A Cripavirus in the brown planthopper, Nilaparvata lugens

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A Cripavirus-like long unique sequence was identified during transcriptome sequencing of the brown planthopper (BPH), Nilaparvata lugens. This unique sequence demonstrated high similarity with the whole-genome sequence of cricket paralysis virus, including 5’ and 3’ untranslated regions; thus we considered it the whole genome of a new virus. We propose that the virus be named Nilaparvata lugens C virus (NlCV). The plus-strand RNA genome spanned 9144 nt, excluding a 3’ poly(A) tail with two large ORFs encoding structural and non-structural proteins, respectively. Detection of NlCV in BPH honeydew raised the hypothesis of horizontal transmission of the virus. Honeydew from viruliferous BPHs was used to feed non-viruliferous insects, the results of which indicated that the BPH could acquire NlCV through feeding and that the virus could multiply in the insect body. A tissue-specific distribution test using real-time quantitative PCR demonstrated that NlCV was mainly present in the reproductive organs, and the virus was detected in eggs laid by viruliferous female insects using nested PCR, indicating the possibility of vertical transmission as well. As no significant symptom was detected in the viruliferous BPH, NlCV is considered a new commensal virus of BPH. Interestingly, this virus was also detected in two other hemipteran insects, the white-backed planthopper and the horned gall aphid, indicating that NlCV might be present in many other hemipteran insects and have a wide host range.

INTRODUCTION

The brown planthopper (BPH, Nilaparvata lugens) is one of the most serious rice pests in East Asia and Southeast Asia. Besides sucking sap from the vascular bundle, it can transmit severe viruses to rice plants, such as rice ragged stunt virus (RRSV) and rice grassy stunt virus (Hibino, 1996). However, a novel reovirus, Nilaparvata lugens reovirus, was found in the BPH that did not cause visible symptoms in either rice plants or insects when compared with RRSV (Nakashima et al., 1996; Noda et al., 1991). Subsequently, several other viruses considered commensal viruses have been characterized in the BPH, i.e. Himetobi P virus (HiPV) (Huang et al., 2013; Nakashima et al., 2006), Nilaparvata lugens commensal X virus (NLCXV) (Nakashima et al., 2006), Nilaparvata lugens honeydew virus (NLHV) (Murakami et al., 2013, 2014) and Nilaparvata lugens endogenous nudi-virus (NIENV) (Cheng et al., 2014).

In our study on the BPH transcriptome, we identified a sequence highly similar to cricket paralysis virus (CrPV). CrPV was first characterized in 1970, and it is severely pathogenic and lethal in crickets (Reinganum et al., 1970). Subsequent investigations demonstrated that CrPV could infect lepidopteran larvae (Plus & Scotti, 1984; Reinganum, 1975), cultured Drosophila cells (Garrey et al., 2010; Moore et al., 1981; Scotti, 1975–1976), and Dacus oleae (Manousis & Moore, 1987; Manousis et al., 1988). CrPV is a plus-strand RNA virus with two long, non-overlapping ORFs in the RNA genome; each ORF is preceded by one internal ribosome entry site (IRES), including one intergenic IRES by which translation can be initiated without involvement of the initiation codon AUG and Met-tRNA (Costantino & Kieft, 2005; Jan et al., 2003; Pestova et al., 2004; Wilson et al., 2000a, b). CrPV also has a 3’ poly(A) tail (Eaton & Steacie, 1980). CrPV became a model for investigating virus–insect interaction (Kerr et al., 2015), as well as a research model of IRES (Schüler et al., 2006; Wilson et al., 2000a). Subsequently,
a new genus, *Cripavirus*, and a new family, *Dicistroviridae*, were established (Bonning & Miller, 2010). We were surprised to find a long, continuous sequence homologous to the CrPV sequence in the BPH transcriptome. Primers were designed to amplify specific fragments from the virus-like sequence, and confirmed the existence of the virus in BPHs. In this paper, this virus is referred to as Nilaparvata lugens C virus (NlCV). In transmission studies, the virus sequence was detected in BPH honeydew and was found to be transmitted horizontally.

