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The recovery of Bt toxin content after temperature stress termination in transgenic cotton

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Abstract

The insecticidal efficacy of Bt cotton under different environments has generated controversy in recent years. The objective of this study was to investigate possible reasons of the conflicting results caused by temperature stress. Two different types of Bt transgenic cotton cultivars (a Bt cultivar, Sikang1, and an hybrid Bt cultivar, Sikang3) were selected. The plants of the two Bt cultivars were exposed to high temperature (37°C), low temperature (18°C), and the control (27°C) for short (24 h) and long (48 h) periods of stress at peak boll stage, and then moved to the glasshouse where the control plants were maintained. The results showed that the leaf insecticidal toxin content fully recovered within 24 h to the level of control after the end of short duration high-temperature treatment, and recovered mostly within 48 h of the termination of 24 h low-temperature stress. Under long duration high temperature treatment the Bt toxin content required longer recovery periods (48 and 72 h for Sikang3 and Sikang1, respectively) to reach the control level. The Bt protein content only recovered partially at 96 h after the end of the long-duration low-temperature stress, and the concentrations of the Cry1Ac protein were 74% and 77% of the corresponding control for Sikang1 and Sikang3, respectively. The different recovery and increase slowly of the leaf nitrogen metabolic rates after ceasing the heat and cold stress may be possible reason for the above difference. In summary, the duration and type of temperature (cold or heat) stress may cause different Bt insecticidal protein recovery rate due to different recovery rate of enzymes.

Additional key words: Bt cotton; Cry1Ac protein; recovery rate; temperature stress.

Introduction

The world cotton (*Gossypium hirsutum* L.) industry regards transgenic (Bt) cotton containing genes for the Cry1Ac protein from *Bacillus thuringensis* as a major component of its integrated insect management strategy for reducing the economic burden of controlling boll worms (*Helicoverpa amigera* and *Helicoverpa zea*) and the environmental consequences of insecticide use (Pmsel *et al.*, 2004; Wu, 2007). In China, single Bt gene cultivars carrying the Cry1Ac gene provide good protection before flowering. However, it is more difficult to control *H. amigera* with Bt proteins from flowering onwards, because Bt protein contents in the plants varies and thus affects insecticidal efficacy (Fitt & Wilson, 2000; Zhen *et al*., 2008; Shen *et al*., 2010). This variation has been attributed to soil, climatic and agronomic factors, as well as variation between individual plants (Benedict *et al.*, 1993; Sachs *et al*., 1998; Greenplate, 1999; Wang *et al.*, 2001). Temperature is one of the environmental factors affecting the efficacy of boll worm control in Bt cotton. In greenhouse studies, Dong & Li (2007) reported that Bt cotton plants lost their insect resistance significantly under both high temperature (above 37°C) and low temperature (below 18°C) conditions. Olsen *et al.* (2006) observed that cool temperatures in the early growing season could markedly reduce the efficacy of Cry1Ac Bt plants. Our previous study also showed that high temperature causes decrement of Bt protein concentration during

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Abbreviations used: Bt (*Bacillus thuringiensis*); DAS (days after sowing); GOT (glutamate oxaloacetate transaminase); GPT (glutamic-pyruvic transaminase); GS (glutamane-synthetase).

the boll filling stage (Chen *et al.,* 2005). In contrast, no significant changes in Cry1Ac expression were observed after short episodes of stress by crop nutrition, population density, and plant growth regulator application in field studies (Adamczyk *et al.*, 2001; Adamczyk & Meredith, 2004; Ian, 2006). It is possible that the non-significant difference observed at different temperatures for CryIAc protein expression in field experiments might be related to the day/night temperature cycle. Temperatures fluctuate, thereby the plants growing in the field are under intermittent temperature stress (for periods of less than 24 h), and thus the efficacy expressed as Cry1Ac protein may probably recover to some extent after a short period of temperature stress.

