Isolation of the Thosea asigna virus (TaV) from the epizootic Setothosea asigna larvae collected in South Sumatra and a study on its pathogenicity to Limacodidae larvae in Japan

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In 2005, natural epizootics were observed during an outbreak of Setothosea asigna (Lepidoptera: Limacodidae) larvae in an oil palm plantation in South Sumatra, Indonesia. The causative agent was filterable, which implied it was a virus. Since preliminary testing using reverse PCR gave a positive result for the Thosea asigna virus (TaV), we experimented with the purification of the viral agent from the diseased larvae. Electron microscopy revealed nonenveloped virus-like particles that were spherical in shape and about 40 nm in diameter. cDNA cloning followed by sequencing demonstrated that RNA purified from the particles contained two large open-reading-frames (ORFs) with a partly shared sequence and extensive homology (>98% identity at the nucleotide level) with ORFs of TaV encoding an RNA-dependent RNA polymerase and the capsid protein, respectively. 3' RACE suggested that there is, like in TaV genomic RNA, no poly(A) tract at the 3'-terminus of the RNA. The pathogenicity of the purified particles against Limacodidae larvae in Japan was demonstrated to be very strong for Monema flavescens and Austrapoda dentata. These results indicated that the agent causing the epizootic disease among S. asigna larvae in the oil palm plantations was TaV which also has potential as a biological control agent for Limacodidae pests in Japan.

Keyword: Oil palm plantation, Limacodidae, Viruses, Tetraviridae, TaV

INTRODUCTION

The increasing significance of oil palm plantations for the Indonesian economy has raised serious concerns. The plantations regularly use herbicides, insecticides and fertilizers in large amounts. There is a great fear that these agricultural practices cause enough soil- and water-pollution to harm animals and also humans.

S. asigna (Lepidoptera; Limacodidae) is a major defoliating pest of oil and coconuts palms, with outbreaks recorded in north-eastern Sumatra, Borneo and western Malaysia (Holloway et al., 1987). Feeding on a wide range of host plants, S. asigna has also become an economically important defoliator of oil palms, Elaeis guineensis. Their life cycle is about 86-115 days, including a larval stage (1st to 9th instar) of 35-49 days (Godfray et al., 1987). In the outbreak, leaf surfaces are rapidly destroyed (up to 50%), resulting in more than a 78% loss of production in the first year and 40% in the second year (Sipayung, 1987).

In South Sumatra, the first outbreak of S. asigna, with a population density of around 13 larvae per frond, was observed in 1999, and continued up to 2002 with more than 25 larvae per frond (Rahmadsyah et al., 2003). In 2006, the population of S. asigna reached 50 larvae per frond. At this population level, only rib leaves remained on the tree. Furthermore, S. asigna can cause caterpillar dermatitis involving serious pain to people in contact with the larvae (Rahmadsyah et al., 2006).

Natural pathogens play an important role in the regulation of S. asigna populations in oil palm plantations, since outbreaks of the insects are not infrequently terminated by epizootic disease in the pests. In Indonesia, epizootic outbreaks of S. asigna in oil palm plantations have been observed since 1996 (Rahmadsyah et al., 2003). Farmers collect the diseased larvae, store them in a freezer and, during outbreaks of S. asigna, spread them on oil palm leaflets. This has provided good control of the populations of pests in South Sumatra (Rahmadsyah et al., 2003), though the causal agent in S. asigna has not been identified.

In this study, we isolated a Thosea asigna virus (TaV) from S. asigna larvae suffering from epizootic disease in oil palm plantations in South Sumatra. Subsequently, we examined the pathogenicity of the purified TaV against Limacodidae larvae in Japan.
MATERIALS AND METHODS

Source of the agent causing epizootic disease

The S. asigna larvae suffering from epizootic disease were collected from oil palm plantations in South Sumatera, and were kept frozen at ~80°C prior to processing for the experiments.

