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ORIGINAL ARTICLE

Knockdown of a putative *alanine aminotransferase* gene affects amino acid content and flight capacity in the Colorado potato beetle *Leptinotarsa decemlineata*

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Abstract Alanine aminotransferase (ALT) plays important physiological and biochemical roles in insect. In this study, a full-length *Ldalt* cDNA was cloned from *Leptinotarsa decemlineata*. It was ubiquitously expressed in the eggs, larvae, pupae and adults. In the adults, *Ldalt* mRNA was widely distributed in thorax muscles, fat body, midgut, foregut, hindgut, Malpighian tubules, ventral ganglion and epidermis, with the expression levels from the highest to the lowest. Two double-stranded RNAs (dsRNAs) (ds*Ldalt*1 and ds*Ldalt2*) targeting *Ldalt* were constructed and bacterially expressed. After adults fed on ds*Ldalt*1- and ds*Ldalt2*-immersed foliage for 3 day, *Ldalt* mRNA abundance was significantly decreased by 79.5 and 71.1 %, and ALT activities were significantly reduced by 64.5 and 67.6 %, respectively. Moreover, silencing *Ldalt* affected free amino acid contents. Lysine was decreased by 100.0 and 100.0 %, and arginine was reduced by 87.5 and 89.4 %, respectively, in the hemolymph from ds*Ldalt*1- and ds*Ldalt*2-ingested beetles, compared with control ones. In contrast, proline was increased by 88.7 and 96.4 %. Furthermore, ingestion of ds*Ldalt*1 and ds*Ldalt*2 significantly decreased flight speed, shortened flight duration time and flight distance. In addition, knocking down *Ldalt* significantly increased adult mortality. These data imply that *Ld*ALT plays important roles in amino acid metabolism and in flight in *L. decemlineata*.

Keywords *Leptinotarsa decemlineata* · Alanine aminotransferase · RNA interference · Enzyme activity · Flight · Mortality

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Introduction

Ala aminotransferase (ALT, E.C. 2.6.1.2), or Glu–pyruvate (Pyr) aminotransferase, is a pyridoxal-5′-phosphate-dependent enzyme that catalyzes the reversible transamination between Ala and α-ketoglutarate (α-KG) to form Pyr and Glu (Miyashita et al. 2007; Weber et al. 1992). By mediating the conversion of the four major intermediate metabolites, ALT plays important roles in gluconeogenesis, amino acid metabolism, nitrogen use efficiency and nitrogen waste detoxification (Inagaki et al. 2012; McAllister et al. 2013; Miyashita et al. 2007).

As for gluconeogenesis, the enzyme is involved in Ala–Pyr conversion in the intercellular carbon shuttle (Felig 1973), and serves as a strategic link between the carbohydrate and protein metabolism (Etebari et al. 2005). As for amino acid metabolism, the widely known example is

the catabolism and biosynthesis of proline (Pro) in some insect species such as *Leptinotarsa decemlineata* (Mordue and De Kort 1978; Weeda et al. 1979, 1980a, b), *Glossina morsitans* (Bursell 1981), *Pachnoda sinuata* (Auerswald and Gäde 1999), *Aedes aegypti* (Scaraffia and Wells 2003) and *Anopheles stephensi* (Giulivi et al. 2008). Moreover, ALT is involved in ammonium assimilation and detoxification in some insects such as *A. aegypti* (Isoe and Scaraffia 2013; Scaraffia et al. 2010). Furthermore, ALT has been documented to be important for producing excitatory neurotransmitter Glu neuromuscular junctions in the brain (DiAntonio 2006; Peng et al. 1991; Sinakevitch et al. 2010).

Due to its importance in many metabolic pathways, ALT is widely spread in Eukarya, Archaea and Eubacteria species (McAllister et al. 2013). Up to now, however, only a few studies reported *alt* genes and/or proteins in insect. From *Sogatella furcifera*, two putative *alt* genes were cloned, and Sf9 cell-expressed *Sf*ALT proteins had ALT activities (Fan et al. 2014). In *Nilaparvata lugens* (Wan et al. 2014a) and *Laodelphax striatellus* (Yang et al. 2012), putative *alt* fragments were identified. Moreover, ALT activities were determined in *L. decemlineata* (Khan and De Kort 1978), *A. aegypti* (Scaraffia and Wells 2003; Scaraffia et al. 2005), *Drosophila melanogaster* (Chen and Baker 1976; Schneider and Chen 1981), *Schistocerca gregaria* (Mane and Mehrotra 1977), *Periplaneta americana* (Storey and Bailey 1978) and *Locusta migratoria* (Khan and De Kort 1978).

Because there is a shortage of information in the literature, in the present paper, we cloned a putative *alt* gene based on a transcriptome data (Shi et al. 2013) of the Colorado potato beetle *L. Decemlineata*, a notorious insect defoliator of potato in Xinjiang Uygur autonomous region in China. We knocked down *Ldalt* using RNA interference to evaluate the possible effects on free amino acid contents in the hemolymph and on adult performance. Our results imply that *Ldalt* encodes a functional ALT enzyme.

Materials and methods

Insect culture

Leptinotarsa decemlineata was routinely reared in an insectary according to a previously described method (Shi et al. 2013), using potato foliage at vegetative growth or tuber initiation stages.

