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Short communication

Chitin synthases are required for survival, fecundity and egg hatch in the red flour beetle, *Tribolium castaneum*

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ABSTRACT

The synthesis of chitin, the β-1,4-linked polymer of *N*-acetylglucosamine, is catalyzed by chitin synthase (CHS). Chitin is essential for the structural integrity of the exoskeletal cuticle and midgut peritrophic membrane (PM) of insects. To study the functions of the two chitin synthase genes, *TcCHS-A* and *TcCHS-B*, during embryonic and adult development in the red flour beetle, *Tribolium castaneum*, RNA interference (RNAi) experiments were carried out. When dsRNA for *TcCHS-A* was injected into male or female pharate adults, all insects died 5–7 d after the adult molt, and the females failed to oviposit prior to death. When ds*TcCHS-A* was injected into young adults 1–2 d post-eclosion, a similar lethal phenotype was obtained after 5 d and no oviposition occurred. When ds*TcCHS-A* injections were delayed until after adult maturation (7–10 d post-eclosion), the treated females did oviposit and the resulting embryos appeared to develop normally. However, the chitin content of the eggs was dramatically reduced, the embryos became twisted and enlarged, and the eggs did not hatch. Adults treated with dsRNA for *TcCHS-B* exhibited little or no chitin in their PM and died about 2 wk after injection. None of the *TcCHS-B*-treated females oviposited, which was probably a secondary effect caused by starvation. These results extend our previous findings that *CHS* genes are required for all types of molt. The present study also demonstrates that these genes have additional roles in embryonic and adult development.

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1. Introduction

In insects, chitin is a major component of the cuticle and tracheae as well as the peritrophic membrane (PM) that lines the midgut. This polysaccharide plays a critical role in strengthening exoskeletal structures and also in protecting insects from environmental stresses and pathogenic microbes (Kramer and Muthukrishnan, 2005). We previously cloned and characterized two chitin synthase (*CHS*) genes, *TcCHS-A* and *TcCHS-B*, from the red flour beetle, *Tribolium castaneum*. These genes were previously referred to as *CHS-1* and *CHS-2*, respectively (Arakane et al., 2004, 2005; Kramer and Muthukrishnan, 2005; Merzendorfer, 2006). *T. castaneum* is an excellent model for performing dsRNA-mediated RNA interference (RNAi) experiments at all developmental stages, including early embryonic stages via vertical transmission of the RNAi effect from the parental generation (Bucher et al., 2002; Tomoyasu and Denell, 2004). We used RNAi to demonstrate (1) that TcCHS-A is responsible for the synthesis of cuticular chitin and (2) that TcCHS-B specializes in the production of PM-associated chitin (Arakane et al., 2004, 2005). Injection of dsRNA for *TcCHS-A* disrupted all types of molts, larval–larval, larval–pupal and pupal–adult, while ds*TcCHS-B* RNAi suppressed chitin synthesis in the PM. However, possible functions of the *TcCHS* genes during embryonic development and after adult eclosion were not investigated. In this study, we performed parental RNAi of the two *CHS* genes in *T. castaneum* and observed phenotypic defects in their progeny as well as decreased adult survival. Interestingly, our results indicate that not only TcCHS-B but also TcCHS-A have critical functions after completion of the adult molt.

2. Materials and methods

The GA-1 strain of *T. castaneum* was maintained under standard conditions (Haliscak and Beeman, 1983) for use in this study. Under these conditions, the pupal stage lasts for 5–6 d. dsRNAs for *TcCHS-A* (ds*TcCHS-A*) and *TcCHS-B* (ds*TcCHS-B*) were synthesized and

Abbreviations: CHS, chitin synthase; PM, peritrophic membrane; Tc, *Tribolium castaneum*; RNAi, RNA interference; PCR, polymerase chain reaction; FITC, fluorescein isothiocyanate; CBD, chitin-binding domain.

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injected into female and male pharate adults as previously described (Arakane et al., 2005). For parental RNAi, ds*TcCHS-A* and ds*TcCHS-B* were injected into young (1–2 d old) and mature (2–3 wk old) adult females. For *TcCHS-A* we used the splice-variant-nonspecific dsRNA as described (Arakane et al., 2005). One day after injection, females were mated with an equal number of untreated males and eggs were collected every 4 d.

