Front. Agric. China 2007, 1(4): 382–388
DOI 10.1007/s11703-007-0064-1

RESEARCH ARTICLE

ZHOU Guisheng, FENG Chaonian, ZHOU Qing

Physiological activities and expression of insecticidal protein in *Bacillus thuringiensis* transgenic cotton leaves subjected to high and low temperatures

Abstract This research was to investigate physiological activities and the expression of insecticidal protein in cotton leaves of Kemian 1, a widely grown cotton cultivar in the lower reaches of the Yangtze River, treated under high (40°C and 35°C) and low (25°C and 20°C) temperatures for 48 h. The main results indicated that the contents of free amino acid and malondialdehyde (MDA) increased during the heat and cold stress with the biggest increments in the first 12 h; and the content of soluble protein, activities of superoxide dismutase (SOD), and glutamic-pyruvic transaminase (GPT) decreased during the stress with the biggest decrements also in the first 12 h; the activity of nitrate reductase (NR) decreased in the first 24 h, and during the remaining 24 h its activity followed a gradual uptrend. The content of insecticidal protein in cotton leaves followed a downtrend during the stress, and the biggest decrements occurred in the first 12 h, suggesting that the expression of insecticidal protein was immediately inhibited during the process of stress. The results also indicated that physiological activities and the expression of insecticidal protein in cotton leaves were closely related. Compared with other stress temperatures, 40°C produced more immediate effects on the physiological activities and insecticidal protein content. In contrast to the peak flowering period, the physiological activities and insecticidal protein expression at the peak boll-setting period were more susceptible to heat and cold stress.

Keywords *Bacillus thuringiensis*, transgenic cotton, insecticidal protein expression, high and low temperatures

Received June 26, 2007; accepted September 7, 2007

ZHOU Guisheng, FENG Chaonian (✉)
The Jiangsu Provincial Key Laboratory of Crop Genetics and Physiology, Yangzhou University, Yangzhou 225009, China
E-mail: gszhou@yzu.edu.cn

ZHOU Qing
Huaiyin Institute of Technology, Huai'an 223001, China

1 Introduction

With the introduced *Bacillus thuringiensis* (Bt) gene, the transgenic cotton producing insecticidal protein has come to dominate world cotton production because of its good resistance to bollworms, less usage of insecticides, and thus reduced negative effects on both cotton farmers and natural environment (Gould, 1988; Gasser and Fraley, 1989; Cui and Xia, 1999). In China, the transgenic cotton over one million acres has been bringing about benefits not only to the cotton farmers but also to the natural environment (Guo et al., 1999; Li and Wang, 1999; Zhao et al., 2000; Xing et al., 2001). However, the unstable expression of insecticidal protein and the less optimistic tolerance effects to bollworms of cotton plants at critical cotton growth stages have been reported frequently (Zhao and Oosterhuis, 1997; Su et al., 1998; Zhang and Feng, 1998). Some other researches also indicated that unfavorable conditions including drought, water-lodging, and extremely high or low temperatures could lead to less tolerance of Bt transgenic cotton to bollworms (Wu et al., 1997, Wang et al., 2000, Wang et al., 2001). Our previous research (Zhou et al., 2003) showed that the expression of cotton insecticidal proteins was greatly affected under high temperature (40°C) at the peak flowering and peak boll-setting stages. To gain a better understanding of the mechanism leading to the inhibited expression of insecticidal protein under the stress conditions, further researches are needed.

With more experiments conducted in the stressed conservatories with different high and low temperatures, the primary objective of this study was to make a probe into the mechanism leading to the unstable expression of insecticidal proteins so as to provide a hint for better application of Bt cotton under unfavorable conditions.

2 Materials and methods

2.1 Stress treatment and the preparation of plant materials

Pot experiment was conducted at Yangzhou University Experimental Farm, Jiangsu Province, China (32°30′N,

119°25′E), during the cotton growing seasons of 2003 and 2004. Seeds of Kemian 1, a widely grown hybrid in the lower reaches of the Yangtze River, were sown in the seedbed covered with a plastic film on April 9, and seedlings were transplanted into the pots containing 20 kg soil (50 cm in diameter and 60 cm in depth) on May 19 in both years. Soil used in pots was the field loam containing 30.5 g/kg organic matter, 123, 19.7 and 80.9 mg/kg available N, P_2O_5 and K_2O, respectively. Fertilizer application including nitrogen, phosphorus and potassium and other cultural practices were in conformity with the recommended local standards.

