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Correspondence and requests for materials should be addressed to K.Y.Z. (kzhu@ksu.edu)

Cholinergic and non-cholinergic functions of two acetylcholinesterase genes revealed by gene-silencing in *Tribolium castaneum*

Yanhui Lu[1,2], Yoonseong Park[1], Xiwu Gao[2], Xin Zhang[1], Jianxiu Yao[1], Yuan-Ping Pang[3], Haobo Jiang[4] & Kun Yan Zhu[1]

[1]Department of Entomology, 123 Waters Hall, Kansas State University, Manhattan, KS 66506-4004, USA, [2]Department of Entomology, China Agricultural University, Beijing 100193, China, [3]Computer-Aided Molecular Design Laboratory, Mayo Clinic, Rochester, Minnesota, 55905, USA, [4]Department of Entomology and Plant Pathology, Oklahoma State University, Stillwater, OK 74078, USA.

We compared biological functions of two acetylcholinesterase genes (*TcAce1* and *TcAce2*) in *Tribolium castaneum*, a globally distributed major pest of stored grain products and an emerging model organism, by using RNA interference. Although both genes expressed at all developmental stages and mainly in the brain, the transcript level of *TcAce1* was 1.2- to 8.7-fold higher than that of *TcAce2*, depending on developmental stages. Silencing *TcAce1* in 20-day larvae led to 100% mortality within two weeks after eclosion and increased larval susceptibilities to anticholinesterase insecticides. In contrast, silencing *TcAce2* did not show insect mortality and significantly affect insecticide susceptibility, but delayed insect development and reduced female egg-laying and egg hatching. These results demonstrate for the first time that *TcAce1* plays a major role in cholinergic functions and is the target of anticholinesterase insecticides, whereas *TcAce2* plays an important, non-cholinergic role in female reproduction, embryo development, and growth of offspring.

Acetylcholinesterase (AChE, EC 3.1.1.7) is an important enzyme that terminates the neurotransmission by rapidly hydrolyzing the neurotransmitter acetylcholine at cholinergic synapses in all animals[1]. It is also involved in many cellular processes in eukaryotes, including apoptosis, modulation of cellular interactions, cell adhesion and synaptogenesis in vertebrates[2–5]. In insects, AChE has been extensively studied because it functions in neurotransmission, serves as a major target for anticholinesterase insecticides (organophosphates and carbamates), and constitutes a common mechanism of insecticide resistance through its reduced sensitivity to the insecticides[6–8].

AChE has long been known to be encoded by a single gene (*Ace*) in vertebrates[1]. In insects, the first *Ace* was cloned from the fruit fly (*Drosophila melanogaster*) in 1986[9] and subsequently other insects were thought to possess a single *Ace*. However, many studies identified insecticide resistance associated with reduced sensitivity of AChE to anticholinesterase insecticides, but were unable to identify any amino acid substitutions of deduced AChE sequences from the resistant strains[6]. This conflict was resolved by the first report of an *Ace* gene in the greenbug (*Schizaphis graminum*) that is paralogous to that of *D. melanogaster* in 2002[10] and subsequent reports of paralogous *Ace* in the cotton aphid (*Aphis gossypii*)[11] and the African malaria mosquito (*Anopheles gambiae*)[12].

It is now known that most insect species have two AChEs[8,14] except for the species in the suborder Cyclorrapha of Diptera including *D. melanogaster* as confirmed by its genome sequence[13], the house fly (*Musca domestica*), and the Australian sheep fly (*Lucilia cuprina*). One AChE (AChE1 or AP-AChE) is encoded by a paralogous *Ace* (*Ace1*) and the other AChE (AChE2 or AO-AChE) is encoded by an orthologous *Ace* (*Ace2*)[8,14]. It is also known that the amino acid substitutions conferring insecticide resistance are associated with AChE1 in the species possessing both *Ace1* and *Ace2* and with AChE2 in the Cyclorrapha species such as *Drosophila* that have a single AChE gene (i.e., *Ace2*)[8].

To date, cDNAs encoding AChEs have been isolated from at least 43 insect species. Of these species, 27 have been reported for both *Ace1* and *Ace2*[15]. However, the functions of the two gene products (AChE1 and AChE2) have hitherto been unclear. There has been scant information on which of the two AChEs is responsible for

cholinergic neurotransmission and therefore a target of anticholinesterase insecticides. We have recently studied both AChE1 and AChE2 genes in the red flour beetle (*Tribolium castaneum*), a globally distributed major pest of stored grain products and an emerging model organism[16] and found that both of the deduced AChE sequences contain all the conserved sequence motifs including a choline-binding site, a catalytic triad, and an acyl pocket[15]. However, the *T. castaneum* AChE1 gene (*TcAce1*) resides on chromosome 5, whereas the *T. castaneum* AChE2 gene (*TcAce2*) is on chromosome 2. Our large-scale protein simulation studies suggest that *T. castaneum* AChE1 is a robust acetylcholine hydrolase, whereas AChE2 is not a catalytically efficient acetylcholine hydrolase[15].

In this context, we compared transcript abundances of *TcAce1* and *TcAce2* and investigated their biological functions using RNA interference (RNAi) in *T. castaneum*. The results of these studies have provided for the first time crucial evidence with regard to which of the two AChEs is responsible for cholinergic neurotransmission and therefore a target of anticholinesterase insecticides and which of the two is responsible for non-cholinergic functions.