Molecular cloning, multiple sequence alignment and phylogenetic analysis

An expressed sequence tag (EST) of the putative *Ldalt* was found from *L. decemlineata* transcriptome data (Shi et al. 2013). To verify the correctness of the sequence, total RNA was extracted from the fourth instars and first-strand cDNA was synthesized using the reverse transcriptase (M-MLV RT) (Takara Bio., Dalian, China) and an oligo (dT)18 primer, and was used as a template for polymerase chain reaction (PCR) using primers in Table 1. The 5′- and 3′-RACE Ready cDNAs were synthesized from the fourth *L. decemlineata* instars following the manufacturer's instructions, primed by oligo (dT) primer and the SMARTer II A oligonucleotide using the SMARTer RACE cDNA amplification kit (Takara Bio., Dalian, China). Antisense and sense gene-specific primers (Table 1) corresponding to the 5′-end and 3′-end of the *alt* sequence, and the universal primer in the SMARTer RACE kit (Takara Bio.) were used to amplify the 5′- and 3′-ends, using the components and the thermal cycling conditions according to the

Table 1 Primers used for RT-PCR, 5′ RACE, dsRNA synthesis, and qRT-PCR

Fragment name	Amplicon size (bp)	Forward sequence (5′–3′)	Reverse sequence (5′–3′)
RT-PCR			
Ldalt	127	CAGGTAATCCTACAGGGCAAGT	GAGCCTTCTGCATAGATATTGT
RACE			
5′ RACE		TTGCCCTGTAGGATTACCTGGATTGATT	
3′ RACE		TCAATCCAGGTAATCCTACAGGGCAAGT	
ORF verification			
Ldalt	1904	TGAATCCGGTCCAGCTACT	ACAACGACCAAGAAATTACAG
dsRNA synthesis			
dsegfp	414	AAGTTCAGCGTGTCCG	CTTGCCGTAGTTCCAC
dsLdalt1	398	CAATCTGTGCCTGAATCCGG	TTCTTCACGCCCTGCTCTAAC
dsLdalt2	392	AGGTATCCGCACCCAGAG	GACGCAATCCAATGTAGC
qPCR			
qLdalt	85	CCACCATTTTCGCACTACAA	AAAGTCGGCGTGGAACTCTC

manufacturer's protocols. After obtaining the full-length cDNA, a pair of primers (Table 1) was designed to verify the complete open reading frame (ORF). The resulting sequence (*Ldalt*) was submitted to GenBank (KJ802126).

The ALT-like proteins from 17 insect species and 1 mammal species were downloaded from NCBI database, and respectively, aligned with the predicted *Ld*ALT using ClustalW2.1 (Larkin et al. 2007). The alignments were used to construct the maximum-likelihood (ML) trees using RAxML version 8.1.3 (Stamatakis 2006) to select the best-fitting model (LG + γ, with empirical frequency) after estimated by ProtTest (Darriba et al. 2011). The reliability of ML tree topology was evaluated by bootstrapping a sampling of 1000 replicates.

Bioassay

The method as described recently was used to express dsRNA (Wan et al. 2014b; Zhou et al. 2013). In brief, ds*Ldalt*1 and ds*Ldalt*2 derived from a 398-bp fragment or a 392-bp fragment of *Ldalt*, and ds*egfp* derived from a 414-bp fragment of *egfp* gene were amplified by RT-PCR with specific primers in Table 1, respectively. The PCR products were individually cloned into the plasmid pET-2P within EcoR I sites to generate the recombinant plasmid. The plasmid was transformed into *E. coli* HT115 (DE3) competent cells lacking RNase III, respectively. Individual colonies were inoculated, and induced to express dsRNA. The expressed dsRNA was extracted and confirmed by electrophoresis on 1 % agarose gel (data not shown). Bacteria cells were centrifuged at 5000×*g* for 10 min, and resuspended in 0.05 M phosphate-buffered saline (PBS, pH 7.4) at the ratio of 10:1 (the final concentration of dsRNA was approximately 0.5 μg/μL) for bioassay.

Bioassay was carried out with the adults 7 days after emergence. The treatments included: (1) PBS-, (2) ds*egfp*-, (3) ds*Ldalt*1-, (4) ds*Ldalt*2-dipped leaves. The test suspensions were individually used to dip fresh potato foliage for 10 s. The treated leaves were dried for 2 h under airflow on filter paper, and then placed in Petri dishes (9.0 cm in diameter and 1.5 cm in height). The adults were starved for at least 4 h prior to the experiment; ten individuals were transferred to each dish. The Petri dishes were kept in the laboratory at 28 ± 1 °C, under a 14 h:10 h light–dark photoperiod and 50–60 % relative humidity. The potato leaves were replaced with corresponding freshly treated ones every day.

Each treatment was replicated 24 times (a total of 240 adults each treatment). Nine replicates were used to extract total RNA for qPCR after continuously fed for 1, 2 and 3 days. After continuously fed for 3 days, three replicates were used to prepare crude enzyme solutions; three replicates to test flight capacity; and three replicates to collect hemolymph for free amino acid analysis. The remaining six replicates were used to record mortalities after continuously fed for 6 days.

Quantitative real-time quantitative PCR (qPCR)

Three groups of samples were prepared and each group was repeated in biological triplicate. The first group was derived from whole bodies of developing eggs, the larvae from the first through fourth instars, wandering larvae (W, 1 day after nonfeeding), pupae (P, 7 days after burring into soil) and sexually mature adults (A, 7 days after emerging). The second was derived from epidermis (EP), thorax muscles (TM), fat body (FB), Malpighian tubules (MT), foregut (FG), midgut (MG), hindgut (HG) and ventral ganglion (VG) of the adults. The two groups were fed on normal potato leaves. The third was from the adults 1, 2 and 3 days after the initiation of the bioassay. The mRNA abundance of *Ldalt* (primers listed in Table 1) in each sample was estimated by qPCR, using internal control genes (*rp18*, *rp4*, *arf1* and *arf4*) according to the published results (Shi et al. 2013). Each sample was repeated in technical triplicate. Data were analyzed by the $2^{-\Delta\Delta CT}$ method (Pfaffl 2001), using the geometric mean of *rp18*, *rp4*, *arf1* and *arf4* for normalization according to the strategy described previously (Pfaffl 2001; Vandesompele et al. 2002).

