Visualization and live imaging analysis of a mosquito saliva protein in host animal skin using a transgenic mosquito with a secreted luciferase reporter system

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Abstract

**Mosquitoes inject saliva into a vertebrate host during
blood feeding. The analysis of mosquito saliva in host
skin is important for the elucidation of the inflamma-
tory responses to mosquito bites, the development of
antithrombotic drugs, and the transmission-blocking
of vector-borne diseases. We produced transgenic
Anopheles stephensi mosquitoes expressing the
secretory luciferase protein (MetLuc) fused to a saliva
protein (AAPP) in the salivary glands. The transgene
product (AAPP-MetLuc) of transgenic mosquitoes
exhibited both luciferase activity as a MetLuc and
binding activity to collagen as an AAPP. The detection
of luminescence in the skin of mice bitten by trans-
genic mosquitoes showed that AAPP-MetLuc was
injected into the skin as a component of saliva via
blood feeding. AAPP-MetLuc remained at the mos-
quito bite site in host skin with luciferase activity for
at least 4 h after blood feeding. AAPP was also sus-
pected of remaining at the site of injury caused by the
mosquito bite and blocking platelet aggregation by
binding to collagen. These results demonstrated the
establishment of visualization and time-lapse analysis
of mosquito saliva in living vertebrate host skin. This**

**technique may facilitate the analysis of mosquito
saliva after its injection into host skin, and the devel-
opment of new drugs and disease control strategies.**

Keywords: mosquito saliva, luciferase, live imaging,
transgenic mosquito, anopheline antiplatelet protein.

Introduction

A mosquito takes blood from animals, including humans
and livestock, for vitellogenesis. It has been shown that
various saliva proteins are injected into host skin during
blood feeding (Ribeiro, 1987); anticlotting molecules,
antiplatelet aggregation molecules, vasodilators and
anaesthetic substances are included in the proteins in
mosquito saliva (Ribeiro, 1987) and mosquitoes perform
blood feeding using these proteins. An allergic reaction is
caused by an immune response to these molecules in
host skin (Billingsley *et al.*, 2006). Furthermore, blood
feeding promotes the transmission of disease, such as
malaria and dengue fever; therefore, analysis of the mos-
quito saliva proteins injected into host skin facilitates the
development of antihaemostasis and anti-inflammatory
drugs as well as transmission-blocking strategies for
vector-borne diseases (Hayashi *et al.*, 2012). Neverthe-
less, the state of these saliva proteins after their injection
into host skin remains elusive. A previous study has inves-
tigated mosquito saliva in the host skin (Choumet *et al.*,
2012) and the development of the latest technique of
imaging *in vivo* with reporter molecules (e.g. fluorescent
protein or luciferase) has facilitated the observation of
various molecular biological events in living animals
(Gross & Piwnica-Worms, 2005). For example, the kinet-
ics of malaria parasites, which are transmitted by
anopheline mosquitoes, have been analysed in animal
skin, the liver and the blood using *in vivo* imaging with the
luciferase protein or fluorescent proteins (Mota *et al.*,
2001; Amino *et al.*, 2006; Franke-Fayard *et al.*, 2006;
Kebaier *et al.*, 2009; Ploemen *et al.*, 2009). We recently
established a system for female salivary gland-specific

gene expression in transgenic *Anopheles stephensi* mosquitoes using the *anopheline antiplatelet protein* (*aapp*) gene promoter region of *A. stephensi* (Yoshida & Watanabe, 2006). Furthermore, this system allowed salivation to be visualized by the expression of a transgene fused to the fluorescence protein gene (Yamamoto *et al.*, 2010, 2012; Sumitani *et al.*, 2013).

In the present study, we produced a transgenic *A. stephensi* mosquito expressing the luminescent protein gene *Metridia longa luciferase* (*metluc*) fused to *aapp* (*aapp-metluc*) in the female salivary glands, and attempted to detect saliva injected into skin using live imaging. MetLuc is a naturally secreted luciferase protein, and may be easily secreted in saliva from the secretory cells of mosquito salivary glands. The AAPP protein is an antiplatelet protein contained in *Anopheles* mosquito saliva. *In vitro* and *ex vivo* analyses showed that AAPP directly bound to collagen, which blocked platelet aggregation (Yoshida *et al.*, 2008; Hayashi *et al.*, 2012, 2013). AAPP may promote blood feeding by preventing platelet aggregation around a sucking proboscis in host skin. People in a hyperendemic area of malaria, the Solomon Islands, were shown to have high levels of antibodies against AAPP, and antibody titers were positively correlated with antibody titers against malarial antigens (Yoshida *et al.*, 2008); therefore, AAPP is important for developing antithrombotic drugs and predicting the emergence of malaria (Yoshida *et al.*, 2008; Hayashi *et al.*, 2012).