Diagnostic analysis using PCR with a primer set targeting a tetravirus

RNA was extracted from S. asigna larvae using Catrimox™ (Takara) according to the manufacturer’s instructions and used as a template for reverse transcription-PCR (RT-PCR) with a One-Step RT-PCR kit (Invitrogen). RT-PCR was performed as recommended by the manufacturer using a primer set targeting the RdRp gene of TaV (Gen Bank accession No. AF 282930), G1 (5'-gagcaattag gagagttactccg-3', nucleotides (nts) 343-364) and G2 (5'-tegggtgtgtttactccct-3', nts 572-597) (Fig. 1). The amplified DNA was then analyzed by agarose gel electrophoresis. The DNA fragments visualized with ethidium bromide staining were extracted from the gel, cloned into a pGEM-T Easy plasmid vector (Promega) and introduced into the competent cells of the E. coli strain DH5α as reported (Yamagishi et al., 1999). Plasmid DNAs prepared from the transformed bacterial colonies (white) in the agar plates containing X-Gal were sequenced using an automatic DNA sequencer (ABI PRISM 3130, Applied Biosystems).

Purification of viruses from the frozen Setotho-sea asigna

The purification of viral particles was carried out according to the method described by Yi et al., (2005) with minor modifications. In brief, the frozen insect larvae (200 g) were thawed and homogenized in 500 ml of extraction buffer (50 mM Tris/HCl, 1 mM EDTA (pH 7.5), and 0.2% 2-mercaptoethanol). The homogenate was clarified by centrifugation at 10,000 × g for 30 min, and the supernatant was then centrifuged at 100,000 × g for 3 h. The pellets were resuspended in 50 ml of TE buffer (50 mM Tris/HCl, and 1 mM EDTA (pH 7.5)), layered on TE buffer containing 30% sucrose (w/v), and then centrifuged at 100,000 × g for 3 h. The pellets were resuspended in 5 ml of TE buffer and subjected to further purification by a sucrose gradient (10 to 40% w/v, in TE buffer) spun using an oligo(dT) primer with a unique sequence at its 3'-terminus supplied by the manufacturer, and then amplified with a gene-specific primer, 3F (5'-ccagaggggtgcctccacagg-3', nts 572-597) targeting the complete coding sequence for the capsid protein gene (CP) of TaV (AF062037). The PCR products were ligated into the pGEM-T Easy vector (Promega), and sequenced as above. Sequences of the inserted DNAs in the clones obtained in this study were analyzed using the computer programs BLAST (http://www.ncbi.nlm.nih.gov/) and Multalin (http://bioinfo.genopole-toulouse.prd.fr/multalin).

3' RACE analysis

The rapid amplification of cDNA ends (RACE) method was used to determine the complete nucleotide sequence of the 3' terminus of the RNA extracted from the particles. The RNA was polyadenylated with poly(A) polymerase (Invitrogen) in the presence of ATP before ligation with the RNA oligo adapter provided by the manufacturer. As a control, we carried out an experiment where polyadenylation at the 3'-terminus of the RNA was skipped. In the 3' RACE, the first strand of cDNA was synthesized using an oligo(dT) primer with a unique sequence at its 3'-terminus supplied by the manufacturer, and then amplified with a gene-specific primer, 3F (5'-ccagaggggtgcactgg-3', nts 2321-2340, AF062037) and GeneRacer™ 3' Primer (5'-gctgctcaacgatactgaagtactgg-3') targeting the unique sequence of the oligo(dT) primer.

The PCR products were ligated into a TA TOPO cloning plasmid vector (Invitrogen), and the ligation mixture was used to transform One Shot® TOP10 Competent Cells stained with 2% (w/v) sodium phosphotungstate (pH 7.2) on formvar-coated grids, and examined with a transmission electron microscope (Hitachi H-800).

