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# Developmental expression and stress induction of glutathione *S*-transferase in the spruce budworm, *Choristoneura fumiferana*

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## Abstract

Developmental and stress-induced expression of *Choristoneura fumiferana* glutathione *S*-transferase (*Cf*GST) mRNA and protein were examined using Northern blots and Western blots. High levels of *Cf*GST mRNA and protein were detected in 1st instar larvae and diapausing 2nd instar larvae. Expression of *Cf*GST gradually decreased during larval development from 3rd to 5th instar, after which the expression increased once again, reaching peak levels in 6th instar larvae. *Cf*GST mRNA and protein were undetectable in the pupal stage. Exposure to low temperature did not induce an increase in *Cf*GST expression. Feeding on balsam fir foliage resulted in an increase in the expression of *Cf*GST as compared to larvae that fed on artificial diet. The bacterial insecticide, *Bacillus thuringiensis* delta-endotoxin (Bt), the non-steroidal ecdysone analog, tebufenozide, and the synthetic pyrethroid, permethrin, induced the expression of *Cf*GST mRNA in 5th instar larvae, whereas the chitin synthesis inhibitor, diflubenzuron, did not have any such effect. These results suggest that *Cf*GST plays an important role in detoxifying various allelochemicals and insecticides in the spruce budworm. The developmental expression pattern strongly suggests that in addition to detoxification, *Cf*GST might be involved in other functions. Crown Copyright © 2000 Published by Elsevier Science Ltd. All rights reserved.

**Keywords:** Detoxification; Insecticide; Allelochemical; *Bacillus thuringiensis*; Tebufenozide; Permethrin; Diflubenzuron

## 1. Introduction

Glutathione *S*-transferases (GSTs) are a group of enzymes that catalyze the conjugation of compounds that have an electrophilic reactive center with the nucleophilic thiol group of the tripeptide, glutathione. They are ubiquitous in nature and have been demonstrated to be involved in detoxification of xenobiotic compounds in vertebrates and invertebrates (for reviews see Clark, 1989; Pickett and Lu, 1989; Armstrong, 1991; Tsuchida and Sato, 1992; Rushmore and Pickett, 1993).

In insects, GSTs play an important role in detoxification of insecticides (Motoyama and Dauterman, 1980; Clark, 1989). It has also been shown that certain insecticides induce GST activity (Clark, 1989). Insecticide-resistant strains of some insect species contain

higher levels of GST activity than susceptible strains (Wang et al., 1991). Elevated levels of GSTs are known to increase an insect's tolerance to insecticides (Wadleigh and Yu, 1988). Insecticidal compounds that are characteristically metabolized by GSTs include several chemical groups, such as organophosphates, organothiocyanates and chlorinated hydrocarbons (Motoyama and Dauterman, 1980). GSTs are also involved in resistance to allelochemicals in phytophagous insects, such as the Southern armyworm (*Spodoptera eridania*) (Abdel-Aal and Roe, 1990), fall armyworm (*Spodoptera frugiperda*) (Yu 1982, 1989; Wadleigh and Yu, 1988), corn earworm (*Heliothis zea*) (Yu, 1989), tobacco budworm (*Heliothis virescens*) (Yu, 1989), cabbage looper (*Trichoplusia ni*) (Wadleigh and Yu, 1988, Yu, 1989), and the velvetbean caterpillar (*Anticarsia gemmatilis*) (Wadleigh and Yu, 1988; Yu, 1989). Besides its detoxification function in insects, no other physiological function has been shown, whereas in mammals GSTs have been demonstrated to be involved in the transport of a

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variety of hormones and endogenous metabolites (Listowsky et al., 1988).

We have cloned a cDNA encoding a GST protein from the spruce budworm, *Choristoneura fumiferana* (CfGST, Feng et al., 1999). In that study, we found that the non-feeding 2nd instar larvae in diapause contained much higher concentrations of GST than those that were feeding. These results suggested that CfGST might have other physiological roles besides detoxification of xenobiotics. To further elucidate the physiological roles of CfGST in the spruce budworm, we studied the developmental expression and induction of CfGST by various types of physical and biochemical stresses, including exposure to low temperatures, feeding on the host plant needles, and ingestion of insecticides. The results of our study reported here indicate that while CfGST is inducible by various stresses and may be involved in detoxification, it is also developmentally regulated and may have other physiological roles besides detoxification.

## 2. Materials and methods

### 2.1. Experimental insects

Spruce budworm (*Choristoneura fumiferana* Clem., Lepidoptera: Tortricidae) were reared on artificial diet (McMorran, 1965). Moths were allowed to lay eggs on balsam fir needles. The eggs were incubated at 22°C and 70% relative humidity and allowed to hatch into 1st instar larvae, which were maintained in balsam fir needles. Five days after emergence, the first instar larvae molted into 2nd instars and spun hibernacula in the mesh of a cheese cloth. The 2nd instar larvae were maintained at 16°C for 2 weeks (pre-diapause) and then moved to 4°C for 27 weeks to satisfy the diapause requirement (diapause) (Grisdale, 1970). During diapause larvae did not feed. At the end of diapause, the larvae were moved to 16°C for 1 week (post-diapause). The larvae were then reared on an artificial diet at 22°C, 70% relative humidity and a photoperiod of 12-h light and 12-h darkness, until reaching the pupal stage.

### 2.2. Cold treatment

Larvae were reared on artificial diet at 22°C until 1 day after ecdysis into the 5th instar. The larvae were then reared at 4°C for various periods. The larvae maintained at 4°C were periodically collected after 0, 1, 2, 4, 6 and 8 weeks in the cold condition. The samples were stored at –70°C until they were extracted for RNA and protein.