The Bt protein is part of the soluble protein pool in Bt cotton plant. The synthesis of Bt protein is also controlled by nitrogen metabolism (Steward, 1965). Our previous work showed that the insecticidal proteins content in leaves was highly correlated with the nitrogen metabolism of the Bt cotton (Chen *et al.*, 2004, 2005; Wang *et al.*, 2009). Therefore, investigating the relationships between the rebound characteristics of the efficacy after terminating temperature stress and the nitrogen metabolism is important for elucidating the process of the efficacy recovery. Therefore, we hypothesized that the insecticidal efficacy might rebound after terminating the temperatures stress, and that the rate of increase in Bt protein concentration may be partly explained by the nitrogen and protein metabolism.

The principal objectives of this study include (1) to study whether there is a rebound effect in Cry1Ac toxin content in the leaves of Bt cotton after the termination of periods of temperature stress of different durations, and (2) to determine the relationship between rebounded level of toxin and nitrogen metabolism.

Material and methods

Plant materials and experimental design

The experiments were conducted in naturally-illuminated, temperature-controlled glasshouses at Yangzhou University, Yangzhou, Jiangsu Province, China (32° 30' N, 119° 25' E), in the cotton growing seasons of 2007 and 2008. Two Bt transgenic cotton cultivars, medium in maturity, 'Sikang 1' (Bt cultivar) and 'Sikang 3' (hybrid Bt cultivar), were planted on April 6th in both years in a warm room covered by plastic mulch. Seedlings were transplanted to pots at 41 days after sowing (DAS) (May 19th for both years). Each porcelain pot (50 cm height, 40 cm diameter, 62.8 L volume) was filled with 30 kg of sandy loam soil (Typic fluvaquents, Entisols) which contained 16.3 g kg^{-1} organic matter and available N, P and K at 113, 36.7 and 80.4 mg kg^{-1}, respectively. One seedling was transplanted to each pot. The plants were watered and fertilized throughout the season according to local management practices. Sikang1 and Sikang3 flowered at DAS90 and DAS91, and first boll opened at DAS138 and DAS139, respectively.

The experiment for each cultivar followed a completely randomized design consisting in three temperature (day/night) treatments: high (37°C), low (18°C), and control (27°C). During boll stage, the plants of both cultivars were maintained in the greenhouse under control conditions (27°C for day/night temperature) for two days, then plants for high and low temperature treatments were moved to other two greenhouses with different temperature settings according to treatments. In 2007, plants were treated with different temperature regimes for 24 h at peak boll stage (DAS126), and then transported back to the glasshouse where the control plants were maintained. In 2008, plants were exposed to different temperature regimes for 48 h at peak boll stage (DAS127) before being moved back to the glasshouse of control conditions. In all three greenhouses, the photoperiod was 12.5-h long (1,200 μmol m^{-2} s^{-1} photosynthetic photon flux density), and relative humidity of 65-75%.

Sample preparation

In 2007, measurements were taken at 0, 24 and 48 h after the termination of temperature stress treatments. In 2008, samples were collected at 0, 24, 48, 72 and 96 h after the end of temperature stress treatments. Each sampling time represented different recovery duration. Each pot was considered as an experiment unit and four replications were used to conduct the experiment. For each cultivar, measurements were taken at each sampling time on 12 plants with 4 plants per treatment, and a new set of 12 plants was used at the next sampling time. Leaf samples were collected from the fourth node below the apex of the main-stem of the plants for quantification of Cry1Ac protein, key enzymes [glutamate oxaloacetate transaminase (GPT), glutamane-

synthetase (GS), and protease] and amino acids. The fourth leaf from the top of the plant was used because this tissue reflects expression differences accurately (Shi, 1980). Leaf samples were frozen in liquid nitrogen, and stored in a freezer (–20°C).

