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Original Article

Production of a Transgenic Mosquito Expressing Circumsporozoite Protein, a Malarial Protein, in the Salivary Gland of *Anopheles stephensi* (Diptera: Culicidae)

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We are producing a transgenic mosquito, a flying syringe, to deliver a vaccine protein to human beings via the saliva the mosquito deposits in the skin while biting. The mosquito produces a vaccine protein in the salivary gland (SG) and deposits the protein into the host's skin when it takes the host's blood. We chose circumsporozoite protein (CSP), currently the most promising malaria vaccine candidate, to be expressed in the SG of *Anopheles stephensi*. To transform the mosquitoes, plasmid containing the CSP gene under the promoter of female SG-specific gene, as well as the green fluorescent protein (GFP) gene under the promoter of 3xP3 as a selection marker in the eyes, was injected into more than 400 eggs. As a result, five strains of GFP-expressing mosquitoes were established, and successful CSP expression in the SG was confirmed in one strain. The estimated amount of CSP in the SG of the strain was 40 ng per mosquito. We allowed the CSP-expressing mosquitoes to feed on mice to induce the production of anti-CSP antibody. However, the mice did not develop anti-CSP antibody even after transgenic mosquitoes had bitten them several times. We consider that CSP in the SG was not secreted properly into the saliva. Further techniques and trials are required in order to realize vaccine-delivering mosquitoes.

Key words: malaria, salivary gland, flying syringe, transgenic mosquito, vaccine

Mosquitoes are nuisance to human beings because they puncture the skin to obtain blood, which they use for their eggs. They deposit saliva in the skin when they take blood, and this causes itching [1]. They also transmit pathogens of diseases such as malaria, filarisis, yellow fever, and dengue fever. People have long tried to prevent mosquitoes from biting using mosquito nets [2] and repellents [3]. Moreover, people have attacked mosquitoes with insecticides to eliminate them [4, 5]. These struggles have continued not only in developing countries but also in advanced countries.

In the last 30 years, developments in genetic engineering have allowed us to add new genes to wild-type cells or to remove protein genes from them [6]. These technologies were used at first in bacteria or yeast to obtain recombinant proteins for experiments and to understand the mechanisms of protein molecules. More recently, many transgenic experiments have been applied to plants and animals, including vertebrates and insects. Recombinant-DNA-modified soybean, cotton, and corn are widely adopted and harvested around the world [7]. Transgenic mice, sheep, dogs, zebra fish, flies, silkworms, and so on have appeared in laboratories as well [8-12]. The objectives of genetic engineering in animals are

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several: to understand cell differentiation, to find molecules responsible for causing specific diseases, and to speed up the production of meat, fish, and silk.

Some attempts have been made to create transgenic mosquitoes to make them non-vectors of germs or to reduce the germs' transmission ability [13, 14]. Anopheline mosquitoes are the primary targets for transgenes, because they transmit malaria parasites. Some laboratories, including ours, have succeeded in producing transgenic mosquitoes with lower levels of malaria parasites in the digestive tract after blood meals on malaria-infected animals [15, 16]. The goal of those attempts is to control disease transmission through genetic modification of the mosquitoes [17].

We here present a new attempt to produce a useful protein in the mosquito salivary gland (SG) by adding a new gene to a mosquito chromosome. When mosquitoes attach to the skin surface, they try to find blood vessels from which they can take blood. At that time, they first deposit their saliva in the skin [1]. Mosquito saliva contains many molecules, such as vasodilators, platelet inhibitors, an anesthetic substance, and so on [18]. These substances dilate the blood vessel, allowing the mosquito to insert its proboscis into the vessel. A mosquito can have a blood meal without the blood coagulating and without being noticed. On the other hand, humans develop anti-saliva protein antibodies after experiencing several mosquito bites [19, 20]. Our idea is to put a gene encoding a useful protein into a mosquito chromosome, causing it to make the useful protein in its saliva and to inject the protein, via the saliva, into animals or human beings upon blood feeding. We expect that the host would develop antibodies to the recombinant protein as a reaction. If these transgenic (TG) mosquitoes, whose saliva contains a vaccine protein against a disease, were spread in an area where people are suffering from the disease, people who are daily bitten by them would develop antibodies to the vaccine protein, ultimately vaccinating the community from the disease. In this situation, mosquitoes would play the role of vaccine deliverers [21].