### 2.3. Foliage treatment

Larvae were reared on the artificial diet at 22°C until they molted into the 5th instar. They were then divided into two groups. One group was allowed to feed on fresh balsam fir foliage for 4 days, while the other group was allowed to feed on artificial diet free from any antibiotics. Larvae were collected from these two groups at days 1, 2, 3 and 4 after their feeding regimen. The samples were stored at –70°C until they were extracted for RNA and protein.

### 2.4. Insecticide treatment

On day 1 5th instar larvae were individually force-fed with sublethal doses of tebufenozide (Mimic®240LV) at the rate of 70 ng/larva (active ingredient, AI), diflubenzuron (Dimilin®4L) at 70 ng/larva (AI), permethrin (Ambush®500EC) at 7 ng/larva (AI), and *Bacillus thuringiensis* (Foray®48B) at  $2.55 \times 10^{-7}$  BIU/larva (AI), respectively. All solutions were offered to the larvae as a 0.2 µl droplet in an aqueous solution containing 1% sucrose, 0.01% Triton-X-100, and 0.01% Red#28 as a marker dye. The control larvae were also individually force-fed with this solution without the insecticides. The larvae were then reared on artificial diet that did not contain any antibiotics. Six larvae were collected for each treatment at days 1, 2 and 3 post treatments, respectively. The samples were stored at –70°C before being used for RNA and protein extraction.

### 2.5. Northern blot analysis

Total RNA was isolated from whole larvae using the guanidinium isothiocyanate–phenol–chloroform extraction method (Chomczynski and Sacchi, 1987). Ten micrograms of total RNA were separated on formaldehyde–agarose (1%) gels and transferred to Hybond N nylon membranes. The Northern blots were hybridized with a CfGST cDNA probe labeled with  $\alpha$ -[<sup>32</sup>P]dATP. Hybridization and washes were conducted as described by Palli et al. (1998). Northern blots were exposed to X-ray film. Quantitative analysis was conducted using Electrophoresis Gel Imaging and Quantitation System (QuantiGel Corp., Madison, WI, USA).

### 2.6. Protein isolation and purification

Larvae were homogenized in homogenization buffer (50 mM Tris, 10 mM EDTA, 15% glycerol, 0.005% phenylthiourea, pH 7.8) using a motor-driven Teflon pestle in 1.5 ml polypropylene microcentrifuge tubes. The homogenate was centrifuged for 5 min at 12,000 g. The supernatant was collected and used either for protein analysis or for further purification.

CfGST protein from 2nd instar larvae that had been

in diapause for 20 weeks was purified using the GST–glutathione affinity system from Pharmacia P-L Biochemicals Inc. (Piscataway, NJ, USA), using the manufacturer's instructions. To 1 ml of the supernatants containing CfGST, 100 µl of a slurry of Glutathione Sepharose 4B (50%) was added. The mixture was incubated with gentle agitation at room temperature for 30 min. The suspension was then centrifuged at 500 g for 5 min. The supernatant was discarded and the sepharose matrix was washed thrice, each time with 10 bed volumes of phosphate buffered saline. After the final wash, 1 ml of glutathione elution buffer (10 mM reduced glutathione in 50 mM Tris–HCl, pH 8.0) was added to one bed volume of the sedimented matrix and incubated at room temperature for 10 min. The suspension was centrifuged at 500 g for 5 min. The supernatant containing CfGST was collected and used for protein analysis. Protein concentrations were measured using the Bio-Rad reagents and bovine serum albumen as the standard.

### 2.7. SDS–PAGE

Protein samples were denatured at 100°C for 5 min in 0.1 M Tris buffer, pH6.8, containing 2% SDS, 0.5% β-mercaptoethanol, 12% glycerol, and 0.002% bromophenol blue. SDS–PAGE was performed on 15% acrylamide gel in Tris–glycine–SDS buffer (10 mM Tris, 50 mM glycine, 0.1% SDS, pH8.0). The gel was stained with Coomassie Blue R-250.

### 2.8. Antibody production

Polyclonal antibodies were produced as described by Pang (1993). Proteins from 2nd instar diapausing larvae were resolved in an SDS–PAGE gel. After electrophoresis, the CfGST protein was excised from the gel. The protein was then eluted by electrophoresis inside a dialysis tube. The protein was mixed with Freund's adjuvant and then injected into a New Zealand white rabbit. Antiserum was collected after administering two-booster immunizations. Serum from the same rabbit collected prior to immunization was used as the control.

### 2.9. Western blot

After electrophoresis, proteins were transferred from SDS–PAGE gels to Hybond C nylon membranes. Immunodetection was performed using rabbit antibodies raised against the partially purified CfGST (Feng et al., 1999) as primary antibodies at a dilution of 1:2000. Sheep anti-rabbit antibodies conjugated with alkaline phosphatase from Sigma Chemical Co. (St Louis, MO, USA.) were used as the secondary antibody at a dilution of 1:2000. The substrates 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium were used for color development. Quantitative analysis was conducted using Electro-

phoresis Gel Imaging and Quantitation System (QuantiGel Corp., Madison, WI, USA).

### 2.10. Statistics

Experiments on the effect of various agents were repeated three times. The GST bands in both Northern and Western blots were scanned and the data were expressed as mean±SD. Student's *t* test was used to evaluate the significance of difference between control and various treatments.