Cry1Ac protein concentration assay

The Cry1Ac protein concentrations in the leaf samples were determined by immunological analysis by means of ELISA (Chen *et al.*, 1997). Three subsamples were excised from each leaf using a standard 2-cm diameter paper puncher. Leaf tissue extracts (ca. 0.5 g) were prepared by homogenizing the frozen leaf tissue in 2 mL extraction buffer (1.33 g Na_2CO_3, 0.192 g DTT, 1.461 g NaCl, and 0.5 g vitamin C dissolved in 250 mL distilled water), then transferring to a 10-mL centrifuge tube; the residue remaining on the wall of the mortar was washed again with 3 mL of the buffer and this was also added to the centrifuge tube. The contents of this tube were shaken by hand, and stored at 4°C for 4 h for extraction, the supernatants were collected after centrifugation of the tube at 10,000 g at 4°C for 20 min, then filtered through a C^{18} Sep-Pak Cartridge (Waters, Milford, MA, USA), and the filtered supernatants were collected for determination. The Cry1Ac concentrations for all the samples were measured using a commercially available kit (Scientific Service, Inc., China Agriculture University, Beijing, China). Microtitration plates were coated with the standard Cry1Ac insecticidal proteins and samples (50 µL), incubated at 37°C for 4 h. The antibodies (50 µL) were added to each well and incubated for another 30 min at 37°C. Then horseradish peroxidase-labelled goat anti-rabbit immunoglubolin (100 µL) was added to each well and incubated for 30 min at 37°C. Finally, the buffered enzyme substrate (orthopenylenediamino, 100 µL) was added, and the enzyme reaction was carried out in the dark at 37°C for 15 min, then terminated using 3 M H_2SO_4 (50 µL). The absorbance was recorded at 490 nm. Calculation of the ELISA data was performed as described by Weiler *et al.* (1981).

Assay of free amino acid and soluble protein content

The leaf samples (0.5 g) used for extraction and analysis of amino acid and assessment soluble protein content were homogenized at 4°C in 5 mL cold water (MilliQ reagent grade) and centrifuged at 800 *g* for 5 min. The supernatant was stored on ice, and the pellet resuspended in 3 mL cold water prior to re-centrifugation (800 *g*) for another 5 min. The supernatants from both centrifugations were pooled and stored on ice, the pellet was resuspended in a further 2 mL of cold water, centrifuged at 800 *g* again, and the supernatants were pooled for analysis. The total free amino acid content was determined by ninhydrin assay (Yemm & Cocking, 1955). The absorbance readings were converted to mg amino acid g^{-1} fw using a 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 concentrations using BSA standard curve.

Glutamic-pyruvic transaminase (GPT) and glutamane-synthetase (GS) assays

Leaf samples (0.5 g) were homogenized in buffered medium (0.05 mM Tris-HCl, pH 7.2), and then centrifuged at 26,100 *g* for 10 min at 0°C. The supernatant was analysed to detect GPT activity. A mixture of 0.5 mL of 0.8 M alanine prepared in 0.1 M Tris-HCl (pH 7.5), 0.1 mL of 2 mM pyriodoxal phosphate, 0.2 mL of 0.1 M 2-oxoglutarate solution and 0.2 mL of the leaf sample preparation was incubated at 37°C for 10 min, terminating the reaction with 0.1 mL of a 0.2 M trichloroacetic acid solution. Then the pyruvate was converted to pyruvate hydrazone with chromogen. The colour intensity of the hydrazone in saturated water toluene was measured at 520 nm. The GPT activity, in terms of pyruvate production, was calculated from authentic pyruvate standards run simultaneously (Tonhazy *et al.*, 1950).

GS activity was determined by the ADP-dependent transferase assay (Valverde & Gabriel, 2003), which was downscaled and adapted to ELISA microplates. Leaves were ground with a Teflon pestle in 1 mL of extraction buffer per 100 mg leaves. The extraction buffer was composed of 100 mM N-morpholino methane sulphonate (MES), 15% (v/v) ethyleneglycol, 2% (v/v) 2-mercaptoethanol, 100 mM sucrose, and 1 mM phenylmethylsulfonylfluoride (PMSF), with a pH of 6.8. Leaf homogenates were centrifuged at 25,000 g for 20 min. The supernatant was transferred to a new microtube and stored on ice for enzyme assays.

