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The effect of high temperature on the insecticidal properties of Bt Cotton

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Abstract

Bt transgenic cotton has not shown the same level of resistance to bollworm in China, as in other major Bt cotton growing areas of the world. The objective of this study was to investigate the effects of high temperature on the CryIA insecticidal protein content and nitrogen metabolism, in the leaf of Bt transgenic cotton. The study was undertaken on two transgenic cotton cultivars, one conventional (Xinyang 822) and the other a hybrid (Kumian No. 1), during the 2001 and 2002 growing seasons at the Yangzhou University Farm, Yangzhou, China.

In the 2001 study, potted cotton plants were exposed to 37 °C for 24 h under glasshouse conditions at three growth stages – peak square, peak flowering and peak boll developing periods. Based on the 2001 results, in 2002 the same two cultivars were exposed to the same temperature for 48 h at two growth stages—peak flowering and boll developing periods. The results of the study indicated that the insecticidal protein content of the leaf was not significantly affected by the stress during the square and flowering periods. However, exposure to high temperature for 24 h during the boll period reduced the CryIA protein content by approximately 51% in the cultivar Kumian No 1, and 30% in Xinyang 822 in the 2001 study, and by approximately 73 and 63% for 48 h with the same cultivars, respectively, in the 2002 study. Glutamic–pyruvic transaminase (GPT) activity, total free amino acid and soluble protein content, and the activity of protease in the leaf, showed relatively little change in response to high temperature in the flowering period. However, exposure to high temperature in the boll period resulted in the following changes - a reduction of GPT activity, a sharp increase in free amino acid content, a significant decrease in soluble protein content, and significant increases in the activity of protease.

The results suggest that high temperature may result in the degradation of soluble protein in the leaf, with a resulting decline in the level of the toxin CryIA. It is believed that this may be the cause of the reduced efficacy of Bt cotton in growing conditions in China, where temperatures during the boll period often reach 36–40 °C.

Keywords: Bt cotton; High temperature; CryIA insecticidal protein; Nitrogen metabolism

1. Introduction

The *Bacillus thuringiensis* (Bt) transgenic cotton are rapidly dominating world agriculture (Traxler and Falck, 1999; Ismael et al., 2002). The introduction of

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commercial cotton varieties producing the insecticidal proteins is expected to improve grower profitability, reduce environmental pollution from synthetic insecticides, increase worker safety (Gould, 1988; Gasser and Fraley, 1989). More than one million acres of insect tolerant cotton were used since commercial planting in China in 1997, the technology reduced both the cost of pesticide applications and exposure to pesticides. (Guo et al., 1999; Zhao et al., 2000; Pray et al., 2002). However, poor performance of the transgenic traits during boll period, variable performance between different regions were reported in China and the other cotton production region of the world (Fitt et al., 1994; Matzke and Matzke, 1995; Zhao et al., 1999; Olsen and Daly, 2000). The loss of the efficacy was associated with a reduction of the insecticidal proteins (Finnegan and Mcelory, 1994; Benedict et al., 1996). Sachs et al. (1998) attributed these differences to somaclonal variation and/or positional effects on CryIA gene expression, some have deduced the introduced genes are silenced or switched off (Stam et al., 1997). Others attributed that production of the toxin is influenced by plant age or reproductive stage, and/or by a variety of environmental factors (Benedict et al., 1993; Wu et al., 1997). High temperature is one of the environmental factors to affect cotton development process (Reddy et al., 1992, 1998), the maximum temperature is usually at 36–40 °C, the period of high temperature occur for 6–10 days during cotton growing season in China (Miao et al., 1998; Zhou, 1999), and resulted in senescence of leaves, abscission of bolls and reduced lint yield (Zhou et al., 1996). The field investigation discovered that the reduction of the insect-resistant efficacy for Bt cotton was after high temperature climate. Therefore, we hypothesized that the changed efficacy might be related with the high temperature. To ensure that resistance management strategies designed for use with transgenic cotton successful, the assessment of the insecticidal proteins expression by high temperature is needed.