2.1.1 Timing of stress treatment

The ten days from July 15 to 25 and the first ten days in August are not only the time in a year in the lower reaches of the Yangtze River that bollworms cause the greatest damage but also the time that cotton plants experience the two most critical growth stages of peak flowering and peak boll-setting. Meanwhile, the temperature during this period is rather unstable because frequent typhoons leading to sudden temperature decreases and long-term droughts may bring about high temperature hikes. So stress experiments were conducted on July 20 (the peak flowering stage) and August 5 (the peak boll-setting stage).

2.1.2 Choices of temperature used under heat and cold stress

The average temperature on July 25 and August 5 in Yangzhou is around 30°C. So the potted cotton plants were treated under cold stress at 20°C and 25°C, which were 10°C and 5°C lower than the daily average temperature. And as for heat stress, 35°C and 40°C were chosen, 5°C and 10°C higher than the average temperature.

2.1.3 Stress treatment and the preparation of plant materials

At around 5 p.m., the potted plants were moved into the conservatories set at the above-mentioned temperatures, and both heat and cold treatments would last for 48 h. Temperatures were kept to meet the above-mentioned temperature requirements by air conditioners and heaters. The light intensity in conservatories was similar to that in the natural environment. Prior to stress and every 12 h during the treatment, cotton leaves were sampled from the first fruit node of the second fruit branch from the top. After collection, these samples were carried in mini-freezer, sent to laboratory within 5 min, frozen immediately in liquid nitrogen for 15 min, and then stored at −30°C for further physiological analysis.

2.2 Physiological measurements

2.2.1 Determinations of free amino acid and soluble protein

The 0.5 g frozen leaf was ground into powder in 5 mL 10% (v/v) acetic acid and then diluted to 100 mL with the purified nitrogen-free water. The dilution (1 mL) was used for further assay. Ninhydrin (3 mL) and 0.1% Vitamin C (0.1 mL) were added into it. Then the sample was boiled for 20 min. After boiling, 10 mL 80% (v/v) ethanol and 10.5 mL purified nitrogen-free water were added. At last the content of free amino acid was determined by ninhydrin assay. The absorbance readings were converted into free amino acid content per gram fresh leaf by using glycine standard curve (Yemm and Cocking, 1955).

The 0.5 g leaf of the frozen sample was used for the determination of soluble protein. It was ground into powder, added with 10 mL cold purified water and centrifuged at 4 000 r/min for 10 min, and then the supernatant was pooled for Bradford (1976) Coomassie Blue dye-binding assay. The absorbance readings were converted into protein content per gram fresh leaf by using BSA as standard curve.

2.2.2 Assays of superoxide dismutase (SOD) activity and malondialdehyde (MDA) content

The SOD activity was assayed by monitoring the inhibition of photochemical reduction of nitro blue tetrazolium (NTB) according to the method of Beauchamp and Fridovich (1971), with the notes taken into account by Beyer and Fridovich (1987). Leaf samples were homogenized in four volumes (w/v) of an ice-cold buffer containing 0.1mol/L Tris–HCl (pH 7.8), 0.1 mmol/L EDTA and 0.05% Triton X-100. The homogenates were filtered through four layers of cheesecloth and centrifuged at 4°C for 30 min at 15 000 × g. The crude extracts were dialyzed for 24 h against half strength extraction buffer without Triton X-100, centrifuged for 20 min at 15 000 × g and the supernatants were used for SOD assay. The reaction mixture contained 50 mmol/L phosphate buffer (pH 7.8), 0.053 mmol/L NTB, 10 mmol/L methionine, 0.005 3 riboflavin and an appropriate aliquot of enzyme extract. The reaction was started by switching on the light and allowed to run for 7 min. One unit of the SOD activity was defined as the amount of the enzyme required to cause 50% inhibition of the reduction of nitrotetrazolium blue chloride (NBT), monitored at 560 nm.

The MDA content was assayed according to the method published in Crop Physiological Analysis (Zhang, 1991).

2.2.3 Assays of activity of nitrate reductase (NR) and glutamic-pyruvic transaminase (GPT)

The 0.3 g leaf of the frozen sample was vacuum infiltrated in 10 mL of incubation buffer containing 0.1 mol/L potassium phosphate (pH 7.5), 0.05 mol/L KNO_3 and 1% (v/v) propanol.

