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Identification and biochemical characterization of *Laodelphax striatellus* neutral ceramidase

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Abstract

Ceramidases are a group of enzymes that catalyze hydrolysis of ceramides to generate fatty acid and sphingosine. In this study, we report the cloning and characterization of the rice small brown planthopper *Laodelphax striatellus* neutral ceramidase (nCDase), LsnCer. LsnCer was identified by sequencing the transcriptome of *Laodelphax striatellus*. LsnCer is a protein of 717 amino acids with a predicted molecular weight of 79.3 kDa. Similar to other known nCDases, LsnCer has a pH optimum at 8.0 and a temperature optimum at 37 °C for its *in vitro* activity. LsnCer activity is inhibited by Zn²⁺ significantly and Fe²⁺ slightly. LsnCer has broad substrate specificity with preference for ceramides with a medium acyl-chain or a mono unsaturated long acyl-chain. Infection with the rice strip virus (RSV) or treatment with insecticides significantly increased LsnCer mRNA expression and its enzymatic activity in *L. striatellus*. These results suggest that LsnCer is a *bona fide* nCDase that may have a role in adaption of *L. striatellus* to environmental stresses.

Keywords

nCDase; LsnCer; activity; mRNA; rice strip virus; insecticide

Introduction

Sphingolipids are essential lipid components of biological membranes and serve as a reservoir of bioactive lipids, such as ceramide, sphingosine and sphingosine-1-phosphate (S1P). Numerous studies demonstrate that ceramide or sphingosine mediates proliferation

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inhibition and programmed cell death (PCD) in mammalian and insect cells in response to various stressful signals (Hannun & Luberto, 2000; Woodcock, 2006). In contrast, S1P mainly promotes cell proliferation and survival (Mao & Obeid, 2008) although its aberrant accumulation may lead to cytotoxicity in insect or mammalian cells.

Ceramide, sphingosine, and S1P are metabolically interconvertible in cells. Ceramide can be hydrolyzed to form sphingosine, which in turn is phosphorylated to form S1P. S1P can be dephosphorylated to form sphingosine, which in turn is recycled back to ceramides. Enzymes responsible for interconversion among ceramide, sphingosine, and S1P may play an important role in biological responses mediated by these bioactive sphingolipids.

Ceramidases (E.C.3.5.1.23) are a group of enzymes that catalyze the hydrolysis of ceramide to form sphingosine. According to their pH optima for *in vitro* enzymatic activity, ceramidases are classified into three subgroups: the acid, neutral and alkaline ceramidase type. To date, 5 distinct ceramidase genes have been identified in human or mice: one acid, one neutral, and three alkaline ceramidase genes. The acid ceramidase, which is localized to lysosomes, is associated with Farber Disease (Bar *et al.*, 2001). NCDase is a critical enzyme in regulating the metabolism of bioactive sphingolipids and apoptosis (Choi *et al.*, 2003; Kono *et al.*, 2006). Alkaline ceramidases have distinct substrate specificity and regulate cell proliferation, apoptosis and adhesion (Hu *et al.*, 2010; Sun *et al.*, 2009; Sun *et al.*, 2010).

In the previous study, sphingolipids have been found to play important role in insect development (Kraut, 2011). In the insect *Drosophila*, two ceramidase genes have been identified: the nCDase (nCDase) and alkaline ceramidase (daCER). The nCDase is necessary for maintaining the photoreceptor homeostasis by regulating rhodopsin endocytosis (Acharya *et al.*, 2004). In our previous study, we demonstrated that nCDase is highly expressed in *Tribolium castaneum* reproductive organs (Zhou *et al.*, 2012), indicating that nCDase may be important in insect reproduction. Our group finds that daCER regulates the antioxidant ability and the lifespan of the *Drosophila* (Yang *et al.*, 2010).

The small brown planthopper *Ladephax striatellus* (Fallen) threatens the rice yield since 1960s (Zhu, 1996; Zhu *et al.*, 2005). *L. striatellus* is also the vector of the rice stripe virus (RSV). RSV is the pathogenic virus of the rice stripe disease. Rice stripe disease, which mainly occurs in temperate and subtropical areas, is one of the most economically devastating rice diseases (Zhu *et al.*, 2005). The control of *L. striatellus* still depends on insecticides, such as imidacloprid, chlorpyrifos and fipronil. The sub-lethal dosage of organic phosphorus insecticides could stimulate the reproduction of planthoppers such as *N. lugens* by up-regulating vitellogenin (Bao *et al.*, 2010). Whether insecticides impact the metabolism of sphingolipids remains unknown. In this study, we report the identification of nCDase in this insect and demonstrate that nCDase is highly elevated in response to the rice stripe virus or insecticide.