Results

Comparison of mRNA levels of *TcAce1* and *TcAce2*. We determined the abundances of *TcAce1* and *TcAce2* transcripts of *T. castaneum* at different developmental stages and the abundances in the brains dissected from late pupae by using quantitative PCR (qPCR) (Table 1). The transcript levels of *TcAce1* in 3-day eggs, 5 and 20-day larvae, 6-day pupae, 2-day adults and late pupal brains were about 8.7-, 5.4-, 1.2-, 3.8-, 3.0-, and 5.2-folds higher than those of *TcAce2*, respectively ($P \leq 0.05$). Apparently, *TcAce1* mRNA was more abundant than *TcAce2* mRNA at all the stages and in the pupal brains. Because the transcript levels of *TcAce1* and *TcAce2* were nearly identical in 20-day larvae, we chose this stage for our RNAi experiments to evaluate the silence specificity and to explore functional differences of the two genes.

Double-stranded RNA-mediated depletion of *TcAce* transcripts and effects on AChE activity. To confirm target specificity of RNAi, we prepared double-stranded RNA (dsRNA) from each gene and injected 20-day larvae with an individual dsRNA (ds*TcAce1* or ds*TcAce2*) or their mixed dsRNAs (ds*TcAce1+2*) at the same amounts. qPCR analyses using primer pairs that did not overlap with the dsRNA regions showed that both ds*TcAce1* and ds*TcAce2* dramatically reduced their respective transcript levels without significantly affecting the non-target mRNA levels on day 4 (Fig. 1A). Specifically, the injections of ds*TcAce1* and ds*TcAce2* suppressed *TcAce1* and *TcAce2* transcripts by 92.3 and 95.2%, respectively, as compared with the control larvae injected with the buffer only (see our rationale in the method section). No significant impact on the transcript levels of the non-target gene was observed. Specifically, the injection of ds*TcAce1* did not significantly reduce the transcript level of *TcAce2*, whereas the injection of ds*TcAce2* did not significantly reduce the transcript level of *TcAce1* in the larvae. As expected, the injection of ds*TcAce1+2* suppressed both *TcAce1* and *TcAce2* transcripts to a similar extent.

Table 1 | Transcript copy numbers of *TcAce1* and *TcAce2* in each egg and insect of different developmental stages and in each brain of late pupa of *T. castaneum**

	TcAce1		*TcAce2*		Ratio
Samples	Mean	±SE	Mean	±SE	*TcAce1/TcAce2*
3-Day egg	77.3	17.0	8.9	2.5	8.7
5-Day larva	263.4	82.2	48.9	13.2	5.4
20-Day larva	8964.0	664.0	7296.4	3895.9	1.2
6-Day pupa	12778.6	2840.6	3377.8	446.5	3.8
2-Day adult	6373.4	2029.8	2153.0	356.8	3.0
Brain	4808.5	2443.9	931.1	657.2	5.2

*The absolute mRNA level of each of *TcAce* genes was determined based on a standard curve constructed from a cloned plasmid DNA containing a *TcAce* fragment. The standard curves for *TcAce1* and *TcAce2* were Y=−2.981X+10.934 and Y=−2.976X+9.974, respectively, where Y represents Ct value and X represents Log [plasmid DNA (ng)]. PCR efficiencies were 116.5% for *TcAce1* and 116.8% for *TcAce2*. The transcript abundance of each *TcAce* gene was converted to the transcript copy number using the equation as previously described[42].