Transgenic mosquitoes expressing AAPP-MetLuc in their salivary glands were successfully used in visualization and time-lapse analysis of saliva injected into vertebrate animal skin. The AAPP-MetLuc protein injected into the skin was proposed to remain and maintain luciferase activity in the same part of the skin for several hours. AAPP may inhibit platelet aggregation by binding to collagen at the sites of injury caused by mosquito bites. The salivary gland-specific expression system of saliva proteins fused to the secreted luciferase protein is expected to be a powerful tool for live-imaging analysis of the saliva proteins injected into host skin and may also facilitate the development of antihaemostasis and anti-inflammatory drugs, and transmission-blocking strategies against vector-borne diseases.

Results and discussion

Establishment of transgenic mosquitoes expressing MetLuc fused to AAPP in the salivary glands

A *Minos*-based transformation vector harbouring a gene cassette consisting of the *Metridia luciferase* gene fused to the *aapp* gene (*aapp-metluc* gene) under the control of the *aapp* promoter (Fig. 1A, Fig. S1) was injected with a *Minos* helper plasmid into *A. stephensi* embryos. Two

independent G1 individuals were obtained, and one of them was established as a strain for >10 generations. A single integration event was confirmed by Southern blot analysis (Fig. 1B). Immunoblot analysis with the anti-AAPP antibody showed that the AAPP-MetLuc protein (Mr = 59 KDa) was expressed in female transgenic mosquito salivary glands (Fig. 1C). The lower band (Mr = 37 KDa) expressed in wild-type salivary glands was shown to be the native AAPP of mosquitoes (Yoshida *et al.*, 2008). Firefly luciferase was previously expressed in the salivary glands of transgenic *Aedes aegypti* using the *apyrase* (*apy*) gene promoter; however, luciferase activity could not be detected using an imaging system because gene expression levels were very low (1–50 pg per mosquito, estimated) (Coates *et al.*, 1999). Although the promoter region of the *aapp* gene as well as the *apy* promoter induced female- and salivary gland-specific expression in *A. stephensi*, the expression level of the transgene using the *aapp* promoter was >1000-fold higher than that using the *apy* promoter (Yoshida & Watanabe, 2006). The amount of AAPP-MetLuc protein expressed in the transgenic line was ~100 ng per pair of salivary glands, as quantified by a comparison with recombinant AAPP (Mr = 49 kDa) (Fig. 1D). This represents ~3–10% of the total content of salivary gland protein (Calvo *et al.*, 2006). This result demonstrated that the *aapp* promoter region is an excellent tool to express the luciferase gene in the salivary glands of mosquitoes. Moreover, the amount of AAPP-MetLuc expressed in transgenic mosquitoes was higher than that of the transgene products (10–40 ng) expressed in salivary glands using the *aapp* promoter described in our previous studies (Yoshida & Watanabe, 2006; Matsuoka *et al.*, 2010, 2012; Yamamoto *et al.*, 2010, 2012; Sumitani *et al.*, 2013). When MetLuc fuses with AAPP, it may be stable in the saliva secreted by the secretory cells of salivary glands of transgenic mosquitoes because it is a natural secretory protein and AAPP is a native saliva protein of *A. stephensi*.

Luminescent activity and binding activity to collagen of AAPP-MetLuc of transgenic mosquitoes

We examined whether AAPP-MetLuc in the salivary glands of transgenic mosquitoes exhibited luciferase activity. Luminescent activity to a *Metridia* luciferase substrate was observed in the salivary gland homogenates of the transgenic line (Fig. 2A). In contrast, these homogenates showed no luminescent activity to firefly Luciferin (data not shown). These results indicated that AAPP-MetLuc expressed in the salivary glands of transgenic mosquitoes exhibited specific luciferase activity to the *Metridia* substrate.

