Article

CHARACTERIZATION OF SOLUBLE AND MEMBRANE-BOUND ALKALINE PHOSPHATASE IN *Nilaparvata lugens* AND THEIR POTENTIAL RELATION TO DEVELOPMENT AND INSECTICIDE RESISTANCE

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Two forms (soluble and membrane-bound) of alkaline phosphatases (ALPs) were found in the brown planthopper, Nilaparvata lugens. In order to further study ALPs in N. lugens, two putative ALP genes (Nl-ALP1 and Nl-ALP2) were identified in this pest. Both Nl-ALP1 and Nl-ALP2 show approximately the same degree of sequence identity (40–50%) to other insect soluble and membrane-bound forms of ALP. Correlation of ALP activity and mRNA levels at different developmental stages, or following application of 20-hydroxyecdysone (20E) and

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insecticide fenvalerate, suggests that Nl-ALP1 and Nl-ALP2 might encode a soluble (sALP) and a membrane-bound ALP (mALP), respectively. Nl-ALP1-specific antibody Nl1-I detected only a specific band in soluble protein preparations and Nl-ALP2 specific antibody Nl2-I only detected a specific band in insoluble protein preparations, which provided conclusive linkages between Nl-ALP1 and a sALP and between Nl-ALP2 and a m ALP. Then, Nl-ALP1 was denoted as Nl-sALP for a sALP and Nl-ALP2 was denoted as Nl-mALP for a mALP. Only sALP activity and Nl-sALP mRNA level were induced by 20E and fenvalerate, which was confirmed by the density of specific band detected by Nl1-I in Sus strain with or without fenvalerate treatment. Additionally, the sALP activity, as well as Nl-sALP mRNA level, was significantly higher in a fenvalerate resistant population, compared with Sus strain. These results indicate that the sALP is more responsive to chemical stimulus, such as hormone and insecticide, and might play dual roles in development and insecticide tolerance. © 2011 Wiley Periodicals, Inc.

Keywords: alkaline phosphatase; *Nilaparvata lugens*; 20-hydroxyecdysone; Fenvalerate; insecticide resistance

INTRODUCTION

Alkaline phosphatases (ALP, EC 3.1.3.1) are abundant enzymes mainly involved in removing phosphate groups from organic molecules. The wide distribution of ALPs suggests their fundamental roles in cells. ALPs have common properties in hydrolase/transferase reactions, dimeric structure, activity dependence on Zn^{2+} and Mg^{2+}, and identical amino acid sequences around the active site (Eguchi, 1995). In insects, ALPs are involved in several biological processes and respond to stress, pathogenesis, or infection (Kucera and Weiser, 1974; Sujak et al., 1978; Chang et al., 1993; Eguchi, 1995; Sukhanova et al., 1996; Miao, 2002). ALP is one important synthesizing enzyme of tyrosine, the precursor of dopamine and octopamine, which are known to take part in the control of levels of insect developmental hormones, juvenile hormone (JH), and 20-hydroxyecdysone (20E) (Wright, 1987; Rauschenbach et al., 2007a,b).

ALP in the silkworm is confined mainly to the gut tissue, from which two ALP isozymes were detected by electrophoresis. The slow migrating band was membrane-bound (mALP) and the fast moving one was a soluble form (sALP) (Eguchi et al., 1972; Eguchi, 1995). Biochemical studies showed different properties for two ALP isozymes, such as pH optima of 10.1 for s-ALP and 11.3 for mALP (Eguchi, 1995). Two silkworm ALP isozymes are encoded by different genes, which are mapped to a small DNA region and organized in tandem (Itoh et al., 2003). Such tandem duplication of ALP genes is also found in the wild silkworm, *Bombyx mandarina* (Itoh et al., 2008). The deduced amino acid sequences of two *Bombyx mori* ALP genes are about 60% identical (Itoh et al., 1991, 1999). The brown planthopper (*Nilaparvata lugens*), a hemipteran insect, is a major rice pest in many parts of Asia. Insecticides have been extensively used to control this pest and other rice insect pests, and resistance to a wide range of insecticides has been reported globally (Liu et al., 2005; Liu and Han, 2006; Wang et al., 2008). Resistance to imidacloprid was a main factor contributing to a large rice loss in China and other Asian countries in 2005. To manage insecticide resistance in

rice insect pests, genetically modified (GM) rice is under study, such as Bt (*Bacillus thuringiensis*) rice. In some insect species, mALP binds with Bt toxin Cry1Ac. ALP may thus be a novel receptor of Bt toxins (McNall and Adang, 2003; Jurat-Fuentes and Adang, 2004; Perera et al., 2009). Although relatively little information is available about ALP as an insecticide resistance mechanism, some evidences suggest an association for ALP with insecticide resistance. For example, a fenvalerate-resistant population of *Helicoverpa armigera* and four diazinon-resistant populations of *Chilo suppressalis* showed higher ALP activity than susceptible populations (Srinivas et al., 2004; Zibaee et al., 2009), and parathion and methomyl application increased ALP activity in *H. armigera* (Gao et al., 1996). We investigated ALPs in *N. lugens* and their potential relation with the development and insecticide resistance in this insect pest. In this report, we show the presence of two forms of ALP in *N. lugens* and identify the genes that apparently encode each type.