Results

Cloning of *L. striatellus* nCDase

To determine if the nCDase is expressed in other insects, we searched the *L. striatellus* transcriptome database with the *Drosophila melanogaster* nCDase sequence as a query. We found that a full-length open reading frame (ORF) of 2154 nucleotides (GenBank accession number JX569799), which was assembled from several cDNA contigs, encodes a putative protein highly homologous to the *Drosophila* nCDase. This ORF was cloned from *L. striatellus* RNA by RT-PCR and its sequence was confirmed by DNA sequencing (Figure 1A). Phylogenetic analysis classified the protein encoded by this ORF into the insect

nCDase cluster and showed that it possesses a high similarity to other known nCDases (Figure 1B), suggesting that this protein may be a *L. striatellus* nCDase (LsnCer).

Cellular location of LsnCer

To better understand the physiological function of LsnCer, we defined its cellular localization. LsnCer was overexpressed in High-five cells and LsnCer was analyzed by Western blot in both the medium and total membranes isolated from high-five cells. LsnCer was detected in the membranes but not the medium (Figure 2A), suggesting that LsnCer is a membrane-bound protein. To determine its subcellular localization, we tagged LsnCer with green fluorescent protein (eGFP) and expressed the LsnCer-eGFP fusion in High-five cells. Confocal microscopy demonstrated that LsnCer-eGFP was co-localized to the plasma membrane (PM) with the PM marker 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiIC18(3)) (Figure 2D), suggesting that LsnCer was localized to the PM.

Tissue-specific expression of LsnCer

To better understand the physiological function of LsnCer, we determined its tissue-specific expression. LsnCer mRNA levels in different organs were analyzed by qRT-PCR. It was found that LsnCer mRNA was expressed in all tissues examined and that its levels are higher in ovary and spermary than in head, midgut or malpighian tube (Figure 3A and B), suggesting that LsnCer is highly expressed in the reproductive organs.

Biochemical properties of LsnCer

To determine whether LsnCer has ceramidase activity, we measured ceramidase activity in membranes isolated from High Five cells transfected with the empty vector (pFast-HTB) or the vector carrying the full-length LsnCer ORF (pFast-HTB/LsnCer) at pH 7.4. Transfection with pFast-HTB/LsnCer increased ceramidase activity significantly compared with transfection with pFast-HTB (Figure 4A), suggesting that LsnCer has ceramidase activity. To biochemically characterize LsnCer, the membranes were prepared from High Five cells infected with pFast-HTB/LsnCer and pFast-HTB viruses. First, membrane-bound ceramidase activity was determined with C₁₂-ceramide (N-lauroyl-D-erythro-sphingosine) as a substrate at different pH values. We found that LsnCer exhibited the highest activity at pH 8.0 (Figure 4B), suggesting that LsnCer has an alkaline pH optimum for its *in vitro* ceramidase activity. Second, its activity in different temperatures ranging from 20 °C to 45 °C was also analyzed. Similar to other insect nCDase, LsnCer has the highest activity around 37 °C (Figure 4C). Little activity was detected at 45 °C. Third, the substrate specificity of LsnCer activity was determined with ceramides carrying different fatty acyl chains as substrates (Figure 4D). LsnCer efficiently hydrolyzed C₁₂-Cer (N-lauroyl-D-erythro-sphingosine), followed by C_{18:1}-Cer (N-oleoyl-D-erythro-sphingosine), C_{16:0}-Cer (N-palmitoyl-D-erythro-sphingosine) and C₆-Cer (N-Hexanoyl-D-erythro-sphingosine) with the highest activity toward C₁₂-Cer (Figure 4D). However, it hydrolyzed C₂₀-Cer (N-arachidoyl-D-erythro-sphingosine) and C₂₄-Cer (N-lignoceroyl-D-erythro-sphingosine) with a much lower efficiency and did not hydrolyze the short chain ceramide C₂-Cer (N-acetyl-D-erythro-sphingosine). These results suggest that LsnCer prefers medium to long-chain ceramides as substrates. Interestingly, we found that LsnCer hydrolyzed C_{18:1}-Cer and C_{24:1}-Cer (N-nervonoyl-D-erythro-sphingosine) more efficiently than C_{18:0}-Cer (N-acetyl-D-erythro-sphingosine) and C_{24:0}-Cer, respectively, suggesting that LsnCer prefers ceramides with unsaturated acyl-chains over ceramides with saturated acyl-chains of the same length. We also found that LsnCer did not use phytoceramides as substrates, suggesting that LsnCer is a ceramide-specific ceramidase. Finally, the cationic effects on LsnCer were investigated. LsnCer activity was determined in the presence of various cations