After the infiltration, the samples were incubated in water bath for 30 min at 30°C in the dark. After the incubation, 0.4 mL of the incubation buffer was diluted with water to 4 mL. Two millimeters of 1% sulfanilic acid in 1.5 mol/L HCl was added to the diluted incubation buffer, followed by 2 mL of *N*-(1-naphthyl) ethylenediamine-HCl (200 mg/L). The samples were incubated once again for over 20 min at the room temperature to allow full color development. At last, the samples were monitored at 540 nm (Ernest et al., 1971).

The 0.5 g leaf was homogenized in buffer containing 0.05 mmol/L Tris-HCl (pH 7.2) and the homogenate was centrifuged at 26 100 × g for 10 min at 0°C. The supernatant was used for GPT analysis. A mixture of 0.5 mL of a 0.8 mol/L alanine in 0.1 mol/L Tris-HCl (pH 7.5) + 0.1 mL of 2 mmol/L pyriodoxal phosphate solution was used, and to this 0.2 mL of a 0.1 mol/L 2-oxoglutarate solution and 0.2 mL of 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 mol/L trichloroacetic acid solution, then the pyruvate with chromogen was converted to pyruvate hydrazone. The color intensity of the hydrazone in water satured toluene was measured at 520 nm. The GPT activity was calculated in terms of the pyruvate production from the authentic pyruvate standards occurring simultaneously (Tonhazy et al., 1950).

2.3 Assay of insecticidal proteins

The content of insecticidal protein was determined by an enzyme-linked immunosorbent assay technique (Chen et al., 1999). The main procedures were the followings.

As for leaf sample preparation, 0.5 g leaf was homogenized in 2 mL buffer (1.33 g Na_2CO_3, 0.192 Dithiothreitol, 1.461 g NaCl, 0.5 g Vitamin C dissolved in 25 mL distilled water). And then the homogenate was centrifuged at 10 000 × g for 15 min at 4°C. The supernatant was used for the analysis of the content of insecticidal protein.

As for coating antibody, the antibody was dilated with 50 mmol/L carbonate buffer (pH 9.5), added 200 μL in each well of a 96-well plate, and then left overnight at 4°C.

The samples were shortly washed twice with Phosphate Buffered Saline + Tween 20 (PBST) solution (pH 7.4), containing 8 mmol/L phosphoric acid buffer, 140 mmol/L NaCl, 2.6 mmol/L KCl and 0.07% Tween 20, tested by adding the standard Bt toxin solution into the wells of the plates, 150 μL for each, and then stored at 4°C for 6 h. Thereafter, the plates were shortly washed three times with the above PBST solution. The enzyme-antibody conjugate was diluted with PBSTO (with 0.5% ovalbumin) and added in the wells 150 μL for each, then put at 4°C overnight. Finally the plates were washed five times, for 3 min each, with the PBST solution.

As for color developing, the substrate solution (pH 5.0, citric acidbuffer 0.1 mol/L, TMB 5 mg/L, hydrogen peroxide 0.006%) was added in the wells, 100 μL for each, with the development lasting for 15 min at 30°C, and then with sulphuric acid of 3 mol/L ended into the wells, 50 μL in each well.

For colorimetry, the optical density (OD) value was measured with a micro-plate reader.

For calculation, the standard curve was obtained by linear regression with the concentration of standard protein and the OD value as the horizontal and vertical coordinates, respectively.

In this study, the percentage recoveries were all above 90%, and all sample extraction dilution curves paralleled the standard curves, indicating the stability and liability of this method.

2.4 Statistical methods

All data were calculated with Excel 2003. Statistical analyses were conducted using SPSS 10.0.

3 Results

3.1 Physiological activities in cotton leaves

3.1.1 Content of free amino acid and soluble protein

Prior to stress, the content of free amino acid was relatively low, less than 600 μg/g fresh weight leaf at both the peak flowering period and the peak boll-setting period. During the stress, the content of free amino acid increased gradually, and when the stress was over, the content increased by more than 100% to over 1 500 μg/g fresh weight leaf. Of all stress temperatures, 40°C produced a more immediate effect, and the biggest increment occurred during the first 12 h (Figs. 1 and 2). During the 40°C stress, the free amino acid content was much higher than other stresses, indicating that a relatively high temperature stress could produce a more significant effect. In contrast to the peak flowering period, the content of free amino acid prior to stress at the peak boll-setting period was lower, but the increment during the stress was bigger, suggesting that free amino acid on August 5 was more susceptible to the outside stress.