## 3. Results

### 3.1. Developmental expression of CfGST

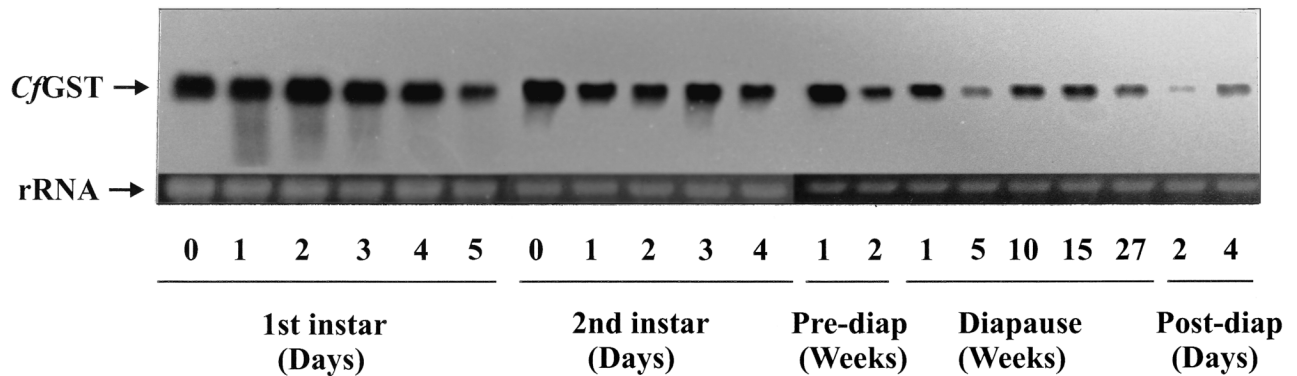
Two methods were used to analyze CfGST expression during larval and pupal development: (1) CfGST mRNA was detected using Northern blots hybridized with a <sup>32</sup>P-labeled CfGST cDNA probe and (2) CfGST protein was detected using SDS–PAGE followed by Western blotting. Northern blots detected a single band of about 700 nucleotides, which was consistent with the cloned 665-nucleotide CfGST cDNA (Feng et al., 1999). The fact that there was only a single mRNA band indicated that there was no polydispersity in CfGST transcription. SDS–PAGE gels and Western blots exhibited a protein band with a molecular mass of 23 kDa. Affinity-purified CfGST from 2nd instar diapausing larvae appeared as a single protein band and was used as a positive control in all gels.

*C. fumiferana* eggs laid on balsam fir needles and maintained at 22°C hatched into 1st instar larvae that lasted for 5 days, during which time both CfGST mRNA [Fig. 1(a)] and protein [Fig. 1(b)] were present at high levels. The mRNA levels appeared to gradually decrease, whereas the protein levels progressively increased.

After spending 5 days as 1st instars, the larvae molted into the 2nd instars at which time they were moved from 22°C to 16°C. Two weeks later, they were moved to 4°C and kept at this temperature for 27 weeks to satisfy diapause requirements. They were then moved from 4°C to 16°C for 1 week for post-diapause adjustment. During these long periods of pre-diapause (22°C and 16°C), diapause (4°C) and post-diapause adjustment (16°C) stages, the larvae did not feed. At the beginning of diapause, the larvae contained CfGST mRNA as high as that in the 1st instar larvae [Fig. 1(a)]. But the levels of CfGST mRNA gradually decreased during diapause and post-diapause periods. However, CfGST protein levels remained high during diapause [Fig. 1(b)].

The levels of CfGST mRNA and protein in 3rd and 4th instar larvae gradually decreased [Fig. 2(a)]. The expression of CfGST mRNA and protein continued to

(a)



(b)

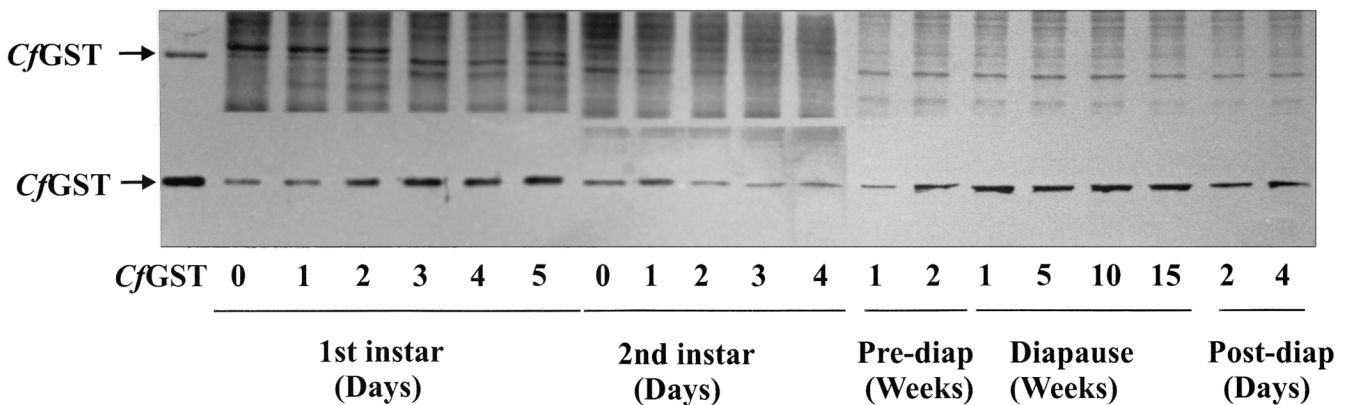


Fig. 1. Developmental expression of *CfGST* mRNA and protein in 1st instar and 2nd instar larvae of *C. fumiferana*. (a) The top panel shows the Northern blot containing 10  $\mu$ g of total RNA hybridized with *CfGST* cDNA probe. The bottom panel shows rRNA stained with ethidium bromide, indicating equal loading of total RNA. (b) The top panel shows the 15% SDS-PAGE gel stained with Coomassie Blue R-250. The bottom panel shows the Western blot stained with the antibodies produced against *CfGST*. Three micrograms of protein were loaded into each lane. The purified *CfGST* was from 2nd instar larvae in diapause for 20 weeks. Pre-diap, pre-diapause; Post-diap, post-diapause.

decrease in 5th instar larvae and only trace levels of *CfGST* mRNA and protein were detected [Fig. 2(b)].

