SUPPLEMENTARY INFORMATION

SUPPLEMENTARY METHODS

Cloning of CLSP genes

For the CLSP1 cloning, a 1,054-bp DNA fragment was obtained using a set of sense and antisense primers (R411 & R412) and used as a probe to screen the cDNA library prepared from previtellogenic A. aegypti female mosquitoes. A total of five CLSP1 cDNA clones were obtained, the longest being 1,343 bp. Sequencing of this clone showed that it contained a 3’-UTR sequence, but lacked the 5’-UTR and six nucleotides of a predicted full open reading frame (ORF) (Supplementary information Fig. S1). Even 5’-RACE analysis failed to fully recover the 5’-UTR and missing ORF. However, PCR cloning and sequence analysis of the CLSP1 full-length ORF (Primers of R1058 & R996) confirmed the integrity of the CLSP1 gene annotation.

For CLSP2 cloning, a 949-bp DNA fragment was initially obtained (primers of R414 & R415) and used to screen the cDNA library. The CLSP2 clone was isolated and contained a 787-bp DNA fragment with a 3’-UTR sequence, but also lacked the 5’-UTR and a part of the ORF (Supplementary information Fig. S2). 5’- and 3’-RACE analyses further characterized an additional 268-bp nucleotide sequence containing a 16-bp 5’-UTR and an additional 31 bp of 3’-UTR with poly(A) sequence, finally revealing a 1,350-bp full-length cDNA of the CLSP2 gene (Supplementary information Fig. S2).

The SP domain of the CLSP2 gene encodes a protein almost identical to a SP encoded by AAEL014387 (282 identical out of 283 amino acids). The CLSP2 CTL domain is completely identical to the 117 amino acids of Aedes CTLGA9 (AAEL014857). Genes encoding SP or CTL are located less than 3 kb apart from each other, suggesting that they are complex linked genes, each of which encodes a component of compound proteins of SP and CTL—and this would be a duplication of the CLSP2 gene.
However, prediction of a combined \textit{AAEL014387} and \textit{CTLGA9} gene was not characterized by our molecular cloning methods. The predicted gene differs from the \textit{CLSP2} gene in its 3’-UTR sequence and lack of a region of amino acids (284–312 aa of \textit{CLSP2}) linking SP and CTL domains. The PCR cloning and 3’-RACE analyses under various conditions (naïve, immune induction, and infectious blood feeding) resulted only in the cloning of \textit{CLSP2} (\textit{AAEL011616}), but not of a combined gene of \textit{AAEL014387} and \textit{CTLGA9} (data not shown). These results suggest that \textit{AAEL014387} and \textit{CTLGA9} may constitute two different genes encoding SP and CTL, respectively. Gene transcripts with a size corresponding to that of \textit{AAEL014387} and \textit{CTLGA9} were not detected by Northern analyses, indicating that the expression levels of both genes were negligible during egg development and after immune challenge (data not shown).

**Primers used**

\textbf{CLSP1 PCR cloning}

R411: 5’- AGCACATTGCGTTACAGTGC -3’
R412: 5’- TTCCATTGTCTGGTTGTCCA -3’

\textbf{CLSP1 5’-RACE}

R1053: 5’- CTTTATCAAAGCTAAATCCGCTGTG -3’

\textbf{CLSP1 3’-RACE}

R1055: 5’- GTTTATCGGTGGCACCAGCTTAG -3’

\textbf{CLSP1 Full-length ORF cloning}

R1058: 5’- CAGTTCGAAAGTGGAAGATGAAC -3’
R996: 5’- TTACGACCAGTTTAGACTAA -3’

\textbf{CLSP1 dsRNA synthesis}

R452: 5’- TAATACGACTCACTATA GGGCCAATGTTCTGAAAGCAGCA -3’
R453: 5’- TAATACGACTCACTATA GGGCCGTCTTGTATCCGATAGGC -3’

\textbf{CLSP1 RT-PCR for confirmation of the dsRNA knockdown experiment}

R995: 5’- CTTCCAGTGTGGAATACGGC -3’
R996: 5’- TTACGACCAGTTTAGACTAA -3’
CLSP2 PCR cloning

R414:  5'- GTACAAGGACAAGGCGAAGC -3'
R415:  5'- CGACCTCGAGACAGTGTTCA -3'

CLSP2 5’-RACE

R1049: 5’- CTTTATCAGAGCTAAATTCACTATG -3’

