Hemolytic C-Type Lectin CEL-III from Sea Cucumber Expressed in Transgenic Mosquitoes Impairs Malaria Parasite Development

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The midgut environment of anopheline mosquitoes plays an important role in the development of the malaria parasite. Using genetic manipulation of anopheline mosquitoes to change the environment in the mosquito midgut may inhibit development of the malaria parasite, thus blocking malaria transmission. Here we generate transgenic Anopheles stephensi mosquitoes that express the C-type lectin CEL-III from the sea cucumber, Cucumaria echinata, in a midgut-specific manner. CEL-III has strong and rapid hemolytic activity toward human and rat erythrocytes in the presence of serum. Importantly, CEL-III binds to ookinetes, leading to strong inhibition of ookinete formation in vitro with an IC₅₀ of 15 nM. Thus, CEL-III exhibits not only hemolytic activity but also cytotoxicity toward ookinetes. In these transgenic mosquitoes, sporogonic development of Plasmodium berghei is severely impaired. Moderate, but significant inhibition was found against Plasmodium falciparum. To our knowledge, this is the first demonstration of stably engineered anophelines that affect the Plasmodium transmission dynamics of human malaria. Although our laboratory-based research does not have immediate applications to block natural malaria transmission, these findings have significant implications for the generation of refractory mosquitoes to all species of human Plasmodium and elucidation of mosquito–parasite interactions.

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Introduction

Malaria, transmitted by anopheline mosquitoes, is among the worst health problems in the world, killing 1–2 million people every year, mostly African children. Lack of an effective vaccine and the emergence of Plasmodium strains resistant to many existing anti-malarial drugs have aggravated this situation. Therefore, the control of vector competence has become a more important target in malaria intervention.

Recent advances in genetic engineering of anopheline mosquitoes have raised hopes for their use as new strategies for malaria control, also the provision of powerful tools for investigating mosquito-parasite interactions. We and others have characterized tissue-specific promoters that drive robust expression of transgenes in the midgut [1,2], hemocoel [3], and salivary glands [4]. The next challenge is to identify “effector” molecules to inhibit development of malaria parasites without competitive cost to the mosquito. To date, several effector molecules have been identified (e.g., single-chain antibody fragments directed against parasite ligands [5,6], the dodecapeptide SM1 [7], PLA2 [8], a cecropin-like peptide [9], and the Vida3 peptide [10]; see reviews [11,12]). Of these, transgenic mosquitoes expressing either SM-1 or PLA2 in a midgut-specific manner were less able to support transmission of the rodent parasite P. berghei [13,14]. However, the SM1 transgenic mosquito was not resistant to the human malaria parasite P. falciparum (M. Jacobs-Lorena, unpublished observations), and the PLA2 transgenic mosquito was significantly less fit than the wild-type [15]. In those transgenic mosquitoes generated so far, no single effector molecule has exhibited a “non-sporozoite” phenotype in the salivary glands, i.e., complete Plasmodium transmission blockade. Therefore, other effector molecules and/or mechanisms are required to generate a transgenic mosquito that is both fit and refractory to all species and strains of human Plasmodium.

Transmission of malaria parasites is absolutely dependent on availability of competent mosquito vectors. Development of Plasmodium in the mosquito begins with ingestion of an infectious blood meal containing gametocytes from a vertebrate host [16]. In the mosquito midgut lumen, female and male gametocytes mature into gametes after exposure to environmental and mosquito-specific factors. These include a drop in temperature of 5 °C and exposure to xanthurenic acid [17]. A signal transduction cascade results in the release of...
calcium in the cytoplasm of the activated gametocyte, initiating development and its escape from the erythrocyte [18]. After fertilization, the zygote matures into a motile ookinete. Anopheline mosquitoes rapidly concentrate the contents of the blood meal 1.5- to 2-fold, resulting in highly viscous gut content. Although little is known about the influence of these changes, we postulated that changes to the midgut environment could inhibit parasite development. We chose to express the CEL-III lectin from the sea cucumber, Cucumaria echinata. CEL-III is a Ca$^{2+}$-dependent (C-type) lectin, that exhibits strong hemolytic and cell-dependent activity [19] as well as cytotoxicity toward some cultured cell lines [20] by forming ion-permeable pores in target cell membranes through oligomerization after binding to carbohydrate chains on the cell surface [21,22]. Furthermore, synthetic peptides derived from the C-terminal hydrophobic region of CEL-III exhibit strong activity against Gram-positive bacteria such as Staphylococcus aureus and Bacillus subtilis [23].