Enzyme activity assays

The beetles were chilled at 4 °C for 30 min, and the wings and legs were removed by scissors and forceps. Each beetle was homogenized in 2 mL 100 mM Tris–HCl pH 7.8 and 15 mM mercaptoethanol and further sonicated for 10 min at 30 °C using an ultrasonic cleaning bath. The homogenate was centrifuged at 100,000×*g* for 30 min at 0 °C and the supernatant used for enzyme assays. ALT activities in the enzyme preparations were measured at 37 °C with Alanine Aminotransferase Activity Assay Kit (Biovison, San Francisco, USA) according to the manufacturer's instructions.

Total protein content of each sample preparation was determined by the Bradford method using bovine serum albumin as the standard (Bradford 1976). The ALT activity of each enzyme preparation was presented as nmol of Ala produced via the transamination of Pyr per min per mg protein.

Free amino acid analysis

The same method as described recently (Wan et al. 2014b) was used. In brief, a total of 2–5 μL of hemolymph was collected from each adult with a 10-μL micropipette after continuous exposure to dsRNA for 3 days, diluted (1:1,

v/v) with cold 0.85 % NaCl containing 0.025 % phenylthiourea in a microcentrifuge tube. Immediately after collection, 5 samples from each replicate were pooled and centrifuged for 15 min at 10,000×*g* and 4 °C. Free amino acid content in the hemolymph was analyzed with a Beckman 6300 Amino Acid Analyzer (Beckman Instruments Inc., Fullerton, CA, USA). The amino acid content was given as μmol per mL hemolymph.

Tethered flight test

All flight tests were done with a computer-interfaced tethered flight system the same as described previously (Xiao et al. 2010). In brief, each adult was anesthetized under carbon dioxide (exposure time was about 1 min), and adhered to one end of a lightweight lever, which was balanced by a small flag attached to the opposite end and was pivoted on a fulcrum. After recovery from anesthesia, the beetle was allowed to fly and the lever rotated around the fulcrum. The rotation cycles of the lever were recorded and the flight duration and distance for each beetle over an 8-h light phase were calculated by the computer.

Data analysis

The data were given as mean ± SE, and were analyzed by one-way ANOVA followed by the Tukey–Kramer test, using SPSS for Windows (SPSS, Chicago, IL, USA).

Results

Molecular cloning and sequence analysis

A full-length cDNA (KJ802126) of a putative *alt* gene in *L. decemlineata* was cloned (*Ldalt*). It contains a complete coding sequence of 1587 bp that encodes a 528-amino acid protein (Fig. 1).

*Ld*ALT contains the complete domains of transaminase Subgroup I, which are conserved among various transaminases including Asp, Ala, Tyr, histidinol-phosphate and Phe aminotransferases (Mehta et al. 1993). ALTs have been known to bind to pyridoxal 5′-phosphate (PLP) by Lys residues via an aldimine bond. The crucial 11 amino acid residues essential for binding the coenzyme PLP and

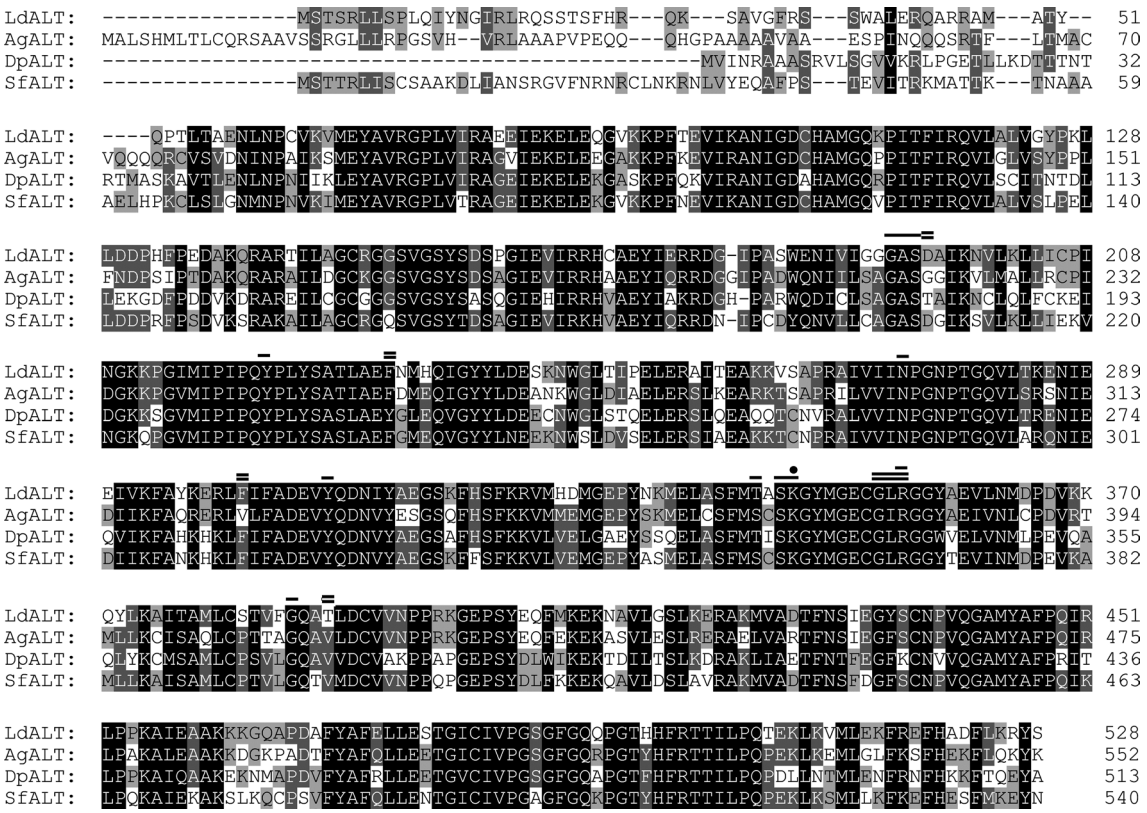

Fig. 1 Amino acid sequence alignment of *Leptinotarsa decemlineata* alanine aminotransferase (*Ld*ALT) with its homologues. ALTs originate from *Anopheles gambiae* (AgamALT, XP_316880), *Danaus plexippus* (DpleALT, XP_004921714) and *Sogatella furcifera* (S_furALT1, EHJ74142), respectively. Identical and similar amino acids are *shaded*. Gaps have been introduced to permit alignment. The pyridoxal-5′-phosphate binding sites are marked with *single solid line* above the residues. The sites of homodimer interface are indicated with *double solid lines*. The pyridoxal-5′-phosphate binding residue is marked with full-filled cycle

for stabilizing the enzyme:substrate transition state (Liepman and Olsen 2003; Mehta et al. 1993; Tanase et al. 1979; Ward et al. 2000) are conserved in *Ld*ALT (Fig. 1).