CLSP2 3’-RACE

R1051: 5’- GTATATCGGTGGCACCAGATTGGG -3’

CLSP2 dsRNA synthesis

R450:  5'- TAATACGACTCACAATAGGGGTACAAGGACAAGGCGAAGC -3’
R451:  5'- TAATACGACTCACTATA GGATACCACGGACCATTCGTGT -3’

CLSP2 RT-PCR for confirmation of the dsRNA knockdown experiment

R997: 5'- TGGAATAAGGCAATACAAAAC -3’
R998:  5'- TTACGACCAGTGAGCTGA -3’

SUPPLEMENTARY FIGURE LEGENDS

Figure S1. Molecular cloning of the CLSP1 gene from the mosquito Ae. aegypti. The nucleotide sequence of CLSP1 was decoded using cDNA library screening and RT-PCR analyses. RT-PCR primers (R411 & R412) were designed based on the VectorBase genome annotation (AAEL011622). The DNA probe obtained by RT-PCR was used to screen a cDNA library from the naïve previtellogenic Ae. aegypti mosquitoes. Finally, full-length ORF RT-PCR (R1058 & R996) confirmed the nucleotide sequence of full-length cDNAs of CLSP1 gene. There are a few variations of nucleotide and deduced amino acid sequences between cloned CLSP1 cDNA and VectorBase annotation, which is indicated by yellow (cloned CLSP1 cDNA) and gray (AAEL011622) highlighted boxes. Red, green, and turquoise colored highlights indicate signal peptide, SP, and CTL domains. The primers used for the CLSP1 cloning and silencing is indicated by underlining and directional arrows, respectively. The first nucleotides of cDNA clone CLSP1-1 and 5’-RACE product are indicated by elbow arrows. The three amino acid sequence, QPD, of the CTL domain, which is a signature of the galactose-specific CTL, is indicated in bold red characters. The putative proteolytic activation site (V) is highlighted in blue.
**Figure S2. Molecular cloning of the CLSP2 gene from the mosquito *Ae. aegypti***. The nucleotide sequence of CLSP2 was decoded using cDNA library screening, RT-PCR, and RACE analyses. RT-PCR primers (R413 & R414) were designed based on the VectorBase genome annotation (AAEL011616). The DNA probe obtained by means of RT-PCR was used to screen a cDNA library from the naïve previtellogenic *Ae. aegypti* mosquitoes. Missing regions of CLSP2 cDNA were fulfilled by 5’- and 3’-RACE analyses. There are a few variations of nucleotide sequences between cloned CLSP2 cDNA and VectorBase annotation, which are marked as yellow (cloned CLSP1 cDNA) and gray (AAEL011622) highlighted boxes. The primers used for the CLSP2 cloning and silencing are indicated by underlining and directional arrows, respectively. The first and last nucleotides of the CLSP2 cDNA clone are indicated by elbow arrows. The three-amino acid sequence, QPD, of the CTL domain, which is a signature of the galactose-specific CTL, is indicated in bold red characters. The putative proteolytic activation site (V) is highlighted in blue.

**Figure S3. Comparison of amino acid sequences of elastase-like protease.** The N-terminal SP domains of *Aedes aegypti* CLSPs share similarity with SP domains of *Drosophila melanogaster* Gastrulation Defective (DGD), *D. melanogaster* ModSP *Manduca sexta* HP14 and with *Carcinoscorpius rotundicauda* factor C. *Tenebrio molitor* and *A. aegypti* orthologues of *D. melanogaster* ModSP were also used in this analysis. Asterisks mark the positions of fully conserved amino acid residues. Dots and colons mark similar amino acid residues. The H, D, and S residues proposed to be a catalytic triad are highlighted in turquoise. Residues that determine the primary specificity binding pocket are highlighted in bright green. The putative proteolytic activation sites are marked by an arrow.

**Figure S4. RNAi-mediated depletion of CLSP1 or CLSP2 did not cause formation of melanotic tumors.** The melanotic masses were found only in the hemocoel cavities of the mosquitoes with Serpin-2 depletion (A), and not in those with control luciferase dsRNA treatment (B), CLSP1 RNAi depletion (C), or CLSP2 RNAi depletion (D). The red arrow indicates the melanotic mass tumor.
Figure S5. Real-time PCR analyses of activation of CLSP genes in mosquitoes fed on either uninfected or Plasmodium-infected chickens. The CLSP2 gene upregulation 48 h PBM on Plasmodium-infected chickens was confirmed by quantitative PCR. PV, previtellogenic; 24h or 48h PBM, UGAL 24 h or 48 h after chicken blood-meal; Non-infectious BM, blood-meal without P. gallinaceum; Infectious BM, infectious blood-meal with P. gallinaceum.