Here we show that CEL-III strongly inhibits ookinete formation in vitro, and transgenic mosquitoes expressing CEL-III in the midgut significantly inhibit oocyst formation and sporozoite production, not only of P. berghei but also P. falciparum. To our knowledge, this is the first demonstration of stably engineered anophelines in which the reduction of vectorial capacity transcends Plasmodium species.

Results

Hemolytic and Hemagglutination Activities of CEL-III Are Directed Toward Human and Rat Erythrocytes, but Not Mouse Erythrocytes

CEL-III has strong Ca$^{2+}$-dependent hemolytic activity toward human and rabbit erythrocytes, but shows only weak hemagglutination of chicken and horse erythrocytes [24]. This species-specific hemolysis is due to the binding of CEL-III to specific carbohydrate receptors on the erythrocyte surface. We examined whether CEL-III has hemolytic and hemagglutination activities toward mouse and rat erythrocytes as hosts for the rodent malaria parasite P. berghei. Figure 1A and 1B

![Figure 1. Hemolytic and Hemagglutination Activities of CEL-III toward Human, Mouse, and Rat Erythrocytes](image-url)
shows that the hemolytic activity of CEL-III was strong toward human and rat erythrocytes at low concentrations (IC50 = 0.3 and 0.8 µg/ml, respectively) in the presence of 5% fetal bovine serum (FBS: a source of Ca2+), whereas there was no hemolytic activity toward mouse erythrocytes. Weak hemolytic activity was observed against human and rat erythrocytes even in the absence of FBS. Similarly, CEL-III exhibited strong hemagglutination activity toward human and rat erythrocytes, but not toward mouse erythrocytes (Figure 1C). Fluorescent microscopic studies also confirmed that CEL-III bound to rat erythrocytes with numerous punctuate dots distributed throughout the cells, whereas no signals were detected in mouse erythrocytes (Figure 1D). These results suggest that carbohydrate chains on the mouse and rat erythrocyte surface may differ.

In Vitro Effect of CEL-III on Ookinete Development

It has been reported that CEL-III is cytotoxic toward some cultured cell lines as well as toward erythrocytes [20]. Therefore, we investigated the effect of CEL-III on ookinete development in vitro. At first, CEL-III was added to cultured ookinetes in the absence of Ca2+. Figure 2A shows that bound CEL-III was observed as small punctuate dots distributed throughout the ookinete (similar the binding of CEL-III to rat erythrocytes as shown in Figure 1D), whereas no signals were detected in the ookinete without CEL-III. Next, CEL-III was incubated with gametocytes in vitro and the number of ookinetes was determined 24 h later. Figure 2B shows that CEL-III (10 µg/ml) inhibited ookinete development by approximately 95%. This inhibition was dose-dependent, with an IC50 of approximately 15 nM.

### CEL-III Transgenic Mosquito

To express CEL-III in the A. stephensi midgut, we made a pAgCP-CEL-III gene cassette consisting of the promoter, 5'-UTR, and signal peptide from the A. gambiae carboxypeptidase A (AgCPA) gene [1] linked to the coding sequence of the CEL-III gene that lacked signal peptide sequence and the anopheles trypsin 1 (Antryp1) putative terminator region (Figure 3A). This gene cassette was inserted into pMinos-EGFP-RfA-F to construct pMinos-EGFP-carboxypeptidaseP-
phagostimulant, the AgCPA CEL-III. In mosquitoes offered a blood-free ATP meal as a
mobilities of these two forms were similar to those of native
sugar-fed transgenic mosquitoes (Figure 4). The relative
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Those of non-transgenic mosquitoes, indicating CEL-III is
hemolytic activity toward human erythrocytes, but not in
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gene [25]. To examine whether CEL-III is secreted into the midgut lumen upon blood
injection, transgenic mosquitoes were offered a serum meal
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Immunoblot analysis detected monomeric (48 kDa) and
oligomeric (>200 kDa) forms of CEL-III in the midguts of sugar-fed transgenic mosquitoes (Figure 4). The relative
mobilities of these two forms were similar to those of native
CEL-III. In mosquitoes offered a blood-free ATP meal as a phagostimulant, the AgCPA promoter is activated [25, 26].

