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 phenoloxidases, laccase and tyrosinase, have been proposed to participate in tanning, but 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 phenoloxidases. Normal phenotypes were obtained after dsRNA-mediated transcript depletion for all phenoloxidases tested, with the exception of laccase 2. Insects injected with dsRNA for the *laccase 2* gene failed to tan, were soft-bodied and deformed, and subsequently died in a dsRNA dose-dependent fashion. The results presented here support the hypothesis that two isoforms of laccase 2 generated by alternative splicing catalyze larval, pupal, and adult cuticle tanning in *Tribolium*.

exoskeleton | sclerotization | pigmentation | *Tribolium castaneum* | RNA interference

Tanning (or sclerotization and pigmentation) is a complex extracellular process first proposed >60 years ago as a mechanism for stabilizing proteinaceous extracellular structures found throughout the animal kingdom (1). Insects and other invertebrates use tanning reactions extensively to effect the hardening and pigmentation of their exoskeletons or cuticles, egg capsules, chorions, oothecae, and silk cocoons. During tanning, cross-links form between adjacent polypeptide chains, causing progressive hardening, dehydration, and close packing of the polymers. This cross-linking occurs as a result of oxidative and nucleophilic reactions between highly reactive tanning agents derived from catechols and nucleophilic side-chain groups of proteins (2–4). Catechol oxidation is catalyzed by phenoloxidases and is an important step not only in tanning but also in other processes, such as wound healing and immune responses in insects (5–7). Until now, it has not been possible to differentiate among proposed roles of individual phenoloxidases in sclerotization, pigmentation, wound-healing, or pathogen response.

The initial steps of tanning in some cuticles involve formation of quinones and quinone methides derived from *N*-acylcatecholamines, followed by their oxidative conjugation with cuticular proteins, leading to changes in mechanical properties and coloration (3, 8). Two multicopper oxidases in the phenoloxidase group, laccase [Gene Ontology function 0008471 (EC.1.10.3.2), *ortho*- and *para*-diphenol:O_2 oxidoreductase] and tyrosinase [Gene Ontology function 0004097 (EC.1.10.3.1), *ortho*-diphenol:O_2 oxidoreductase], have been detected in insect cuticles (4), but evidence presented to date regarding the identity of the enzyme(s) responsible for tanning has been inconclusive (2, 4). Whereas tyrosinases, which are oxidases that contain two copper ions and catalyze the oxidation of *ortho*-diphenols, have been studied extensively for their roles in melanization, wound healing, and insect immunity (7, 9–11), less information is available about the importance in insect developmental physiology of laccases, which are oxidases that contain four copper ions and have *ortho*- and *para*-diphenol oxidizing activities (4, 12). To establish the involvement of either or both of these phenoloxidase genes in insect cuticle tanning, RNA interference (RNAi) 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′-GGNACNCAYTTYTGGCA-3′ and 5′-CCRTGNARRTGRAANGGRTG-3′ for *TcLac2* and 5′-AAYYTNCAYCAYTGGCAYTGG-3′ and 5′-CKRTCRAANGGRWANCCCAT-3′ for *TcTyr1* and *TcTyr2*. The highly conserved amino acid sequences chosen for designing these primers for PCR were GTHFWH and HPFHLHG for *TcLac2* and NLHHWHW 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′-TCAACCCCACAACATAAACAG-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. 2*B*), 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-nonspecific 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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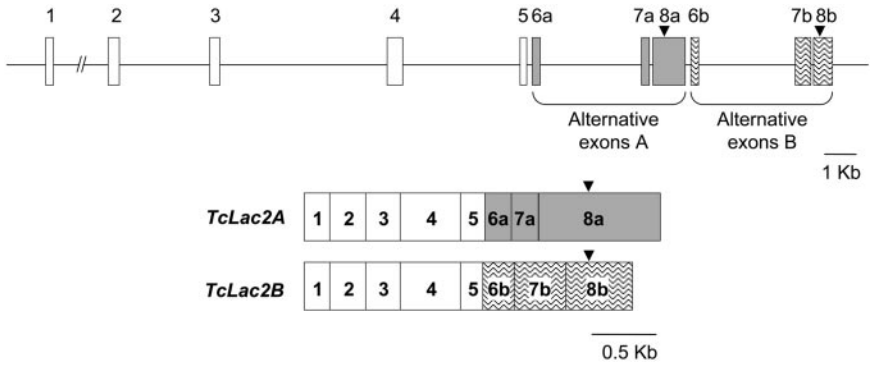