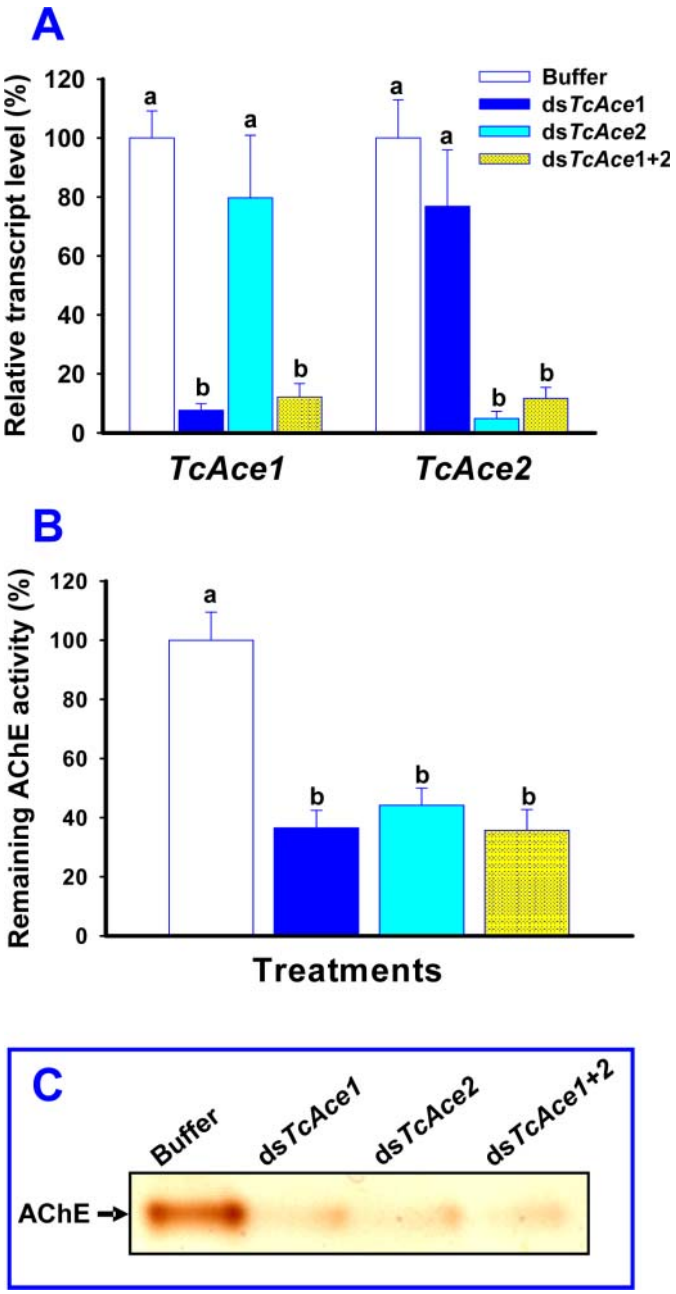

Figure 1 | The ds*TcAce1*, ds*TcAce2* and ds*TcAce1+2* mediated suppressions of *TcAce1* and *TcAce2* transcripts as determined by qPCR (A) and AChE activity as determined by enzyme assays (B) and non-denaturing polyacrylamide gel electrophoresis (PAGE) (C) on day 4 after 20-day larvae of *T. castaneum* were injected with buffer (control), ds*TcAce1*, ds*TcAce2*, or ds*TcAce1+2*. The results are presented as the mean and standard errors of three replicates (each was performed with a RNA sample prepared from four insects). Different letters above the standard error bars indicate significant differences based on the one-way ANOVA followed by Fisher's LSD multiple comparison test ($P \leq 0.05$). For PAGE analysis, 55 μg of total protein were loaded in each well. The gels were stained for AChE activity by using ATC as substrate.

We further evaluated the effect of RNAi on the enzyme levels on day 4 by measuring total AChE activity (Fig. 1B) and performing non-denaturing polyacrylamide gel electrophoresis followed by staining for the enzyme activity (Fig. 1C). Both methods demonstrated significant reductions in the enzyme levels. However, because *TcAce1* and *TcAce2* encode two different AChEs with similar predicted molecular weights[15], we were not able to separate the contribution of RNAi for each gene to the reduced AChE activities. Nevertheless, our study clearly showed that injections of ds*TcAce1*, ds*TcAce2*, or ds*TcAce1+2* dramatically decreased the levels of respective transcripts which, in turn, led to the reduced enzyme levels four days after the treatments.

Effects of RNAi of *TcAce1* and *TcAce2* on pupation and emergence. The injections of ds*TcAce1* and ds*TcAce2* to 20-day larvae significantly delayed the pupation and emergence of *T. castaneum*. For the larvae injected with buffer alone (control), 100% pupation were observed nine days after the injection (Fig. 2A). In contrast, only 87.6, 72.2, and 65.0% of pupation rates were observed when larvae were injected with ds*TcAce1*, ds*TcAce2*, and ds*TcAce1+2*, respectively. For the adult eclosion, 100% eclosion was achieved 16 days after 20-day larvae were injected with buffer alone (Fig. 2B). In comparison, only 81.2, 71.4 and 8.0% of the normal eclosion rates were observed when the larvae were injected with ds*TcAce1*, ds*TcAce2* and ds*TcAce1+2*, respectively. There was an enhanced effect on the adult eclosion when both *TcAce1* and *TcAce2* were simultaneously silenced.

The injection of either ds*TcAce1* alone or ds*TcAce1+2* in 20-day larvae resulted in 100% mortality within two weeks after adult eclosion or 19 days after the injection (Fig. 2C). Many pupae treated with ds*TcAce1+2* in the larval stage died even before adult emergence. In contrast, the injection of buffer (control) or ds*TcAce2* alone did not lead to any significant mortality even though the remaining *TcAce2* transcript level was only 4.8% of the control (Fig. 1). In addition, complete adult mortality was observed 27 days after the injection of ds*TcAce1+2*. This was four days earlier than the adults whose larvae were injected with ds*TcAce1* alone (Fig. 2C).

Effects of RNAi of *TcAce2* on egg-laying, egg hatching, and offspring development. Since the injection of ds*TcAce2* in 20-day larvae had a negligible effect on insect survival in the same generation, we further investigated: 1) whether the injection of ds*TcAce2* in 20-day larvae would affect reproduction of adult females, 2) whether the RNAi effect would be carried over to the next generation, and 3) which gender does that if so (Fig. 3A). Although the injection of ds*TcAce2* in 20-day larvae did not lead to the mortality of the insects, we observed dramatic decreases in the egg-laying (Fig. 3B), egg hatching (Fig. 3C), and larval body weight of the following generation (Fig. 3D) when a female was from the larva injected with ds*TcAce2*. In contrast, such effects were not associated with any injections of ds*TcAce2* in the males. Thus, our observed effects of RNAi for *TcAce2* were clearly carried through the female rather than the male. As consequences of these RNAi effects, we observed relatively lower numbers of pupae and adults in the offspring when a female was from a larva injected with ds*TcAce2* than those when a female was from a larva injected with buffer alone (Fig. 3E).