at 5 mM or in the absence of any cation. We demonstrated that among cations examined, Zn^{2+} and Fe^{2+} inhibited LsnCer activity by 80% and 20%, respectively (Figure 4E).

LsnCer expression in response to stresses

We established the rice strip virus (RSV)-infected *L. striatellus* colony and RSV-free colony in our lab. RNA was isolated from *L. striatellus* in different life stages/forms including nymph, female macropter, female brachypter, male macropter, and male brachypter. The RSV-infected *L. striatellus* and RSV-free insect were validated by PCR with RSV-specific primers. qRT-PCR analysis showed that LsnCer mRNA levels were significantly higher in the RSV-infected insect in every life stage/form compared to the RSV-free insect (Figure 5A). The most significant difference was found in the nymphal stage. To confirm the qRT-PCR results, we compared the ceramidase activity between RSV-infected and RSV-free *L. striatellus*. The membranes isolated from *L. striatellus* nymphs were assayed for ceramidase activity. Consistent with the qRT-PCR results, the ceramidase activity in RSV-infected nymphs was higher than that in RSV-free nymphs (Figure 5B).

We investigated how LsnCer responds to insecticide treatment. 4-instar nymphs were treated with imidacloprid, chlorpyrifos, or fipronil for different time periods before total RNA and membranes were isolated from these insects. qRT-PCR results showed similar changes in LsnCer mRNA expression in response to treatment with the three different insecticides. LsnCer mRNA expression began to rise at 1 h after treatment with each of the three insecticides and reached the highest level at 4 h. LsnCer mRNA levels began to decline at 12 h post insecticide treatment and reached the lowest levels at 24 h. At 48 h, the impact of each of the tested insecticides on LsnCer mRNA expression was diminished (Figure 6A). The change of ceramidase activity in the nymph followed a similar trend to the change of LsnCer mRNA levels in response to imidacloprid treatment (Figure 6B).

Discussion

Subcellular location of LsnCer

In our study, we cloned the *L. striatellus* nCDase. Sequence analysis showed that it had high similarities to nCDases of various organisms especially insects. Western blot analysis found that LsnCer is associated with membranes and that no LsnCer is secreted to medium, suggesting that LsnCer is a membrane-bound protein. Confocal microscopy revealed that LsnCer is mainly located to the plasma membrane. We previously demonstrated that the nCDase of *Tribolium castaneum* is also localized to the plasma membrane (Zhou *et al.*, 2010). These results suggest that the cellular location of nCDase is conserved among different insects.

NCDases from different insects have distinct biochemical properties

Biochemical characterization reveals that LsnCer has distinct biochemical properties from other insect nCDases. LsnCer has an alkaline pH optimum at pH 8 whereas nCDases from *Drosophila melanogaster*, mouse, rat, and humans have a neutral pH optimum (El Bawab *et al.*, 2000; Yoshimura *et al.*, 2002; Yoshimura *et al.*, 2004). LsnCer is also distinct in the pH optimum from the nCDase of *Tribolium castaneum*, which shows a broad pH optimum for its activity (Zhou *et al.*, 2010). Similar to the nCDase from *T. castaneum*, LsnCer has a temperature optimum at 37 °C for its in vitro enzymatic activity. This suggests that insect nCDases may have low cellular activity at rearing temperature. Similar to nCDase activity from *D. melanogaster*, mammals, and fungi (Mitsutake *et al.*, 2001; Ohlsson *et al.*, 2007; Okino *et al.*, 2010; Thayyullathil *et al.*, 2011; Yoshimura *et al.*, 2002), LsnCer activity is significantly inhibited by Zn^{2+} . Fe^{2+} inhibits LsnCer activity slightly whereas previous studies showed that Fe^{2+} significantly inhibits the activity of the nCDase of *D.*

melanogaster, *T. castaneum* and rice (Pata *et al.*, 2008; Yoshimura *et al.*, 2002; Zhou *et al.*, 2010). These results suggest that LsnCer is regulated by cations in a different manner from other nCDases. LsnCer can hydrolyze various ceramides with different acyl chains. It prefers medium to long-chain ceramides as substrates. Similar to fungi nCDases (Okino *et al.*, 2010), LsnCer also hydrolyzes ceramides with unsaturated acyl-chain more efficiently than ceramides with saturated acyl-chains of the same chain-length, suggesting that LsnCer and other nCDases prefer unsaturated ceramides as substrates. The physiological significance behind this substrate specificity remains unclear.