Figure S6. Three independent experiments on the effect of CLSP RNAi depletions on development of oocysts of Plasmodium gallinaceum in Aedes aegypti female mosquitoes. Midguts from dsRNA-treated mosquitoes were dissected and the oocysts in each midgut were scored 8 days after blood feeding on the P. gallinaceum-infected chicken. The number of fully developed oocysts in each midgut is shown as a circle. Mean parasite oocyst numbers per group are indicated by black bars.
R1055 3’-RACE ➔

1081  GTTTATCGGTGGCACCGACTTAGGATATCTACACCAATGA


← R453 RNA1  ← R412 RT-PCR cloning

1141  GCCTATCGGATACAAGACGGGATACCTAAATTATTCACCTGGACAACCAGACAATGGAA


Galactose-type signature

1201  AGGAATTGAAAACTGTCTGAGATGGTCAGTTGGGTTGCAGACTTTGGGAATGATGTGCC


← R996 full-length

1261  ATGCGATGCAAGCTACGCTATTTGTGAATCTGTTAGTCGAAACTGGTGCCGTAATAT

Figure S2
Figure S2

R1051 3’-RACE ➔
1081 TGGCACGGATGGGGAATGAAAGGACACTTTTGGATATCTACCAATAACGGCTGTCGG
1141 ACACGAGACAGGATAACCATCAACTTTTACTCCGGACAACCAGACAATTACAGAGAAAATGA

R415 PCR cloning T
Galactose-type signature
1201 ACACTGTCTCGAGGTCGCTCGTTGGGGTGGTGTAAAATGGAATGATGTACATTGTCATGC

SPL2-2 cDNA clone
1261 AAGATCACGCTACATTGTGAAACTGTCAGTCACACTGTCGTCACTTCGAATGTATTACT
416  R--S--R--Y--I--C--E--T--V--S--P--H--W--S--*-

1321 ATGAAAATAATAATCATTTAGTGGTTTAT(A)
Figure S3

Aa CLSP2: TRSLITNAYNVQPDGYPWHTAIYQ----------VVPVRYICGGTLVQSVIITAACS
Aa CLSP1: TRSLITNAYNVQPDGYPWHTAIYQ----------VVPVRYICGGTLVQSVIITAACS
Dm GD: ESDDSADSPSITRSGWPDALAAVYVN----------NLTLDFCCGSLVSAVRIIACS
Ms HP14: GTELVLGGERAFQGELPWQAGZYTYT----------KNTRPMQICGGLISSSTVLVLAACS
Aa modular SP: AAYIIIGRNATIEPVHTHGTYRNLEATIDLRSEDWQYICGGTLTERLVSAACS
Tm Modular SP: AQTLYINGKKGDPWVPDVALYTNL----------DKE----------GAGGGVIIQVVTIAC
Dm Modular SP: IKQFSSYYTINNTVPHVHGLVYWH----------NEKYDHFQCGGSLLTFDVLVTIAC
Cr factor C: RSPFVWGNSTIEGQWPAGISRWL----------ADHNNFILQCGSSLNEKWITVLAAC

** Figure S3 **

Aa CLSP2: TVQGGEARDIDELVIKVGKH-LLNVKSEFE---LERELSSIIVHSEFS--SDKHNDIACS
Aa CLSP1: TVPGLGARDIDELVIKVGKH-LLNVKSEFE---HERELSSIIVHGSGF--FDKHNDIACS
Dm GD: KLFN--KRYTSNEVFLRGLRNKWWNEEGS---LAAPVDSGNYHPDNFSQSSLVDYAC
Ms HP14: VWANDAVTFGEE-YAVALAKLQMPQKVPMKQGKEIDHISFYFLGFRNNQTYAC
Aa modular SP: WDVTSFPHDLR--SFIVTAGKYRRELNAVESLVP-AQILRVRELIAQPQYQDFSGYYNLDIACS
Tm Modular SP: TD-DKGKLLSKENYMVAVGKYYRPFNDSRNEAQFSEVKHMFFPELYKSTQNYVGRDIACS
Dm Modular SP: YDEGTRLPSYDTRFVIAAFKRYNYGETTPE--KRDVRLIEIAPYGRKRTENYQDLIACS

