Anopheline Anti-Platelet Protein from Mosquito Saliva Regulates Blood Feeding Behavior

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DISSERTATION

ハマダラカ唾液由来Anopheline Anti-Platelet Proteinは蚊の吸血行動を制御する

Anopheline Antiplatelet Protein from Mosquito Saliva Regulates Blood Feeding Behavior

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Dedicated to DEAR PARENTS

I have worked with your flower
And nourished it to grow
I am going to return it now
Love it with handful care
It’s bloom new with every day,
In August it just a bud
Grow up such wonderful way
Then January it will lovely blossom
Though it’s yours rightfully
Part will always be with me!!!

SUMMARY

Hematophagous arthropods saliva is enriched with a composite mixture of bioactive components, can efficiently block the host homeostatic responses. However, the biological implication of many saliva components has yet to be unwavering. Anopheline anti-platelet protein (AAPP) from malaria vector mosquito *An. stephensi* exhibits strong anti-platelet activity when bound directly to host collagen by its GE-rich C-terminal domain and through its N-terminus with calcium-binding activity. To explore the role of AAPP on mosquito physiology in inspect of their ecological attributes, we applied a transgenesis-based protein inactivation approach to generate transgenic *Anopheles stephensi* expressing anti-AAPP mAb single chain fragment (scFv) in their salivary glands. The AAPP-specific collagen-binding activity was almost completely abolished by AAPP─scFv complex formation in the saliva of TG mosquitoes. Probing and prediuresis time, feeding success, blood meal size, and fecundity, which are all fitness parameters, were significantly reduced in the TG mosquitoes. However, the oocysts number in these mosquitoes were not significantly reduced following blood meal intake from *Plasmodium berghei*- infected mice. These outcomes reveal that AAPP facilitates in obtaining a blood meal but does not have deleterious effects on malaria vectorial capacity (sporogonic development) in our laboratory model, but its high fitness cost would pose a survival risk for parasite-infected mosquitoes in nature. Moreover, our transgenesis-based protein inactivation and protein-protein interaction approaches provide an exclusive opportunity to understand the complex interactions occurring between multifaceted saliva proteins and host homeostasis or pathogen transmission *in vivo*.

LIST OF CONTENTS

SUMMARY………………………………………………………………………….. i

LIST OF CONTENTS…………………………………………………………… ii

LIST OF FIGURES……………………………………………………………….. iii

LIST OF TABLE……………………………………………………………….… iv

ABBREVIATIONS……………………………………………………………….. v─vi

INTRODUCTION……………..………………………………………………... 1─3

AIMS OF THE STUDY………………………………………………………… 4

MATERIALS AND METHODS……………………………………………… 5─15

Ethical statement……………………………………………………………… 5

Animals, parasites, and mosquitoes…………………………………………... 5

AAPP-collagen binding inhibition assays……………………………………. 6

Protein separation and Ca^{2+} binding assays…………………………………….. 6

Minos vector construction and embryo microinjections……………………... 7

Selection of TG homozygous mosquitoes……………………………………. 8

Genomic DNA extraction and qPCR………………………………………… 9

Production of the anti-mDsRed polyclonal antibody………………………… 10

Immunoblotting and Ni-NTA pull-down assays……………………………... 10

Saliva collection……………………………………………………………… 11

Quantification of AAPP levels……………………………………………….. 11

Probing time analysis………………………………………………………… 12

Prediuresis time analysis……………………………………………………... 13

Blood feeding success on mice and artificial membranes ………………....................... 13

Analysis of the amounts of blood ingested…………………………………… 14

Malaria infection assays…………………………………………………….... 14

Fitness assessment……………………………………………………………. 14

Statistical analysis……………………………………………………………. 15

RESULTS………………………………………………………………………. 16─29

DISCUSSION…………………………………………………………………… 30─33

CONCLUSION…………………………………………………………………. 34

REFFERENCES……………………………………………………………….. 35─39

ACKNOWLEDEMENTS………………………………………………………40─41

LIST OF FIGURES

Figure 1. Expression and purification of truncated AAPPs and Ca^{2+}-binding assay……… 16

Figure 2. Specific inhibition of AAPP─collagen binding by 8H7 mAb…………………... 17

Figure 3. Establishment of the mDsRed-8H7scFv TG mosquito lines……………………..19

Figure 4. Gene Dose Ratio (GDR) of the transgene in TG mosquito genome……………...20

Figure 5. Western blot analysis and Ni-NTA pull-down assay…………………………......21

Figure 6. Observation of saliva under fluorescence microscope……………………………22

Figure 7. AAPP depletion prolongs probing and prediuresis time in TG mosquitoes……..24

Figure 8. Feeding success of TG mosquitoes on mice and artificial membranes…………..25

Figure 9. Malaria infection assay in TG mosquitoes……………………………………….27

Figure 10. Comparison of hatchability and longevity between TG and WT mosquitoes......28

LIST OF TABLE

Table 1. AAPP–scFv complexation depletes AAPP bioactivity in TG mosquitoes............23

ABBREVIATIONS

AAPP	Anopheline anti-platelet protein
Ab	Antibody
An. stephensi	*Anopheles stephensi*
BF	Blood feeding
BSA	Bovine serum albumin
Ca^{2+}	Calcium ion
CI	Confidence interval
E. coli	*Escherichia coli*
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
Fab	Fragment antigen binding
gDNA	Genomic DNA
GDR	Gene dose ratio
GE-rich	Glycine-glutamic acid rich
GFP	Green fluorescent protein
His-tag	Histidine-tag
HRP	Horseradish peroxidase
kDa	Kilo Dalton
mAbs	Monoclonal antibodies
mDsRed	Monomeric DsRed
MFA	Membrane feeding assay
N.D.	Not detected
Ni-NTA	Nickel-nitrilotriacetic acid
pAAPP	aapp promoter
pActin	actin5c promoter
PBS	Phosphate-buffered saline

PBST	Phosphate-buffered saline/Tween
P. berghei	*Plasmodium berghei*
PfCSP	*Plasmodium falciparum* circumsporozoite protein
pI	Isoelectric point
qPCR	Quantitative real-time PCR
rAAPPs	Recombinant anopheline anti-platelet proteins
RNAi	RNA interference
scFv	Single-chain fragment variable
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TG	Transgenic
Trx	Thioredoxin
V_H	Heavy chain
V_L	Light chain
WT	Wild type
2-ME	2-mercaptoethanol

INTRODUCTION

Blood-feeding behavior of mosquitoes is part of their intrinsic character, as blood proteins are essential nutrients for egg production and reproductive fitness. Adult female mosquitoes utilize a diverse array of feeding strategies on their hosts, which enables them to counteract the obstacles associated with host homeostasis (1). Mosquitoes search a suitable blood vessel moving their mouthparts in to the skin during the probing phage. Successful probing along with the injection of salivary components known to enable the mosquito to obtain a blood meal and counteract host homeostasis (2). Several studies have reported on the potent and pleiotropic effects of saliva proteins from hematophagous arthropods, which include anti-coagulation, vasodilation and anti-inflammation (3). The roles played by these proteins in blood feeding have been demonstrated through the use of *in vitro* biochemical analyses even though the biological relevance of these saliva proteins on mosquito function, such as blood feeding propensity, fecundity, egg hatching rates, and viability remains largely unknown. Furthermore, salivary components delivered to the bite site with vector-borne pathogen have been shown to modulate vertebrate immune responses (4). For example, tick derived saliva factors appear to inhibit inflammatory cytokine secretion(5); SAAG-4 from *Aedes aegypti* mosquito saliva has the potential to alter the Th-profile of the bite-induced immune responses(6); sand fly saliva has a caspase-dependent, pro-apoptotic effect on neutrophils(7). The main effect of immunomodulatory saliva components in regard to infection is appear to be temporary and local that allow the vector to feed and resulting into establish an infection(8).

In mosquitoes, transgenesis-based gene silencing and saliva protein inactivation approaches are potentially being used for analyzing the effects of saliva proteins on mosquito physiology. We have established a female salivary gland-specific transgene expression system

in *Anopheles stephensi* mosquitoes using the promoter region of the anopheline antiplatelet protein (AAPP) gene, which encodes an inhibitor of collagen-induced platelet aggregation in the salivary glands (9-11). Using this system, several foreign effector genes encoding the SP15 saliva protein of the *Phlebotomus papatasi* sand fly (12), the repeat region of the *Plasmodium berghei* circumsporozoite protein (CSP) (13, 14) and the anti-*P. falciparum* circumsporozoite protein (PfCSP) single-chain Ab (scFv)(15) have been functionally expressed in the salivary glands as a component of saliva. This system could also be used for gene silencing or protein inactivation for studying the effects of saliva proteins on the ecological attributes of mosquitoes. Recently, Chagas *et al*. (2014) used RNA interference (RNAi) in *Aedes aegypti* silencing the *Aegyptin* gene, which encodes a homolog of AAPP, Aegyptin, significantly reduced its cognate mRNA and protein levels in the salivary glands of female mosquitoes, resulting in prolonged probing time and reduced feeding quality and quantity(16).

In the present study, instead of transgenic RNAi, we used a transgenesis-based protein inactivation approach to explore the functions of AAPP, which is the predominant saliva protein in the main Asian malaria vector mosquito *An. stephensi* (17). AAPP and Aegyptin belong to a member of 30-kDa GE-rich salivary gland protein family with comparable modes of collagen-binding action (10, 18, 19). We have previously reported (20) that AAPP possesses four cysteine residues at its C-terminus and exhibits strong antiplatelet activity. AAPP is also an acidic secretory protein with an isoelectric point (pI) of 3.8, and a GE-rich region containing 10 unique repeats of a 6-amino acid unit (GEEGGA) and related sequences at its N-terminus. Because several Ca^{2+}-binding proteins are known to have clusters of acidic amino acids(21), AAPP might possess Ca^{2+}-binding properties. Therefore, we generated transgenic (TG) *Anopheles stephensi* mosquito lines that express anti-AAPP mAb single chain fragment (scFv)

in their salivary glands. Functional inactivation of AAPP caused by the scFv in the salivary glands was found to almost completely abolish the collagen-binding activity of AAPP. Consequently, a significant increase in probing and prediuresis times, reduction in feeding success, blood meal size, and fecundity were observed in the TG mosquitoes, as compared with their wild-type (WT) counterparts. Sporogonic development in the TG mosquitoes showed no significant reduction in terms of oocysts numbers following blood meals on the *P. berghei*-infected mice. These results indicate that AAPP plays an important role in facilitating blood feeding, but impairment of blood feeding behavior did not affect the malaria vectorial capacity (sporogonic development) in our laboratory model.