MATERIAL AND METHODS

Insect Culture and Treatment with 20E or Fenvalerate

N. lugens-susceptible strain (Sus) is a laboratory strain originally obtained from the China National Rice Research Institute in September 2001. A fenvalerate-resistant strain (R_{FEN}), with 153-fold resistance ($LD_{50} = 852.6 \pm 64.3$ ng/female compared with 5.6 ± 0.5 ng/female for Sus strain), was selected from the Sus strain in laboratory for 17 generations. The Sus strain was used unless stated otherwise. Insects were kept indoors at 25 (± 1)°C and 70–80% relative humidity under a 16/8-h light–dark cycle. Under laboratory conditions, the duration of *N. lugens* life cycle is about 30–35 days, in which the duration of last-larval (fifth instar) stage is 3–4 days. 20E (Sigma, St. Louis, MO) was dissolved in reagent-grade methanol at a concentration of 1 mg/ml and stored at −20°C until use. The treatment method followed the micro-topical application technique reported by Nagata (1982) with some modification. Under CO_2 anesthesia, a droplet (0.1 µl) of 20E methanol solution was applied topically to the prothorax notum of the fifth instar macropterous female nymphs (soon after ecdysis) with a hand microapplicator (Burkard Manufacturing Co Ltd, Rickmansworth, UK). Controls used methanol alone. The sex identification of the fifth instar nymphs at the first day is based on the morphology of the external genitalia and the seventh to ninth abdominal segments, as described previously (Zhang, 1980). Fenvalerate (95%; Ruize Agrochemical Co Ltd, Jintan, China) was dissolved in methanol at 8 µg/ml, which approximated the LD_{20} dose (0.8 ng/insect) for the Sus strain when 0.1 µl droplet was topically applied as above. Fenvalerate was applied topically to the pronotum of the fifth instar macropterous female nymphs (soon after ecdysis) from Sus strain.

Alkaline Phosphatase Activity Assay

The separation of two ALP proteins was carried out as previously described but with some modifications (Gu et al., 2009). Thirty milligrams of insect whole bodies (fifth instar macropterous females or macropterous female adults) was homogenized in 1 ml of 100 mM glycin–sodium hydroxide buffer (GSB, pH 8.5–11.0) at 0°C followed by sonication for 30 sec. The cuticle debris was removed by centrifugation at 1,000*g* at 4°C for 10 min. The homogenates were then centrifuged at 105,000*g* at 4°C for 60 min.

The resulting supernatant and precipitate were regarded as the fractions containing sALP and mALP, respectively. The supernatant was subjected directly to activity assay, whereas the precipitate was resuspended in GHB for activity measurement. Protein content was determined using Bio-Rad DC protein assay kit (Bio-Rad, Hercules, CA) with bovine serum albumin as the standard.

ALP activity measurement was carried out as described by Sukhanova et al. (1996) using α-naphthyl phosphate (α-NP; Sigma) as a substrate and fast blue RR salt (Sigma) as a staining agent. The reaction mixture (200 μl) consisted of 40 μl of enzyme solution, 0.46 μl of 10% MnCl, 0.46 μl of 10% MgCl, 0.2 mg of α-NP, 0.2 mg of fast blue RR salt, 1 mg of polyvinylpyrrolidone (Sigma), 4 mg of NaCl and make up the volume to 200 μl by GSB with corresponding PH of enzyme solution used. Incubation was carried out at 25°C in the dark for 25 min and the reaction was stopped by adding 600 μl of ice-cold distilled water. In 5 sec, the amount of product formed was measured at 470 nm with a double beam spectrophotometer UV-2401PC (Shimadzu Corporation, Kyoto, Japan).

Amplification of Two Alkaline Phosphatase Genes

Total RNA was isolated from 10 individuals of the fifth instar female using a Trizol kit (Invitrogen, Foster, CA). Synthesis of first-strand cDNAs was carried out according to the reverse transcriptase XL (AMV) (TaKaRa, Dalian, Liaoning, China) protocol with oligo dT_{18}. The first strand cDNA (1 μl) was used as a template for PCR. Searching the BPH EST Database (http://bphest.dna.affrc.go.jp/; Noda et al., 2008) with the amino acid sequences of two *B. mori* ALPs (sALP and mALP) gave two homolog fragments (Clone ID: NLMA5211 and C_NLMA1028) with 40–50% identity at the amino acid level to *B. mori* sALP and mALP. Based on these two fragments, gene-specific primers (GSPs) were designed and the rapid amplification of cDNA ends (RACE) technique was used to amplify the full-length cDNAs for two fragments according to Generacer™ Kit (Invitrogen). The GSPs for the first fragment were NL1-5 (GAG GCA TGT GTC ACA CGA GTG GTT G) and NL1-3 (GAT GAG GCT ATC ATG AAA GCA GTG G). The GSPs for the second fragment were NL2-5 (CTT GTC ATC CAA CCA CTC CTG GAT C) and NL2-3 (GTT CAT TCA TTC CAC TGA GTG AGT G). The amplified product was separated by agarose gel electrophoresis and purified using the Wizard PCR Preps DNA Purification System (Promega, Madison, WI). Purified DNA was ligated into the pGEM-T easy vector (Promega) and several independent subclones were sequenced from both directions.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

mRNA levels were measured by qRT-PCR using the One Step SYBR PrimeScript RT-PCR Kit (Takara). Total RNA from *N. lugens* was treated with DNase I (Sigma) and then used as a template. qRT-PCR was performed in a 25-μl total reaction volume containing 5 ng of total RNA, 0.5 μl of primer mix containing 10 μM each of forward and reverse GSPs, 0.5 μl of Ex TaqTM HS (5 U/μl), 0.5 μl of PrimeScript RT Enzyme Mix, 12.5 μl of 2 × One Step SYBR RT-PCR Buffer, and 8.5 μl of H_2O. The GSPs for the first ALP were: NL1-F (GTT TAT CTG GAA TAG GGA CGA CCT G) and NL1-R (GAT GGT TTC ATC CAG TGC GAT CTG). The GSPs for the second ALP were NL2-F (GTT GAA GGA GCT CTC ATT GAC AAA G) and NL2-R (GTC GAG TAG CTA TAC TTC ATG TCG TG). Two kinds of negative controls were set up: nontemplate reactions (replacing total RNA by H_2O) and minus reverse transcriptase controls (replacing PrimeScript RT Enzyme Mix by H_2O). qRT-PCR was carried out