In the 6th larval instar, *CfGST* mRNA levels increased again, beginning on day 1 in the 6th instar, and these higher levels of *CfGST* mRNA were maintained until head capsule slippage [Fig. 3(a)]. The *CfGST* mRNA level decreased again and became undetectable at the prepupal stage. *CfGST* protein was also detected during the 6th instar at higher levels than in 3rd, 4th and 5th instar larvae, but lower than that in the first and diapausing 2nd instar larvae [Fig. 3(b)]. As larvae molted into the pupal stage, *CfGST* mRNA and protein were undetectable once again (Fig. 3).

In summary, 1st instar larvae had the highest levels of *CfGST* mRNA whereas 2nd instar diapausing larvae had the highest levels of *CfGST* protein. After diapause, both *CfGST* mRNA and protein levels continued to decrease until the 6th instar, during which expression of *CfGST* mRNA and protein increased again.

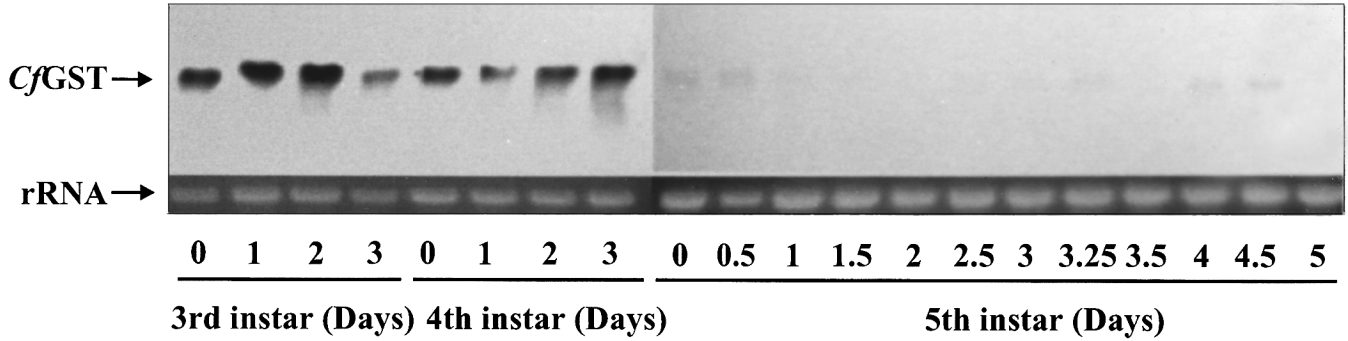
### 3.2. Effect of low temperature on expression of *CfGST*

Because 2nd instar diapausing larvae at low temperature contained significantly high levels of *CfGST* protein and mRNA (Fig. 1), we studied the effect of low temperature on the expression of *CfGST*. As 5th instar larvae possess the lowest levels of *CfGST*, they were used in the study of low temperature effect. Molted 5th instar larvae were shifted from 22°C to 4°C and assayed for *CfGST* mRNA and protein. As shown in Fig. 4, there was no increase in *CfGST* mRNA and protein levels during 8 weeks exposure to cold [Fig. 4(a) and (b)].

### 3.3. Induction of *CfGST* by balsam fir foliage

Spruce budworm eggs are normally laid on balsam fir foliage and are allowed to hatch into 1st instar larvae on the needles, which serves as the natural food for the insect. Normally, the 1st instar larvae are maintained on

(a)



(b)

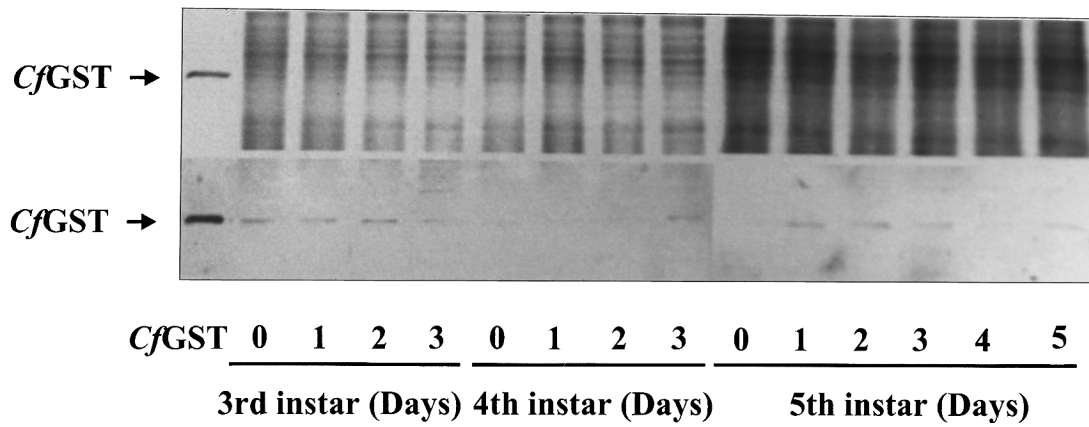
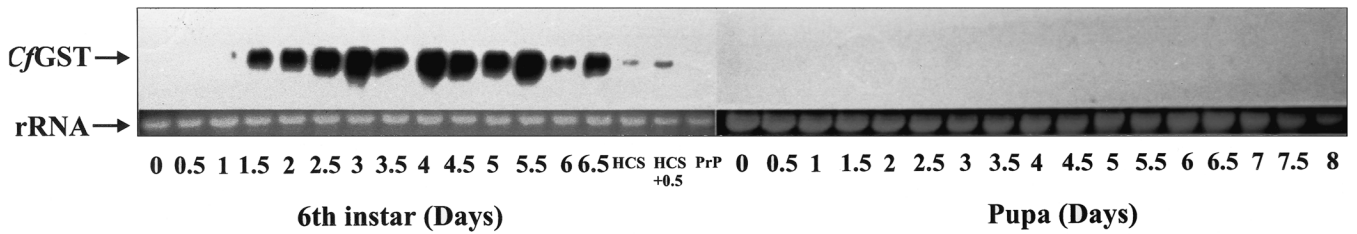


Fig. 2. Developmental expression of *CfGST* mRNA and protein in 3rd, 4th and 5th instar larvae of *C. fumiferana*. Refer to Fig. 1 for further explanation.

