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Flying vaccinator; a transgenic mosquito delivers a *Leishmania* vaccine via blood feeding

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Abstract

'Flying vaccinator' is the concept of using genetically engineered hematophagous insects to deliver vaccines. Here we show the generation of a transgenic anopheline mosquito that expresses the *Leishmania* vaccine candidate, SP15, fused to monomeric red fluorescent protein (mDsRed) in its salivary glands. Importantly, mice bitten repeatedly by the transgenic mosquitoes raised anti-SP15 antibodies, indicating delivery of SP15 via blood feeding with its immunogenicity intact. Thus, this technology makes possible the generation of transgenic mosquitoes that match the original concept of a 'flying vaccinator'. However, medical safety issues and concerns about informed consent mitigate the use of the 'flying vaccinator' as a method to deliver vaccines. We propose that this expression system could be applied to elucidate saliva–malaria sporozoite interactions.

Keywords: mosquito, salivary gland, malaria, *Leishmania*, SP-15.

Introduction

Stable germline transformation systems have been achieved in a number of insect species within the last decade (O'Brochta & Handler, 2008). These systems provide the basis for the development of powerful tools in hematophagous insect vectors for the control of parasite transmission and management of insect populations in the field. Mosquitoes are vectors of pathogens such as malaria, dengue and yellow fever, and recent advances in the genetic engineering of mosquitoes have raised expectations for their use in new strategies for infectious disease control (Chen *et al.*, 2008). The generation of anopheline mosquitoes refractory to malaria by transgenesis has been intensively studied (Marshall & Taylor, 2009), and some mosquito lines expressing malaria-refractory molecules to rodent and avian malaria parasites have been established (Ito *et al.*, 2002; Moreira *et al.*, 2002; Jasinskiene *et al.*, 2007). Recently, we reported that a transgenic mosquito expressing the haemolytic C-type lectin CEL-III from sea cucumber impairs the *Plasmodium* transmission dynamics of human malaria (Yoshida *et al.*, 2007). These studies support the use of such transgenic mosquitoes in population replacement strategies (Terenius *et al.*, 2008). Alternately, 'release of insects carrying a dominant lethal (RIDL)' has been proposed as a new sterile insect technique (SIT) for the control of insect populations using genetically engineered insects (Heinrich & Scott, 2000; Thomas *et al.*, 2000; Fu *et al.*, 2007; Alphey *et al.*, 2008).

An intriguing use of transgenesis technology was proposed as a concept, termed the 'flying vaccinator'. This idea shifts a hematophagous insect from a pest to a beneficial insect (Crampton *et al.*, 1999). The 'flying vaccinator' strain was not developed previously in mosquitoes because until recently, a salivary gland-specific expression system was not available. We identified and characterized an abundant protein in the salivary glands of the malaria vector mosquito, *Anopheles stephensi*. As the saliva protein binds directly to collagen and inhibits collagen-induced platelet aggregation (Yoshida *et al.*, 2008), it was named anopheline anti-platelet protein (AAPP). The upstream region of the *aapp* gene open reading frame has the capacity to direct the strong female salivary gland-specific and blood meal-inducible expression of a reporter gene. A transgenic *A. stephensi* mosquito harbouring a gene encoding tetrameric red fluorescent protein (tDsRed) under the control of the *aapp* promoter specifically expressed tDsRed protein as a cytoplasmic form in the secretory cells of the distal–lateral lobes of the female salivary glands (Yoshida & Watanabe, 2006). These transgenic mosquitoes

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facilitated investigations of the mechanisms of sporozoite invasion into the salivary glands using *in vivo* imaging. Thus, we have established a robust salivary gland-specific expression system in an anopheline mosquito.

We report here the development of 'flying vaccinator' strains of transgenic *A. stephensi* that secrete a transgene product as a component of saliva. We chose the gene encoding the SP15 antigen, which is a 15 kDa saliva protein of the sand fly *Phlebotomus papatasi*, as the expressed vaccine candidate molecule. *P. papatasi* saliva enhances *Leishmania* infection when the parasites are transmitted to their vertebrate hosts by sand fly feeding (Kamhawi *et al*., 2000). Valenzuela and colleagues showed in a murine model that vaccination with SP15 conferred protection to a subsequent *Leishmania* infection (Valenzuela *et al*., 2001) and are developing a DNA vaccine containing the *sp15* cDNA. We generated transgenic mosquito lines expressing SP15 fused to a secretory form of monomeric DsRed (mDsRed) in the salivary glands. Mice bitten repeatedly by the transgenic mosquitoes raised anti-SP15 antibodies, indicating the achievement of the original concept of the 'flying vaccinator'. Although it is unlikely that this concept will be developed as a public health measure for delivering vaccines, the salivary gland-specific expression of relevant proteins is expected to be a powerful tool for the elucidation of saliva–malaria sporozoite interaction.