Fig. 1. Schematic diagram of the organization of the *TcLac2* gene. cDNA sequences of *TcLac2A* and *TcLac2B* were compared to the genomic sequences gleaned from the BeetleBase database to define the exons (including alternative exons) and introns. The boxes and lines indicate exons and introns, respectively. A translation start codon is located in exon 1. The arrowheads indicate the translation termination positions for *TcLac2A* and *TcLac2B*.

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 ds*TcLac1* (nucleotides 1060–1548), ds*TcLac2* (nucleotides 1321–1646) and ds*TcTyr* were 489, 326, and 426 bp, respectively. By using the AmpliScribe T7-Flash transcription kit (Epicentre 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 RNeasy 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* polyubiquitin gene (18) were used as an internal control for normalization of equal sample loading: 5′-GACCGGCAAGACCATCACT-3′ and 5′-CGCAGACGCAAAACTGAAGG-3′. For *TcLac2* RNAi, ds*Lac2* was injected into prepupae, and total RNA was isolated 5–6 d after pupation (6–7 d after injection). For *TcLac1* or *TcTyr* RNAi, ds*TcLac1* or ds*TcTyr* (ds*TcTyr1*) 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 ds*Lac2A* or ds*Lac2B* or a mixture of 100 ng each of ds*Lac2A* and ds*Lac2B* 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). SIGNALP 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 terminus 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*.

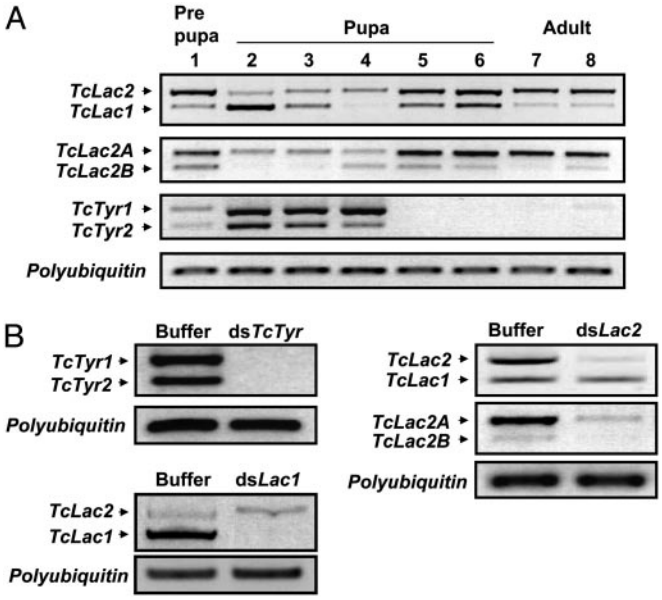

Fig. 2. Developmental patterns of expression of phenoloxidase genes and dsRNA-mediated down-regulation. (*A*) The developmental profiles of expression for *TcLac1*, *TcLac2A*, *TcLac2B*, *TcTyr1*, and *TcTyr2* from the prepupal stage through the adult stage were determined by using RT-PCR (24 cycles) and total RNA prepared from pools of three insects at each stage. Lane 1, prepupae; lane 2, 0- to 1-d-old pupae; lane 3, 1- to 2-d-old pupae; lane 4, 2- to 3-d-old pupae; lane 5, 3- to 4-d-old pupae (pharate adults); lane 6, 4- to 5-d-old pupae (pharate adults); lane 7, 0-d-old adults; lane 8, 5- to 6-d-old adults. (*B*) To analyze the knock-down levels of transcripts of *TcLac1*, *TcLac2*, and *TcTyr*, total RNA was isolated from 5- to 6-d-old pupae (pharate adults) (6–7 d after injection) for *TcLac2* RNAi or from 0- to 1-d-old pupae (6–7 d after injection) for *TcLac1* and *TcTyr* (*TcTyr1*) RNAi. The RT-PCR analysis of *Tribolium* polyubiquitin transcripts with the same cDNA templates served as an internal control for normalization of equal sample loading.

