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Inhibitory effects of plant latex on trehalase activity and trehalase gene expression in the red flour beetle, *Tribolium castaneum* (Coleoptera: Tenebrionidae)

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Abstract. Plant latex contains proteins and other components that defend plants against herbivorous insects. We determined the inhibitory activity of methanolic extracts of latex obtained from three species of plant: mulberry, *Morus alba*; jackfruit, *Artocarpus heterophyllus*; and weeping fig, *Ficus benjamina*, against trehalase in the red flour beetle, *Tribolium castaneum*. We also determined the changes that occurred throughout the life of the insect in the enzymatic activities of soluble and membrane-bound trehalase and the expression profiles of the genes encoding the two types of trehalase. Soluble trehalase activity was higher than membrane-bound trehalase activity in larvae and adults, whereas there was little difference in eggs, prepupae and pupae. The expression of the trehalases, *TcTre-1* and *TcTre-2*, changed during insect development but did not coincide closely with changes in enzymatic activity, indicating that these changes did not necessarily depend on gene expression. All of three plant latices tested inhibited the activities of both the soluble and membrane-bound trehalase. At the gene expression level, these latices reduced the expression of *TcTre-1* but not *TcTre-2*, indicating that the latices contain component(s) that selectively inhibit gene expression or at least differentially inhibit these two trehalase genes. The inhibition of trehalase activity resulted in a 140% increase in the concentration of trehalose in the beetle and a decrease in glucose concentration to 72% of the control. These findings show that the latices tested contain components that inhibit trehalase activity and *TcTre-1* expression and thus, may contribute to the plants' defense against herbivorous insects.

INTRODUCTION

Plant latex is a white sap that is exuded from a damaged part of a plant (leaf, shoot, young branch, fruit or bark). Latex is produced by between 12,000 and 36,000 species of plants. Latex is thought to defend plants against herbivorous insects (Dussourd & Denno, 1991; Konno et al., 2004; Mithofer & Boland, 2012).

Defense chemicals and proteins in plant latex include, for example, cardenolides in that of milkweed, *Asclepias syriaca*; cysteine proteases in that of papaya, *Carica papaya* and fig, *Ficus virgatalatex*; and carbohydrase inhibitors and chitinase-like proteins in that of mulberry, *Morus alba* (Malcolm, 1991; Konno et al., 2004; Hirayama et al., 2007). *M. alba* belongs to the family Moraceae, which contains approximately 1,000 species that mainly occur in tropical and subtropical regions. Mulberry latex contains chitinase-like proteins that play a crucial role in the defense against dipteran larvae, possibly by hydrolyzing chitin (Kitajima et al., 2010), and inhibiting the growth of caterpillars of the cabbage armyworm, *Mamestra brassicae* and the Eri silkworm, *Samia cynthia ricini* (Wasano et al., 2009).

Jackfruit, *Artocarpus heterophyllus* and weeping fig, *Ficus benjamina*, are economically important species belonging to the family Moraceae that produce milky latex, but there are few studies of the defensive components in these plant latices. Proteases have been purified from *A. heterophyllus* latex (Siritapetawee et al., 2012) and that of *F. benjamina* contains a protein similar to the chitinase-like protein in the latex of *M. alba* (Chen et al., 2000).

Trehalase (α-glucoside-1-glucohydrolase, EC 3.2.1.28) is an enzyme that catalyzes the hydrolysis of trehalose to glucose. Trehalose is the main haemolymph sugar in insects, is stored in the larval haemolymph during the feeding period and serves as an energy source for adult differentiation in pupae. Thus, trehalase activity changes during the course of insect growth and development (Terra & Ferreira, 1994; Silva et al., 2004, Tatun et al., 2008).

The inhibition of trehalase activity prevents the hydrolysis of trehalose to glucose and induces abnormal biological events such as hypertrehalosemia, hyperglycemia, loss of the ability to move and fly in the locust *Locusta migratoria* and house fly *Musca domestica* (Kono et al., 1994; Takahashi et al., 1995; Wegener et al., 2003), and death in the migratory locust *L. migratoria*, oriental leaf worm moth *Spodoptera litura* and *M. brassicae* (Asano et al., 1990; Kono et al., 1993; Ando et al., 1995; Wegener et al., 2003). However, despite extensive efforts to develop latex-derived bioinsecticides, there is no information on whether there are trehalase inhibitor(s) in plant latex.

Here, we report the effects of latices of three species of plants belonging to different genera of the family Moraceae: *M. alba*, *A. heterophyllus* and *F. benjamina*,

on trehalase activity in the red flour beetle, *T. castaneum*. As insects contain two types of trehalase, soluble and membrane-bound trehalase (Terra & Ferreira, 1994; Becker et al., 1996; Thompson, 2003; Tatun et al., 2008), we prepared soluble and membrane-bound trehalase samples and separately examined the inhibitory effects on the two types of trehalase and their gene expression. The results indicate that the methanolic extracts of these three plant latices inhibited trehalase activity and gene expression to different degrees, and that the two types of trehalase exhibited different levels of sensitivity to the extracts.