*Ld*ALT had the greatest identity (70 %) with that from *Dendroctonus ponderosae*, followed by those from *Pediculus humanus humanus* (65 %), *Acyrthosiphon pisum* (65 %), *A. aegypti* (65 %), *Anopheles gambiae* (65 %), *Culex quinquefasciatus* (64 %), *Danaus plexippus* (59 %), *B. mori* (57 %) and *Nasonia vitripennis* (56 %). However, the identities of *Ld*ALT with six mammal ALTs from *Homo sapiens*, *Mus musculus* and *Bos taurus* were low, only 48 and 54 %, respectively.

The evolutionary relationship of ALT-like proteins derived from 17 insect species and 3 mammal species was evaluated (Fig. 2). The phylogenetic tree showed that the ALTs formed an insect and a mammal clusters. The two clusters were well segregated from each other. Among insect cluster, ALT-like proteins formed a Hymenoptera clade, a Lepidoptera clade, a Diptera clade, a Coleoptera clade and a Hemiptera–Siphonaptera clade, supported by 100, 100, 100, 56 and 17 % of bootstrap values, respectively. Between mammal cluster, ALT1 clade and ALT2 clade were joined together with 100 % bootstrap value. As expected, *Ld*ALT protein belonged to the Coleoptera clade (Fig. 2).

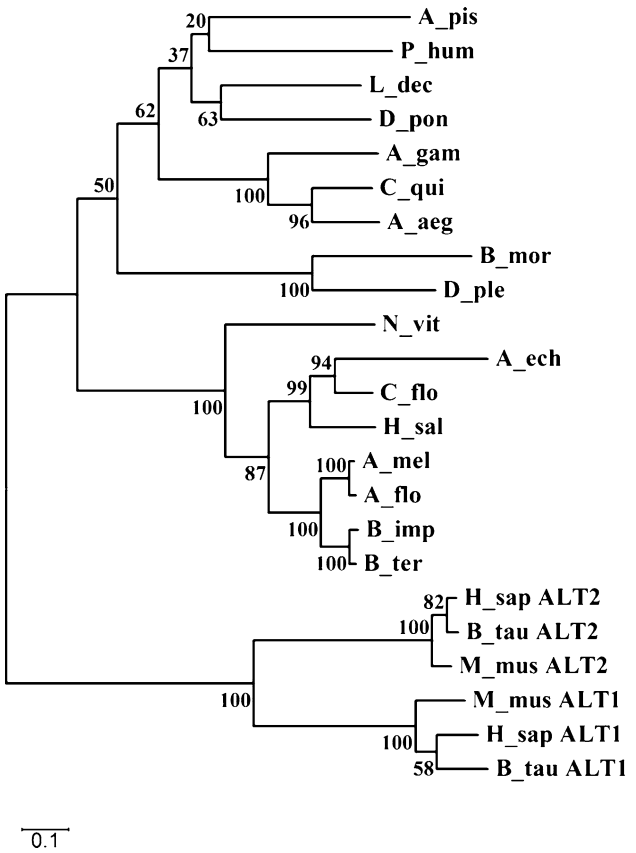

Fig. 2 The phylogenic analysis of alanine aminotransferases (ALTs). An unrooted phylogenetic tree is constructed by the maximum-likelihood method (the best-fitting model, LG + γ, with empirical frequency) based on the protein sequence alignments. The ALT-like sequences originate from coleopteran species *Leptinotarsa decemlineata* (L_dec) and *Dendroctonus ponderosae* (D_pon, ENN78579); dipteran species *Aedes aegypti* (A_aeg, XP_001660470), *Anopheles gambiae* (A_gam, XP_316880 and *Culex quinquefasciatus* (C_qui, XP_001864663); lepidopteran species *Danaus plexippus* (D_ple, EHJ74142) and *Bombyx mori* (B_mor, XP_004924185); hymenopteran species *Nasonia vitripennis* (N_vit, XP_003425526), *Acromyrmex echinatior* (A_ech, EGI66290), *Camponotus floridanus* (C_flo, EFN75006), *Harpegnathos saltator* (H_sal, EFN89499), *Apis mellifera* (A_mel, XP_392720, *Apis florea* (A_flo, XP_003692587), *Bombus impatiens* (B_imp, XP_003489354) and *Bombus terrestris* (B_ter, XP_003403160); siphonapteran species *Pediculus humanus humanus* (P_hum, XP_002430266), hemipteran species *Acyrthosiphon pisum* (A_pis, XP_001948711); and mammal species *Homo sapiens* (H_sapALT1, NP_005300; H_sapALT2, NP_597700), *Mus musculus* (M_musALT1, XP_877957; M_musALT2, NP_776291) and *Bos taurus* (B_tauALT1, NP_001077209.1; B_tauALT2, XP005218795). The percentiles of bootstrap values (1000 replicates) are indicated. The *scale bar* represents the amino acid divergence

Temporal and spatial transcript profiles

Ldalt was widely expressed in all developmental stages including eggs, first through fourth instars, wandering larvae, pupae and sexually mature adults. The highest expression peak of *Ldalt* occurred at the fourth-instar larvae. It was moderately expressed in the second instars, the third instars, and eggs, and lowly expressed in the newly molted neonates, wandering larvae and adults. The trough of *Ldalt* was observed in the pupae (Fig. 3a).