Leaf extract (5 µL) was added to 100 mL of reaction mixture (20 mM Tris-acetate, 8.75 mM hydroxylamine, 1 mM EDTA, 2.25 mM $MnCl_2$, 17.5 mM NaH_2AsO_4, 2.75 mM ADP, and 35 mM glutamine, pH = 6.4) and incubated at 37°C for 3 to 5 h. Reaction was terminated by adding 100 mL of ferric reagent (3.2% (w/v) $FeCl_3$, 4% (w/v) TCA, 0.5 N HCl). Control reactions had no glutamine added. Production of γ-glutamyl hydroxamate was measured with an ELISA microplate reader at 530 nm. The GS unit was defined as the amount of enzyme that catalyses the production of 1 µmol γ-glutamyl hydroxamate per minute (unit g^{-1} fw).

Protease activity assay

Leaf samples (0.8 g) were homogenized at 4°C in 1 mL of 0.05 M β-mercaptoethanol extraction buffer (a mixture of 0.02 M ethylene glycol, 0.5 M sucrose, and 0.05 M phenylmethyl sulfonyl fluoride, pH = 6.8). Cell debris was removed by centrifugation, and the supernatant placed on ice and immediately used to estimate the leaf protease. Protease activity was determined spectrometrically at 400 nm using azocasein as substrate (Vance *et al.*, 1979) and expressed as mg protein g^{-1} leaf fw h^{-1}.

Statistics analysis

The statistical significance of differences between means was analyzed by analysis of variance (ANOVA) and multiple comparisons were evaluated by Tukey's HSD (α = 0.05) in SAS 6 (SAS Institute, 1989).

Results

The rebound levels of leaf endotoxin over time after short stress duration (24 h)

In 2007, at 0 h of recovery time, the 24 h high temperature and low temperature stress treatments had significant effects on leaf Bt protein content at peak boll stage, with a reduction of 17.8% and 16.5% for Sikang1 and Sikang3, respectively under 37°C, and of 35.9% and 34.1% under 18°C, compared to the control. However, a rebound effect after termination of the temperature stress was observed for both cultivars (Table 1). In comparison to the control, the leaf toxin content recovered fully within 48 h for both stress temperatures, with the high temperature treatment exhibiting a faster recovery compared to low temperature treatment. At 24 h after terminating the temperature stress, no significant difference was found for Cry1Ac protein concentration between the high temperature treatment and the control, but the leaf insecticidal protein content in low temperature treated plants was significantly lower than the protein content of the control and the lower content was noted until the end of measurement at 48 h.

Leaf soluble protein and amino acid content after short stress duration (24 h)

Leaf soluble protein was significant reduced by 24 h high and low temperature treatments, but recovered within 48 h (in 2007) after ceasing temperature stress (Table 1). However, the recovery rate differed between

Table 1. Leaf insecticidal protein content, soluble protein and amino acid content over time after the end of hot and cold temperature stress treatments lasting for 24 hours, in two Bt transgenic cotton cultivars at peak boll stage

Cultivar	Treatment	Bt protein (ng g^{-1} fw)			Soluble protein (mg g^{-1} fw)			Amino acid (mg g^{-1} fw)		
		0	24	48	0	24	48	0	24	48
		(hours after terminating stress)								
Sikang1	37°C	635.1^{b}	705.2^{b}	715.0^{a}	8.71^{b}	9.98^{b}	10.65^{a}	85.2^{c}	49.3^{b}	47.5^{a}
	18°C	495.2^{a}	553.5^{a}	745.3^{a}	6.97^{a}	7.87^{a}	10.13^{a}	35.1^{a}	37.9^{a}	47.2^{a}
	Control (27°C)	772.5^{c}	720.0^{b}	780.3^{a}	10.55^{c}	10.41^{b}	10.45^{a}	47.6^{b}	48.1^{b}	48.0^{a}
	Significance (p)	< 0.001	< 0.001	0.1498	< 0.001	< 0.001	0.1247	< 0.001	0.008	0.4804
Sikang3	37°C	705.6^{b}	845.7^{b}	860.5^{a}	10.4^{b}	12.3^{b}	12.4^{a}	88.7^{c}	52.2^{b}	51.6^{a}
	18°C	556.8^{a}	625.2^{a}	835.1^{a}	8.7^{a}	9.5^{a}	11.3^{a}	37.3^{a}	40.6^{a}	51.0^{a}
	Control (27°C)	845.0^{c}	860.9^{b}	851.3^{a}	12.5^{c}	12.3^{b}	12.6^{a}	51.5^{b}	51.9^{b}	51.4^{a}
	Significance (p)	< 0.001	< 0.001	0.2405	< 0.001	0.0013	0.1135	< 0.001	0.0002	0.3609