Figure 4. CEL-III Expression in Midguts of Transgenic Mosquitoes

Transgenic (CEL-III) and non-transgenic (WT) mosquitoes were allowed to
feed on naïve mice. After 6 or 24 h, midguts of engorged mosquitoes
were dissected and lysed, then electrophoresed on 8% SDS-PAGE. As a
control, midguts of sugar-fed mosquitoes (S) were dissected and lysed.
CEL-III expression level was examined by western blotting using mouse
anti-CEL-III antisera. Each lane contains protein lysates equivalent to
two midguts. The source of protein is indicated at the top of each lane (6,
24 h). For quantitative estimation of CEL-III per midgut, native CEL-III
isolated from C. echinata body fluid was analyzed by western blotting.
The amount of native CEL-III (5, 25, 100 ng) is indicated at the top of each
lane. Arrows indicate the positions of monomeric and oligomeric forms
of CEL-III.

CEL-III-antryp1T, then transformed into the germ line of A. stephensi embryos. A total of 876 embryos were injected and 22
fertile G0 matings were obtained. From these, one mating produced transgenic offspring expressing the egfp selectable
marker. A transgenic homzygous line was obtained and propagated. A single integration event was confirmed by
Southern blot analysis using genomic DNA from G0 adults (data not shown).
The transgenic line has been stably maintained by blood feeding on mice or rats for over 30
generations, with no difference in reproductive fitness between transgenic and non-transgenic mosquitoes (i.e.,
number of eggs and hatched larvae; data not shown).

Expression profiles of the CEL-III transgene were investi-
gated by real-time (RT)-PCR (Figure 3B). CEL-III mRNA was
present in the midguts of sugar-fed mosquitoes and was
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Figure 5. Hemolysis of Human Blood in Mosquito Midgut

Mosquitoes were allowed to feed on a human volunteer. Representative
photomicrographs of engorged mosquito gut sections 24 h after a blood
meal are shown (HE staining, ×40 magnification for [A and B], and ×1,000
magnification for [C and D]). Midgut of non-transgenic mosquitoes was
filled with intact erythrocytes (A and C), with many spaces between
erthrocytes. In contrast, no space is observed in the midgut of
transgenic mosquitoes (B and D). Erythrocytes appear to be completely
hemolyzed, and HE-stained lymphocytes cells are detectable (arrows).
Scale bars in (A and B) and (C and D) are 500 μm and 10 μm, respectively.
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Under these conditions, a slightly enhanced expression of the
oligomeric form was observed 24 h after the meal. Compared
to the native CEL-III, we estimate 5–10 ng of CEL-III
accumulated in a single midgut after the ATP meal.

Transgenic Mosquitoes Completely Hemolyze Human
Erythrocytes 24 h after a Blood Meal

We confirmed hemolysis of human erythrocytes 24 h after a
blood meal in midgut sections of a transgenic mosquito. Mosquitoes
were allowed to feed on a human, then, 24 h after the
blood feeding, gut sections were prepared for histology
and stained with hematoxylin and eosin (HE). Compared to
non-transgenic midguts which were filled with intact eryth-
rocytes (Figure 5A and 5C), erythrocytes in the midgut of
transgenic mosquito were extensively hemolyzed (Figure 5B
and 5D). Lymphocytes were clearly contrasted in the midgut
of transgenic mosquito (Figure 5D), but not amongst
the intact erythrocytes in the midgut of non-transgenic mosquito
(Figure 5C). These results are consistent with the data shown
in Figure 3C, where the secretion of CEL-III into the midgut
lumen caused effective hemolysis.