A search of the genomic databases of *Drosophila melanogaster* and *A. gambiae* identified *TcLac2* orthologs in both species. These orthologs have, respectively, three and two sets of alternative exons encoded in C-terminal regions corresponding to the alternatively spliced region of *TcLac2* [see also Dittmer *et al.* (12)]. However, the sizes of the *Drosophila* and *Anopheles* alternative exons do not correspond well with those of *TcLac2*, and the arrangement of exons is more complex in the two dipteran species (data not shown).

The two tyrosinase genes of *Tribolium* identified by TBLASTN analysis of the *Tribolium* genome sequence assembly correspond to the two cDNA clones that we had amplified by RT-PCR. These genes encode proteins of 684 and 683 aa and have an amino acid sequence identity of 71% (Fig. 7 and Table 3). There are no alternative exons in these genes. The amino acid sequence identity of TcTyr1 and TcTyr2 to other insect tyrosinase-like proteins is ≈50%.

Expression of Laccase and Tyrosinase Transcripts During Development. To determine the proper timing of dsRNA injections for effective disruption of the target gene function, the developmental patterns of expression of the two tyrosinase genes and *TcLac1* and the two alternatively spliced transcripts of the laccase 2 gene, namely *TcLac2A* and *TcLac2B*, were determined by RT-PCR analysis of RNA prepared from prepupal, pupal, and adult stages (Fig. 2*A*). The highest levels of *TcLac2* transcripts were detected in prepupae and pharate adults just before the pupal and adult molts, respectively, whereas *TcLac1* transcript levels were the highest in young pupae and pharate adults and the lowest in the interval between these two stages. *TcLac2A* 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. 2*B*). The knockdown of both tyrosinase transcripts by dsRNA for *TcTyr1* may be attributed to short regions of high sequence similarity between these two genes. In contrast, injection of dsRNA for *TcLac1* reduced the levels of its transcripts substantially without affecting those of *TcLac2* (Fig. 2*B*). dsRNA for *TcLac2* was similarly selective in its action, and the levels of *TcLac1* transcripts were unaffected after injection of ds*TcLac2*. 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. 3*A*. 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. 3*A*). We did not observe any lethality from injection of ds*TcTyr*, ds*TcLac1*, 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 elytra 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 the larval–larval molt, animals treated with buffer showed normal cuticle tanning, whereas ds*Lac2*-treated larvae exhibited no cuticle tanning (Fig. 4*A*). The larvae became shorter in length and died before any subsequent molting. One-day-old pupae that were treated as larvae with ds*Lac2* also exhibited no pupal cuticle

