Laccase 2 is the phenoloxidase gene required for beetle cuticle tanning

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Cuticle tanning (or sclerotization and pigmentation) in invertebrates involves the oxidative conjugation of proteins, which renders them insoluble and hardens and darkens the color of the exoskeleton. Two kinds of phenoxodisases, laccase and tyrosinase, have been proposed to participate in tanning, but lack of proof of the true identity of the enzyme(s) responsible for this process has been elusive. We report the cloning of cDNAs for laccases and tyrosinases from the red flour beetle, Tribolium castaneum, as well as their developmental patterns of expression. To test for the involvement of these types of enzymes in cuticle tanning, we performed RNA interference experiments to decrease the levels of individual phenoxodisases. Normal phenotypes were obtained when both of these phenoxodisase genes in insect cuticle tanning, RNAi (RNA interference) experiments were performed by using the red flour beetle, Tribolium castaneum, an economically important agricultural pest species that is exquisitely sensitive to dsRNA-mediated posttranscriptional gene silencing (13, 14). The results presented here demonstrate that a specific laccase gene plays the major if not exclusive role in the tanning of larval, pupal, and adult cuticles of T. castaneum.

Materials and Methods

Insects. T. castaneum strain GA-1 (15) was used in this study. Insects were reared at 30°C under standard conditions (16).

Cloning the cDNAs. The degenerate primers used were 5′-GGNACNCAAYTTYGGCA-3′ and 5′-CCTGNGARTTGRAANGGRTG-3′ for TcLac1 and 5′-AYAYNTNAYCTYTG-GCAYTGGG-3′ and 5′-CKRTCAANGGRWANCATT-3′ for TcTyr1 and TcTyr2. The highly conserved amino acid sequences chosen for designing these primers for PCR were GTHFWH and HPFLHLHG for TcLac2 and NLHWHW and MGYPFDR for TcTyr1 and TcTyr2 (Figs. 6 and 7, which are published as supporting information on the PNAS web site). The PCR amplifications yielded products of 1,088 bp for TcLac2 and 1,325 bp for TcTyr1 and TcTyr2. To obtain the full-length cDNA sequences of TcLac2A, TcLac2B, TcTyr1, and TcTyr2, 5′- and 3′-RACE was performed according to the manufacturer’s (Invitrogen) instructions. The sequences are available in GenBank with the following accession numbers: TcLac2A, AY884061; TcLac2B, AY884062; TcTyr1, AY884063; and TcTyr2, AY884064. Querying the Tribolium genome with Manduca sexta lac1 (GenBank accession no. AY135185) and Anopheles gambiae lac1 (GenBank accession no. AY135184) through the BeetleBase database (http://bioinformatics.ksu.edu/BeetleBase/index.html) identified another laccase gene, TcLac1. A partial cDNA of TcLac1 was cloned from pupal cDNA by using the primers 5′-CCCTTGCGCAAGAAAATGTG-3′ and 5′-ATGACTCCTCCTCCTACAT-3′, and then 5′- and 3′-RACE was performed to obtain the full-length cDNA (GenBank accession no. AY884065).

Synthesis of dsRNAs and Injection Protocol. For dsRNA-mediated transcript depletion experiments (for a summary, see Fig. 2B), we targeted the most diverse regions of TcLac1 and TcLac2 and the least divergent regions of TcTyr1 and TcTyr2. The region chosen for synthesis of TcLac2 exon-non-specific dsRNA included a portion of exon 5, which is common to TcLac2A and TcLac2B (see Fig. 1); therefore, the dsRNA was expected to

Abbreviation: RNAi, RNA interference.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AY884061–AY884065).