(a)



(b)

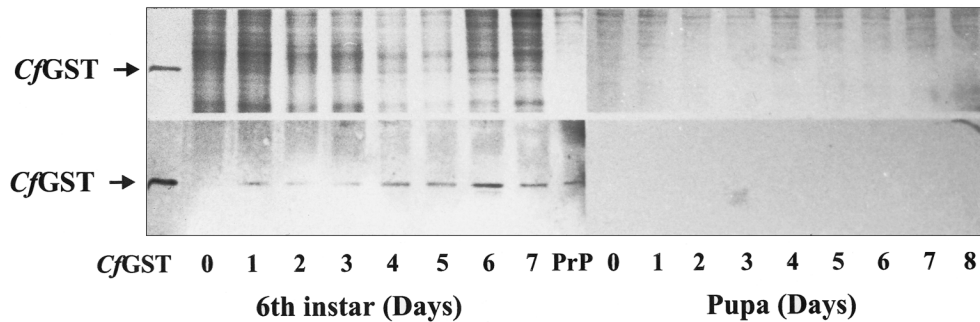


Fig. 3. Developmental expression of *CfGST* mRNA and protein in 6th instar larvae and pupae of *C. fumiferana*. Refer to Fig. 1 for further explanation. HCS, head capsule slippage; PrP, pre-pupa.

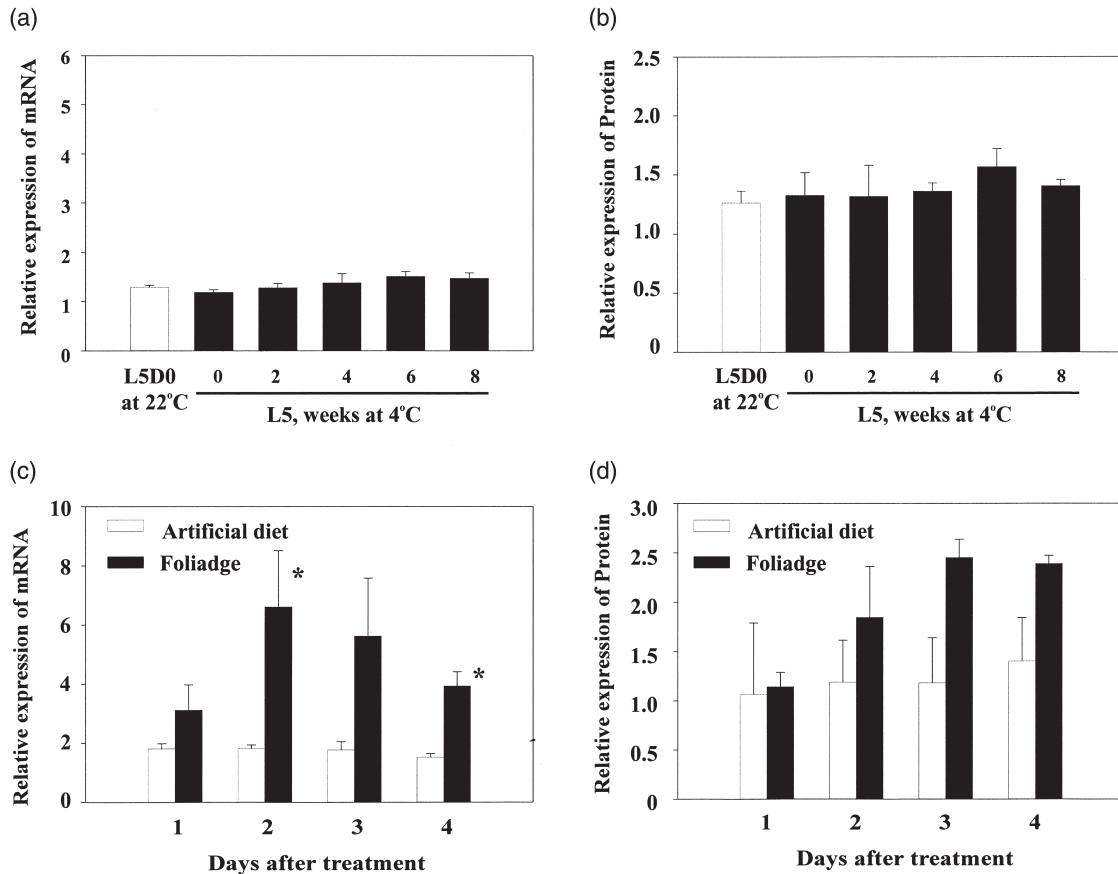


Fig. 4. Expression of *CjGST* mRNA (a) and protein (b) in 5th instar larvae reared at 4°C. Expression of *CjGST* mRNA (c) and protein (d) in 5th instar larvae days after feeding balsam foliage or the artificial diet. The signals from *CjGST* mRNA bands on three Northern blots and the signals from protein band recognized by *CjGST* antibodies in the Western blots were quantified as described in Section 2. Mean $\pm$ SD for three independent experiments are presented. Asterisks indicate the significant difference between treatment and control,  $P < 0.05$ .

balsam fir needles until the larvae enter diapause, after which the larvae are reared on artificial diet. Because 1st and diapausing 2nd instar larvae contained higher levels of *CjGST* than the larvae in later stages, we hypothesized that the difference in *CjGST* mRNA and protein levels may be due to feeding on different diets. To verify this hypothesis, we fed fresh balsam fir foliage to the 5th instar larvae, which normally contain the lowest levels of *CjGST*. The results showed that balsam fir needles induced the expression of *CjGST* mRNA within 2 days [Fig. 4(c)]. Levels of *CjGST* protein also increased at days 3 and 4 after feeding on balsam fir needles [Fig. 4(d)].

