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Original Article

Viral infection induces different detoxification enzyme activities in insecticide-resistant and -susceptible brown planthopper *Nilaparvata lugens* strains

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This study aimed to describe the relationship between viral infection in *Nilaparvata lugens* (Stål), the brown planthopper (BPH), and different insecticide susceptibilities. BPH-resistant strains were selected using fenthion (an organophosphate) or etofenprox (a pyrethroid); a susceptible strain was used as the baseline colony before insecticide selection. All strains were infected with rice ragged stunt virus (RRSV) or rice grassy stunt virus (RGSV), after which the activities of three detoxification enzymes, cytochrome-P450-monooxygenase (P450), glutathione *S*-transferase (GST), and carboxylesterase (CE), were compared. Males of the strains selected for both insecticides showed high P450 and GST-CDNB activities. The activity of all enzymes was higher in males than in females, as a whole. However, males of the susceptible strain infected with RRSV showed decreased CE and GST-CDNB activities. BPH with low susceptibility to etofenprox showed a marked increase in P450 activity after RRSV infection; the GST-CDNB activity of females in the insecticide-resistant strain increased. RGSV infection induced high CE and P450 activities in etofenprox-selected females. The RRSV infection rate, but not the RGSV, decreased in etofenprox-selected strains.

Keywords: rice grassy stunt virus, rice ragged stunt virus, carboxylesterase, glutathione *S*-transferase, cytochrome P450 monooxygenase, fenthion, etofenprox.

Introduction

Nilaparvata lugens (Stål), the brown planthopper (BPH), is a monophagous hemipteran insect that causes damage to rice plants by ingesting fluids from the phloem of the stem.[1)] Large populations cause the death of rice plants in concentric circular patterns in rice fields (commonly called "hopper burn") and can induce plant mortality across an entire rice field.[1–3)] BPH is able to migrate over long distances[3–5)] and is well-distributed across Asia.[6)] BPH populations are controlled mainly by insecticides, but the unregulated use of insecticides in many areas have resulted in insecticide tolerance and the outbreak of BPH in tropical Asian countries,[7)] including frequent outbreaks in the 1960s and 1970s after the Green Revolution.[8)] Such accelerated disease outbreaks in turn prompt farmers to use insecticides.

Several studies have indicated decreasing susceptibility to various insecticides, including pyrethroids, organophosphates, carbamates, and neonicotinoids,[9–13)] which is caused largely by the induction and activation of detoxification enzymes[14)] such as carboxylesterase (CE)[15)] and cytochrome-P450-monooxygenase (P450) against organophosphates and carbamates,[16)] glutathione *S*-transferase (GST) against pyrethroids,[17–19)] and P450 against neonicotinoid insecticides.[20)] The genetic changes in these enzymes occur at the target site of the insecticides, such as on acetylcholinesterase.[21)] CE[15)] catalyzes hydrolysis reactions in chemicals with endo- and exogenous ester bonds and acts against organophosphates and carbamates.[22)] GST enzymes catalyze additional reactions to use glutathione as an antioxidant, resulting in various signaling cascades or stress responses against toxins, harmful metabolites, or oxidation.[23)] P450 has an important role in metabolizing xenobiotics, such as drugs, insecticides, and plant toxins. Insecticide-selected BPH individuals overexpress cytochrome P450 (*CYP*) genes encoding P450 enzymes[24–28)] and also show promoter polymorphism,[29)] wherein the *CYP* and *CE* genes are overexpressed in response to the organophosphate chlorpyrifos.[16)]

On the other hand, BPH acquires and transmits rice ragged stunt virus (RRSV; *Reoviridae*, *Fijivirus*)[30–33)] and rice grassy stunt virus (RGSV; *Tenuivirus*)[33,34)] by feeding on infected plants, accelerating the severe damage on growing rice plants. Although the role of detoxification enzymes in insecticide resistance has

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been clarified, the effects of virus infection on the activities of key detoxification enzymes such as CE, GST, and P450 in vector insects remain undescribed.

In this study, the activities of CE, GST, and P450 in infected and uninfected BPH, as well as in BPH strains selected using fenthion and etofenprox, were compared with those of control BPH individuals.

Materials and Methods

1. Rice seedlings

Rice seedlings with no resistance to BPH (MinoNishiki, Nouken Co., Ltd., Japan) were placed in six paper pots (Nippon Beet Sugar Manufacturing Co., Ltd., Tokyo, Japan) filled with Polus culture soil (Kyodo Hiryo Co., Ltd., Japan), which were then placed in a small plastic case (2.1 cm height, 11.2 cm length, 8.2 cm width). These pots were then placed under a 20 W lamp and supplied with water until the seedlings reached a height of 4–5 cm. They were then moved to a wire-framed cage (18 cm height, 25 cm width, 20 cm length) covered with a 0.4 mm mesh cloth. The ambient climate was set at 25±1°C with 16 hr light and 8 hr dark.