In field conditions of malaria-endemic areas, for instance, nearly 100 mosquitoes bite an individual per night, but more than 50% of the mosquitoes are nulliparous females (non-oviposit females) [22]. Even in malaria-holoendemic areas, the percentage of mosquitoes infected with malaria parasites in the SG is less than 4% among collected Anopheline mosquitoes [23]. Indeed, it is important to reduce malaria-infective mosquitoes, but people in malaria-endemic areas are bitten by thousands of non-infective mosquitoes each year. These non-infective mosquitoes could work as vaccine distributors.

We discovered one molecule of a platelet inhibitor in the SG of a model mosquito, *Anopheles stephensi*, cloned the gene, and named it the Anopheline antiplatelet protein (AAPP) [24]. This molecule is expressed only in the female SG. We used the upper stream area of the AAPP gene (*aapp*) as a promoter for the vaccine protein. For the vaccine protein, we chose circumsporozoite protein (CSP) of the malaria parasite. This molecule has been studied as a malaria vaccine candidate [25, 26]. In a rodent malaria model, a monoclonal antibody to CSP neutralizes the infectivity of sporozoite, an infective form of malaria parasite from the mosquito [27], and a synthetic peptide induces sufficient antibodies to protect immunized mice from sporozoite challenge [28]. CSP is now thought to be a promising vaccine candidate molecule [29], and recombinant proteins composed of CSP are being evaluated in malaria-endemic areas [30]. Thus, we decided to produce a TG mosquito containing the promoter area of *aapp* followed by the CSP gene (*csp*). We expected that the TG mosquitoes would express CSP in the SG and inject the recombinant CSP (rCSP) into the skin of mice when the TG mosquitoes fed on them.

Materials and Methods

Mosquitoes, mice, and parasites. *Anopheles stephensi* SDA 500 strain was reared in our laboratory under conditions of 26℃ room temperature, 50–70% relative humidity, and light control of 14 h bright and 10 hours dark. Female BALB/c mice were purchased from SLC (Shizuoka, Japan). A rodent malaria parasite, *Plasmodium berghei* ANKA strain, was maintained by cyclical passage through BALB/c mice and *An. stephensi* mosquitoes.

Recombinant CSP of P. berghei (rPbCSP). The rPbCSP was expressed as His-tagged at the C-terminal by the baculovirus expression system, and purified with a nickel column (Qiagen, Hilden, Germany) as described elsewhere [31]. The rPbCSP is expressed with two molecular weight sizes, 70 and

82 kDa [31]. When mice are immunized with rPbCSP three times, they become protected against parasite challenge (unpublished data). The protein concentration of rPbCSP was measured using a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA). Bovine serum immunoglobulin was used as a standard for the protein concentration.

Preparation of anti-CSP antibody. For anti-CSP antibody production, 0.5 ml of rPbCSP solution (50 μg/ml) and 0.5 ml of alum, $Al(OH)_3$ (16 mg/ml), were well mixed and 0.2 ml was injected intraperitoneally into each of four BALB/c mice. Injection was done 3 times at intervals of 2 weeks. Anti-CSP antibody was monitored by ELISA, as shown below, and the mice were sacrificed to take their sera. The sera were used as the anti-CSP antibody.

Preparation of anti-saliva antibody. To prepare anti-saliva antibody, 4 female BALB/c mice were each bitten by 100 female mosquitoes every 2 weeks for a 10-week period (5 biting sessions). Development of an anti-saliva antibody in the mice was confirmed by ELISA as shown below, and sera were taken from the mice. The sera were used as the anti-saliva antibody.

Evaluation for antibody titer by ELISA. For the evaluation of anti-CSP antibody, 8 wells of a 96-well assay plate were coated with 100 μl of rPbCSP (1 μg/ml) in 0.05 M carbonate buffer (pH 9.6), and 8 wells were filled with 100 μl of carbonate buffer without antigen. The plate was incubated at 4℃ overnight.

