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Methods paper

Two conserved Z9-octadecanoic acid desaturases in the red flour beetle, *Tribolium castaneum*

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ABSTRACT

Z9 Desaturases catalyse the formation of a *cis*-unsaturated bond in the Δ9 position of the saturated fatty acids stearate and palmitate. They are considered essential enzymes in eukaryotic organisms as their Z9 unsaturated fatty acid products are required for homeostatic roles such as maintenance of membrane fluidity. Two putative Z9 acyl Coenzyme-A desaturase genes were identified in the red flour beetle, *Tribolium castaneum*, genome (*TcasZ9desA* and *B)* based on their similarity to acyl CoA-desaturases of other insects. *TcasZ9desA* and *B* share 75% nucleic acid sequence identity and appear to be functionally conserved; the genes were cloned and expressed in the yeast strain *Saccharomyces cerevisiae* (*ole1*); both genes complemented the yeast requirement for Z9 fatty acids and produced substantial quantities of Z9 desaturated products with a stearate>palmitate chain length preference. Quantitative PCR analysis of transcripts in RNA obtained from adult, larval and pupal stages of the beetles show *TcasZ9desA* and *B* are expressed at similar levels in all stages, with the pupal stage having the lowest expression.

1. Introduction

The endogenous production of Z9 fatty acids is vital in eukaryotes for maintaining correct cell membrane fluidity (Tiku et al., 1996) and for other homeostatic roles including as a precursor to polyunsaturated fatty acid and pheromone syntheses. Eukaryotes possess at least one essential and conserved fatty acid desaturase which introduces a double bond in the Z *(cis)* orientation at the Δ9 position of the 18-carbon chain length saturated fatty acid, stearate, or the 16-carbon homologue palmitate. In insects, Z9 acyl CoA desaturase gene expression has been shown to be responsive to temperature and feeding following starvation. In the onion maggot, *Delia antiqua,* Z9 desaturase expression was upregulated in pupae in response to cold (Kayukawa et al., 2007) resulting in increased cold tolerance and content of unsaturated fatty acid in brain phospholipids. Further, Riddervold et al. (2002) showed significantly increased Z9 desaturase expression in response to feeding in newly eclosed adult crickets following a period of starvation.

Over recent years there has been intensive investigation of acylCoA desaturases from insects among moths, flies and crickets that perform homeostatic and specialist roles such as pheromone synthesis (Dallerac et al., 2000; Eigenheer et al., 2002; Labeur et al., 2002; Liu et al., 2004; Park et al., 2008; Riddervold et al., 2002; Rosenfield et al., 2001; Xue et al., 2007; Zhou et al., 2008). However, the conserved Z9 desaturases from Coleoptera (beetles), the largest order of eukaryotic organisms, have not been isolated and functionally characterised. *Tribolium castaneum*, the red flour beetle, is a well-developed model in developmental genetics and an economically important stored grain pest. The genome of *T. castaneum* has been sequenced and will play an important role in understanding the molecular physiology of this beetle. Here, we describe the cloning and functional characterisations of two conserved Z9 acylCoA desaturases from *T. castaneum* and examine their gene expression across developmental stages. Identification of two apparently homeostatic Z9 desaturases with the same activity was unexpected especially with the finding that they are expressed at similar levels in each developmental stage.

2. Materials and methods

2.1. Gene prediction

All full-length insect desaturase protein sequences (obtained from the Pfam database—http://www.sanger.ac.uk/Software/Pfam/index.shtml) were aligned using the Clustal X program (Thompson et al., 1997). From this sequence alignment, a highly conserved sequence after the third histidine box was observed (HNYHHAYPW-DYKAAEIGMPLNSTASLIRLCASLGWAYDLKSV) and used in a tBLASTn

Abbreviations: BLOSUM, Blocks of amino acid substitution matrix; CoA, Coenzyme A; Cq, quantification cycle; CSIRO, Commonwealth Scientific and Industrial Research Organisation; DMOX, dimethyloxazoline; d*N*/d*S*, nonsynonymous to synonymous ratio; FAME, fatty acid methyl ester; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MEGA, Molecular Evolutionary Genetics Analysis; qPCR, quantitative PCR.