MATERIALS AND METHODS

Insect cultures

The *Tribolium castaneum* (Coleoptera: Tenebrionidae) cultures were reared on a basic diet consisting of wheat flour containing 5% yeast at 30 ± 1°C and 70 ± 5% relative humidity. In the current study, we used 1-week old adults for assaying the inhibition of trehalase activity.

Plant latex preparation

The latices of *M. alba*, *A. heterophyllus* and *F. benjamina* were collected by cutting the petioles of young leaves. Methanol (60 ml) was added to 15 ml of latex, which was then kept at 4°C for 48 h. After centrifugation at 14,000 *g* for 30 min at 4°C, the resulting supernatant was dried in an evaporator at 40°C. The residue was dissolved in 15 ml of distilled water and stored at –20°C.

Treatment of latices

The methanol extracts of latices were subjected to dialysis, heating and trypsin digestion. The methanol extracts were dialyzed using a dialysis tube with a 6–8 kDa cut-off (Spectrum Laboratories, Rancho Dominguez, CA, USA) surrounded by 500 times its volume of distilled water for 24 h at 4°C, and the dialysate was used directly in the trehalase inhibition assay. To determine the thermal stability of the methanol extracts they were heated to 100°C for 5 min, cooled to 4°C for 10 min and centrifuged at 10,000 *g* for 5 min at 4°C. The resulting supernatant was used directly for assaying inhibitory activity. For trypsin digestion, the methanol extracts were added to 10 µl of trypsin (10 mg/ml, BioBasic Inc., Markham, Ontario, Canada). The solution was incubated for 5 h at 37°C. The reaction was halted by adding 10 µl of trypsin inhibitor (10 mg/ml, soybean trypsin inhibitor, BioBasic). Then, the solution was centrifuged at 10,000 *g* at 4°C for 10 min and the supernatant used immediately for assaying trehalase inhibition.

Preparation of trehalase samples

To determine the inhibitory activity of each plant latex we used three different enzyme preparations in the trehalase activity assay. The first preparation was the crude preparation of trehalase. Fifty larvae (20–22 days old) were homogenized in 50 ml of 20 mM sodium phosphate buffer (NaH_2PO_4 / Na_2HPO_4; PB), pH 6.0, filtered through cheesecloth and centrifuged at 10,000 *g* at 4°C for 10 min and the resulting supernatant stored at –20°C. The supernatant contained the crude preparation of trehalase, which consisted of both the soluble and membrane-bound trehalases. To separately prepare the soluble and membrane-bound trehalases according to the procedure described by Hirayama et al. (2007), 5 adults (1 week old) were homogenized in 500 µl of 20 mM PB, pH 6.0, sonicated for 30 seconds and filtered through cheesecloth. The homogenates were centrifuged at 23,000*g* at 4°C for 30 min and the supernatant used as the soluble trehalase fraction. To confirm that the soluble fraction was not contaminated with membrane-bound trehalase, the sample was then centrifuged at 105,000 *g* at 4°C for 30 min. The trehalase activity in the resulting supernatant did not differ from that of the soluble fraction obtained by centrifugation at 23,000 *g* at 4°C for 30 min. The precipitate was suspended in 500 µl of 20 mM PB, homogenized with a plastic homogenizer and then centrifuged at 23,000 *g* for 15 min at 4°C. The supernatant was discarded and the resulting precipitate was suspended in 500 µl of 20 mM PB and used as the membrane-bound trehalase fraction.

Measurement of trehalase activity

Trehalase activity was determined according to Hirayama et al. (2007). Briefly, the reaction mixture consisted of 62.5 µl of 40 mM trehalose (Sigma, St. Louis, MO, USA) in 20 mM PB, 10 µl of crude enzyme solution and 177.5 µl PB to adjust the final volume to 250 µl. When the soluble or membrane-bound trehalase fractions were used instead of the crude enzyme solution, 50 µl of the soluble or membrane-bound trehalase fraction and 137.5 µl of PB were added. The mixture was incubated at 37°C for 60 min and the reaction halted by heating at 100°C for 5 min. The reaction mixture was centrifuged at 12,000 *g* for 10 min at 4°C to remove coagulated proteins and an aliquot of the supernatant was subjected to a glucose assay using the modified form of the hexokinase-glucose-6-phosphate dehydrogenase method cited by Bergmeyer et al. (1974). The reaction was performed in a 1-ml reaction mixture containing 50 units of hexokinase, 100 units of glucose-6-phosphate dehydrogenase, 2 mM NADP and 2.8 mM ATP (Roche Diagnostics GmbH, Mannheim, Germany). Trehalase activity was determined based on a glucose standard curve and the enzymatic activity expressed in nmol of glucose/µg of protein/min. The protein concentration in each sample solution was determined using the protein dye-binding method (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as the standard.