Effects of RNAi of *TcAce1* and *TcAce2* on insect susceptibility to insecticides. To assess whether the reductions of *TcAce1* and *TcAce2* transcript levels and total AChE activity by RNAi can lead to a change of insect response to anticholinesterase insecticides, we injected 20-day larvae with ds*TcAce1*, ds*TcAce2*, or ds*TcAce1+2* and performed the bioassay with each of four anticholinesterase insecticides including two organophosphates (dichlorvos and malathion) and two cabamates (carbaryl and carbofuran) on day 4 after the injection (Fig. 4). Although the accumulative larval

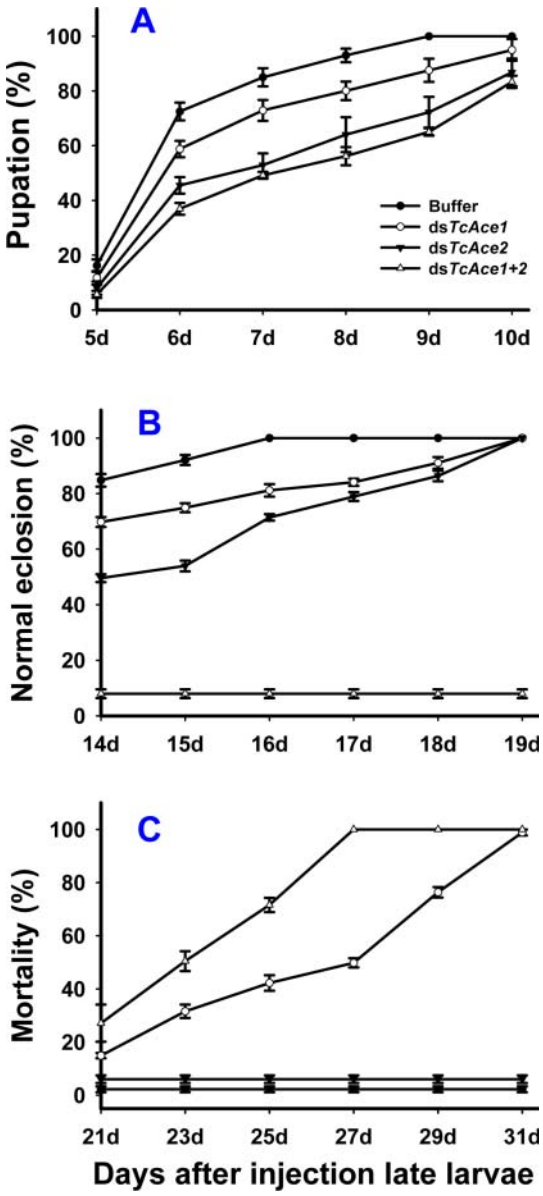

Figure 2 | The effect of gene-silencing mediated by RNAi on the pupation (A), eclosion (B), and mortality (C) after 20-day larvae of *T. castaneum* were injected with buffer (control), ds*TcAce1*, ds*TcAce2*, or ds*TcAce1+2*. The results are presented as the mean and standard errors of three replicates (each replicate was performed with at least 30 insects).

mortalities increased in all the insects injected with buffer, ds*TcAce1*, ds*TcAce2*, and ds*TcAce1+2* from 24 to 72 h due to insecticide exposures, significant increased mortalities, as compared with the controls, were observed in larvae injected with ds*TcAce1* and ds*TcAce1+2*. Generally, we did not find significantly increased mortalities in the larvae injected with ds*TcAce2* alone as compared with the larvae injected with the buffer when all the larvae were later treated with each of the four insecticides. Slightly increased mortalities in the ds*TcAce2*-injected larvae were only found at 72 h for carbofuran, and at 24 and 72 h for dichlorvos (Fig. 4).

Discussion

The canonical biological function of AChE is to terminate impulse transmission at cholinergic synapses by rapidly hydrolyzing the neurotransmitter acetylcholine in animals[1]. However, the discovery of a paralogous gene (*Ace1*) in many insect species prompted us to determine which of the two AChE genes is responsible for the cholinergic