2. Measuring the LC_{50} value

Three or four rice seedlings were dipped into chemical solutions for 30 sec adjusted to 5.0×10^2 ppm, 1.0×10^3 ppm, 2.0×10^3 ppm, 4.0×10^3 ppm of etofenprox (Tore-bon, Mitsui Chemicals Agro, Ltd.), or fenthion (Baycid, Kumiai Chemical Industry Co., Ltd.), and then dried and placed in a plastic cup. Then, 10 females and males (two days post-adult emergence) were released onto the seedlings. Dead insects were counted 24 hr after treatment. This process was repeated three times. A control group was treated only with My-Rino solvent (polyalkylene glycol alkyl-ether 27%, Nihon Nouyaku Co., Ltd.).

3. Development of insecticide-selected strains

Insecticide-susceptible BPH individuals were reared for one week with rice seedlings treated with the LC_{50} concentration of each insecticide. These insects were then moved to untreated rice seedlings until the population reached sufficient numbers for the next insecticide treatment. This was repeated at 1–2-month intervals. The strain surviving after ten generations was assumed to be the insecticide-selected strain, and was used in subsequent experiments. The susceptibility of BPH to insecticides increased 1.5-fold in etofenprox and 3.68-fold in fenthion as compared to that of the first baseline control.

4. Partial purification of rice grassy stunt virus (*RGSV*)

RGSV-infected leaves were obtained from Dr. T. Uehara-Ichiki, Central Region Agricultural Research Center, and homogenized in PBS at 0.1 M, pH 7.4. This was filtered through cheesecloth, and chloroform was added at a ratio of 4 : 1 homogenate : chloroform, and then mixed for 2 min. After centrifugation at 8000×*g* at 4°C for 15 min, the water-soluble layer was drawn off. Polyethylene glycol (PEG 6000, MW 6000) and Triton X-100 were added to become 4% (w/v) and 0.1% (v/v) of the final concentration, respectively. This solution was mixed and centrifuged at 8000×*g* and 4°C for 15 min, and then the pellet was resuspended in phosphate buffer (0.1 M, pH 7.4) with 10% carbon tetrachloride (CCl_4) and agitated for 10 min, and then spun at 3000×*g* for 15 min. The supernatant was removed to a new tube, and CCl_4 at 10% was added and agitated for an additional 10 min, and then centrifuged again at 130,000×*g* for 2 hr. Following this, the precipitate was suspended with a small volume of 0.01 M phosphate buffer (pH 7.4) and centrifuged for a final time at 8000×*g* for 15 min. The remaining supernatant was used as a crude purified virus solution.

5. Partial purification of rice ragged stunt virus (*RRSV*)

RRSV-infected leaves crushed in PBS were added to $MgCl_2$ at a final concentration of 0.01 M and filtered with cheesecloth, followed by the addition of 20% CCl_4, and homogenized for 2 min. This homogenate was then centrifuged for 15 min at 3000×*g* and 4°C. The supernatant was recovered, and PEG 6000 was added to adjust to 6% (w/v) of the final concentration; NaCl was added at 0.3 M, and Triton X-100 was added to become 1% (v/v). The solution was mixed for 40 min, and then spun for 15 min at 6000×*g* and 4°C. The precipitate was then suspended with 0.1 M histidine buffer (pH 7.0) containing $MgCl_2$ and adjusted to a final concentration of 0.01 M. This solution was then incubated for 30 min and centrifuged for 15 min at 3000×*g* and 4°C. CCl_4 (10%) was added, and the solution was mixed for 2 min, followed by centrifugation at 96,000×*g* and 4°C for 40 min. The supernatant was discarded, and the pellet was resuspended with 0.1 M phosphate buffer (pH 7.0) containing $MgCl_2$ adjusted at a final concentration of 0.01 M. This was centrifuged at 3000×*g* and 4°C for 15 min, and the remaining supernatant was used as a virus solution.

6. Injection of each virus fluid and verification of virus infection

Less than 0.1 μL of each virus solution was injected into the abdomens of BPHs at 2–3 instars that had been chilled for 5 min to produce temporary paralysis (Nanoject II, Drummond Scientific, USA). After injection, BPH individuals were placed in a plastic petri dish with healthy rice seedlings and grown to the 4–5 instar stages before being separated and individually placed in a plastic tube with a healthy seedling. The long-winged morphotype was used for experiments at two days after adult emergence. BPH individuals injected with buffer only and without injections were used as controls for comparisons and reared under the same conditions.