**RESULTS**

**Organization of NICV RNA genome**

Using the annotation information on the BPH transcriptome (see Methods), a unique CrPV-like sequence was extracted and compared with the CrPV nucleic acid sequence (accession number NC_003924) through the BLASTN program. The nucleic acid identity was 83%. According to comparison through the BLASTP program, there was 92% identity and 95% similarity between the structural protein amino acid sequences of NICV and CrPV, and 92% identity and 95% similarity between their non-structural protein sequences. Compared with the CrPV genome sequence, we found that the first nucleotide of NICV at the 5′ end mapped to the 5′ untranslated region (5′UTR) first site of CrPV. The 5′UTR of NICV was from 1 to 664 nt in length and that of CrPV was from 1 to 708 nt. The nucleic acid identity of the two 5′UTRs was 87% with 44 gaps. The 3′ terminal region of NICV ended with a poly(A) tail. The NICV genome sequence has been submitted to GenBank (accession number KM270560).

The NICV genome sequence comprised 9144 nt, excluding the 3′ poly(A) tail, with 37.8% GC content. The coding sequence (CDS) of the virus genome was predicted through the FGENESV0 program in SoftBerry (http://linux1.softberry.com/), and two long, non-overlapping ORFs were obtained. To investigate the actual CDS region, the structural and non-structural protein sequences of CrPV were mapped to the NICV deduced amino acid sequence, and finally we identified the 665–5977 nt region as ORF1, coding non-structural protein, and the 6170–8860 nt region as ORF2, coding structural protein (Fig. 1). The two ORFs were preceded by their respective IRES, by which the ORF initiated translation. The initial triplet code of ORF2 was GCU.

**Phylogenetic analysis**

ORF1 contains the RNA-dependent RNA polymerase (RdRp) domain, and ORF2 encodes the virus capsid protein. According to the phylogenetic tree generated from the RdRp deduced amino acid sequences of members of the order *Picornavirales*, NICV was placed in the family *Dicistroviridae* (Fig. 2a). This result is consistent with the NICV characteristic of having two large ORFs in the genome. Using the deduced amino acid sequences of the non-structural protein to construct the phylogenetic tree of *Dicistroviridae*, NICV was located in the genus *Cripavirus* (Fig. 2b). NICV was closely related to CrPV while being quite distant from HiPV, another *Cripavirus* detected in BPH.

**Transmission of NICV**

To examine the virus carrier in different BPH laboratory colonies, three BPH populations reared in isolated rooms were tested. PCR determined that the HZ1 and FY populations were viruliferous, while HZ2 was non-viruliferous (Fig. S1, available in the online Supplementary Material). These populations were mixed, consisting of different sexes and instars. The virus was also detected in adult females and males, nymphs and egg samples (Fig. 3b). The results from the three control groups, which are described in Methods, indicated that the expected fragment was derived from NICV genomic RNA in BPH (Fig. 3c). In addition, an intense band, indicating a virus-specific fragment (Fig. S1e), was detected in two different laboratory colonies of the white-backed planthopper (*WBPH*), *Sogatella furcifera*, and its sequence was consistent with NICV.

Honeydew collected from HZ1 underwent RNA extraction (pure water underwent RNA extraction as the negative control). A band of the expected size was detected (Fig. S1d), indicating that NICV can be excreted in honeydew. This raised the hypothesis that NICV could be transmitted horizontally through honeydew. In order to test this hypothesis, the non-viruliferous HZ2 population was fed with honeydew collected from the viruliferous HZ1 population. The electrophoresis results (Fig. 4) demonstrated that the HZ2 BPHs were non-viruliferous at day 0, but a weak band indicating the expected sequence was detected in one of ten individuals at day 1 (Fig. 4, lane 6). A significant difference between the treatment and control groups was detected at day 7, when an obvious band indicating the NICV sequence was detected from nine of ten
individuals from the treatment group, while the control group remained non-viruliferous. This result indicated that NICV can be transmitted through honeydew and can be obtained through feeding. It is possible that BPHs ingest the virus when feeding on rice plants contaminated by honeydew from viruliferous insects. The results of day 1 and day 7 indicated that NICV can multiply in the insects.

To further confirm the replication of virus in insects, we designed a gene-specific primer, NS1, for negative-strand RNA reverse transcription and set up a control group for reverse transcription without primer. The virus-specific band was detected in the viruliferous samples using reverse transcription PCR (RT-PCR) and nested PCR (data not shown), indicating the presence of the negative strand of the virus.