The spatial mRNA levels of *Ldalt* were also measured in the adults. *Ldalt* was highly expressed in the thorax muscles and fat body. It was moderately expressed in the midgut, foregut, hindgut, Malpighian tubules and ventral ganglion. In contrast, it was lowly expressed in the epidermis (Fig. 3b).

Effect of dsRNA on *Ldalt* expression, ALT activities and free amino acid contents

Two dsRNAs (ds*Ldalt*1 and ds*Ldalt2*) targeting *Ldalt* were constructed and bacterially expressed. After the adults were fed on ds*Ldalt*1- and ds*Ldalt*2-immersed foliage for 1, 2 and 3 days, *Ldalt* mRNA abundance in the resulting beetles was decreased by 53.1 % and 44.0 % (Fig. 4a), 72.1 and 65.3 % (Fig. 4b), and 79.5 and 71.1 % (Fig. 4c), respectively, compared with blank control. In contrast, *Ldalt* mRNA level varied little in ds*egfp*-ingested adults. ANOVA analysis revealed that the *Ldalt* mRNA levels in ds*Ldalt*1- and ds*Ldalt*2-ingested beetles were significantly lower than those in control and ds*egfp*-ingested ones (Fig. 4a–c).

After ingested normal, ds*egfp*-, ds*Ldalt*1- and ds*Ldalt*2-dipped foliage for 3 days, the ALT activities in the enzyme preparations from the resulting beetles were 894.3,

948.5, 317.1 and 290.0 nmol/min/mg protein, respectively. ANOVA analysis revealed that the enzyme preparations from control and ds*egfp*-ingested adults showed significantly higher activities than those from ds*Ldalt*1- and ds*Ldalt*2-ingested beetles (Fig. 4d).

The concentration of the 20 free amino acids in the hemolymph of the resulting beetles, after continuous ingestion of normal, ds*egfp*-, ds*Ldalt*1- and ds*Ldalt*2-immersed foliage for 3 days, was respectively, 129.75, 134.06, 132.05 and 133.04 μmol/mL. Four amino acids, Pro, Cys, Gln and Asn, were most abundant (Table 2).

Silencing *Ldalt* affected the contents of several amino acids. Among them Lys was decreased by 100.0 and 100.0 %, and Arg was reduced by 87.5 and 89.4 %, respectively, in the hemolymph from ds*Ldalt*1- and ds*Ldalt*2-ingested beetles, compared with control beetles. In contrast, Pro was increased 88.7 and 96.4 % in the hemolymph of ds*Ldalt*1- and ds*Ldalt*2-ingested beetles. ANOVA analysis revealed that ingestion of ds*Ldalt* significantly reduced free Lys and Arg concentrations, but significantly increased Pro content.

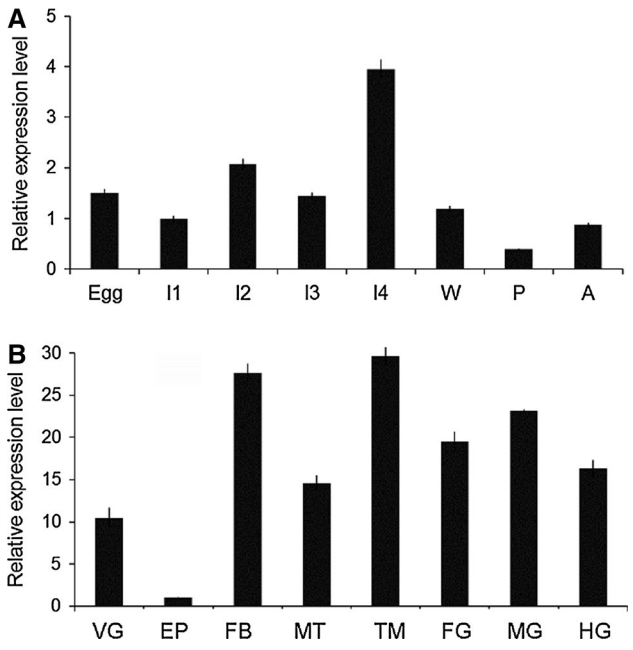

Fig. 3 Temporal (**a**) and spatial (**b**) expression patterns of the putative *Ldalt*. cDNA templates are derived from the first-, second-, third- and fourth-instar larvae (I1, I2, I3, I4, I5), wandering larvae (W), pupae (P) and sexually mature adults (A), or from epidermis (EP), thorax muscles (TM), fat body (FB), Malpighian tubules (MT), foregut (FG), midgut (MG), hindgut (HG) and ventral ganglion (VG) of the adults. For each sample, 3 independent pools of 5–10 individuals are measured in technical triplicate using qPCR. The values are calculated using the $2^{-\Delta\Delta Ct}$ method. The *columns* represent averages with *vertical bars* indicating SE

Effect of dsRNA on adult performance

Continuous ingestion of normal, ds*egfp*-, ds*Ldalt*1- and ds*Ldalt*2-immersed foliage for 3 days affected flight capacities. The flight speeds of the resulting beetles were 10.98, 10.67, 0.01 and 0.01 m/min, respectively, the former two were significantly faster than the latter two (Fig. 5a). Moreover, the total flight durations, on an average, were 10.4, 11.3, 5.6 and 4.7 min, respectively, with those from normal and ds*egfp*-dipped foliage sustained for significantly longer time periods (Fig. 5b). As a result, the flight distances of the resulting beetles were 113.86, 120.60, 0.04 and 0.07 m, respectively, the former two were significantly longer than the latter two (Fig. 5c).