Assessment of inhibitory activity in vitro

The crude trehalase (10 µl) was mixed in separate microcentrifuge tubes with 137.5 µl of the following methanol extracts of plant latices: dialyzed, trypsin-treated and heated extracts. The mixtures were incubated at 37°C for 30 min. The preincubated mixture was added to 62.5 µl of 40 mM trehalose and 40 µl of 20 mM PB and then incubated at 37°C for 60 min. In the control reaction, the mixture consisted of the crude trehalase (10 µl), 62.5 µl of 40 mM trehalose, 40 µl of 20 mM PB and the final volume was adjusted to 250 µl by adding of 137.5 µl of distilled water. The reaction was halted by heating in boiling water for 5 min. The mixture was centrifuged at 12,000 *g* for 10 min at 4°C and an aliquot of the supernatant was used to assess the amount of glucose as described above. The amount of glucose in the methanolic extract of each of the latices was determined in the same manner. The amount of glucose released in each assay was calculated by subtracting the amount of glucose in the methanolic extract from that in the mixture after the reaction.

Assessment of inhibitor activity in vivo

The methanolic extracts of the plant latices (0.5 ml) were mixed with 5 g of the basic diet (the treated diet). A control diet was prepared by only adding 0.5 ml of distilled water to the basic diet. The diet was then dried at 40°C for 48 h. Thirty adults were placed together in plastic containers, 4.5 cm in diameter and 6.5 cm high, containing the control or treated diet. Adults were collected 5 days after the beginning of the treatment and used for determining trehalase activity, the amounts of trehalose and glucose and the expression of the *trehalase* genes.

Quantity of trehalose and glucose in the beetles

Five adults were weighed and homogenized in 400 µl of distilled water, boiled for 10 min, chilled on ice and centrifuged at 15,000 *g* for 10 min. The supernatant was mixed with 4 volumes of cold methanol, incubated at 4°C for 2 h and centrifuged at 15,000 *g* for 10 min to remove precipitates. The supernatant was then transferred to a new tube and the same volume of hexane added. The tube was vigorously mixed and centrifuged briefly to separate the hexane and aqueous methanol layers. The lower aqueous layer was transferred to a new tube, the solvent was evaporated and the residue was dissolved in 20 mM PB. The resulting solution was divided equally into two different tubes to quantify the amounts of trehalose and glucose. First, 50 µl of the trehalase solution was added to the sample solution and incubated at 37°C for 24 h to completely digest the trehalose in the solution to glucose. The amount of glucose was determined using the hexokinase-glucose-6-phosphate dehydrogenase method as described above. Second, the amount of glucose in another tube was determined using the same method as above, and the amount of trehalose in the first tube was calculated by subtracting the amount of glucose in the second tube from that in the first tube. The glucose concentrations were expressed as nmol of glucose/mg wet weight.

Trehalase gene expression

The total RNA was extracted from eggs, larvae, pupae and adults using the EZ-RNA II Total RNA Isolation Kit (Bioind, Kibbutz Beit-Haemek, Israel). RNA samples were treated with DNaseI (Fermentas, Harrington, Ontario, Canada) followed by phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation. Two hundred nanograms of each RNA sample were reverse-transcribed using oligo dT primers and reverse transcriptase (TakaraBio, Otsu, Japan) according to the manufacturer's recommendations. The primers were designed based on *T. castaneum Trehalase* genes (GenBank accession nos. XM_968859 and XM_967517). The thermal cycling conditions were as follows: one cycle for 2 min at 94°C; 35 cycles of 15 s at 94°C, 30 s at 52°C and 30 s at 72°C; and a final extension step of 5 min at 72°C. The PCR products were subcloned, and the sequence data for the *Trehalase-1* (*TcTre-1*, soluble trehalase) and *Trehalase-2* (*TcTre-2*, membrane-bound trehalase) genes of *T. castaneum* were deposited in GenBank (accession nos. JX099777 and JX099778, respectively). The expression levels of *TcTre-1* and *TcTre-2* were determined by semi-quantitative RT-PCR using the cDNA samples (1 µl) prepared from 200 ng of total RNA sample as template and the gene-specific primer sets 5'-GTGTATTGCCAAGGCAACCT-3' and 5'-CATGCCTTTAACCGTCTGGT-3' for the *TcTre-1* gene and 5'-ACTGGAACGCGGTTCTCTTA-3' and 5'CGTCGTACT TTTCAAACA-3' for the *TcTre-2* gene. The *T. castaneum ribosomal protein 49* gene (*TcRp49*) was used as an internal control and was amplified using the primer set 5'-CAGGCACCAGTCTGACCGTTATg-3' and 5'-GCTTCGTTT TGGCATTGGAGC-3' (Minakuchi et al., 2009). PCR was performed using a Hybaid Px2 Thermal Cycler (Thermo Scientific, Waltham, MA, USA) and the following thermal cycling conditions: one cycle for 2 min at 94°C; 28 cycles for *TcTre-1* and *TcTre-2* or 25 cycles for *TcRp49* of 15 s at 94°C, 30 s at 55°C and 30 s at 72°C; and a final extension step of 5 min at 72°C. The PCR products were separated by electrophoresis on a 1.2% agarose gel and visualized under UV light after ethidium bromide staining. The gel images were captured digitally and the densitometric values of the individual bands analyzed using NIH imaging software (Scion Image). The band intensities of *TcTre-1* and *TcTre-2* in a sample were corrected to the intensities of *TcRP49* and there were five replicates of each experiment.