with the following cycling regime: initial incubation at 42°C for 5 min and 95 °C for 10 sec; 40 cycles at 95°C for 5 sec, 60°C for 20 sec, and 72°C for 15 sec. Standard curves were obtained using a 10-fold serial dilution of pooled total RNAs from 20 individuals. mRNA levels were quantified in relation to the expression of β-actin (**EU179846**; Liu et al., 2008). The GSPs for β-actin were β-F (TGG ACT TCG AGC AGG AAA TGG) and β-R (ACG TCG CAC TTC ATG ATC GAG). The primer pair for each gene was designed to amplify a 200- to 300-bp PCR product, which was in each case verified by nucleotide sequencing. Only data that showed good efficiency ($\geq 85\%$) and correlation coefficient ($\geq 95\%$) were included in the analysis. Means and standard errors for each time point were obtained from the average of three independent sample sets.

Western Blotting Detection

Polyclonal antisera (Nl1-I and Nl2-I) specific for the Nl-ALP1 and Nl-ALP2 putative proteins were raised against bacterially expressed fusion proteins containing N-terminal peptide sequences of Nl-ALP1 (Asp34-Gln53) and Nl-ALP2 (Asp48-Asn67). Fusion proteins were produced and affinity purified on nickel–nitrilotriacetic acid resin under denaturing conditions as described by the manufacturer (Qiagen, Valencia, CA). The purified fusion proteins were used to produce polyclonal antibodies in rabbits. The soluble and insoluble proteins were prepared as described above. The soluble or insoluble protein (250 μg) was separated by SDS/PAGE and then electroblotted onto Hybond-C nitrocellulose membranes (Amersham BioSciences, Little Chalfont, Buckinghamshire, UK). Membranes were blocked and then incubated with 1:1,200 dilution of one specific antibody (Nl1-I or Nl2-I) for 1 h at room temperature. The nitrocellulose membrane was washed thoroughly, incubated with 1:1,200 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG, and processed by the ECL detection system, following the manufacturer's instructions (Millipore, St. Charles, MO).

Statistical Analysis

Differences were analyzed by one-way ANOVA with at least three repeats. Differences between values were analyzed using a LSD pairwise comparison of means. The level of significance for results was set at $P < 0.05$. The big-PI Predictor (http://mendel.imp.ac.at/gpi/gpi_server.html) tool was used to analyze both ALP sequences for GPI-anchored sites prediction.

RESULTS

Detection of the Two Types of Alkaline Phosphatase and pH Optimum Determination

Ultracentrifugation at 105,000*g* was used to divide the whole body homogenates from the macropterous female adult *N. lugens* (soon after ecdysis) into two fractions, supernatant and precipitate. Because the pH optimum for sALP and mALP is often different, such as 10.1 for sALP and 11.3 for mALP in *B. mori* (Eguchi, 1995), insect sample was homogenized and ALP activities in two fractions were measured in GSB across a range of pH values (9.0–11.0). The results showed that pH 9.3–9.4 and 10.5–10.6 are the most active pH values for the soluble fraction (Fig. 1A) and for the insoluble fraction (Fig. 1B). At the pH optimum, activity was 0.093 ± 0.006 μmol mg protein^{-1} min^{-1} for the soluble fraction and 0.214 ± 0.025 μmol mg protein^{-1} min^{-1} for the insoluble fraction, indicating

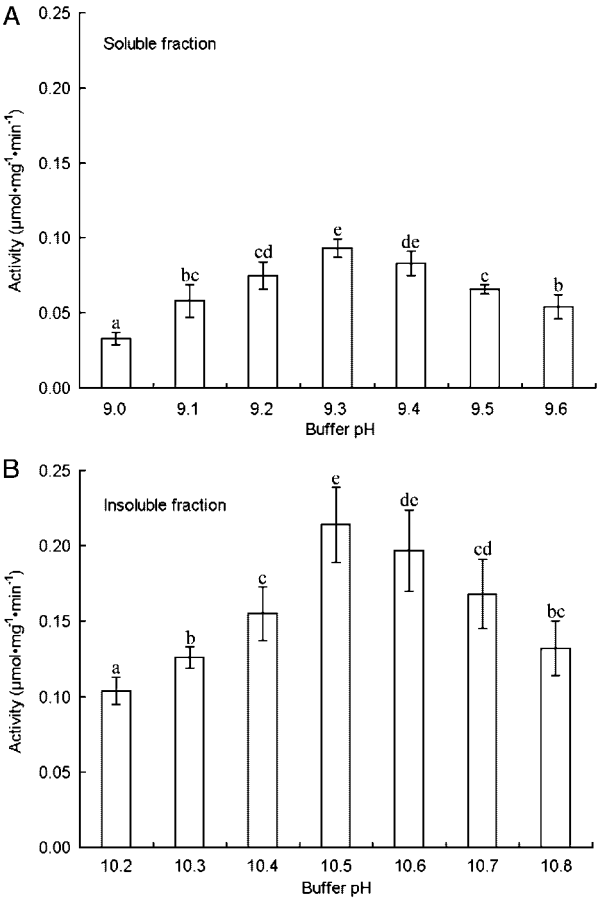

Figure 1. Determination of pH optimum for *Nilaparvata lugens* soluble (soluble fraction, **A**) and membrane-bound (insoluble fraction, **B**) ALP activities. The data represent mean values ± SE of at least three repeats. Different lowercase letters above the columns indicate significant differences at $P < 0.05$ level. ALP, alkaline phosphatase.

that whole body samples from macropterous female adults contain both sALP and m ALP forms. The results also showed, at pH optimum, that the majority of ALP activity was present in the insoluble fraction, which accounted for about 70% of the total activity. In the following experiments, ALP activity in two fractions was always measured at their pH optimum (pH 9.3 for soluble fraction and pH 10.5 for insoluble fraction).