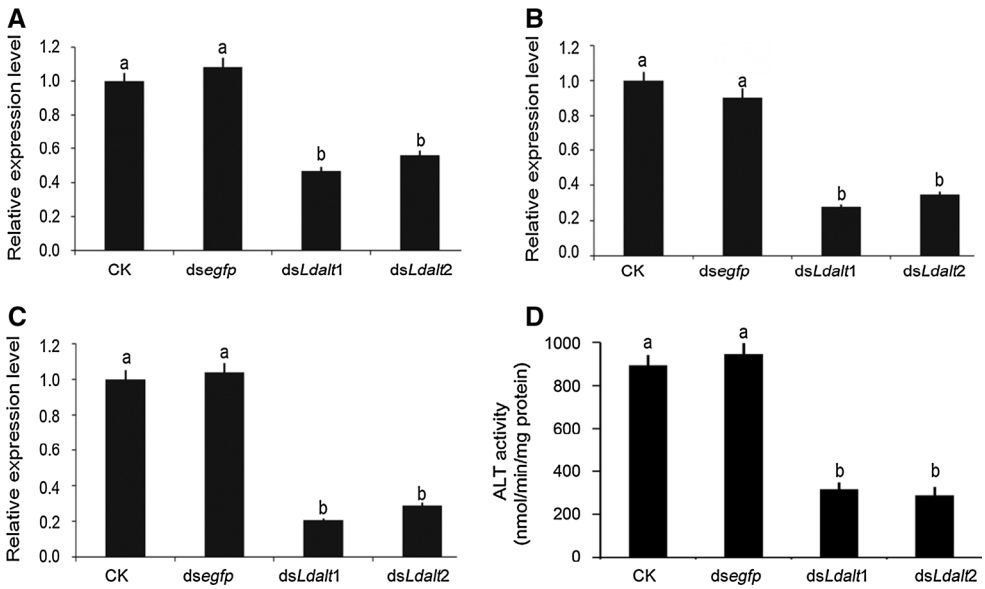

Fig. 4 The mRNA levels of *Ldalt* after exposed to dietary dsRNA for 1 (**a**), 2 (**b**) to 3 (**c**) days and the ALT activities of enzyme preparations (**d**). Three biological replicates are conducted, and the mean ± SD ($n = 3$) is calculated to measure the relative transcript levels using the $2^{-\Delta\Delta CT}$ method. The *columns* represent averages with *vertical bars* indicating SE. *Bars* that do not share the *same letter* are significantly different at *P* value of 0.05

Table 2 Content of free amino acids in the hemolymph of *Leptinotarsa decemlineata* adults fed on PBS, ds*egfp*, ds*Ldalt*1 and ds*Ldalt*2-immersed foliage for 3 days

Amino acid	Concentration (μmol/mL)			
	PBS	ds*egfp*	ds*Ldalt*1	ds*Ldalt*2
Alanine (Ala)	6.53 ± 0.44 a	7.81 ± 0.37 a	6.45 ± 0.39 a	6.58 ± 0.47 a
Arginine (Arg)	4.32 ± 0.25 A	4.15 ± 0.21 A	0.54 ± 0.04 B	0.46 ± 0.05 B
Aspartic acid (Asp)	1.22 ± 0.10 a	1.41 ± 0.08 a	1.33 ± 0.06 a	0.94 ± 0.05 a
Asparagine (Asn)	10.58 ± 1.01 a	11.87 ± 0.52 a	11.40 ± 0.81 a	10.34 ± 0.44 a
Cysteine (Cys)	16.8 ± 0.68 a	17.3 ± 0.72 a	14.15 ± 0.58 a	21.54 ± 1.12 a
Glutamate (Glu)	1.15 ± 0.15 a	1.47 ± 0.06 a	1.08 ± 0.08 a	0.88 ± 0.04 a
Glutamine (Gln)	12.32 ± 1.14 a	11.4 ± 1.01 a	13.48 ± 0.79 a	17.36 ± 0.80 a
Glycine (Gly)	7.83 ± 0.56 a	8.36 ± 0.75 a	7.93 ± 0.60 a	6.88 ± 0.37 a
Histidine (His)	2.83 ± 0.21 a	3.06 ± 0.18 a	2.43 ± 0.11 a	2.68 ± 0.19 a
Isoleucine (Ile)	5.48 ± 0.44 a	6.04 ± 0.37 a	5.42 ± 0.25 a	4.31 ± 0.47 a
Leucine (Leu)	7.28 ± 0.43 a	8.68 ± 0.40 a	7.67 ± 0.57 a	5.72 ± 0.31 a
Lysine (Lys)	8.00 ± 0.43 A	5.98 ± 0.55 A	0.00 ± 0.00 B	0.00 ± 0.00 B
Methionine (Met)	0.02 ± 0.00 A	0.01 ± 0.00 A	0.00 ± 0.00 B	0.00 ± 0.00 B
Phenylalanine (Phe)	3.87 ± 0.25 a	4.09 ± 0.38 a	3.67 ± 0.26 a	2.74 ± 0.18 a
Proline (Pro)	18.2 ± 1.24 b	17.3 ± 1.13 b	34.35 ± 1.65 a	33.98 ± 1.91 a
Serine (Ser)	7.83 ± 0.50 a	8.36 ± 0.32 a	7.92 ± 0.58 a	6.88 ± 0.66 a
Threonine (Thr)	6.17 ± 0.61 a	6.26 ± 0.52 a	5.20 ± 0.50 a	4.70 ± 0.38 a
Tryptophan (Trp)	0.03 ± 0.00 A	0.02 ± 0.00 A	0.00 ± 0.00 B	0.00 ± 0.00 B
Tyrosine (Tyr)	3.75 ± 0.27 a	3.77 ± 0.28 a	3.72 ± 0.22 a	2.96 ± 0.17 a
Valine (Val)	5.54 ± 0.51 a	6.72 ± 0.58 a	5.32 ± 0.63 a	4.09 ± 0.41 a
Total	129.75 ± 10.44 a	134.06 ± 11.83 a	132.05 ± 10.14 a	133.04 ± 9.97 a