In our previous study, AAPP was shown to bind directly to collagen (type I and III), and it also inhibited platelet
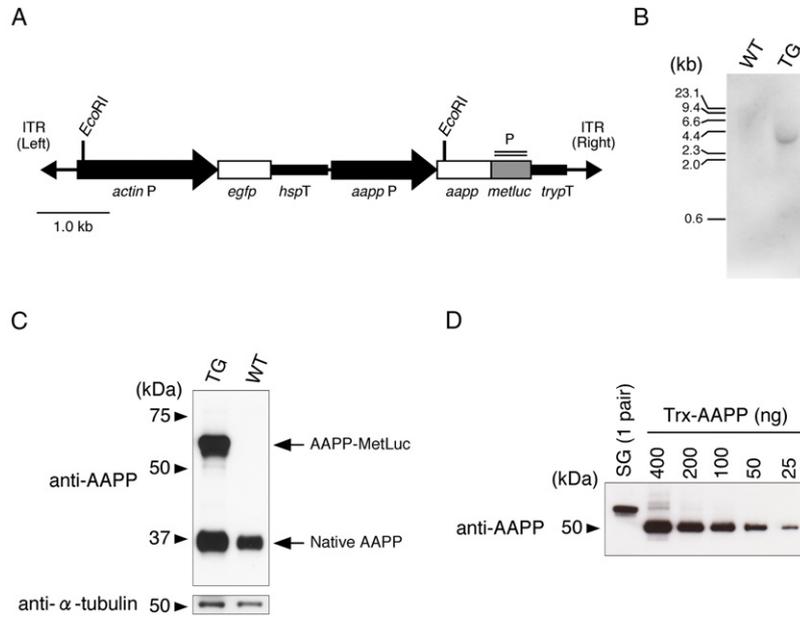
Figure 1. Establishment of the AAPP-MetLuc transgenic (TG) mosquito. (A) Schematic representation of the genomic structure of the AAPP-MetLuc mosquito. ITR, *Minos*-inverted terminal repeat; *actin*P, *Drosophila melanogaster actin5c* promoter; *egfp*, EGFP-coding sequence; *hsp*T, *D. melanogaster heat shock protein 70* terminator sequence; *aapp*P, *Anopheles stephensi aapp* regulatory elements; *aapp*, anopheles antiplatelet aggregation protein containing the signal peptide at the N-terminus-coding sequence; *metluc*, *Metridia longa* luciferase-coding sequence; trypT, *Anopheles gambiae trypsin* terminator sequence. A double line represents the probe region used for Southern blot analysis. (B) Southern blot analysis of the AAPP-MetLuc mosquito. Genomic DNAs from the AAPP-MetLuc (TG line) or wild-type (WT) mosquitoes were digested with *Eco*RI, and hybridized with a 0.7-kb DNA fragment encoding the MetLuc protein. (C) Immunoblotting of the AAPP-MetLuc protein in the salivary glands. Two pairs of female salivary glands from TG line or WT mosquitoes were analysed using anti-AAPP and anti-alpha-tubulin antibodies. Arrows show AAPP-MetLuc and a native AAPP. (D) Estimation of the amount of AAPP-MetLuc in the salivary glands. One pair of female salivary glands and recombinant AAPP proteins (thioredoxin-AAPP: Trx-AAPP) were analysed using the anti-AAPP antibody. The amount of Trx-AAPP (400, 200, 100, 50, 25 ng) is indicated at the top of each lane.
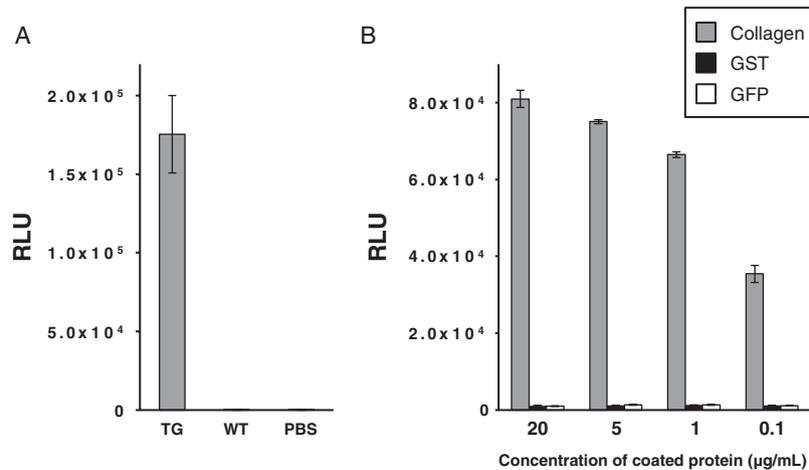

Figure 2. *In vitro* luminescent assay of AAPP-MetLuc in the salivary glands of transgenic (TG) mosquitoes. (A) *In vitro* luminescent assay of AAPP-MetLuc. Luminescent levels (relative light unit, RLU) in the homogenate of one pair of salivary glands [TG or wild type (WT)] were measured using a microplate reader. All data represent the mean ± SE obtained from three independent experiments. (B) Binding ability to collagen of AAPP-MetLuc. The indicated concentrations of collagen, recombinant glutathione-S-transferase (GST), and recombinant green fluorescent protein (GFP) coating the 96-well microtiter plate. After blocking each well, the homogenate of 0.5 pairs of salivary glands of TG mosquitoes was added to each well and incubated for 1 h. Luminescent levels (RLU) were measured after washing each well with phosphate-buffered saline (PBS). All data represent the mean ± SE obtained from three independent experiments.

adhesion to collagen (Yoshida *et al.*, 2008). A luminescent assay was performed using collagen-, recombinant glutathione-S-transferase (rGST)-, and recombinant green fluorescent protein (rGFP)-coated plates to test whether AAPP-MetLuc had the ability to bind to collagen. Figure 2B shows that AAPP-MetLuc exhibited binding ability to collagen. This result raised the possibility that AAPP-MetLuc inhibited platelet adhesion to collagen as well as the native AAPP of mosquitoes.