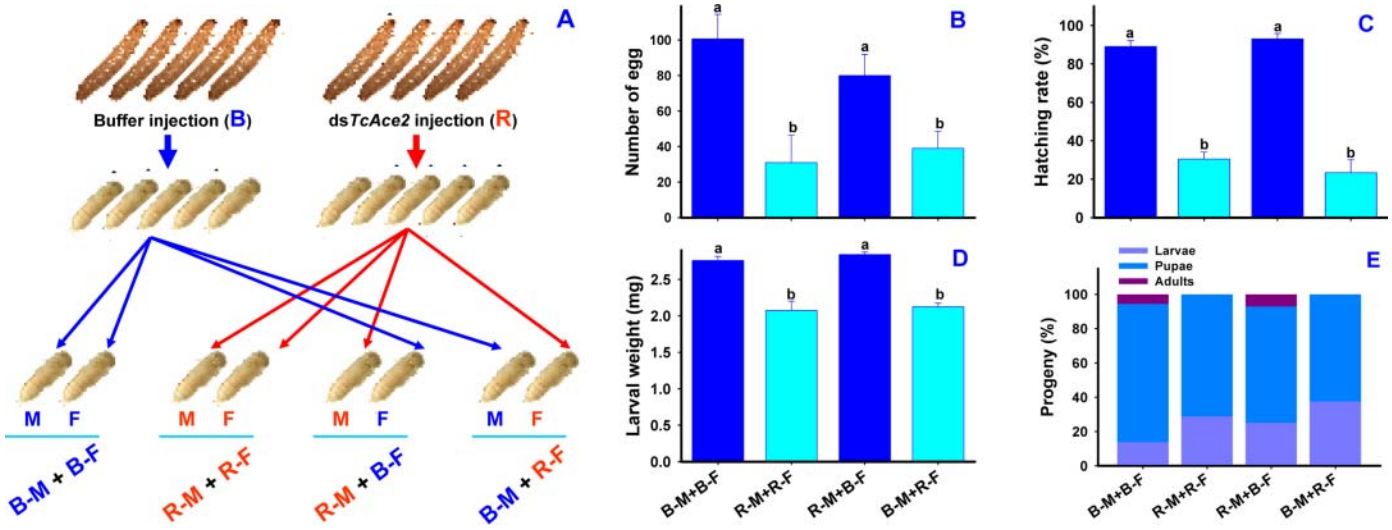

Figure 3 | The gender-dependent effect of RNAi for *TcAce2* (A) on female egg laying (B) and egg hatching (C), and larval body weight (D) and distribution of different stages of the offspring (D) after 20-day larvae of *T. castaneum* were injected with ds*TcAce2*. B-M+B-F: a male injected with buffer mating with a female injected with buffer; R-M+R-F: a male injected with ds*TcAce2* mating with a female injected with ds*TcAce2*; R-M+B-F: a male injected with ds*TcAce2* mating with a female injected with buffer; B-M+R-F: a male injected with buffer mating with female injected with ds*TcAce2*. An aliquot of 400 ng of ds*TcAce2* was used in injection in all the treatment. The results are presented as the mean and standard errors of three replicates (each was performed with eight pairs of the female and male). Different letters above the standard error bars indicate significant differences based on the one-way ANOVA followed by Fisher's LSD multiple comparison test ($P \leq 0.05$).