Free amino acid content in the hemolymph is analyzed with a Beckman 6300 Amino Acid Analyzer. Difference of the content of each amino acid among treatments is analyzed by ANOVA followed by the Tukey–Kramer test. Data that do not share the same lowercase or uppercase letter are significantly different at *P* values of 0.05 or 0.01

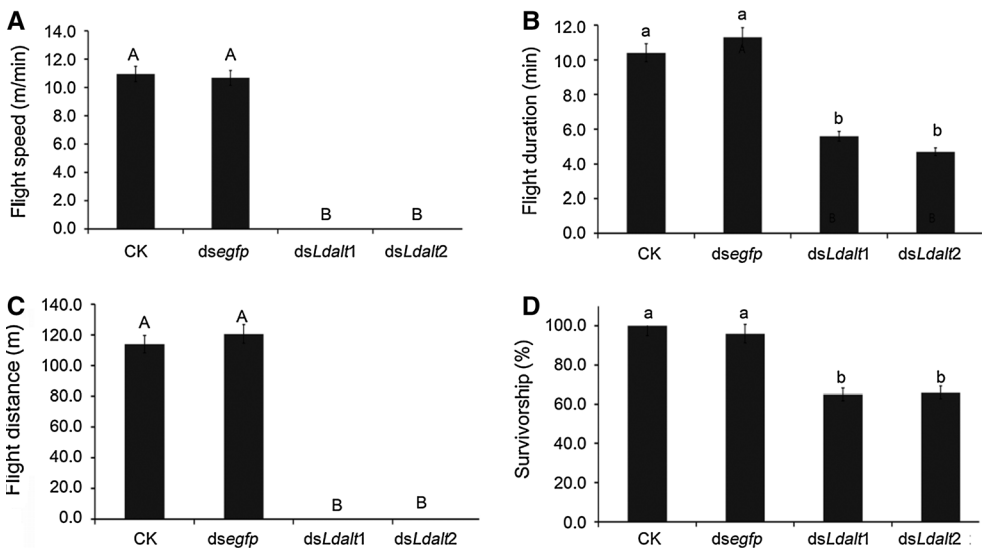

Fig. 5 Ingestion of ds*Ldalt* on adult flight performance and mortality. The flight abilities are estimated by flight speed (**a**), flight duration (**b**) and flight distance (**c**) after 3 days of exposure to ds*Ldalt*. The survivorships are calculated after 6 days of exposure to ds*Ldalt*. The *columns* represent averages with *vertical bars* indicating SE. *Bars* that do not share the same *lowercase* or *uppercase letter* are significantly different at *P* values of 0.05 or 0.01

Continuous ingestion of ds*Ldalt*-immersed foliage significantly increased adult mortality. Six days after exposure to ds*Ldalt*1 and ds*Ldalt*2, the adult mortalities reached up to 35 and 34 %, respectively. In contrast, less than 5 % of the adults on normal and ds*egfp*-dipped leaves died (Fig. 5d).

Discussion

ALT plays important roles in a number of cellular processes. In mammals, two ALT enzymes were found to, respectively, locate in cytoplasm and mitochondria, and were named as ALT1 and ALT2 (Jadaho et al. 2004; Sohocki et al. 1997; Yang et al. 2009). From insect genome data, however, only one *alt* gene was discovered in *A. pisum*, *A. aegypti*, *A. mellifera*, *A. gambiae*, *B. mori*, *C. quinquefasciatus*, *D. plexippus*, *D. melanogaster*, *P. humanus humanus* and *N. vitripennis*. In *S. furcifera*, although two full-length *alt*s were cloned, one (*Sfylsalt*) was suggested to come from yeast-like symbionts whereas the other (*Sfalt1*) originated from the planthopper (Fan et al. 2014). Moreover, while two *alt* fragments were cloned from both *N. lugens* (Wan et al. 2014a) and *L. striatellus* (Yang et al. 2012), both fragments might originate from a single gene. In the present paper, we cloned a putative *Ldalt*. The phylogenetic result indicated that *Ld*ALT was distantly related to other insect ALT-like proteins, while it was well segregated from six mammal ALTs. It appears that *Ldalt* in *L. decemlineata* is orthologous with other insect *alt* genes and all the insect *alt*s have evolved from a common ancestral gene.

In the present paper, we provided four lines of experimental evidence to support that the putative *Ldalt* encoded a functional ALT enzyme that catalyzed the reversible transfer of an amino group from Ala to α-KG to form Pyr and Glu. Firstly, *Ld*ALT showed typical protein domains and temporal expression patterns that were associated with a functional enzyme. *Ld*ALT in *L. decemlineata* has typical Subgroup I primary structure features (Tanase et al. 1979), containing eleven invariant residues essential for binding the coenzyme PLP and for stabilizing the enzyme:substrate transition state (Liepman and Olsen 2003; Mehta et al. 1993; Ward et al. 2000). Moreover, *Ldalt* was ubiquitously expressed in *L. decemlineata* eggs, larvae, pupae and adults. Similarly, both *alt*s were expressed in *S. furcifera* nymphs and adults (Fan et al. 2014). Consistent with this, we found that the ALT activity in the adults was 894.3 nmol/min/mg protein. Comparably, the ALT activity in the fat body of *L. decemlineata* adults was 74.5 μmol/h/mg protein (1281.7 nmol/min/mg protein) (Weeda et al. 1980b). The ALT activity in *L. decemlineata* was higher than that in *P. americana* (96.7 nmol/min/mg protein) (Storey and Bailey 1978) and *L. migratoria* (383.3 nmol/min/mg protein) (Khan and De Kort 1978).