Transgenic Mosquitoes Impair P. berghei Oocyst
Formation in Both Rat and Mouse Models

To investigate the effect of CEL-III expression on P. berghei
development, both transgenic and non-transgenic mosquito
to were allowed to feed on the same P. berghei–infected rat
and the number of oocysts formed was counted. In three
experiments, the infection rate (prevalence) of transgenic
mosquitoes (10.5%) was markedly reduced compared to non-
transgenic mosquitoes (63.6%) (transmission blockade of
TBp; 83.5%, p < 0.01). The oocyst numbers were
consistently and strongly lower in transgenic mosquitoes
Table 1. Rat–P. berghei Experiments

| Experiment | Mosquitoes | Prevalence* | TBp** | Number Oocysts/Gut^ (Range) | TBI* |
|------------|------------|-------------|-------|-----------------------------|------|
| 1          | Control    | 72.7% (8/11) | —     | 4.3 ± 1.5 (0–22)            | —   |
|            | CEL-III    | 9.4% (3/32)  | 87.1% | 0.4 ± 1.6 (0–8)            | 90.7% |
| 2          | Control    | 58.3% (14/24)| —     | 4.0 ± 7.5 (0–28)           | —   |
|            | CEL-III    | 14.3% (2/14) | 75.5% | 0.4 ± 1.2 (0–8)            | 90.0% |
| 3          | Control    | 66.7% (6/9)  | —     | 4.7 ± 6.6 (0–20)           | —   |
|            | CEL-III    | 9.1% (1/11)  | 86.4% | 0.1 ± 0.3 (0–1)            | 97.9% |
| Average    | Control    | 63.6% (28/44)| —     | 4.2 ± 6.9 (0–28)           | —   |
|            | CEL-III    | 10.5% (6/57) | 83.5% | 0.4 ± 1.3 (0–8)            | 90.5% |

Table 2. Mouse–P. berghei Experiments

| Experiment | Mosquitoes | Prevalence* | TBp** | Number Oocysts/Gut^ (Range) | TBI* |
|------------|------------|-------------|-------|-----------------------------|------|
| 1          | Control    | 83.3% (15/18)| —     | 50.2 ± 78.9 (0–300)         | —   |
|            | CEL-III    | 44.0% (11/25)| 47.2% | 10.6 ± 22.3 (0–100)        | 78.9% |
| 2          | Control    | 75.0% (12/16)| —     | 58.3 ± 68.3 (0–217)        | —   |
|            | CEL-III    | 33.3% (4/12) | 55.6% | 3.3 ± 7.1 (0–24)           | 94.3% |
| Average    | Control    | 79.4% (27/34)| —     | 54.0 ± 73.1 (0–300)        | —   |
|            | CEL-III    | 40.5% (15/37)| 49.0% | 8.2 ± 18.9 (0–100)         | 84.8% |

Transgenic (CEL-III) and non-transgenic (control) mosquitoes were fed on the same P. berghei–infected rats. On day 15, the guts were dissected and the number of oocysts per gut was determined.

aPercentage of infected mosquitoes (actual numbers in parentheses).
bTransmission blockade of prevalence (TBp) = 100 – (prevalence of transgenic mosquitoes/prevalence of control mosquitoes) × 100.
cTransmission blockade of intensity (TBI) = 100 – (average oocyst number per gut in transgenic mosquitoes/ average oocyst number per gut in control mosquitoes) × 100.
dStatistical significance (p < 0.01), as calculated by the Mann-Whitney U test.

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Transgenic Mosquitoes Impair Oocyst Formation of P. falciparum

To investigate the effect of CEL-III expression on human Plasmodium development, both transgenic and non-transgenic mosquitoes were allowed to feed on mature P. falciparum gametocyte cultures by membrane feeding, followed by determination of the number of oocysts formed (Table 5). In Experiment 1, oocyst formation in transgenic mosquitoes was significantly impaired (TBI 76.6%). In Experiment 2, TBI was 57.1%, and there was no statistically significant difference between transgenic and non-transgenic mosquitoes. Most likely, the low infection prevalence (30%) and low oocyst number (0.7 ± 1.4) in Experiment 2 affected the statistical analysis. Overall, with the two experiments combined, transgenic mosquitoes significantly impaired P. falciparum oocyst numbers (TBI 69.1%, p < 0.05), although TBp was only 7.8%.