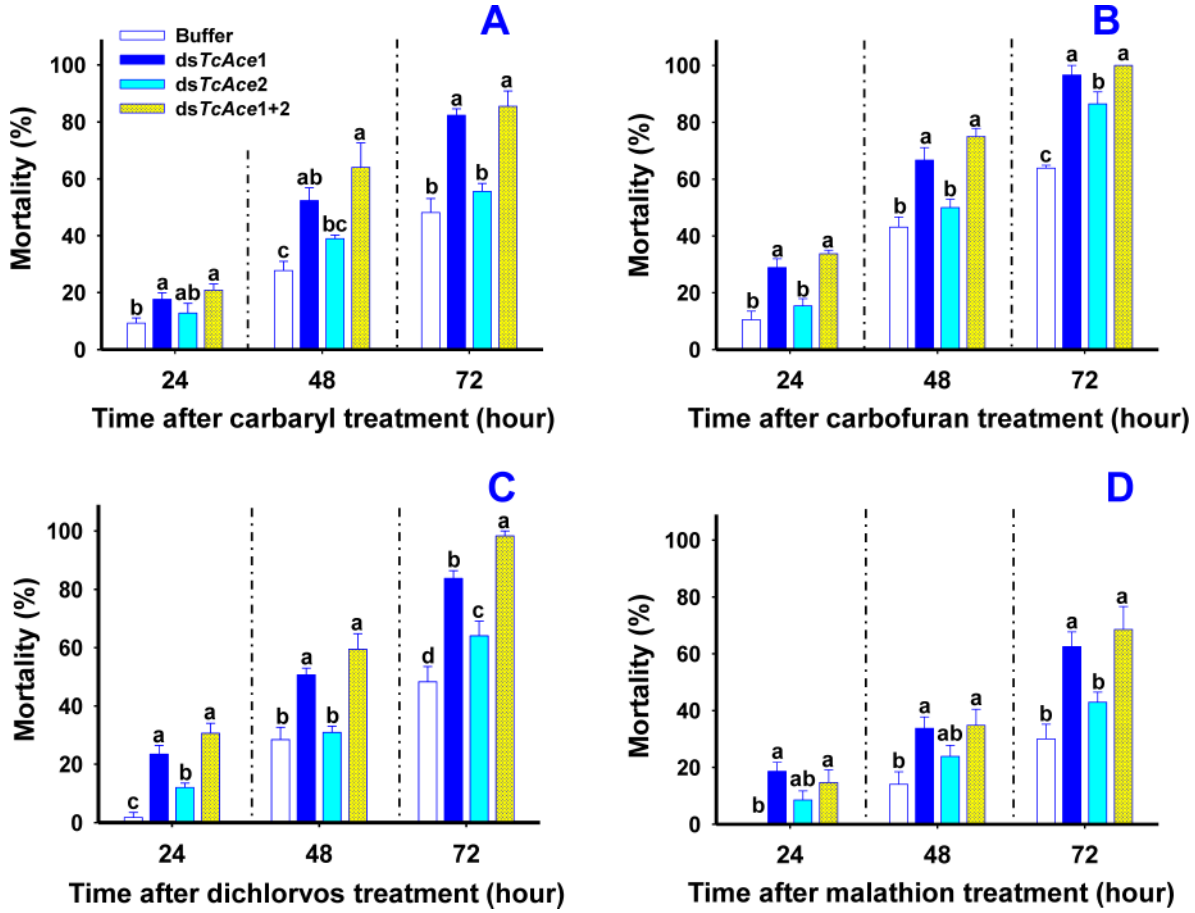

Figure 4 | The effect of gene-silencing mediated by RNAi on the susceptibility to each of four anticholinesterase insecticides: carbaryl (A) and carbofuran (B), dichlorvos (C) and malathion (D) after 20-day larvae of *T. castaneum* were injected with buffer (control), ds*TcAce1*, ds*TcAce2*, or ds*TcAce1+2*. After the larvae were injected with buffer or dsRNA, they were treated with each of the four insecticides on day 4. The larval mortality was observed at 24, 48, and 72 h after the insecticide treatment. The results are presented as the mean and standard errors of three replicates (each was performed with about 15 larvae). Different letters above the standard error bars indicate significant differences among the treatment of the same time point based on the one-way ANOVA followed by Fisher's LSD multiple comparison test ($P \leq 0.05$).

function and insecticide resistance as well as which of the two is for non-cholinergic functions.

Two *Ace* genes reportedly display significant differences in tissue-specific expressions and molecular properties in the German cockroach (*Blattella germanica*)[17–19], the silkworm (*Bombyx mori*)[20], the cat flea (*Ctenocephalides felis*)[21], and *T. castaneum*[15]. Significant differences of biochemical properties between the two Ace products heterologously expressed in the baculovirous-infected cells have also been reported in the African malaria mosquito (*Anopheles gambiae*)[22], the wild silkworm (*Bombyx mandarina*)[23], and *C. felis*[21]. Recent studies suggested that AChE is involved in larval growth and development as observed by RNAi-mediated down-regulation of *Ace* transcript levels in the cotton bollworm (*Helicoverpa armigera*)[24], *B. germanica*[25], and the Asiatic rice borer (*Chilo suppressalis*)[26]. Functional differences of the two genes have not been adequately analyzed, although, based on selective and irreversible inhibition studies of aphid AChEs, it has been suggested that AChE2 does not contribute significantly to the overall AChE activity in aphids[27].

In this study, we first determined the transcript abundances of *TcAce1* and *TcAce2* in *T. castaneum* at different developmental stages by using qPCR in 3-day eggs, 5-day larvae, 20-day larvae, 6-day pupae, 2-day adults, and in brains from the late pupae (Table 1). The transcript levels of *TcAce1* at these stages and in the brain were 1.2- to 8.7- fold higher than those of *TcAce2* ($P \leq 0.05$), indicating that *TcAce1* transcript is more abundant than *TcAce2* at all the developmental stages and in the brains. Our data are consistent with those found in other insect species, including *B. germanica*[18], the diamondback moth (*Plutella xylostella*)[28], the oriental tobacco budworm (*Helicoverpa assulta*)[29], and the mosquito (*Culex pipiens*)[30]. Nevertheless, the relative transcript abundances of the two genes vary significantly among the insect species and different tissues of the same species, ranging from 13- to 250-fold in the different tissues of *P. xylostella*[28] and 3-fold in the nerve cord of *B. germanica*[18]. The mRNA level of *Ace1* is generally more abundant than that of *Ace2* in insects.

Because *T. castaneum* has robust RNAi responses at all developmental stages and in different cell types[31,32], we took advantage of RNAi to systematically investigate cholinergic and non-cholinergic functions of each AChE gene. We observed 100% mortality within about two weeks after adult eclosion when 20-day larvae were injected with ds*TcAce1* or ds*TcAce1+2*. In contrast, the injection of buffer (control) or ds*TcAce2* alone did not lead to any significant mortality even though the *TcAce2* transcript level was reduced to only 4.8% of the control (Fig. 2C). These results clearly indicate that AChE1 is essential for insect survival, presumably by regulating cholinergic neurotransmission, whereas AChE2 does not seem to play a major role in the neurotransmission in *T. castaneum*. The respective cholinergic and non-cholinergic functions of AChE1 and AChE2 are consistent with our large-scale protein simulation studies suggesting that *T. castaneum* AChE1 is a robust ACh hydrolase, whereas *T. castaneum* AChE2 is not a catalytically efficient ACh hydrolase, because the entrance of the active site of the AChE2 model refined by multiple molecular dynamics simulations appeared to be reversed relative to that of the corresponding AChE1 model and because ACh does not adopt the fully extended conformation and its carbonyl oxygen atom is not placed in the oxyanion hole in the AChE2 model[15].

Interestingly, the injection of ds*TcAce2* in 20-day larvae led to significantly delayed development of the insect, reduced egg-laying, egg hatching, and growth of the offspring. Further examination of the ovaries in the adult females indicated relatively fewer mature ovarioles in the ds*TcAce2*-treated insects than the control (data not shown). This phenomenon appears to be similar to the incomplete ovary development and subsequent premature regression that correlates with changes in activity of the "acetylcholinesterase cells" of the pars distalis in the bird (*Zonotrichia leucophrys gambelii*)[33]. These results indicate that *T. castaneum* AChE2 plays an important, non-cholinergic role in insect embryonic development, growth, and reproduction, and are consistent with the observations that the expression of *Ace* in insects, including *T. castaneum*, was detected during early embryonic developmental stages long before the nervous system starts functioning[15]. These results are also consistent with the reports that *Ace2* may confer non-cholinergic activities in most insect species possessing both *Ace1* and *Ace2*[34,35] and that, although still in debate[36], AChE may have non-cholinergic functions such as regulation of cell differentiation and neural formation in vertebrates and *D. melanogaster*[3–5].