Because the synthesis of the Bt protein and its cycle in plant are also the physiological process of nitrogen metabolism, which were controlled by several key enzymes such as NR, GPT, GOT, protease and peptidase (Steward, 1965). Our recent work showed that the leaf insecticidal proteins content of Bt cotton had close correlation with NR, GPT and protease activity (Chen et al., 2003), indicating that the nitrogen metabolism of the Bt cotton affected the Bt protein content. Therefore, investigating the relationships between the toxin levels and the nitrogen metabolism under high temperature condition is important to illustrate the cause of the efficacy reduction.

The primary objective of the research reported here was to characterize the expression of a modified CryIA gene insecticidal proteins in Bt cotton cultivars after exposure to high temperature condition that similar to the high temperature weather of cotton growth season in China. A secondary objective was to examine relationship between the toxin level and nitrogen metabolism under high temperature condition.

2. Materials and methods

2.1. Plant material and experimental design

The experiment was conducted in glasshouses at Yangzhou University, Yangzhou, China (32°30′N, 119°25′E), during 2001–2002 cotton growing season. Two Bt transgenic cotton cultivars (medium in maturity, *Gosyipium hirsutum L*) ‘Kumian No.1’ (hybrid) and ‘Xinyang822’ (conventional) were used. The seeds of the two cotton cultivars were planted in warm room covered by plastic film, and sowing date for both was on April 4. The seedlings were transplanted to the pots at 43 days after sowing. Each porcelain pot (50-cm height, 40-cm diameter, 62.8-l volume) was filled with 20 kg sandy loam soil [Typic fluvaquents, Entisols (U.S. taxonomy)] which contained 18.5 g kg^{-1} organic matter and available N–P–K at 108, 40.5 and 82.0 mg kg^{-1} respectively. On the day of transplanting (17 May), 1.6 g N as urea, 0.6 g P as single superphosphate and 2.4 g as KCl were mixed into the soil of each pot, and one seedling was transplanted in each pot. At 48 days after transplanting, 1.54 g N as urea, 0.6 g P as single superphosphate and 2.4 g as KCl were top-dressed into each pot. At 70 days after transplanting, 1.9 g N as urea were top-dressed into each pot. Each variety were transplanted to 40 pots respectively, the plants were watered daily by hand to maintain a soil water content close to field capacity, Kumian No.1 and

Xingyang822 flowered at 89 and 92 days after sowing (DAS), first boll opened on 137 DAS and 139 DAS respectively.

The high temperature of 37 °C was imposed for 24 h at peak square period (77 DAS), flowering period (106 DAS) and peak boll developing period (129 DAS) respectively in 2001. The temperature was regulated by air conditioner, and the glasshouse maintained humidity close to air humidity outside. The pots for both cultivars were allocated in four replications. There were controls (non-stress) with the same cultivars in four replications growing in another glasshouse where the temperature was between 25 and 32 °C.

On the basis of the results in 2001, the experiment was further conducted during 2002 cotton growth season. The planting pattern was same as 2001, the same two cultivars were exposed to 37 °C for 12, 24, 36 and 48 h at flowering period (102 DAS) and peak boll developing period (124 DAS) respectively, pots were also allocated in four replications for each treatment time. There were also non-stress treatments (controls) in four replications growing in another glasshouse where temperature was 25–32 °C.

2.2. Preparation of samples

Leaf samples were collected from the fourth leaves from the top of the plants after 24 h high temperature treatment in 2001, and the controls were sampled at same time. Same leaf samples were harvested after exposing to 37 °C for 12, 24, 36 and 48 h respectively, in 2002. All the samples were frozen with liquid nitrogen, and stored in freezer.