To ensure the virus infection of each BPH individual, the insects' heads were used because both viruses are most active in the salivary glands of infected BPH individuals. After confirmation of virus infection, the protein extracted from each thorax-abdomen was used to measure the activity of each enzyme. Protein with undetectable PCR bands that showed no virus infection was discarded from the data. RNA extracted from each head was used to reverse transcribe cDNA, which was used as

a template to confirm the PCR band detected under conditions mentioned in subsequent sections.

7. Extraction of virus RNA

Each head part was homogenized with 200 μL of TRIzol reagent (Invitrogen Corporation, Carlsbad, USA) using a polytron (Hitachi, Japan). After 5 min of incubation at 30°C and the addition of 40 μL chloroform, the solution was mixed vigorously for 15 sec. The solution was then centrifuged at 13,200×g and 4°C for 20 min, and the supernatant was removed to a fresh tube. An equal volume of isopropanol was added, and the sample was incubated at 30°C for 10 min. The treated samples were incubated at −20°C for at least 30 min, and then centrifuged at 13,200×g at 4°C for 15 min to obtain the total RNA. The pellet was washed in cold 75% ethanol and dried, and then resuspended in a small amount of 0.01 M Tris–EDTA buffer (pH 7.4) and incubated at 50°C for 10 min. The RNA quantity was measured using SimpliNano (GE Healthcare, USA).

8. Synthesis of cDNA

Two micrograms of total RNA was used to produce 20 μL cDNA using SuperScript® Reverse Transcriptase kit (TaKaRa Bio Inc., Japan) in accordance with the manufacturer's protocol. The RNA template was decomposed using RNaseH (Sigma-Aldrich Corporation, USA).

9. PCR

PCR was performed using cDNA synthesized as a template from adult BPH individuals 2 days after infection. Each 0.5 μL template was added to 6.3 μL 2X AmpliTaq Gold® or 2X AmpliTaq Gold® 360 (Applied Biosystems Corporation, USA), 0.5 μL of each forward/reverse primer (10 μM), and 5.7 μL of Milli-Q. PCR reactions were carried out in a thermocycler per the following program: denaturation at 94°C for 30 sec, annealing at 53°C for 30 sec, and extension at 72°C for 60 sec, with 40 cycles. This was followed by a 72°C final extension step. The PCR products were visualized on 1.5% agarose gel. The primer sequence was as follows:

For RGSV_S5-sense: 5′-TAG CCA GGT TGA CAA AAC CC-3′,
S5-antisense: 5′-GCT GGA GAC AAT GCT GTT GA-3′.
For RRSV_S7-sense: 5′-CGT ACC ACC ATC GCC TTA CT-3′,
S7-antisense: 5′-CGT AAT CGT CAC TCC ACC CT-3′.

10. Measurement of carboxylesterase (CE) activity

Each thorax-abdomen sample was homogenized with a proteinase inhibitor cocktail (0.8 mg/mL each of chymostatin, aprotinin, leupeptin, and pepstatin in DMSO) for a few seconds using an ultrasonic disruptor (TOMY, UD-200) in 0.1 M Pipes-NaOH buffer (pH 7.4), and then placed on ice. The samples were then centrifuged at 20,128×g and 4°C for 15 min, and the supernatant was used as an enzyme solution. Each sample was incubated with 100 μL of 0.3 mM alpha-naphthyl acetate as a substrate for 10 min, and then 20 μL of 1% Naphthanil Diazo Blue B with 5% sodium dodecyl sulfate was added. CE activity was measured colorimetrically at 595 nm in a 96-well microplate using a microplate reader (BioRad Laboratories, Inc., USA).

11. Measurement of cytochrome P450-monooxygenase (P450) activity

Each sample was homogenized in 1 mL of precooled 0.1 M Na/K-phosphate buffer (pH 7.8) containing 1 mM EDTA, 1 mM DTT, 1 mM PTU, and 1 mM PMSF. The homogenized solution was spun at 20,128×g and 4°C for 20 min, and enzymatic activity was measured in the supernatant. Ninety microliters of the supernatant solution was added to 100 μL of 2 mM p-NA and incubated at 27°C for 2 min in a 96-well microplate. Subsequently, 10 μL of 9.6 mM NADPH was added to each well. P450 activity was measured at intervals of 25 sec at 405 nm absorbance in a microplate reader.