To provide further evidence that AChE1 is the enzyme responsible for cholinergic neurotransmission and the target of anticholinesterase insecticides, we performed RNAi for both *TcAce1* and *TcAce2* in 20-day larvae followed by insecticide bioassay. Our bioassay results showed significantly increases in larval susceptibility to all four insecticides at 24, 48 and 72 h after the larvae were injected with ds*TcAce1* or ds*TcAce1+2*. In contrast, no major changes were observed when the larvae were injected with buffer (control) or ds*TcAce2*. Because RNAi for *TcAce1* caused the depletion of its transcript, which ultimately led to a reduced translation of AChE1 enzyme, the outcomes of *TcAce1* RNAi were expected to be similar to those caused by anticholinesterase insecticides that inhibit AChE activity. Thus, the depletion of *TcAce1* transcript will make the larvae more susceptible to any insecticides if AChE1 encoded by *TcAce1* is a target of the insecticides as examined in this study. Our results further support that *T. castaneum* AChE1 is a key enzyme involved in cholinergic neurotransmission. Decrease in *TcAce1* transcript level can ultimately lead to insect mortality as was observed in insecticide bioassay. Furthermore, since RNAi for *TcAce2* did not significantly increased susceptibility of the larvae to the insecticides, *T. castaneum* AChE1 is hence a target of the anticholinesterase insecticides.

The present work has not only delineated the functional differences of the two AChE genes in *T. castaneum* but also offered insight into developing environmentally-safe insecticides for insect pest control. In view of the cholinergic and non-cholinergic functions of two AChEs in *T. castaneum*, we suggest that any insect-specific anticholinesterase insecticides designed for insect pest control should target the AChE encoded by *Ace1* rather than *Ace2* unless the insect species possessing only *Ace2*. This notion is promoted by our findings that AChE1 in *T. castaneum* and many other insect species possesses a cysteine residue at the opening of the AChE active site but this cysteine residue is absent in fish and mammalian AChEs and insect AChE2[15,27,37,38]. This insect AChE1-specific cysteine residue allows design of new chemicals that irreversibly inhibit AChE1 for insect pest control by conjugation of the chemicals to the cysteine residue[27]. Indeed, several recent studies have demonstrated promising potencies of some synthetic chemicals in irreversibly inhibiting the total AChE activity of insects including *S. graminum* and *A. gambiae* but virtually no or limited inhibition to AChE from humans[27,39]. Such chemicals could potentially lead to the development of novel and environmentally-safe insecticides that are toxic to most insect species possessing *Ace1* that is now known to be responsible for cholinergic neurotransmission[40].

In summary, our studies have for the first time delineated cholinergic and non-cholinergic functions of two AChE genes (*TcAce1* and *TcAce2*) in *T. castaneum*. AChE1 is an essential enzyme involved in cholinergic neurotransmission and is the target of anticholinesterase insecticides. As such, insecticide resistance conferred by reduced sensitivity of AChE to insecticides would be expected to be due to genetic modifications of *Ace1* rather than *Ace2* in the insect species possessing both *Ace1* and *Ace2*. In this context, we propose that AChE1 should be used as a target for designing new anticholinesterase insecticides for insect control. In contrast, AChE2 plays important but non-cholinergic roles in insect growth, female reproduction,

and embryo development. Such non-cholinergic functions of *TcAce2* are carried through the female rather than the male.

Methods

Insects. The Georgia-1 (GA-1) strain of *T. castaneum* was reared on whole-wheat flour containing 5% (w/w) of brewers' yeast at 30°C and 65% RH under standard conditions[41].

Analysis of *TcAce1* and *TcAce2* transcript abundances. Total RNA was isolated from 3-day eggs, 5-day larvae, 20-day larvae, 6-day pupae, 2-day adults or the brains dissected from late pupae by using TRIzol reagent (Invitrogen). First strand cDNA synthesis and qPCR were carried out using gene-specific primers[15]. Plasmid DNA containing a *TcAce* fragment was used to generate a standard curve and the transcript abundance of each *Ace* gene was converted to the copy number as previously described[42].

RNAi of *TcAce1*, *TcAce2*, and *TcAce1+2*. dsRNA was synthesized based on the greatest sequence divergence between *TcAce1* and *TcAce2* using MEGAscript® RNAi Kit (Ambion). Each 20-day larva was injected with 400 ng of ds*TcAce1*, ds*TcAce2* or a total of 800 ng of the ds*TcAce1* and ds*TcAce2* mixture (i.e., 400 ng of ds*TcAce1* and 400 ng of ds*TcAce2*). The mortality owing to injection damage was <10%. Our preliminary experiments showed very gene-specific phenotypes (i.e., insect mortality for *TcAce1* vs. no mortality for *TcAce2*) after the expressions of these genes were individually suppressed by injecting their corresponding dsRNA in the larvae. Such gene-specific RNAi effects indicated that our observed phenotypes were not due to non-specific effect of exogenous nucleic acids (i.e., dsRNA). Thus, we used the buffer alone as a negative control in our injection experiments. After the injection, the insects were reared under standard conditions for visually monitoring of phenotypes and further analyses of the remaining transcript levels by qPCR. Three replications were carried out with at least 30 insects in each control or treatment.