Isolation of cDNAs Encoding Two ALPs

Searching the BPH EST Database (http://bphest.dna.affrc.go.jp/) with the amino acid sequences of *B. mori* sALP and mALPs gave two homologous fragments (Clone ID: NLMA5211 and C_NLMA1028) with 40–50% identity at the amino acid level. The full-length of *N. lugens* cDNAs corresponding to these two fragments were obtained using RACE technique. The first cDNA (Nl-ALP1; **FJ175380**) has an open reading frame (ORF) of 1,674 bp and 558 deduced amino acids, whereas the second cDNA (Nl-ALP2; **FJ175381**) has an ORF of 1,665 bp and 555 deduced amino acids. The predicted molecular masses for translated cDNAs of Nl-ALP1 and Nl-ALP2 were 62.7 and

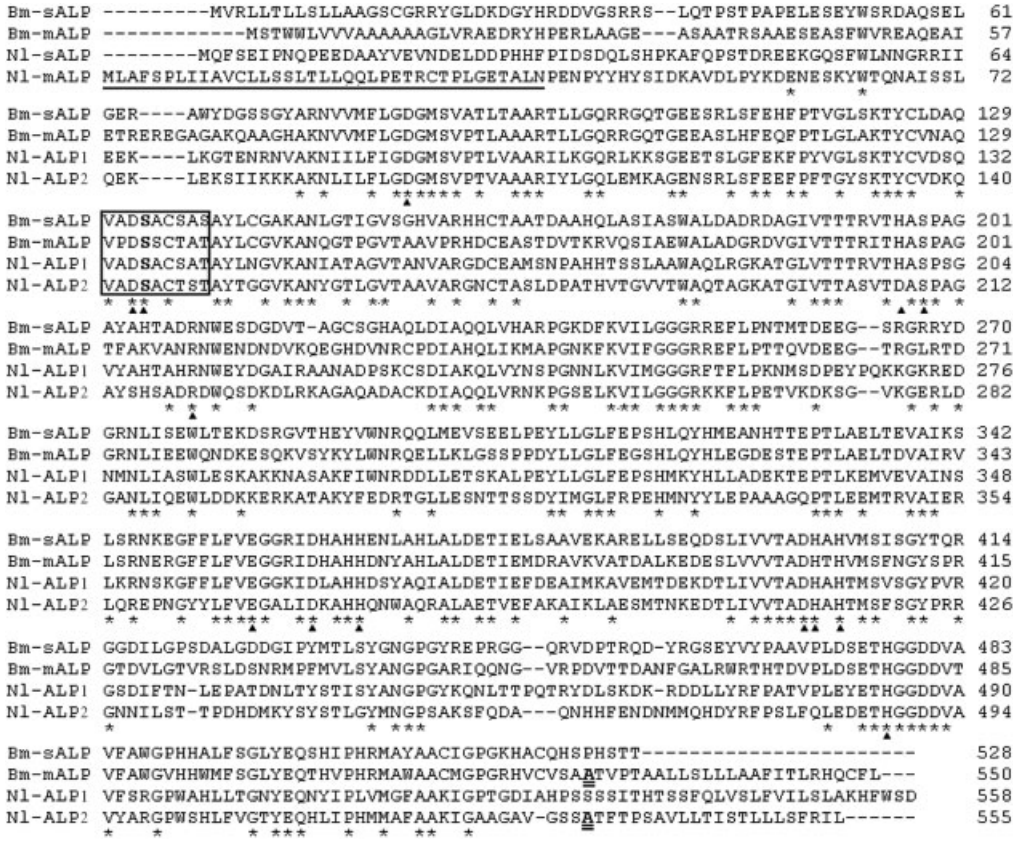

Figure 2. The alignment of amino acid sequences of soluble and membrane-bound ALPs from *Nilaparvata lugens* and *Bombyx mori*. Numbers on the right side of the alignment indicate the position of residues in the sequence of each protein. Identical amino acid residues are indicated by asterisks below the sequences. The signal peptide is underlined. Putative ALP signature region is included in a square, with the predicted active site in bold. Thirteen functionally important amino acids for ALP are indicated by triangles. Predicted GPI-anchor sites are in bold and doubly underlined. Bm-sALP and Bm-mALP (BAG41976 and BAG41977), *B. mori* soluble and membrane-bound ALPs; Nl-ALP1 and Nl-ALP2 (ACN29682 and ACN29683), two putative ALPs from *N. lugens*. ALP, alkaline phosphatase.

60.2 kD. In the deduced amino acid sequence of each cDNA (Fig. 2), there is one putative ALP signature motif and all 13 functionally important amino acids for ALPs (Sowadski et al., 1985). Both Nl-ALP1 and Nl-ALP2 show approximately the same degree of sequence identity (40–50%) to other insect sALP and mALP, although Nl-ALP2 contains a putative glycosylphosphatidylinositol (GPI) anchor (Fig. 2), which makes it difficult to predict which cDNA encodes a sALP or mALP.