In vivo *detection of mosquito saliva injected into host skin by mosquito biting*

We examined whether the luminescence of saliva released from transgenic mosquitoes could be detected. Luminescence was observed around the proboscis inserted in a luciferase substrate solution with the *in vivo* imaging system (IVIS; PerkinElmer, Boston, MA, USA; Fig. 3A). This result demonstrated that AAPP-MetLuc was

released as a component of saliva, and could be detected with the luminescent imaging system. Moreover, a reduction in the luminescent activity of the salivary gland of blood-fed mosquitoes compared with that of non-blood-fed mosquitoes suggested that ~70% of AAPP-MetLuc equivalent to 70 ng was injected during blood feeding (Fig. 3B). It is considered that half of the mosquito salivary protein is discharged during blood feeding (Calvo *et al.*, 2006). In *A. aegypti*, the enzyme activity of the salivary protein apyrase is decreased by ~50–75% after blood feeding (Marinotti *et al.*, 1990; Reno & Novak, 2005); therefore, our estimation is plausible.

We detected the luminescence of mosquito saliva in mouse skin bitten by transgenic mosquitoes. The MetLuc substrate (400 μg/kg) was injected into mice via the tail vein immediately after blood feeding. Luminescence was observed at the blood-sucking site of the skin of mice bitten by transgenic mosquitoes (Fig. 3C). We could not detect luminescence in mice injected with a lower concen-
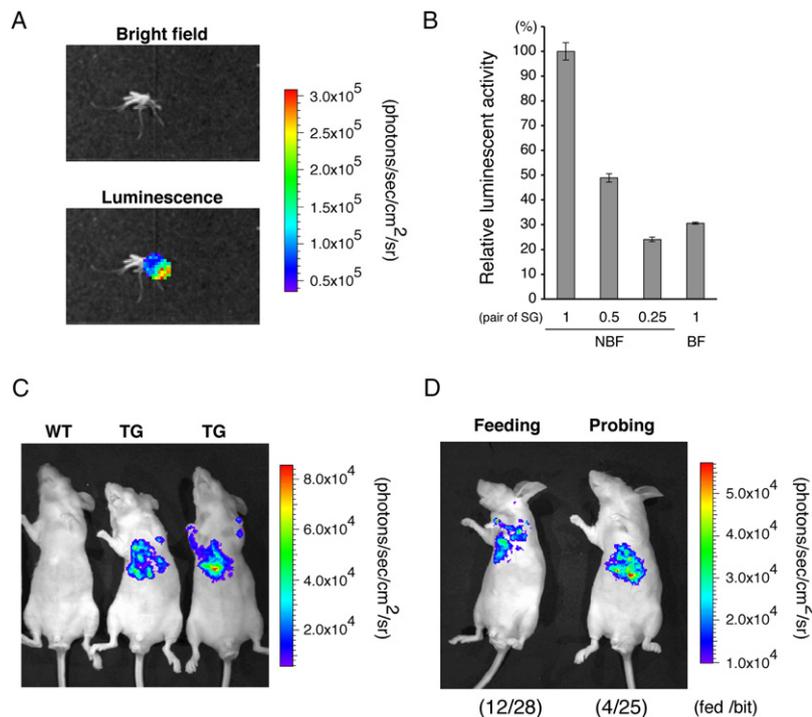