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knock down both alternatively spliced transcripts of this gene, which it did. The nucleotide sequence identity between TcTyr1 and TcTyr2 in the targeted region (nucleotides 1430–1855 for TcTyr1 and 1430–1858 for TcTyr2 with all nucleotides numbered from the translation start site) is 68% but includes several identical stretches of 20 nt or more. We anticipated that dsRNA corresponding to this region of TcTyr1 might be capable of causing depletion of transcripts for both tyrosinase genes. The lengths of the regions chosen for production of dsRNAs for dsTcLac1 (nucleotides 1060–1548), dsTcLac2 (nucleotides 1321–1646) and dsTcTyr were 489, 326, and 426 bp, respectively. By using the AmpliScribe T7-Flash transcription kit (Epitenc Technologies, Madison, WI) with the appropriate DNA as template and a pair of primers containing T7 promoter sequences at the 5’ end, dsRNAs spanning the desired regions of the template were generated. For RNAi experiments, ≈0.002–0.2 μg of the indicated dsRNA (0.01–1 μg/μl dissolved in 0.1 mM sodium phosphate, pH 7, containing 5 mM KCl) was injected into penultimate instar larvae, last-instar larvae, or prepupae (13). After injection, insects were kept at 30°C for the indicated periods for visual monitoring of phenotypes and other analyses.

Analysis of Expression by RT-PCR. To analyze the transcription patterns of TcLac1, TcLac2A, TcLac2B, TcTyr1, and TcTyr2 during development, total RNA was isolated from whole insects (prepupae, pupae, or adults) by using the RNaseasy Mini kit (Qiagen). First-strand cDNA synthesis and RT-PCR were done as described in ref. 17 using the primers listed in Table 1, which is published as supporting information on the PNAS web site. The following primers designed for the Tribolium polypepillquinin gene (18) were used as an internal control for normalization of equal sample loading: 5’-GACCGGCAAGCCATCTACAT-3’ and 5’-CCGACAGCACAACCTGAAGG-3’. For TcLac2 RNAi, dsLac2 was injected into prepupae, and total RNA was isolated 5–6 d after pupation (6–7 d after injection). For TcLac1 or TcTyr RNAi, dsTcLac1 or dsTcTyr (dsTcTyr1) was injected into late-stage larvae, and the resulting 0- to 1-d-old pupae (6–7 d after injection) were harvested for RNA isolation. For exon-specific TcLac2 RNAi, 100 ng of dsLac2A or dsLac2B or a mixture of 100 ng each of dsLac2A and dsLac2B were injected into prepupae, and total RNA was isolated 5–6 d after pupation (6–7 d after injection) before RT-PCR.

Protein Sequence Analysis. Protein sequences were aligned with CLUSTALW software (19). SIGNAL 2.0 software was used to predict putative signal peptides. NETNGLYC 1.0 and NETOGLYC 2.0 were used to identify the potential N and O glycosylation sites. These programs are available on the Expert Protein Analysis System Proteomics server of the Swiss Institute of Bioinformatics (http://us.expasy.org).

**Results**

Characterization of Laccase and Tyrosinase Genes. Before the recent release of Version 1 of the Tribolium genome sequence assembly (www.hgsc.bcm.tmc.edu/projects/tribolium) and its incorporation into the Tribolium genome database (BeetleBase; www.bioinformatics.ksu.edu/BeetleBase), we had cloned and characterized full-length cDNAs for laccases (TcLac2A and TcLac2B) and tyrosinases (TcTyr1 and TcTyr2) from this species. Initially, partial cDNA fragments were obtained by PCR amplification using pupal cDNA as template and degenerate primers designed from highly conserved amino acid sequences of laccases and tyrosinases derived from several other insect species (12, 20–22). Complete sequences of cDNAs were assembled by 5’- and 3’-RACE, as described in Materials and Methods. After the release of the Tribolium genome assembly, we used the TBLASTN program to identify genes corresponding to these four previously characterized phenoloxidase cDNAs and another putative laccase gene, TcLac1. A partial cDNA for TcLac1 was amplified from the pupal cDNA by using gene-specific primers, and then 5’- and 3’-RACE was performed to obtain the full-length cDNA. The TcLac2 gene gave rise to two cDNA clones, TcLac2A and TcLac2B, as a result of alternative splicing (see below). No additional phenoloxidase genes were identified in the genome sequence assembly. The amino acid sequences of the three laccases and the two tyrosinases were deduced from the PCR-amplified cDNA sequences. Conceptual translations of the three laccase and two tyrosinase genes exhibited good alignments and sequence similarities with other insect laccases and tyrosinases, respectively (Figs. 6 and 7 and Tables 2 and 3, which are published as supporting information on the PNAS web site).