Developmental Expression Patterns and the Regulation by 20E of Two Alkaline Phosphatase Genes. The developmental changes in enzyme activity for two ALP forms and developmental expression of the corresponding genes were compared, with or without 20E application (Fig. 3). From the ecdysis (first day) of the fifth instar macropterous female nymphs, the activities of both sALP and mALP were increased and peaked at the third day (the last day of the fifth instar). Both activities had a steep decline at the fourth day (the first day of adult), after which the mALP activity quickly

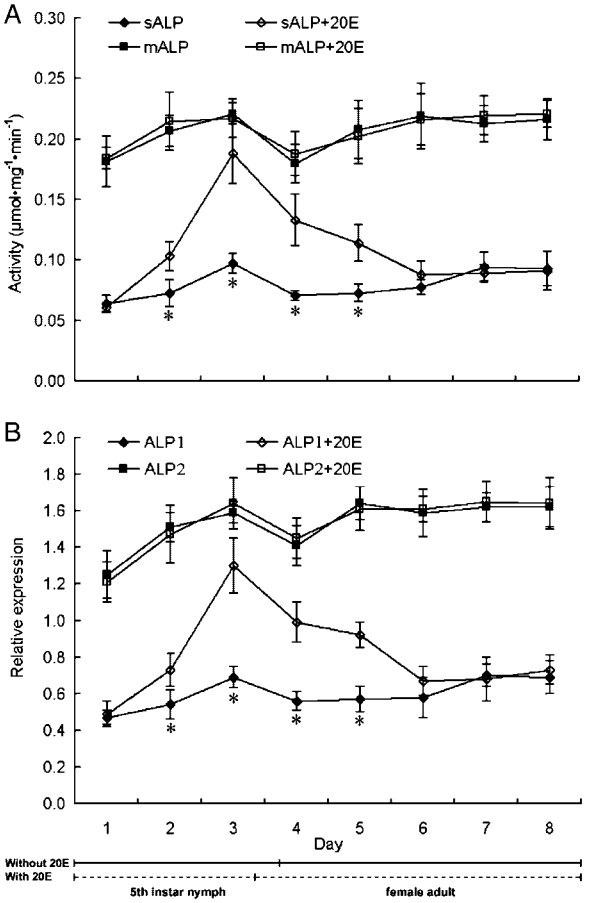

Figure 3. Developmental changes of ALP activities and gene mRNA levels and their regulation by 20E. (**A**) Activities of soluble and membrane-bound ALP. (**B**) Levels of two *Nilaparvata lugens* ALP mRNAs detected by the quantitative real-time RT-PCR. The dose of 20E was 8 ng/insect, applied to the fifth instar macropterous female nymphs (soon after ecdysis). Under the *X*-axis, the durations of fifth instar nymph and adult stages are indicated by single line for control (without 20E) and dotted line for 20E treatment (with 20E). The data represent mean values ± SE of at least three repeats. The mRNA level is normalized relative to β-actin transcript levels. *Indicates the significant differences at $P < 0.05$ level between nontreatments and 20E treatments. ALP, alkaline phosphatase; 20E, 20-hydroxyecdysone.

returned to similar level of the third day in following days (fifth to eighth days), but the sALP activity increased slowly from the fifth day and reached the peak again at the seventh day (Fig. 3A). Nl-ALP1 and Nl-ALP2 mRNA levels showed similar developmental patterns as the activities of both sALP and mALP, such as the peak at the third day and decline at the fourth day (Fig. 3B), with the calculated correlation coefficient of 0.96 between Nl-ALP1 mRNA levels and sALP activities and of 0.89 between Nl-ALP2 mRNA levels and mALP activities. After the fourth day, Nl-ALP1 mRNA level showed similar pattern as the sALP activity, such as the slow increase from the fifth day and reached the peak again at the seventh day. In contrast, Nl-ALP2 mRNA level showed similar pattern as the mALP activity after the fourth day, such as the quick increase at the fifth day. In all samples, the mALP activity was much higher than that of soluble enzyme, and the level of Nl-ALP2 mRNA was much higher than

Nl-ALP1, which is consistent with (but does not prove) that Nl-ALP1 encodes a sALP while Nl-ALP2 encodes a mALP.

When 8 ng of 20E/insect was applied to the fifth instar macropterous female nymph (soon after ecdysis), a large increase in both sALP activity and the level of Nl-ALP1 mRNA was observed at the third day (1.94-fold increase for the activity and 1.86-fold for mRNA level), which decreased gradually after the sixth day (Fig. 3). After 20E application, the correlation coefficient between Nl-ALP1 mRNA levels and sALP activities was 0.94. In contrast, no increase in mALP activity and Nl-ALP2 mRNA level was observed under the same conditions. These results provide additional circumstantial evidence that Nl-ALP1 encodes a sALP and Nl-ALP2 encodes a mALP. These results also indicate that the regulation of the sALP activity occurs mainly at the level of gene transcription. It was found that the 20E-treated nymphs had a slightly shorter duration of the fifth instar (3.39 ± 0.26 days) than the untreated nymphs (3.67 ± 0.32 days), although the difference was not significant at the $P < 0.05$ level.

Detection of Putative Nl-ALP1 and Nl-ALP2 Proteins in Different Populations

The above results suggested that Nl-ALP1 and Nl-ALP2 mRNAs might encode different ALP proteins. In order to confirm such suggestion, specific antibodies (Nl1-I and Nl2-I) were generated against bacterial fusion proteins containing N-terminal peptide sequences of Nl-ALP1 and Nl-ALP2. In soluble protein preparation from Sus strain, A specific band (expected molecular weight 64 kD) was detected by Nl-ALP1 specific antibody Nl1-I (Fig. 4, lane A), which was absent in lane C (insoluble protein preparation from Sus strain detected by Nl1-I). When Sus strain nymphs were treated with fenvalerate, the specific band was also found in soluble protein preparation (Fig. 4, lane B), which was with more density than that in lane A. In contrast, Nl-ALP2 specific antibody Nl2-I only detected a specific band (expected molecular weight 61 kD) in insoluble protein preparation (Fig. 4, lane D), but not in soluble protein preparation (lane F). The treatment of fenvalerate did not change the density of specific bands detected by Nl2-I in insoluble protein preparations from Sus strain (Fig. 4, lane D and E). The protein detection in soluble and insoluble protein