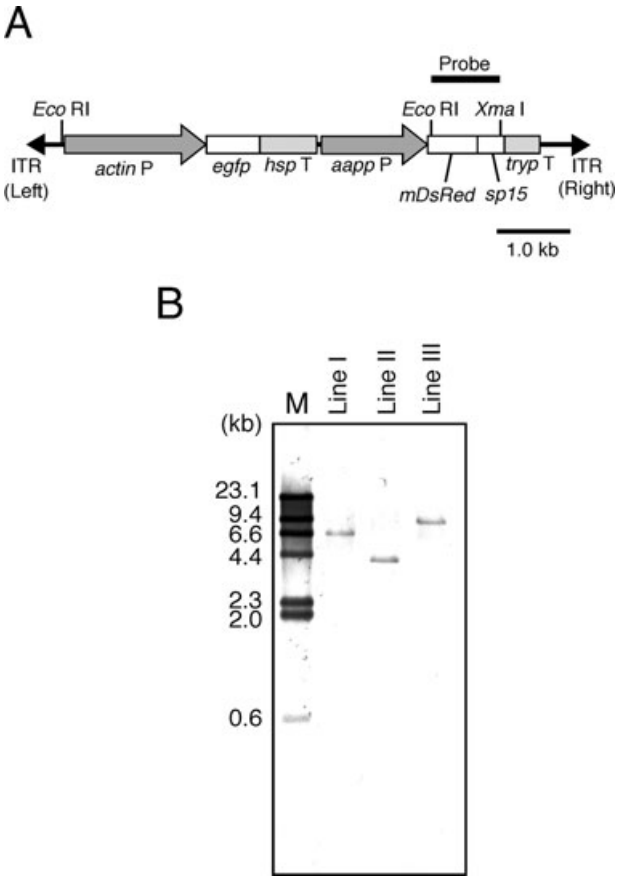

Figure 1. Structure of the monomeric DsRed (*mDsRed*) gene and Southern blot analysis of the mDsRed-SP15 mosquito. (A) Schematic representation of genomic structure of the mDsRed-SP15 mosquito. ITR, *Minos* inverted terminal repeat; *actin* P, *Drosophila melanogaster actin5c* promoter; *egfp*, enhanced GFP (EGFP)-coding sequence; *hsp* T, *D. melanogaster* terminator sequence; *aapp* P, *Anopheles stephensi aapp* promoter; *mDsRed*, mDsRed-coding sequence; *sp15*, *Phlebotomus papatasi* SP15-coding sequence; *tryp* T, *Anopheles gambiae trypsin* terminator sequence. A black bar represents the probe used for Southern blot analysis. (B) Southern blot analysis of the mDsRed-SP15 mosquito. Genomic DNAs from three independent mDsRed-SP15 transgenic lines were digested with *Eco*RI, and hybridized with a 0.9 kb *Eco*RI-*Xma*I DNA fragment partially encoding the mDsRed-SP15 protein. Leftmost lane shows size markers (kb).

Results and discussion

Generation of transgenic lines

A *Minos*-based transfer vector comprising a gene cassette consisting of the *sp15* gene fused to the *mDsRed* gene (*mDsRed-sp15*) under the control of the *aapp* promoter (Fig. 1A) was injected together with a *Minos* helper plasmid into *A. stephensi* embryos. One-hundred and thirty-five embryos of the 505 injected (27%) hatched normally G_0 generation and 54 adults were eclosed. Three independent transgenic lines were established. A single integration event was confirmed by Southern blot analysis (Fig. 1B). The three transgenic lines have been stably maintained by blood meals on mice for over 10 generations.

Expression of mDsRed-SP15 in the salivary glands

Immunoblot analysis of all three transgenic lines with anti-DsRed and anti-SP15 antibodies verified the presence of expressed mDsRed-SP15 protein [molecular mass (Mr) = 44 kDa] in the salivary glands (Fig. 2). Anti-DsRed antibody recognized a protein (Mr = 28 kDa) in the salivary glands of the tDsRed transgenic mosquitoes, which express the *tDsRed* gene without the *sp15* gene, and no signal was detected with anti-SP15 antibody. The expression levels of mDsRed-SP15 protein in the salivary glands were indistinguishable among the three lines following calibration with anti-AAPP antibody-recognition of native AAPP (Mr = 37 kDa). This is consistent with the interpretation that there are no gross position effects based on this analysis. The amount of mDsRed-SP15 protein expressed is estimated at ~20 ng per pair of salivary glands, as quantified by comparison with commercially available tDsRed protein. This expression level was equivalent to as much as 4% of the total salivary gland protein, which was similar to that of tDsRed protein in the corresponding transgenic mosquitoes (25 ng per pair of salivary glands; Yoshida & Watanabe, 2006).