The values are means (n = 4). Means followed by the same letter are not significantly different according to Tukey HSD test (p = 0.05).

Table 2. Leaf insecticidal protein content over time after end of 48 hours of hot or cold temperature stress at peak boll stage in cotton

Cultivar	Treatment	0	24	48	72	96
		(hours after ceasing stress)				
Sikang1	37°C	557.3[b]	620.1[b]	705.5[b]	715.6[b]	720.4[b]
	18°C	420.3[a]	345.1[a]	490.7[a]	555.2[a]	565.4[a]
	Control (27°C)	760.1[c]	775.4[c]	775.4[c]	765.3[b]	765.9[b]
	Significance (p)	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Sikang3	37°C	645.2[b]	685.1[b]	760.7[b]	781.5[b]	790.0[b]
	18°C	490.7[a]	465.4[a]	560.5[a]	625.8[a]	644.9[a]
	Control (27°C)	824.8[c]	839.7[c]	829.3[b]	819.8[a]	834.1[b]
	Significance (p)	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

The values are means (n = 4). Means followed by the same letter are not significantly different according to Tukey HSD (p = 0.05).

high and low temperature treatments. The average leaf soluble protein content increased by 14.58% and 18.27% for Sikang1 and Sikang3, respectively, at 24 h after terminating the high temperature treatment, and by 12.91% and 9.20% for Sikang1 and Sikang3, respectively, at 24 h after terminating the low temperature treatment. At 48 h after the temperature treatments, no difference on leaf soluble protein content was observed between the high temperature treatment and the control. In contrast, the leaf soluble protein content of the low temperature treatment was significantly lower than the control. This profile was in agreement with the recovery pattern for Bt protein content in cotton leaves with low temperature treated plants exhibiting slower recovery compared to the high temperature treated ones.

The amino acid content in leaves was significantly increased by 24 h high temperature treatments, and it was reduced after ending high temperature stress, but reached control levels within 24 h. In contrast, 48 h were needed for leaf amino acid concentration rebound to the control level after low temperature treatment (Table 1).

The rebound levels of leaf endotoxin over time after long stress duration (48 h)

High and low temperature treatments with longer duration (48 h) reduced leaf insecticidal protein expression greater than temperature treatments with short duration (24 h) (Tables 1 and 2). For Sikang1 and Sikang3 respectively, the Bt protein concentration reduced by 26.7% and 21.8% under 37°C, and by 44.7% and 40.5% under 18°C after 48 h temperature stress compared to the control. Thus, the recovery periods of the insecticidal toxin content after 48 h stress exposure were longer than 24 h stress exposure for both studied cultivars. However, a faster recovery of leaf toxin content was observed after high temperature treatment. Although the leaf Cry1Ac protein levels were numerically lower than the corresponding control, no significant differences in the leaf Cry1Ac protein were detected between the treatment and the control at 48 h for Sikang1 and 72 h for Sikang3 after high temperature treatment. On the contrary, the reduced Bt toxin levels caused by the low temperature treatment did not recover to the level of the control within 96 h. The leaf endotoxin levels in low temperature treated plants declined within 24 h after treatment, and then slowly increased afterwards, but the leaf endotoxin content was still lower than the control at 96 h after treatment (73.9% and 77.2% of the corresponding control for Sikang1 and Sikang3, respectively).