2.3. The CryIA protein concentration assay

The CryIA protein concentrations in cotton leaf extracts were determined by immunological analysis by means of ELISA (Chen et al., 1997). Leaf tissue extracts (0.8–1.2 g) were prepared by homogenizing the frozen leaf tissue in 2 ml extraction buffer (Na_2CO_3 1.33 g, DTT 0.192 g, NaCl 1.461 g, Vc 0.5 g dissolved in 250 ml distilled water), the contents was moved to 10 ml centrifuged tube, the residue was washed with 3 ml of the buffer and this was added to the centrifuged tube. The contents of this tube were shaken with hand, and stored at 4 °C for 4 h. The extracts were collected after centrifugation at 10,000 × *g* at 4 °C for 20 min, then passed through a C18 Sep-Pak Catridge (Waters, Milford, MA), and the supernatant was collected for determination. Microtitration plates were coated with the standard CryIA insecticidal proteins and samples, incubated at 37 °C for 4 h. The antibodies were added to each well and incubated further 30 min at 37 °C. The antibodies against the CryIA insecticidal protein were obtained as described by Weiler et al. (1981). Then horseradish peroxidase-labelled goat antirabbit immunoglubolin was added to each well and incubated for 30 min at 37 °C. Finally, the buffered enzyme substrate (orthopenylenediamino) 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. The absorbance recorded at 490 nm. Calculation of the ELISA data were performed as described by Weiler et al. (1981).

2.4. Glutamic–pyruvic transaminase (GPT) assay

The leaf samples (0.5 g) were homogenized in buffered medium (0.05 mM Tris–HCl, pH 7.2), and the homogenate was centrifuged at 26,100 × *g* for 10 min at 0 °C. The supernatant was analysed for GPT activity. A mixture of 0.5 ml of 0.8 M alanine in 0.1 M Tris–HCl (pH 7.5) + 0.1 ml of a 2 mM Pyriodoxal Phosphate solution was used, and to this 0.2 ml of a 0.1 M 2-oxoglutarate solution and 0.2 ml the enzyme preparation were added, the reaction mixture was incubated at 37 °C for 10 min followed by termination of reaction with 0.1 ml of a 0.2 M trichloroacetic acid solution, then the pyruvate with chromogen was converted to pyruvate hydrazone. The colour intensity of the hydrazone in saturated water toluene was measured at 520 nm. The GPT activity, in term of pyruvate production, was calculated from authentic pyruvate standards run simultaneously (Tonhazy et al., 1950).

2.5. Protease activity assay

The leaf samples (0.8 g) were homogenized at 4 °C in 1 ml of β-mercaptoethanol extraction buffer, pH 6.8, containing ethylene glycol, sucrose, and phenylmethyl sulfonyl fluoride (Jessen et al., 1987, 1988). Cell debris was removed by centrifugation, and the supernatant placed on ice and immediately used to estimate the leaf protease, Protease activity was determined using azocasein as substrate (Vance et al., 1979) and

expressed as change in absorbance (400 nm) mg protein g^{-1} leaf fresh weight.

2.6. Assay of free amino acid and soluble protein content

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

The total soluble protein content was determined by the Coomassie Blue dye-binding assay of Bradford (1976), absorbance readings were converted using BSA as standard curve.

2.7. Statistics analysis

Student *t*-test was conducted for each characteristics of nitrogen metabolism between high temperature treatment(different period) and the control (non-stress treatment), using Sigmaplot.

3. Results

3.1. Leaf insecticidal protein level in response to the high temperature

In comparison to the control, there were different effects for the high temperature on the contents of the leaf insecticidal proteins at the three period for the two cultivars in 2001 (Table 1), the values were not significant different between the treatments and the controls at peak square period (77 DAS), and during the flowering period (106 DAS). However, the contents decreased sharply ($P < 0.01$) at peak boll developing period (129 DAS), the value reduced by 51.1% for Kumian No1, and by 30.4% for Xinyang822.

3.2. The leaf GPT and protease activity in response to high temperature

The results of the high temperature on the leaf GPT (in 2001) showed that the enzyme activity reduced comparing to the control, however, there were no significant difference between the treatment and the control at peak square (77 DAS) and at flowering periods (106 DAS), and diminished drastically at peak boll developing period (129 DAS). The average enzyme activity decreased by 9.6% at 77 DAS, 10.2% at 106 DAS, and 47.4% at 129 DAS for Kumian No.1; 12.2, 18.2 and 41.5% at 77, 106 and 129 DAS for Xingyang822 respectively (Table 2).