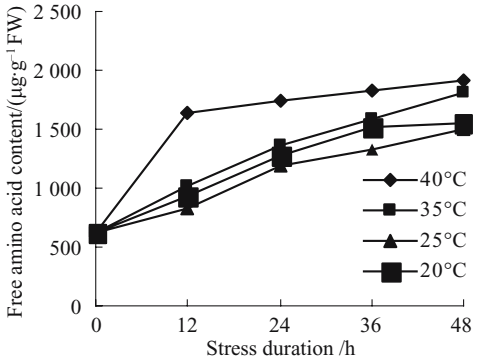

Fig. 1 Free amino acid content in cotton leaves during stress at the peak flowering period

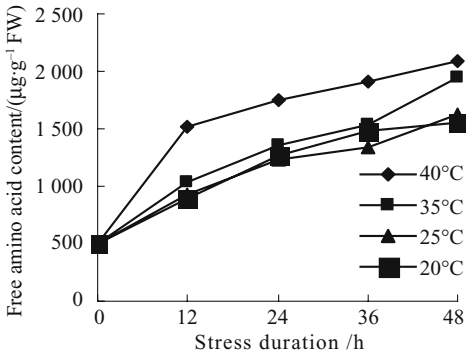

Fig. 2 Free amino acid content in cotton leaves during stress at the peak boll-setting period

Following a trend contrary to free amino acid, the content of soluble protein in cotton leaves declined drastically during the first 12 h under both heat and cold stresses, and during the following 36 h the decrement became much smaller (Figs. 3 and 4). Of the four temperatures, 40°C produced the most significant effect on the decreased content of soluble protein, indicating that the soluble protein was more susceptible to 40°C heat stress than to other treatments. In contrast with the peak flowering period, the content of soluble protein prior to stress at the peak boll-setting period was much lower while the decreasing rate during the remaining 36 h was much higher.

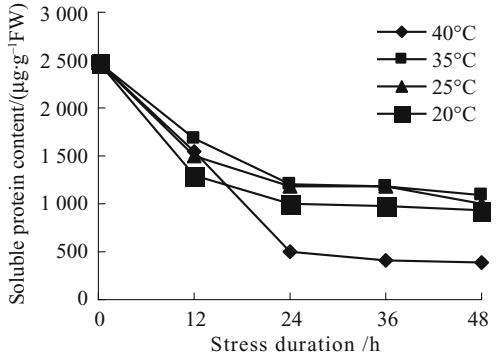

Fig. 3 Soluble protein content in cotton leaves during stress at the peak flowering period

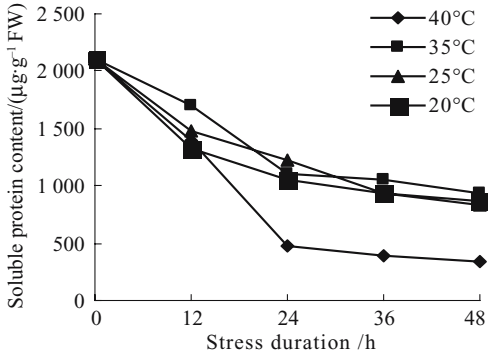

Fig. 4 Soluble protein content in cotton leaves during stress at the peak boll-setting period

Further statistical analysis indicated that both the free amino acid and the soluble protein were significantly negatively correlated in contents (–0.889 3** on July 20 and 0.936 7** on August 5, respectively), indicating a very close relationship between the free amino acid and the soluble protein.

3.1.2 SOD activity and MDA content

It was observed that SOD activity decreased sharply as both heat and cold stress were applied (Figs. 5 and 6), and the biggest decrement occurred during the first 12 h. During the remaining 36 h the decreasing trend became more gradual. The stress temperature 40°C produced the most significant effect, indicating that the SOD activity was more easily affected by the high temperature stress.

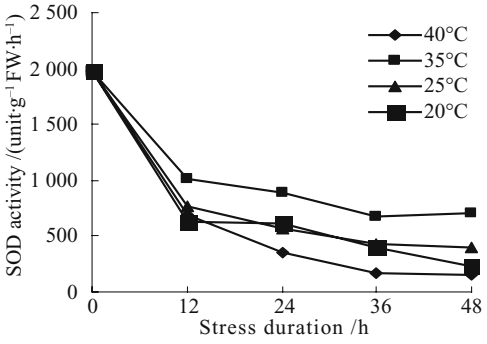

Fig. 5 SOD activity of cotton leaves during stress at the peak flowering period

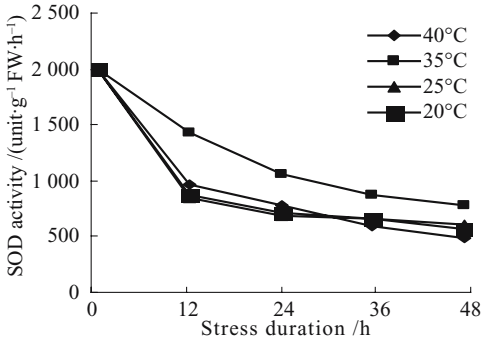

Fig. 6 SOD activity of cotton leaves during stress at the peak boll-setting period