**Fig. 2.** Phylogenetic tree of NICV. The tree was constructed using MEGA 5 software with the neighbour-joining method; bootstrap analysis of 1000 replicates was performed. (a) Phylogenetic tree generated from deduced amino acid sequence of RdRp. (b) Phylogenetic tree generated from structural protein sequence.
**NlCV virion detection**

The honeydew of the BPH HZ1 population was collected for NlCV virion observation. Spherical virions about 30 nm in diameter were detected (Fig. 5), which were similar in type and size to those of cripaviruses characterized in previous studies (Kerr *et al.*, 2015; Manousis & Moore, 1987; Reinganum, 1975). We tested for two other viruses suspected to be present in honeydew, i.e. NLHV (Murakami *et al.*, 2013) and HiPV (Huang *et al.*, 2013), using RT-PCR, and did not detect virus-specific fragments (data not shown).

**Virus distribution test in different tissues and eggs**

Real-time quantitative PCR (qPCR) was conducted to investigate the distribution of NlCV in different BPH tissues. An equal ratio of samples from males and females was tested and the result demonstrated that the ovary contained more NlCV than the other tissues, followed by the testis (Fig. 3a). This result indicates the possibility of vertical transmission of the virus.

As a further test of vertical transmission, we collected viruliferous female BPHs carrying eggs and put them on new rice seedlings in a glass tube. After laying, the eggs were picked out and RNA was extracted. In total, 140 eggs were collected, half of which were washed three times with 10 % NaClO solution for 10 min each time to remove possible virus contamination on the egg surface. The results of RT-PCR demonstrated that neither the washed nor the unwashed egg sample contained virus, whereas the female insects laying these eggs were detected to be viruliferous (Fig. 3b). Nested PCR was used to further test for NlCV in egg samples, and this time both washed and unwashed egg samples showed a virus-specific band (Fig. 3c). This result indicated that eggs laid by viruliferous female insects contained NlCV, probably at a very low titre.

**NlCV detection in rice plants**

We tested NlCV in rice seedling samples that were not exposed to insects, samples fed on by insects, and samples fed on by insects and then washed with 10 % NaClO.
solution. After RNA extraction and RT-PCR amplification, the results showed that rice seedlings not exposed to insects did not contain NlCV, whereas seedlings fed on viruliferous insects showed a virus-specific band that disappeared on washing (Fig. 6b). Using nested PCR to further test the washed seedling samples also gave a negative result. This indicated that NlCV originated from insects and not rice plants and that the virus was found mainly on the surface of the plant.

As a further test, we designed an experiment in which viruliferous BPHs fed on clean rice seedlings. In this experiment the seedlings were divided into five groups (Fig. 6a). The results showed that seedlings of Groups 1 and 3 were infected with NlCV on day 1, while the virus could not be detected in the Group 2 sample, which was washed with NaClO solution. The virus was not detected in the control group. Subsequently, the insects in Groups 1 and 2 were removed. On day 7, the virus in Groups 1 and 2 seedling samples was no longer detectable, which indicated that the virus could not multiply in the absence of viruliferous insects. The Group 3 sample showed virus as expected, while no virus was detected in the Group 4 sample, which had been washed (Fig. 6c). Using nested PCR to further test Groups 2 and 4 and the control group, the virus was still not detectable (data not shown). All the results indicated that NlCV was not able to replicate in rice.

**DISCUSSION**

To our knowledge, the fact that the virus was detected in excreta such as honeydew means that a large amount of virions are present in the BPH gut. NLHV, proven to be transmitted horizontally through honeydew, belongs to **Iflaviridae**, which infect the midgut of insects (Murakami *et al.*, 2013). However, the qPCR results showed that the ovary had the highest virus content, while the content of the gut was significantly lower. The virus content was higher in the reproductive organs and in eggs laid by viruliferous female insects, indicating the possibility of vertical transmission of the virus and a diversity of transmission pathways.

When CrPV was first identified in the cricket, it was found in the egg, epidermis, gut and ganglion (Reinganum *et al.*, 1970). Subsequent research demonstrated that CrPV could
infect lepidopteran larvae (Plus & Scotti, 1984; Reinganum, 1975) and the dipteran insect *Dac. oleae* (Manousis & Moore, 1987; Manousis et al., 1988), and could also multiply in *Drosophila* cells (Scotti, 1975–1976). In this study, a virus highly similar to CrPV was first detected in the hemipteran insects BPH and WBPH. In addition, in our study of the transcriptome of the horned gall aphid, *Schlechtendalia chinensis*, collected from fields in Xianju, Zhejiang, PR China, we found a unique 7380 nt sequence that was almost the same as that of NICV (unpublished results), and a band of the expected size was detected through PCR using the primer pair NICVF and NICVR (Fig. S1f). This result indicates that NICV might be present in other hemipteran insects and have a wide host range. The question is how these insects acquire the virus initially. A previous study demonstrated that crickets could be infected by CrPV through feeding on viruliferous lepidopteran larvae, raising the hypothesis that CrPV came from lepidopteran insects and that it is transmitted to crickets through the food chain (Reinganum, 1975). Based on our study, we presume that planthoppers acquire NICV by feeding on plants contaminated by the excreta of viruliferous insects.