The protease activity increased after 24 h high temperature treatment (Table 2), however, there were no significant difference between the treatment and the

Table 1
The leaf insecticidal protein content exposed to 37 °C for 24 h at three growth stage in cotton (*Gossipium Hirsutum.L*)

Cultivar	Treatment	Days after sowing		
		77	106	129
		fresh wt. (ng g^{-1})		
Kumian No.1	37 °C	320.3 ± 3.7	186.0 ± 6.4	145.0 ± 5.7
	Control	326.0 ± 4.8	203.7 ± 5.2	296.7 ± 9.8
	t-test *P* value	0.40	0.08	0.00
Xingyang822	37 °C	225.7 ± 5.0	143.8 ± 8.6	120.3 ± 3.5
	Control	236.0 ± 8.4	159.9 ± 3.2	172.9 ± 3.6
	t-test *P* value	0.35	0.10	0.00

The value are means ± S.E. ($n = 4$).

Table 2
The leaf GPT and protease activity exposed to 37 °C for 24 h at three growth stage in Bt cotton (*Gossipium Hirsutum.L*)

Cultivar		Days after sowing					
		77		106		129	
		GPT (μmol g^{-1} h^{-1})	Protease (mg g^{-1} h^{-1})	GPT (μmol g^{-1} h^{-1})	Protease (mg g^{-1} h^{-1})	GPT (μmol g^{-1} h^{-1})	Protease (mg g^{-1} h^{-1})
Kumian No.1	37 °C	0.59 ± 0.01	0.66 ± 0.04	0.49 ± 0.01	2.63 ± 0.17	0.51 ± 0.01	5.27 ± 0.16
	Control	0.65 ± 0.03	0.50 ± 0.05	0.55 ± 0.02	2.28 ± 0.05	0.97 ± 0.06	3.03 ± 0.11
	t-test *P* value	0.11	0.07	0.05	0.15	0.00	0.00
Xingyang822	37 °C	0.54 ± 0.02	0.59 ± 0.03	0.48 ± 0.03	2.89 ± 0.25	0.60 ± 0.08	5.23 ± 0.14
	Control	0.62 ± 0.05	0.55 ± 0.01	0.59 ± 0.03	2.33 ± 0.01	1.03 ± 0.01	2.94 ± 0.02
	t-test *P* value	0.23	0.24	0.06	0.11	0.01	0.00

The value are means ± S.E. (n = 4).

control at peak square (77 DAS) and at flowering periods (106 DAS), the activity bolstered significantly at peak boll developing period. In comparison to the control, the values increased by 73.9% for Kumian No.1, by 77.8% for Xingyang822 at 129DAS. These results suggest that the decrease of Bt insecticidal protein in the leaf results from reducing amino acid synthesis and bolstering the protein degradation under the high temperature during boll developing stage.

3.3. Duration of the high temperature on the leaf insecticidal protein

The results of duration of high temperature treatment on the leaf insecticidal protein in 2002 showed further that the contents reduced slightly, but not significant (student *t*-test) for the two Bt cultivars when exposed to 37 °C for 48 h at 102 DAS in flowering period (Fig. 1A), the contents were from 197.5 to 178.0 ng g^{-1} fresh weight for Kumian No.1, and 182.1 to 165.2 for xinyang822, decreased by 9.8 and 7.5% respectively. However, the leaf insecticidal proteins contents decreased significantly when exposed to 37 °C for 48 h at 124 DAS in peak boll developing period (Fig. 1B), the contents were from 288.8 to 77.8 ng g^{-1} fresh weight for Kumian No.1, and 159.2–59.7 for xinyang822, decreased by 73.1% for kumian No.1, and 62.5% for xinyang822.