12. Measurement of glutathione S-transferase (GST) activity

GST activity was measured using CDNB (1-chloro-2,4-nitrobenzene) as a substrate for GST 1 and 2 and DCNB (1,2-dichloro-4-nitrobenzene) as a substrate for GST 3 and 4. To measure GST activity using CDNB, 10 μL of enzyme solution and 130 μL of 10 mM reduced-form glutathione were mixed into 1020 μL of 0.1 M potassium phosphate buffer (pH 8.0). Ten microliters of 150 mM CDNB was then added, and the mixed solution was incubated at 28–30°C and measured colorimetrically at 340 nm at intervals of 25 sec over a 10 min period. For DCNB, 200 μL of enzyme solution and 130 μL of 10 mM reduced-form glutathione were added to 1100 μL of 0.1 M potassium phosphate buffer (pH 8.0), and the reaction was initiated by the addition of 10 μL 150 mM DCNB. Activity was measured at 344 nm over the same time scales as described above.

13. Measuring protein content

Bovine serum albumin (BSA) at concentrations of 0, 0.2, 0.4, 0.6, 0.8, and 1.0 μg/μL was used as a standard solution. Protein assay reagent dye (BioRad Laboratories, Inc.) was added to each standard, and the absorbance was measured at 595 nm in a 96-well plate to generate a standard curve, as per the manufacturer's protocol. Experimental samples were then measured at 595 nm, and the protein concentration was determined along this calibrated curve.

14. Statistical analysis

Excel Tokei (Bell Curve) was used in addition to the analysis of variance performed on all data followed by the Tukey–Kramer multiple comparison test at the level of $p<0.05$.

Results

The higher activity of P450 and GST in strains resistant to fenthion or etofenprox significantly increased in male BPHs in comparison to intact controls (Table 1, $p<0.05$). The activity of all enzymes was higher in male BPHs than in females, likely because of the following reasons: the total enzyme activity was calculated per mg of protein; the amount of total protein extracted

Table 1. Each detoxification enzyme activity in nonselected and each selected strain[a)]

	Treatment	CE activity (nmol/min/mg protein)	P450 activity (nmol/min/mg protein)	GST activity with CDNB (nmol/min/mg protein)	GST activity with DCNB (nmol/min/mg protein)
Male	Control (Susceptible)	103.6±20.9	81.1±19.0	191.2±19.4	0.22±0.03
	Strain selected with etofenprox	93.8±1.9	118.6±17.8[b)]	276.5±5.3[b)]	0.17±0.06
	Strain selected with fenthion	90.7±7.8	117.1±14.5[b)]	278.0±40.3[b)]	0.11±0.05
Female	Control (Susceptible)	48.1±9.4	64.1±10.8	86.4±16.1	0.05±0.01
	Strain selected with etofenprox	39.6±7.8	46.8±10.7	94.0±10.9	0.08±0.03
	Strain selected with fenthion	48.4±11.4	49.8±14.0	71.2±12.8	0.03±0.02

[a)] CE: carboxyesterase, P450: cytochrome p450 monooxygenase, GST: glutathione-*S* transferase, CDNB: 1-chloro-2,4-nitrobenzene, DCNB: 1,2-dichloro-4-nitrobenzene. [b)] Means significantly difference when compared to control value in each enzyme (n=5, p<0.05, Turkey–Kramer)

from female adult abdomens was slightly heavier than that of males.

Infection rates differed between the two viruses in spite of the same infection-by-injection technique. Successful RGSV infection occurred in less than 30% of injected individuals, while successful RRSV infection occurred in more than 50% of individuals (Table 2). Insecticide-susceptible BPH strains were significantly more likely to become infected with RRSV than insecticide-selected strains (n=40 for each, p<0.05, Tukey–Kramer test); however, this was not observed with RGSV infection (Table 2).

Table 2. Infection rate of two virus on BPH

Treatment	Number	Rice ragged stunt virus (RRSV) %	Rice grassy stunt virus (RGSV) %
Control	40	62.9±4.4	21.9±1.3
Etofenprox	40	48.5±5.0[a)]	21.2±1.1
Fenthion (MPP)	40	56.5±7.3	25.5±7.7

[a)] Infection rate was significantly different to the value of control (p<0.05, Turkey–Kramer)

