Suppression of *Laccase 2* severely impairs cuticle tanning and pathogen resistance during the pupal metamorphosis of *Anopheles sinensis* (Diptera: Culicidae)

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**Abstract**

**Background:** Phenol oxidases (POs) catalyze the oxidation of dopa and dopamine to melanin, which is crucial for cuticle formation and innate immune maintenance in insects. Although, Laccase 2, a member of the PO family, has been reported to be a requirement for melanin-mediated cuticle tanning in the development stages of some insects, whether it participates in cuticle construction and other physiological processes during the metamorphosis of mosquito pupae is unclear.

**Methods:** The association between the phenotype and the expression profile of *Anopheles sinensis* Laccase 2 (*AsLac2*) was assessed from pupation to adult eclosion. Individuals showing an expression deficiency of *AsLac2* that was produced by RNAi and their phenotypic defects and physiological characterizations were compared in detail with the controls.

**Results:** During the dominant expression period, knockdown of *AsLac2* in pupae caused the cuticle to be unpigmented, and produced thin and very soft cuticles, which further impeded the eclosion rate of adults as well as their fitness. Moreover, melanization immune responses in the pupae were sharply decreased, leading to poor resistance to microorganism infection. Both the high conservation among Laccase 2 homologs and a very similar genomic synteny of the neighborhood in *Anopheles* genus implies a conservative function in the pupal stage.

**Conclusions:** To our knowledge, this is the first study to report the serious phenotypic defects in mosquito pupae caused by the dysfunction of *Laccase 2*. Our findings strongly suggest that *Laccase 2* is crucial for *Anopheles* cuticle construction and melanization immune responses to pathogen infections during pupal metamorphosis. This irreplaceability provides valuable information on the application of *Laccase 2* and/or other key genes in the melanin metabolism pathway for developing mosquito control strategies.

**Keywords:** *Anopheles sinensis*, *Laccase 2*, Phenol oxidases, Cuticle tanning, Melanization, Immunity, Pupal metamorphosis
**Background**

Melanin is not only the substrate used for cuticle tanning in insects, but it is also involved in innate immune responses against exogenous pathogen infections through melanotic encapsulation [1–8]. Thus, melanin metabolism determines whether these two essential physiological processes can be normally activated and affect the typical development of insects. For the Mosquitoes, the adults pose a threat to human health because they transmit malignant diseases by biting humans [9–11]. During the pre-developmental stage of the adult, the development status of the mosquito pupae directly affects the eclosion rate, and further affects the population growth rate of adults.

During pupal development, the cuticle gradually darkens and sclerotizes, which provides enough support for pupae to break out of the puparium, protects them from mechanical injuries, and facilitates the emergence of adults; in addition, some intermediates of melanin metabolism are known to be involved in encapsulation, which is helpful for the survival of the pupae in natural ecosystems [7, 11–16]. Therefore, melanin metabolism during the pupal stage is crucial for mosquito development, survival, and reproduction.