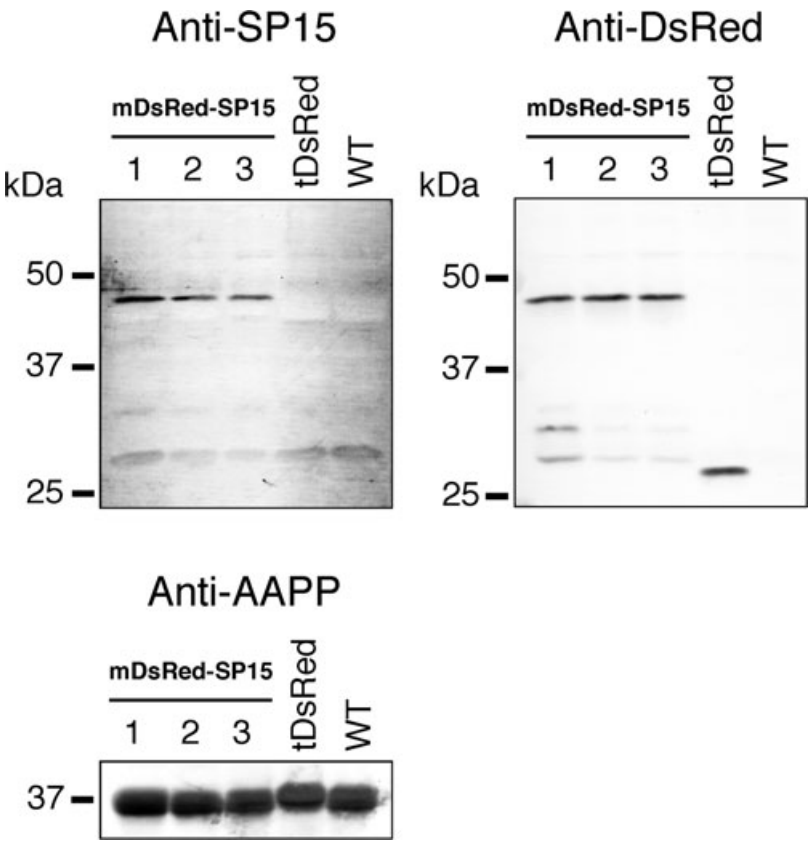

Figure 2. Immunoblotting of mDsRed-SP15 protein in the salivary glands. The female salivary glands of three mDsRed-SP15 transgenic lines (mDsRed-SP15, 1–3), tetrameric DsRed transgenic (tDsRed) and wild-type (WT) mosquitoes were analysed using anti-SP15, anti-DsRed and anti-AAPP antibodies.

Location of mDsRed-SP15 protein in the salivary glands

Strong red fluorescence was observed in the distal–lateral lobes of dissected female salivary glands of both the tDsRed and mDsRed-SP15 transgenic mosquitoes. This is consistent with the endogenous expression profile mediated by the *aapp* promoter (Fig. 3A–D). A detailed analysis using 3D confocal microscopy revealed distinct differences in the distribution of DsRed proteins between the two transgene constructs. mDsRed-SP15 protein was observed in the cytoplasm and secretory acini of the cells in the lobes (Fig. 3E), whereas tDsRed protein was detected only within the secretory cells (Fig. 3F). Furthermore, incubation of the distal–lateral lobes with SYTO-13 green-fluorescent dye, which stains DNA and RNA, facilitated visualization of the shape of the secretory cells (Figs 3G–J, S1). Overlaying the red and green fluorescence patterns showed that tDsRed protein was located in the secretory cells (yellow), indicating a cytoplasmic form. In contrast, mDsRed-SP15 protein was located in the secretory cavities and ducts of the salivary glands, consistent with secretion into the acini.

To obtain supportive data on the localization of the expressed mDsRed proteins, the dissected salivary glands of the transgenic mosquitoes were physically ruptured under fluorescent microscopy. Although a trace of tDsRed protein spilt out from the sites of rupture, the intensity of red fluorescence remained strong (Fig. 4, Videos S1 and S2). In contrast, a large amount of mDsRed-SP15 protein was clearly released into the dissecting medium following damage. These results are consistent with the interpretation that mDsRed-SP15 protein is secreted and accumulates in the secretory acini and duct as a component of saliva. The release of saliva with red fluorescence during salivation from the proboscis supports this conclusion (Fig. 5A and Video S3). In contrast to mDsRed transgenic mosquitoes, saliva with red fluorescence was not released from proboscis of tDsRed transgenic mosquitoes. As a result, mice bitten repeatedly by tDsRed transgenic mosquitoes induced no antibody response to DsRed (data not shown). The difference in the localization between the two transgene products is likely to be because of protein structure, including a signal leader peptide, and conformation. tDsRed protein is a tetramer with no signal leader sequence, resulting in a cytoplasmic form with low solubility. mDsRed-SP15 protein is a monomer with a signal leader peptide, resulting in a secreted form with high solubility. Transgenic mosquitoes harbouring the gene encoding nonfusion SP15 with the same signal peptide under the control of the *aapp* promoter expressed undetectable levels of SP15, although the SP15 transcripts