Figure 3. *In vivo* imaging of AAPP-MetLuc released from transgenic (TG) mosquitoes. (A) The imaging of luminescence from saliva released from the AAPP-MetLuc strain. Rainbow images of bioluminescence are shown in total flux (photons/s/cm²/sr). (B) Reduction in luminescent activity of salivary gland after blood feeding. The salivary glands (SG) of TG mosquitoes within 1 h after blood feeding (BF) and non-blood-fed TG mosquitoes (NBF) were dissected and used for luminescent assay. Mosquitoes were allowed to bite and fully feed on mouse blood. Ten pairs of SG were homogenized and diluted with phosphate-buffered saline (PBS). The amount of SG is shown at the bottom of the graph. The relative percentages of luminescent activity were obtained with reference to the luminescent activity of one pair of SG of NBF mosquitoes being 100%. All data represent the mean ± SE obtained from four independent experiments. (C) *In vivo* imaging of AAPP-MetLuc in skin of mice bitten by mosquitoes. Rainbow images of luminescence in mice. The abdominal skins of mice were bitten by 40 TG mosquitoes and wild-type (WT) mosquitoes. Mice were injected with the luciferase substrate immediately after blood feeding for 5 min. Bioluminescence is shown in total flux (photons/s/cm²/sr). (D) Detection of luminescence of AAPP-MetLuc in mouse skin by probing. The abdominal skins of mice were bitten by TG mosquitoes. Blood feeding: a mouse was kept on a cage and TG mosquitoes were allowed to feed on its blood for 15 min. Probing: a mouse was lifted up every 15 s for 15 min to interrupt TG mosquitoes from taking blood. The number of mosquitoes that bit and fed on the blood of a mouse is indicated at the bottom of the panel. Bioluminescence is shown in total flux (photons/s/cm²/sr).
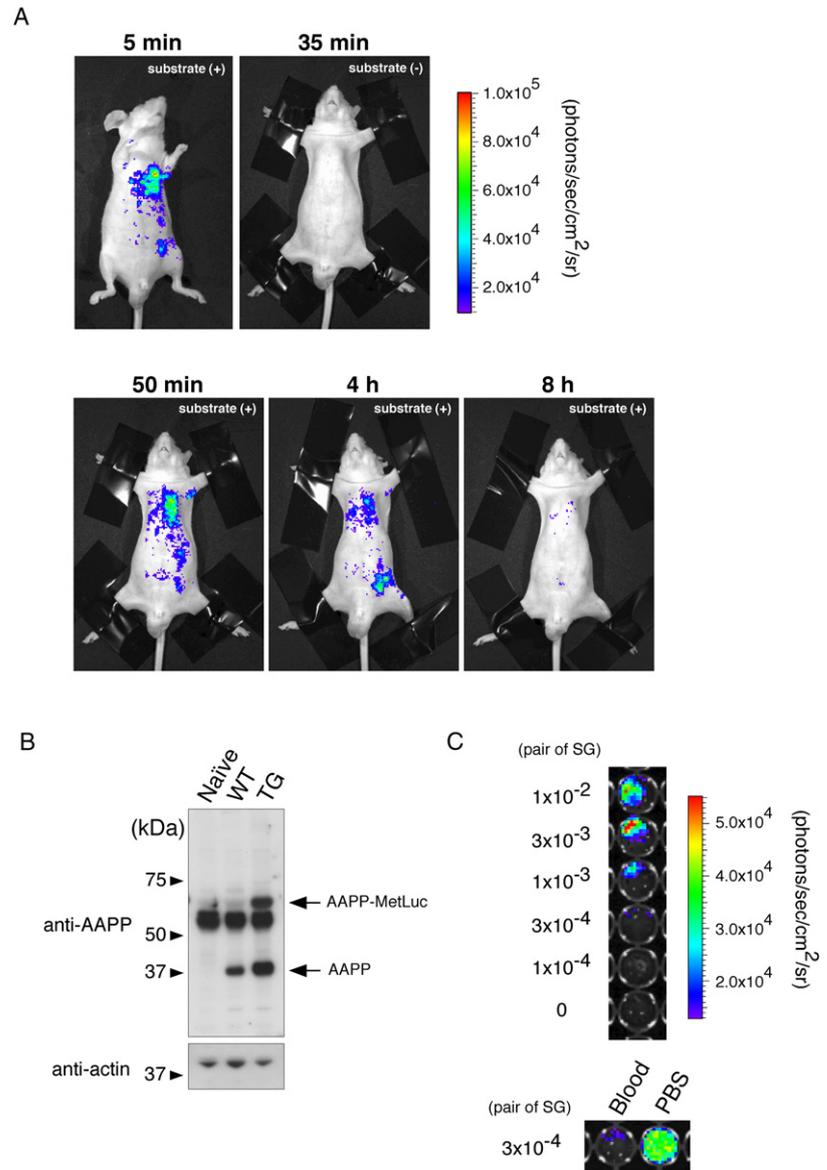
Figure 4. Time-lapse imaging of AAPP-MetLuc in mouse skin after mosquito bites. (A) Rainbow images of luminescence in a mouse bitten by 50 transgenic (TG) mosquitoes. A mouse was injected with the luciferase substrate just after blood feeding for 5 min. The time after blood feeding is indicated at the top of the panels. Substrate (+) and (−) indicate immediately and 30 min after administration of the substrate, respectively. Bioluminescence is shown in total flux (photons/s/cm²/sr). (B) Immunoblotting of the AAPP-MetLuc protein in mouse skin 4 h after 40 mosquito bites. Mouse skin bitten by TG lines and wild-type (WT) mosquitoes was analysed using anti-AAPP and anti-actin antibodies. (C) *In vitro* imaging of the salivary gland homogenate of TG mosquitoes in naïve mouse blood. Top panel: Luminescence of serially diluted homogenate of the salivary glands of TG mosquitoes in naïve mouse blood. The amount of salivary glands is shown at the left of the panel. Bottom panel: Comparison of the luminescence of AAPP-MetLuc in the blood and in phosphate-buffered saline (PBS). Rainbow images of bioluminescence are shown in total flux (photons/s/cm²/sr).