The metabolism of melanin with the hydroxylation of tyrosine to dihydroxy phenylalanine (dopa) by the rate-limiting enzyme tyrosine hydroxylase (TH), followed by the decarboxylation of dopa to dopamine by the dopa decarboxylase (DDC) [5, 17–19]. These melanin precursors must be further oxidized by phenol oxidases (POs) to quinones and quinone methides [19–25]. Subsequently, the quinones and quinone methides conjugate with cuticular proteins to construct and tan the cuticle, and are also involved in melanotic encapsulation [3, 5, 14, 15, 17, 18, 22–25]. Laccase, a member of the phenol oxidase family, conservatively contains three cupredoxin-like domains and four copper ions that reside in a T1 copper site and a T2/T3 tricopper center [25]. Laccases oxidize a broad range of substrates, including polyphenol, methoxy-substituted phenol, aminophenol, and phenylenediamine [5, 27–29]. There are two major types of Laccase genes, *Laccase 1* and *Laccase 2*, that have been identified in many insects [30–44]. Laccase 1 has been reported to likely be involved in cuticular sclerotization [33, 35, 37], while Laccase 2 is mainly expressed in the cuticle or egg shell and is directly involved in melanin-mediated cuticle tanning [38–44]. In the red flour beetle, stinkbug, and honey bee, the pupal cuticle or newly-molted adult becomes white and more flexible due to the depletion of Laccase 2 [38, 40, 42]. Additionally, the dysfunction of Laccase 2 in mosquito eggs results in pale and fragile eggshells, which further causes the eventual collapse of the eggs [39]. This evidence suggests that Laccase 2 is an important regulator in melanin synthesis and deposition. Its dysfunction results in melanin precursors that fail to be oxidized, and impairs the pigmentation and sclerotization of the cuticle or exoskeleton. Although the expression of Laccase 2 is enriched in the epidermis, we speculated that it is also expressed in immune tissues, such as hemolymph or fat body, and may be involved in melanin synthesis, which participates in melanotic encapsulation immune responses. However, knowledge of Laccase 2 functions in cuticle tanning and pathogen resistance during mosquito pupal development is still limited.

**Methods**

**Insect rearing**

The *Anopheles sinensis* LS-WX strain was reared at 27 °C with 80% humidity under a 12 h/12 h (light/dark) photoperiod. The larvae in the different developmental stages were fed fry food in clean water, and the adults were provided 10% glucose solution.

**Identification and cloning of Laccase 2**

BLAST analysis was performed to search homologous Laccase from the *Anopheles sinensis* genome and transcriptome databases using three insect Laccase 2 proteins (Protein ID: AGAP006176-PB, NP_001034487, and BAG70891) as queries. Fragments from the transcriptome data [45] were assembled using the SeqMan program (https://www.dnastar.com/). The signal peptide was predicted by SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP) and conserved domains were analyzed by SMART (http://smart.embl-heidelberg.de/). Total RNA was extracted from the pupae (after pupation 32 h) using the TRizol reagent (Invitrogen, Shanghai, China) according to the manufacturer’s instructions. Total RNA (1 μg) was reverse-transcribed with random primer using the First-Strand cDNA Synthesis Kit (Takara, Dalian, China). Five pairs of primers were designed based on the putative *AsLac2* sequence to obtain the full open reading
frame (ORF). The sequence of 3’UTR was obtained by the rapid amplification of cDNA ends (RACE) technique using the GeneRacer kit (Invitrogen, Shanghai, China). PCR products were isolated and subcloned into PMD-19 vector (Takara, Dalian, China) for sequencing. The primers are listed in Additional file 1: Table S1.

**Phylogenetic and genome synteny analysis**

Homologous Laccase were searched in the NCBI database (http://www.ncbi.nlm.nih.gov/) and Vectorbase (http://www.vectorbase.org/) using the BLASTP program. Amino acid sequences of the divergence part in the C-terminus were aligned with MUSCLE (http://www.ebi.ac.uk/Tools/ma/ma/muscle/). The best-fit evolutionary model (WAG + G) and the genetic distance were estimated by MEGA 5.0 (http://www.megasoftware.net/) [46]. Maximum likelihood phylogenetic analysis was conducted by MEGA 5.0 and bootstrap values were obtained based on 1,000 bootstrap replications. Additionally, the *An. gambiae Laccase* 2 gene and its adjacent genes were used as the templates to search for Laccase 2 homologs in other insect genomes, and their genome locations and distributions were compared in detail. We selected 7.5 kb upstream DNA sequences of the *Laccase 2* gene to predict cis-acting regulatory elements would respond to hormonal signals during pupal development using JASPAR (http://jaspar.genereg.net/) with 90% confidence settings.