### 3.4. Induction of *CjGST* by insecticides

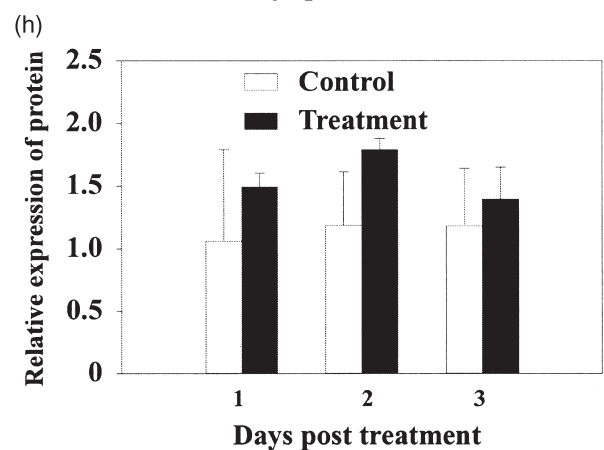
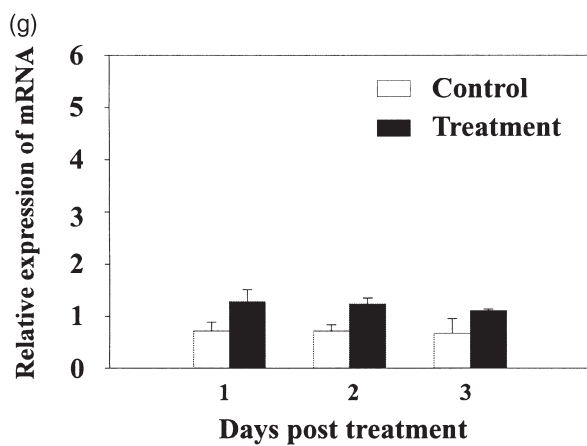
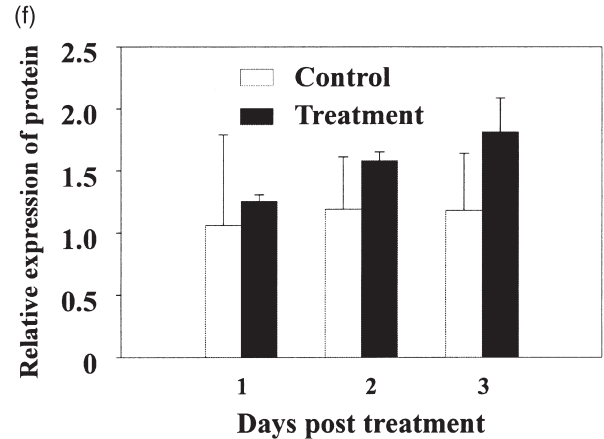
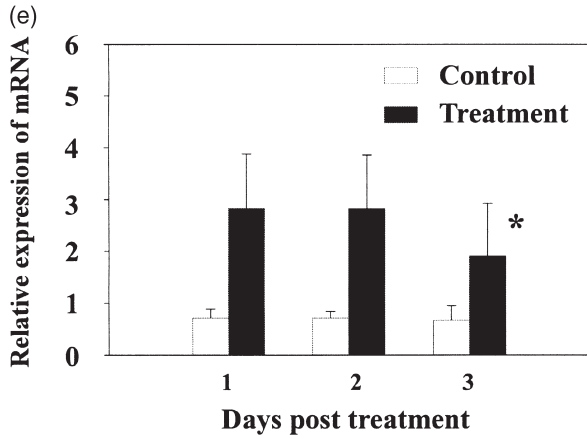
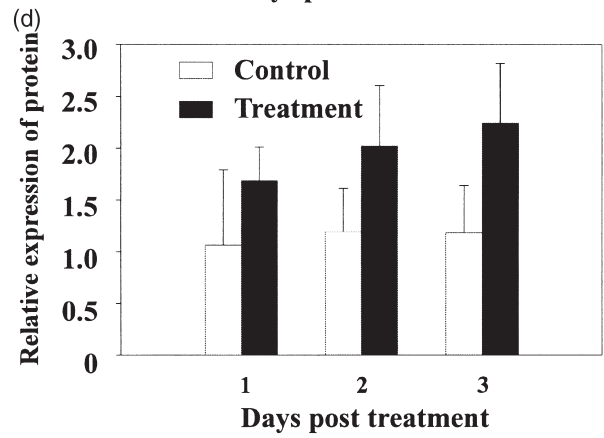
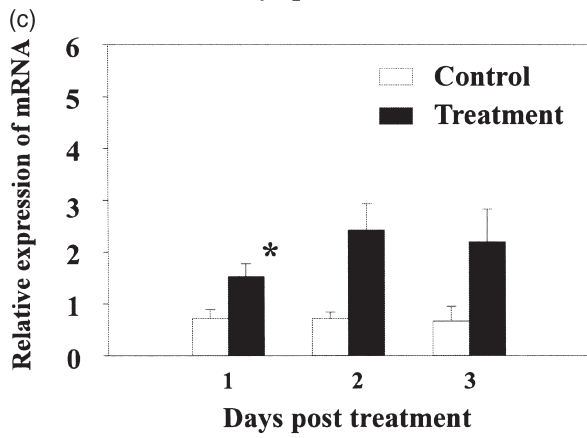
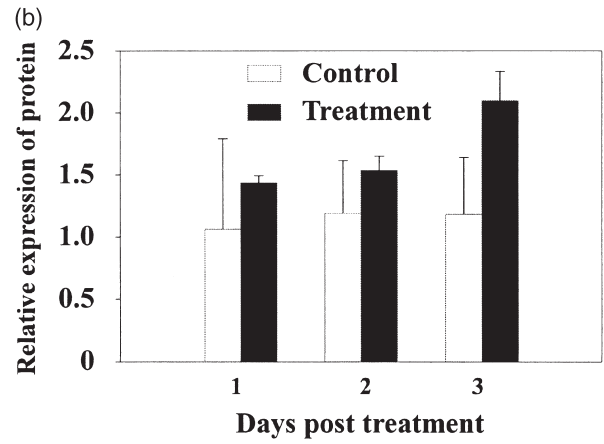
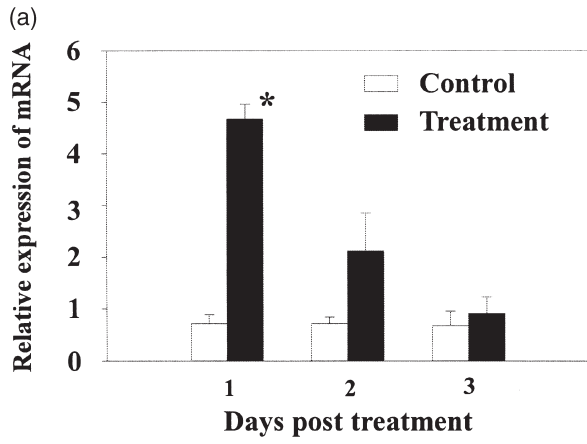
A bioinsecticide, *B. thuringiensis* delta-endotoxin (Bt) and three synthetic chemical insecticides, permethrin, tebufenozide and diflubenzuron, were tested for their effects on *CjGST* expression in 5th instar larvae. After the larvae were force-fed with Bt, expression of *CjGST* mRNA increased within the first day post treatment (p.t.) followed by gradual decrease at days 2 and 3. However,