Cr factor C: TYSATEIIDPNQFMXLYNKGRY---DDSRRDDYVQVREALEIYHNPYD---FGMNNFIAC

** Figure S3 **

Aa CLSP2: LMITKEPLEYKGFQVACLPT-FSLTRDNAVGN-----IVGWGFT----------AC
Aa CLSP1: LMITKEPYKGFQVACLPT-FSLTSRDAVGN-----IVGWGFT----------AC
Dm GD: VILRKEVRFNTFIRPVACWS-GSSKTEYIVGERGI--VIGWSDTRNTRQKLSSLELP
Ms HP14: VVILETTIVYKPHIRPVCLNPFDIQFEKELYVGSLGKVAGWGIKDE----------AC
Aa modular SP: IIIVLDIFVKSFRICLRELTRDLTSEKKIKPNSLGRVAGWVTLS
Tm Modular SP: ILVTRVTLLSRSKVRPCVIDYGLKTYSTYN--FGY--VTGWYTLQ--
Dm Modular SP: LLTLDEPFELSHVIRPICTVFPSFAEKESTFDVQG--KFAFWEIN
Cr factor Ct: LIQLKTPVTLTRTVQICLPTDITLTREHLEGTLAV--VTGWGLNE----------AC

** Figure S3 **

Aa CLSP2: -NKKSISNVKAANAPIVSRALCVK-SNPSVSSTSLLTNEMFCAGYRN----------GTVIACS
Aa CLSP1: -KKKSISNVKAANAPIVSRALCVK-SNPSVSSTSLLTNEMFCAGYRN----------GTVIACS
Dm GD: GKKSTDAPKVKVAPIVNGAEFCRAN-AHHFLSSSNRTFCAGIQAERDTHQGSA3TY
Ms HP14: --AGNPSQFVVLKPVLYPDVQI--QSPQAFLRPIYGDKICAG--FANG----------AC
Aa modular SP: --GGELSPNLKVDIPTVQVLQCFPSE--KRRQTVLIEIAPYGRKRTENYQDLIACS
Tm Modular SP: --NDKPSVLAKELKVPVSTEQCSS-AIPEHDYYLTHDKLCAQYLD----------GTVIACS
Dm Modular SP: --KHEIQFVAVKLDSSNSVCR---NLQIDIAKFCQCFQG----------GTVIACS
Cr factor C: --NNTYSETIQQAVLPVAACTECEEGYKEADLPVLTVENFMCAGYK--GTVIACS

** Figure S3 **

Aa CLSP2: NOSGQFFFRFVEG------NWVLVGVITSTFIAKQO-------NENLCSSTD-YAFIDVVKYKRIENNSVSFG---
Aa CLSP1: NOSGQFFFRFVEG------NWVLVGVITSTFIAKQO-------DENICSSTD-YAYIDVVKYKRIENNSVSFG---
Dm GD: TGQAGAGLFRHRMRRWVPAVTKAPRS transporting protein--KRMLRGTVAIGLSPAEHSHHLSKCNKOY-YIYADVAKFLDITAFVI--
Ms HP14: KGQQGGLSFAVPARRNLYGIVSTHTHTSN----------EACNAMAF-LFVTNLILSEHHFPIERWTDEY--
Aa modular SP: QCQGDGGFLAKIEGETYVFLYGVWSSPARSA---SGSCDNTKQY-LAFTEVNYQIMPALESFVPI--
Tm Modular SP: QCQGDGGFLVFKFDQG--------RYYVYTGVLSLRQAS--TGCGCDTQO-YLITYKVGTYISDFIKETRSQFRP--
Dm Modular SP: QCQGDGGFFSSELPSTANSTANTARHFLDVFVSN-------------APNADQCMAHS-ILVMTRNQFEMDILMAMNRSVETS
Cr factor C: QCQGDGGPLVFADDSTERRRVLWLEGIVSVWS---SGCGKANQYFGKTGFVFVLSWRFQI--

** Figure S3 **
Figure S5

- **SPL1**
- **SPL2**

![Graph showing relative mRNA amount for SPL1 and SPL2 in different time points (PV, 24h, 48h PBM) for Non-infectious BM and Infectious BM. The graph indicates a significant difference (P<0.05) between the groups.](image-url)
Figure S6

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Significance:
P<0.001
P<0.05
P<0.01