AIMS OF THE STUDY

The overall aim of this thesis was to establish an effective transgenesis-based protein inactivation approach for analyzing the relevance AAPP on mosquito blood feeding and malaria transmission in the Asian malaria vector mosquito *Anopheles stephensi*. In addition, the specific aim of this study were:

- To investigate whether the transgenesis could drive the expression of anti-AAPP mAb scFv in the salivary glands of female mosquito
- To examine whether scFv could form a complex with AAPP (AAPP─scFv) in female mosquito salivary glands
- To observe the neutralizing effects of AAPP whether interfering in probing or prediuresis time or blood feeding quantity and quality
- To evaluate whether AAPP has deleterious effect on sporogonic development in the mosquito
- To assess whether AAPP dysfunction could impact on mosquito viability

MATERIALS AND METHODS

Ethics statement

All animal care and handling procedures were approved by the Animal Care and Ethical Review Committee of Kanazawa University (no. 22118–1) and Jichi Medical University (no. 16092), Japan. All experimental procedures were performed in accordance with the Guidelines for Animal Care and the Ethical Review Committee of Kanazawa University (no. 22118–1) and Jichi Medical University (no. 16092), Japan. All efforts were made to minimize animal suffering during the experiments.

Animals, mosquitoes, and parasites

The female inbred BALB/c, slc: ICR or ddY mice obtained from Japan SLC (Hamamatsu, Shizuoka, Japan) were used in all experiments at 7–8 weeks of age. Mice were housed under pathogen-free conditions in Laboratory of Vaccinology and Applied Immunology, Kanazawa University.

The *Anopheles stephensi* mosquitoes strain SDA500 was reared at 27 °C and 50–70% relative humidity under 12 h-light/12 h-dark conditions. Adult mosquitoes were maintained on 5% fructose diet containing 0.05% p-aminobenzoic acid (Nacalai tesque, Inc., Kyoto, Japan) solution. For the eggs production, mosquitoes were fed on mouse blood. Two days post blood meal, an oviposition cup placed to lay eggs. The laid eggs were collected and allowed to hatch in a tray of dechlorinated water and newly hatched larvae were fed on Liquifry (Interpret, Surrey, England). Adult larvae were maintained on a tropical fish food (Kyorin, Himeji, Japan).

P. berghei ANKA parasites constitutively expressing green fluorescent protein (GFP)-*P. berghei* (Pb-conGFP) (22) were maintained by cyclical passaging through slc: ICR mice and *An. stephensi* (SDA 500 strain) according to a standard protocol (14, 23).

AAPP-collagen binding inhibition assays

8H7 and 28B8 anti-AAPP mAbs have been described previously (24). The collagen-AAPP binding inhibition assay with 8H7 and 28B8 mAbs was performed as described previously(10) In brief, 96-well enzyme immunoassay (EIA) plates were collagen-coated by immobilization of soluble type-I collagen (0.3 mg/ml diluted with HCl, pH 3.0; Becton Dickinson, Franklin Lakes, NJ, USA) followed by blocking with phosphate-buffered saline (PBS) containing 1% BSA. Serial dilutions of 8H7 and 28B8 mAbs were incubated with 4 nM of thioredoxin (Trx)-$AAPP_{ex3-4}$(20) for 1 h, and then applied to the collagen-coated plates and incubated for 1 h. Binding of AAPP to collagen was detected using an anti-His Ab conjugated to horseradish peroxidase (HRP) (Qiagen, Hilden, Germany). The AAPP-collagen binding inhibition rate was determined by comparison with the non-mAb control.

Protein separation and Ca^{2+}-binding assays

Recombinant proteins were solubilized with Laemmli buffer containing 2% 2-mercaptoethanol (2-ME) and then boiled for 5 min. The proteins were separated by 10% sodium dodecyl sulfate polyacrylamide (SDS-PAGE), and the gels were either stained using the Rapid CBB KANTO Kit (KANTO Chemical Co., Ltd, Tokyo, Japan) or the Stains-All metachromatic cationic carbocyanine dye (WAKO Chemical Inc., Tokyo, Japan), as described in the literature(25). A Ca^{2+} binding assay was performed as described in the literature(26).

Briefly, a nitrocellulose membrane was pre-equilibrated for 30 min in a buffer solution consisting of 60 mM KCl, 5 mM $MgCl_2$ and 10 mM imidazole-HCl (pH 6.8) and then air-dried at room temperature for 2 h. The recombinant proteins, ovalbumin, and bovine brain calmodulin, each at a concentration of 1 μg/μl per recombinant protein (Calbiochem, San Deigo, CA, USA) were dissolved in ddH_2O and then spotted in triplet onto the membane in equimolar amounts. Calmodulin was used as the reference Ca^{2+}-binding protein, and a 1:15 dilution of it was also spotted onto the membrane as described above. The membrane was air-dried at room temperature for 2 h and then incubated with 10 ml of a buffer containing 1 μCi/ml $^{45}Ca^{2+}$ (Amersham Biosciences Ltd, Buckinghamshire, UK) for 1 h. After incubation, the membrane was rinsed for 5 min with the buffer and air dried at room temperature for 2 h before autoradiography and exposed to X-ray film for 9 days at –70 °C. Densitometric analysis of the autoradiograph was performed using the SCAN ANALYSIS software program (BIOSOFT, Great Shelford, Cambridge, UK).

Minos vector construction and embryo microinjections

The gene fragments encoding 8H7 V_H and V_L have already been cloned from 8H7 hybridoma cells(24). The DNA sequences for the V_H and V_L genes have been deposited in GenBank™ database under the accession numbers AB903029 and AB903030, respectively. The gene encoding 8H7scFv, which consists of the V_H and V_L genes linked to a hinge sequence encoding $(Gly_4Ser)_3$, was inserted into the EcoRI/SphI restriction sites of pENTR-aappP-mDsRed-SP15-antryp1T(12) to generate pENTR-aappP-mDsRed-8H7scFv-antryp1T. The pMinos-EGFP-aappP-mDsRed-8H7scFv-antryp1T transformation plasmid was generated by incubating pMinos-EGFP-RfA-F(9) and pENTR-aappP-mDsRed-8H7scFv-antryp1T in the

presence of LR Clonase (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), as described previously(9). Microinjection of transformation plasmid together with a *Minos* helper plasmid into *Anopheles stephensi* embryos (SDA 500 strain) was carried out as described by Catteruccia *et al.* 2000 (27). Microinjection of 267 mosquito embryos resulted in 36 surviving G_0 mosquitoes. Surviving G_0s were outcrossed to mosquitoes of the opposite sex, and female mosquitoes were fed on mice and eggs were collected and reared as previously described (28) to produce offspring. Progeny larvae (G_1) were screened for GFP expression in their midgut by using fluorescence microscope (BZ-X700; Keyence Corp., Osaka, Japan) equipped with a GFP filter set. Only GFP expressing larvae were separated and inter-crossed, and their progeny (G_2) were obtained. Three independent transgenic lines (TG-03, TG-30 and, TG-37) were established and maintained by intercrossing transgenic siblings.

Selection of transgenic homozygous mosquitoes

To establish the homozygous lines, 15 TG hemizygous females (2-5 days old) were mated with 25 hemizygous TG males of the same age. The females were offered a single blood meal and after 2 days, fed mosquitoes were placed individually in single cups and maintained on a 5% fructose meal. Two days later, wet filter papers were placed in each cup as an oviposition site to lay eggs. Egg deposition was monitored at 72 hours post blood meal and allowed to hatch larvae and reared under the conditions described above. The fourth-instar F_1 larvae were placed and reared individually in a single cup. After eclosion, 25 mating pairs (1 transgenic F_1 virgin female and 1 transgenic F_1 male) were set up. Progeny from individual F_1 pairs were reared as one population to the F_2 generation. Individual F_2 adults were examined under a fluorescent microscope to ensure all mosquitoes were transgenic that carried GFP in their

midguts. To ensure all of the mosquitoes were homozygous, a bioassay was conducted by crossing between transgenic males with virgin nontransgenic (WT) females. Because GFP marker is dominant, all individuals in the offspring population from homozygous transgenic lines should carry GFP. Finally, three homozygous lines (TG-03, TG-30, and TG-37) were obtained.

Genomic DNA extraction and qPCR

The copy numbers of the transgenes to be inserted in the three TG mosquito-lines genomes were determined by qPCR using the AAPP promoter (*pAAPP*) and 8H7 gene as the targets for PCR amplification. Genomic DNA (gDNA) from WT mosquitoes was used as the control. One copy of *pAAPP* per genome is expected in the WT mosquitoes and two or more in the TG mosquitoes. gDNA was extracted from whole mosquito larvae using the QIAamp® DNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's instructions. The extracted DNA was stored at −20ºC until use. gDNA concentrations were estimated by measuring the nucleic acid absorbance at 260 nm. Sample purity was estimated from the 260/280 nm ratio for each preparation, the concentrations of which were adjusted to be equal. The pENTR-pAAPP-mDsRed-G4S1-8H7scFv-polyA plasmid DNA harboring the *pAAPP* and 8H7 genes was used to produce a 10-fold diluted standard curve ranging from 1 to 10^6 copies.

Quantitative analysis of the gDNA was performed using Real-time PCR with SYBR® Green Premix Ex Taq™ (Takara, Tokyo, Japan) and the following primer sets: pAnSG-F14 (5′-ACGGTTCCGAGGTCCGACGATCGTC-3′) and pAnSG-R3 (5′-AAGCTTCATCGTTTATTCACCTG-3′), p8H7-F1 (5′-CCGAAGCGTCTGATCTACCT-3′) and p8H7-R1 (5′-GGCTGATCTTCAGGGTGAAA-3′). Samples were run in triplicate. Three

qPCRs were performed, each with a freshly prepared DNA dilution. Standard curves were generated and the number of copies of each gene in each sample was determined and the average values were calculated.

Production of the anti-mDsRed polyclonal antibody

The gene encoding monomeric DsRed (mDsRed) was excised from plasmid pDsRed-monomer-C1 by digestion with NcoI and SalI, and then cloned into the NcoI/XhoI sites of pET32b (29) to generate pET32b-mDsRed. The recombinant mDsRed protein, created as a fusion protein with Trx, was expressed in *E. coli* and then purified using a Ni-NTA affinity column (Qiagen, Valencia, CA, USA). BALB/c mice were intraperitoneally immunized with the mDsRed protein with Imject® Alum (Thermo Scientific, Waltham, MA, USA) three times at 3-weekly intervals. Three weeks after the last immunization, whole blood from the immunized mice was collected by cardiac puncture and sera was harvested, stored at −20ºC and used later for the immunoblotting experiments.