Leaf nitrogen metabolic characteristics after long stress duration (48 h stress)

Leaf amino acid and soluble protein content

Leaf amino acid content was significantly enhanced by the 48 h high temperature treatment, but reduced by low temperature. During the recovery period, the amino acid content decreased after the high temperature treatment and increased gradually after low temperature stress (Fig. 1a). The leaf amino acid content was increased right after the high temperature stress ceased,

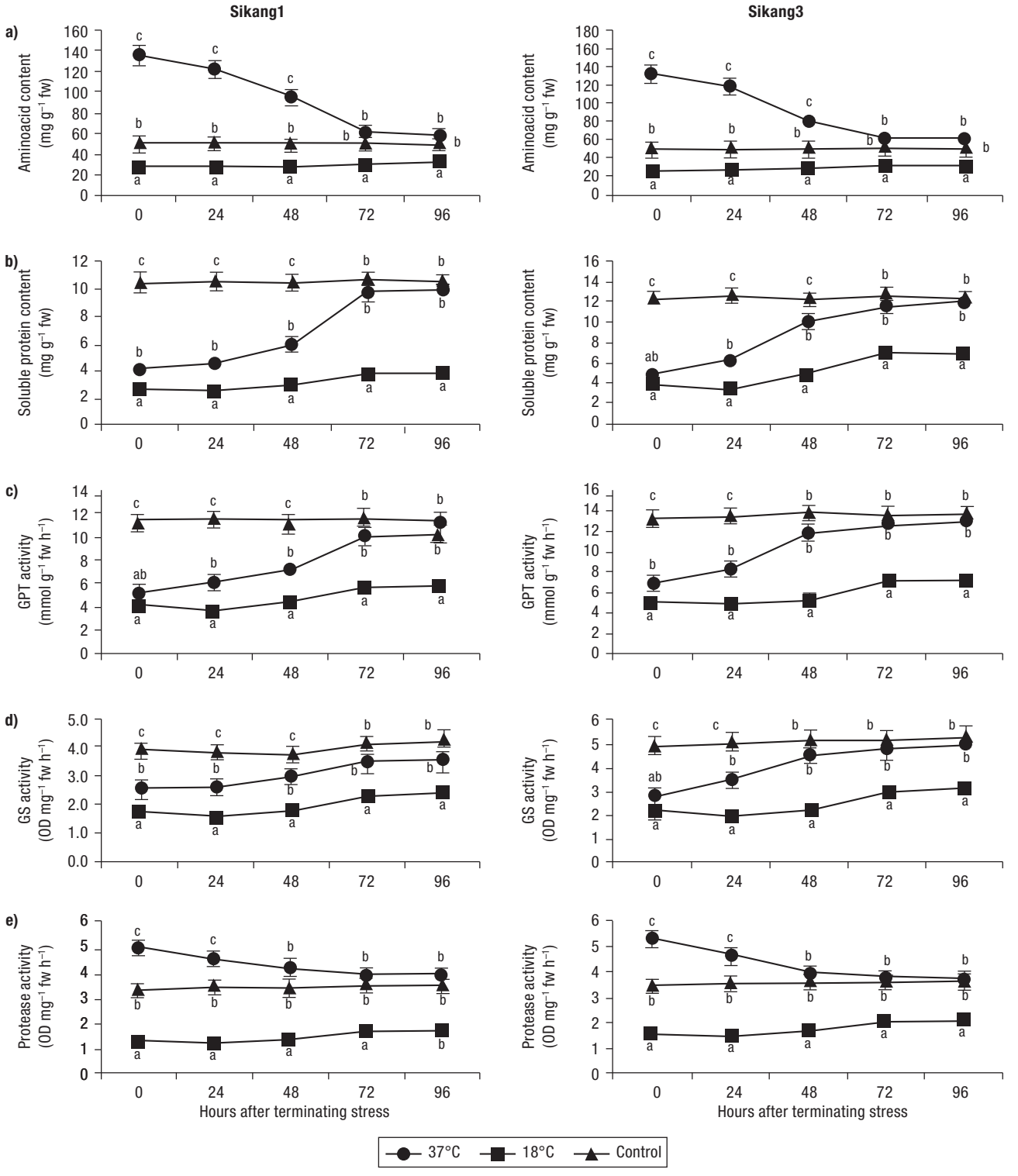

Figure 1. Free amino acid content (a), soluble protein content (b), GPT activity (c), GS activity (d) and protease activity (e) in leaves of the Bt cotton cultivars over time after end of 48 h of hot or cold temperature stress for Bt cultivar Sikang1 (left) and hybrid Bt cultivar Sikang3 (right). Symbols represent the mean values for the different stress treatments, terminated at time 0. From end of stress onwards all plants were kept at the same temperature. Error bars represent SE of the mean (n = 4), shown when value exceeds the size of the symbol. Means followed by the same letter within each recovery time are not significantly different (HSD test at 0.05 significance level).

but quickly decreased by 57.9% and 54.5% compared to the content of the control at 96 h after stress termination for Sikang1 and Sikang3, respectively. In contrast, the leaf amino acid content after low temperature treatment increased by 21.9% and 32.7% compared to the content of the control at 96 h after stress termination for Sikang1 and Sikang3 respectively. In addition, the greatest decrements of the leaf amino acid content were observed from 48 to 72 h for Sikang1, and from 24 to 48 h for Sikang3 after high temperature treatment; the largest increments of the leaf amino acid content were observed from 48 to 72 h for both cultivars after low temperature treatment. Thus, high temperature treated plants had a quicker recovery rate of amino acid pool.