tration of the substrate (40 μg/kg or 200 μg/kg) via the tail vein (data not shown), whereas the luminescence of AAPP-MetLuc, injected into host skin by the mosquito's probing, was fully detected (Fig. 3D). We examined how long the luciferase activity of AAPP-MetLuc could be monitored in the skin. Although luciferase luminescence was not detected in mice 30 min after administering the MetLuc substrate, luminescence was detected again by injection of the substrate (Fig. 4A). These results suggested that AAPP-MetLuc was injected into skin as a component of mosquito saliva and remained at that site, and the disappearance of the initial luminescence was attributable to the absence of substrate because of degradation. Furthermore, when the substrate was again injected into the mouse 4 h after blood feeding, lumines-

cence was detected at the same site (Fig. 4A). Immunoblotting analysis showed that MetLuc remained fused with AAPP, which had binding ability to collagen on the skin at this time (Fig. 4B); however, despite the recent administration of substrate, luminescence was not detected 8 h after blood feeding (Fig. 4A). These results indicate that AAPP-MetLuc remained in the skin for several hours, maintaining luciferase activity.

Vertebrate hosts are known to be immunized to mosquito saliva proteins by repeated bites, and to develop antibodies (IgG or IgE) to them (Billingsley *et al.*, 2006); however, the mosquito saliva proteins in host skin remain unknown. Mosquito saliva was recently visualized in the skin by immunohistochemistry with antibodies against whole saliva (Choumet *et al.*, 2012); however, the direct

detection of mosquito saliva has not been completed in living hosts. In this study, we succeeded in establishing live imaging of the mosquito saliva proteins expressed as the luciferase-fusion protein in mouse skin by mosquito transgenesis. AAPP-MetLuc possesses binding activity to collagen. In our previous study, *in vitro* analysis revealed that AAPP inhibited platelet aggregation by binding to collagen (types I and III; Yoshida *et al.*, 2008; Hayashi *et al.*, 2012); therefore, AAPP injected by the mosquito is thought to remain at the mosquito bite site by binding to collagen (Yoshida *et al.*, 2008). Time-lapse analysis showed that AAPP-MetLuc was considered to remain at the mosquito bite site. This finding occurred as a consequence of the function of AAPP.

In the present study, mosquito saliva was detected in the skin of mice bitten by transgenic mosquitoes, whereas saliva was not detected in the bloodstream under the skin tissue. The amount of AAPP-MetLuc in the bloodstream of mice was considered to be too small for *in vivo* detection. Moreover, MetLuc activity was shown to be inhibited by serum albumin levels in the blood (Hiramatsu *et al.*, 2005). The luminescent image of salivary gland homogenate in the blood, including the luciferase substrate equivalent of the concentration used for the *in vivo* assay (10 µg, substrate/2 ml, total blood of mouse), showed that the detection of transgenic mosquito saliva at amounts $<3 \times 10^{-4}$ pair of salivary glands, equivalent to 30 pg of AAPP-MetLuc in 50 µl of blood, may be difficult in our system (Fig. 4C). The luminescence of AAPP-MetLuc was lower in blood than in phosphate-buffered saline (PBS) (Fig. 4C); therefore, the detection of AAPP-MetLuc in the bloodstream under the skin may be difficult. These results suggest that our system is appropriate for the specific detection of saliva in the skin. Furthermore, AAPP-MetLuc mosquitoes could be useful for investigating the amount of saliva injected in the skin, depending on the condition of blood feeding, such as the number of mosquitoes feeding, probing frequency, and time allowed to feed.

The saliva of blood-sucking animals, including mosquitoes, contains various proteins including antihaemostasis molecules, vasodilators and anaesthetic substances. Some of these molecules have recently been used for therapeutic applications (Furlan *et al.*, 2006; Warkentin *et al.*, 2008; Hayashi *et al.*, 2012). Some saliva proteins are promising candidate molecules for markers of exposure to mosquito bites and may also predict the emergence of malaria or dengue virus (Yoshida *et al.*, 2008; King *et al.*, 2011; Rizzo *et al.*, 2011; Doucoure *et al.*, 2012). Moreover, blood-sucking arthropod saliva has been shown to enhance the infectivity of pathogens, including the malaria parasite, and several molecules in saliva have the potential to be vaccine targets for the prevention of pathogen transmission (Titus *et al.*, 2006; Mecheri, 2012). Since the saliva of AAPP-MetLuc mosqui-