no significant change in protein levels was detected [Fig. 5(b)].

Expression of *CjGST* mRNA was also increased by tebufenozide, starting on day 1 p.t. [Fig. 5(c)], while protein levels were not significantly changed [Fig. 5(d)]. Permethrin strongly induced expression of *CjGST* mRNA, also starting on day 1 p.t. [Fig. 5(e)]. The mRNA levels then gradually decreased. No significant changes in protein levels were detected [Fig. 5(f)]. Unlike permethrin and tebufenozide, diflubenzuron did not significantly induce expression of *CjGST* mRNA and protein [Fig. 5(g) and (h)]. No significantly increased levels of *CjGST* mRNA and protein were detected in diflubenzuron-fed larvae during 3 days p.t.

## 4. Discussion

We recently cloned a *CjGST* cDNA from the spruce budworm and expressed it in a baculovirus expression system to produce an active enzyme (Feng et al., 1999). In this study, we attempted to understand how *CjGST* levels change during larval development, the factors that



regulate *Cf*GST expression and what other possible roles the *Cf*GST may play in the spruce budworm.

We first studied the developmental expression of *Cf*GST. The results indicated that there were two peaks of *Cf*GST expression during the larval development: the first peak occurred during the 1st instar and diapause, and the second peak occurred during the 6th instar. The expression profile of *Cf*GSTs levels is similar to that of the diapause associated proteins, *Cf*DAP1 and *Cf*DAP2 (Palli et al., 1998). The mRNA expression of *Cf*DAP1 and *Cf*DAP2 peaks prior to diapause and during the 6th instar stadium prior to pupation, and the corresponding proteins are present in high concentrations throughout diapause and the last larval stage. These proteins have been implicated in storage function. Unlike these diapause associated proteins, GST is apparently not a storage protein and its main function has been demonstrated to be detoxification of xenobiotics. The question is why the diapausing larvae store such high concentrations of a detoxifying enzyme when they do not feed. There are at least two possible explanations. First, the natural diet, balsam fir foliage, may contribute to the first peak. First instar larvae were maintained on balsam fir needles on which they have been shown to feed (Retnakaran et al., 1999). The larvae then entered diapause at a lower temperature. When 5th instar larvae were reared on artificial diet, they normally contained low levels of *Cf*GST mRNA and protein; they showed an increase in *Cf*GST expression when they were allowed to feed on balsam fir foliage. This indicates that the foliage might be reason for the observed induction and may explain in part the high levels of *Cf*GST mRNA and protein present in 1st instar and 2nd instar diapausing larvae. Second, in addition to the detoxification function, *Cf*GST may play other unknown roles during diapause. In fact, GSTs have been found to protect cells from oxidative stress (Daniel, 1993) and engage in intracellular transport of a variety of hormones and endogenous metabolites (Listowsky et al., 1988). Before the larvae become inactive there is usually a build up of reserve food substances, particularly in the fat body, where GST is mainly present (Feng et al., 1999), with a consequent reduction in the proportion of water in the body. It is possible that GST may have functions in addition to detoxification during this stage.

This hypothesis is supported by the fact that the

*Cf*GST expression peak in the 6th larval instar prior to pupation appears not to be related to toxic compounds from balsam fir needles. Profound metamorphic changes take place at this crucial larval stage when insects change from the larval form to the pupal form. Our results on developmental expression of *Cf*GST indicate that the levels of *Cf*GST gradually decreased from the 3rd to the 5th instar, but increased again in the 6th instar, suggesting that in addition to detoxification, *Cf*GST may play other key functions which are not clear at present. Yeast strains deleted for GST exhibit increased sensitivity to heat shock (Choi et al., 1998). In the rat ovary GST is involved in intracellular transport of steroid hormones (Singh and Pandey, 1996). It has been shown to bind thyroid hormone (Ishigaki et al., 1989). The GST in rat brain is involved in hydrocortisone transport (Tansey and Cammer, 1991). It is conceivable that *Cf*GST might have some such non-detoxifying role as an additional function in the spruce budworm.

