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*.

**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′-GGNACNAYTTYGGCA-3′ and 5′-CCRTGNAARTTGRAANGRTG-3′ for *TcLac1* and 5′-AAYYNTNACAYTG-GCAAYTG-3′ and 5′-CRRCTRAANGRWANCCTAT-3′ for *TcTyr1* and *TcTyr2*. The highly conserved amino acid sequences chosen for designing these primers for PCR were GTHFWH and HPHFHLHG for *TcLac2* and NLHHHW and MGYPFD 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 BtBase database (http://bioinformatics.ksu.edu/BtBase/index.html) identified another laccase gene, *TcLac1*. A partial cDNA of *TcLac1* was cloned from pupal cDNA by using the primers 5′-CCCTTGCGCAAGAAAATGTG-3′, 5′-AACCGAATTCGCTGTGAATG-3′, 5′-CCRTGNAARTTGRAANGRTG-3′, and 5′-CCRTGNAARTTGRAANGRTG-3′ for *TcLac2* 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...

**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...
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 (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′-CGCAGACGCCAAAATCAGAGG-3′. For TcLac2 RNAi, dsLac2 was injected into prepupae, and total RNA was isolated 5–6 d after injection (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 injection (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. NETMGLYC 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 laccase2 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 larval stage in Tribolium 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 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. 2B). 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 TcTyr1, 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. 3A). 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 elytra that were deformed and legs 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–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

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 homology 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. 2A). 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

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- to 3-d-old adults; lane 8, 3- 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 TcLac2A and TcLac2B 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.
TcLac2 plays a critical role in the sclerotization and pigmentation of larval and pupal cuticles and adult cuticle. We performed exon-specific RNAi by using dsRNAs for TcLac2, TcLac1, or TcTyr (200 ng per insect) to inject late-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, TcLac2B (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.

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. 8A, which is published as supporting information on the PNAS web site). Injection of dsLac2A reduced the TcLac2A transcript level without reducing that of TcLac2B. Conversely, the introduction of dsLac2B reduced the TcLac2B transcript level without affecting that of TcLac2A. The resulting phenotypes are shown in Figs. 3B and 8B. 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...
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.

### 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 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 new types of cross-linked biopolymers based on that chemistry 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).

We thank Drs. Sonny Ramaswamy, Stevin Gehrike, Peter Dunn, Neal Dittmer, and Maureen Gorman for helpful discussions and reviews of the manuscript and Sue Haas for beetle husbandry. This work was supported by National Science Foundation Grants MCB-0236039 and IBN-0316963. This work is a cooperative investigation between Kansas State University (Contribution 05-262-J from the Kansas Agricultural Experiment Station) and the United States Department of Agriculture.

### References