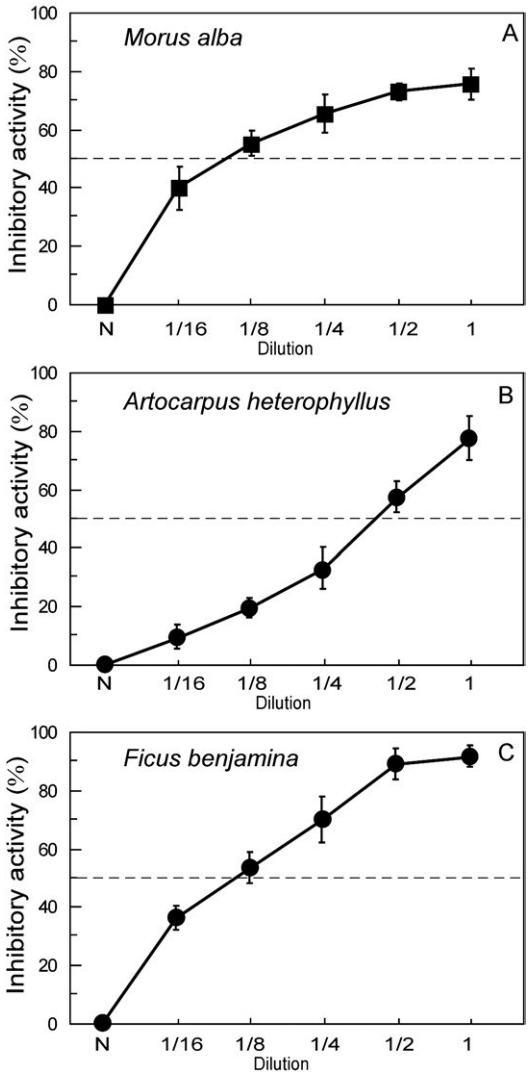

Fig. 1. The inhibition of trehalase from *Tribolium castaneum* by the latex of three species of plants recorded in vitro. A crude preparation of trehalase extracted from larvae was incubated with serially diluted extracts of latex and the activity presented as a percentage, where 100% indicates no glucose generation. N, reaction mixture without the addition of latex, which was designated 0% of inhibitory activity. The error bars indicate the SD (n = 5).

Statistical analysis

A one-way-ANOVA was used in all the statistical analyses.

RESULTS

Trehalase inhibitory activity

The methanolic extracts of the latex produced *M. alba*, *A. heterophyllus* and *F. benjamina* strongly inhibited the trehalase activity of *T. castaneum* in vitro (Fig. 1). Under standard assay conditions, the crude enzyme samples prepared from the 20–22-day-old larvae exhibited an enzymatic activity of 252.1 nmol glucose/µg protein/min (Fig. 2A). Adding the methanol extracts of latex from *M. alba*, *A. heterophyllus* or *F. benjamina* to the reaction mixture

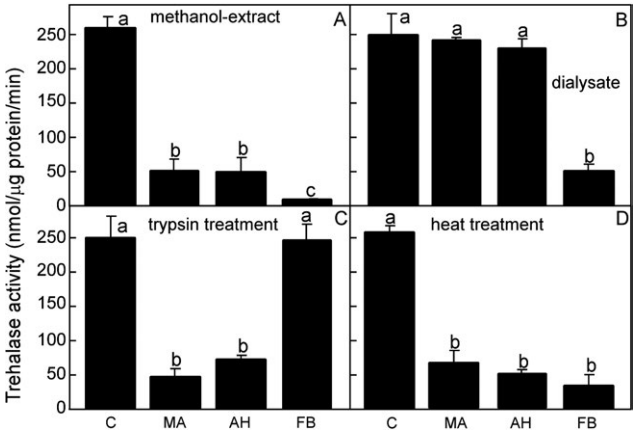

Fig. 2. Changes recorded in the activity of trehalase from *Tribolium castaneum* after incubating it with plant latices. The trehalase activity was determined using crude trehalase extracted from larvae. Trehalase activity was measured after the enzyme was incubated with extracts of the latices. A – methanolic extracts of plant latices; B – dialysates of extracts; C – trypsin treated extracts; D – extracts heated to 100°C for 5 min. MA – latex of *Morus alba*; AH – latex of *Artocarpus heterophyllus*; FB – latex of *Ficus benjamina*. The enzyme activity is expressed as nmol of glucose generated/μg of protein/min. The error bars indicate the SD, and the values labelled with different letters are significantly different ($P < 0.05$) (n = 5).

reduced the enzymatic activity to 57.78, 50.04 and 11.22 nmol/μg protein/min, respectively, which is equivalent to 21.4%, 18.5% and 4.1% of the control activity, respectively. Trehalase activity decreased in a concentration-dependent manner (Fig. 1), with an 11.1-fold dilution required for 50% inhibition (IC_{50}) for the latex of *M. alba*, 2.5-fold for that of *A. heterophyllus* and 10-fold for *F. benjamina*. These results indicate that the latex of each of these three species of plant contain methanol-extractable factors that inhibit trehalase activity.