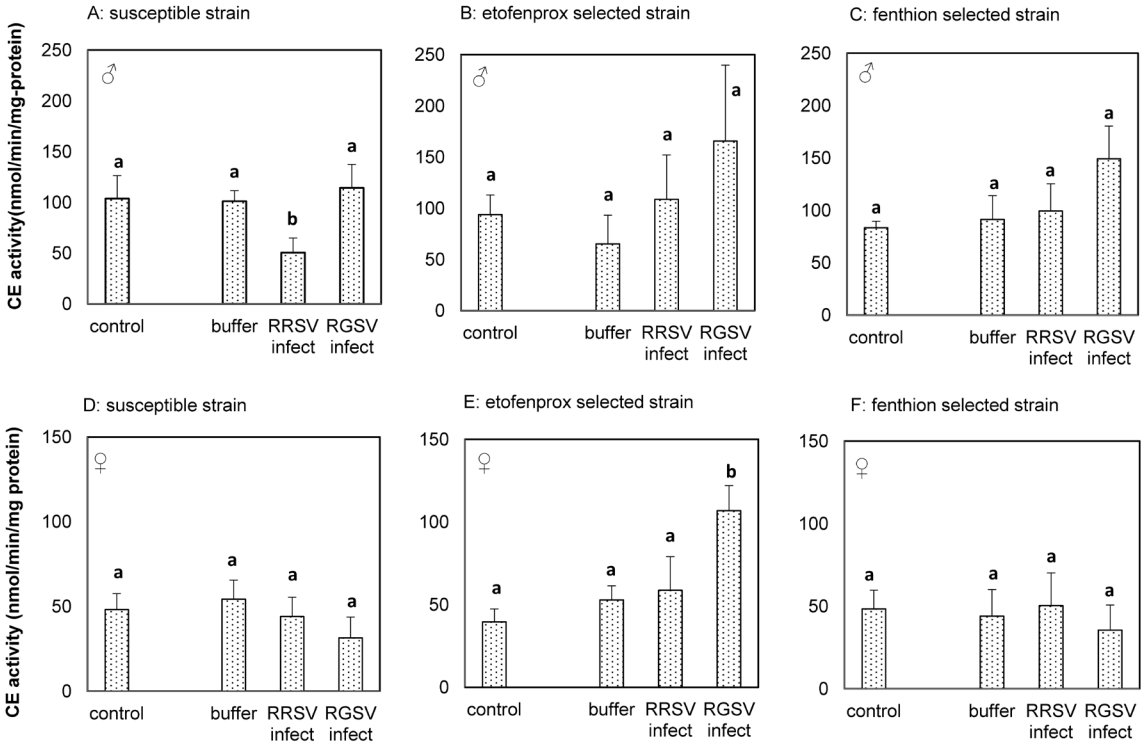

Fig. 1. Enzyme activity of carboxylesterase (CE) in males (♂) and females (♀) of each strain. Each rice ragged stunt virus (RRSV) or rice grassy stunt virus (RGSV) solution was injected in the abdomen of BPH 2–3 instars after they had been paralyzed on ice for 5 min. Adults 2 days post-ecdysis were used for measuring enzyme activity (N≧5). Untreated individuals were used as controls. Buffer indicates injections of buffer only. The substrate used for enzymes was 0.3 mM alpha-naphthyl acetate. Different letters on each bar indicate statistically significant differences (Tukey–Kramer, p<0.05).