The concentration of leaf soluble protein was reduced under longer duration of high and low temperature stress. However, they increased significantly from 0 to 96 h after terminating temperature stress (Fig. 1b). Higher increments were observed after high temperature treatment than after low temperature treatment. The leaf soluble protein values for Sikang1 and Sikang3 were enhanced by 140.1% and 142.4% after high temperature treatment, but only by 47.3% and 75.2% after low temperature treatment within 96 h compared to the control, respectively. In addition, the soluble protein content of both cultivars recovered to 93.8-95.6% of the control at 96 h after high temperature treatments and to 36.4-54.8% of the corresponding control after low temperature treatment. Furthermore, the greatest increase was from 48 to 72 h for Sikang1 (161.7%), and from 24 to 48 h for Sikang3 (163.4%) after end of high temperature treatment. For both cultivars, the biggest increase was observed from 48 to 72 h after end of low temperature treatment (127.2% and 135.8% compared to the control, respectively).

Activities of leaf GPT, GS and protease

Leaf GPT and GS activity decreased significantly under both high and low temperature stress. However, leaf GPT activity increased from 0 to 96 h after termination of high temperature (37°C) and up to 48 h after low temperature stress (18°C). Larger increments were observed within 96 h after high temperature treatment than after low temperature treatment: the values for Sikang1 and Sikang3 increased by 98.0% and 85.5% after high temperature treatment, and by 43.6% and 47.9% after low temperature, respectively, compared to the corresponding contents at stress termination. In addition, the GPT activities of both cultivars recovered to 87.8-93.4% of the corresponding control level after high temperature treatment; and to 48.7-51.8% of the corresponding control level after low temperature treatment. Furthermore, the period of peak increment for the leaf GPT activities was similar to that of leaf soluble protein for both cultivars studied. The values increased by 39.4% from 48 to 72 h for Sikang1 and 42.2% from 24 to 48 h for Sikang3 after high temperature treatment, and by 27.9% and 32.7% from 48 to 72 h after low temperature treatment (Fig. 1c). Similar profiles were observed for GS activity after terminating the extreme temperature stresses (Fig. 1d).