Secondly, knocking down *Ldalt* using RNAi inhibited ALT activity. Ingestion of ds*Ldalt*1 and ds*Ldalt*2 decreased 79.5 and 71.1 % of *Ldalt* mRNA abundance, and reduced 64.5 and 67.6 % of ALT activities. The similar reductions of *Ldalt* mRNA levels and ALT activities in ds*Ldalt*-ingested beetles suggested that *Ld*ALT identified in this study might be the only functional ALT enzyme in *L. decemlineata* adults.

Thirdly, RNAi-mediated knockdown of *Ldalt* significantly reduced the contents of Arg and Lys. ALT plays an important role in ammonium assimilation in insects (Scaraffia et al. 2010). In *A. aegypti*, the midgut mainly fixes ammonia through reactions catalyzed by Gln synthetase, Glu dehydrogenase and ALT, whereas, the fat body mainly assimilated ammonia using a Gln synthetase/Glu synthase pathway, pyrroline-5-carboxylate synthase, pyrroline-5-carboxylate reductase, Glu dehydrogenase and ALT (Scaraffia et al. 2010). In *L. decemlineata* adults, we found that *Ldalt* mRNA was abundant in the midgut, foregut, hindgut and Malpighian tubules. Higher *Ldalt* mRNA levels in the digestive canal and related organ may be involved in ammonium assimilation. Thus, silencing *Ldalt* may inhibit ammonium assimilation and cause ammonia deficiency. Lack of ammonia then affects the biosynthesis of amino acids, especially those possessing several amino groups. Among the 20 protein amino acids, Lys and Arg are the basic amino acids. Besides the α-amino group, Lys has an ε-amino group, and Arg possesses a complex guanidinium group at the distal end which has three amino group. Thus, biosynthesis of Lys and Arg needs more ammonia, and ammonia deficiency in the *Ldalt* RNAi hypomorphs seriously affects their contents. As for other two amino acids possessing more than one amino group, Gln and Asn, they may still function in ammonium assimilation in the *Ldalt* RNAi hypomorphs. Ammonia in the gut was mainly used to biosynthesize Gln and Asn. Therefore, the contents of Gln and Asn did not change much in ds*Ldalt*-ingested adults. Similarly, in *A. aegypti* midgut mainly fixed ammonia into Gln and Ala, and fat body mainly assimilated ammonia into Gln and Pro (Scaraffia et al. 2010). In *N. lugens*, ammonia may be assimilated into Gln and Asn (Wan et al. 2014a).

The fourth line of experimental evidence was that silencing *Ldalt* inhibited the flight capacity of the resulting beetles. We found that *Ldalt* was highly expressed in the thorax muscles and fat body. Moreover, a metabolic pathway of Pro has been proposed in *L. decemlineata* during flight (Mordue and De Kort 1978; Weeda et al. 1979, 1980a, b). In the flight muscles, Pro is converted to Glu. Glu subsequently serves as the substrate for ALT, providing Ala and α-KG. The latter is further oxidated in the tricarboxylic acid cycle to generate malate. Malate is then decarboxylated to produce Pyr, which is subsequently converted into Ala in the presence of Glu by ALT. Ala, in turn, is released to the hemolymph and transported to the fat body, where it is catalyzed by ALT to transfer an amino group to α-KG to form Glu and Pyr. Glu is converted to Pro. Newly biosynthesized Pro is then released from the fat body to the hemolymph,

transported to and absorbed by the flight muscles (Candy et al. 1997; Gäde 2004; Gäde and Auerswald 2002; Weeda et al. 1980b). Thus, *Ld*ALT is involved in catabolism of Pro in flight muscles and biosynthesis of Pro in fat body. The highest expression level of *Ldalt* in the thorax muscles and fat body supports the Pro metabolism hypothesis outlined above in *L. decemlineata.*

Furthermore, in this study, we found that the content of Pro in the hemolymph from ds*Ldalt*-ingested adults significantly increased. This suggested that knockdown of *Ldalt* more seriously inhibit catabolism of Pro, and subsequently affected flight capacity. Consistent with this suggestion, we found that ingestion of ds*Ldalt*1 and ds*Ldalt*2 significantly decreased flight speed, and shortened flight duration time and flight distance. Thus, we provide the fourth line of experimental evidence to support that the putative *Ldalt* encoded a functional ALT enzyme.

In this study, we found that the flight speed, flight duration and flight distance of the control beetles were 10.98 m/min, 10.4 min and 113.86 m, respectively, during an 8-h light period. Thus, *L. decemlineata* adults used in this study are weak flyers. It is well known that *L. decemlineata* adults exhibit a season-dependent flight (Baker et al. 2001). Spring emergers often fly a long distance from overwintering locations to mate, feed and lay eggs (Baker et al. 2001). In contrast, the summer and autumn beetles tend to move by walking (Noronha and Cloutier 1999). In this study, we used summer beetles, and therefore the flight capacity was initially weak. In fact, each adult reared under a long-day photoregime (similar to the summer beetle) could biosynthesize 400–500 μg Pro per hour (Weeda et al. 1980b). However, the rate of Pro oxidation by isolated mitochondria from the flight muscle of a beetle is 1650 μg per hour calculated from the rate of oxygen consumption (Weeda et al. 1980a). This mean that flight duration in the summer beetle is limited by availability of Pro.

ALT also serves as a strategic link between carbohydrate and protein metabolism (Etebari et al. 2005). In addition, ALT has been reported to be important for generating excitatory neurotransmitter Glu (DiAntonio 2006; Peng et al. 1991; Sinakevitch et al. 2010). Consistent with the neurological role, our results revealed that *Ldalt* was also moderately expressed in the ventral ganglion of *L. decemlineata* adults. Moreover, knockdown of *Ldalt* significantly increased adult mortality. Therefore, providing more support that *Ld*ALT identified in this study is a functional enzyme in *L. decemlineata*.

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Conflict of interest The authors state that there is no conflict of interests.

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