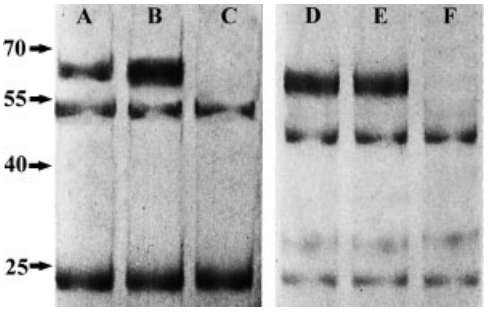

Figure 4. Specific detection of soluble and membrane-bound ALPs by Nl-ALP1- and Nl-ALP2-specific antibodies. Nl-ALP1-specific antibody Nl1-I was used to detect specific band in soluble protein preparation from Sus strain (lane **A**), soluble protein preparation from Sus strain treated with fenvalerate (lane **B**) and insoluble protein preparation from Sus strain (lane **C**). Nl-ALP2-specific antibody Nl2-I was used to detect specific band in insoluble protein preparation from Sus strain (lane **D**), insoluble protein preparation from Sus strain treated with fenvalerate (lane **E**) and soluble protein preparation from Sus strain (lane **F**). ALP, alkaline phosphatase.

preparations provided strong linkage of Nl-ALP1 and a sALP and of Nl-ALP2 and a mALP. Hence, Nl-ALP1 and Nl-ALP2 were denoted as Nl-sALP and Nl-mALP here.

Differential Expression of Two Alkaline Phosphatase Genes in Fenvalerate Susceptible and Resistant Strains

Enzyme activity of two different ALPs and mRNA level of the corresponding genes were measured and compared in fenvalerate-susceptible (Sus) and -resistant (R_{FEN}) strains (Fig. 5). The measurement was performed both at the second day of the fifth instar macropterous female nymphs (Figs. 4C, 5A) and in macropterous female adults at the day after eclosion (Figs. 5B and D). The differences were found in sALP activity (Figs. 5A and B) and Nl-sALP mRNA level (Figs. 5C and D), but not in mALP activity and Nl-mALP mRNA level.

Effects of fenvalerate application on enzyme activity and mRNA level were also examined after topical application of fenvalerate (0.8 ng/pest) to fifth instar macropterous female nymphs (soon after ecdysis) from Sus strain. Higher activity of sALP and higher Nl-sALP mRNA level were found in treated samples from the third to fifth days, with the biggest increases at the fourth day (1.59-fold increase in enzyme activity and 1.67-fold in mRNA level) (Fig. 6). In contrast, no increase in mALP activity and Nl-mALP mRNA level was observed under the same conditions. These results indicate that fenvalerate induces sALP activity, which occurs mainly at the level of gene

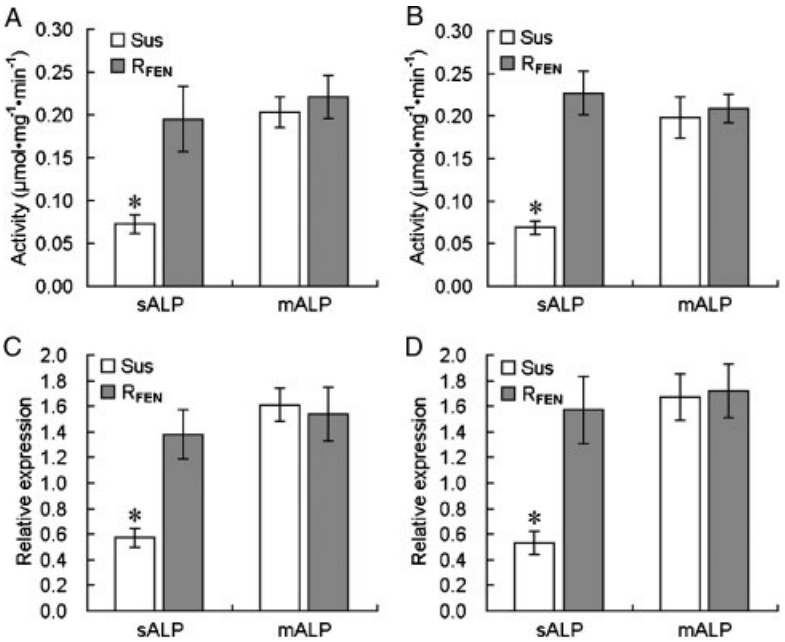

Figure 5. Comparison of soluble and membrane-bound ALP activities and the corresponding gene mRNA levels between fenvalerate susceptible (Sus) and resistant (R_{FEN}) populations. The data represent mean values ± SE of at least three repeats. ALP activities were determined both in the fifth instar macropterous female nymphs (**A**) and macropterous female adults at the day after eclosion (**B**). For comparison, mRNA levels were also tested in the fifh instar macropterous female nymphs (**C**) and macropterous female adults at the day after eclosion (**D**). *Indicates the significant differences at $P < 0.05$ level between Sus and R_{FEN} Strains. ALP, alkaline phosphatase.