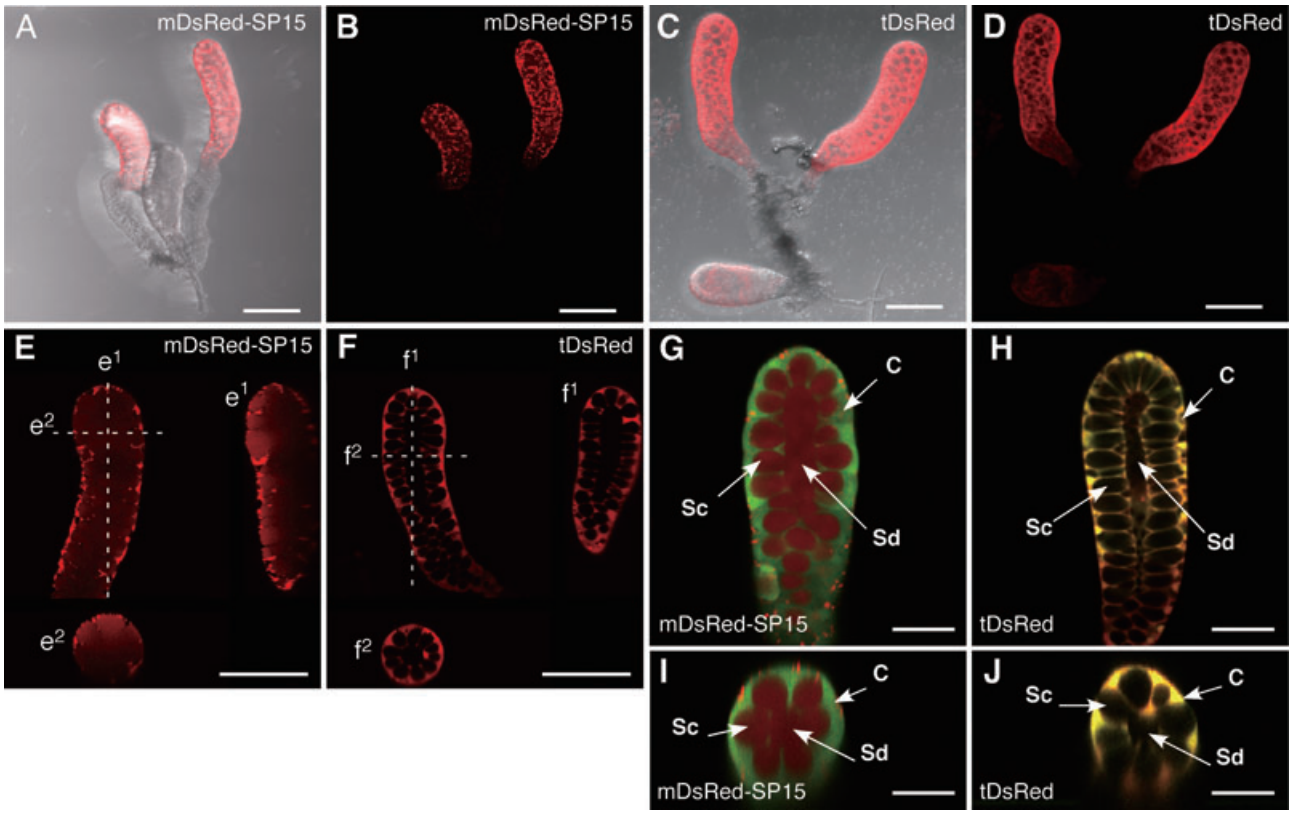

Figure 3. 3D confocal imaging of the dissected salivary gland from female mDsRed-SP15 and tDsRed mosquitoes. Red fluorescence was observed in the distal–lateral lobes of the female salivary glands of both transgenic mosquitoes. (A) Overlay image of the DsRed fluorescence and differential interference contrast of the salivary gland of mDsRed-SP15 mosquito. (B) DsRed fluorescence image of the salivary gland of mDsRed-SP15 mosquito. (C) Overlay image of the DsRed fluorescence and differential interference contrast of the salivary gland of tDsRed mosquito. (D) DsRed fluorescence image of the salivary gland of tDsRed mosquito. (E) Section of the distal–lateral lobe of mDsRed-SP15 mosquito. e^1 and e^2 are transverse sections indicated by the lines e^1 and e^2, respectively. (F) Section of distal–lateral lobe of tDsRed mosquito. f^1 and f^2 are transverse sections indicated by the lines f^1 and f^2, respectively. A longitudinal section (G and H) and cross-section (I and J) of the distal–lateral lobe stained with SYTO-13, of each transgenic mosquito line. C, secretory cell; Sc, salivary cavity; Sd, salivary duct. Scale bars = 100 μm (A–D) and 50 μm (E–J).

were detected in the salivary glands by real time-PCR (Fig. S2). So far, we have failed to express non-mDsRed fusion constructs at levels equivalent to the mDsRed-SP15 protein. These results support the conclusion that the mDsRed-fusion expression system facilitates not only the monitoring of secretion of transgene products but also stabilizes and/or chaperones mDsRed-fusion proteins prior to their secretion.