Total RNA extraction, first-strand cDNA synthesis and PCRs were carried out as described by Arakane et al. (2005). Real-time PCR was performed using SYBR Premix Ex Taq (Takara) and the Mx3000PTM Real-Time PCR System (Stratagene). The threshold cycle number (Ct) was determined and used for comparative quantitative analyses. The following pairs of primers were used: 5′-GGTGGTCGATCC GGATTATTACGA-3′ and 5′-GTTGACTTGCGTTTCGTCGTCGTT-3′ for *TcCHS-A*, 5′-TGCAGTGGTTTCGAAAGGGACAAC-3′ and 5′-AATGAGG AGCCAGATCCATTGCAC-3′ for *TcCHS-B*, and 5′-ACGCAAGTCAGT TAGAGGGTGCAT-3′ and 5′-TCCTGTTCGCCTTTACGCACGATA-3′ for *TcrpS6* that served as an internal control to normalize differences in recoveries between samples. Chitin content was measured using a modified Morgan–Elson method as described previously (Arakane et al., 2005).

3. Results and discussion

To date, only two chitin synthase genes, *CHS-A* (encoding a class-A CHS) and *CHS-B* (encoding a class-B CHS), have been found by BLAST searches of the fully sequenced genomes of several insect species, including *Drosophila melanogaster*, *Anopheles gambiae*, *Aedes aegypti*, *Nasonia vitripennis*, *Apis mellifera* and *T. castaneum* (Kramer and Muthukrishnan, 2005; Merzendorfer, 2006; unpublished data). We have cloned and characterized the two *CHS* genes from *T. castaneum*, *TcCHS-A* and *TcCHS-B* (Arakane et al., 2004). Injection of dsRNAs for *TcCHS-A* disrupted the larval–larval, larval–pupal and pupal–adult transformations, whereas administration of dsRNA for *TcCHS-B* into larvae significantly reduced the chitin content of the larval midgut, suggesting that TcCHS-A contributes to the synthesis of chitin in the exoskeleton and TcCHS-B is utilized for synthesis of PM-associated chitin in the midgut (Arakane et al., 2005). These studies, however, did not investigate whether *CHS* genes have any role(s) during embryonic development or in adults.

We previously reported that *TcCHS-A* transcripts (two splice variants) are expressed in embryos, larvae and pupae of *T. castaneum*, whereas *TcCHS-B* transcripts are found in larval stages only, but not in embryonic and pupal stages (Arakane et al., 2005). To assess whether *TcCHS-A* and *TcCHS-B* genes are expressed during early adult development, we quantified transcript levels for these genes by real-time PCR (Fig. 1). Indeed, the transcript levels of *TcCHS-A* were substantially increased after molting to the adult (0–1 d old), but the levels then declined after 1 wk. Levels of *TcCHS-B* transcripts, on the other hand, increased dramatically 1 wk after the adult molt, coincident with the period of active feeding when PM synthesis is expected to occur.

To determine whether *TcCHS-A* transcription is essential for early adult development, we injected dsRNAs for *TcCHS-A* into male and female pharate adults (5 d-old pupae). These insects molted to adults the day after injection, but the adults died prematurely 5–7 d after eclosion. The dsRNA-treated females failed to oviposit. Dissection of these animals indicated the absence of eggs in the ovaries (data not shown). Anal leakage of fluid was observed in the moribund adults, which resulted in the formation of an anal plug of dietary flour paste (Fig. 2A). A similar lethal phenotype and no oviposition also were observed after ds*TcCHS-A* was injected into 1–2 d-old female adults, suggesting that chitin deposition is still required after the final molt to fully develop the cuticle, tracheae and/or internal organs such as the linings of foregut and hindgut. Negative control insects treated as pharate adults with dsRNA for *T. castaneum vermilion* (*Tcver*) had normal longevity in both sexes, and females oviposited normally.