For the evaluation of anti-saliva antibody, 10 pairs of SG were collected from female mosquitoes, destroyed in 1.0 ml of carbonate buffer by sonication (1 sec × 5 times), and centrifuged (8,000 rpm for 3 min). The supernatant was used as the SG antigen. Eight wells were each coated with 100 μl of SG antigen.

Two microliters of blood was collected from the tail of each mouse 7 days after the injection of rPbCSP or mosquito biting. The blood was mixed with 0.8 ml of phosphate-buffered saline containing 1% bovine serum albumin (BSA/PBS) and centrifuged at 8,000 rpm for 3 min. The supernatant was used as an 800-fold-diluted serum.

After the antigen solution was removed and washed twice, the wells of an ELISA plate were blocked with 150 μl of 1% BSA/PBS for 30 min. After removing the blocking solution, 100 μl of the mouse serum diluted 800-fold was distributed into each of 2 antigen wells and 2 no-antigen wells and incubated for 2 h. As the second antibody, rabbit anti-mouse IgG conjugated with horseradish peroxidase (HRP) (Bio-Rad Laboratories) diluted 3,000-fold was distributed into each of the same wells and incubated for 1 h. After the plate was washed, the substrate solution, a mixture of 0.04% 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (Sigma-Aldrich, St. Louis, MO, USA), 0.05% H_2O_2 in 0.05 M phosphate, and 0.1 M citrate buffer (pH 4.5) was added. After 20–60 min, a green color appeared. The absorption was measured at a wavelength of 405 nm by a microplate reader, Spectra Max M5 (Molecular Devices, Tokyo, Japan). The mean absorbance of antigen wells minus the mean absorbance of no-antigen wells was taken as the OD value for the antigen.

Plasmid construction (Fig. 1). The anopheline anti-platelet protein gene (*aapp*) promoter region and its signal peptide coding region of *An. stephensi* [24] (1,746 bp) were amplified with a primer pair of AAPP-F-*AscI* (5'-*GGCGCGCC*TTATAAGACGGAGCTCATTGTCGCTCGTC-3') and AAPP-R-*SmaI* (5'-*CCCGGG*CGGCCGTGCGGATACGATCAGCGCAAGGC-3'), then cloned into the pCR-BluntII TOPO vector (Invitrogen, Carlsbad, CA, USA) (plasmid a). The open reading frame of the *P. berghei CSP* gene without a signal peptide coding region or a C-terminal GPI-anchor domain coding region was amplified with a primer pair of PbCS-F-*SmaI* (5'-*CCCGGG*CAAAATAAAATCATCCAAGCCCAAAGGAAC-3') and PbCS-R-*SpeI* (5'-*ACTAGT*TATTTATCCATTTTACAAATTTCAGTATCAATATC-3'), then cloned into the vector (plasmid b). The 3' non-coding region of the AAPP gene was amplified with a primer pair of 3UTR-F-*SpeI* (5'-*ACTAGT*GAAACACACCGTTAACGACAC-3') and 3UTR-R-*XbaI* (5'-*TCTAGA*TATTCAAAGGTCCACAAATGTC-3'), then cloned into the vector (plasmid c). The inserts of plasmids a, b, and c were serially cloned into the pENTR4 vector (Invitrogen) (donor vector).

The pBac [3xP3-EGFP] vector [11] and helper plasmid were kindly provided by Professor A. S. Raikhel (Department of Entomology, University of California at Riverside, USA). The pBac [3xP3-EGFP] vector was digested with *AscI*, blunted with a