LsnCer is highly expressed in reproductive organ

We demonstrated that similar to nCDase from *T. castaneum*, LsnCer is a ubiquitously expressed nCDase with the highest expression in ovary and spermary. Other enzymes responsible for sphingolipid metabolism have been shown to be critical for *D. melanogaster* reproduction. Sphingosine-1-phosphate lyase mutant is abnormal in reproductive structure (Phan *et al.*, 2007). Fecundity was diminished in a *Drosophila* mutant deficient in the sphingosine kinase 2 gene (Herr *et al.*, 2004). These results suggest that the metabolism of sphingolipids is instrumental in insect reproduction. Because LsnCer hydrolyzes ceramides for generate sphingosine and fatty acid and sphingosine can be phosphorylated to form sphingosine-1-phosphate, LsnCer may regulate the levels of ceramides, fatty acids, and sphingosine-1-phosphate and thereby biological responses mediated by these bioactive lipids. The very-long-chain fatty acids are required in cleavage-furrow ingression during early telophase in *Drosophila* spermatocytes (Szafer-Glusman *et al.*, 2008). Sphingosine-1-phosphate protects ovarian cells from apoptosis (Morita *et al.*, 2000). The high expression of LsnCer in ovary and spermary may be important in the development of reproductive organs of *L. striatellus*.

The expression of LsnCer is affected by rice stripe virus

Rice stripe virus (RSV) is known to be transmitted mainly by *L. striatellus*, but the mechanism by which RSV infect *L. striatellus* is not fully understood. It has been reported that respiratory syncytial virus can stimulate nCDase in mammalian lung epithelial cells to enhance viral survival (Monick *et al.*, 2004). The wheat nCDase is also up-regulated upon infection by stripe rust fungus (Yu *et al.*, 2011). We demonstrated that both LsnCer mRNA and enzymatic activity are elevated by RSV infection, suggesting that like other nCDases, LsnCer may play a role in *L. striatellus* infection by RSV. This hypothesis is under investigation.

Insecticide stimulates LsnCer expression and LsnCer activity

Ceramide can accumulate in different species in a stressful environment. Exposure of rat renal mesangial cells to nitric oxide or tumor necrosis factor (TNF)- α stimulates rapid increase in ceramide level and ceramidase activity (Huwiler *et al.*, 1999). We are the first to report that nCDase is increased in response to insecticide (LD50). When *L. striatellus* is treated by insecticides, LsnCer mRNA level is increased immediately. It is significantly increased at 4 h after treatment and then falls back. LsnCer response to insecticide stress may change sphingolipid metabolism, which in turn may regulate adaptation of the insect to a stressful environment induced by the insecticide. This hypothesis is also under investigation. Sphingolipid signaling has been proven to play role in the disease development in mammalian (Rhome & Del Poeta, 2010) and pathogenic defense in plants (Shah, 2005). We demonstrate here that LsnCer takes part in *L. striatellus* adaption to the rice stripe virus infection and modulating the response to the insecticide stress. The role of sphingolipids in insect-virus and insect-insecticide interaction needs further research.

Experimental procedures

Insect and chemicals

RSV-infected and RSV-free small brown planthopper (SBPH) *L. striatellus* colonies were reared in the rice seedling at 25 °C with 16 h photoperiod at constant humidity in separated cages in insectary. High Five insect cells were grown in TNM-FH medium containing 10% FBS at 28 °C in a non-CO₂ incubator. Ceramides and *D-e*-Sphingosine with 18 carbons (C18SPH) and 17 carbons (C17SPH) were purchased from Avanti Polar Lipids. The following insecticides of technical grade were used: Imidacloprid (96.3%), Chlorpyrifos (97%), Fipronil (88%).

