and national boundaries.

Physiological measurements

Determination of Cry1Ac endotoxin content

The concentrations of the Cry1Ac endotoxin in the boll shell, fiber, and seed extracts were determined by immunological analysis using enzyme-linked immunosorbent assay (ELISA) [25]. Tissue extracts were harvested by homogenizing the frozen tissue (1.5 g) in 2 mL of extraction buffer (Na_2CO_3 1.33 g, DTT 0.192 g, NaCl 1.461 g, and Vc 0.5 g dissolved in 250 mL of distilled water). The extracts were then transferred to 10-mL centrifuge tubes. The tubes were shaken by hand and stored at 4 °C for 4 h. After centrifugation at 10,000×g, the extracts were collected, and the filtered supernatants were collected for determination. Microtitration plates were coated with the standard Cry1Ac insecticidal endotoxin and samples, then incubated at 37 °C for 4 h. Antibodies against the Cry1Ac insecticidal endotoxin were added. After that, horseradish peroxidase-labelled goat anti-rabbit immunoglobulin was added, and the samples were incubated for 30 min at 37 °C. Finally, the buffered enzyme substrate (1,2-phenylenediamine) was added. Fifteen minutes later, the reaction was terminated using 50 µL of H_2SO_4 (3 mol L^{-1}). The results from absorbance measurements at 490 nm were recorded.

Assay of soluble protein (SP) and free amino acid (AA) content

Fresh seed samples (0.5 g) were used for the extraction and analysis of soluble protein and free amino acid content. The samples were homogenized at 4 °C in 3 mL cold water (Milli-Q reagent grade) and centrifuged at 800 × *g* for 5 min. The supernatant was stored on ice. The total free AA content was determined by the ninhydrin assay according to Yemm et al. [51]. The total soluble protein content was determined by the Coomassie Blue dye-binding assay of Zou [52]. The absorbance readings were converted into protein concentrations using bovine serum albumin in the standard curve.

Activities of glutamic-pyruvic transaminase (GPT) and glutamic oxaloacetate transaminase (GOT) assay

Fresh seed samples were homogenized in 0.05 mmol L^{-1} Tris-HCl (pH 7.2), the homogenate was centrifuged, and the supernatant was analyzed for GOT activity. A mixture of 0.5 mL of 0.8 mol·L^{-1} alanine in 0.1 mol·L^{-1} Tris-HCl (pH 7.5) together with 0.1 mL of 2 mmol·L^{-1} pyridoxal phosphate solution was used, and 0.2 mL of 0.1 mol·L^{-1} 2-oxoglutarate solution and 0.2 mL of the prepared enzyme were added to this mixture. The reaction mixture was incubated at 37 °C for 10 min followed by termination of the reaction with 0.1 mL of 0.2 mol·L^{-1} trichloroacetic acid solution, following which the pyruvate with chromogen was converted to pyruvate hydrazone. The color intensity of the hydrazone in saturated water toluene was measured at 520 nm. GOT activity was calculated simultaneously from authentic pyruvate standards. 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) was substituted for 0.5 mL of 0.1 mol·L^{-1} buffered aspartate solution in the reaction mixture, and aniline citrate addition was omitted [53].

Activities of protease and peptidase assay

Fresh seed samples were homogenized at 4 °C in 1 mL of β-mercaptoethanol extraction buffer (pH=6.8). Cell debris was removed by centrifugation, and the supernatant was placed on ice, and the protease activity determined spectrophotometrically at 400 nm using azocasein as a substrate. Peptidase activity was determined as described by Hu et al. [54]. A total of 0.1 mL extract was added to 1-mL buffer containing 50 mM Tris-HCl (pH 8.0), 1 m Mol L^{-1} MnCl2, and 5 m Mol L^{-1} peptide, and then 25 mL of the reaction mixture was incubated at 37 °C for 30 min in 1 mL of a 1% ninhydrin solution

containing 100 mg of cadmium acetate, 85 mL of ethanol, and 15 mL of acetic acid in a total volume of 100 mL. The optical density was measured at 505 nm.

Data processing and statistics

Experimental data were analyzed using SPSS 25 (IBM Corp., Armonk, NY, USA). The significance of the differences between different treatments was tested with the least significant difference ($P \leq 0.05$), and the Pearson correlation coefficient was used to measure their correlations. The tables and figures were processed and plotted with Excel 2016 (Microsoft Corp., Redmond, USA) and Origin 2023 (OriginLab, Northampton, Massachusetts, USA).

Path analysis can decompose the direct correlation between the independent variables and the dependent variables, study their direct and indirect importance, and provide a theoretical basis for accurate statistical decisions [55, 56]. In this paper, SPSS software, Pearson method, physiological indicators related to nitrogen metabolism (soluble protein, SP content, AA content, GOT activity, GPT activity, protease activity, and peptidase activity) as the independent variable (X), Bt protein content as the dependent variable (Y), and the direct and indirect effect of physiology related to nitrogen metabolism on Bt protein content.

$$\mathrm{Pyi}=\mathrm{bisi}/\mathrm{sy};\ \mathrm{Pyij}=\mathrm{rij}\ \times\ \mathrm{Pyi}$$

Where Pyi and Pyij represent the direct diameter coefficient and the indirect diameter coefficient rij are the correlation coefficient between independent variable i and dependent variable j; bi is the partial regression coefficient of dependent variable y on independent variable i; si and sy represent the standard deviation of i and y respectively.

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Author contributions

Y.C., X.Z., D.C. and Z.L. designed the study. S.D., Y.D. and Z.L. performed the experiments. Y.C. and Z.L. analyzed the data and wrote the manuscript. Y.C., Y.L.C. and Z.L. checked and revised the manuscript. All authors reviewed the manuscript.

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Data availability

All data generated or analyzed during this study are included in this published article.

Declarations

Ethics approval and consent to participate

We all declare that manuscript reporting studies do not involve any human participants, human data, or human tissue. Plant samples were collected from university research area. Study protocol must comply with relevant institutional, national, and international guidelines and legislation. Our experiment follows with the relevant institutional, national, and international guidelines and legislation.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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