Characterization of the inhibitors in latex

To broadly characterize the inhibitors in latex, the methanol extracts were subjected to dialysis against distilled water, trypsin treatment and heating. When the latex was dialyzed against distilled water, the inhibitory activity remained in the dialysate of the latex from *F. benjamina* but not in those of the latices from *M. alba* and *A. heterophyllus* (Fig. 2B). Next, we treated the methanol extracts with trypsin (Fig. 2C). This treatment did not reduce the inhibitory activity of the extracts of the latices from *M. alba* and *A. heterophyllus* (50.12 and 81.22 nmol/μg protein/min, respectively), but abolished the inhibitory activity of that from *F. benjamina* (248.1 nmol/μg protein/min). Heating the latex of all three plants at 100°C for 5 min did not reduce its inhibitory activity (Fig. 2D). These findings indicate that the inhibitor in *F. benjamina* latex could be a heat-stable protein, whereas that in the latex from *M. alba* and *A. heterophyllus* might be non-peptidic heat-stable small molecule or trypsin-insensitive peptide.

Changes that occur during development in trehalase activity

The activities of soluble and membrane-bound trehalase were determined for all stages of the *T. castaneum* life cycle (Fig. 3). The activity of soluble trehalase was undetectable in eggs, just detectable in newly emerged, 0-day-old larvae, marked in 2 day old larvae and remained high with appreciable fluctuations until the prepupal stage. Soluble trehalase activity then decreased rapidly to a very low level in the early prepupal stage and remained low throughout the pupal stage. The activity increased after adult eclosion and peaked 5 days after eclosion.

The membrane-bound trehalase activity was detectable but remained very low during the egg stage and increased in larvae 2 days after hatching. The activity remained at levels ranging between 30.7–94.5 nmol/μg protein/min during the larval period, with lower levels in the prepupal and pupal stages. In the adult stage, the activity appeared somewhat higher than that in the pupal stage. The activity of membrane-bound trehalase was approximately 4 times lower than that of soluble trehalase in the larval stage, and its activity did not abruptly increase after eclosion as did that of soluble trehalase.

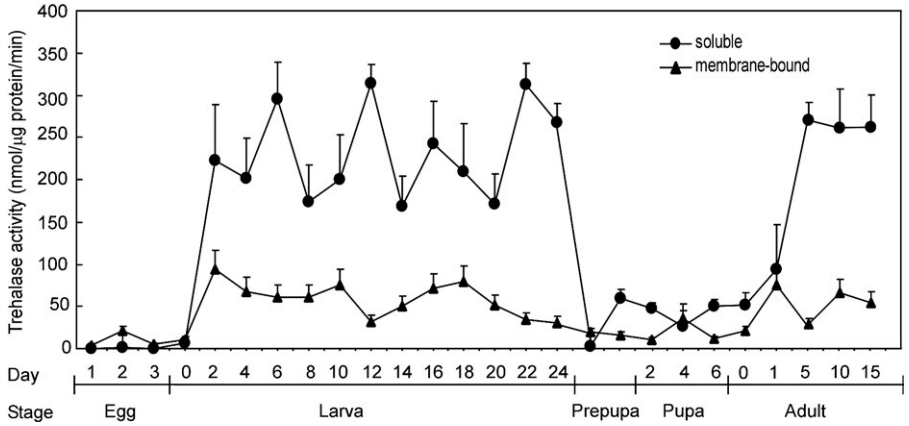

Fig. 3. Changes recorded in trehalase activity throughout the development of *Tribolium castaneum.* Circles, soluble trehalase; triangles, membrane-bound trehalase. The trehalase activity is expressed as in Fig. 1. The error bars indicate the SD (n = 5).