Discussion

This study demonstrates a novel “proof-of-concept” showing that transgenic mosquitoes expressing C-type lectin CEL-III significantly impairs development of both P. berghei and P. falciparum. We hypothesized that an environmental change in the midgut of anopheline mosquitoes by genetic manipu-
Transgenic (CEL-III) and non-transgenic (control) mosquitoes were fed on the same *P. berghei*-infected rat. To measure transmission, three to six mosquitoes were fed on individual naïve mice 21 days after the infectious blood meal. Six mosquitoes were allowed to feed on individual naïve mice 21 days after ingesting the infectious blood meal. Of six mosquitoes, at least three mosquitoes were observed to feed on each mouse.

The infection status of each mouse was established by examining a smear of tail vein blood on alternate days. Mice that had no parasites by day 30 were considered to be non-infected.

Control CEL-III

% Infected Miceb
(Number of Infected/Total)

| Vector competencec | Control | CEL-III |
|-------------------|---------|---------|
| (3 ≤ mosquito bites/mouse ≤ 6) | 100% (10/10) | 20% (1/5) |

| % Infected mosquitoes (Number infected/total) | Control | CEL-III |
|---------------------------------------------|---------|---------|
| 100% (10/10) | 24 | 18 |
| 90% (9/10) | 12 | 1 |
| 75% (7/10) | 19 | 1 |
| 50% (5/10) | 5 | 5 |
| 25% (2/8) | 0 | 0 |

Immediately after blood feeding (Table 3), engorged mosquitoes (20 transgenic and 60 non-transgenic mosquitoes) were picked up and the salivary glands were dissected and the number of sporozoites per salivary gland was determined.

| Number of Sporozoites (spz)/Salivary Glandsa | Number of Mosquitoes |
|---------------------------------------------|---------------------|
| 0 | Control | CEL-III |
| 1 ≤ spz ≤ 500 | 24 | 18 |
| 500 ≤ spz ≤ 5,000 | 12 | 1 |
| 5,000 < spz | 5 | 5 |
| % Infected mosquitoes (Number infected/total) | 60% (36/60) | 10% (2/20)c |

In the transgenic mosquitoes, CEL-III is constitutively expressed prior to blood meal ingestion and accumulates in the midgut. Expression level of CEL-III was enhanced and expressed prior to blood meal ingestion and accumulates in the midgut before a blood meal is likely to cause hemolysis of mouse erythrocytes, oocyst formation was also significantly reduced in the mouse model suggesting that direct parasite toxicity may be the dominant impact of the peptide. Preliminary observations suggest CEL-III reduces the efficiency of fertilization (S.Y. unpublished data). Additionally CEL-III bound to cultured ookinetes correlating with a strong killing effect on the parasites (IC50 = 15 nM) at 100- to 1,000-fold lower concentrations in vitro, when compared to other reported effector molecules, such as cecropin-like peptide [9], defensin [31], Vida3 [10], SM1 [7], and PLAZ [8]. In the rat model, the higher TBI (90.5%) may be due to additional hemolysis compared to that of the mouse model (84.8%). Although the binding specificity and mechanism by which CEL-III kills parasites in mosquitoes is unknown, findings from this study suggest that CEL-III may cause lethal damage to the female gamete and ookinete by pore formation following oligomer formation.

For *P. berghei*, the key property, as proposed in 1968 by Curtis [32], of vectorial competence was demonstrably and severely impaired, as measured by the relative inefficiency of transgenic mosquitoes to infect naïve mice compared to wild-type. Importantly, CEL-III transgenic mosquitoes impair sporogonic development of *P. falciparum*. To our knowledge, this is the first demonstration of stably engineered anophelines that affect the human *Plasmodium* transmission dynamics of a human malaria. Compared to the *P. berghei*-rat model, the TBI of *P. falciparum* is numerically lower (69.1%). One possible explanation for the lower TBI is that membrane feeding of in vitro cultured *P. falciparum* gametocytes does not contain leukocytes that may remain active in the mosquito blood meal and kill or phagocytose the liberated extracellular parasites [33,34].