cDNA cloning

We sequenced *L. striatellus* transcriptome, in which, several contigs homologous to nCDase were found. The full-length coding sequence of *L. striatellus* nCDase (LsnCer) was obtained by sequence assembly. Total RNA was isolated from 6 adult *L. striatellus* with Trizol (Invitrogen). The first strand cDNA of LsnCer was synthesized using PrimeScript™ 1st Strand cDNA Synthesis Kit (Takara). The open reading frame (ORF) of LsnCer was amplified with the forward primer “TCTAGAATGACA CGCTTCACATTT” and reverse primer “AAGCTTCTATTCCTCTTTGGTATT” under the condition of one cycle of 94 °C 3 min, 35 cycles of 94 °C 30 s, 53 °C 30 s, 72 °C 2 min 30 s. The resulting PCR product was cloned into the pGEM-T vector (Promega, Madison, WI, USA), and the resultant LsnCer construct (pGEM-T/LsnCer) was sequenced to confirm the correctness of LsnCer ORF.

LsnCer expression construct

pGEM-T/LsnCer was digested by XbaI and HindIII to release the LsnCer ORF, which was subcloned into the vector pFast HTB. The resulting LsnCer expression construct (pFast-HTB/LsnCer) was sequenced before being transformed into *Escherichia coli* DH10Bac according to the manufacturer's instruction to obtain the bacmid used for transfection.

LsnCer overexpression in High Five Cells

The recombinant bacmid of pFast-HTB/LsnCer and pFast-HTB (control) were prepared and transfected with lipofectamine 2000 (Invitrogen) into High Five cells to obtain *Autographa californica nuclear polyhedrosis* (AcNPV). All the procedures for the production of viral particles were performed according to the manufacturer's instructions. Briefly, 1×10⁶ High Five cells were seeded in cell culture plates (Corning) one day before transfection. The first stock of recombinant baculoviruses was harvested 72 h after transfection and infected the next generation of High Five Cell at a multiplicity of infection (MOI) of 0.1. This was repeated for 5 times to obtain a baculoviral stock with high titer, which was maintained at 4 °C in a light-tight container. To express the recombinant LsnCer with a His tag, High Five cells grown in several 75 cm² cell bottles were infected with the viral stock.

Membrane preparation

The infected High Five Cells were harvested at 60 h post-infection. The cells were sonicated on ice in lysis buffer (100mM Tris, 25mM source, pH 7.4) containing protease inhibitor (Complete Mini EDTA Free, Roche). For preparing the membranes from *L. striatellus*, the nymphs were homogenized and sonicated in lysis buffer containing protease inhibitor. The cell lysates were centrifuged at 1,000 g for 10 min to discard the nuclear fraction, and the supernatant was centrifuged at 100,000 g for 1 h to obtain total membranes, which were resuspended in an assay buffer (25mM Tris, pH 7.4). The protein concentration was measured using bicinchoninic acid (BCA) protein assay kit (Pierce) according to manufacturer's instruction.

Western blotting

Proteins (40 µg per lane) from the membrane fraction were resolved on a 10% SDS-polyacrylamide gel before being transferred onto a nitrocellulose membrane. After being blocked in 5% skim milk, the nitrocellulose membrane was probed with anti-His antibody followed by anti-mouse IgG antibody conjugated to HRP. HRP-labeled antibody was detected by ECL Plus™ Western blotting detection reagent (ECL, Amersham Biosciences). Chemifluorescent signal was detected by FluorChem®FC2 (Alpha Innotech).

Subcellular localization study in High Five cells

To express LsnCer tagged with GFP (eGFP/LsnCer fusion), the expression construct pFast-HTB-eGFP/LsnCer was generated. The Bacmid containing pFast-HTB-eGFP/LsnCer was transfected into High Five cells as described earlier, and recombinant baculoviruses were harvested to re-infect High Five cells for 24 h. The infected cells were fixed with 4% paraformaldehyde and stained with plasma membrane dye 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI18(3)) before being examined under a confocal fluorescence microscope (Leica TCS SP5, Germany).

Ceramidase activity assay

LsnCer activity was measured essentially as described in our previous study. (Yang *et al.*, 2010)..

Insecticide treatment

Insecticide was diluted to a series of concentration in acetone. A droplet (0.051 ul) of insecticide solution was applied topically to the prothorax notum of *L. striatellus* 4-instar nymphs with hand microapplicator (provided by Nanjing Agriculture University). The treated nymphs were kept at standard condition (25 °C, 16L 8D) with rice seedlings in glass tubes. Median lethal dose (LD50) was determined at 24 h after insecticide treatment and analyzed by PoloPlus (provided by International Rice Research Institute). 4-instar nymphs from RSV-free *L. striatellus* colony were treated with each insecticide at LD50 for 1, 4, 12, 24, 36 and 48 h before LsnCer mRNA levels and ceramidase activity were determined.