3.4. Duration of the high temperature on the leaf GPT activity

The trends of leaf GPT activity over the duration of the high temperature for the two cultivars were similar. The results in Fig. 2A showed that leaf GPT activity increased from 0 to 12 h and decreased from 12 to 36 h slightly, boosted slightly again after 36 h when exposed to 37 °C at 102 DAS (flowering period), but the changes were not significant. the leaf GPT activity

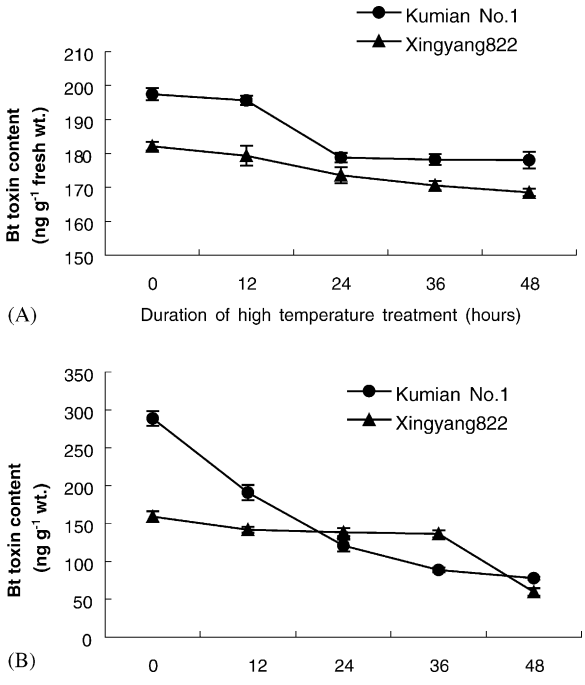

Fig. 1. The leaf insecticidal proteins contents of cotton cultivars exposed to 37 °C for period of 12–48 h: (A) at 102 DAS (flowering) and (B) at 124 DAS (boll period). Symbol Kumian No1 and Xingyang822 are the name of the two Bt cultivars, vertical bar represent S.E. of the mean ($n = 4$), when value exceeds the size of the symbol. The value at 0 h was level of the control.

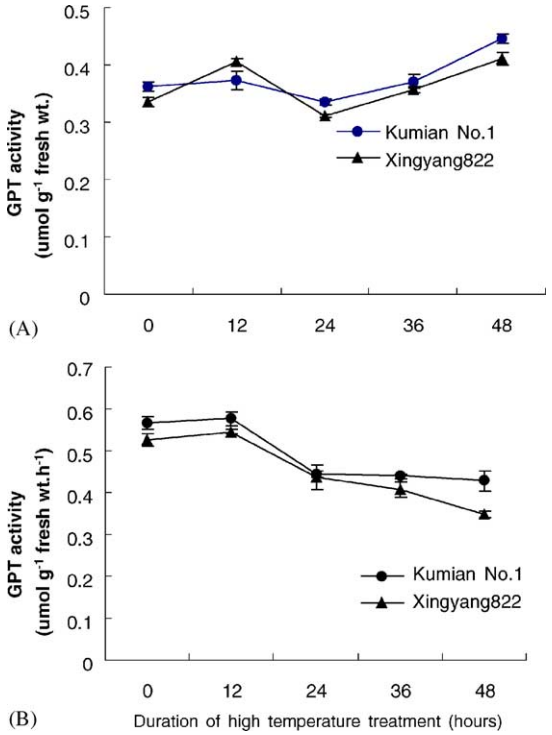

Fig. 2. The leaf GPT activity of the Bt cotton cultivars exposed to 37 °C for period of 12–48 h: (A) at 102 DAS (flowering) and (B) at 124 DAS (boll period). Symbol Kumian No.1 and Xingyang822 were the name of the two cultivars, vertical bar represent S.E. of the mean ($n = 4$), when value exceeds the size of the symbol. The value at 0 h was level of the control.