Contrary to the dynamics of SOD activity, the MDA content in cotton leaves showed significant hikes during the first 12 h (Figs. 7 and 8). Compared with the effects produced by 40°C and 20°C stress, the effects produced by 35°C and 25°C were more insignificant. As also can be seen in Fig. 8, SOD activity on August 5 was more susceptible to heat and stress.

Both SOD activity and MDA content are important indices for the physiological activities of cotton leaves. The declined activity and the enhanced MDA content at the same time indicated that the physiological activities of cotton leaves had

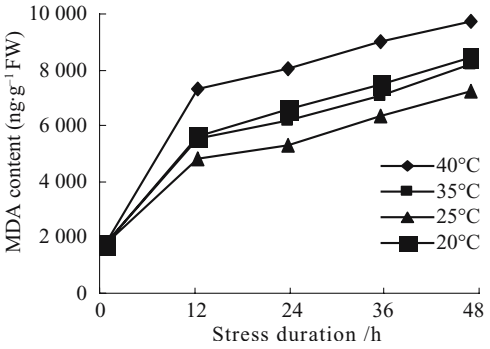

Fig. 7 MDA content in cotton leaves during stress at the peak flowering period

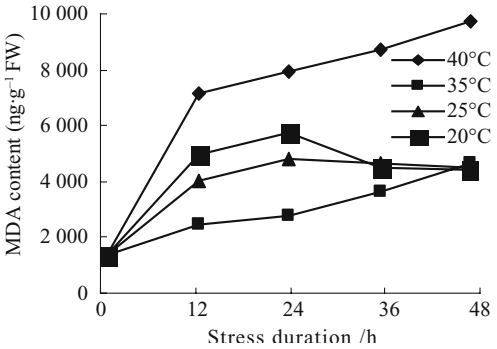

Fig. 8 MDA content in cotton leaves during stress at the peak boll-setting period

been seriously damaged under both the heat and cold stress conditions, especially during the first 12 h.

3.1.3 Leaf NR activity and GPT activity

Both NR and GPT are critical enzymes in the plant nitrogen assimilation and metabolism. Thus, both are closely related to protein synthesis including the synthesis of insecticidal protein. As can be seen in Figs. 9 and 10, NR activities in cotton leaves declined during the first 24 h, and then went up during the remaining 24 h. In contrast to August 5, NR activities on July 20 prior to stress were higher.

The GPT activities in cotton leaves were also susceptible to both heat and cold stress conditions (Figs. 11 and 12). At the stage of peak flowering, the GPT activity under 40°C decreased drastically, while other treatments showed milder declines, suggesting 40°C produced more serious effects on the nitrogen assimilation and metabolism. At the peak boll-setting period GPT activity declined drastically during the first 24 h, and then the decline became rather mild.