Antibody responses to mDsRed-SP15 in mice exposed to transgenic mosquitoes

Immunoblot analyses showed that sera from four individual mice bitten repeatedly by ~1500 mDsRed-SP15 mosquitoes recognized Glutathione S-transferase (GST)-fusion mDsRed-SP15 protein (Fig. 5B). Anti-SP15 antibody titres were determined by enzyme-linked immu-

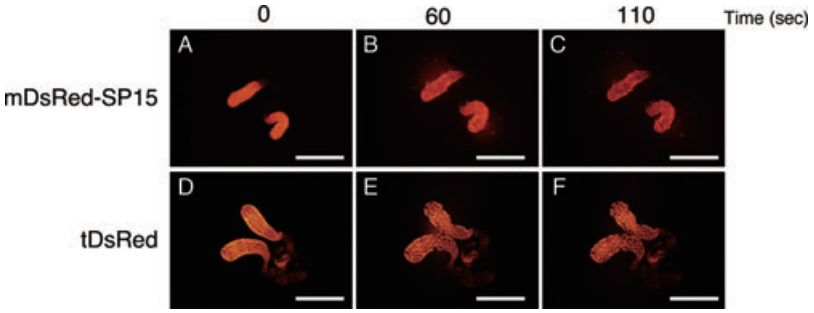

Figure 4. The observation of physical rupture of the salivary gland in two transgenic lines using a fluorescent microscope. (A–C) mDsRed-SP15 mosquito; (D–F) tDsRed mosquito. Numbers indicate the time after the rupture of the salivary glands that were put between a slide and cover slip and pressure applied using tweezers(s). Scale bars = 200 μm.