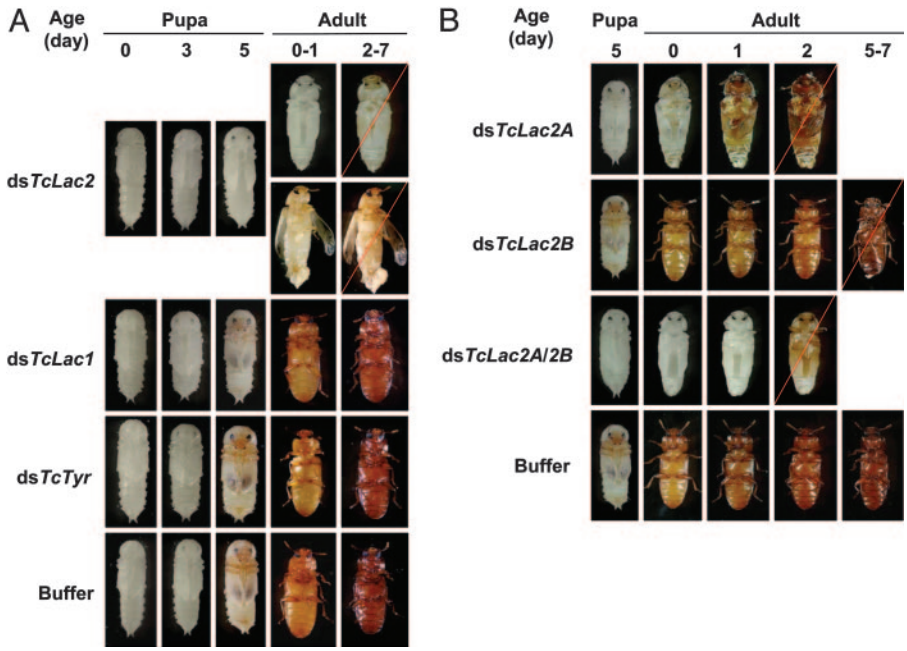

Fig. 3. The effect of dsRNAs for *TcLac2*, *TcLac1*, and *TcTyr* on pupal and adult development and cuticle tanning of *Tribolium*. (*A*) dsRNAs for *TcLac2*, *TcLac1*, or *TcTyr* (200 ng per insect) were injected into last-instar larvae or prepupae as indicated in Fig. 2 ($n = 40$, two replicates of 20 insects each). All ds*TcLac2*-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 ds*Lac1*, ds*TcTyr* (ds*TcTyr1*), 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*, *TcLac2B* (100 ng per insect), and *TcLac2A/2B* (mixture of ds*Lac2A* and ds*Lac2B*, 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.

tanning (Fig. 4*B*). No tanning was observed in pupa-specific cuticular structures, including setae, urogomphi, or gin traps. Gin traps were markedly distended, probably because the softness associated with the absence of sclerotization rendered them more sensitive to the swelling effect of internal hemolymph pressure (Fig. 4*C*). These results clearly demonstrated that *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 dsRNAs for the 3′ UTRs and extreme 3′ ends of C-terminal coding regions of the two alternatively spliced transcripts. The length of dsRNAs for *TcLac2A* and *TcLac2B* were 596 and 424 bp, respectively. The knockdown levels of the transcripts of *TcLac2A* and *TcLac2B* were examined by RT-PCR (Fig. 8*A*, which is published as supporting information on the PNAS web site). Injection of ds*Lac2A* reduced the *TcLac2A* transcript level without reducing that of *TcLac2B*. Conversely, the introduction of ds*Lac2B* reduced the *TcLac2B* transcript level without affecting that of *TcLac2A*. The resulting phenotypes are shown in Figs. 3*B* and 8*B*. Insects injected with dsRNA for *TcLac2A*, which is the major transcript of this gene, exhibited little tanning of the pupal and pharate adult cuticles. Injection of dsRNA for *TcLac2B*, which is the minor transcript, caused only a delay in adult cuticle tanning. Insects treated with a mixture of both dsRNAs showed phenotypes similar to those of insects injected with exon-nonspecific *TcLac2* dsRNA. Little or no cuticle tanning was observed during the pupal, pharate adult, or adult stages. All of the beetles treated with exon-specific dsRNAs, either alone or in combination, died within a week after adult eclosion. These results indicated that both of the laccase 2 isoforms, 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

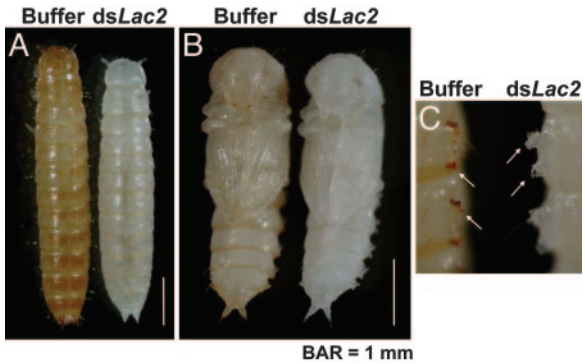

Fig. 4. The larval and pupal phenotypes produced by injection of dsRNA for Tc*Lac2*. dsRNA for *TcLac2* was injected into late larvae to observe the effect on larval and pupal cuticle tanning. (*A*) Last-instar, 1-d-old larvae, injected 3 d earlier with buffer or *dsLac2*. (*B*) One-day-old pupae injected 7 d earlier with buffer or *dsLac2*. No tanning was observed in the larval or pupal cuticle. (*C*) 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.