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analysis (Altschul et al., 1997) against the *T. castaneum* genome (http://www.bioinformatics.ksu.edu/BeetleBase/; Tribolium genome sequencing consortium, 2008). From this analysis, 15 putative desaturase sequences were obtained. Each of the contigs was then subjected to a gene prediction program in Softberry (http://www.softberry.com/cgi-bin/programs/gfin/fgenesh) using parameters for *Brugia malaya* and *Caenorhabditis elegans*.

2.2. Amplification of desaturases

Adult *T. castaneum* TC4 strain were obtained from long term cultures at CSIRO Entomology reared on wholemeal flour with brewers yeast, at 25 °C and 55% relative humidity, in the dark. Total RNA was extracted from adult beetles using the Trizol® reagent according to the manufacturer's instructions (Invitrogen Corporation, Carlsbad, CA, USA) and further purified using the RNeasy mini protocol for RNA cleanup (QIAgen, Valencia, CA, USA). Two desaturase genes with high homology to other insect Z9 desaturases, *TcasZ9desA* and *B*, were amplified from 37 ng of *T. castaneum* total RNA using the SuperscriptIII RT/Platinum *Taq* DNA polymerase kit (Invitrogen) according to the manufacturer's instructions. *TcasZ9desA* was amplified using the primers Z9desAfor (5′ GGATCCATGCCACCCTATGTGTCC) and Z9desArev (5′GCATGCTTAATTAAAATCGTCAGA) containing a *Bam*HI site and an *Sph*I site, respectively (underlined). *TcasZ9desB* was amplified using the Z9desBfor (5′ GGATCCATGACACCAAATGCTTCA) and Z9desBrev (5′GAATTCCTACGCACTCTTCCTATG) containing a *Bam*HI site and an *Eco*RI site, respectively (underlined). Approximately 1 kb amplicons were obtained from each RT-PCR and purified using the QIAquick PCR purification kit (QIAgen). The *TcasZ9desA* and *B*-containing fragments were cloned into pGEM T Easy (Promega), to generate pGEM-TcasZ9desA and pGEM-TcasZ9desB, respectively, and the sequences confirmed. BLASTn analysis was conducted with *TcasZ9desA* and *B* against the nucleotide collection in Genbank.

2.3. Sequence and phylogenetic analysis

Deduced amino acid (aa) sequences for TcasZ9desA and B and characterised Z9 desaturases from insects were aligned using ClustalW (Thompson et al., 1994) and their sequence identities/similarities were calculated using the BLOSUM62 similarity matrix. BLASTp analyses (Altschul et al., 1997) of TcasZ9desA and TcasZ9desB proteins were conducted against the non-redundant protein database of NCBI. Predictions for transmembrane regions in the *T. castanuem* sequences were made using TMpred (Hofmann and Stoffel, 1993). The non-synonymous to synonymous changes and transition/transversion ratio were determined using DIVERGE (Accelrys-GCG).

Phylogenetic analyses were conducted on a wide range of insect desaturases with representatives from five insect orders and activities that included Δ9, Δ10–13, Δ12 and Δ14 desaturation. Protein sequences were aligned using ClustalW (Thompson et al., 1994) and then a phylogenetic tree was constructed using the neighbour-joining method of Saitou and Nei (1987) using the package *MEGA* version 4 (Tamura et al., 2007). Bootstrap analysis with 1500 replicates was carried out to determine statistical support for tree branches. The insect orders and full species name of organisms whose protein sequences were used in the analysis are as follows: Orthoptera: *Acheta domesticus* Diptera: *Delia antiqua*, *Drosophila melanogaster*, *Musca domestica, Anopheles gambiae* Lepidoptera: *Bombyx mori, Choristoneura parallela, Epiphyas postvittana, Helicoverpa zea, Mamestra brassicae, Manduca sexta, Ostrinia nubilalis, Planototrix octo, Spodoptera littoralis, Thaumetopoea pityocampa, Trichoplusia ni* Hymenoptera: *Apis mellifera* Coleoptera: *Diaprepes abbreviatus*, *Tribolium castaneum* (TcasZ9desA HM234671 and TcasZ9desB HM234672).