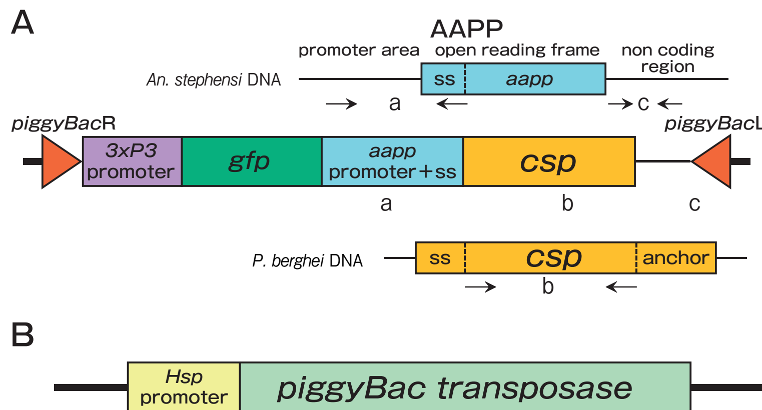

Fig. 1 Construction of plasmids injected into mosquito eggs. **A**, Two DNA fragments (a and c) were amplified from *A. stephensi* DNA. One DNA fragment (b) was amplified from *P. berghei* DNA. Three fragments were inserted in tandem in a plasmid vector downstream from the GFP gene. A promoter, 3*xP*3, was adopted to induce GFP expression in the eyes of mosquitoes as a selection marker. AAPP: anopheline anti-platelet protein. ss: signal sequence. CSP: circumsporozoite protein; **B**, To improve uptake of the recombinant plasmid in the mosquito chromosome, a piggyback helper plasmid was added when microinjection was performed. Hsp: heat-shock protein.

Klenow fragment, and dephosphorylated. The reading frame cassette A (Invitrogen) was then cloned into the vector (destination vector). The insert in the donor vector was cloned into the destination vector by Gateway LR clonase reaction (Invitrogen). The resulting plasmid (pBac-AAPP-PbCSP: Fig. 1A) was expected to drive CSP gene expression under the AAPP promoter. The pBac-AAPP-PbCSP plasmid was mixed with piggyBac helper (Fig. 1B) and microinjected into the eggs of *A. stephensi* as described below.

Microinjection of the plasmids into mosquito eggs. The mosquito microinjection was performed as described elsewhere [32]. In brief, blood-fed *An. stephensi* mosquitoes were allowed to lay eggs on a wet filter sheet 72–84 h after a blood meal. Eggs were laid and injected with plasmids within 90 min after oviposition. Injection was done by glass needles (Eppendorf, Hamburg, Germany) with a mixture of the pBac-AAPP-PbCSP (500 ng/μl) and piggyBac helper (300 ng/μl) in injection buffer (5 mM KCl, 0.1 mM Na_2HPO_4, pH 6.8). After injection, the eggs were placed in water and observed for hatching. Hatched larvae were analyzed on a fluorescence microscope at a wavelength of 490 nm to detect GFP expression.

Selection of transgenic mosquito. We selected GFP-expressing larvae and made them emerge (G0). One G0 adult female (or male) expressing GFP was put in a cage containing 5 wild-type males (or females). After mating and blood feeding, each female was allowed to lay eggs individually. Hatched larvae were observed under a fluorescence microscope, and GFP-expressing larvae were isolated as a G1 strain. Among the same batch of G1, mosquitoes were allowed to mate, feed, and lay eggs. Hatched larvae were observed and GFP-expressing larvae were isolated as the G2 of the strain.

Selection of CSP-expressing strain. Ten pairs of SG were collected from G2 female adults in each strain, destroyed in 1.0 ml of carbonate buffer by sonication (1 sec × 5 times), and centrifuged (8,000 rpm for 3 min). The supernatant was used as the SG antigen. Then, 100 μl of SG antigen was distributed into each of four wells of an ELISA plate and allowed to stand at 4℃ overnight. After blocking with 1% BSA/PBS, anti-CSP antibody diluted 800-fold was poured into 2 wells, and normal mouse serum diluted 800-fold was poured into 2 other wells. Incubation with the secondary antibody and the subsequent procedures were the same as described above.