Extraction of RNA from the purified particles, cDNA cloning and nucleotide sequencing

RNA was extracted from the purified viral particles using TRIzol (Invitrogen) reagent according to the manufacturer’s instructions, except for the addition of glycogen to enhance RNA precipitation. The RNA pellet was resuspended in 10 μl of DEPC-treated water. The integrity of the RNA extracted from the particles was examined by electrophoresis on a 1% agarose gel containing formaldehyde (final concentration, 2.2 M). Complementary DNA (cDNA) against the RNA was then generated with random primers using a cDNA synthesis kit (Takara) according to the manufacturer’s instructions. The resulting cDNAs were used as templates for the subsequent PCR. Two primer sets were used in the PCR. One set, 5'PRdRp (5'-ctcctttagagggtgtgc-3', nts 1-19) and 3'PRdRp (5'-cgtccctctctctctgct-3', nts 3752-3768), targeted the complete coding sequence for the RNA-dependent RNA polymerase gene (RdRp) of TaV (Gen Bank accession No. AF282930). The other set, CP1 (5' cgaatgatagtgcttttg 3', nts 85-102), and CP2 (5'-atcattttggaaaaaggtgcgc-3', nts 2461-2482), targeted the complete coding sequence for the capsid protein gene (CP) of TaV (AF062037). The PCR products were ligated into the pGEM-T Easy vector (Promega), and sequenced as above. Sequences of the inserted DNAs in the clones obtained in this study were analyzed using the computer programs BLAST (http://www.ncbi.nlm.nih.gov/) and Multalin (http://bioinfo.genopole-toulouse.prd.fr/multalin).
Pathogenicity of the purified virus against Limacodidae in Japan

Limacodidae larvae of *M. flavescens* and *A. dentata* were collected from Maple, Cherry and Plum trees in Hokkaido University. Six larvae of each Limacodidae (various larval stages) were fed on the plum tree leaves (1 cm × 1 cm) treated with water, the water suspension of purified particles (1% v/v) and the water suspension of the diseased *S. asigna*, a homogenate of the moribund *S. asigna* larvae (10% w/w).

**RESULTS**

Identification and purification of TaV from carcasses of *S. asigna* larvae

In a preliminary experiment, RT-PCR followed by agarose gel electrophoresis yielded only one PCR product (Fig. 1 A). The DNA fragment was estimated to be about 250 nts.
250 nts long, corresponding to the size of the target sequence in the TaV genomic RNA. A subsequent analysis revealed that the insert was 250 nts long and had a sequence that coincided with the target sequence of TaV (Fig. 1 B). The sequences obtained from three independent clones were the same.

Negative staining revealed that the purified particles had a spherical shape and no envelope, and were about 40 nm in diameter which was estimated from the width of tobacco mosaic virus (TMV) used as an internal size indicator (Fig. 2). These structural traits were similar to those reported for insect picorna-like viruses and members of the family Tetraviridae such as the *Nudaurelia capensis* β virus, TaV and *Dendrolimus punctatus* tettavirus (Bailey, 1976; Gordon et al., 1999; Hanzlik and Gordon, 1997; Hendry et al., 1968, 1995; Morris et al., 1999; Pringle et al., 1999; Tinsley and Melnick, 1973; Yi et al., 2005).

**Sequencing analysis**

Using the RNA extracted from the purified virus-like particles as a template, RT-PCR was carried out with the primer sets to amplify the RNA-dependent RNA-coding sequence (RdRp) and the capsid protein-coding sequence (CP) of TaV, resulting in the amplification of DNA fragments of expected sizes, 3858 nts (tentative RdRp) and 2482 nts (tentative CP) (data not shown). Subsequent sequencing revealed that the nucleotide sequence of the tentative RdRp and CP was about 99% identical to that of RdRp (ORF: 3883 nts) and CP (ORF: 2270 nts) of TaV, respectively (Fig 3). In addition, the overlapping of 627 nts was observed between the two sequences as is the case in TaV (Hanzlik and Gordon, 1997). A comparison of the TaV sequences showed that there were three nucleotide substitutions in the RdRp ORF, two of which resulted in amino acid substitutions (Fig. 3). Four nucleotide substitutions were observed in the CP ORF and one in the 3' terminal non-coding region. One of the five in the CP ORF was found to cause an amino acid substitution (Fig. 3).