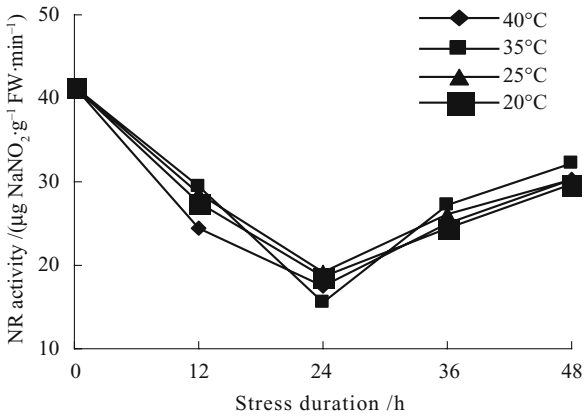

Fig. 9 NR activity in cotton leaves during stress at the peak flowering period

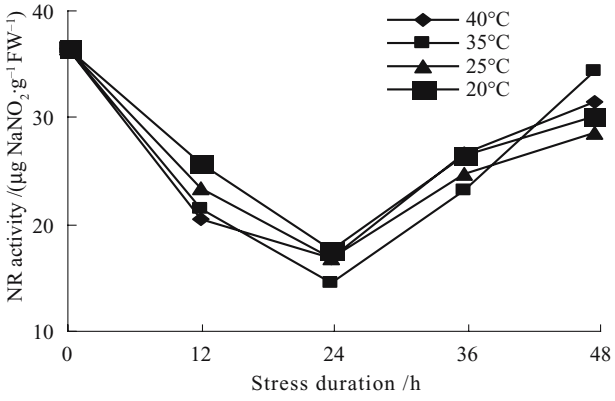

Fig. 10 NR activity in cotton leaves during stress at the peak boll-setting period

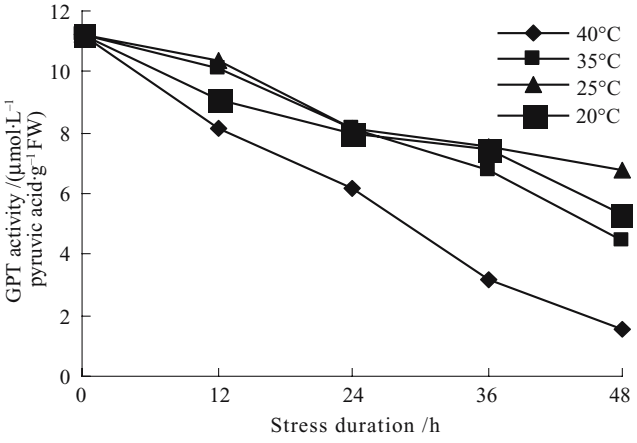

Fig. 11 GPT activity in cotton leaves during stress at the peak flowering period

3.2 Expression of insecticidal protein content

As can be seen in Figs. 13 and14, both heat and cold stresses produced an immediate inhibiting effect on the expression of insecticidal protein, especially during the first 12 h; and during the remaining 36 h the effect became much milder, suggesting that in order to maintain the stability of expression of insecticidal protein cultivation measures must be conducted as early as possible. Compared with other stress temperatures, the extreme high and low temperatures (40°C and 25°C) brought about the most negative effects on the insecticidal protein content. Compared with that at the peak

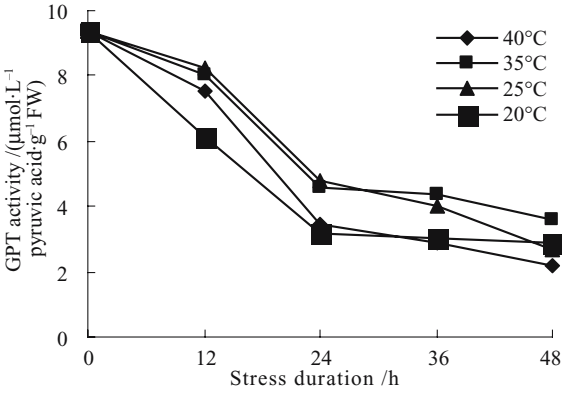

Fig. 12 GPT activity in cotton leaves during stress at peak boll-setting period

flowering period, the insecticidal protein content on August 5 was much lower.

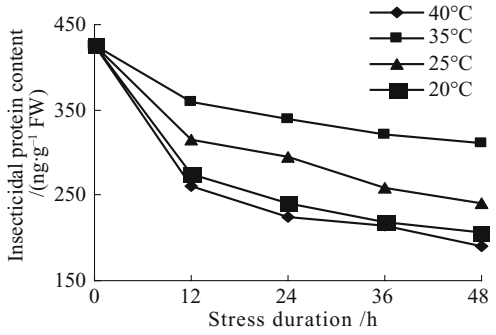

Fig. 13 Content of insecticidal protein in cotton leaves during stress at the peak flower period

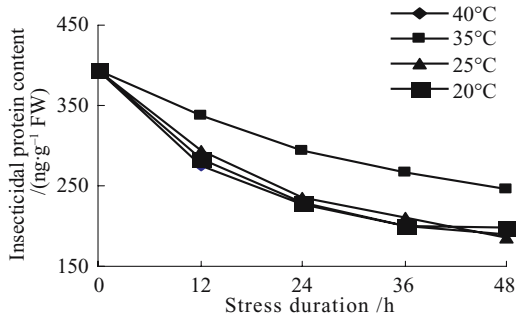

Fig. 14 Content of insecticidal protein in cotton leaves during stress at the peak boll-setting period

4 Discussion

4.1 Relationship between physiological activities and insecticidal protein expression

The contents of free amino acid (FAA), soluble protein (SP) and MDA, and the activities of SOD, NR and GPT are all the important indices used in determining physiological activities in plant leaves, especially under stress conditions. In this research both heat and cold stresses had significant effects on these physiological parameters.