Western blotting. Ten pairs of salivary glands were collected from both TG female mosquitoes and wild-type female mosquitoes. Samples were separated on a 10% SDS-polyacrylamide gel under reducing conditions (with 2% 2-mercaptoethanol), transferred onto a nitrocellulose (NC) sheet, and probed with mouse anti-CSP antibody diluted 800-fold. The NC sheet was next incubated with anti-mouse IgG conju-

gated with HRP (Bio-Rad Laboratories) diluted 3,000 fold, then reacted with substrate, SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Rockford, IL, USA). Positive bands were visualized in a Lumino Image Analyzer (LAS-1000) (Fuji Film, Tokyo, Japan).

Immunization of mice via biting by CSP-expressing transgenic mosquitoes. Two female BALB/c mice were bitten by each of 50 TG female mosquitoes every 2 weeks over a 10-week period (5 biting sessions). Two other mice were bitten by wild-type female mosquitoes under the same protocol. Two microliters of mouse blood was collected from the tail vein 7 days after the final mosquito biting. Antibody assay to CSP and SG was carried out by ELISA as described above.

Comparison of CSP contents in the SG before and after blood feeding. A pair of SG of the TG female mosquitoes before blood feeding was transferred in 0.5 ml of carbonate buffer, destroyed, and centrifuged as above. Another TG female mosquito was dissected within 10 min after full blood feeding, and a pair of SG were isolated and treated as described above. Each antigen solution was added to 4 wells, 100 μl per well. Anti-CSP antibody (800-fold) was reacted with the 2 wells and anti-saliva antibody (800-fold) was reacted with the other 2 wells. Incubation with the secondary antibody and the subsequent procedures were the same as described above. Eight SG samples in each group were tested.

Results and Discussion

Isolation of TG mosquito. We injected recombinant plasmid into 461 mosquito eggs, from which 109 larvae hatched and 103 adults emerged (G0 adults: 56 males and 47 females). We crossed these G0 males with wild-type females and allowed them to lay eggs. We also crossed G0 females with wild-type males and allowed them to lay eggs. GFP-expressing larvae were selected and allowed to emerge. From the G0 male group we established 1 strain, and from the G0 female group we established 4 strains. The rate of establishment for 5 strains from the 461 egg injections (1.1%) was reasonable compared with our previous experiments [16, 33] and with the results obtained by other groups [15, 32].

As shown in Fig. 2, larvae and adult TG mosquitoes expressed GFP in the eyes as expected, because the *3xP3* promoter works specifically in the eyes [11]. Unexpectedly, GFP was expressed not only in the eyes but also in the SG (Fig. 2C). 3xP3 marker protein is typically used for *Drosophila* (a small fly) transgenesis, and expression of a transgene in the SG of *Drosophila* has not been confirmed. Thus, this is the first observation that the *3xP3* promoter works in the SG of a mosquito. The SG cells may contain some transcriptional factors to attach to the *3xP3* promoter. GFP expression in female SG was observed among all strains of CS1 to CS5. In the SG, the highest expression of GFP was observed in the distal region of the lateral lobes in all SGs (Fig. 2C). In contrast, GFP was not always expressed in the median lobes. This tendency was similar to the results of a previous report [33].

Recombinant CSP expression in the SG. We expected that rCSP would be expressed in the SG of TG mosquitoes. ELISA was performed to confirm rCSP expression. Ten pairs of SG from each strain of TG female mosquitoes were collected and used as ELISA antigens. Only one of the 5 strains (CS2) showed strong reactivity against anti-CSP antibody (Fig. 3). This means that the *3x3P* promoter worked in all 5 strains, but that the *aapp* promoter worked only in strain CS2. The exact reason for this is unknown, but may be related to the insertion site in the mosquito chromosome. The insertion site may allow the *3xP3* promoter, but not the *aapp* promoter, to work. A possible explanation for this is that some of the transcriptional factors that attach to the *aapp* promoter to start AAPP expression react mostly with the intrinsic *aapp* promoter. The inserted *aapp* promoter cannot react with these factors, and thus rCSP may be expressed in only small amounts.