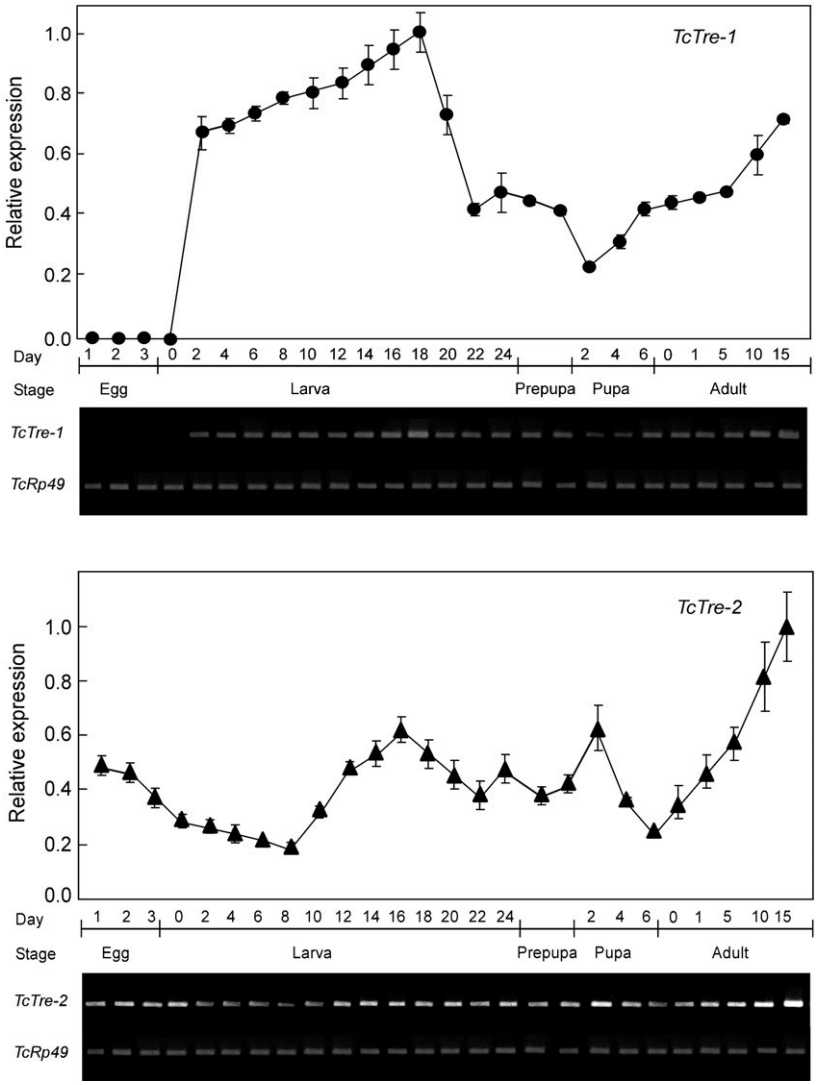

Fig. 4. The relative expression of *TcTre-1* (A) *and TcTre-2* (B) recorded during the development of *Tribolium castaneum* using RT-PCR. Samples were obtained from eggs, larvae, prepupae, pupae and adults. The PCR amplification of *TcTre-1* and *TcTre-2*, with *TcRp49* as an endogenous control (lower panel); the relative expression of *TcTre-1* and *TcTre-2* after normalization to *TcRp49* expression (upper panel) (n = 5).

Changes during development in trehalase gene expression

Semi-quantitative PCR was performed to determine the expression of *TcTre-1* and *TcTre-2* throughout the life of *T. castaneum* (Fig. 4). *TcTre-1* transcripts were not detected in eggs. The expression increased abruptly 2 days after hatching, gradually increased to a peak on day 18 of the larval stage and then decreased gradually during the last larval stage. *TcTre-1* expression was consistently low in the prepupa to pupa and then increased gradually in the adult stage. In contrast, *TcTre-2* expression was recorded throughout the life cycle, with substantial fluctuations until adult eclosion after which there was a steady increase.

Inhibition of trehalase activity and trehalase gene expression by latex extracts

As trehalase activity in *T. castaneum* adults was high and stable, we used adults to determine the inhibitory activity of latex extracts in vivo (Fig. 5A and B). Adults (1 week old) were fed either control or extract-treated diets and collected 5 days after the beginning of the feeding period. The enzymatic activities of soluble trehalase in adults fed on the three treated diets were significantly lower than that of the control adults. Starvation decreased the soluble trehalase activity to a level similar to that in adults fed with the diets containing extracts of the lattices of *A. heterophyllus* and *F. benjamina* (Fig. 5A). Similarly, when fed on the extract-treated diets there was a significant reduction in the activity of membrane-bound trehalase. In contrast to soluble trehalase activity, starvation increased membrane-bound trehalase activity (Fig. 5B).

Next, we examined the in vivo effects of latex extracts on the gene expression of two types of trehalase. *TcTre-1* expression in adults fed on the treated diets was significantly lower than that in control and starved insects ($P < 0.05$) (Fig. 5C). In contrast, *TcTre-2* expression was not