Leaf protease activities were significantly enhanced by the long high temperature treatment, but reduced by the long low temperature treatment. On the contrary, the enzyme activities for both cultivars decreased over time after high temperature treatment, and increased after low temperature treatment (Fig. 1e). Protease activity decreased significantly from 4.94 to 3.90 mg g^{-1} fw h^{-1} for Sikang1, and from 5.27 to 3.74 mg g^{-1} fw h^{-1} for Sikang3 within 96 h after the high temperature treatment, but increased slightly from 1.23 to 1.7 mg g^{-1} fw h^{-1} for Sikang1, and from 1.54 to 2.07 mg g^{-1} fw h^{-1} for Sikang3 within 96 h after the low temperature treatment. In addition, the protease activity of both cultivars decreased to the same level of the corresponding control at 96 h after high temperature treatment, but recovered to only about half the level of the corresponding control after low temperature treatment.

Discussion

This study examined the impact of the duration (24 and 48 h) of high (37°C) and low (18°C) temperature treatments on the Cry1Ac toxin content of two Bt cotton cultivars at peak boll period. Both leaf toxin levels and their recovery rate were influenced by the duration of extreme temperatures. Our results indicate that the toxin content of Bt cotton could recover completely after a short period (24 h) extreme temperature stress (either high or low temperature). The insecticidal efficacy can recover almost completely after long-duration high-temperature treatment (48 h), but it is unlikely to recover to the same level as the control after long-duration low-temperature treatment. For plants growing in the field, the temperature stress is usually short (less than 24 h) because of the day/night cycle.

Thus, a large insecticidal efficacy reduction at boll period should not be expected because of rebound of the endotoxin content after the end of short-term stress (Adamczyk *et al.*, 2001; Adamczyk & Meredith, 2004; Ian, 2006). However, extreme temperature, especially low temperature stress for longer period, can contribute to the decrease in Cry1Ac protein because of long lasting after-effect and weak recovery (Meyer & Heidmannm, 1994; Xia & Guo, 2004; Chen *et al.*, 2005; Olsen *et al.*, 2006).

Hence, current management strategies for cotton plants at boll stage may also need to be reexamined and optimized to ensure the most efficient utilization of transgenic traits after low temperature stress caused by variable climate.

The high temperature treatment significantly increased leaf amino acid content and protease activity, but decreased leaf GPT, GS activity and soluble protein contents. However, with the increased activity of leaf GPT and GS and decreased protease activity after terminating high temperature treatment at peak boll stage, soluble protein contents increased and amino acid content decreased sharply. It is evident that the leaf soluble protein degradation is reduced, and synthesis is enhanced markedly during recovery after high temperature stress. These results suggest that both decreased degradation and enhanced synthesis of leaf soluble protein after high temperature may result in the recovery of the soluble protein levels. The process of nitrogen metabolism is associated with the level of the insecticidal protein in Bt cotton (Dong *et al.*, 2000; Chen *et al.*, 2004; Zhang *et al.*, 2007). There was a significant positive correlation between the leaf insecticidal protein and soluble protein content after 24 h and 48 h high temperature treatments (r = 0.958**, 0.929**, respectively), and a negative correlation between the leaf insecticidal protein and free amino acid content at the boll stage (r = –0.772, -0.825*, respectively). Thus, rebound in leaf insecticidal protein, which is part of the total soluble protein, was probably related to overall decreased protein degradation and enhanced protein synthesis.

There was a greater impact on the reduction of the efficacy expressed in Cry1Ac under low temperature condition (Greenplate *et al.*, 2000; Olsen *et al.*, 2006). Our results indicate further that the recovery effect of key enzyme activities and nitrogen metabolism were slower after 48 h low temperature treatment. The leaf GPT, GS activity, amino acid and soluble protein content slightly increased, and protease activity decreased. And significant positive correlations were observed between the leaf insecticidal protein and soluble protein content after 24 h and 48 h low temperature treatments (r = 0.953**; 0.908**, respectively). Positive correlations were also detected between the leaf insecticidal protein and free amino acid content at the boll stage (combined r = 0.995**) after the two low temperature treatments. This result suggests that weakened synthesis of soluble protein by leaves after low temperature treatment may result in slow recovery of the soluble protein, which causes low rebound in the level of the toxin reflected as low Cry1Ac protein.

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