All three of the laccase isoforms encoded by the two Tribolium laccase genes have putative signal peptides and are presumed to be secreted proteins with multiple glycosylation sites. A comparison of the sequences of TcLac2A and TcLac2B cDNAs and of the TcLac2 locus revealed the intron–exon organization of this gene (Fig. 1). The genomic sequence encoding the C-terminal of TcLac2 consists of two sets of alternative exons with three exons in each set, which results in the production of two alternatively spliced transcripts corresponding to the two laccase 2 cDNAs that we had isolated. These transcripts encode proteins of 717 and 712 aa (Figs. 1 and 6). The amino acid sequence identity is 74% in the variable C-terminal regions of these two isoforms (266 and 261 aa for TcLac2A and TcLac2B, respectively). The gene for the other laccase, TcLac1 has no alternative exons and the encoded protein shares only ≈35% amino acid sequence identity with either of the laccases encoded by TcLac2.
transcripts were more abundant than TcLac2B transcripts at almost all time points examined, but the relative amount of each alternatively spliced transcript varied during development. In contrast, transcripts of the tyrosinases were abundant during early pupal development and were essentially undetectable in pharate adults and in adults. These differences in temporal patterns of expression during development suggest that the two types of phenoloxidases have different, specialized functions. The timing of expression of TcLac2 just before the initiation of the cuticle tanning process and the strong expression of the orthologous laccase 2 in M. sexta pharate pupal epidermis and its reduced expression in young pupal epidermis (12, 23) are observations consistent with the presumed role of laccases 2A and/or 2B in cuticle tanning.

dsRNA-Mediated Depletion of Laccase and Tyrosinase Transcripts. To obtain direct evidence for the involvement of specific phenoloxidases in the tanning process, we used dsRNA-mediated transcript depletion, which has been demonstrated to be very efficient for other genes in Tribolium (13, 14). dsRNAs were injected into dorsal abdomens at least 3 d before the time of maximal accumulation of the targeted transcript. Administration of dsRNA for TcTyr1 at the late larval stage knocked down transcripts for both tyrosinase genes, TcTyr1 and TcTyr2, at the early pupal stage, as shown by RT-PCR analysis (Fig. 2B). The knockdown of both tyrosinase transcripts by dsRNA for TcTyr1 may be attributed to short regions of highly conserved sequences within these two genes. In contrast, injection of dsRNA for TcLac1 reduced the levels of its transcripts substantially without affecting those of TcLac2 (Fig. 2A). dsRNA for TcLac2 was similarly selective in its action, and the levels of TcLac1 transcripts were unaffected after injection of dsTcLac2. Both of the alternatively spliced forms of TcLac2 were down-regulated by injection of this exon-nonspecific dsRNA, because of shared identical sequences in these transcripts. In no case did dsRNA injections result in detectable changes in levels of polyubiquitin transcripts that were used as a control for equal sample loading and for monitoring of nonspecific effects of dsRNA.

RNAi Phenotypes. The phenotypes of the animals obtained from these experiments are shown in Fig. 3A. In beetles injected with buffer alone, tanning was evident by day 5 in the pupal and pharate adult cuticles and in <1-day-old adult cuticle. The adult cuticle tanning process was completed after 3 d, not only in buffer-injected controls but also in animals injected with dsRNA (>0.2 μg per insect) for TcTyr, demonstrating that down-regulation of the TcTyr1 and TcTyr2 transcripts had no effect on cuticle tanning. Similarly, insects treated with dsRNA for TcLac1 also showed normal pupal and adult cuticle tanning (Fig. 3D). We did not observe any lethality from injection of dsTcTyr, dsTcLac1, or buffer. In contrast, prepupae injected with dsRNA for TcLac2 exhibited little or no tanning on the last day of the pupal stage and relatively little tanning of the pharate adult or adult cuticle. These adults had soft colorless cuticles and abnormally enlarged bodies with erytra that were deformed and leg sockets that were oversized. All of the beetles treated with dsRNA for TcLac2 (n = 40) died within several days after adult eclosion.