CrPV was characterized as pathogenic and lethal to the host cricket (Reinganum et al., 1970) and *Dac. oleae* (Manousis & Moore, 1987). However, we did not observe any visible symptoms in BPHs infected by NICV, nor did we detect significantly different death rates between the treatment and control groups in the virus-feeding experiment (data not shown). This indicates that NICV might be a commensal virus in the BPH, and the effect of NICV on planthoppers warrants further study. Various commensal viruses have been detected in BPH, as described above, multiplying in the insect without negative effects on the host (Cheng et al., 2014; Huang et al., 2013; Murakami et al., 2013, 2014; Nakashima et al., 2006; Noda et al., 1991). Although the function of commensal viruses in BPHs remains a mystery, these viruses might reflect a tendency of virus–host coevolution in the invertebrate world. Is non-pathogenicity an evolutionary trend of viruses during virus–host interaction? A commensal virus in *Heliothis armigera* was characterized as beneficial to the host (Xu et al., 2014). The discovery of the DNA virus NIENV, which integrates into the BPH genome, provided a new perspective of virus–host coevolution (Cheng et al., 2014). The reason why and mechanism by which the BPH coexists with numerous viruses is unclear, but this phenomenon indicates that the BPH can host many viruses. With the sequencing and analysis of the BPH genome (Xue et al., 2014), which has provided a considerable amount of information on BPH, this insect can be a good model for investigating the coevolution of viruses in hemipteran insects.

**METHODS**

**Insects.** A laboratory colony of BPHs was reared on rice seedlings under constant 25 ± 1 °C temperature and 16 h of daily illumination. Three populations collected from different locations and reared in isolated rooms were used in this study. HZ1 was collected from rice fields on Huajiachi Campus of Zhejiang University, Hangzhou, Zhejiang, PR China. HZ2 was collected from rice fields on Zijingang Campus of Zhejiang University, Hangzhou, Zhejiang, PR China. FY was collected from rice fields in Fuyang, Zhejiang, PR China.

**RNA-Seq and phylogenetic analysis.** The HZ1 population, which was collected in 2009 and reared in our laboratory, was used as the source of total RNA for transcriptome sequencing. Raw data were obtained through the Illumina HiSeq 2000 sequencing platform and were assembled into unigenes using Trinity software. Unigenes were annotated using BLASTX against main databases such as NCBI-nr and Swiss-Prot. Based on the annotation information, a CrPV-like sequence was found and extracted (GenBank accession number KM270560).

The deduced amino acid sequences of many members of the order *Picornavirales* were downloaded from the NCBI database (Table S1). Multiple alignments of amino acid sequences were generated using CLUSTAL_X software. The phylogenetic analysis was performed using MEGA5 and the neighbour-joining method.

**PCR examination of NICV.** Total RNAs of BPHs and rice seedlings were extracted using TRIzol reagent (TaKaRa). First-strand cDNA was prepared using ReverTra Ace qPCR RT Master Mix kit including a gDNA remover reagent kit (Toyobo), using 1 μg total RNA template in a 10 μl reaction following the manufacturer’s protocol. To detect the negative-strand RNA of NICV, the primer NS1 (5’-GCCAGGC-ATAGCTGGATCGTCAATCTCA-3’), derived from the 5’UTR of the positive-strand RNA, was used for reverse transcription, using the First Strand cDNA Synthesis kit (Toyobo). RNA samples were treated with DNase to remove genomic DNA before reverse transcription. After reverse transcription, the products were amplified by PCR and nested PCR.
Specific primers were designed using the Primer Premier 5 program. The primer pair NlCV (5’-TAGTGAATGCCACGGTTACGC-3’) and NlCVR (5’-CCTGAACCCATCCAATCCTGT-3’) was used to amplify a 597 bp sequence fragment to verify the presence of NlCV. Nested PCR was further performed using the primer pair NlCVnestF (5’-AACATCAGAGTCGGTCAGGC-3’) and NlCVnestR (5’-AAATCGTCGTCACCAGTTCC-3’) when the RT-PCR results were negative. Three control groups were used to ensure the reliability of the results: (1) RNA template without insect sample; (2) normal RNA template but without reverse transcriptase during cDNA synthesis; and (3) no cDNA template when PCR was conducted. To monitor the quality of the template prepared, primers for eukaryotic elongation factor 1A (eEF1A) of BPH, i.e. BPHeEF1A-f and BPHeEF1A-r (Murakami et al., 2013), were used for control PCR amplifications of the same batch of RNA templates. To confirm that the fragment was derived from NlCV, it was purified, ligated with T-vector and transformed into TG1-competent cells to be cloned and sequenced.