**Temporal-spatial expression analysis of AsLac2**

Three individuals at each sampling point were collected at different development stages (pupae were collected at 0, 8, 16, 24, 32 and 40 h after pupation and the adults were collected at 0, 3, 6, 9, 12, 24, 48 and 72 h after the eclosion) for phenotype observations (Olympus, Japan). All of the tissue treatments and slice preparations were carried out as previously described [12]. The thickness of each cuticle layer was measured as the mean thickness in one biological repeat. Four equal spaced measurements for each dorsal plate section, and the average value was calculated as the mean thickness in one biological repeat. Four biological replicates were performed in the *dsLac2*- and the *dsRed*-injected groups, respectively.

**Pathogen infection and in vivo melanization assays**

The pathogens, *Serratia marcescens* (*Sm*) and *Bacillus bombyseptie* (*Bb*), were incubated to the logarithmic phase (OD$_{600}$ = 0.6–0.8) in LB medium at 37 °C. Individuals in the *dsLac2*- (failed to pigment) and the *dsRed*-injected groups were sampled according to the melaninization degree, and then injected with 0.12 μl of bacteria solution at 26 h after pupation, respectively (the colour of the pupa cuticle in the *dsRed* group started to darken, while the cuticle of *Lac2*-silenced pupae failed to tan). The pupal survival rate was recorded every 2 h after being infected. Six pupae at the same developmental stage of the same size were homogenized at 4 °C with 400 μl of PBS (pH = 7.0) and centrifuged (500× g for 5 min at 4 °C). Total protein concentrations (8 mg/ml) were determined using the Bradford method (BBI, Hong Kong, China). Melanization reactions were incubated at 30 °C for 3 h, followed by adding 1 mM of phenylthiourea (PTU) to terminate the reactions and A$_{490}$ nm values were measured to estimate the amount of melanin. Each sample was used to perform three biological repeats.

**Results**

**Characteristics of AsLac2**

The *Anopheles sinensis* Laccase 2 (*AsLac2*) is 4,391 bp (without poly A) with a 2,265 bp ORF encoding 754 through the back of the dorsal plate within 2 h after the pupation according to the temporal expression pattern of *AsLac2*. A red fluorescent protein gene (*dsRed*) was used as the control. The tanning degree of the pupae was checked at 38 h after *dsLac2* or *dsRed* injection, while the checking time for adults was 3 h after the eclosion. Three pupae or adults in the *dsLac2*- and *dsRed*-injected groups at each sampling point were also collected for qRT-PCR analyses [12]. Each sample was used to perform three biological repeats. The *Ribosomal protein L49* (*RPL49*) gene was used as the internal control. The primers used for dsRNA synthesis are listed in Additional file 1: Table S1.
expression of A be significantly increased with the tanning degrees. The involved in melanin synthesis and cuticle tanning should concluded that the expression levels of key genes in-
pation (Fig. 2a) and subsequently, strong melanization. The cuticle colour of the female pupae is a little trans-
Besides, the cuticle tanning pattern is the same in the male and female pupae [12]. Therefore, we concluded that Aslac2 is closely linked to pupal cuticle tanning.

The AsLacase 2 expression pattern is in good agreement with pupal cuticle tanning degree
Both male and female Anopheles sinensis reared in our laboratory required about 40–42 h to complete the eclo-
sion. According to our observations of pupae phenotype in different developmental stages, the cuticle tanning pattern is the same in the male and female pupae [12]. The cuticle colour of the female pupae is a little transparent with a slight yellowish tint within 24 h after pupation (Fig. 2a) and subsequently, strong melanization and hardening were observed until the late pupal stage, especially at 32 h after pupation (Fig. 2a). Therefore, we concluded that the expression levels of key genes involved in melanin synthesis and cuticle tanning should be significantly increased with the tanning degrees. The expression of Aslac2 was almost undetectable at 0 h after pupation, while its expression started to be upreg-
ulated at 8 h, was remarkably increased at 16 h, and was maintained to 40 h after pupation (Fig. 2b). This expression pattern positively correlated well with the cuticle melanization and sclerotization process, but negatively correlated with the derived hemolymph ec-
dysone titer during the mosquito pupal stage [47] (Fig. 2b). Interestingly, a retarded expression pattern of Aslac2 was observed when compared with the up-
stream, rate-limited gene AsITH [12], indicating that the catalytic function of Aslac2 occurred after the accumu-
lation of catecholamine. Furthermore, we found that the expression intensity of Aslac2 in the pupae (from the early-middle to the last stage) was significantly stronger than that in the adults (Fig. 2a), implying its importance in normal pupal development (Fig. 2).