In malaria endemic areas, multiple infections with *Plasmodium* species and strains are often observed. Those effector gene products must inhibit development of all species and strains of *Plasmodium* in the mosquito. As CEL-III targets erythrocytes, the “vehicles” for this parasite, as well as ookinetes, this transgenic mosquito may prove to be refractory to all species and strains of *Plasmodium*, including *P. falciparum* and *P. vivax*. Transgenic mosquitoes must have a minimal fitness cost, as such costs would reduce the effectiveness of the genetic drive mechanisms used to introduce transgenes into field mosquito populations. To date, there have been no single or cumulative toxic effects.
observed from CEL-III production in mosquitoes for fecundity (eggs laid per female). Further studies are nevertheless required to address the ability of CEL-III transgenic mosquitoes to compete with their non-transgenic siblings.

While we have demonstrated it is possible to create mosquitoes with impaired vectorial competence for more than one species of malarial parasites, we recognize there are numerous other scientific and ethical problems to be overcome before such a control strategy could be implemented.

### Materials and Methods

Mosquitoes, animals, and parasites. *A. stephensi* mosquito strain SDA 500 was maintained at Jichi Medical University and Imperial College London. Female BALB/c mice were obtained from SEASCO (Saitama, Tokyo, Japan) and used at 7 to 8 weeks of age. Female brown Norwegian rats were obtained from SEASCO and used at 7 to 8 weeks of age. *P. berghei* strain ANKA 294 was maintained by cyclical passage through BALB/c mice and *A. stephensi* using standard methods [35]. *P. falciparum* strain 3D7 was maintained in asynchronous culture as described elsewhere [36].

#### Hemolytic and hemagglutination and assay

CEL-III was purified from *C. echinata* body fluid as previously described [22]. Hemolytic activity was measured in the absence or presence of 5% FBS either by visual examination of lysis of erythrocytes or by measurement of hemoglobin release from erythrocytes using absorbance at 540 nm, as previously described [24]. Hemagglutination activity of CEL-III toward human, mouse, and rat erythrocytes was measured in the presence of 10% Dextran 4 (an osmotic protectant: SERVA, Heidelberg, Germany) as previously described [19].

**In vitro ookinete inhibition assay**. *P. berghei*-infected mouse blood was diluted in 5 vol ookinete medium ( RPMI 1640, 10% FCS, 50 μg/ml hypoxanthine, 0.024 M NaHCO₃ 5 μg/ml penicillin and 5 μg/ml streptomycin, final pH 8.5) in 24-well plates with different concentrations of CEL-III and control containing the same buffer. The plate was then incubated at 19 °C on a slow moving shaker for 24 h. After 24 h, the culture was then smeared and fixed with methanol. Air-dried slides were stained with Giemsa, and then the number of ookinetes was counted in a sample of 2,000 or 5,000 RBCs.

**Binding of CEL-III to erythrocytes and ookinetes**. Mouse and rat erythrocytes were prepared from whole bloods by washing five times with PBS. In vitro cultured ookinetes were purified as previously described [37]. Mouse erythrocytes, rat erythrocytes, or ookinetes were incubated with 25 μg/ml of CEL-III at room temperature for 1 h in PBS, and then washed five times with PBS. Bound CEL-III was detected by fluorescence microscopy with goat FITC-labeled anti-mouse IgG (Biosource) following mouse anti-CEL-III antiserum [19].