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**Fig. 2.** Negative staining of particles purified from *S. asigna* by sucrose density gradient centrifugation. The insert shows the rod-shaped tobacco mosaic virus (TMV) used as an internal size indicator (→).

**Fig. 3.** A schematic representation of the genome's organization predicted from the sequence obtained in this study (TaVI). Boxes show the ORFs coding for RdRp and CP. The positions of a methyltransferase domain (●) and the nucleotide substitutions (※; synonymous and nonsynonymous) in comparison to the sequence of TaV (AF062037 and No. AF282930) (shadowed) are presented. The numbers (●) refer to the nucleotide number in the TaV.
Characteristics of the 3' terminus

To analyze the 3' terminal structure of the RNA extracted from the purified particles, the RACE method was adopted. When the RNA directly annealed with the RNA oligo adapters was used, no band was obtained in the subsequent PCR amplification for the 3' terminus (data not shown). However, polyadenylation at the 3'-terminus allowed amplification of two bands in the subsequent PCR, one with about 270 nts and the other with around 200 nts (Fig. 4). The sequence of the DNA fragment (272 nts) coincided with the sequence expected from the TaV genome reported previously (Gorbalenya et al., 2002; Pringle et al., 1999). The sequence of 200 nts could not be specified. These results strongly suggested that the RNA molecule extracted from the purified particles had no poly(A) stretch at the 3' terminus.

We also carried out 5' RACE (Fujita et al., 2006) to analyze the 5' terminal structure but obtained unreliable results (data not shown).

Pathogenicity of the isolate against Limacodidae larvae in Japan

The inoculation experiment showed that the isolate has the ability to infect Limacodidae larvae in Japan, *M. flavescens* and *A. dentata*. *M. flavescens* larvae and *A. dentata* larvae inoculated with the crude homogenate of infected *S. asigna* larvae developed symptoms on the 1st and 3rd day post-inoculation, respectively. The same symptoms were observed in the larvae of both species on the 1st and 8th days after inoculation with the purified particles, respectively (Fig. 5).

The symptoms in the inoculated larvae were similar to those observed in the epizootic *S. asigna* larvae such as brown coloring, flaccidity and finally moribundity. In *M. flavescens*, the inoculation of the purified virions led to a moribund state without flaccidity, but that of the crude homogenate resulted in flaccid symptoms prior to moribundity (Fig 5). Flaccidity was observed for *A. dentata* even on infection with the purified virions.

**DISCUSSION**

Farmers in Indonesia use moribund *S. asigna* larvae collected in epizootic oil palm fields as a pesticide to control *S. asigna*. The method of application is very simple. About 100 grams of moribund larvae was homogenized, mixed with 12 liters of water and then sprayed on palm trees in the field. This method is cheaper than using commercially available pesticides. However, the epizootic agents in diseased larvae have not been identified. An aim of this study was to identify the causal agent of *S. asigna* infections in South Sumatera, Indonesia, in order to develop a biological pesticide for integrated pest management (IPM) in oil palm plantations.

Preliminary experiments demonstrated that the causal agent was filterable (data not shown), implying that it was a virus. We suspected that the epizootic infection was caused by TaV, the only virus reported for *S. asigna* in Indonesia (Pringle et al., 1999). Subsequent RT-PCR experiments using a primer set targeting a methyltransferase domain of the TaV RdRp gene resulted in the amplification of a DNA fragment with the size expected from the sequence of TaV. This result strongly supported the prediction about the causal agent. Using the procedure for purifying tetraviruses described by Yi et al., (2005), we obtained a fraction containing spherical virus-like particles around 40 nm in diameter which corresponded to TaV.