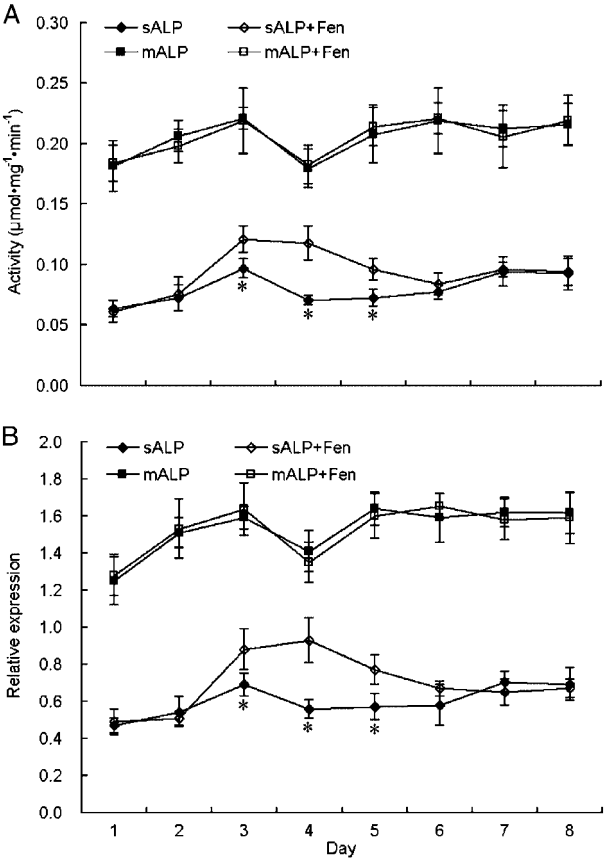

Figure 6. The regulation of ALP activities and gene mRNA levels by insecticide fenvalerate in Sus strain. (**A**) Activities of soluble and membrane-bound ALP. (**B**) Levels of two *Nilaparvata lugens* ALP mRNAs detected by the quantitative real-time RT-PCR. The dose of fenvalerate was 0.8 ng/pest (which was close to LD_{20} dose against Sus strain), applied to 5th instar macropterous female nymphs (soon after ecdysis) from Sus strain. The data represent mean values ± SE of at least three repeats. *Indicates the significant differences at $P < 0.05$ level between nontreatments and fenvalerate treatments. ALP, alkaline phosphatase.

transcription. These results also provide additional circumstantial evidence that Nl-sALP encodes a sALP and Nl-mALP encodes a mALP.

In order to find out whether fenvalerate application could induce ALP activities and mRNA levels in fenvalerate-resistant strain (R_{FEN}), the activities of two ALP forms and mRNA levels of two ALP genes were determined in R_{FEN} strains after fenvalerate treatment. Because significant induction in Sus strain was found only from second to fifth days, ALP activities and mRNA levels were determined only in these days for the fifth instar macropterous female nymphs from R_{FEN} strains after the fenvalerate treatment (Fig. 7). For ALP activities and mRNA levels, there was not any significant difference in mALP between the treated and untreated R_{FEN} nymphs, which is similar to the finding in Sus strain. In contrast, higher sALP activities and Nl-sALP mRNA levels were found in the fenvalerate-treated R_{FEN} nymphs than that in the untreated R_{FEN} nymphs, but a significant difference existed only at the fourth day. At the fourth day, the sALP activity and Nl-sALP mRNA level were induced by fenvalerate application to 1.25-fold and 1.21-fold of that in R_{FEN} strain, which was much lower

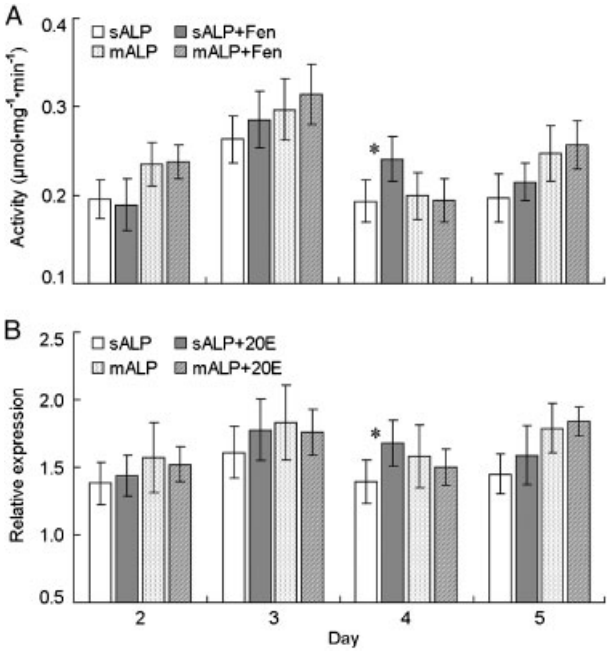

Figure 7. The regulation of ALP activities and gene mRNA levels by insecticide fenvalerate in fenvalerate resistance strain R_{FEN}. (**A**) Activities of soluble and membrane-bound ALP. (**B**) Levels of two *N. lugens* ALP mRNAs detected by the quantitative real-time RT-PCR. The dose of fenvalerate was 75.0 ng/pest (which was close to LD_{20} dose against R_{FEN} strain), applied to the fifth instar macropterous female nymphs (soon after ecdysis) from R_{FEN} strain. Because significant induction in Sus strain was only found from second to fifth days, ALP activities and mRNA levels were only determined in these days for the fifth instar macropterous female nymphs from R_{FEN} strains after the fenvalerate treatment. The data represent mean values ± SE of at least three repeats. *Indicates the significant differences at $P < 0.05$ level between nontreatments and fenvalerate treatments. ALP, alkaline phosphatase.

than that in Sus strain (1.69-fold and 1.61-fold for sALP activity and Nl-sALP mRNA level, respectively).