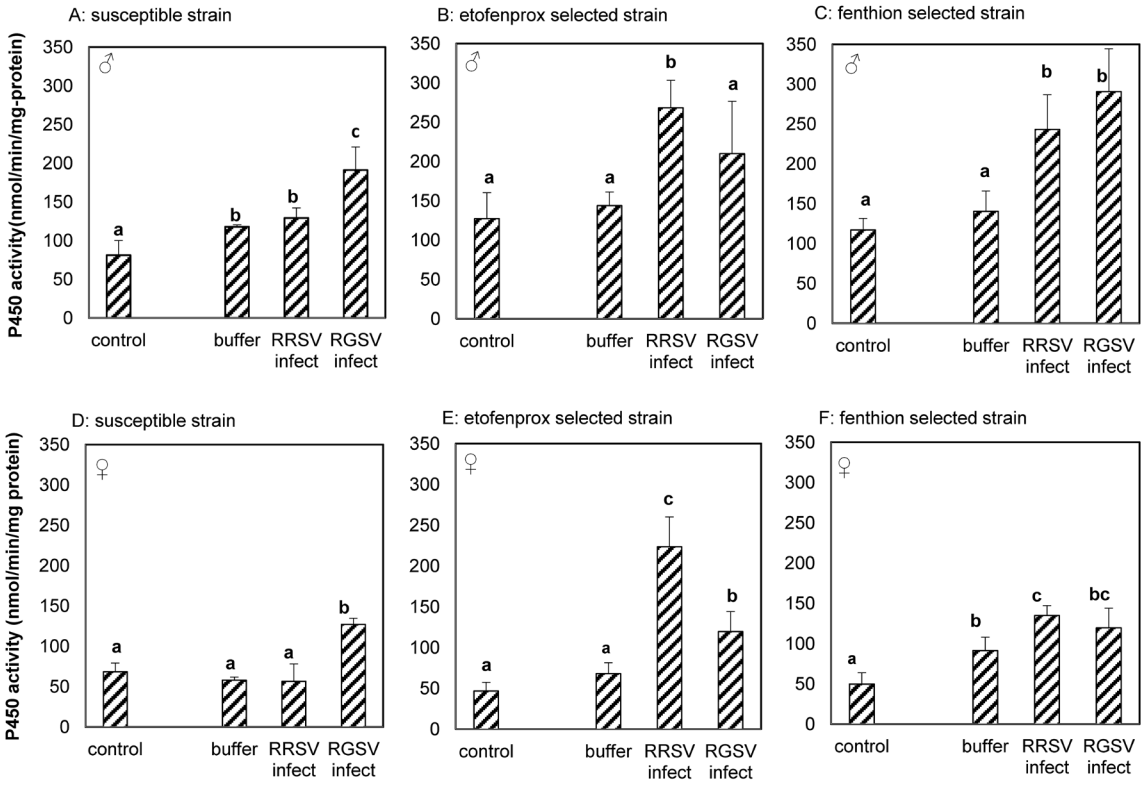

Fig. 2. Cytochrome P450 monooxygenase activity in males (♂) and females (♀) of each strain. Enzyme activity was measured in individuals 2 days post adult ecdysis at intervals of 25 sec using p-NA as a substrate at 405 nm absorbance (N≧5). Different letters on each bar indicate statistically significant differences (Tukey–Kramer, $p<0.05$).

1. Carboxylesterase (CE) activity

Insecticide-susceptible RRSV-infected males showed significantly lower CE activity ($n=3$–5, $p<0.05$, Turkey–Kramer test), but comparable females showed no difference (Fig. 1). Etofenprox-selected females infected with RGSV showed increased CE activity (Fig. 1E). Males in both etofenprox- and fenthion-selected strains showed no significant differences in viral infection, as compared to the susceptible strain.

2. Cytochrome P450 monooxygenase (P450) activity

P450 activity in insecticide-susceptible males was significantly greater in RGSV-infected BPH individuals as compared to buffer-injected controls and those infected with RRSV ($n=3$–5, $p<0.05$, Turkey–Kramer test, Fig. 2A); both the buffer-injected controls and RRSV-infected BPH individuals showed greater P450 activity, as compared to untreated controls ($n=3$–5, $p<0.05$, Turkey–Kramer test). There was no statistically significant difference in the P450 activity between etofenprox-selected males infected with RGSV and uninfected controls, although this value seems to be higher in the former. On the other hand, RRSV-infected males and females tolerant to both insecticides showed significantly higher P450 activity (Fig. 2B, C and 2E, F).

BPH individuals with low susceptibility to etofenprox or fenthion showed greater P450 activity when infected by RRSV or RGSV, as compared to uninfected controls.

3. Glutathione S-transferase (GST) activity

Susceptible RRSV-infected males showed significantly lower GST activity (Fig. 3A), although virus-infected male BPH individuals showed no difference in GST activity as measured using CDNB as a substrate for both selected strains. Insecticide-susceptible females showed no difference in GST activity; however, higher GST activity was observed in RRSV-infected female resistant to both etofenprox and fenthion (Fig. 3E, F).

GST activity, as measured using DCNB (Fig. 4D, E), showed no significant difference between susceptible and etofenprox-selected individuals. Although the use of DCNB as a substrate instead of CDNB was of little consequence, both males and females tolerant to fenthion showed greater GST activity when infected with RRSV but not with RGSV (Fig. 4C, F). However, no difference between RRSV-infected individuals and those injected with buffer indicates the improbability of RRSV infection resulting in an increase in the GST activity but merely as a response to the injection.