Immunoblotting and Ni-NTA pull-down assays

Groups of 20 pairs of female mosquito salivary glands from WT and TG mosquitoes were homogenized using a plastic homogenizer with 50 μl of Laemmli buffer containing 2 % 2-ME, and then boiled at 95 °C for 5 min. Each sample was separated on a 12% SDS-PAGE gel and transferred to an Immobilon FL® PVDF membrane (Merck Millipore, MA, USA). Anti-AAPP mAb and anti-mDsRed polyclonal antibody were used as the primary antibodies. Prior to immunoblotting, the membranes were blocked with 5 % skimmed milk (WAKO Chemical Inc., Tokyo, Japan) in 0.1% Tween-20 in PBS (PBST). The membranes were incubated with

the primary antibodies, washed, probed with a secondary antibody conjugated to IRDye 800 (Rockland Immunochemicals, Gilbertsville, PA, USA), and then visualized under an infrared imager (Odyssey *LI-COR*, Lincoln, NE, USA). For the Ni-NTA pull-downs, homogenates of the dissected salivary glands from both TG and WT mosquitoes were lysed with Cellytic^{TM} M (Sigma-Aldrich, St. Louis, MO, USA). Ni-NTA beads (Qiagen, Valencia, CA, USA) (100 µl aliquots) were added to the mixtures, followed by incubation on ice for 1 h with shaking. The Ni-NTA beads allowed the recombinant proteins to be pelleted, and after washing each pellet with PBS three times with intermittent centrifugation, the proteins were eluted by boiling the preparations in 50 µl of Laemmli buffer with 2% 2-ME, followed by storage at −20ºC until they were used for the immunoblotting experiments.

Saliva collection

Twenty female mosquitoes (5-7 days old, never blood feed), both TG and WT, were used for collecting saliva as described salivation protocol (30) with some modifications. Briefly, the mosquitoes were chilled and immobilized, and each mosquito proboscis was placed into 50 µl PBS (pH 7.4) for 3 min. Saliva in the PBS was observed with a fluorescence microscope (BZ-X700; Keyence Corp., Osaka, Japan) under a DsRED filter and then stored at −20ºC until further analysis.

Quantification of AAPP levels

AAPP in saliva or salivary gland homogenates was quantified by ELISA as described previously (20, 24). Briefly, soluble type-I collagen (0.3 mg/ml diluted in HCl, pH 3.0; Becton Dickinson, Franklin Lakes, NJ, USA) was immobilized in 96-well EIA/RIA polystyrene plates (Corning Inc.; Corning, NY, USA) at 100 µl/well (7.5µg/ml), blocked with blocking buffer

(1% BSA in PBS) for 1 h at room temperature, after which recombinant Trx-AAPP$_{ex3-4}$, saliva solutions or salivary gland homogenates were applied to the collagen-coated plates and incubated at room temperature for 1 h. After washing with excessive PBST followed by PBS (three times each), the anti-AAPP antibody, diluted 2,000 fold in blocking buffer, was added and incubated for 1 h. AAPP–collagen binding was detected using an HRP-conjugated anti-His antibody (Bio-Rad Inc., Hercules, CA, USA).

Probing time analysis

Probing time in the TG and WT mosquitoes was measured according to the method described previously (31). Probing time is defined as the time from initial insertion of the proboscis into the host's skin until the initial observable ingurgitation of blood. Mosquitoes, 5–7 days old, never blood fed, were sugar-starved the night before the tests, and individually caged in polystyrene vials (Thermo Fisher Scientific, Waltham, MA, USA) (70 x 120 mm) with the hole (20 mm diameter) sealed by a cotton net. Four groups of 35 mosquitoes each (TG-03, TG-30, TG-37, and WT) were used. Mice were anesthetized with ketamine (100 mg/kg, intramuscular, i.m.; Daiichi Sankyo, Tokyo, Japan) and xylazine (10 mg/kg; i.m.; Bayer, Tokyo, Japan) (32). Their backs were shaved and placed in direct contact with the mosquito-containing cages. Probing times were measured for three different naïve mice, alternating the mosquitoes within each group. Mosquitoes that did not initiate probing or showed no interest in the host after 5-min exposure were eliminated from the analysis. Probing-time observations were terminated after 420 s, and the times recorded were used in the analyses. Data were analyzed using Dunnett's multiple comparisons test vs the control with a 95% confidence interval (CI).

Prediuresis time analysis

Prediuresis times in TG and WT mosquitoes were determined according to the method described previously (31). Prediuresis time was defined as the time taken from initial insertion of proboscis into the host skin until the first droplet of prediuresis liquid was observed. Briefly, female mosquitoes (5─7 days old, never blood fed) were individually caged in transparent polystyrene vials and starved overnight before the experiments commenced. The shaved back of an anesthetized naïve mouse made direct contact with a cage containing the mosquitoes. Prediuresis times were measured on three different mice, alternating single mosquitoes from each group. The times recorded were used in the analyses. Data were analyzed using the Dunnett's multiple comparisons test vs the control with a 95% CI.

Blood feeding success on mice and artificial membranes

Feeding success in the TG and WT mosquitoes was determined using anesthetized mice or a MFA, as previously described (16). Four groups (TG-3, TG-30, TG-37, and WT) of 30 5–7-days-old female mosquitoes were caged and deprived of sugar the day before the experiments were performed. To each cage was offered an anesthetized naïve mouse and the mosquitoes were allowed to feed for 10 min. The number of fed and unfed mosquitoes was scored. Females with either their abdomens fully or partially engorged were considered as fed. For the MFAs, the same four groups of mosquitoes (30 females each), were allowed to feed for 10 min on an artificial membrane feeder (Chemglass Life Sciences, Vineland, NJ, USA) covered with a stretched parafilm membrane (Fuji film Corp., Tokyo, Japan). The blood meal consisted of human defibrinated blood. Membrane feeders were kept at 37 °C during feeding, and the mosquitoes were assessed as fed or unfed as per the direct feeding experiments in mice.

Analysis of the amounts of blood ingested

The blood meal size ingested by the TG and WT mosquitoes from mice or the MFA was assessed. The abdomens of the engorged mosquitoes were dissected and homogenized in 50 µl of PBS (pH 7.4). The homogenates were centrifuged at 10000 ×g for 5 min at 4 °C, and the supernatants were collected and kept on ice. Total hemoglobin in their abdomens was quantified using the hemoglobin colorimetric assay kit (Cayman, Ann Arbor, MI, USA). From each sample, 20 µl was added in duplicate to a 96-well microtiter plate followed by 180 µl of kit's hemoglobin detector. The microtiter plate was incubated while protected from light at room temperature for 15 min, and the absorbance was measured at 560 nm in a microtiter plate reader (Multiskan FC, Thermo Scientific, Waltham, MA, USA).

Malaria infection assays

TG and WT mosquitoes, 5–7 days after eclosion, were allowed to feed on the same *P. berghei* (Pb-conGFP) infected mouse for 30 min according to a standard protocol (14, 23). Unfed mosquitoes were counted and discarded. Only fed mosquitoes were kept on a 5% fructose diet at 21 °C. On days 10–12 the midguts were dissected, and the number of oocysts per midgut was counted under a phase contrast microscope. On days 19–21 the salivary glands were dissected, and the number of sporozoites per salivary gland was counted.

Fitness assessment

Egg hatchability and the adult survivorship were assessed in the TG mosquitoes and their WT siblings as described in literature (33, 34). Briefly, group of 35 newly eclosed TG males and females were selected and used to set up pairwise mating for each of the 4 experimental

lines (TG-03, TG-30, TG-37, and WT). Female mosquitoes were then exposed to anesthetized mouse to obtain a blood meal without time limit. Fed females (n=20) were put individually into oviposition cup and maintained on a 5% fructose meal. Egg deposition was monitored at 72 hours post blood meal and the number of eggs laid by each female was counted and recorded as an index of fecundity, excluding females that laid no eggs. To assess the hatchability, the laid eggs were collected on a wet filter paper and kept in a wet Petri dish. After two days at 27°C, the number of larvae and no hatched eggs was counted with the aid of microscope. The numbers of larvae hatching from the laid eggs were recorded as a measure of fertility. To estimate adult survivorship, 20 adults from each lines of both sexes were caged separately and fed on 5% fructose or anesthetized mice without time restriction. The total surviving adults in the case was recorded daily till all died. Insectary procedures were standardized to ensure that all mosquitoes were treated similarly.

Statistical analysis

Experimental data were analyzed with Prism version 7.0a (GraphPad Software Inc., La Jolla, CA, USA) and plotted as bar graphs or scatter plots. Dunnett's multiple comparison tests (95% CI) and two-way analysis of variance were performed in the comparisons analysis. p values of < 0.05 were considered statistically significant. A *Chi*-square test (95% CI) was used to compare the feeding success calculation. The Kruskal-Wallis and the log rank test (95% CI) were performed to analyze the infection rate and survival curves respectively.

RESULTS

AAPP is a Ca^{2+}-binding protein

AAPP, a predominant saliva protein in the malaria vector *An. stephensi* (10), exhibits strong antiplatelet activity by binding directly to collagen and subsequently blocking platelet aggregation via its C-terminus conformational domain, which contains four cysteine residues (20). AAPP also has another characteristic feature of acidic secretory proteins (pI =3.8) in that, as mentioned above, it contains a GE-rich region containing 10 unique repeats of a 6-amino acid unit (GEEGGA) or related sequences at the N-terminus. Therefore, as several Ca^{2+}-binding proteins are known to have clusters of acidic amino acid residues, we examined whether AAPP has Ca^{2+} binding properties. Because the AAPP gene is encoded by four exons,