We also studied the effect of various stresses on the expression of *Cf*GST. The 5th instar larvae exposed to low temperatures for up to 8 weeks did not show any increased expression of *Cf*GST mRNA and protein. Thus, low temperature treatment did not appear to induce *Cf*GST either at the transcription level or at the translation level.

Feeding balsam fir foliage increased the expression of *Cf*GST in 5th instar larvae. This result is consistent with the observations that plant allelochemicals can induce GST activity after the insects feed on host plants (Yu 1982, 1984; Wadleigh and Yu, 1988). When the fall armyworm was allowed to feed on parsnip, GST activity was increased 39 fold over larvae fed on artificial diet. The inducer of GST activity in this instance was identified as xanthotoxin (Yu, 1984). Activity of GSTs can be induced by the compounds such as coumarins, indoles, flavones, xanthotoxins and monoterpenes. We have not identified the chemicals in the balsam fir needles that contribute to the increase of *Cf*GST expression in the spruce budworm. High levels of expression of *Cf*GST induced by feeding balsam fir foliage may be beneficial to insects for detoxifying the toxic compounds in the leaves. Increases in GST activity induced by plant allelochemicals may also result in increased tolerance to insecticides (Yu, 1982; Wadleigh and Yu, 1988). This may explain in part why some insecticides, e.g. Bt, usually have better insecticidal effect in laboratory conditions than during field application.

We tested four insecticides for their effects on *Cf*GST expression and found that three of them can increase *Cf*GST mRNA expression. These insecticides are quite different in structure, chemical properties, toxicity and mode of action. Bt is now the most widely used bioinsecticide in forestry. Its insecticidal activity is attributed to the parasporal crystals that consist of protoxins. The mode of action of the toxic proteins involves creating

Fig. 5. Expression of *Cf*GST mRNA (a, c, e, g) and protein (b, d, f, h) in 5th instar larvae days after they were force-fed with *B. thuringiensis* at  $2.55 \times 10^{-7}$  BIU/larva (AI) (a and b), tebufenozide at 70 ng/larva (AI) (c and d), permethrin at 7 ng/larva (AI) (e and f) and diflubenzuron at 70 ng/larva (AI) (g and h), respectively. The signals from *Cf*GST mRNA bands on three Northern blots and the signals from protein band recognized by *Cf*GST antibodies in the Western blots were quantified as described in Section 2. Mean  $\pm$  SD for three independent experiments are presented. Asterisks indicate the significant difference between treatment and control,  $P < 0.05$ .



ion channels or pores on membranes of midgut cells (Schnepf et al., 1998). The present study shows for the first time that Bt can induce expression of *CfGST*. We had earlier stated that *CfGST* is synthesized in the fat body (Feng et al., 1999). It would be interesting to know how Bt acting on midgut cells (Schnepf et al., 1998) causes increase in GST expression in the fat body.

Tebufenozide is a non-steroidal ecdysteroid agonist that mimics 20-hydroxyecdysone (20E) in a variety of insects, especially the insects belonging to order Lepidoptera (Retnakaran et al. 1995, 1997; Dhadialla et al., 1997). Its insecticidal activity involves disturbing the insect molting process. This is the first report to show that this compound can induce GST expression.

Permethrin is a synthetic pyrethroid that interferes with the function of the nervous system (Ruigt, 1985). Permethrin-resistant *Aedes aegypti* express more GST activity throughout their larval and pupal development (Grant and Matsumura, 1989). Permethrin was found to significantly induce GST activity in honey bees (Yu et al., 1984).

While mRNA levels in larvae fed on these insecticides were significantly higher than those in control larvae, the protein levels are not significantly different in these two groups. This could be due to abundance and stability of GST protein. It might take longer than 4 days to see differences in protein levels.

Diflubenzuron is an inhibitor of chitin synthesis. Its insecticidal action is due to inhibition of chitin synthesis and deposition in the endocuticular region of the integument (Retnakaran and Wright, 1987; Nakagawa et al., 1993; Nakagawa and Matsumura, 1994). Like our present results from the spruce budworm, Yu et al. (1984) also observed that diflubenzuron can not induce GST activity in honey bee. It is difficult to explain why tebufenozide and permethrin can induce GST expression, whereas diflubenzuron can not, on the basis of their chemical structure. The only common feature is that all of them have aromatic rings. Similarly, both the bioinsecticide Bt and the chemical insecticides tebufenozide and permethrin can stimulate expression of GST, even though they are unrelated in their chemical structure. In fact, a large number of insecticides that have different chemical structures have been found to induce GST activity and to be metabolized by GST (Clark, 1989). The hypothesis that reactive oxygen is the transduction signal that mediates activation of *c-fos* and *c-jun* gene expression provides an explanation for the inducible expression of GST genes by a wide variety of structurally unrelated compounds (Wattenberg, 1985; Daniel, 1993). Chemical stress may first cause production of reactive oxygen species, such as superoxide anion, O<sub>2</sub>, H<sub>2</sub>O<sub>2</sub>, hydroxyl radical HO·, organic peroxides, or radicals, resulting in oxidative stress on cells. Active oxygen induces expression of *c-fos* and *c-jun* genes, which form an Activator Protein-1 (AP-1) com-

plex. AP-1 binds to the antioxidant responsive element (ARE) of the GST gene promoter and then activates GST gene expression (Pinkus et al., 1993). Experiments are planned to verify whether this is indeed how insecticides induce expression of *CfGST* mRNA.

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