Discussion

This study demonstrated that three different detoxification enzymes involved in insecticide resistance are affected by viral infection. Insecticide-susceptible BPH males showed a significant decrease in CE and GST enzyme activity when infected by RRSV. Buffer-injected uninfected male BPH individuals showed no difference in comparison to intact controls, indicating that

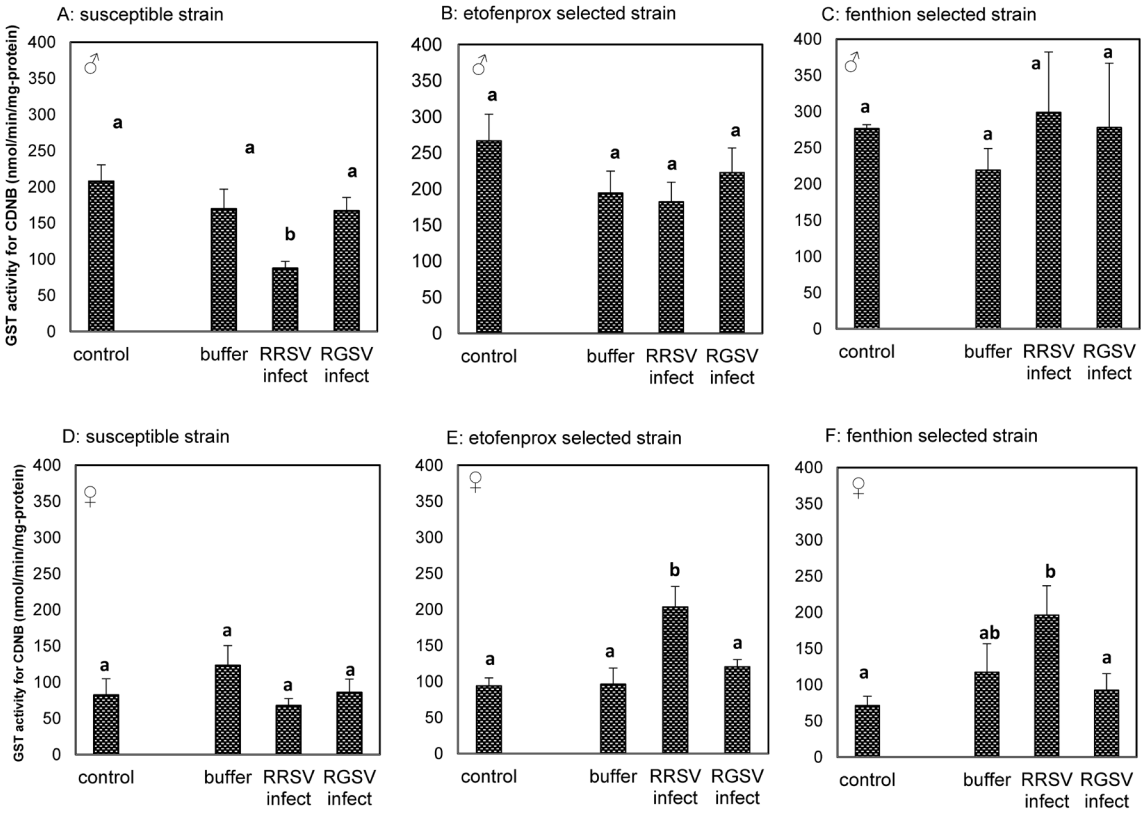

Fig. 3. Glutathione *S*-transferase activity using CDNB as a substrate in males (♂) and females (♀) of each strain. GST activity was measured at 28 to 30°C at 340 nm at 25 sec intervals over 10 min. Different letters on each bar indicate statistically significant differences (Turkey–Kramer, $p<0.05$).