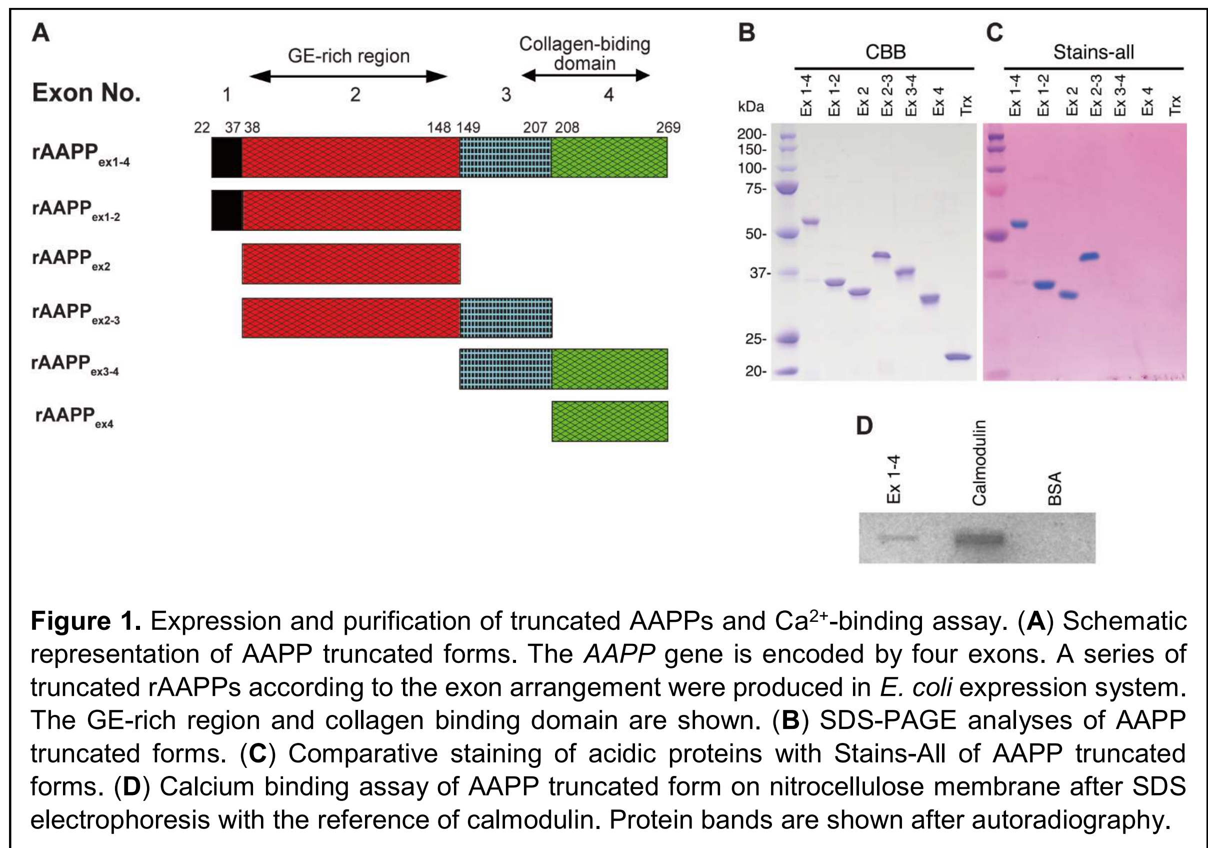

Figure 1. Expression and purification of truncated AAPPs and Ca^{2+}-binding assay. (**A**) Schematic representation of AAPP truncated forms. The *AAPP* gene is encoded by four exons. A series of truncated rAAPPs according to the exon arrangement were produced in *E. coli* expression system. The GE-rich region and collagen binding domain are shown. (**B**) SDS-PAGE analyses of AAPP truncated forms. (**C**) Comparative staining of acidic proteins with Stains-All of AAPP truncated forms. (**D**) Calcium binding assay of AAPP truncated form on nitrocellulose membrane after SDS electrophoresis with the reference of calmodulin. Protein bands are shown after autoradiography.

the *Escherichia coli* expression system was used as reported previously to produce a series of

truncated AAPP recombinant proteins (rAAPPs) according to the exon arrangement (20) depicted in Fig. 1A. Purified rAAPPs were stained with Stains-All reagent, which has previously been used to identify Ca^{2+}-binding activity (35). Truncated $rAAPP_{ex1-4}$, $rAAPP_{ex1-2}$, $rAAPP_{ex2}$ and $rAAPP_{ex2-3}$ were clearly stained blue with Stains-All, unlike $rAAPP_{ex3-4}$ and $rAAPP_{ex4}$ (Fig. 1B, C). This result indicates that the highly negatively charged GE-rich region encoded by exon 2 contains Ca^{2+} binding sites (Fig. 1A). The $^{45}Ca^{2+}$ overlay assay directly evidences the Ca^{2+} binding property of $rAAPP_{ex1-4}$, which is estimated to be eight times lower than that of calmodulin when equimolar amounts were tested (Fig. 1D). No Ca^{2+}-binding property was observed for the bovine serum albumin (BSA) control.

8H7 mAb, potentially inhibits the binding of AAPP to collagen

We have previously tested several recombinant truncated genes carrying different

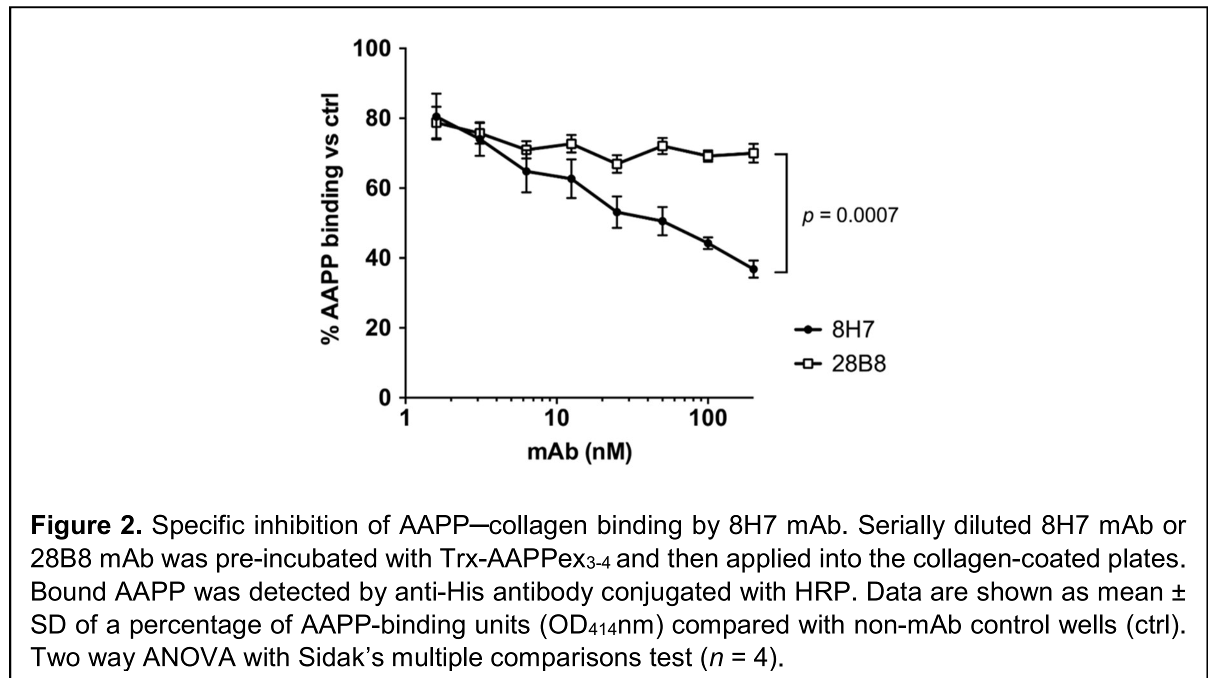

Figure 2. Specific inhibition of AAPP–collagen binding by 8H7 mAb. Serially diluted 8H7 mAb or 28B8 mAb was pre-incubated with Trx-$AAPPex_{3-4}$ and then applied into the collagen-coated plates. Bound AAPP was detected by anti-His antibody conjugated with HRP. Data are shown as mean ± SD of a percentage of AAPP-binding units (OD_{414}nm) compared with non-mAb control wells (ctrl). Two way ANOVA with Sidak's multiple comparisons test (n = 4).

combinations of the four exons to locate the precise collagen binding sites in AAPP. Of them, exons 3 and 4 are absolutely required for collagen-AAPP interactions (20). We also obtained

a series of anti-AAPP monoclonal Abs (mAbs) (24) from which the 8H7 mAb was found to be a potent inhibitory Ab capable of blocking the binding of AAPP to collagen, while the 28B8 mAb (Fig. 2) was not. The crystal structure of the collagen-binding domain of AAPP has been solved with bound 8H7 Fab (24). The heavy chain (V_H) and light chain (V_L) gene fragments encoding the 8H7 mAb were cloned from 8H7 hybridoma cells by reverse-transcription-PCR as described previously (24). The nucleotide sequence data for the V_H and V_L genes have been deposited in GenBank® database (https://www.ncbi.nlm.nih.gov/genbank/) under the accession numbers AB903029 and AB903030 respectively.

Establishing transgenic mosquitoes

A *Minos*-based transformation vector harboring a gene cassette encoding 8H7scFv fused