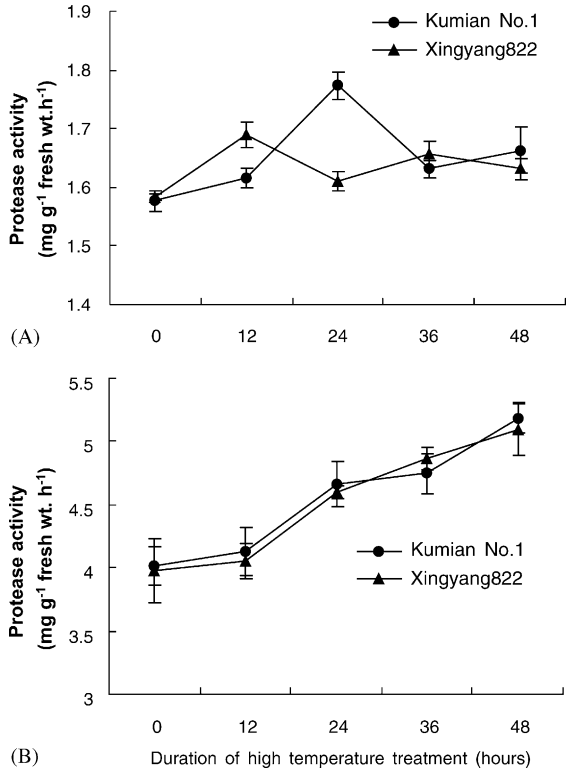

Fig. 3. The leaf Protease activity of the two cultivars exposed to 37 °C for period of 12–48 h: (A) at 102 DAS (flowering) and (B) at 124 DAS (boll period). Symbol Kumian No.1 and Xingyang822 are the name of the two varieties, vertical bar represent S.E. of the mean ($n = 4$), when value exceeds the size of the symbol. The value at 0 h was level of the control.

increased slightly (not significant with *t*-test) from 0 to 12 h, When exposed to 37 °C at 124 DAS in peak boll developing period (Fig. 2B), however, decreased significantly.

3.5. The duration of the high temperature on the leaf protease

The results of the leaf protease activity showed that it changed slightly (not significant with *t*-test) when exposed to 37 °C for the two cultivars at 102 DAS in flowering period (Fig. 3A), however, increased significantly when exposed to 37 °C at boll developing period (Fig. 3B). The activity of protease varied between 1.5 and 1.8 mg g^{-1} fresh weight h^{-1} from 0 to 48 h at flowering period, but increased significantly over duration of the high temperature treatment at 129 DAS, which was 4.02–5.17 mg g^{-1} fresh weight h^{-1} from 0 to 48 h for kumian No.1, and 3.98 to 5.07 mg g^{-1} fresh weight h^{-1} for xinyang822.

3.6. Duration of the high temperature on leaf amino acid and soluble protein

The contents of leaf amino acid and soluble protein were different over the duration of the high temperature treatment at the two growth periods (Fig. 4). When exposed to 37 °C at 102 DAS (flowering period), the contents of amino acid increased significant (*t*-test) from 0 to 24 h for Kumian No.1, which was 0.30 to 0.64 mg g^{-1} fresh weight, however, the values remained stable after 24 h (Fig. 4A); The content of amino acid also bolstered significantly during 0 to 36 h for xinyang822, which was from 0.25 to