When we postponed ds*TcCHS-A* injection into females until after adult maturation (2–3 wk post-eclosion), there was no adult lethality and oviposition was normal. The resulting embryos appeared to complete their development, but they exhibited a twisted and enlarged phenotype, and the eggs did not hatch, presumably as a consequence of an abnormally soft, chitin-deficient cuticle (Fig. 3A). dsRNA for *T. castaneum vermilion* (*Tcver*) was injected as a positive/negative control (Arakane et al., 2005). Embryos from ds*Tcver*-treated control adult females lacked eye pigmentation (positive control for dsRNA-mediated parental RNAi) but otherwise were normal in appearance and in their hatching ability (negative control for parental RNAi) (Fig. 3A). RT-PCR demonstrated that both alternatively spliced transcripts of *TcCHS-A*, namely *TcCHS-A-8a* and *TcCHS-A-8b* (Arakane et al., 2004, 2005), were significantly down regulated in the embryos from ds*TcCHS-A*-treated females (Fig. 3B). Furthermore, the total chitin content of those eggs was dramatically reduced when compared to that of control (ds*Tcver*) embryos (Table 1). The failure of the eggs to hatch may reflect a lack of mechanical strength of the newly synthesized embryonic cuticle. Moreira et al. (2007), however, identified a chitin-like component in *A. aegypti* eggshells. When *T. castaneum* eggs were stained with FITC (fluorescein isothiocyanate)-conjugated chitin-binding protein (Arakane et al., 2005), fluorescence was observed in wild-type eggshells but not in those from ds*TcCHS-A*-treated females (unpublished data). Thus, TcCHS-A apparently contributes not only to synthesis of chitin in embryonic cuticle but also to chitin synthesis in the eggshell. The effect of parental ds*TcCHS-A* treatment on the resulting embryos is reminiscent of the embryonic phenotype in homozygotes of the *D. melanogaster* chitin synthase-1 mutant (*CS-1*/*kkv*, class-A). Such embryos fail to hatch

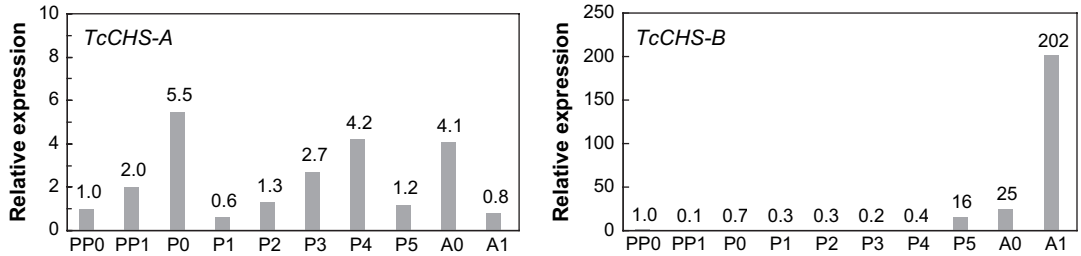

Fig. 1. Expression profiles of *TcCHS-A* and *TcCHS-B* genes during development of *T. castaneum*. Total RNAs extracted from whole insects ($n = 5$) at the pharate pupal through young adult stages were treated with RNase-free DNase I. The concentration of each total RNA was measured by UV absorbance (NanoDrop, Thermo Scientific) and then 500 ng of each RNA sample was used to synthesize first-strand cDNA. PP0, 0–1 d-old pharate pupae; PP1, 1–2 d-old pharate pupae; P0, 0 d-old pupae; P1, 1 d-old pupae; P2, 2 d-old pupae; P3, 3 d-old pupae; P4, 4 d-old pupae; P5, 5 d-old pupae; A0, 0 d-old adults; A1, 1 wk-old adults. Real-time PCR of *Tribolium* ribosomal protein S6 (*rpS6*) transcripts with the same cDNA template served as an internal control to normalize differences in template levels between samples. Expression levels for *TcCHS-A* and *TcCHS-B* relative to the levels of expression in pharate pupae (PP0) are shown.