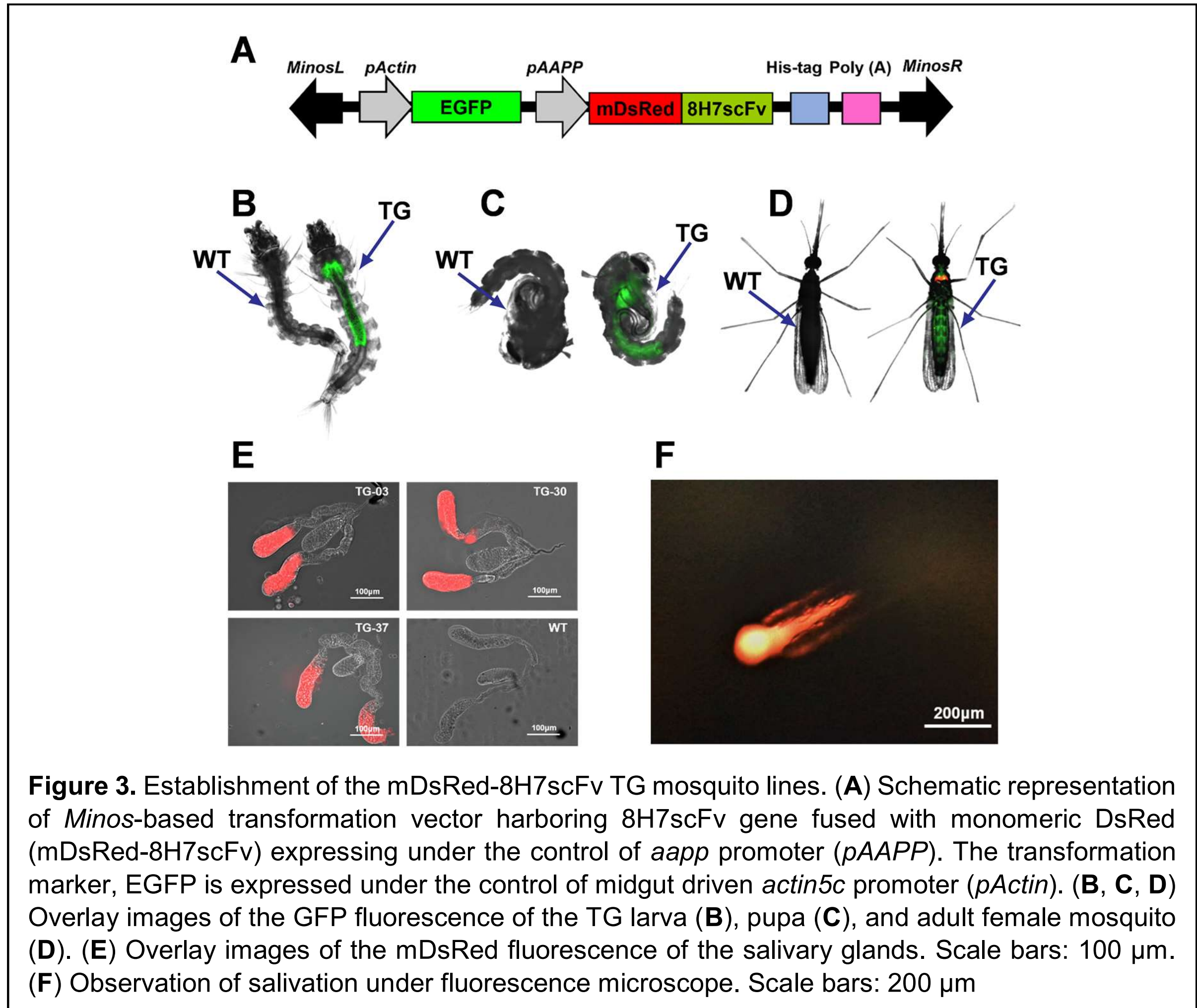

Figure 3. Establishment of the mDsRed-8H7scFv TG mosquito lines. (**A**) Schematic representation of *Minos*-based transformation vector harboring 8H7scFv gene fused with monomeric DsRed (mDsRed-8H7scFv) expressing under the control of *aapp* promoter (*pAAPP*). The transformation marker, EGFP is expressed under the control of midgut driven *actin5c* promoter (*pActin*). (**B**, **C**, **D**) Overlay images of the GFP fluorescence of the TG larva (**B**), pupa (**C**), and adult female mosquito (**D**). (**E**) Overlay images of the mDsRed fluorescence of the salivary glands. Scale bars: 100 μm. (**F**) Observation of salivation under fluorescence microscope. Scale bars: 200 μm

to mDsRed through $Gly_4Ser \times 3$ linkers with a hexahistidine-tag at the C-terminus under the control of the female salivary gland-specific *aapp* promoter, was constructed (Fig. 3A) and injected together with a *Minos* helper plasmid (14) into *An. stephensi* embryos. Three independent TG mosquito lines (TG-03, TG-30, and TG-37) were established and stably maintained by intercrossing with the transgenic siblings. Midgut specific GFP expression observed in the TG mosquitoes almost all of the developing stages that includes TG larvae (Fig. 3B), TG pupa (Fig. 3C), and TG adults (Fig. 3D) respectively. Fluorescence microscopy revealed strong red fluorescence in the distal-lateral lobes of the dissected female salivary

glands from the TG mosquitoes (Fig. 3E). We also observed using fluorescence microscopy that the mDsRed-8H7scFv protein was released from the proboscis as a saliva component (Fig. 3F).This expression pattern is consistent with our previous transgenic lines established using the same *aapp* promoter (12, 14, 15).

Gene dose determination in transgenic mosquitoes

The copy numbers of the transgenes to be inserted into the genomes of three TG mosquito lines was determined by quantitative real-time PCR (qPCR). The salivary gland-specific AAPP

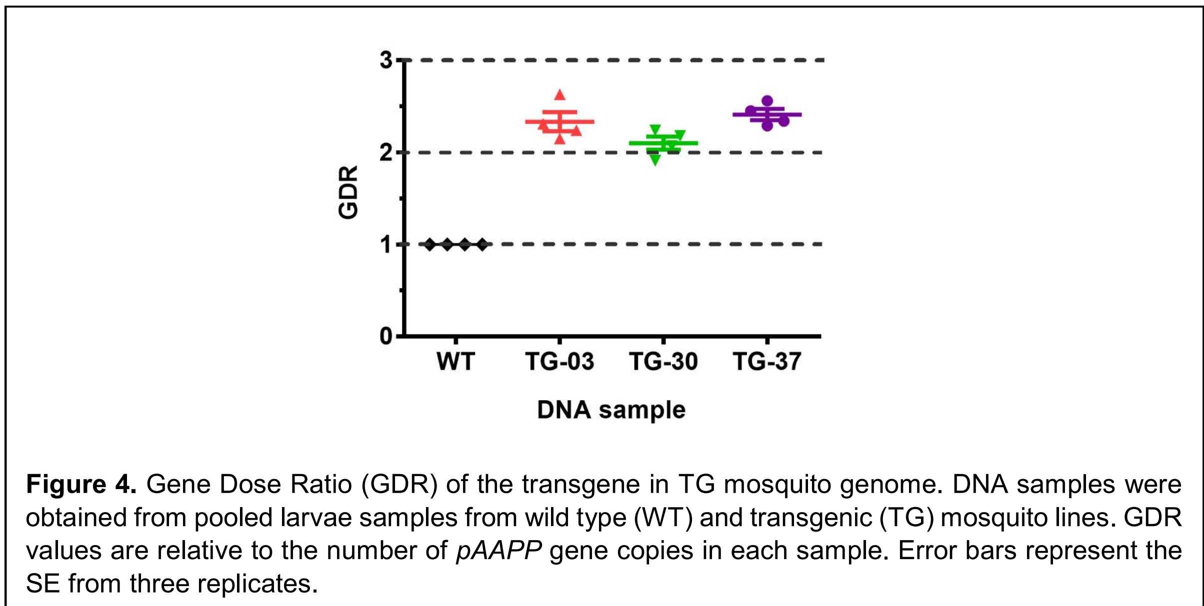

Figure 4. Gene Dose Ratio (GDR) of the transgene in TG mosquito genome. DNA samples were obtained from pooled larvae samples from wild type (WT) and transgenic (TG) mosquito lines. GDR values are relative to the number of *pAAPP* gene copies in each sample. Error bars represent the SE from three replicates.

promoter gene *(pAAPP)* was selected as an internal reference single-copy gene because it is highly conserved. The gene dose ratio (GDR) values obtained are summarized in the Fig. 4. As can be observed, the *aapp* promoter gene was the same (i.e., remained a single copy) in the WT mosquitoes, while duplicate gene copies were clearly separated in the TG mosquito lines. These results indicate that there was single copy insertion per genome in all the TG mosquito lines. Moreover, we have not observed non-transgenic mosquitoes from any of the 3 TG lines during the subsequent 2 years (observation of more than 25 generations with more than 500

adults per generation). This observation confirmed that the mosquitoes were homozygous for the insertion and that the transgene is stably integrated.

Complex formation between 8H7scFv and AAPP in the salivary glands

Immunoblots probed with the 28B8 mAb revealed that AAPP (M*r* = 37 kDa) was detectable in the salivary gland lysates from both WT and TG mosquitoes at similar amounts

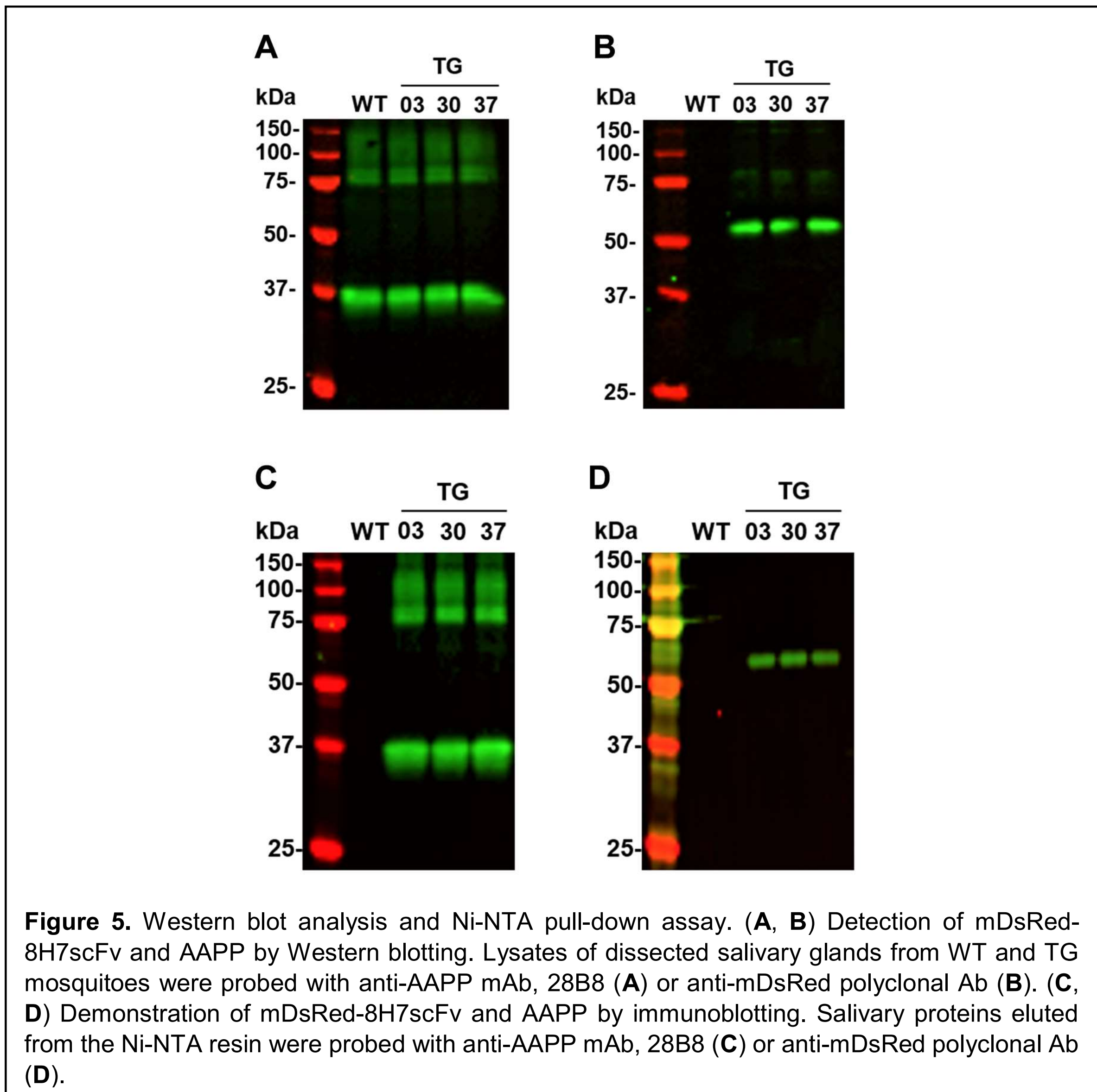

Figure 5. Western blot analysis and Ni-NTA pull-down assay. (**A**, **B**) Detection of mDsRed-8H7scFv and AAPP by Western blotting. Lysates of dissected salivary glands from WT and TG mosquitoes were probed with anti-AAPP mAb, 28B8 (**A**) or anti-mDsRed polyclonal Ab (**B**). (**C**, **D**) Demonstration of mDsRed-8H7scFv and AAPP by immunoblotting. Salivary proteins eluted from the Ni-NTA resin were probed with anti-AAPP mAb, 28B8 (**C**) or anti-mDsRed polyclonal Ab (**D**).