To investigate the virus distribution in different BPH tissues, real-time qPCR was conducted using the primer pair CVF (5’-GTTCAAGTTCTCTTCAAGACGC-3’) and CVR (5’-TCCTTCTCACAGTTGGAATCGAC-3’). Six types of tissues were analysed: gut, fat body, testis, ovary, salivary gland, and integument. Sample dissection, complementary DNA preparation, and qPCR manipulation were performed as described by Ye et al. (2015); three technical replicates and three biological replicates were performed. We used three housekeeping genes – 18S, actin 1 and mitochondrial ribosomal protein S11 (RPS11) – as endogenous references in real-time qPCR. The results showed that the Cq values of 18S and RPS11 were stable in insects.
with or without NICV, and in different tissues. Finally, the BPH housekeeping gene 18S rRNA (GenBank accession number JN662398.1) was used as the endogenous reference gene. Relative quantitative variation was evaluated using the 2^{-\Delta\Delta Ct} method (Livak & Schmittgen, 2001).

**Collection of BPH honeydew.** Parafilm was used to form a closed chamber surrounding the base of large rice seedlings (Fig. 2). About 200 BPHs were placed in each chamber and they fed on the rice for 24 h. Honeydew drops attached to the film were collected by pipette. We used 60–100 μl pure water to wash off the residual honeydew drops on the film.

**Surface cleaning of samples.** Sodium hypochlorite (NaClO) had been used previously for sterilization of eggs to remove the virus on the surface (Guy et al., 1992). In our experiment, 10 % NaClO solution was used to wash the surface of insect eggs and rice seedlings. Samples were put into 1.5 ml centrifuge tubes, NaClO was added and washing took place for 10 min. The washing step was repeated three times. Then samples were rinsed three times in pure water.

**Electron microscopic detection of NICV virions.** To detect NICV virions, honeydew from 200 viruliferous BPHs was collected as described above and diluted in 15 ml pure water. The samples were ultracentrifuged at 4 °C and 159 800 g for 2 h. The supernatant was discarded and the sediment was suspended using 40 μl pure water. The sample was negatively stained with 2 % phosphotungstic acid and observed with an electron microscope.

**Transmission test.** The method of feeding non-viruliferous insects with honeydew from viruliferous insects to verify horizontal transmission of the virus was described by Murakami et al. (2013). Honeydew was collected from about 500 viruliferous BPHs and diluted to 4 ml using 0.09 g ml^{-1} sucrose solution. Non-viruliferous adult BPHs were confined within a glass tube modified according to the method of Fu et al. (2001), and each tube contained 10 adult BPHs fed with the sucrose solution through stretched Parafilm. The treatment group contained 80 adult BPHs fed with sucrose solution containing honeydew, while the 20 adult BPHs in the control group were fed with sucrose-only solution; both groups were cultured at 26 °C and 80 % humidity for 24 h. Before treatment, the RNA of 10 individual insects was extracted to test for virus presence, and this was recorded as day 0. After the 24 h virus acquisition, BPHs were placed on rice seedlings. Ten individuals from the treatment group were used to test for virus presence, and this was recorded as day 1. At day 7, 10 individuals from each of the treatment and control groups were examined for NICV by RT-PCR.

**Virus testing in rice plants.** To test whether NICV can multiply in rice plants, clean rice seedlings that had not been exposed to insects were used to feed viruliferous insects. In this experiment, five groups were set up (Fig. 6). Each group consisted of four rice seedlings and ten insects from the viruliferous colony. On day 1, two rice seedlings in Groups 1, 2 and 3 and the control group were picked out, and RNA was extracted in order to test for virus. Samples from Group 2 were washed with NaClO solution before RNA extraction. Then the insects in Groups 1 and 2 were removed. On day 7, each group of rice seedlings was tested for virus by RT-PCR; those of Groups 2 and 4 were washed with NaClO solution before RNA extraction.

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