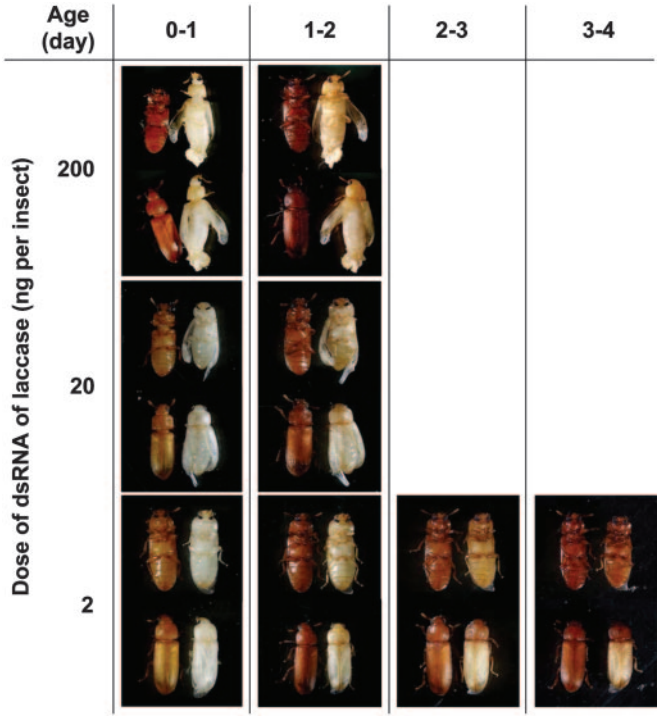

Fig. 5. Inhibition of cuticle tanning is correlated with the concentration of *TcLac2* dsRNA injected. Shown are phenotypes (ventral and dorsal views) produced by injection of 2–200 ng of dsRNA for *TcLac2* into prepupae. In each panel, the individual on the left is a control injected with buffer only and the one on the right was injected with *TcLac2* dsRNA. All of the insects ($n = 20$ per group) injected with 200 and 20 ng of dsRNA died at the last time point shown. The insects injected with 2 ng of dsRNA developed into slightly malformed adults with longevity and appearance more similar to control insects.

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, foregut, 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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1. Pyror, M. G. M. (1940) *Proc. R. Soc. London Ser. B* **128,** 393–407.
2. Hopkins, T. L. & Kramer, K. J. (1992) *Ann. Rev. Entomol.* **37,** 273–302.
3. Kramer, K. J., Kanost, M. R., Hopkins, T. L., Jiang, H., Zhu, Y. C., Xu, R., Kerwin, J. L. & Turecek, F. (2001) *Tetrahedron* **57,** 385–392.
4. Andersen, S. O. (2005) in *Comprehensive Molecular Insect Science*, eds. Gilbert, L. I., Iatrou, K. & Gill, S. (Elsevier, Oxford, U.K.), Vol. 4, pp. 145–170.
5. Locke, M. & Krishnan, N. (1971) *Tissue Cell* **3,** 103–126.
6. Ashida, M. & Brey, P. T. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 10698–10702.