TaV is a RNA virus first isolated from *S. asigna* collected in an oil palm plantation in Malaysia (Reinganum et al., 1978). Further characterization including the nucleotide sequencing of TaV RNA was performed using TaV isolated in North Sumatera, Indonesia (Pringle et al., 1999). The nucleotide sequence for the cDNAs against the RNA extracted from the purified virus-like particles was almost the same as the nucleotide sequence reported for TaV (Gene Bank accession number: AF 282930) with the same organization consisting of two large open reading frames corresponding to the RdRp- and CP-coding regions of TaV which was overlapped by 627 nts (Fig. 3), indicating that the virus-like particles purified in this study were TaV.
Eight nucleotide substitutions were observed in the ORFs, three in RdRp and five in CP. One of the three observed in RdRp was a synonymous substitution and the other two resulted in the replacement of asparagine (Asn) with serine (Ser) and arginine (Arg) with lysine (Lys) (Fig. 3). These substitutions occurred among amino acids with similar physicochemical properties, suggesting they caused no significant change in the structure of RdRp whose function was critical to the viral replication. In CP, only one of the five substitutions caused an amino acid change, from alanine (Ala) to threonine (Thr) (Fig. 3), which resulted in a change from a hydrophobic to hydrophilic residue. Though this substitution may have a functional effect on CP, there is not enough information to predict the possible functional change at present.

The 3’ RACE was successful only when a poly(A) tail was added at the 3’ terminus, suggesting that there was no poly(A) tract at the 3’ terminus of the viral RNA, consistent with a previous report for TaV (Pringle et al., 1999). On the other hand, the 3’ terminal sequences of the genomic RNAs of tetraviruses for which complete nucleotide sequences are available can be folded into 3’-tRNA-like secondary structures (Gordon et al., 1995). The poly(A) tail at the 3’ terminus protects the RNA from degradation by exonucleases (Gordon and Hanzlik, 1998), and is involved in the initiation of translation (Beelman and Parker, 1995; Dreytus and Philippe, 2002). Considering these observations, the lack of a poly(A) tail in TaV RNA, like the hepatitis A virus (HAV) (Kusov et al., 2005), implied a unique mechanism of replication/multiplication among tetraviruses. The 5’ end of TaV remains to be characterized (Gorbalenya et al., 2002).

TaV and other members of the family Tetraviridae are characterized by the ability to infect midgut cells (Gordon and Hanzlik, 1998), probably related to the prompting of infected S. asigna larvae such as feeding—termination and liquefying. TaV shows greater infectivity in the early stages of larval development than the later stages (Entwistle, 1987; Rahmadsyah et al., 2003). These traits suggest that TaV has a good potential as a viral insecticide in Integrated Pest Management (IPM) (Hendry and Agrawal, 1993; Christians et al., 2001). Interestingly, TaV showed strong pathogenicity against Limacodidae larvae in Japan, M. flavescens and A. dentata, suggesting it to be a novel biological control agent for the Limacodidae pest which feeds on economically important plants in Japan such as cherry, plum and maple trees. This is the first report of the ability of TaV to infect insect species other than S. asigna. These observations also suggested that TaV has an excellent potential as a control agent against Limacodidae insects.

Deep insight into the molecular mechanism of their replication should facilitate the effective/safe application of viruses. The Tetraviridae include the Betatetravirus and Omegatetravirus, the former has a monopartite RNA genome and the latter a bipartite genome, suggesting a difference in the replication mechanisms between them (Agrawal and Johnson, 1992; Hanzlik et al., 1993, 1995, 2005). A recent study showed that the Euproctis chrysorrhoea virus (EeV) is closely related to TaV and the two share properties distinguishing them from prototypic tetraviruses, including a predicted conserved pseudonot at the
3’-end, a structurally unique RdRp with a permuted active site (pRdRp) and a putative VPg signal mediating the priming of RNA synthesis in dsRNA Birnaviridae (Zeddam et al., 2010). These observations suggested unique replication mechanisms of TaV and EeV among tetraviruses. However, the replication mechanisms of TaV/EeV remain to be delineated as well as other members of Tetraviridae, mainly because there is a lack of susceptible cell lines with the exception of the Providence virus (PrV) (Pringle et al., 2003).

In conclusion, the virus isolated from the epizootic S. asigna larvae collected in South Sumatra was a TaV, which also showed strong pathogenicity against Limacodidae larvae in Japan. These results will greatly facilitate the study of TaV as a promising biological agent to control Limacodidae pests in oil palms and other plants.

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