Western blotting analysis and estimation of rCSP expression. Western blotting was performed with 10 pairs of SG dissected from transgenic mosquitoes of the CS2 strain. A band of 38 kDa reacted with anti-CSP in the TG mosquito lane (Fig. 4). The molecular size was as we expected for rCSP. This indicates that we succeeded in producing a foreign protein of rCSP in the mosquito SG. A comparison of the density of the 38 kDa band with those of the positive control bands of a series of different amounts of rPbCSP revealed that the amount of rCSP in the CS2 lane was equivalent (in reactivity

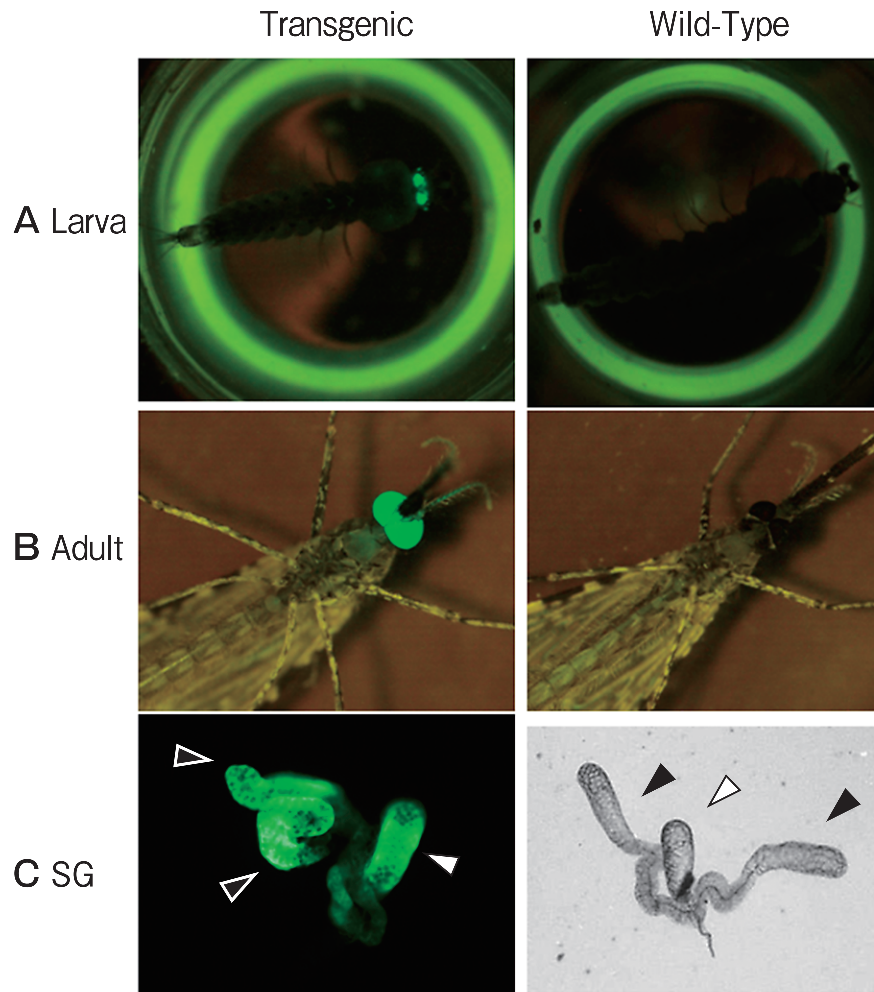

Fig. 2 Transgenic mosquito of *A. stephensi* expressing GFP. A, Larvae expressing GFP in the eyes. Since each larva was placed into its own wells of a 96-well plate, auto-fluorescence appeared from the well edge; B, Adult female expressing GFP in the eyes; C, Unexpectedly, GFP was expressed in the SG of transgenic mosquitoes. As a reference, the SG of a wild-type mosquito was taken at the phase condition. Arrow heads (▶) show the lateral lobes of SG, and the other arrow heads (▷) show the median lobes of SG. No GFP expression was observed in the SG of wild-type mosquitoes.

with the anti-CSP antibody) to 400 ng rPbCSP. This, in turn, indicated that approximately 40 ng of rCSP was expressed in one pair of SG in the TG mosquito.