Quantitative real time PCR (qRT-PCR)

Total RNA was isolated from different organs of *L. striatellus* adults of RSV-free and subjected to qRT-PCR analysis using SYBR® PrimeScript® RT-PCR Kit (Perfect Real Time) (Takara) according to the manufacturer's instructions. The actin forward primer was CCGCCTCCTCCAGTTCAC, the reverse primer was TGTCCAC GTCGCACTTCAT. The LsnCer forward primer was CGCACCACGAACGCTCTT, reverse primer was CGGCACGCACACTATCACAA. qRT-PCR was carried out on ABI PRISM 7300 PCR SYSTEM under condition of one cycle of 95 °C 30s, 40 cycles of 95 °C 5s, 60 °C 31s. A melt curve was performed at the end of each reaction to verify PCR product specificity. $\Delta\Delta\text{Ct}$ method was used to analyze the result. Changes in mRNA level of LsnCer for each treatment were normalized to that of actin.

Acknowledgments

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1 ATGACACGCTTCACATTTTGTTCCTTATTTTCACAACCTCTGCTTCAAGTGACGAAAATTTCAAGTAGGGCTGCCGAATCATTGTACACCATTTGGAGTTGGTATCGCAGACATAACTGGC
 1 M T R F T F L F L I F T T L L Q V T K I S S R A A E S L Y T I G V G I A D I T G
 121 CCACCAAGTGAAGTAGCCTTTATGGGCTACGCTAGAATGGACAGCGGGAGATGGTCTTCATCTTCGTGAGTTTCAAGAGCATTCATTCTGGATGATGCCATGAGCGCTTTGTATTT
 41 P P V E V A F M G Y A R M D Q R G D G L H L R Q F S R A F I L D D G H E R F V F
 241 GTGACAGCAGATTGCGGCATGATGGGAACAGGAGTGAGAGAAGCTATTTAAGAAAATAGAAAGCAGTTACCACGGACTGTATAACGAACAGAAATGTAATGCTGAGTGGAAACACATACG
 81 V T A D C G M M G T G V R E A I L K K L E S S Y H G L Y N E Q N V M L S G T H T
 361 CATTCCGCTCCAGGCGCTTTCTGATGCATTGATATTCGATTGAATACAATGGGATTGTGCCTGAAACATTCACAGCTCTTTGTTGATGAATAACCTGAGCATCATCCGAGCACAC
 121 H S A P G G F L M H L I F D L N T M G F V P E T F T A L V D G I T L S I I R A H
 481 GACAGACTGACTGGCGGCGCTGTTTCATGGCCAAGGCGGAGGTATTGGTGCTAGCATCAATCGCAGTCCGCAATCCTATCTACAGAATCCGCGCAGGAGCGGCCAAATACAAGGAT
 161 D R L T G G R L F M A K G E V F G A S I N R S P Q S Y L Q N P P Q E R A K Y K D
 601 GATGTGGACAAGACGCTGGTTACGCTTCAGTTGGAAGACTCTTCAGGAAGCCGTTGGTGCAATCTCTGGTTTGCACTGCATCCAGTCAGTATGACAAACCAATCGACTTGTATCC
 201 D V D K T L V Q L Q F E D S S G K P L G A I S W F A V H P V S M N N T N R L V S
 721 AGTGACAACATGGGCTTTGAGCATTACTTTTCGAGAAGAAAATGAACCTGGTTCTTTCTCGGGAAGGAGAGTTCTGTCGAGGTTCCGCTGAGCAATCTGGGAGACGTGTCGCC
 241 S D N M G F M A L F E K K M N P G S F P G K E F V A G S P
 841 AACGTGCGGGGACCGCTGCTCCGCTGTCGGCGAGCGGTGTGACGGAGTGAAGCTTGTGTCGGAACGCGATGCGTGTGTGGCGGCGGCGCGGCGGAGACATGTTTGACAGCACT
 281 N V R G P R C S V S G E P C D G V S S S C P N G D A C V A A G P G Q D M F D S T