toes can be visualized, this line may be used to analyse the effect of loss-of-function of other saliva proteins on the release of saliva. Moreover, transgenic *Plasmodium* expressing a reporter gene has been used to analyse sporozoite injection by mosquitoes and movement in living animal skin (Vanderberg & Frevert, 2004; Engelmann *et al.*, 2006; Jin *et al.*, 2007; Kebaier *et al.*, 2009; Voza *et al.*, 2012). There may be progress, therefore, in the elucidation of mosquito saliva–malaria sporozoite interactions using the combination of the transgenic mosquitoes and transgenic malaria parasites. This expression system can be applied to other saliva proteins in mosquitoes, so visualization and kinetic analysis of these proteins may be possible in vertebrate hosts. Thus, this expression system may facilitate elucidation of the individual functions of mosquito saliva proteins, and the development of new drugs or disease control strategies.

Experimental procedures

Ethics statement

All animal procedures were approved by the Animal Ethics Committee of Jichi Medical University, Japan.

Animals

The *A. stephensi* mosquito strain SDA500 was maintained at Jichi Medical University according to previously described methods (Yamamoto *et al.*, 2012). Male BALB/c and BALB/c-*nu* strain mice were obtained from Japan SLC (Hamamatsu, Shizuoka, Japan) and used at 8 weeks of age.

Minos *vector construction and germline transformation*

PCR reactions were performed with *Pfu* DNA polymerase (Stratagene GmbH, Heidelberg, Germany). A DNA fragment of the *metluc* gene was amplified from pMetLuc2-Control (Clontech Laboratories, Inc., Mountain View, CA, USA) using primers pMetluc2-F3: 5′-CACCTCGAGAAGAGCACCGAGTTCGACCCC AACATC-3′, and pMetluc2-R1: 5′-GCATGCAGTGAAAAAAATG CTTTATTTGTGAAATTTGTG-3′. The PCR product was cloned into pENTR/D-TOPO (Invitrogen, Carsbad, CA, USA) to generate pENTR-MetLuc. A DNA fragment of the *aapp* gene was amplified from the salivary gland cDNA of *A. stephensi* using primers pAnSG-F19: 5′-CACCGAATTCACTACGGATCAAGAATCATCG ACCGAGCTA-3′, and pAnSG-R1: 5′-CTCGAGTGCGGCCGCC TCTGAATCACGCTTTTCGACGAT-3′. The PCR product was cloned into pENTR/D-TOPO (Invitrogen) to generate pENTR-aapp. The *metluc* gene fragment was excised from pENTR-MetLuc by digestion with *Xho*I and *Asc*I, and then cloned into the *Xho*I/*Asc*I sites of pENTR-aapp to generate pENTR-AAPP-MetLuc. The *aapp-metluc* gene fragment was excised from pENTR-AAPP-MetLuc by digestion with *Eco*RI and *Sph*I, and then cloned into the *Eco*RI/*Sph*I sites of pENTR-aapppP-mDsRed-SP15-antryp1T (Yamamoto *et al.*, 2010) to generate pENTR-aapppP-aapp-MetLuc-antryp1T. The transformation plasmid pMinos-EGFP-aapppP-aapp-MetLuc-antryp1T (Fig. 1A) was generated by incubating pMinos-EGFP-RfA-F (Yoshida & Watanabe,

2006) and pENTR-aappP-aapp-MetLuc-antryp1T in the presence of LR Clonase® (Invitrogen) as described previously (Yoshida & Watanabe, 2006). Procedures for the microinjection into embryos, screening of transgenic individuals, and generation of homozygous lines have been described previously (Catteruccia *et al.*, 2000).

Southern blot analysis

Southern blot analysis was performed as described previously (Yamamoto *et al.*, 2008). A total of 5 μg of genomic DNA from mosquitoes extracted using the DNeasy Kit (Qiagen, Valencia, CA, USA) was digested with *Eco*RI, separated on 0.8% agarose gels, and then transferred to a Hybond N+ membrane (GE Healthcare Ltd, Little Chalfont, UK). Probe labelling, hybridization, washing, and the detection of signals were performed using Roche DIG-High Prime DNA labelling and detection reagent (Roche, Indianapolis, IN, USA) and CDP-star (Roche).

Immunoblotting

Rabbit anti-AAPP antiserum (Yoshida *et al.*, 2008), rabbit anti-alpha-tubulin monoclonal antibody (11H10 mAb, #2125, Cell Signalling), and rabbit anti-actin antibody (Sigma, St. Louis, MO, USA) were used as primary antibodies.