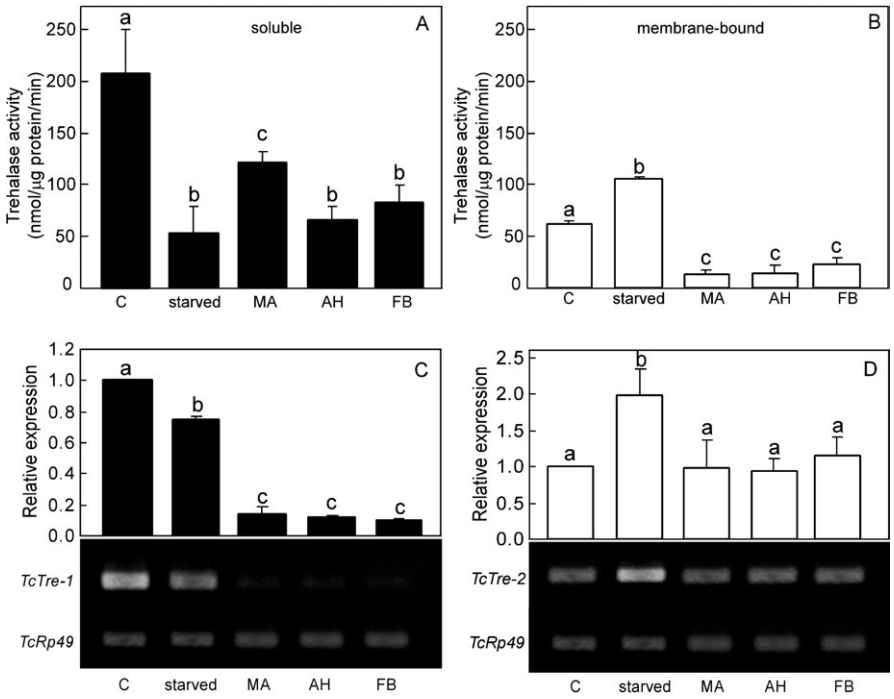

Fig. 5. Inhibition of trehalase activity and trehalase gene expression in *Tribolium castaneum* fed on diets containing methanol extracts of plant latices. One-week-old adults were fed a diet containing a methanolic extract of the latex of *Morus alba*, *Artocarpus heterophyllus* or *Ficus benjamina* for 5 days and then the soluble (A) and membrane-bound (B) enzyme samples and RNAs were prepared. Expression of *TcTre-1* (C) *and TcTre-2* (D) were determined using RT-PCR. The PCR amplification of *TcTre-1* and *TcTre-2*, with *TcRp49* as an endogenous control (lower panel); the relative expression of *TcTre-1* and *TcTre-2* after normalization to *TcRp49* expression (upper panel) and the normalized value of band intensities of the control sample was designated as one. MA – *Morus alba*; AH – *Artocarpus heterophyllus*; and FB – *Ficus benjamina.* C – adults were fed a control diet; starved, adults were kept without food for 5 days. The error bars indicate the SD, and the values labelled with different letters are significantly different ($P < 0.05$) (n = 5).

affected by any of the treated diets (Fig. 5D). When adults were starved, *TcTre-1* expression was slightly decreased, whereas that of *TcTre-2* was substantially increased (Fig. 5C vs. D).

Effects of plant latices on trehalose and glucose levels

Because the methanolic extracts of the latex produced by these three species of plants equally inhibited the enzymatic activities of the two types of trehalase both in vitro and in vivo, we determined whether the trehalose and glucose contents of adults were affected by the extracts (Fig. 6). After adults were fed on a treated diet for 5 days, the average trehalose concentration was 5.12 mM, which was approximately 1.5 times the concentration in adults fed on the control diet (Fig. 6A). The glucose concentration in treated adults was lower than that in control adults (Fig. 6B), whereas that in starved adults was much lower than in the adults fed on control and treated diets.

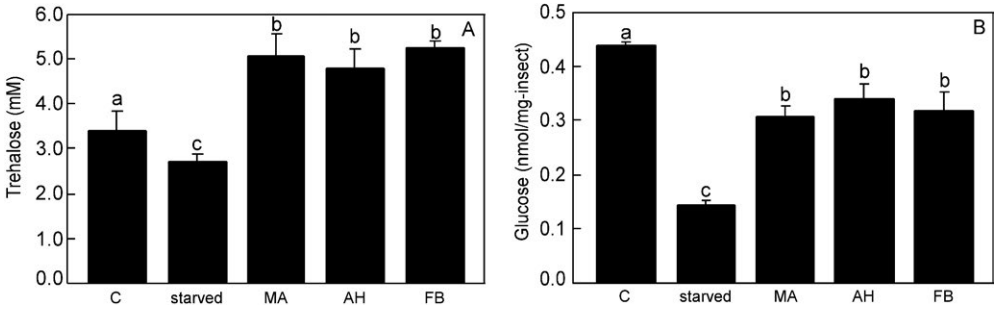

Fig. 6. Effect of feeding adults of *Tribolium castaneum* on a latex-treated diet on the concentration of trehalose (A) and glucose (B). One-week-old adults were fed a latex-treated diet for 5 days and then the amounts of trehalose and glucose were measured. MA – *Morus alba*; AH – *Artocarpus heterophyllus*; FB – *Ficus benjamina*. Samples were collected 5 days post-treatment. Abbreviations are the same as in Fig. 5. The error bars indicate the SD, and the values labelled with different letters are significantly different ($P < 0.05$) (n = 5).