AsLacase 2 depletion results in pupal cuticle tanning
defects and eclosion retardation
Normally, the pupal cuticle darkens and gradually hardens within 24 h of development, and becomes much darker at 38 h after pupation. In this study, no pig-
mentation blocking was observed in the dsRed-injected individuals as they exhibited a normal development and tanning process (Fig. 3a). In contrast, dsLac2-injected pupae exhibited very little or even no melanism at 38 h after pupation, and were accompanied by very soft cuti-
cles (Fig. 3a). At this time point, the expression level of Aslac2 was much lower than that in the dsRed-injected group (Fig. 3a). To avoid the off-target effect, we de-
signed another pair of primers (dsLas2-2) without overlapping with dsLac2. There were 80 and 66% of indi-
viduals whose cuticle was not tanned and flexible that were obtained in the dsLac2 and dsLac2-2 groups, respectively (Fig. 3a, Additional file 4: Table S4). In the dsRed-injected group, the cephalothorax was almost completely tanned at 3 h after adult emergence (Fig. 3b); however, the dsLac2-injected pupae were extremely struggling to emerge and the adults displayed a very light body colour and soft cephalothorax (Fig. 3b). Moreover, we found that the thickness of the dorsal plate section of the cephalothorax in the dsLac2-injected pupae (very fragile and easy to break) (Fig. 4b) was only about 54% (Fig. 4c) of that in the control group (Fig. 4a). The expression level of dsLac2 was also significantly lower than in the control group (Fig. 3b). Our present data indicates that Aslac2 dysfunction can lead to cuticle defects in the melanization and sclerotization processes, suggest-
ing that it is crucial for cuticle tanning (Fig. 3, Fig. 4a, b and c). In the control group, the pupae started to emerge at 37 h and completed the pupation at 41 h with the eclosion rate reaching 79% (Fig. 4d). However, only a 50% eclosion rate was obtained from 45 h to 49 h after pupation in the dsLac2-injected group (Fig. 4d). Obvi-
ously, there was an 8-h eclosion retardation in the dsLac2-injected groups (Fig. 4d). Some of the remaining pupae barely emerged, were physically weak and had wing vibration defects, which distinctly impaired the adults’ ability to survive. The obvious time lag, low emer-
gence rate, and physical weakness definitely affected the mosquito population.
**Fig. 1** Gene structure of AsLac2, phylogenetic analysis of Laccase 1 (LAC1) and Laccase 2 (LAC2) proteins among representative insect species, and genome syntenies of the neighborhood of Laccase 2. 

**a** Solid red boxes represent amino acid coding sequences and fold lines represent the introns. Solid black box represents the 3′UTR. Green square brackets (horizontal) represent the Cu-oxidase domains.