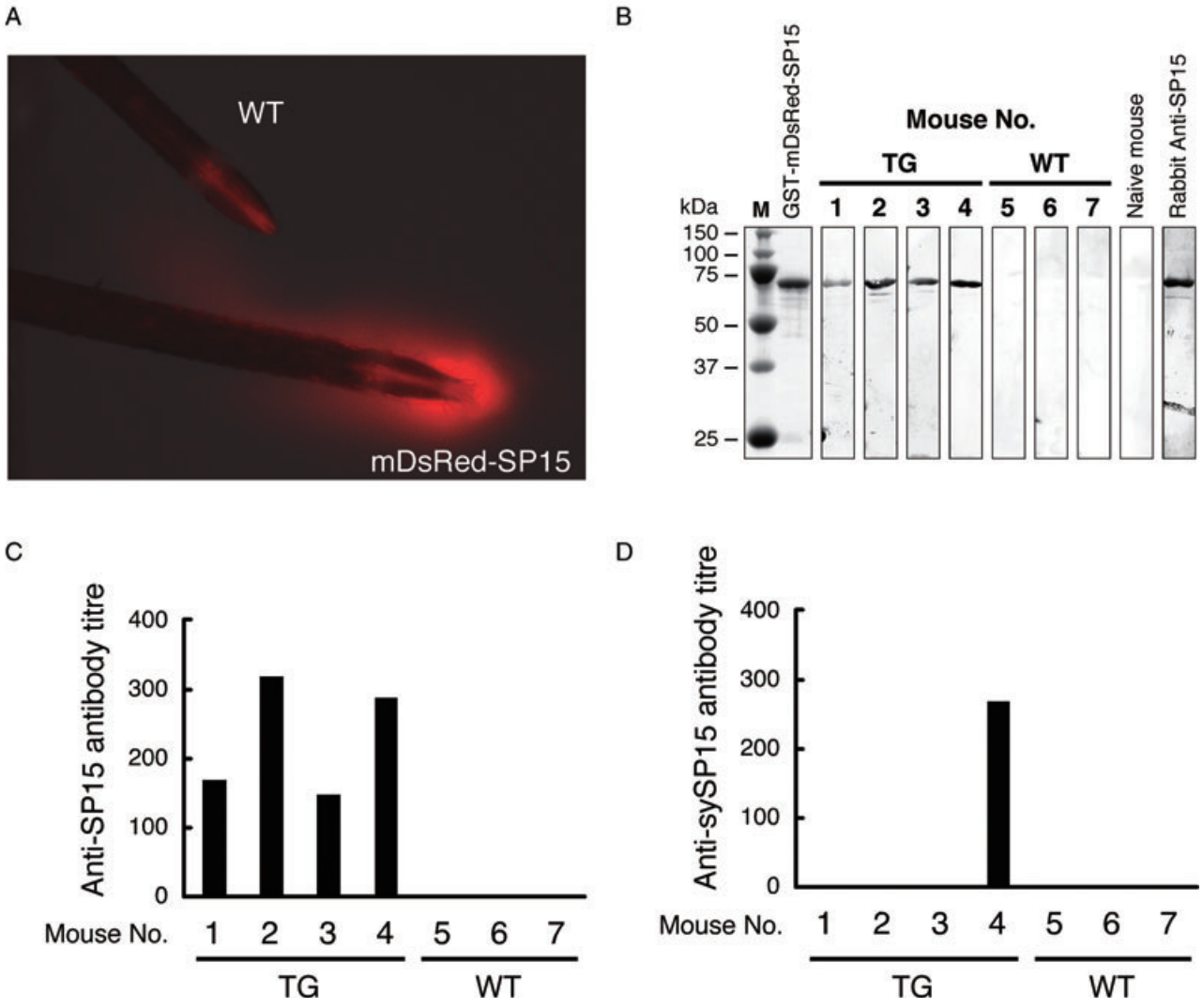

Figure 5. Release of mDsRed-SP15 and anti-SP15 antibody responses in individual mice bitten repeatedly by mDsRed-SP15 mosquitoes. (A) Saliva with red fluorescence during salivation from the proboscis of mDsRed-SP15 mosquitoes (mDsRed-SP15) was released, whereas no red fluorescence was observed in the saliva from wild-type mosquitoes (WT). (B) Immunoblotting of individual sera. A total of about 1500 mDsRed-SP15 (TG) or WT mosquitoes were allowed to feed on each mouse for over five months. Numbers indicate individual mice bitten by TG (nos 1–4) or WT (nos 5–7) mosquitoes, respectively. Sera diluted to 1:300 were used to react with Glutathione S-transferase (GST)-mDsRed-SP15 protein. The left panel is GST-mDsRed-SP15 protein stained with Coomassie Brilliant Blue. Rabbit anti-SP15 antibody was used as a positive control (right lane). M, protein standards. (C–D) Anti-SP15 antibody titres of sera obtained from mice nos 1–7 specific for rSP15 protein (C) and sySP15 (D). Bars indicate the means of anti-SP15 antibody titres.

nosorbent assay (ELISA) using recombinant SP15 protein (rSP15) and SP15 synthetic peptides (sySP15) as antigens. Levels of anti-SP15 antibodies induced in all of the four sera were not high, but statistically different from sera obtained from mice bitten by wild-type (WT) mosquitoes, which had undetectable levels of anti-SP15 antibodies (Fig. 5C). One of the four experimental mice (mouse no. 4) had sera that reacted with the sySP15 (Fig. 5D). These combined results support the conclusions that immunogenically active mDsRed-SP15 was injected as a component of saliva via blood feeding and induced SP15-specific antibodies.

A critical question remains to be answered for the present study concerning the flying vaccinator. It is whether mice exposed to mDsRed-SP15 mosquitoes are protected when challenged by *Leishmania* infection. Challenge studies using *Leishmania*-infected *P. papatasi* are required to answer this question. The mice immunized with SP15 conferred protection to *Leishmania major* infection, and SP15 is recognized by people naturally exposed to sand flies in endemic areas of *Le. major* (Valenzuela *et al*., 2001). Additionally, following exposure to another sand fly, *Lutozomia longipalpis*, lymphocytes reduced the infection rate of *Leishmania* in human macrophages

in vitro (Vinhas *et al*., 2007). Therefore, exposure to mDsRed-SP15 mosquitoes may confer resistance in humans to *Leishmania* infection. We are now proceeding to evaluate vaccine efficacy induced by the mosquito bites against challenge with *Leishmania*-infected *P. papatasi* in a murine model.

In summary, we have demonstrated that transgenic mosquitoes secreting a foreign protein as a component of saliva can elicit immune responses, and therefore demonstrate proof-of-principle for the concept of the 'flying vaccinator'. Although the model we developed targets a pathogen, *Leishmania*, for which a candidate vaccine exists, mosquitoes secreting antigens to combat malaria would be preferred. However, the concept of a 'flying vaccinator' transgenic mosquito is not likely to be a practicable method of disease control, because a 'flying vaccinator' is an unacceptable way to deliver vaccine without issues of dosage and informed consent against current vaccine programmes. These difficulties are further complicated by the issues of public acceptance to release of transgenic mosquitoes. Therefore, we intend only that the present study makes available a model system using a salivary gland-specific promoter as a potential tool to elucidate the saliva–malaria sporozoite interactions.