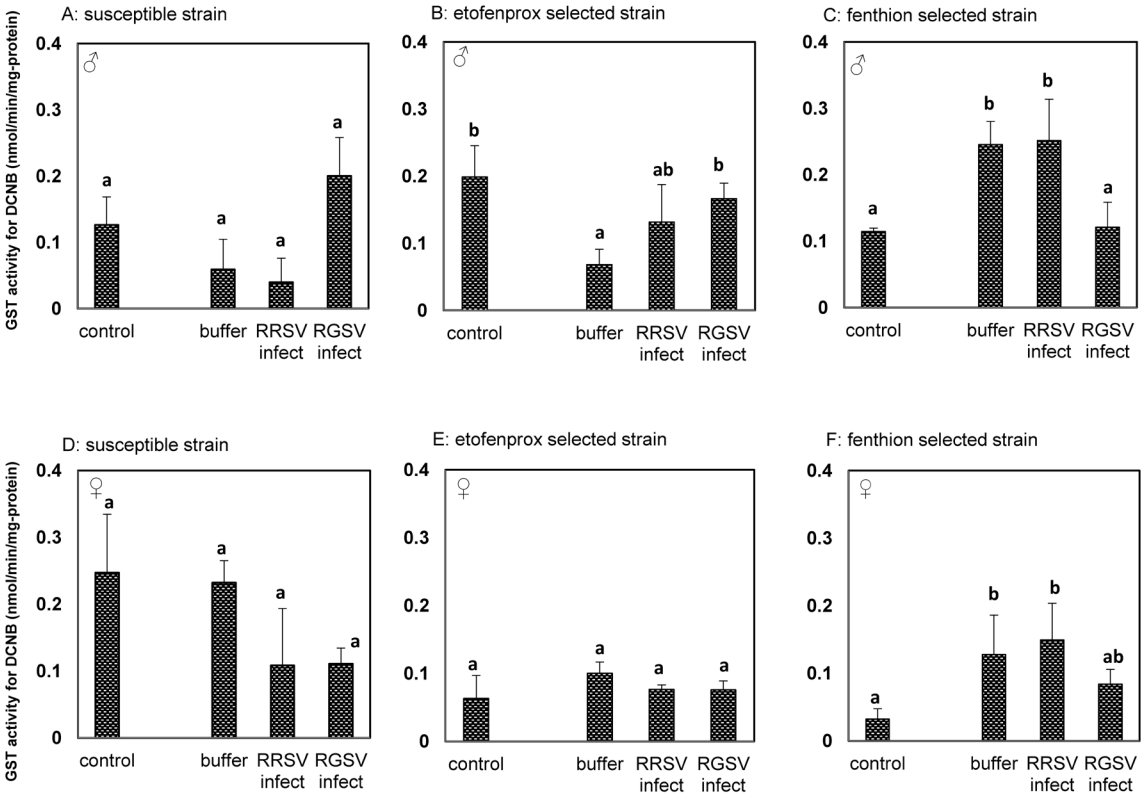

Fig. 4. Glutathione *S*-transferase activity using DCNB as a substrate of males (♂) and females (♀) of each strain. DCNB-substrate enzyme activities were measured at 344 nm at 25 sec intervals over 10 min at 28–30°C. Different letters on each bar indicate statistically significant differences (Turkey–Kramer, $p<0.05$).

RRSV infection induced the decrease in both enzyme activities. Decreases in GST and P450 activities have been reported in cases of bacterial infection, but not in cases of virus.[36,37] The Asian citrus psyllid, *Diaphorina citri*, infected with the Gram-negative bacteria *Candidatus* Liberibacter asiaticus (Las) shows significantly lower activity as compared to uninfected ones, suggesting that the defense response against the xenobiotic may be suppressed by the invading virus. Although there have been no reports that direct virus infection induced high activity of detoxification enzymes, it is reported that the p450 and GST of the detoxification enzyme increased in parasitized hosts with infection by a symbiotic virus, which is injected into the host hemocoel with the eggs of an endoparasitoid, *Vestalis* (=*Cotesia*) *plutellae*–*Plutella xylostella*, host system.[38] Endoparasitoid wasps have a symbiotic DNA virus, a polydnavirus (PDV), which regulates the host immune system by the gene expression of the polydnavirus in the host cells when it is injected into a suitable lepidopteran host along with an egg.[39,40]

Although P450 activity increased in susceptible males infected with RRSV, no significant difference was observed relative to buffer-injected individuals. However, RGSV infection increased the P450 activity in both males and females, indicating that infection by RGSV induced the increase in P450 activity. The P450 activity in etofenprox- and fenthion-selected males was significantly greater as compared to that in susceptible controls (but not in females), indicating that the P450 activity increased as a whole in insecticide-selected BPH individuals for detoxification, and that viral infection may produce significantly higher P450 activity. CYP has been reported as having multiple functions aside from the detoxification of toxic chemicals.[41,42] The increase in P450 activity might cause a decrease in insecticide sensitivity or may represent a defensive reaction to a virus infection.

GST also showed a slight increase in insecticide-selected individuals as compared to insecticide-susceptible controls, while a decrease in GST activity was observed in insecticide-susceptible individuals infected with RRSV. Conversely, GST activity increased in etofenprox-selected individuals infected with RRSV. Increased GST activity has been reported in some insects that are resistant to pyrethroids.[43] Here, etofenprox-selected individuals showed increased GST activity and a lower rate of RRSV infection (Table 1). These findings indicate that P450 and GST may be related to the defense response against RRSV as a xenobiotic. GST activity of *Bombyx mori* also varies in the case of infection with the polyhedrosis virus or the densonucleosis virus; GST genes are induced during the administration of the insecticides.[44] These suggest the possibility that the increase of GST activity could be related to viral defense in BPHs.

This experiment demonstrates the relationship between virus infection and insecticide susceptibility in terms of the activity of the detoxification enzymes CE, GST, and P450. An examination of detoxification enzyme gene expression and the knockdown of the expressed genes would be advisable to further clarify the relationship between detoxification enzymes and viral infection.

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