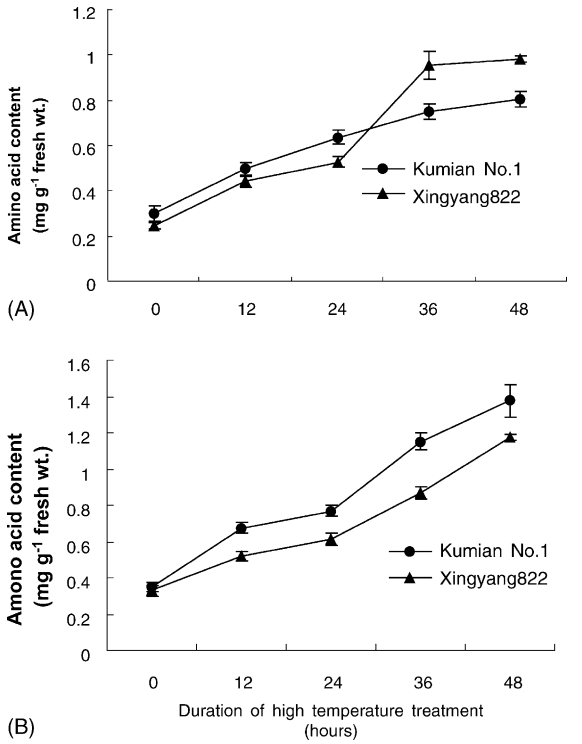

Fig. 4. Leaf free amino acid content of the Bt cotton cultivars exposed to 37 °C for period of 12–48 h: (A) at 102 DAS (flowering period) and (B) at 124 DAS (boll period). Symbol Kumian No.1 and Xingyang822 are the name of the two varieties, vertical bar represent S.E. of the mean ($n = 4$), when value exceeds the size of the symbol. The value at 0 h was level of the control.

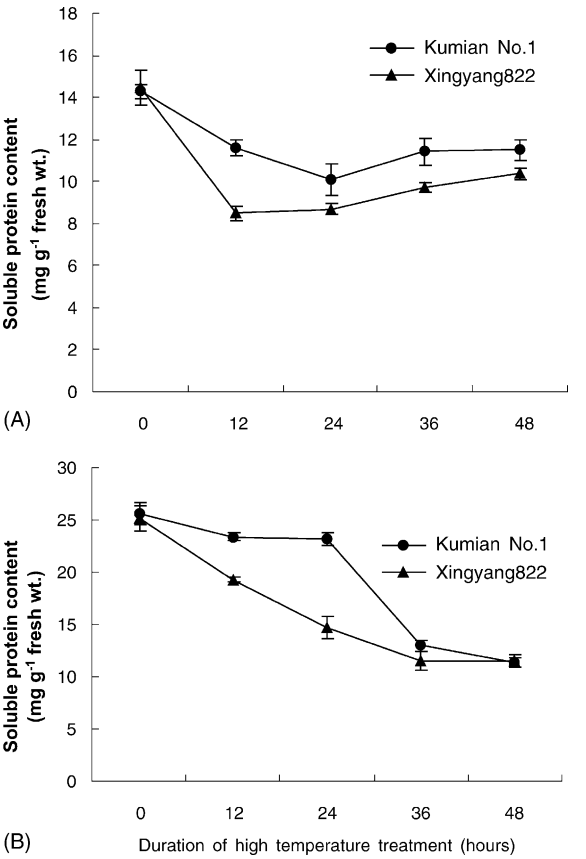

Fig. 5. Soluble protein content of the leaf for the two cultivars exposed to 37 °C for period of 12–48 h: (A) at 102 DAS (flowering period) and (B) at 124 DAS (boll period). Symbol Kumian No.1 and Xingyang822 are the name of the two cultivars, vertical bar represent S.E. of the mean ($n = 4$), when value exceeds the size of the symbol. The value at 0 h was level of the control.

0.95 mg g^{-1} fresh weight, and it also remained stable after 36 h. However, the contents of amino acid increased significantly from 0 to 48 h when exposed to 37 °C at 124 DAS (peak boll developing period), which was 0.35–1.38 mg g^{-1} fresh weight for Kumian No.1, and 0.33–1.18 mg g^{-1} fresh weight for xinyang822, boosted 4.0- and 3.6-fold respectively (Fig. 4B).

In contrast to the contents of leaf amino acid, the contents of leaf soluble protein decreased significantly from 0 to 24 h for Kumian No.1, from 0 to 12 h for Xingyang822, when exposed to 37 °C at 102 DAS, which was 14.29–10.39 mg g^{-1} fresh weight for Kumian No.1, and 14.48–8.67 mg g^{-1} fresh weight for xinyang822, and the contents remained relative stable thereafter (Fig. 5A). However, the soluble protein declined significantly when exposed to 37 °C at 124 DAS, which decreased 55.6% from 0 to 48 h for Kumian No.1, and 47.5% for xinyang822 (Fig. 5B).