In addition to monitoring adult cuticle tanning, we wished to test whether TcLac2 is also critical for tanning of the larval and pupal cuticles of Tribolium. dsRNA for TcLac2 (≈0.2 μg per insect) was injected into larvae that were presumed to be a mixture of penultimate and last instars. As shown in Fig. 4, two different phenotypes were observed. One day after larval–pupal molt, animals treated with buffer showed normal cuticle tanning, whereas dsLac2-treated larvae exhibited no cuticle tanning (Fig. 4A). The larvae became shorter in length and died before any subsequent molting. One-day-old pupae that were treated as larvae with dsLac2 also exhibited no pupal cuticle
pressure (Fig. 4). More sensitive to the swelling effect of internal hemolymph, the absence of sclerotization rendered them less able to cuticular structures, including setae, urogomphi, or gin traps. To evaluate the function(s) of the two alternatively spliced isoforms of laccase 2, TcLac2A and TcLac2B, in pupal and adult cuticle tanning. TcLac2 plays a critical role in the sclerotization and pigmentation of larval and pupal cuticles and adult cuticle.

To evaluate the function(s) of the two alternatively spliced isoforms of laccase 2, TcLac2A and TcLac2B, in pupal and adult cuticle tanning, we performed exon-specific RNAi by using dsRNA for TcLac2A and TcLac2B, play different but indispensable roles in pupal and/or adult cuticle tanning, with TcLac2A making the major contribution to tanning at all of the stages. Although injection of dsTcLac2B only delayed the rate of adult tanning, the animals did not survive for more than 1 week after adult emergence. The effect of administration of exon-nonspecific dsRNA for TcLac2 on the phenotype was dose- and time-dependent. Administration of 200 ng of dsRNA per prepupa resulted in nearly complete inhibition of tanning in adults on day 1, and only a small degree of tanning was observed by day 2 (Fig. 5). All of these insects died on day 2 or 3 and exhibited severe developmental abnormalities. Reducing the dose of dsRNA 10-fold to 20 ng per prepupa resulted in fewer developmental abnormalities but did not result in substantial improvement in tanning or survival. At a dose of 2 ng.

Fig. 3. The effect of dsRNAs for TcLac2, TcLac1, and TcTyr on pupal and adult development and cuticle tanning of Tribolium. (A) dsRNAs for TcLac2, TcLac1, or TcTyr (200 ng per insect) were injected into last-instar larvae or prepupae as indicated in Fig. 2 (n = 40, two replicates of 20 insects each). All dsTcLac2-injected pupae developed without tanning, did not eclose normally, and died after several days. Two different terminal phenotypes observed after injection of dsRNA for TcLac2 are shown. Injection of dsLac1, dsTcTyr (dsTcTyr1), or buffer had no effect on cuticle tanning, with all pupae and adults developing normally. The red slash line indicates that the insect has died. (B) Exon-specific RNAi using dsRNAs for TcLac2A (100 ng per insect), and TcLac2A/2B (mixture of dsLac2A and dsLac2B, 100 ng of each dsRNA per insect) were injected into prepupae (n = 40, two replicates of 20 insects each). All of the animals treated with dsRNA died within a week after eclosion. The red slash line indicates that the insect has died.

Fig. 4. The larval and pupal phenotypes produced by injection of dsRNA for TcLac2. dsRNA for TcLac2 was injected into late larvae to observe the effect on larval and pupal cuticle tanning. (A) Last-instar, 1-d-old larvae, injected 3 d earlier with buffer or dsLac2. No tanning was observed in the larval or pupal cuticle. (B) Dorsal view of pupal cuticle-specific gin traps (arrows) displayed in pupae shown in B magnified 3 times. No tanning was observed in the larval or pupal cuticle. (C) B = 1 mm
per prepupa, the treated insects developed into more normal-looking adults, but they still had dented pronota, malformed wings, and incompletely tanned elytra, and the tanning process was considerably slowed, taking several additional days to complete, suggestive of a progressive loss of RNA interference after administration of low levels of dsRNA. In contrast to insects treated with higher doses of dsRNA, these insects were more viable, indicating substantial recovery from the effects of the lowest dosage of dsRNA administered.