To examine effects of ds*TcAce2* injection on insect growth and development, and gender-dependent effect of the RNAi, a large number of 20-day larvae were injected with ds*TcAce2* or buffer as controls. After the injected larvae developed into pupae, the males and females were separated and paired as follows: 1) both a male and a female adults from the larvae injected with buffer; 2) both a male and a female adults from the larvae injected with ds*TcAce2*; 3) a male adult from the larva injected with ds*TcAce2* but a female adult from larva injected with buffer; and 4) a male adult from the larva injected with buffer but a female adult from larva injected with ds*TcAce2*. Each treatment consisted of eight pairs of a male and a female, and each treatment was repeated three times. Suppression of *TcAce2* transcript was examined after four days following the injections of ds*TcAce2* using total RNA prepared from pools of four individuals. Eggs were collected for five days after mated 13 days post-eclosion, whereas egg hatchability was examined five days after the eggs were collected.

Determinations of *TcAce* transcript levels after RNAi. Total RNA was isolated from a pool of four insects in each control or treatment on day 4 after 20-day larvae of *T. castaneum* were injected with buffer (control), ds*TcAce1*, ds*TcAce2* or ds*TcAce1+2* by using TRIzol reagent (Invitrogen). The RNA was treated with DNase I (Fermentas) and the first-strand cDNA was synthesized by using First Strand cDNA Synthesis Kit (Fermentas) with oligo $(dT)_{18}$ as primer. qPCR was used to detect *TcAce* transcript levels after RNAi. A pair of *TcRps3* primers was used as an internal control to monitor equal loading of cDNA for analysis of *TcAce* transcript levels.

AChE assays. Five larvae that were collected from the control and each ds*TcAce*-injected replication on day 4 were homogenized in 300 µl of ice-cold 0.1 M phosphate buffer (pH 7.5) containing 0.3% (vol./vol.) Triton X-100. The homogenates were centrifuged at 15,000 g for 15 min at 4°C and the supernatant was used as enzyme source. AChE activity was measured using model substrate acetylthiocholine (ATC, Sigma) according to the method of Ellman et al.[43] with some modifications[44]. Specific activity was expressed as nmol of ATC hydrolyzed/min/mg protein. Remaining AChE activity was expressed as a percentage in relation to the activity of each control. Protein content of each enzyme preparation was determined by the BCA method[45] using bovine serum albumin as protein standard. Each assay consisted of three biological replicates. Each reaction mixture including 50 µl AChE preparation, 0.25 mM ATC and 0.4 mM 5′5 dithio-bis (2-nitrobenzoic acid) (DTNB, Sigma) in 150 µl of 0.1 M phosphate buffer (pH 7.5). The enzyme activity expressed by Vmax mOD/min was determined using enzyme kinetic microplate reader (Molecular Devices) at 405 nm. AChE activities were expressed as nmol ATC hydrolyzed per min per mg protein using the extinction coefficient of 1.36×10^4 M^{-1} cm^{-1}.

Analysis of AChE using non-denaturing electrophoresis. Non-denaturing polyacrylamide gel electrophoresis (non-denaturing PAGE) was performed on 4–20% Tris-glycine gels (Invitrogen). The running buffer contained 0.3% (vol./vol.) Triton X-100. The same volume of each AChE preparation containing same concentration of total protein was loaded onto each well. The gel was run at 150 V for 90 min in a cold chamber. The AChE band was visualized after the gel was stained for AChE activity using ATC as substrate[46].

Insecticide bioassay. All four insecticides including dichlorvos (purity 99%), malathion (99.8%), carbaryl (99%) and carbofuran (98%) were obtained from Chem Service (West Chester, PA) and dissolved in acetone for bioassays on day 4 after 20-day larvae were injected with dsRNA. The concentration of each insecticide at LC_{50} (median lethal concentration) was used to treat 15 injected larvae in 50 µl of insecticide solution for 20 s. After the treated larvae were placed on a Whatman filter paper for drying in the air, they were transferred into an 8-ml glass vial and kept under the standard conditions as previously described. Control larvae were treated with acetone only. Each treatment was repeated three times. Mortality was recorded at 24, 48 and 72 h after insecticide treatment.

Statistical analysis. For the data obtained from qPCR, percent relative expression levels were calculated by dividing the relative expression value (REV) of each gene in the ds*TcAce*-injected larvae by the REV of the same gene in the buffer-injected larvae. The percent data of the relative *TcAce* expression were transformed using arcsine square root transformation, and then the transformed data were subjected to ANOVA followed by Fisher's least significant difference (LSD) multiple comparisons to separate the means among the treatments by using ProStat software (Poly Software International). For the data obtained from AChE assay and insecticide bioassay, the percent data were first transformed using arcsine square root transformation, and then subjected to ANOVA followed by Fisher's LSD multiple comparisons to separate the means among the treatments.

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Author contributions

Y.L., Y.P., X.G. and K.Y.Z. designed experiments; Y.L. performed experiments; Y.P., H.J. and K.Y.Z. contributed materials and analytic tools; Y.L., Y.P., X.Z., J.Y. and K.Y.Z. analyzed data; Y.L., Y.P., X.G., X.Z., J.Y., Y.-P.P., H.J. and K.Y.Z. wrote the paper.

Additional information

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