Although it was very difficult to make a clear physiological explanation to the relationship between the above-mentioned physiological indices and the expression of insecticidal protein, related coefficients were calculated in the present paper (Table 1). The FAA content was negatively correlated to insecticidal protein content (significant at the 0.05 or 0.01 levels). So was the MDA content. The SP was positively correlated to insecticidal protein in content (significant at the 0.05 or 0.01 level). The SOD and GPT activities were also positively correlated with the insecticidal protein (significant at the 0.05 or 0.01 level). NR activity was also significantly correlated to the insecticidal protein content, but the correlation was not significant at both 0.01 and 0.05 levels. These statistical analyses indicated that the declined physiological activities in cotton leaves subject to the heat and cold stresses had produced serious negative effects on the expression of insecticidal protein.

4.2 Stress effects of different temperatures on physiological activities and insecticidal protein content

As can be seen from the above analyses, both heat and cold stresses could bring about negative effects on the physiological activities and the insecticidal protein content in cotton leaves. But the stress effects under different temperatures were different. As for heat stress, the higher the stress temperature was, the more seriously negative effects it produced. And as far as the cold stress was concerned, the lower the stress temperature was, the more seriously negative effects it produced.

4.3 Different effects of heat and cold stresses on the insecticidal protein content at the peak flowering period and the peak boll-setting period

The above-mentioned analyses on physiological activities in cotton leaves subject to the heat and cold stresses indicated that the physiological activities and the insecticidal protein expression at the boll-setting period were more susceptible to heat and cold stresses. These were in accordance with the cotton growing conditions at these two periods. At the peak flowering period, more nutrition was still absorbed by vegetative organs including cotton leaves, which made them more tolerant to the stress; while at the peak boll-setting period, the reproductive organs were more capable of taking in nutritional materials. Less strong physiological activities in cotton leaves at the peak boll-setting period prior to and during the stress were the factors inducing the declined insecticidal protein content.

4.4 Cultural practices for improving physiological activities and insecticidal protein expression

In Jiangsu Province, the weather in July and August is unstable because frequent typhoons lead to a sudden decrease of

Table 1 Coefficients between physiological activities and insecticidal protein content in cotton leaves under heat and cold stresses

Date	Insecticidal protein content							
	7/20				8/5			
	40°C	35°C	25°C	20°C	40°C	35°C	25°C	20°C
FAA	–0.995**	–0.967**	–0.933*	–0.929*	–0.995**	–0.989**	–0.987**	–0.983*
SP	0.946*	0.984**	0.982**	0.995**	0.971**	0.980**	0.994**	0.995**
SOD	0.997**	0.982**	0.976**	0.991**	0.993**	0.993**	0.939*	0.966**
MDA	–0.997**	–0.996**	–0.997**	–0.990**	–0.988**	–0.964**	–0.926*	–0.806
NR	0.771	0.586	0.704	0.805	0.454	0.237	0.584	0.603
GPT	0.888*	0.887*	0.912*	0.907*	0.943*	0.970*	0.954*	0.989*

Note: ** and * stand for the significant correlation at the 0.01 level and at the 0.05 level, respectively.

temperature by more than 10°C, and frequent droughts bring about continuous hot weather that usually lasts for over a week. Therefore, the time from July 15 to 25 as well as the first ten days in August are not only the time in a year in Jiangsu Province that bollworms cause the greatest damage but also the time that cotton plants experience the most critical growth periods of peak flowering and peak boll-setting. A sudden up-and-down temperature change causing a great disorder of physiological activities of cotton leaves and lower expression of insecticidal protein, has become one of the factors inducing bollworms outbreaks in the province. So it is pretty necessary to establish preventing cultivation practices to stabilize the physiological activities and the expression of insecticidal protein in cotton leaves under the heat and cold conditions. Because both the heat and cold stress effects were immediate, it is better to take preventing cultivation practices prior to the stress occurrence. Our further researches will be focused on these preventing practices.