Besides the 38 kDa band, some other faint bands appeared in the CS2 lane. The molecular sizes of these bands were 26, 48, 64, and 80 kDa. Even in the reducing conditions (with 2% 2-mercaptoethanol), some intermolecular re-binding may have occurred. There is another possibility that rCSP binds to some molecules of the SG components such as ubiquitins, proteosome-associated cofactors [34].

Anti-CSP antibody in mice bitten by TG mosquitoes. Two Female BALB/c mice were each bitten by 50 TG female mosquitoes of the CS2 strain every 2 weeks over a 10-week period. The mice developed anti-SG antibody but did not develop anti-CSP antibody (Fig. 5). In our prediction, 20% of rCSP (about 8 ng of rCSP per mosquito) in the SG should be injected into a mouse during a single blood feeding. In each blood feeding, a mouse received 50 TG mosquito bites and received a total of 400 ng of rCSP in the skin. As these blood feedings were conducted 5 times, each mouse was injected with 2,000 ng of rCSP. From these injections, the mice

should have developed anti-CSP antibody in the serum. However, no antibody against rPbCSP was detected in the mice. Only anti-SG antibody was detected in the mouse serum. In our experience, 2 injections each of 1,000 ng rPbCSP are sufficient to develop anti-rPbCSP in mice (unpublished observation). Thus, we consider the possibility that rCSP was not secreted as we had expected in the saliva of the TG mosquito and thus was not injected into the skin during blood feeding.

Comparison of CSP contents in the SG before and after blood feeding. We performed ELISA with antigens of the SG before and after blood feeding. The amount of rCSP in the SG after blood feeding did not decrease, although the amount of total saliva protein did decrease (Fig. 6). This indicates that the TG mosquitoes consume a lot of saliva during blood feeding and that the saliva does not contain rCSP.

We used the *aapp* promoter for rCSP expression

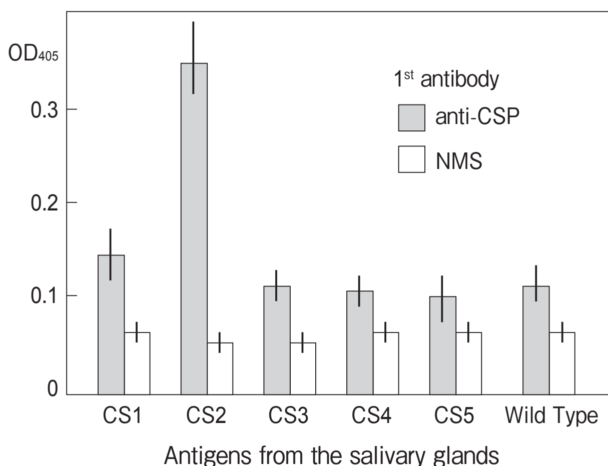

Fig. 3 Evaluation of CSP expression in SG among 5 mosquito strains. SG was taken from the 5 strains and tested as an antigen. Anti-CSP was used as the first antibody. SG from the CS2 strain reacted highly to anti-CSP. NMS (normal mouse serum) was used as a control antibody. The test was conducted 3 times.

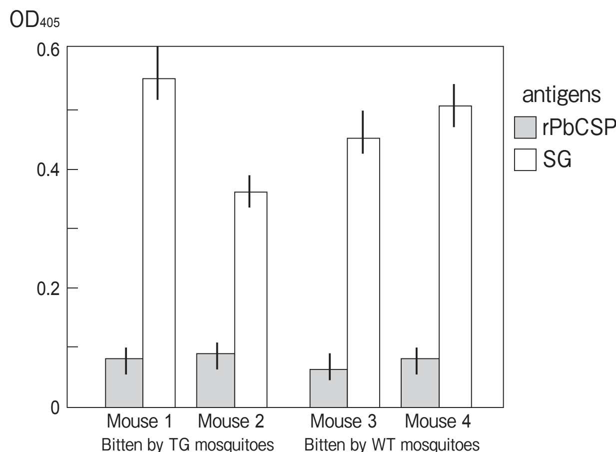

Fig. 5 Anti-CSP antibody and anti-saliva antibody evaluated by ELISA. Mice 1 and 2, which were bitten by TG mosquitoes of the CS2 strain, did not develop anti-CSP antibody. All 4 mice developed anti-saliva antibody. The test was conducted 3 times.