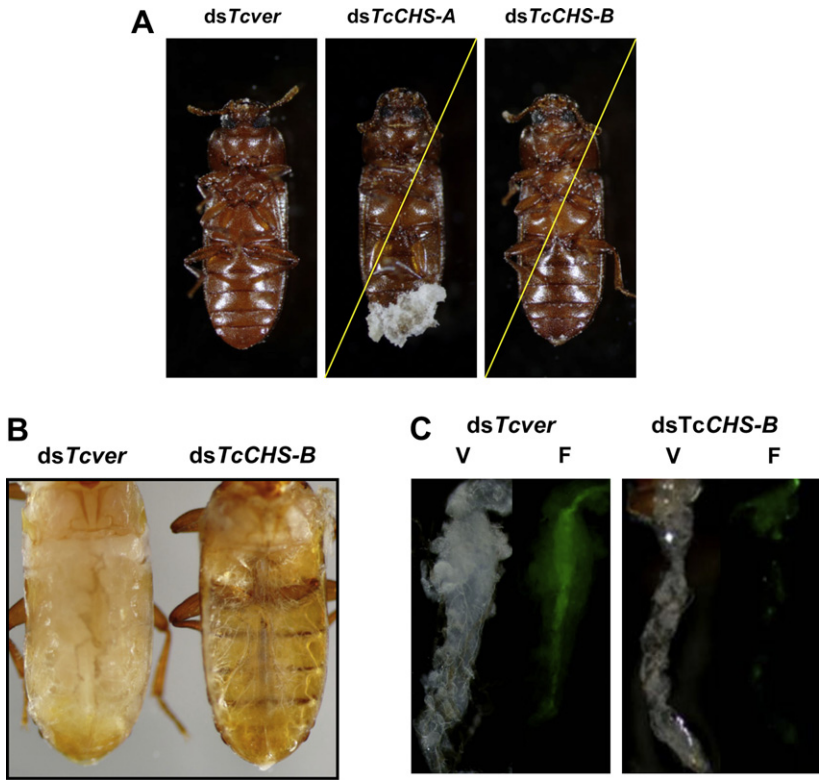

Fig. 2. Terminal phenotypes produced by injection of dsRNAs for *TcCHS-A* and *TcCHS-B* into pharate adults of *T. castaneum*. (A) dsRNAs (200 ng per insect) for *TcCHS-A* or *TcCHS-B* were injected into pharate adults (4–5 d after molt to pupae, $n = 40$). The resulting adults from the ds*TcCHS-A* treatment died 5–7 d after the adult molt. Anal leakage of fluid occurred in these insects, resulting in a plug of flour paste attached to the lower abdomen. (B) dsRNA (200 ng per insect) for *TcCHS-B* was injected into mature adults (2–3 wk old). In contrast to ds*Tcver*-treated control adults, little or no fat body was observed in ds*TcCHS-B* treated adults 5–7 d post-injection. In the latter, depletion of (opaque) fat body revealed the abdominal ventral plates and tracheal system, which were visible through the clear abdominal dorsal cuticle. (C) Midguts from ds*TcCHT-B*- and ds*Tcver*-treated adults (5–7 d old) were dissected and PM-associated chitin was stained with FITC-conjugated chitin-binding protein (FITC-CBD) (Arakane et al., 2005). Midguts from ds*TcCHS-B*-treated insects exhibited little or no FITC fluorescence compared to that of ds*Tcver*-treated control insects. V: visible light, F: fluorescent light. The yellow slashes indicate moribund insects. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and have been described as blimp-shaped with defects in the head skeleton (Ostrowski et al., 2002). They had defects both in the chitinous procuticle (endocuticle and exocuticle) and also in the chitin-deficient epicuticle as well as defects in epidermal cell morphology. These observations support the hypothesis that chitin is critical for cuticle assembly, a process regulated in *Drosophila* by the apical plasma membrane-associated chitin synthase, knickkopf and retroactive proteins (Moussian et al., 2005, 2007). Future studies will be needed to show how knockdown of *TcCHS-A* affects the morphology of functional layers of *Tribolium* embryonic cuticle.

Injection of ds*TcCHS-B* into male and female pharate adults (4–5 d after pupation) had no effect on the pupal–adult molt, but it had severe effects on reproduction, and all of the resulting adults died prematurely at approximately 2 wk post-injection with no