**b** Maximum likelihood phylogenetic tree of Laccase 1 and Laccase 2 among different insects. Amino acid sequence information are as follow: Anopheles gambiae (LAC1: AGAP003738-PA; LAC2: AGAP006176-PB), Anopheles coluzzii (LAC1: ACOM034942-PA; LAC2: ACOM024569-PA), Anopheles arabiensis (LAC1: AARA010056-PA; LAC2: AARA007346-PA), Anopheles funestus (LAC1: AFUN001510-PA; LAC2: AFUN004369-PA), Anopheles stephensi (LAC1: ASTE005343-PA; LAC2: ASTE006211-PA), Anopheles darlingi (LAC1: ADIR002561-PA; LAC2: ADIR007990-PA), Anopheles sinensis (LAC1: ADAC007761-PA; LAC2: ADAC006306-PA), Aedes aegypti (LAC1: AAEL007802-PA; LAC2: AAEL007415-PA), Culex quinquefasciatus (LAC1: CPIJ012357-PA; LAC2: CPIJ010466-PA), Bombyx mori (LAC1: XP_012552135; LAC2: BAG70891), Manduca sexta (LAC1: AY135185; LAC2: AAN17507), Drosophila melanogaster (LAC1: NP_609287; LAC2: NP_724412), and Tribolium castaneum (LAC1: NP_001034514; LAC2: NP_001034487).

**c** Genomic or scaffold syntenies analysis of the neighborhood of Laccase 2 in different insects using the Anopheles gambiae genome as the reference. Solid horizontal lines represent the chromosomes or scaffolds. Orthologue genes are represented by the swallowtail symbols with the same colour (numbers represent gene IDs (The prefix is AGAP0061)). The small triangles linked with the swallowtail represent intron-located genes. The tip of the swallowtail symbols indicates the direction of transcription. Black and white interval lines represent the insertion of multiple genes.
Dysfunctional AsLaccase 2 caused the resistance of pathogen reduced sharply in the pupae

In the dsLac2-injected group, pupa death started at 2 h after Bb challenge, then the mortality increased with the time going from 2 to 10 h and all individuals died at 10 h after the injection (Fig. 5a). In contrast, large-scale mortality in the dsRed group occurred at 20–24 h after the bacteria challenge, which was significantly delayed compared to those in the dsLac2-injected group ($P < 0.01$) (Fig. 5a). Although, the mortality rate in the dsRed- and dsLac2-injected groups caused by Sm was not significantly more severe that those infected by Bb, the survival and life span of the dsLac2-injected pupae were still obviously decreased by Sm ($P < 0.01$) (Fig. 5a). The in vivo assay results revealed that the melanization degree of dsLac2-injected pupal humor was significantly lower than those in the controls after Bb or Sm infection, suggesting that the melanization immune response was severely attenuated in the dsLac2-injected pupae (Fig. 5b). In conclusion, it is worth noting that dsLac2-injected pupae died rapidly and severely after Bb or Sm infections (Fig. 5). These results verified that the AsLac2 gene is involved in Anopheles sinensis’ innate immunity. AsLac2 dysfunction greatly suppressed the melanization immune responses to bacterial infections, which further caused quick and severe death.

Discussion

Laccase 2 is mainly synthesized in epidermis cells and secreted to the procuticle [5, 34, 38, 40–44]. Subsequently, it catalyzes the oxidation of catecholamine to quinones and/or quinone methides, which are involved in cuticle pigmentation, as well as stabilizes and reinforces the cuticle structure via catalyzing the cross-linking among various cuticular components, such as proteins–proteins, proteins–melanin, or proteins–other quinones and/or quinone methides [3–5, 44, 50]. This implies that its normal function controls the supply of important materials for cuticle construction and maintenance. Therefore, the expression pattern of Laccase 2 in a specific developmental stage should reflect the characteristics and changes of the cuticle. In the present study, the expression of AsLac2 was significantly upregulated in the middle and the late pupal stage (Fig. 2). The good agreement of this expression pattern and rapid cuticle tanning strongly supports this view. Serious defects in the pupal cuticle caused by AsLac2 silencing in the high expression period undoubtedly imply that it plays a decisive role in the maintenance of cuticle integrity during mosquito pupal metamorphosis.