(Fig. 5A, lanes WT, and TG). A 54.6 kDa protein corresponding mDsRed-8H7scFv was

detected in lysates from the dissected salivary glands from the TG mosquitoes using the anti-mDsRed polyclonal Ab (Fig. 5B, lane; TG). We next performed a pull-down assay to detect mDsRed-8H7scFv-AAPP in its complexed form. Because the mDsRed-8H7scFv protein has a hexahistidine-tag at its C-terminus, Ni-NTA resin was used to perform the pull-down assays with the salivary gland lysates. After elution from the Ni-NTA resin, AAPP (Fig. 5C, TG lanes) and mDsRed-8H7scFv (Fig. 5D, TG lanes) were detected in TG mosquitoes by the anti-AAPP mAb (28B8) and the anti-DsRed polyclonal Ab, respectively, whereas no signals were detected in the WT mosquitoes (Fig. 5C and D; WT lanes). These results clearly revealed specific binding between the two proteins and resulted in forming a complex.

Complexation of mDsRed-8H7scFv and AAPP severely impairs AAPP–collagen binding

To investigate whether the mDsRed-8H7scFv–AAPP complex could block the collagen-binding activity of AAPP and abrogate its function(s), we collected saliva from the proboscises of 20 female mosquitoes during salivation (Fig. 3F). Salivary gland homogenates were also

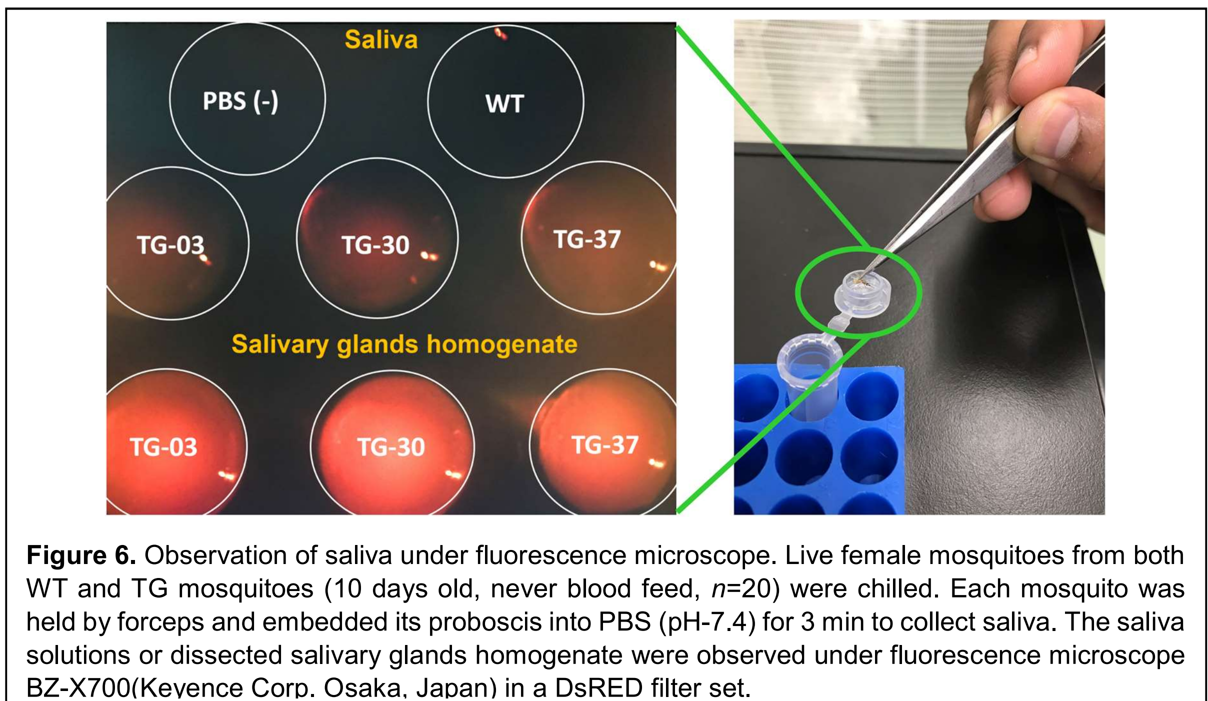

Figure 6. Observation of saliva under fluorescence microscope. Live female mosquitoes from both WT and TG mosquitoes (10 days old, never blood feed, *n*=20) were chilled. Each mosquito was held by forceps and embedded its proboscis into PBS (pH-7.4) for 3 min to collect saliva. The saliva solutions or dissected salivary glands homogenate were observed under fluorescence microscope BZ-X700(Keyence Corp. Osaka, Japan) in a DsRED filter set.

prepared from dissected mosquitoes. Red fluorescence was observed in both the saliva and

Table 1. AAPP–scFv complexation depletes AAPP bioactivity in TG mosquitoes[a]

Sample \ Group	WT	TG-03	TG-30	TG-37
Saliva (ng)	28.4 ± 0.57	N.D.	N.D.	N.D.
Salivary glands (ng)	232.7 ± 1.87	11.37 ± 0.95	9.95 ± 0.38	10.5 ± 0.30

[a]A live female mosquito was held by forceps and its proboscis was placed into PBS (pH 7.4) for 3 min to collect saliva. Alternatively, the salivary glands were dissected and homogenized. Saliva and salivary gland lysates were obtained from a total of 20 mosquitoes per group, and their red fluorescence was observed (Figure 3). These samples were applied for collagen-binding ELISA to measure the amount of AAPP capable of binding to collagen. Trx-$AAPP_{ex1-4}$ was used for quantification of AAPP.
N.D., Not detected (detection limit <0.069 ng)

salivary gland homogenates (Fig. 6). The collagen-binding enzyme-linked immunosorbent assay (ELISA) showed that the saliva from 20 mosquitoes from each TG line completely abrogated the AAPP-collagen binding activity (detection limit <0.069 ng), whereas the saliva from 20 WT mosquitoes retained AAPP-mediated collagen-binding activity (28.4 ± 0.57 ng) (Table 1). The salivary gland homogenates from each TG line retained a trace of collagen-binding activity (9.95-11.37 ng), which is 23-fold lower than that for WT mosquitoes (232.7 ± 1.87 ng), indicating that minute amounts of unbound AAPP existed in the TG lines.

Functional depletion of AAPP prolongs probing and prediuresis times

To evaluate the ecological functions of AAPP, we examined probing and prediuresis times in both TG and WT mosquitoes using a mouse model. The TG mosquitoes had significantly

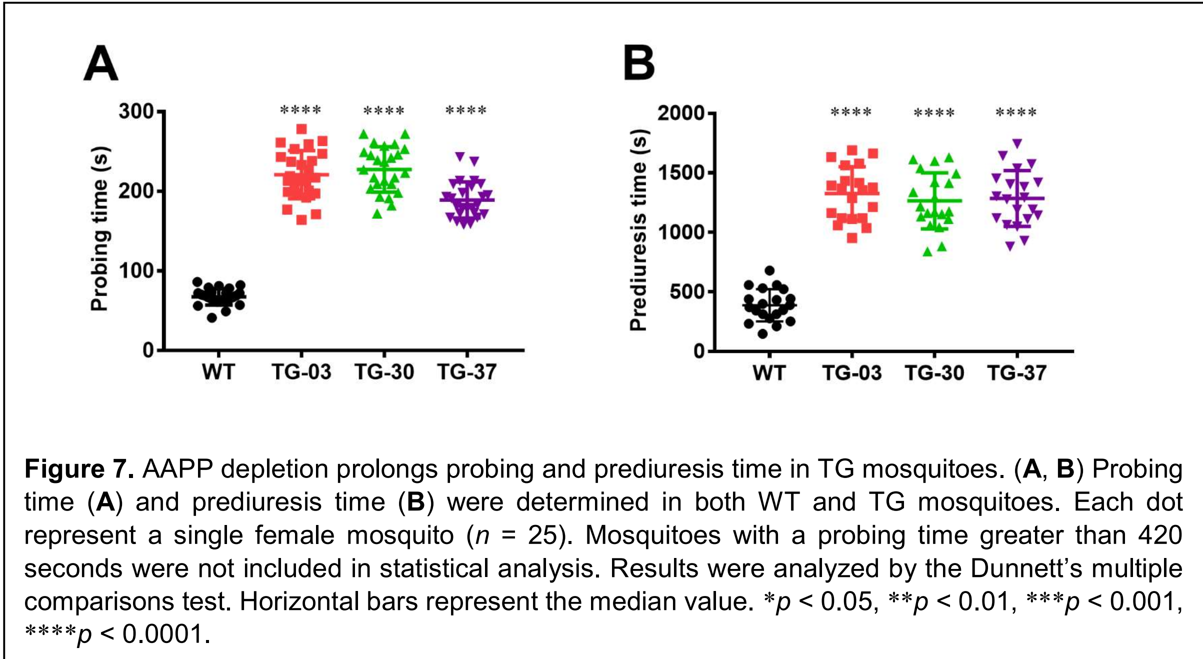

Figure 7. AAPP depletion prolongs probing and prediuresis time in TG mosquitoes. (**A**, **B**) Probing time (**A**) and prediuresis time (**B**) were determined in both WT and TG mosquitoes. Each dot represent a single female mosquito (*n* = 25). Mosquitoes with a probing time greater than 420 seconds were not included in statistical analysis. Results were analyzed by the Dunnett's multiple comparisons test. Horizontal bars represent the median value. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

longer probing times (range, 158–278 s) than those of the WT mosquitoes (range, 41–86 s) (Fig. 7A). The TG mosquitoes also displayed significantly longer prediuresis times (range, 839–1742 s) than those of the WT mosquitoes (range, 147–679 s) (Fig. 7B). The prolonged probing and prediuresis times observed in the TG mosquitoes might be related to 8H7scFv–AAPP complex formation in the salivary glands which probably in disabling of collagen-induced platelet aggregation during the blood meal.