7. Ashida, M. & Brey, P. T. (1997) in *Molecular Mechanisms of Immune Responses in Insects*, eds. Brey, P. T. & Hultmark, D. (Chapman & Hall, London), pp. 135–172.
8. Willis, J. H., Iconomidou, V. A., Smith, R. F. & Hamodrakas, S. J. (2005) in *Comprehensive Molecular Insect Science*, eds. Gilbert, L. I., Iatrou, K. & Gill, S. (Elsevier, Oxford, U.K.), Vol. 4, p. 79–110.
9. Ashida, M. & Yamazaki, H. I. (1990) in *Molting and Metamorphosis*, eds. Ohnishi, E. & Ishizaki, H. (Springer, Tokyo), p. 239–265.
10. Sugumaran, M. & Kanost, M. R. (1993) in *Parasites and Pathogens of Insects*, eds. Beckage, N. E., Thompson, S. N. & Frederici, B. A. (Academic, San Diego), Vol. 1, p. 317–342.
11. Marmaras, V. J., Charalambidis, N. D. & Zervas, G. C. (1996) *Arch. Insect Biochem. Physiol.* **31,** 119–133.
12. Dittmer, N. T., Suderman, R. J., Jiang, H., Zhu, Y. C., Gorman, M. J., Kramer, K. J. & Kanost, M. R. (2004) *Insect Biochem. Mol. Biol.* **34,** 29–41.
13. Tomoyasu, Y. & Denell, R. E. (2004) *Dev. Genes Evol.* **214,** 575–578.
14. Arakane, Y., Muthukrishnan, S., Kramer, K. J., Specht, C. A., Tomoyasu, Y., Lorenzen, M. D., Kanost, M. R. & Beeman, R. W. *Insect Mol. Biol.*, in press.
15. Haliscak, J. P. & Beeman, R. W. (1983) *J. Econ. Entomol.* **76,** 717–722.
16. Beeman, R. W. & Stuart, J. J. (1990) *J. Econ. Entomol.* **83,** 1745–1751.
17. Arakane, Y., Hogenkamp, D., Zhu, Y. C., Kramer, K. J., Specht, C. A., Beeman, R. W., Kanost, M. R. & Muthukrishnan, S. (2004) *Insect Biochem. Mol. Biol.* **34,** 291–304.
18. Lorenzen, M. D., Brown, S. J., Denell, R. E. & Beeman, R. W. (2002) *Insect Mol. Biol.* **11,** 399–407.
19. Thomas, J. D., Higgins, D. G. & Gibson, T. J. (1994) *Nucleic Acids Res.* **22,** 4673–4680.
20. Kawabata, T., Yasuhara, Y., Ochiai, M., Matuura, S. & Ashida, M. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 7774–7778.
21. Jiang, H., Wang, Y., Ma, C. & Kanost, M. R. (1997) *Insect Biochem. Mol. Biol.* **27,** 835–850.
22. Muller, H.-M., Dimopoulos, G., Blass, C. & Kafatos, F. C. (1999) *J. Biol. Chem.* **274,** 11727–11735.
23. Thomas, B. R., Yonekura, M., Morgan, T. D., Czapla, T. H., Hopkins, T. L. & Kramer, K. J. (1989) *Insect Biochem.* **19,** 611–622.
24. Kramer, K. J., Hopkins, T. L. & Schaefer, J. (1995) *Insect Biochem. Mol. Biol.* **25,** 1067–1080.
25. Suderman, R. J., Andersen, S. O., Hopkins, T. L., Kanost, M. R. & Kramer, K. J. (2003) *Insect. Biochem. Mol. Biol.* **33,** 331–343.
26. Hopkins, T. L., Morgan, T. D., Aso, Y. & Kramer, K. J. (1982) *Science* **217,** 364–366.
27. Kramer, K. J., Morgan, T. D., Hopkins, T. L., Christensen, A. M. & Schaefer, J. (1989) *Insect Biochem.* **19,** 753–757.
28. Kramer, K. J. & Muthukrishnan, S. (2005) in *Comprehensive Molecular Insect Science*, eds. Gilbert, L. I., Iatrou, K. & Gill, S. (Elsevier, Oxford, U.K.), Vol. 4, p. 111–144.
29. Schaefer, J., Kramer, K. J., Garbow, J. R., Jacob, G. S., Stejskal, E. O., Hopkins, T. L. & Speirs, R. D. (1987) *Science* **235,** 1200–1204.
30. Xu, R., Huang, X., Hopkins, T. L. & Kramer, K. J. (1997) *Insect Biochem. Mol. Biol.* **27,** 101–108.
31. Miessner, M., Peter, M. G. & Vincent, J. F. V. (2001) *Biomacromolecules* **2,** 369–372.