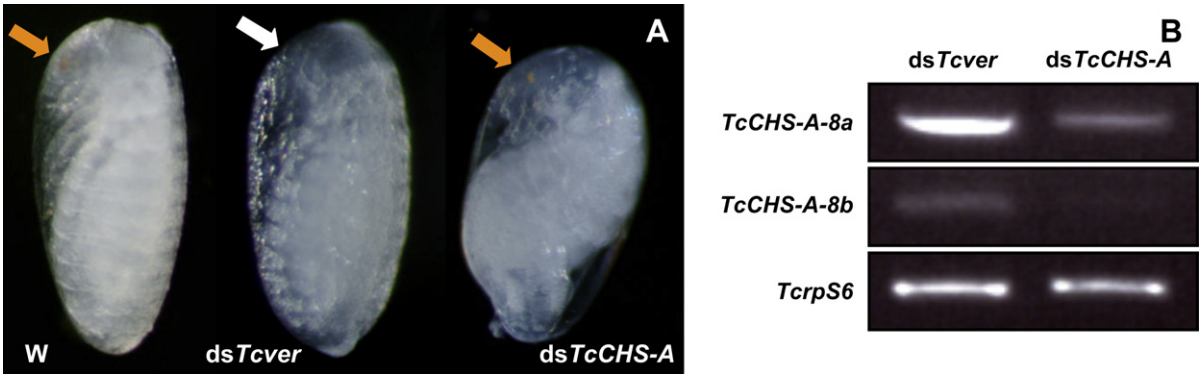

Fig. 3. Effect of injection of dsRNA for *TcCHS-A* into adult females of *T. castaneum* on egg hatch. (A) dsRNA for *TcCHS-A* (200 ng per insect) was injected into 2–3 wk-old adult females ($n = 20$), followed by mating with an equal number of untreated males. dsRNA for *Tribolium vermilion* (ds*Tcver*) was injected as a control. The eye color and morphology of developing embryos were monitored. Orange arrows point to normally pigmented eyespots of embryos from wild-type and ds*TcCHS-A*-treated females. Embryos from ds*Tcver*-treated animals lack eye pigmentation (white arrow). Embryos from the ds*TcCHS-A* treatment had a twisted and enlarged blimp-like phenotype and died without hatching. W: wild-type embryo. (B) RT-PCR showed that, relative to animals injected with ds*Tcver*, both alternatively spliced forms of *TcCHS-A* (Arakane et al., 2004) were significantly down regulated by ds*TcCHS-A* injection. The ds*TcCHS-A* sequence includes a region common to both alternatively spliced forms. Transcript for *Tribolium* ribosomal protein 6 (*TcrpS6*) was used as an internal loading control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Effect of *TcCHS-A* parental RNAi on chitin content of *Tribolium* eggs[a]

	Number of eggs	Mean (nmol GlcNAc/mg sample) ± SD
ds*TcCHS-A*	153	0.78 ± 1.28
	210	1.81 ± 1.81
	165	0.50 ± 0.87
ds*Tcver*	158	35.69 ± 0.20
	153	35.28 ± 4.26
	195	42.54 ± 0.62

[a] Eggs from three batches of 20 females treated with dsRNA for *TcCHS-A* were collected as described in Fig. 3. The chitin content of 4 d-old eggs was analyzed using a modified Morgan–Elson method (Arakane et al., 2005). dsRNA for *Tribolium vermilion* (ds*Tcver*) was injected as a control. GlcNAc = *N*-acetylglucosamine.

oviposition by the females (Fig. 2A). In contrast to the results obtained after injection of ds*TcCHS-A*, lack of oviposition and high adult lethality were observed approximately 2 wk after injection of ds*TcCHS-B* into mature adults. We suspect that these results are an indirect effect of starvation due to the lack of a functional PM, leading to a disruption of digestion and a shut-down of physiological investment in nonessential functions such as reproduction. To assess the presence or absence of chitin in the midgut in these insects, the PM-associated chitin was stained with FITC-CBD. Midguts from ds*Tcver*-treated control insects had high FITC fluorescence, whereas midguts from adults treated with ds*TcCHS-B* exhibited little or no fluorescence, demonstrating that there were substantially lower levels of chitin in the PMs after ds*TcCHS-B* treatment (Fig. 2C). The PM is critical for digestion of food, recycling/circulation of gut enzymes, and protection of gut tissues from environmental hazards (Terra, 2001; Bolognesi et al., 2001). The high incidence of adult lethality observed after injection of ds*TcCHS-B* appears to be the result of interruption of digestion and resulting starvation. Indeed, ds*TcCHS-B*-treated adults exhibited little or no fat body 5–10 d after injection (Fig. 2B). A similar phenotype was observed in untreated adults that had been starved for 5–7 d (data not shown), suggesting that in both cases the beetles were not absorbing nutrients from the gut and that fat body was being mobilized as an emergency nutrient source.

Our previous studies had showed that down-regulation of transcripts for *TcCHS-A* affected every molt (larval–larval, larval–pupal and pupal–adult) and cuticular chitin synthesis, while *TcCHS-B* down-regulation affected only the larval–larval molt and PM-associated chitin synthesis (Arakane et al., 2005). The present study has revealed additional roles for TcCHS-A and TcCHS-B in early adult development as well as in maintaining fecundity. Using parental RNAi, we have demonstrated that down-regulation of *TcCHS-A* transcripts in *Tribolium* during the early adult stage leads to detrimental effects on male and female adult survival and female fecundity. Results obtained from *TcCHS-A* RNAi using mature females revealed in the progeny embryos exhibiting a twisted and elongated embryonic blimp-like phenotype. Chitin deficiency apparently leads to a weakened but more flexible cuticle and failure to hatch. Adults treated with ds*TcCHS-B* starve to death, apparently because of a chitin deficiency in the PM. TcCHS-B, therefore, is required for adult PM synthesis and assimilation of food, and secondarily for maintaining fecundity.

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