 961 AAGCTGATCGCAAGCGCATCTCAATACGGCATGGGAAGTTTGGAAAGGCGAAGAGAGGAAGTGCCTGGCCGAATCAAAGTCATTACCAATTTGCTGACTTATCAAAAATGTCAACT
 321 K L I A K R I F N T A W E V W K G E R E E V R G R I K V I H Q F A D L S K M S T
 1080 ACCTATGTGGATCCTAGTAACGGGCTAGAGCGAATGGTGGAGGGTTGCCTGCCAGCTATGGGTTACAGTTTTCGCGCGGGAACACAGATGGGCGGGCTCTTTCTCATTAAACAGGCC
 361 T Y V D P S N G L E R M V E G C L P A M G Y S F A A G T T D G P G S F S F K Q A
 1211 ACACGCACCACGAACGCTCTTTGGAACGCATTCAGAACTTCATTACTGGGCAAGTTCAAGCAAGTGAATGCCAGAGTCCTAAACCGATTATTATGGCTACTGGAGAGATGAACCTT
 401 T R T T N A L W N A F R N F I T G P S S K Q V E C Q S P K P I L L A T G E M N F
 1331 CCGTTCAATGGCAGCCGTCAGTGGTGTCCACACAGCTGGCGTTTGTGCTGAGTTTGTGATAGTGTGCGTGC CGGGCGAGTTACAAACATGAGCGGCGCGCGGCTGCGGAGAACACTG
 441 P F E W Q P S V V S T Q L A F V G Q F V I V C V P G E F T T M A G R R L R R T L
 1451 CAGCAGACCATCAACCTTCCACCGAGCAACATGTATCATTTGCCGGTCTCTGCAACACCTACAGCGACTATGTCACCACACCTGAAGAGTACAATTTGCAAAAGTACGAAGGAGCTTCC
 481 Q Q T I N L P T E Q H V I I A G L C N T Y S D Y V T T P E E Y N L Q R Y E G A S
 1571 ACTATTTATGGCCCTCATACTCTCACACTCTACATGCATCATTACAACGAATGGTTGTTCTTCGCAACAGAAAAGCCATTGGACAGTGGTCCGAGACCTGCACAACCTATTGAATGAT
 521 T I Y G P H T L T L Y M H H Y K R M V V S S Q Q K S H L D S G P R P A Q L L N D
 1691 CTCATATCTCTGATCCGCGCTGTGCTGTATGACCGCTCTCCATGGTTCTCATCGTTCGGGACGTTGTAGTGCAGCCTCCATCCGAAGTGGCGCTGGAGACACAGTGAAGTCACTTTT
 561 L I S L I P P V L Y D R S P W F S S F G D V V V Q P P S E V R P G D T V Q V T F
 1811 GTGTCTGGCCATCCTCGAAATAATTTGAAACATGGAGAAACGTACTTGTGGTAGAGCGGTTAGTTGGAGATGATTGGGTGAACATAGCTACTGACGCAGATTGGGAGACAAAGTTTATG
 601 V S G H P R N N L K H G E T Y L L V E R L V G D D W V T I A T D A D W E T K F M
 1931 TGGGAGCAACTTCAACATTGCTGGGCACGAGCGAAGCCAAAGTTGAGTGGATTATCCCGGTGACGCAGAACCCGGCGACTACCGAATAAGGCACTTCGGACACTCCAAGTACTTATTT
 641 W E R T S T L L G T S E A K V E W I I P G D A E P G D Y R I R H F G H S K Y L F
 2051 GGAAACATAGAGCCCTACAATGGCACTTCGAAAATTTTCAAGGTCTTGAGTGACGCAATTGAGTCTAAGGAACCGCCGAACGGAATTTACAGACACAAATACCAAGAGGAATAG
 681 G N I E P Y N G T S K I F K V L S D A I E S K E P P N G I S D T N T K E E *

Figure 1. Sequence analysis of LsnCer

(A) The LsnCer coding and protein sequences. The deduced amino acid sequence of LsnCer is shown in one letter symbols below the nucleotide sequence. (B) Phylogenetic analysis of LsnCer. Classification of fungi, plant, insect, and mammalian nCDases was based on amino acid sequence similarities. Neighbor-joining tree was generated using MAGE 4 software. The numerals represent the confidence level from 1000 replicate bootstrap samplings.