2.4. Yeast expression of TcasZ9desA and TcasZ9desB

The pGEM T Easy clones, pGEM-TcasZ9desA and pGEM-TcasZ9desB were digested with *Bam*HI–*Sph*I and *Bam*HI–*Eco*RI, respectively. The desaturase-encoding fragments were ligated with similarly-digested pYES2 (Invitrogen Corporation, yeast expression plasmid) to create pYES-TcasZ9desA and pYES-TcasZ9desB, respectively. Positive clones were determined by restriction analysis and retained for yeast transformation. The pYES-TcasZ9desA and pYES-TcasZ9desB were transformed into *Saccharomyces cerevisiae ole1* strain (*MATa, ole1Δ*:LEU2, leu2-3, leu2-112, trp1-1, ura3-52, his4) using the Sigma Yeast Transformation kit. Transformants were selected on minimal media lacking uracil (Kaiser et al., 1994) and containing 1 mM *cis*-10-heptadecenoic acid and 1% tergitol (NP-40). To test for complementation of the OLE1 phenotype, transformants were patched onto YP medium (20 g l^{-1} peptone, 10 g l^{-1} yeast extract) containing 2% galactose. A control transformant, containing pYES2, was also prepared and tested.

To examine the production of desaturase products, *S. cerevisiae OLE1*/pYES-TcasZ9desA and *OLE1*/pYES-TcasZ9desB and control *OLE1*/pYES were cultured in 10 ml minimal medium without uracil, containing 2% galactose to induce gene transcription, 1% raffinose, and 0.5 mM Z-11-heptadecenoic acid added as a 1% tergitol solution (for *OLE1*/pYES control). The induced culture was grown for 72 h at 20 °C with shaking. Cells were collected by centrifugation (2000 *g*) and stored at −20 °C.

2.5. Lipid analysis of recombinant yeast and whole adult insects

Yeast cells were washed sequentially with 1% tergitol and MilliQ water with centrifugation at 1500*g* for 5 min at +4 °C and dried in a Savant SpeedVac Plus SC110A concentrator/dryer. Cells were directly treated with methanol/hydrochloric acid/chloroform (10:1:1) in a sealed test tube with heating at 90 °C for 60 min to convert lipids to fatty acid methyl esters (FAME). When cool, saline and hexane were added with shaking, and the hexane layer containing FAME was transferred to a vial for analysis by Varian 3800 gas chromatograph fitted with a BPX70 capillary column (Phenomenex 30 m × 0.32 mm × 0.25 μm). Injections were made in the split mode using helium as the carrier gas and an initial column temperature of 60 °C, raised at 20 °C/min until 170 °C, held for 5 min, then raised at 50 °C/min until 255 °C. Confirmation of FAME and dimethyloxazoline (DMOX) derivatives was obtained using a Varian 3800 gas chromatograph/1200 single quadrupole mass spectrometer. Mass spectra were acquired under positive electron impact in full scan mode between 50 and 400 amu at the rate of 2 scans per sec. Confirmation of the identity of a fatty acid was achieved by the conversion of the fatty acid to its DMOX derivative using the method of Yu et al. (1988) and comparison with DMOX mass spectra described in Dobson and Christie (2002) and references within.

2.6. Quantitative PCR analysis of TcasZ9desA and TcasZ9desB in various life stages

Total RNA was isolated from separately pooled larvae, pupae and adult *T. castaneum* lifestages using a RiboPure Kit (Ambion, Foster City, CA, USA) and quantified using a Nanodrop Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). RNA was DNase treated using SV Total RNA Isolation System (Promega, Madison, WI, USA). cDNA was then generated from 1 μg of total RNA using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). A no-reverse transcription control was included to test for DNA contamination. Purified RNA was visualized using electrophoresis and quantified. The ratio at 260/280 nm for the RNA samples was 2.0 and the ratio at 230/260 nm was 2.3.