**Minos vector construction and germline transformation**. PCR reactions were performed with *Pfu* DNA polymerase (Stratagene GmbH). A gene fragment encoding amino acids 11–342 of CEL-III was amplified from plasmid *pGEM-CEL-III* [38] by PCR using primers pCEL-III-F1 and -R1 (Table S1). The PCR product was cloned into pENTR/D-TOPO (Invitrogen) to generate pENTR-CEL-III. A 2,311-bp DNA fragment of the putative promoter region of the *AgCPA* gene and its signal sequence was obtained from *A. gambiae* genomic DNA by PCR using primers pAgCPA-F2 and -R2 (Table S1). A 392-bp DNA fragment of the putative terminator region of *Antryp1* [39] was obtained from *A. gambiae* genomic DNA by PCR using primers pAgAntrp1-F1 and -R1 (Table S1). The *AgCPA* promoter and *Antryp1* terminator were assembled by overlapping PCR using primers pAgCPA-F2 and pAgAntrp1-R1, then cloned into pENTR-D-TOPO (Invitrogen) to generate pENTR-carboxypeptidaseP-antryp1T. The gene fragment encoding CEL-III was excised from pENTR-CEL-III by digestion with BglIII and SpHl, then cloned into the BamHISphl sites of pENTR-carboxypeptidaseP-antryp1T to generate plasmid pENTR-carboxypeptidaseP-CEL-III-antryp1T. Transformation plasmid pMiNos-EGFP-carboxypeptidaseP-CELIII-antryp1T was generated by incubation of pMinos-EGFP-RIA-F [4] and pENTR-carboxypeptidaseP-CELIII-antryp1T in the presence of LR Clonase (Invitrogen) according to the manufacturer’s instructions. Primer sequence information is available in Table S1.

**Embryo microinjection of the transformation and helper plasmids**, screening of EGFP-expressing G0–G2 larvae, and generation of a homozygous line were performed as previously described [4].

**RT-PCR**. We have cloned and sequenced a gene fragment encoding a part of the *AsCPA* gene from the midgut mRNA of *A. stephensi* by RT-PCR using primers, pAgCPA-F1 and pAgCPA-R1 (Table S1), designed for the *AgCPA* gene. Total RNA was isolated from mosquito midguts using an RNeasy Mini column (Qagen). Gene-specific primers for the *CEL-III*, *AsCPA*, and ribosomal protein S7 genes were pCEL3-RT-F1/pCEL3-R2, pAsCPA-F1/pAsCPA-R1, and pAgS7-F1/pAgS7-R1, respectively (Table S1). Aliquots of cDNA representing 0.2 μg total RNA were amplified by PCR using the primer sets for detection of these genes. PCR products were separated by electrophoresis on a 2% agarose gel then visualized by ethidium bromide staining. PCR products of the S7 gene were used as controls for quality of the different mRNA preparations used in the RT-PCR analysis.

**Hemolytic assay for mosquito midgut contents**. Mosquitoes were offered RPMI1640 medium containing 50% FBS through a Parafilm membrane warmed to 37 °C with a glass-watered jacket. Six h after the meal, engorged midguts from 5 mosquitoes were dissected in TBS-Ca (10 mM Tris-HCl [pH 7.5], 150 mM NaCl and 10 mM CaCl₂), then homogenized in a small volume of TBS-Ca buffer, and supernatants were removed by centrifugation. The supernatants were added to human erythrocytes, and then hemolytic activity was measured by visual examination of lysis of erythrocytes as described above.

**Immunoblot analysis**. Mosquitoes were offered protein-free ATP solution (1 mM ATP, 150 mM NaCl, 10 mM NaHCO₃ [pH 7.0]) through a Parafilm membrane warmed to 37 °C with a glass-watered jacket. This protein-free ATP solution was used to minimize background in subsequent western blots as previously described [25]. Engorged midguts were dissected 6 or 24 h after the meal in phosphate buffered saline (PBS), then solubilized with Laemmli buffer containing 1% 2-mercaptoethanol. The equivalent of 2 guts was separated on an 8% SDS-PAGE, electroblotted to Immobilon Transfer Membrane (Millipore), then probed with mouse anti-CEL-III polycional antibody. Bound antibodies were subsequently detected as previously described [5]. Native CEL-III was used for quantification of CEL-III expression per gut.