Moreover, in most insects, Laccase 2 is irreplaceable and dominantly expressed in pupae, while other melanin metabolism genes, such as TH or Yellow [38, 40, 51–53], are not always dominantly expressed in the pupal stage.
Therefore, we thought that the pupae could protect itself from external adverse factors mainly through passive defenses during pupal and/or eclosion periods. The high expression of \textit{Laccase 2} in this stage can promote quinone and/or quinonemethide accumulation to enhance, harden, and steady the pupal cuticle (Figs. 2, 3 and 4). It is also beneficial for adult cuticle formation to adapt to the environment. On the other hand, ecdysone titer peaks during the early and the middle pupa developmental stage further trigger apolysis and promote the formation of the new pupal cuticle [47–49]. The upregulation of \textit{AsLac2} in this period verifies the general rule that the formation of the new cuticle and the expression patterns of \textit{Laccase 2} are modulated by the ecdysone titer and their functional conservations in the insect evolutionary processes.

Two alternative isoforms of \textit{Laccase 2}, \textit{Lac2A} and \textit{Lac2B}, were identified in some insects [34, 38]. They share the same N-terminal structure and three Cu-oxidase domains, but the C-terminals are quite divergent. Previous studies showed that \textit{Lac2A} was definitely up-regulated in the cuticle, but not \textit{Lac2B} [34, 38]. Although the expression pattern and abundance were different in vivo, no significant differences in substrate preferences were observed between the recombinant \textit{Lac2A} and \textit{Lac2B} [54]. In this study, two alternative splicing forms of \textit{Laccase 2} were also predicted in the \textit{Anopheles sinensis} genome (Additional file 5: Figure S1; Additional file 6: Dataset 1). The phylogenetic analysis revealed that insect LAC2s were grouped into two distinct clads (Additional file 5: Figure S1). Lac2As are highly conserved with high bootstrap supports, but Lac2Bs are quite divergent (Additional file 5: Figure S1). This result implies that Lac2B may be more diverse in functions. However, no transcripts of \textit{AsLac2B} were detected in our analysis; this may be because their expression is undetectable or they are dominantly expressed in other development stages or tissues. Although the dsRNA fragment was designed according to the common region of \textit{Lac2A} and predicted \textit{Lac2B}, no expression of \textit{Lac2B} was detected in

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig3.png}
\caption{Effects of silencing \textit{AsLac2} on pupa and adult cuticle tanning. \textbf{a} Effects of two non-overlapping \textit{dsLac2} on pupal cuticle tanning at 38 h after the pupation (unpaired t-test: $t_{48} = 78.86$, $P < 0.0001$ for \textit{dsLac2}; $t_{48} = 47.87$, $P < 0.0001$ for \textit{dsLac2-2}; $n = 3$). \textbf{b} The molting and cuticle tanning in \textit{AsLac2}-silenced, newly emerged adult (3 h after emergence, unpaired t-test: $t_{48} = 51.84$, $P < 0.0001$, $n = 3$). \textit{AsRPL49} gene was used as the internal control. Scale-bars: 500 μm.}
\end{figure}
the high expression period and the tissues selected for Lac2A silencing. Moreover, the defective phenotype caused by RNAi was due to the dramatic downregulation of Lac2A expression, which is in good agreement with previous reports [38, 40–42, 44]. Therefore, we concluded that the defective phenotype was caused by Lac2A, but not Lac2B. Further studies will be conducted to investigate the expression pattern of Lac2B in other development stages (such as the adult stages) and more fine tissues, which can further help to understand its functions.