Experimental procedures

Animals

The *A. stephensi* mosquito strain, SDA500, was maintained at Jichi Medical University. Female C57/BL6 mice were obtained from Japan SLC (Hamamatsu, Shizuoka, Japan) and used at 7–8 weeks of age.

Minos *vector construction and germline transformation*

PCR reactions were performed with *Pfu* DNA polymerase (Stratagene GmbH, Heidelberg, Germany). A 366-bp DNA fragment of the *SP-15* gene was amplified from pcDNA-SP15 (kindly provided by J. G. Valenzuela) using primers pSP15-F2 and pSP15-R2. The PCR product was cloned into pENTR/D-TOPO (Invitrogen, Carlsbad, CA, USA) to generate pENTR-SP15. A 675-bp DNA fragment of the *mDsRed* gene was amplified from pDsRed-monomer-C1 (BD Biosciences Clontech, Mountain View, CA, USA) using primers pmDsRed-F1 and pmDsRed-R1. The PCR product was cloned into pENTR/D-TOPO (Invitrogen) to generate pENTR-mDsRed. The *mDsRed* gene fragment was excised from pENTR-mDsRed by digestion with *Eco*RI and *Mun*I, and then cloned into the *Eco*RI site of pENTR-SP15 to generate pENTR-mDsRed-SP15-XhoI. A 1.1 kb DNA fragment of the *mDsRed-sp15* gene was excised from pENTR-mDsRed-SP15-XhoI by digestion with *Eco*RI and *Xho*I, then cloned into the *Eco*RI/*Xho*I sites of pENTR-aappP-tDsRed-antryp1T (Yoshida & Watanabe, 2006) to generate pENTR-aappP-mDsRed-SP15-antryp1T. The transformation plasmid pMinos-EGFP-aappP-mDsRed-SP15-antryp1T (Fig. 1A) was generated by incubation of pMinos-EGFP-RfA-F (Yoshida & Watanabe, 2006) and pENTR-aappP-mDsRed-SP15-antryp1T in the presence of LR Clonase (Invitrogen) as described previously (Yoshida & Watanabe, 2006). Primer sequence information is available in Table S1. Procedures for microinjection into embryos, screening of EGFP-expressing G_1 larvae and generation of homozygous lines have been described previously (Catteruccia *et al*., 2000).

Southern blot analysis

A total of 10 μg of genomic DNA extracted from female mDsRed-SP15 mosquitoes was digested with *Eco*RI, and separated on 0.8% agarose gels, and then transferred to a Hybond N+ membrane (GE Healthcare Bioscience, Buchingamshire, England). For hybridization, a 0.9 kb *Eco*RI-*Xma*I DNA fragment partially encoding mDsRed-SP15 protein was used as a probe. Probe labelling, hybridization, washing and detection of the signals were performed under high stringency conditions using a DIG-High Prime (Roche, Indianapolis, IN, USA) according to the supplier's protocol.

Immunoblotting

Anti-SP15 antiserum was obtained from rabbit immunized with a synthetic SP15-peptide (sySP15: AKKVKKQAREDSHWLNC). Anti-AAPP antiserum was obtained from rabbits immunized with recombinant AAPP protein (Yoshida *et al*., 2008). Rabbit anti-DsRed polyclonal antibody was purchased from Clontech Laboratories, Inc. (Mountain View, CA, USA).

Groups of 20 pairs of female salivary glands were homogenized by a plastic homogenizer with 40 μl Laemmili buffer (Laemmli, 1970) containing 5% 2-mercaptoethanol, and then boiled at 95 °C for 5 min. Ten microlitres of each sample (equivalent to five pairs of salivary glands) was separated on a 10 or 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis gel, and transferred to Immobilon Transfer Membrane (Millipore, Bedford, MA, USA). The membrane was incubated either with anti-sySP15, anti-AAPP antiserum, or anti-DsRed antibody. Polypeptides recognized by the antibodies were detected with biotinylated anti-mouse or anti-rabbit immunoglobulin G (IgG) (Vector Laboratories, Burlingame, CA, USA), followed by colour development with 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt/nitroblue tetrazolium chloride substrate (Roche) as described previously (Yoshida *et al*., 1999).

Fluorescent microscopy

Following dissection in phosphate-buffered saline (PBS), the female salivary glands were immediately stained with 0.2 mM SYTO-13 (Invitrogen) in PBS for 5 min, and then washed twice with PBS. Stained samples were mounted on a glass slide. DsRed and SYTO-13 fluorescence in the salivary glands were observed by confocal microscopy [FV1000 (Olympus, Tokyo, Japan) and TCS SP5 (Leica, Wetzlar, Germany)].

For the observation of physical rupture of the salivary gland in two transgenic lines, the female salivary glands were dissected in PBS and put between a slide and cover slip, and then pressure was applied using tweezers under a fluorescent microscope AX80R (Olympus). Real-time imaging was monitored and recorded by DP controller software (Olympus).