DISCUSSION

The brown planthopper, *N. lugens*, belongs to the Order Hemiptera, in which the biochemistry and molecular biology of ALPs are poorly studied. Although the distribution of ALP in the silkworm is confined mainly to the gut tissue and midgut is often used as material source in molecular biological studies (Eguchi, 1995), we used whole body samples to clone the cDNAs and to determine ALP activity and mRNA levels, because this insect is small and it is difficult to dissect the tissues, such as midgut, ovary, and fat body, from the nymphs or adults. We used ultracentrifugation to separate two forms of ALP, soluble and membrane-bound, finding that the membrane-bound form (mitochondrial+microsomal) of the enzyme accounted for the majority (close to three-quarters) of the total activity in both nymphs and adults. With sequence data from BPH EST Database (http://bphest.dna.affrc.go.jp/), we were able to clone two cDNAs, Nl-ALP1 and Nl-ALP2, encoding ALPs from *N. lugens*. Analysis of the deduced amino acid sequences of Nl-ALP1 and Nl-ALP2 indicated the presence of one

putative ALP signature and all 13 functionally important amino acids for ALP (Sowadski et al., 1985). In addition, Nl-ALP2 contains a putative GPI anchor site, which is absent from Nl-ALP1. However, it is difficult to judge which cDNA encodes a sALP or mALP, because both Nl-ALP1 and Nl-ALP2 show approximately the same degree of sequence identity (40–50%) to other insect sALP and mALPs. Developmental changes of enzyme activity and mRNA level might give some indirect information to distinguish two cDNAs between soluble and membrane-bound forms. The sALP activity and Nl-ALP1 mRNA level had similar developmental changes from the ecdysis of the fifth instar macropterous female nymphs, and mALP activity and Nl-ALP2 mRNA level had similar change patterns. 20E and fenvalerate application increased sALP activity and induced Nl-ALP1 expression, but not mALP activity and Nl-ALP2 expression. Taking together these data, it suggested that Nl-ALP1 might encode a sALP, while Nl-ALP2 might correspond to a mALP. In order to approve such suggestion, Nl-ALP1- and Nl-ALP2-specific antibodies were raised using N-terminal peptide sequences of the deduced amino acid sequences of these two genes. Nl-ALP1-specific antibody Nl1-I could only detect a specific band in the soluble protein preparation. In contrast, Nl-ALP2 specific antibody Nl2-I could only detect a specific band in the membrane protein preparation. These results provide conclusive linkages between Nl-ALP1 and a sALP and between Nl-ALP2 and a mALP. Hence, in this study, we propose to denote Nl-ALP1 as Nl-sALP (a sALP) and Nl-ALP2 as Nl-mALP (a mALP).

When 20E was topically applied to the fifth instar macropterous female nymphs, a large increase in sALP activity was observed at the third day, but not in mALP activity. mRNA level determination showed that only Nl-sALP expression was induced by 20E, which indicated that the regulation of the sALP activity occurred mainly at the level of gene transcription. In our previous study (Gu et al., 2009), the application of exogenous 20E regulated the enzyme activity of soluble trehalase, but not membrane-bound trehalase, which also occurred mainly at the level of gene transcription. The significant change in soluble trehalase activity (and also the mRNA level of the corresponding gene) was found at the fifth day after 20E application to fifth instar macropterous female nymphs (soon after ecdysis). However, in this study, significant change in sALP activity by 20E application was first observed at the second day and peaked at the third day, which indicated that the induction by 20E on sALP was much quicker than that on soluble trehalase. In *Drosophila melanogaster* and *Drosophila virilis*, JH and 20E led to a substantial increase in ALP activity indirectly via regulating dopamine level (Gruntenko et al., 2005; Rauschenbach et al., 2007a,b). The regulation of soluble trehalase activity by 20E was also thought to be indirect, perhaps being mediated by another hormone or substance (Gu et al., 2009). Although the regulation of soluble trehalase and ALP activity both would be indirect, they might be through different mechanisms, such as via regulating different hormone or signaling molecules, which make the response time (to 20E application) different in soluble trehalase and sALP.

Although P450 monooxygenases, esterases, and glutathione-S-transferases are defined as the most important detoxification enzymes against insecticides, some studies also reported the higher ALP activity in insecticide-resistant insects and induction of ALP activity by insecticides (Gao et al., 1996; Srinivas et al., 2004; Zibaee et al., 2009). In this study, the induction of ALP activity by the insecticide fenvalerate was also observed, which also occurred mainly at the level of gene transcription as with 20E. The induction by fenvalerate was also only observed in sALP activity. Different from 20E induction, the increase in sALP activity and Nl-sALP mRNA level was first

observed at the third day and peaked at the fourth day, which were with 1 day later than the induction by 20E. Because of the induction of ALP activity by fenvalerate, sALP and mALP activities (and the mRNA levels of the corresponding genes) were compared in fenvalerate-susceptible and -resistant strains. Higher sALP activity and Nl-sALP mRNA level, but not mALP activity and Nl-mALP mRNA level, were observed in a fenvalerate-resistant strain (R_{FEN}) than those in the susceptible strain (Sus). These results suggested that the sALP might be associated with fenvalerate resistance in *N. lugens*. In several diazinon-resistance strains of *C. suppressalis*, ALP activities were found significantly higher than that in a susceptible strain (Zibaee et al., 2009). It is easy to interpret the effects of ALPs on insect resistance to organophosphate insecticides, which contain phosphate molecules in their structures. However, it is difficult to explain the reason for the effects of ALPs on insect resistance against the pyrethroid insecticides. Hence, more direct evidence should be obtained to confirm the roles of ALPs in pyrethroid resistance in insects and the possible action modes.

In conclusion, here we have identified two forms of ALP and the corresponding genes from *N. lugens*, which is as far as we know the first identification of ALP genes in a hemipteran species. Although mALP activity accounted for the majority (about three-quarters) of the total enzyme activity, only sALP activity and the corresponding gene Nl-sALP mRNA were responsive to 20E and insecticide fenvalerate induction, and this ALP form was significantly different between fenvalerate-susceptible and -resistant strains. The possible role of sALP in insecticide resistance will be investigated in future. The study on *N. lugens* mALP will provide useful data for the development of GM rice against *N. lugens* and other related insect species, such as the small brown planthopper (*Laodelphax striatellus*) and the white-backed planthopper (*Sogatella furcifera*), especially at the progressive stage of GM rice development in China.

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