Melanin generated from the precursors of dopa and dopamine by the activated phenol oxidases (POs) is important for immune responses [5, 7–9, 12, 14–16, 19, 23, 24, 55, 56]. Genes (TH, DDC etc.) required for melanin synthesis are distributed in immune tissues including fat body and hemolymph [12, 57, 58]. Thus, immune tissues can utilize these precursors in melanization immune reactions catalyzed by phenol oxidases, such as tyrosinase or prophenoloxidases. These reactions are crucial for pathogen resistance. Laccase 2, a member of the phenol oxidase family, can catalyze the precursors to form melanin. If this gene can be expressed in some other immune tissues besides the cuticle of invertebrates [31, 34], theoretically, it can catalyze the terminal oxidation reaction for melanin synthesis. Our results revealed that AsLac2 was expressed in the pupal immune tissues (Additional file 7: Figure S2); this is similar to Laccase 2 expression being slightly increased in Anopheles gambiae after bacterial infection [34]. Combined with our results

![Image](image.png)

**Fig. 4** Effects of silencing AsLac2 on physical characteristics of the adult cuticle and emergence rate. Observation and thickness measurement of the dorsal plate cuticle of individual adults in dsLac2-injected (b) and dsRed-injected groups (a). Insets represent enlarged images from the boxed region. c Graphical representation of cuticle thickness for the two groups. An unpaired t-test was performed to test the difference in cuticle thickness between the two groups (t(6) = 9.67, P < 0.0001, n = 4). d Comparison of emergence time and emergence rate between dsLac2- and dsRed-injected groups (N, represents the sample size; log rank test, **P < 0.01). Scale-bars: a, b, 100 μm; insets, 50 μm
showing that the melanization in the AsLac2-silenced pupae was significantly impaired and the resistance to bacteria challenges was dramatically decreased (Fig. 5), we notice the close relationship between the expression of AsLac2 and melanization immune responses. We speculate that AsLac2 may exert prophenoloxidase activity and involve melanotic immune responses. The dysfunction of AsLac2 can result in insufficient melanin synthesis and further impair immune responses to bacterial pathogens. Additionally, it is unclear whether immune responses are impaired by the feedback inhibition to catecholamine synthase caused by the temporarily accumulation of catecholamine, which is due to the silencing of AsLac2. This feedback inhibition may reduce the efficiency of melanin metabolism, in which the formation of the melanin precursors is further affected. To test this hypothesis, in vitro assays will be conducted to test the activity of total phenoloxidases in melanization and to determine the catecholamine content in dsLac2-injected pupae in subsequent research.

Conclusions

To our knowledge, the present results are the first to illustrate that the suppression of Laccase 2 results in serious adverse effects, which are almost lethal for the wild mosquitoes during the pupal development of Anopheles sinensis. As a rate-limiting enzyme in the final step of melanin metabolism, Laccase 2 has a similar expression pattern as the initial rate-limiting enzyme gene TH in Anopheles sinensis [12]. Silencing these two genes produced similar physiological defective phenotypes, further suggesting that Laccase 2 and TH are crucial for normal pupal development. Our studies further corroborated that melanin metabolism is indispensable for the normal development of mosquito pupae. Therefore, the key genes and/or their regulatory elements in the melanin metabolism pathway may be valuable targets for mosquito prevention and control.

Additional files

**Additional file 1:** Table S1. Primers used in this study. (XLSX 11 kb)
**Additional file 2:** Table S2. Amino acid sequence identity of Cu-oxidase domains of LAC2 orthologs. (PDF 109 kb)
**Additional file 3:** Table S3. Prediction of cis-acting transcriptional regulatory elements upstream. 7.5 kb of Laccase 2. (XLSX 9 kb)
**Additional file 4:** Table S4. Statistical analysis of pupal cuticle tanning degree in the RNAi experiment at 38 h after pupation. (DOC 28 kb)
Abbreviations

AsLa2: Anopheles sinensis Laccase 2; Bb: Bacillus bombyseptie; DDC: DOPA decarboxylase; dopa: Dihydroxyphenylalanine; LAC: Laccase 1; LAC2: Laccase 2; POs: phenol oxidases; Sm: Serratia marcescens; TH: tyrosine hydroxylase

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Availability of data and materials

The data supporting the conclusions of this article are included within the GenBank database under accession number KY132102.

Authors’ contributions

LQ and BC conceived and designed the experiments; BC supplied the internal control. (PDF 72 kb)

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

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