AAPP dysfunction impairs mosquito feeding behavior

We compared the feeding success of the TG mosquitoes in the presence (direct mouse feeding) or absence (membrane feeding assay; MFA) of collagen-induced platelet aggregation after 10 min of blood feeding. Under these conditions, the TG mosquitoes were significantly less successful in obtaining a blood meal when fed on mice (presence) compared with MFA

(absence), $p < 0.05$, χ^2 test; (Fig. 8A). We next examined the ingested blood meal size of the TG and WT mosquitoes after 10 min of blood feeding. The abdomen of each fed mosquito was

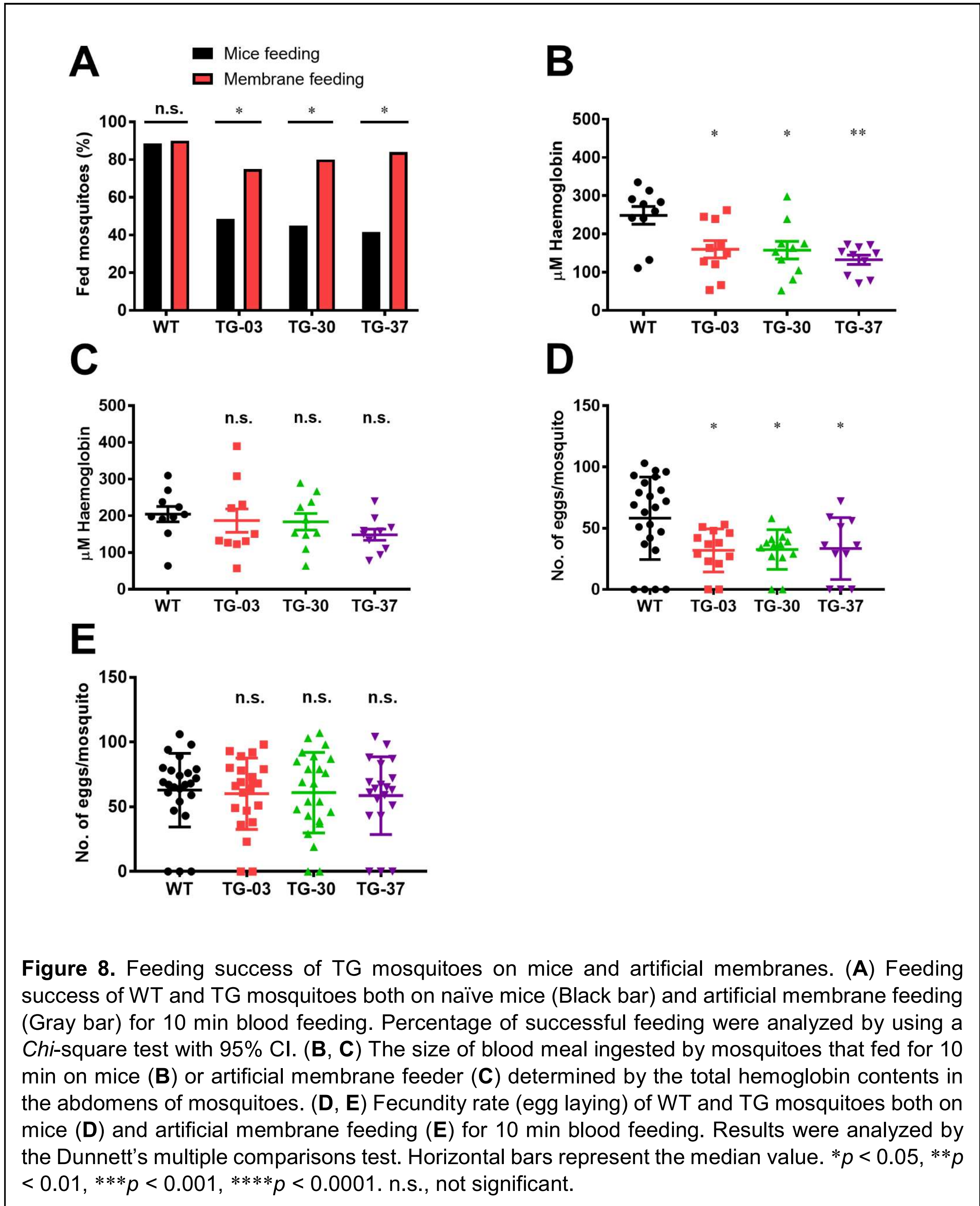

Figure 8. Feeding success of TG mosquitoes on mice and artificial membranes. (**A**) Feeding success of WT and TG mosquitoes both on naïve mice (Black bar) and artificial membrane feeding (Gray bar) for 10 min blood feeding. Percentage of successful feeding were analyzed by using a *Chi*-square test with 95% CI. (**B**, **C**) The size of blood meal ingested by mosquitoes that fed for 10 min on mice (**B**) or artificial membrane feeder (**C**) determined by the total hemoglobin contents in the abdomens of mosquitoes. (**D**, **E**) Fecundity rate (egg laying) of WT and TG mosquitoes both on mice (**D**) and artificial membrane feeding (**E**) for 10 min blood feeding. Results were analyzed by the Dunnett's multiple comparisons test. Horizontal bars represent the median value. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. n.s., not significant.

homogenized and total hemoglobin was quantified with a colorimetric assay (31).

The blood volume ingested by the TG mosquitoes was significantly reduced compared with the WT mosquitoes when fed on mice (Fig. 8B). No significant difference in blood meal size was observed between the TG and WT mosquitoes when fed using MFA (Fig. 8C). Probably related to the blood meal size, fecundity was lower in the TG mosquitoes than in the WT mosquitoes when fed on mice (Fig. 8D), whereas the TG mosquitoes did not show any significant reduction in their fecundity when fed by MFA (Fig. 8E). Thus, AAPP appears to impact in blood feeding success and plays an important role in facilitating blood feeding and subsequent egg production.

Parasite development in transgenic mosquitoes is not impaired

To investigate the effect of depleting AAPP activity on parasite development, TG and WT

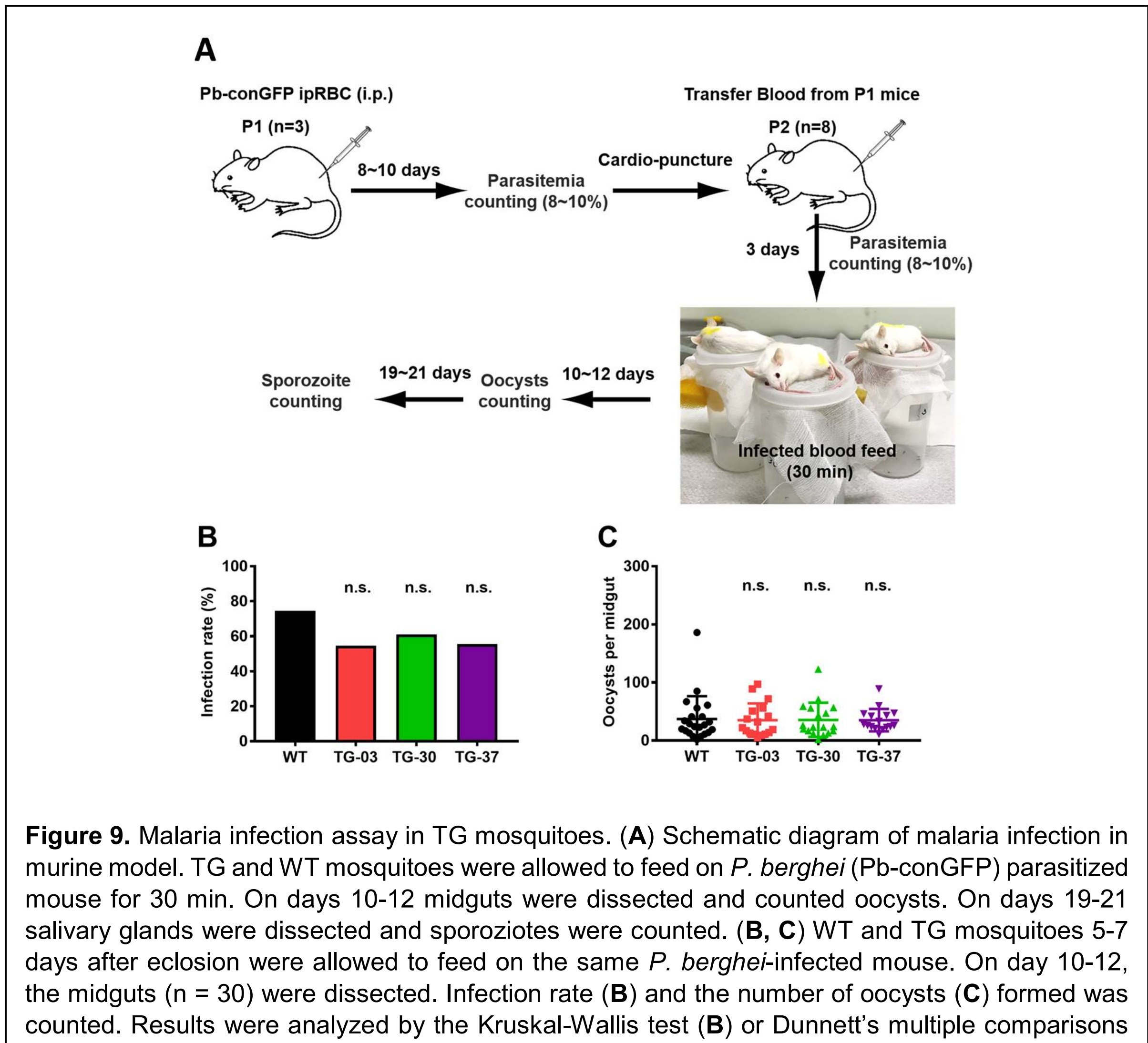

Figure 9. Malaria infection assay in TG mosquitoes. (**A**) Schematic diagram of malaria infection in murine model. TG and WT mosquitoes were allowed to feed on *P. berghei* (Pb-conGFP) parasitized mouse for 30 min. On days 10-12 midguts were dissected and counted oocysts. On days 19-21 salivary glands were dissected and sporoziotes were counted. (**B, C**) WT and TG mosquitoes 5-7 days after eclosion were allowed to feed on the same *P. berghei*-infected mouse. On day 10-12, the midguts (n = 30) were dissected. Infection rate (**B**) and the number of oocysts (**C**) formed was counted. Results were analyzed by the Kruskal-Wallis test (**B**) or Dunnett's multiple comparisons

mosquitoes were allowed to feed on the same mouse that had previously been infected with *P. berghei* (Fig. 9A). There was no statistical difference in the infection rate (Fig. 9B) or oocyst number (Fig. 9C) between the TG and WT mosquitoes, indicating that AAPP has no deleterious effect on sporogonic development in the mosquito.

Fitness assessments

Since, the TG mosquitoes showed significant increase in probing time and reduced feeding quality and quantity, we next sought to the neutralizing effects of AAPP on mosquito viability.