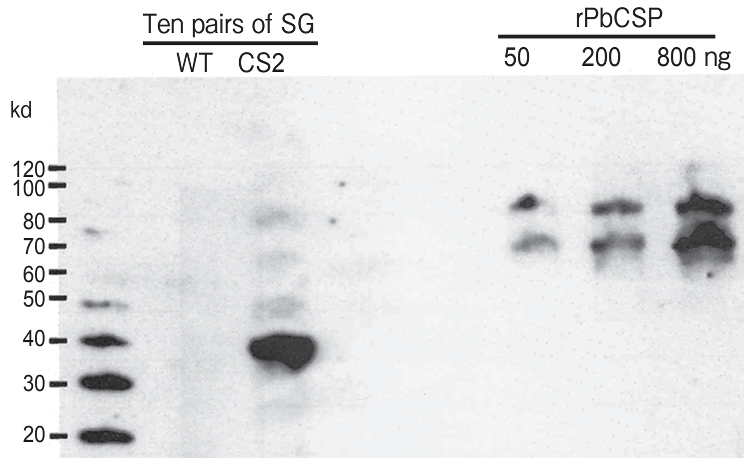

Fig. 4 Western blotting analysis of CS2 strain salivary glands. A 38 kDa band appeared after the reaction with anti-CSP antibody. The left lane contains molecular weight markers. Series of rPbCSP (70 kDa and 82 kDa) with different amounts were run on the same gel in the right 3 lanes.

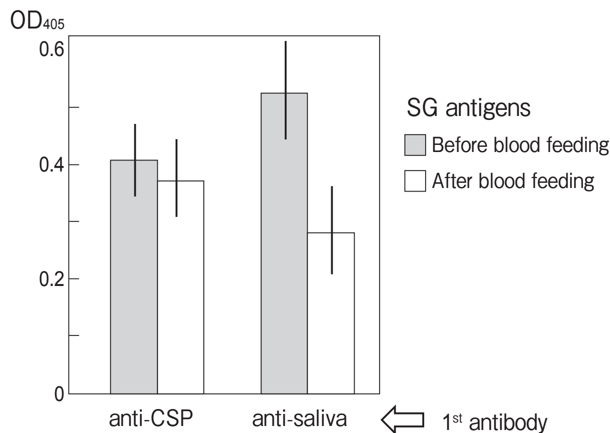

Fig. 6 Comparison of CSP contents in the SG before and after blood feeding. Both SG antigens (n = 8) were filled in the ELISA plate. The reactivity of anti-CSP to both SGs was similar, whereas the reactivity of anti-saliva to the SGs after blood feeding was significantly reduced ($p < 0.05$).

because AAPP was confirmed to be secreted in the saliva and injected into the skin during blood feeding [24]. However, we obtained the disappointing result that the TG mosquito did not properly release the foreign protein into the mouse skin. One possible explanation is that rCSP may have been captured by some components of the SG cells, such as ubiquitins [35]. As shown in Fig. 4, rCSP was expressed as some larger molecules in Western blot analysis. This figure suggests the possibility of "captured rCSP" in the SG. According to this hypothesis, rCSP cannot be secreted in the saliva because of its molecular characteristics, even if it has a secretion signal. We are preparing a new construct using the *aapp* promoter and *aapp* secretion signal with other foreign protein genes to test this hypothesis.

In summary, we presented a transgenic mosquito expressing a foreign protein in the SG. The expressed protein was rCSP, a promising malaria vaccine candidate protein. The amount of rCSP in one pair of SG was estimated to be 40 ng. If 20% of rCSP in the SG is injected into a mouse during a single blood feeding band there are 50 mosquito bites on the mouse, 8×50 ng of CSP should be injected and anti-CSP antibody should be developed in the mouse after several episodes of blood feeding. Since we did not succeed in preparing such a TG mosquito, we will develop new constructs for producing alternative TG mosquitoes.

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