Groups of 20 pairs of female salivary glands were homogenized using a plastic homogenizer with 40 μl of sample buffer (Nacalai Tesque, Kyoto, Japan) containing 5% 2-mercaptoethanol, and then boiled at 95 °C for 3 min. The recombinant thioredoxin-AAPP protein (Yoshida *et al.*, 2008) was serially diluted with sample buffer containing 5% 2-mercaptoethanol, and then boiled at 95 °C for 3 min. Fifty milligrams of skin dissected from mice bitten by mosquitoes was homogenized using a Biomasher (Nippi, Tokyo, Japan) with 200 μl of PBS, equally diluted with sample buffer containing 5% 2-mercaptoethanol, and then boiled at 95 °C for 3 min. Each sample was separated on an 8–10% sodium dodecyl sulphate polyacrylamide gel, and transferred to Hybond™ ECL Membrane (GE Healthcare). Immunoblotting was carried out as previously described (Yamamoto *et al.*, 2013).

Luminescent assay of the salivary glands

The luminescence of AAPP-MetLuc in the salivary glands was measured in a 96-well microtiter plate using SpectraMax M5 (Molecular Devices, Sunnyvale, CA, USA). A group of 10 pairs of female salivary glands was homogenized using a plastic homogenizer with 100 μl of PBS, and then diluted with 400 μl of PBS. Then, 50 μl (equivalent to 1 pair of salivary glands) of each sample was added into each well, and the luciferase substrate was added according to the manufacturer's protocol. The *Metridia* luciferase substrate was obtained from the Ready-To-Glow Secreted Luciferase Reporter Assay (Clontech).

AAPP-MetLuc binding assay

The binding ability of AAPP-MetLuc to collagen was measured in a 96-well microtiter plate coated with collagen type-I (Cell matrix; Nitta Gelatin, Osaka, Japan), rGST protein, or rGFP. The plate was blocked with 1% Block Ace, a blocking reagent (DS Pharma Biomedical, Tokyo, Japan), for 1 h at room temperature. Groups

of 20 pairs of female salivary glands were homogenized using a plastic homogenizer with 100 μl of PBS, and then diluted with 1.9 ml of PBS. Then 50 μl of sample (equivalent to 0.5 pairs of salivary glands) was added to each well, and then incubated for 1 h at room temperature. Nonadherent samples were removed, and wells were washed twice with PBS. The luciferase assay was performed as described above.

Imaging luminescence of salivation

The procedure for monitoring salivation was as described previously (Yamamoto *et al.*, 2010). The proboscis of a mosquito on a glass slide was inserted into *Metridia* luciferase substrate solution and covered with a coverslip. Luciferase activity was acquired using the IVIS (IVIS 100; PerkinElmer) with a 10-cm field of view (FOV), medium binning factor, and exposure time of 20 s using the IVIS. Imaging data were analysed using Living Image® 2.5 (PerkinElmer).

In vivo *imaging of mouse skin*

The luciferase activity of saliva injected from mosquitoes into mice was detected using the IVIS. Mice were anaesthetized with pentobarbital (50 mg/kg, intraperitoneally), and a part of the ventral skin of mice was allowed to contact the cage containing mosquitoes. To reduce the difference in the amount of released saliva between individual mosquitoes, adult female mosquitoes at days 7–10 after eclosion were allowed to feed on blood for 5 min. To avoid anaemia, < 50 mosquitoes were allowed to bite the mice. The *Metridia* luciferase substrate was diluted in substrate buffer and injected via the tail vein (100 μg/ml, 4 ml/kg, intravenously). The *Metridia* luciferase substrate and substrate buffer were obtained from the Ready-To-Glow Secreted Luciferase Reporter Assay (Clontech). Luciferase activity was acquired immediately after injecting the substrate with a 15-cm FOV, medium binning factor, and exposure time of 1–5 min. Imaging data were analysed using Living Image® 2.5 (PerkinElmer). At 4 or 8 h after blood feeding, mice were anaesthetized by inhalation of 30% isoflurane diluted with propylene glycol, and observed using the IVIS. The procedure to probe a mouse by mosquitoes was as described previously (Matsuoka *et al.*, 2002).

In vitro *imaging of the luminescence of AAPP-MetLuc in mouse blood*

A group of 10 pairs of female salivary glands was homogenized using a plastic homogenizer with 100 μl of PBS, and then serially diluted with PBS. Ten microliters of each sample was added into each well containing 50 μl of naïve male BALB/c mouse blood or PBS in a 96-well microtiter plate. The *Metridia* luciferase substrate (100 μg/ml, 2.5 μl/50 μl blood) was used as described above. Luciferase activity was acquired using the IVIS with a 15-cm FOV, medium binning factor, and exposure time of 60 s. Imaging data were analysed using Living Image® 2.5 (PerkinElmer).

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. The DNA sequence and its deduced amino acid sequence of the *aapp-metluc* gene. The DNA sequences of the *aapp* gene and *metluc* gene are shown by red and blue letters, respectively.