Figure 2. *LsnCer* was localized to the plasma membrane

(A) Membranes and medium were prepared from pFastBac-HTB/*LsnCer* (*LsnCer*) and pFastBac-HTB (vector) cells and subjected to Western blot analysis with the anti-His $\times$ 6 antibody (1:1,000). (B) *LsnCer*-eGFP fusion protein was observed in green under confocal fluorescence microscope. (C) Plasma membrane was stained by 1,1'-diiodo-3,3',3',3'-tetramethylindocarbocyanine perchlorate (DiIC18(3)). (D) Merged. Extensive co-localization of *LsnCer*-eGFP with plasma membrane marker was observed in yellow.

Figure 3. Tissue specific expression of LsnCer

(A) mRNA levels of female adult *L. striatellus* nCDase of different organs. (B) mRNA levels of male adult *L. striatellus* nCDase of different organs. Transcript abundance was calculated based on the difference in threshold cycle (Ct) values between LsnCer and actin transcripts based on the normalized relative quantification $2^{-\Delta\Delta C_t}$ method. Data represent the mean value $\pm$ SE of three independent experiments performed in duplicate.

Figure 4. Biochemical properties of LsnCer

(A) The total membrane fractions from cells transfected with pFast-HTB/LsnCer (LsnCer) or cells transfected with pFast-HTB (vector) were assayed for ceramidase activity at pH 7.4. (B) LsnCer activity in the membranes of pFast-HTB/LsnCer and pFast-HTB cells were assayed at different pH values. pH was adjusted by adding the following buffer: NaAc (pH 5–6), Tris-HCl (pH 7–8), Glycine (pH 9–10). Ceramidase activity of the recombinant LsnCer at each pH value was computed by subtracting ceramidase activity in pFast-HTB membranes from that in pFast-HTB/LsnCer membranes. The LsnCer ceramidase activity at pH 8 was highest and set as 100%, and ceramidase activity at other pH values was expressed as % of the maximal activity. (C) Temperature dependence of LsnCer. The membranes were assayed for ceramidase activity at indicated temperature. Ceramidase activity of the recombinant LsnCer at each temperature was computed as mentioned. The LsnCer ceramidase activity at 37 °C was highest and set as 100%, and ceramidase activity at other temperature values was expressed as % of the maximal activity. (D) Substrate specificity of LsnCer. The membranes of pFastBac HTB/LsnCer and pFastBac HTB were assayed for ceramidase activity using indicated ceramide as substrate. Ceramidase activity was computed as mentioned. The LsnCer ceramidase activity was highest with substrate of C₁₂ ceramide and set as 100%, and ceramidase activity on other substrates was expressed as % of the maximal activity. (E) LsnCer activity was inhibited by different cations. The membranes of pFastBac HTB/LsnCer and pFastBac HTB were assayed for ceramidase activity in the presence of indicated cations. The LsnCer ceramidase activity with no cation added was highest and set as 100%, and ceramidase activity in the presence of different

cations was expressed as % of the maximal activity. All data represent the mean value of three independent experiments performed in duplicate.

Figure 5. Higher LsnCer mRNA level and nCDase activity were found in rice stripe virus infected *L. striatellus*

(A) mRNA level of LsnCer was higher in different life stage and forms of rice stripe virus infected *L. striatellus*. Transcript abundance was calculated based on the difference in threshold cycle (Ct) values between LsnCer and actin transcripts based on the normalized relative quantification $2^{-\Delta\Delta Ct}$ method. Data represent the mean value $\pm$ SE of three independent experiments performed in duplicate. (B) NCDase activity of rice stripe virus infected *L. striatellus* nymphs was higher than that of rice stripe virus free one. The ceramidase was assay as mentioned at pH 8.

Figure 6. Insecticide stimulated the LsnCer expression

(A) 4-instar nymphs were treated with fipronil, imidacloprid and chlorpyrifos with LD50. Nymphs treated with acetone were used as control. The relative mRNA level of LsnCer was analyzed by qRT-PCR at indicated time points. Transcript abundance was calculated based on the difference in threshold cycle (Ct) values between LsnCer and actin transcripts based on the normalized relative quantification $2^{-\Delta\Delta C_t}$ method. The mRNA level of acetone treated nymphs was set as 1 at each time point, the relative mRNA level of each insecticide treated insects was calculated by comparing with mRNA level of acetone treated ones. (B) The nCDase activity of imidacloprid treated 4-instar nymphs analyzed at indicated time points. Ceramidase activity was assayed at pH 8. The nCDase activity in acetone-treated nymphs

was set as 100% at each time point. Ceramidase activity of imidacloprid treated nymphs was expressed as % of the activity of the acetone-treated insects. Data represent the mean value $\pm$ SE of three independent experiments performed in duplicate.