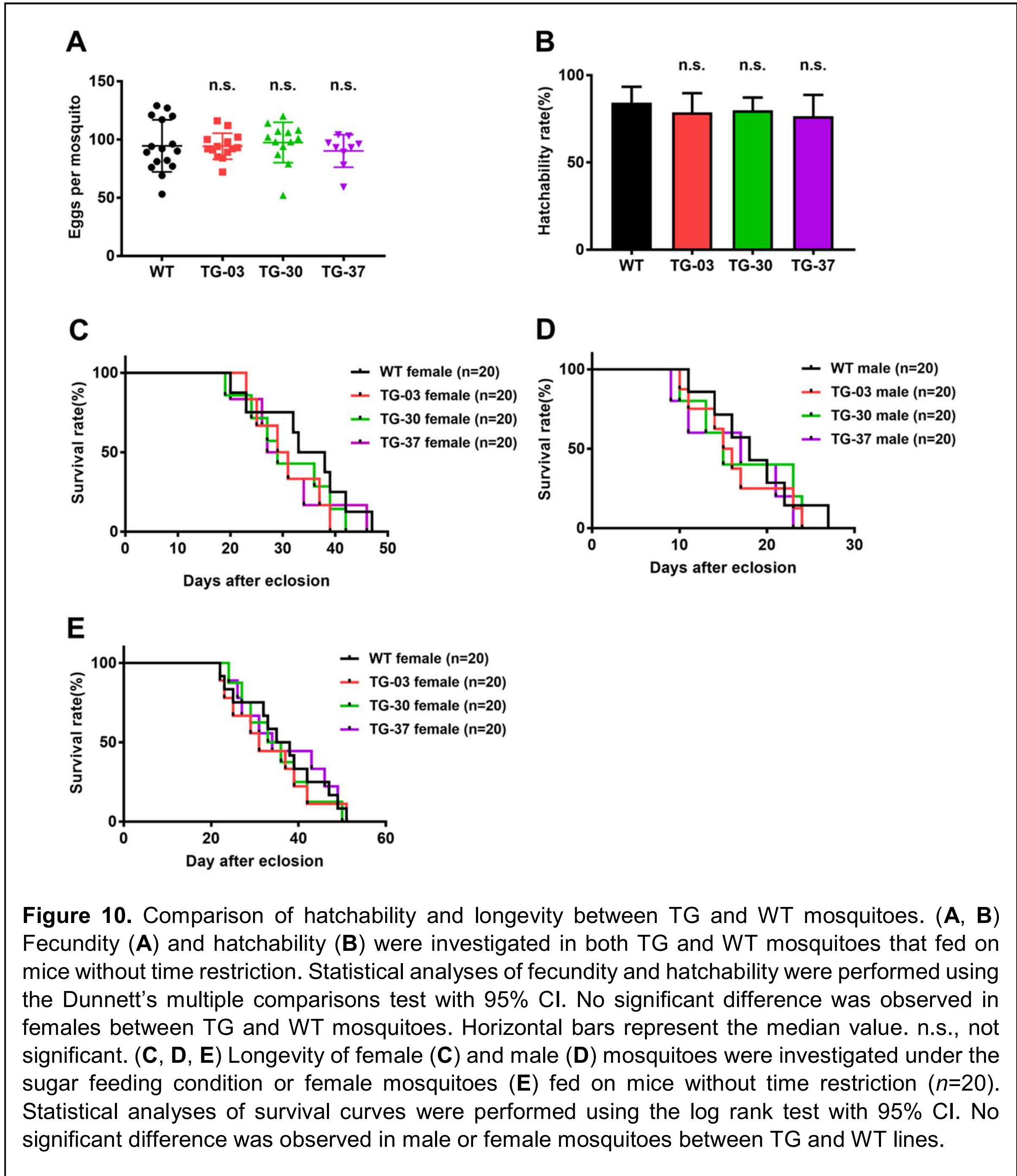

Figure 10. Comparison of hatchability and longevity between TG and WT mosquitoes. (**A**, **B**) Fecundity (**A**) and hatchability (**B**) were investigated in both TG and WT mosquitoes that fed on mice without time restriction. Statistical analyses of fecundity and hatchability were performed using the Dunnett's multiple comparisons test with 95% CI. No significant difference was observed in females between TG and WT mosquitoes. Horizontal bars represent the median value. n.s., not significant. (**C**, **D**, **E**) Longevity of female (**C**) and male (**D**) mosquitoes were investigated under the sugar feeding condition or female mosquitoes (**E**) fed on mice without time restriction (*n*=20). Statistical analyses of survival curves were performed using the log rank test with 95% CI. No significant difference was observed in male or female mosquitoes between TG and WT lines.

Fitness was assessed in TG mosquitoes and their WT siblings using the following parameters:

fecundity (numbers of eggs laid per mosquitoes), fertility as a function of egg hatchability (number of larvae/number of eggs). No significant difference was observed in fecundity (Fig.10A) or hatchability (Fig. 10B) between the TG and WT mosquitoes when fed on mice without time restriction. We next monitored the longevity of the female and male mosquitoes under the sugar feeding or female mosquitoes fed on mice without time limit. Under these conditions, TG female (Fig. 10C) or male (Fig. 10D) mosquitoes under sugar feeding and TG female (Fig. 10E) fed on mice did not show any significant reduction in the longevity. These results suggest that there is no unequivocal impact of the *Minos*-based construct at the specific genomic insertion in these TG mosquito lines.

DISCUSSION

We have previously generated a malaria-refractory transgenic *Anopheles* mosquito line that expresses anti-PfCSP scFv in its salivary glands (15). The anti-PfCSP scFv binds to transgenic sporozoites expressing PfCSP in the salivary glands, impairing their infectivity to mice. Using this scFv-mediated inhibition approach, in the present study we generated transgenic *An. stephensi* lines expressing anti-AAPP scFv in their salivary glands to investigate the role of AAPP in blood feeding and malaria transmission, with the aim of subsequently evaluating the ecological functions of this protein in mosquitoes. The data presented here show that the collagen-binding activity of AAPP was almost completely abolished by the scFv secreted in the saliva of the TG mosquitoes. Probing and prediuresis time, feeding success, blood meal size, and fecundity or hatchability assessments in respect of fed on mice for a restricted time (10 min BF), we found that the TG mosquitoes showed a significant reduction in these parameters when compared with the WT mosquitoes. On the other hand, TG female mosquitoes exhibited no significant differences as compared with their wild-type (WT) counterparts, during assessing the fitness when exposed to an anesthetized mouse to obtain a blood meal without time limit. However, a rapid mechanism capable of defeating host homeostasis at the probing site is a prerequisite for hematophagous arthropod survival. Moreover, prolonged probing and prediuresis time can alert vertebrate hosts to biting, resulting in feeding termination or death for blood-sucking arthropods. Therefore, faster probing or feeding times reduces the vector-host association period and increases the survival of blood-sucking arthropods.

AAPP, Aegyptin and their homologs, which are only conserved among mosquito species as members of the GE-rich protein family, contain a collagen-binding domain located at the C-terminus (20). Sequence homology analysis of the C-terminus reveals that four cysteine residues are completely conserved, although the amino acid identity in this region is only around 89% between each protein (20). As blood feeding behavior such as probing time length and salivary gland antihemostatic activities vary among *Anopheles*, *Aedes* and *Culex* mosquitoes (36), it would be interesting to examine the relationship between the collagen-binding activities of the GE-rich protein family members and the blood feeding behaviors among the various mosquito species. Because the GE-rich domain in AAPP possesses the characteristic feature of Ca^{2+}-binding activity, thus it can prevent a collagen-induced increase in intracellular Ca^{2+}, which is the second messenger in the platelet activation cascade (37), and has evolved to recruit translocating platelets into the developing aggregates. Additionally, AAPP may act as a Ca^{2+} chelator after its bind to collagen, resulting in enhanced inhibition of platelet aggregation.

Reducing the abundance of Aegyptin at the protein level using transgenic RNAi (16) showed similar results to the ones we have described here. In contrast to silencing the *Aegyptin* gene, our TG mosquitoes produced AAPP without collagen-binding activity, at similar amounts as AAPP in the WT mosquitoes. Although the AAPP-collagen binding ELISA showed that the scFv secreted through the saliva causes almost complete abrogation of AAPP-collagen binding activity from each line of TG mosquitoes (Table-1), further investigation will be required to validate the loss of the function of AAPP caused by scFv. Despite the shortage of collagen-binding activity, AAPP in TG mosquitoes may still retain its Ca^{2+}-binding activity in the GE-rich region. We found no apparent phenotypic difference between AAPP protein

inactivation and Aegyptin knock-down, suggesting other functions such as AAPP may participate in forming oligomers or multimers which elucidated in immunoblotting. AAPP multimers and protein bands with high molecular weights were recognized by the anti-AAPP mAb in the salivary gland lysates (Fig. 5A and C). The oligomerization might enable AAPP to increase the number of collagen binding sites. Clearly, further studies are needed to elucidate the precise function of the Ca^{2+} binding properties of AAPP.

We further examined whether the behavioral changes in the TG lines would affect sporogonic development of the malaria parasites. No significant reductions in oocyst numbers were observed in the TG mosquitoes following their blood meals on *P. berghei*-infected mice, indicating that oocyst formation is not likely to be affected by the blood meal size. This result suggests that inactivating a single saliva protein does not affect sporogonic development of the parasite. A recent study also reported that the number of oocysts developing in the mosquito midgut depends on the density of malaria gametocytes in the host's blood (38). Nevertheless, it is also of great interest to address the effect of saliva protein on malaria transmission from mosquito to vertebrate host. It has been documented that in hematophagous arthropods, the saliva components not only facilitate in the acquisition of the blood meal but also modulate vertebrate immune responses resulting in a local microenvironment that favors the establishment of a vector-borne disease (39). Therefore, vertebrate host immunity that blocks the pharmacological action of the salivary constituents has the potential to affect a vector's feeding ability and transmission of vector-borne pathogens. It has been reported that transgenic *An. stephensi* mosquitoes producing extremely low amounts of saliva enabled to ingest blood and the resulting phenotypes showed a significant reduction in the ability to transmit malaria parasites and salivary glands homogenate exhibited reduced exflagellation *in vitro* (31). This

raises the possibility of the development of a saliva protein-based vaccine that targets malaria transmission stages (40).

CONCLUSION

In conclusion, we confirmed that AAPP involves in enabling blood acquisition in *Anopheles stephensi*, does not have adversarial effects on malaria vectorial capacity (sporogonic development) in our laboratory model. Our studies reinforce current knowledge of hematophagous arthropods saliva components and their crucial role in blood feeding. Moreover, our transgenesis-based protein inactivation and protein-protein interaction approaches provide an exclusive opportunity to understand the complex interactions occurring between multifaceted saliva proteins and host homeostasis or pathogen transmission *in vivo*.

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