**Histology of midgut sections**. Mosquitoes were allowed to feed on a healthy Japanese volunteer. 24 h after a blood meal, engorged mosquitoes were fixed with 10% buffered formalin, then embedded

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**Table 5. *P. falciparum* Gametocyte Membrane Feeding Experiments**

| Experiment | Mosquitoes | Prevalence* | TPb | Number Oocysts/Gut† (Range) | TBo|
|------------|------------|-------------|-----|-----------------------------|-----|
| 1          | Control    | 68.0% (34/50) | —   | 7.7 ± 8.8 (0–28)           | —   |
| 2          | CEL-III    | 56.5% (48/85) | 17.0% | 1.8 ± 2.5 (0–11)        | 76.6% |
|            | Control    | 30.0% (15/50) | —   | 0.7 ± 1.4 (0–6)          | —   |
|            | CEL-III    | 26.0% (13/50) | 13.3% | 0.3 ± 1.6 (0–3)         | —   |
|            | Average    | 49.0% (49/100) | —   | 4.2 ± 7.2 (0–28)        | —   |
|            | CEL-III    | 42.5% (61/135) | 7.8% | 1.3 ± 2.1 (0–11)        | 69.1% |

*Prevalence, TPb, intensity, and TBo are described as Table 1.*

*Statistical significance (p < 0.05), as calculated by the Mann-Whitney U test.*

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**CEL-III Transgenic Mosquito**
in paraffin wax. Each block was cut into 4-μm sections, and then stained with HE. The Japanese volunteer gave his written consent to be included in this study after detailed explanation of the research project.

Oocyst inhibition assay for P. berghei. Transgenic and sibling non-transgenic mosquitoes were mixed in the same container then allowed to feed on a single infected rat or mouse. Blood-fed mosquitoes were separated after 24 h, then sorted into transgenic and non-transgenic mosquitoes using a fluorescence stereomicroscope SXZ7 (Olympus) with GFP filter (excitation/emission at 480 nm/515 nm). EGFP in the abdomen of transgenic mosquitoes allowed them to be distinguished from non-transgenic mosquitoes. The two species of mosquitoes were separately housed in pots at 21 °C with 5% fructose solution. On day 15, midguts were dissected, then number of oocysts per midgut was determined. Prevalence, Tpb, the mean number of oocysts in the midgut (intensity), and Tbi were calculated as previously described [5,40]. Data were analyzed using the Mann-Whitney U test.

Oocyst inhibition assay for P. falciparum. Mature gametocytes of P. falciparum (3D7) were produced in vitro as previously described [41]. Briefly, mature gametocyte cultures (0.3 to 0.4% final gametocytemia) were fed for 30 min at 37 °C to transgenic and non-transgenic mosquitoes through a Parafilm membrane. Engorged mosquitoes were separated by pools of 25 °C and 60%–80% relative humidity. On day 10, midguts were dissected and number of oocysts per midgut was determined. The prevalence was analyzed as above.

Sporozoite transmission assay. Transgenic and non-transgenic mosquitoes were allowed to feed on the same naive mouse. Immediately after a blood meal, engorged mosquitoes (20 of 30 transgenic and all non-transgenic mosquitoes) were separated after 24 h and housed in pots at 21 °C with 5% fructose solution. To measure transmission, 6 mosquitoes per group were allowed to feed on individual naive mice 21 days after ingesting the infectious blood meal. Of 6 mosquitoes, at least 5 mosquitoes were observed to feed on each mouse. Immediately after a blood meal, engorged mosquitoes (20 of 30 transgenic and all 60 non-transgenic mosquitoes) were picked up and the salivary glands were excised, placed on a microscope slide, squashed under a cover slip, and then examined by phase-contrast microscopy (×400). Numbers of sporozoites per salivary gland (intensity) was determined using a gland index based on Collins et al. [1977] [43]: 0; 1: 1–499; 2: 500–4999; 3: >5000. The infection status of each mouse was established by examining a smear of tail blood on alternate days. Mice that had no parasites by day 30 were considered to be uninfected.

Supporting Information
Table S1. Primer Sequences
Found at doi:10.1371/journal.ppat.0030192.s0001 (40 KB DOC).

Accession Numbers
The GenBank (http://www.ncbi.nlm.nih.gov/Genbank/) accession numbers for the genes discussed in this paper are AB553072 and CEL-III (AB109017).

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Author contributions. SY conceived and designed the experiments. All authors performed the experiments. SY, MJL, and RES analyzed the data and wrote the paper.

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