To monitor the release of saliva with DsRed fluorescence from the proboscis, mosquitoes were kept on ice, their legs and maxillary palps were removed, and their abdomens were immobilized

on a glass slide with adhesion bond. Salivation was observed under a Biorevo fluorescent microscope (Keyence, Osako, Japan) immediately after inserting the proboscis into 70% glycerol.

Immunization of mice with transgenic mosquito bites

mDsRed-SP15 transgenic and WT mosquitoes were allowed to repeatedly feed on individual mice 42 times at 4-day intervals with a total of approximately 1500 bites.

Recombinant proteins

The *sp15* gene was excised from pENTR-SP15 by digestion with *Nco*I and *Xho*I, and cloned into the *Nco*I/*Xho*I sites of pET32-b(+) (Novagen, Madison, WI, USA) to generate pET32-SP15. A recombinant SP15 protein (rSP15), created as a fusion protein with thioredoxin, was expressed in *Escherichia coli*. A 684-bp DNA fragment of the glutathione s-transferase (*gst*) gene was amplified from pGEX-6P-1 (GE Healthcare Biosciences) by PCR using primers pGEX6P-F2 and pGEX6P-R2. Primer sequence information is available in Table S1. The PCR product was cloned into pENTR/D-TOPO (Invitrogen) to generate pENTR-GEX6P2. The *gst* gene fragment was excised from pENTR-GEX6P2 by digestion with *Nde*I and *Nco*I, and then cloned into the *Nde*I/*Nco*I sites of pET22-b (+) (Novagen) to generate pET22-GSX6P2. A 1.1 kb DNA fragment of the *mDsRed-SP15* fusion gene was excised from pENTR-mDsRed-SP15-XhoI by digestion with *Eco*RI and *Xho*I, and then cloned into the *Eco*RI/*Xho*I sites of pET22-GSX6P2 to generate pET22-GEX6P2-mDsRed-SP15. A recombinant mDsRed-SP15 protein, created as a fusion protein with GST, was expressed in *E. coli*. Both proteins were purified using a Ni-NTA affinity column (Qiagen, Valencia, CA, USA) as described previously (Daly & Long, 1993), and used as antigens for immunoblotting and ELISA.

Antibody assay

Sera from individual mice bitten repeatedly either by mDsRed-SP15 or WT mosquitoes were collected from the tail vein. rSP15- and sySP15-specific antibody titres were quantified by ELISA. Pre-coated 96-well plates with either rSP15 or sySP15 at 100–300 ng/well in carbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) were incubated with serially diluted sera. Specific IgGs were detected using horseradish peroxidase-conjugated goat anti-mouse IgG (H+L) (Bio-Rad, Hercules, CA, USA). The plates were developed with a peroxidase substrate solution [H_2O_2 and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonate)]. The optical density at 414 nm of each well was determined using a Multiskan BICHROMATIC microplate reader (Thermo Labsystems, Vantaa, Finland). End-point titres were expressed as the reciprocal of the highest sample dilution for which the optical density was equal to or greater than the mean optical density of naive mouse sera.

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Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference: DOI 10.1111/j.1365-2583.2010.01000.x

Figure S1. The localization of the expressed transgene products in the salivary gland from both female monomeric DsRed (mDsRed)-SP15 and tetrameric DsRed (tDsRed) transgenic mosquitoes. 3D confocal imaging of a longitudinal section of distal–lateral lobe stained with SYTO-13, of each transgenic mosquito line. SYTO-13 green-fluorescent dye stains DNA and RNA, facilitated visualization of the shape of the secretory cells. Overlaying the red and green fluorescence images showed that the tDsRed protein was located in the secretory cells and the mDsRed-SP15 protein was located in the secretory cavities and ducts of the salivary glands. C, secretory cell; Sc, salivary cavity; Sd, salivary duct. Scale bars = 100 μm.

Figure S2. Expression of the *sp15* gene in the salivary glands and carcasses of nonfusion SP15 (SP15) male and female mosquitoes. The *sp15* mRNA specifically expressed in the female SP15 salivary glands but not in the male SP15 or wild-type salivary glands or their carcasses.

Video S1. Video of the physical rupture of the salivary gland in the mDsRed-SP15 mosquito. The experimental procedures are described in the Experimental procedures of Supporting Information.

Video S2. Video of the physical rupture of the salivary gland in the tDsRed mosquito. The experimental procedures are described in the Experimental procedures of Supporting Information.

Video S3. Video of the release of saliva with red fluorescence during salivation from the proboscis of mDsRed-SP15 mosquito (bottom). No red fluorescence was observed in saliva from nontransgenic (wild-type) mosquito (top). The experimental procedures are described in the Experimental procedures of Supporting Information.

Table S1. List of primers.

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