Acknowledgements We would like to express thanks to the National Natural Science Foundation of China (Grant No. 30771272), Natural Science Foundation of Jiangsu Educational Department (No. MK0410196), and Natural Science Foundation of Yangzhou University (No. 20063152) for financial support.

References

Beauchamp C, Fridovich J (1971). Superoxide dismutase: Improved assay and an assay applicable to acrylamide gels. Anal Biochem, 44(1): 276–287

Beyer W, Fridovich I (1987). Assaying for superoxide dismutase activity: Some large consequences of minor changes in conditions. Anal Biochem, 161: 559–566

Bradford M M (1976). A rapid method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem, 72: 248–254

Chen S, Wu J Y, Cheng D R, Zhang R X (1999). On the enzyme-linked immunosorbent assay of *Bacillus thuringiensis* insecticidal protein expressed in transgenic cotton. Acta Gossypii Sinica, 11(5): 259–267 (in Chinese)

Cui J J, Xia X Y (1999). Studies on the resistance dynamic of the Bt transgenic cotton on cotton bollworm. Acta Gossypii Sinica, 11(3): 141–146 (in Chinese)

Ernest G (1971). Nitrate reductase assay in intact plant tissues. Biochemical and Biophysical Research Communications, 43(6): 1274–1279

Gasser C S, Fraley R T (1989). Genetically engineering plants for crop improvement. Science, 244: 1,293–1,299

Gould F (1988). Evolutionary biology and genetically engineered crops. Bioscience, 38: 26–33

Guo X M, Liu H T, Zhang Y S, Qiang A D (1999). The technique and achievement of Bt transgenic cotton breeding in China. China Cotton, 26(7): 2–5 (in Chinese)

Li Q S, Wang W X (1999). The preliminary study on the resistance of transgenic cotton to cotton bollworm. Journal of Henan Agricultural University, (2): 17–18 (in Chinese)

Su C E, Sun H W, Bai L X, Huang J Q, Li S (1998). Toxic response of cotton bollworm to various parts of Bt-transgenic cotton in different growing stages. Acta Gossypii Sinica, 10(3): 131–135 (in Chinese)

Tonhazy N E, White N G, Umbriet W W (1950). Colorimetric assay of glutamic-pyruvic transaminase. Arch Biochem Biophys, 28: 36–38

Wang J B, Wang L M, Shen F F, Zhang X K, Yang J, Liu R Z (2000). Effect of environment elements on Bt-protein content in transgenic Bt cotton. Shandong Agricultural Sciences, 6: 4–6 (in Chinese)

Wang L M, Wang J B, Shen F F, Zhang X K, Liu R Z (2001). Influences of waterlogging and drought on different transgenic Bt cotton cultivars. Acta Gossypii Sinica, 13(2): 87–90 (in Chinese)

Wu J Y, He X L, Shu C E, Chen S, Fu C X, Huang J Q (1997). Influence of waterlogging on the bollworm resistance of Bt cotton Jiangsu. J of Agr Sci, 13(4): 231–233 (in Chinese)

Xing C Z, Jing S R, Cui X, Guo L F, Guo L P, Wang H L, Yuan Y L (2001). The spatial temporal distribution of Bt insecticidal protein in Bt transgenic cotton and its effect on Bt transgenic cotton resistance to bollworm. Acta Gossypii Sinica, 13(1): 11–15 (in Chinese)

Yemm E W, Cocking E C (1955). Determination of amino acid with ninhydrin. Analyst, 80: 209–213

Zhang B H, Feng R (1998). Achievements, problems and strategies of transgenic insect resistant cotton. Acta Agronomica Sinica, 24: 248–256 (in Chinese)

Zhang X Z (1991). Research Methods of Crop Physiology. Beijing: China Agricultural Press, 215–216 (in Chinese)

Zhao D L, Oosterhuis D (1997). Physiological response of growth chamber-grown cotton plants to the plant growth regulator PGR-IV under water-deficit stress. Environmental and Experimental Botany, 38(1): 7–14

Zhao K J, Zhao J J, Fan X L, Rui C H, Mei X D (2000). Temporal and spatial dynamics of transgenic Bt cotton toxins to cotton bollworm in North China. Journal of Agricultural Biotechnology, 8(1): 49–52 (in Chinese)

Zhou G S, Feng C N, Zhou Q, Zhang W D, Chen H Q (2003). Effect of heat stress on toxin protein content in Bt transgenic cotton leaves. Journal of Yangzhou University (Agricultural and Life Science Edition), 24(4): 76–77 (in Chinese)