**Discussion**

Insect cuticles vary considerably in stiffness, hardness, and pigmentation, depending on the evolutionary dictates of their specific anatomical and physiological roles. Tanning agents, namely oxidized catechols and their derivatives, could be generated by any one of several phenoloxidases or combinations thereof. The identification of the particular phenoloxidase(s) involved in cuticle tanning from among several candidate isoforms of laccases and tyrosinases had been a matter of debate until now. Our data unambiguously demonstrate that only two of the several phenoloxidases of *Tribolium*, namely laccases 2A and 2B, are required for larval, pupal, and adult cuticle sclerotization and pigmentation. Both of these isoforms, which are generated from a single gene, *TcLac2*, as a result of alternative splicing, are indispensable because knock-down of their transcripts at the late larval stage results in incomplete tanning of the larval, pupal, and adult cuticles and, subsequently, death. In contrast, neither laccase 1 nor the two isoforms of tyrosinase are required for tanning. Presumably, these three phenoloxidases have other functions unrelated to cuticle tanning, although a minor, dispensable role in cuticle sclerotization and pigmentation cannot be completely ruled out. Consistent with this interpretation, laccases 2A and 2B are expressed maximally in the epidermis just before the periods of pupal and adult cuticle tanning, when they catalyze the oxidation of endogenous catechols that serve as precursors for cuticle tanning agents (4, 24). Although the dsRNA-mediated down-regulation of *TcLac2* was incomplete, beetles injected with TcLac2 dsRNA failed to tan normally, were soft-bodied, enlarged, deformed, and unable to walk, and they subsequently died prematurely in a dsRNA dose-dependent fashion. The morphological abnormalities and mortality are probably secondary consequences of loss of structural integrity of the cuticle in the absence of tanning. By catalyzing the formation of cross-linking agents from catechols for cuticle sclerotization, the two laccases encoded by the *TcLac2* gene help to generate the supramolecular architecture of the exoskeleton, which provides not only the requisite mechanical properties for muscle attachment and locomotion but also stability against cuticle-degrading enzymes widely distributed in entomopathogenic microorganisms.

The data presented here clearly demonstrate that laccase 2 is involved in the tanning of not only pupal and adult cuticles and associated structures but also larval cuticle. The laccase 2A isoform appears to play the major role in the tanning process, affecting pupal and adult cuticle tanning and probably larval pigmentation as well. The laccase 2B isoform apparently makes a much smaller contribution compared with laccase 2A because it affects only the rate of adult cuticle tanning and has no effect on pupal tanning. However, this contribution is nonetheless indispensable, as indicated by structural abnormalities and premature death, which occur in young adult beetles when the transcripts of this particular isoform are depleted. It is possible that the requirement for the laccase 2B isoform may be related to morphogenesis and tanning of tracheae, forogut, hindgut, and/or other cuticle-containing tissue types exclusive of the exoskeleton during development of the pharate adult.

Progress in deciphering the supramolecular structure of tanned cuticle and the mechanisms of its assembly has been relatively slow because of the intractable nature of the “finished” product and the irreversibility of the tanning process. However, with the results reported here and elsewhere about structural cuticle proteins (4, 8, 25), catechols (26, 27), oxidative enzymes (2, 4, 12), and chitin (14, 17, 28), as well as their interactions (3, 24, 29, 30), researchers should in the future be able to determine more precisely how insects use these components to stabilize their exoskeletons and other tanned structures. A more complete understanding of the biochemistry of cuticle tanning, a process specific to arthropods, may reveal other targets besides laccases 2A and 2B for biorational agents that could be used for controlling agricultural pests and vectors of animal and human diseases. In addition, when the biochemical mechanisms responsible for cuticle tanning are better understood, new types of cross-linked biopolymers based on that chemistry could be produced, some of which may have medical or industrial applications (31).

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