DISCUSSION

This study showed that the latex of *M. alba*, *A. heterophyllus* and *F. benjamina* inhibited trehalase activity in *T. castaneum*. The inhibitors were extractable with methanol and highly heat stable (Fig. 1). The inhibitors in the latex of *M. alba* and *A. heterophyllus* were trypsin insensitive, and their molecular masses were probably less than 6–8 kDa. This indicates that the inhibitor(s) may be non-peptidic molecules. In addition, the similarities in the physical attributes of the inhibitory factor(s) in the latex of both *M. alba* and *A. heterophyllus* indicate that their latices may contain similar trehalase-inhibiting molecules.

M. alba latex contains the sugar-mimicking alkaloids D-AB1 and 1-deoxynojirimycin (DNJ), which inhibit the soluble and membrane-bound trehalases in *S. ricini* (Hirayama et al., 2007). Because DNJ in the latex of *M. alba* passes from the gut lumen into the haemolymph of the silkworm, *Bombyx mori* (Nakagawa et al., 2010; Yin et al., 2010), some factors in latex may inhibit trehalase activity in hemolymph and thus interfere with sugar metabolism. This finding suggests that the latex of *M. alba* and possibly also *A. heterophyllus*, may play an important role in defending plants against insects. However, the latex of breadfruit, *Artocarpus altilis*, which belongs to the same genus as *A. heterophyllus*, did not inhibit trehalase extracted from *T. castaneum* (unpubl. data), indicating that the latex of *A. heterophyllus* does not contain molecules that inhibit trehalase. This indicates that plants belonging to the same genus may use different defense chemicals to protect themselves from attacks by insect pests.

The inhibitor present in the latex of *F. benjamina* may belong to a different class of molecules than those in the latex of *M. alba* and *A. heterophyllus*. The inhibitor(s) in latex of *F. benjamina* was non-dialyzable, trypsin-sensitive and heat-stable, indicating that the inhibitor may be a thermally stable protein. Thermostable serine proteases occur in many plants and that in *F. benghalensis* is stable and active at high temperatures and high pHs (Sharma et al., 2009). Thus, the inhibitor present in the latex of *F. benjamina* may be similar to these proteins.

The activity of soluble trehalase and expression of *TcTre-1* mRNA were high in the larvae of *T. castaneum*, indicating that *TcTre-1* was dominant during the larval stage and in mature adults. In contrast, soluble trehalase activity was not detected in the egg stage. The most distinct difference between soluble trehalase activity and that of membrane-bound trehalase is that the latter was low but detectable and *TcTre*-2 expression appreciably higher in the egg stage. In the ovaries of the pupae of *B. mori* the only trehalase activity is that of the membrane-bound fraction and the quantity of *BmTreh-2* mRNA is approximately 1,000-fold greater than that of *BmTreh-1* mRNA (Kamei et al., 2010). Recently, studies on *Rhodnius prolixus* revealed the presence of membrane-bound trehalase activity in the ovary, which changed during oogenesis, but that of the expression of the gene *RpTre-2* did not change (Santos et al., 2012). These findings for different species of insects suggest that membrane-bound trehalase may play a role in carbohydrate metabolism in the reproductive system and that the activity of this enzyme may be controlled by post-transcriptional mechanisms.

The methanolic extracts of the latex of three species of plant inhibited the activity of soluble and membrane-bound trehalases. These extracts also inhibited the expression of *TcTre-1* in *T. castaneum* adults but not that of *TcTre-2*. This difference in the response of these two genes is also recorded for the bamboo borer, *Omphisa fuscidentalis*, in which 20-hydroxyecdysone alters the expression of the soluble trehalase gene (*OfTreh-1*) but not that of the membrane-bound trehalase gene (*OfTreh-2*) (Tatun et al., 2008). The response to the trehalase inhibitor and 20-hydroxyecdysone are totally different in quality, which may reflect their distinct roles in insects.

The application of trehalase inhibitor causes an increase in trehalose concentration (100–200% increase) and a simultaneous reduction in glucose concentration (by over 90%) in insects, including *B. mori*, *P. americana*, *L. migratoria* and *M. domestica* (Kono et al., 1993, 1994; Wegener et al., 2003). The effects of feeding *T. castaneum* on diets treated with methanolic extracts were, however, not as marked as the concentration of trehalose was only increased by approximately 40% and glucose concentration reduced by 78% of the control value. This relatively weak effect may indicate that inhibition is incomplete when fed on diets containing extracts of latex.

This study is the first to focus on insect trehalase as a target of the insect-defense chemicals manufactured by latex-producing plants. It is uncertain whether the inhibition of trehalase adversely affects animals other than insects, as the role of trehalose in vertebrates, including mammals, is not well known. Nevertheless, the present findings indicate it may be possible to use plant latices to develop new chemicals that disrupt carbohydrate metabolism, which can be used to control populations of stored-grain insects and other insect pests.

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