4. Discussion

4.1. The high temperature reduced the leaf insecticidal protein contents and the extent of the reduction related with the growth period of the Bt cultivars

Wu et al. (1997) reported that the Bt cotton plants lost their insect resistance significantly during waterlogging condition. Wang et al. (2001) reported further

that waterlogging and drought reduced insecticidal protein content of leaves and squares. In the present experiment, we found that the high temperature reduced the leaf insecticidal protein contents and the extent of the reduction related with the growth period of the Bt cultivars. In comparison to the control, the leaf insecticidal protein contents reduced slightly at 77 DAS (peak square period) and 106 DAS (peak flowering period), however, when exposed to 37 °C at boll period, the insecticidal protein declined significantly from 0 to 48 h. These results suggest that the high temperature had little effect on the reduction of the insect-resistance before blooming period for the Bt cotton cultivars, but had significant effect on the decrease of insect-resistance at boll period. The findings indicated that the efficacy reduction of the Bt cotton cultivars related with plant age or reproductive stage under high temperature condition. The highest temperature is usually from the end of June (square period) to August 15 (boll period) in the cotton production region in China, which was between 36 and 40 °C for 6–10 days (Zhou, 1999). The reduction of the efficacy for Bt cotton was usually observed at boll period, especially after the high temperature weather. It concludes that reduced the insect-resistance of Bt cotton is close correlation with the high temperature weather during boll period because the high temperature can increase the probability of the gene silencing (Meyer and Heidmann, 1994). Such results are consistent with the previous reports of speculation on the causes of insect-resistant reduction at boll period (Kaiser, 1996; Benedict et al., 1996; Sachs et al., 1998; Olsen and Daly, 2000).

4.2. The high temperature may result in the degradation of leaf soluble protein, including the Bt insecticidal protein

The process of nitrogen metabolism is associated with the level of the insecticidal protein in Bt cotton (Dong et al., 2000; Chen et al., 2003). The results of high temperature treatment on Bt cotton at different growth stage showed that the high temperature had significant effect on the leaf GPT activity, amino acid and soluble protein contents at boll period. The leaf GPT activity and soluble protein contents reduced, and amino acid content increased sharply. In our present experiment, the correlation analysis showed further that there was significant positive correlation between the leaf insecticidal protein and soluble protein content ($r = 0.75^*$), and negative correlation between the leaf insecticidal protein and free amino acid content at the boll period ($r = -0.79^*$). It is evident that the leaf soluble protein degraded under high temperature condition, the conclusion was proved further by the bolster of activity of protease (Fig. 3B). These results suggest that the high temperature may result in the degradation of the leaf soluble protein in the leaf, with a resulting decline in the level of the toxin CryIA.

The high temperature had a significant effect on the reduction of the leaf Bt toxin content in the boll development period rather than in squaring and flowering period, it may relate with physiological metabolism characteristics of the cotton plant development. The nitrogen nutrient supplied dominantly for the growth of vegetative organs before boll period (Chen et al., 2000), there were less competition for nitrogen between the growth of vegetative and reproductive organs (Mason, 1922; Hearn and Constable, 1984; Guinn, 1986), the characteristics suggest that the N metabolism intensity can recover after some time under the high temperature stress during flowering period because of enough nitrogen supply. The changes of the enzymes activity related with nitrogen and content of the nitrogen compounds in our experiments provided strong evidence, the GPT activity increased at some extent after 24 h (Fig. 2A), the protease activity remained stable after 36 h (Fig. 3A), the results were consistent with the stable contents of free amino acid and soluble protein after 36 h (Figs. 4A and 5A). However, the leaf nitrogen nutrient deficits could develop as a result of competition between growth of fruiting organs and vegetative organs during boll period because of increasing fruit load (Wadleigh, 1944; Radin and Mauney, 1986). The effect implicates that the high temperature reduced the nitrogen metabolism intensity and could not recovered because of supply of deficient nitrogen nutrient for the leaf. Our experiment results also proved it, the GPT activity decreased sharply after 24 h (Fig. 2B), the protease activity increased with the stress time (Fig. 3B), the results were also consistent with the significant increase of the free amino acid contents and the decrease of the soluble protein content (Figs. 4B and 5B).

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