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Molecular and functional characterization of CYP6BQ23, a cytochrome P450 conferring resistance to pyrethroids in European populations of pollen beetle, *Meligethes aeneus*

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ABSTRACT

The pollen beetle (*Meligethes aeneus* F.) is widespread throughout much of Europe where it is a major coleopteran pest of oilseed rape (*Brassica napus*). The reliance on synthetic insecticides for control, particularly the pyrethroid class, has led to the development of populations with high levels of resistance. Resistance to pyrethroids is now widespread throughout Europe and is thought to be mediated by enhanced detoxification by cytochrome P450ś and/or mutation of the pyrethroid target-site, the voltage-gated sodium channel. However, in the case of cytochrome P450 mediated detoxification, the specific enzyme(s) involved has (have) not yet been identified. In this study a degenerate PCR approach was used to identify ten partial P450 gene sequences from pollen beetle. Quantitative PCR was then used to examine the level of expression of these genes in a range of pollen beetle populations that showed differing levels of resistance to pyrethroids in bioassays. The study revealed a single P450 gene, *CYP6BQ23*, which is significantly and highly overexpressed (up to ~900-fold) in adults and larvae of pyrethroid resistant strains compared to susceptible strains. *CYP6BQ23* overexpression is significantly correlated with both the level of resistance and with the rate of deltamethrin metabolism in microsomal preparations of these populations. Functional recombinant expression of full length CYP6BQ23 along with cytochrome P450 reductase in an insect (Sf9) cell line showed that it is able to efficiently metabolise deltamethrin to 4-hydroxy deltamethrin. Furthermore we demonstrated by detection of 4-hydroxy *tau*-fluvalinate using ESI-TOF MS/MS that functionally expressed CYP6BQ23 also metabolizes *tau*-fluvalinate. A protein model was generated and subsequent docking simulations revealed the predicted substrate-binding mode of both deltamethrin and *tau*-fluvalinate to CYP6BQ23. Taken together these results strongly suggest that the overexpression of CYP6BQ23 is the primary mechanism conferring pyrethroid resistance in pollen beetle populations throughout much of Europe.

1. Introduction

Oilseed rape (*Brassica napus*) is a crop of global economic importance and, particularly in the case of winter oilseed rape, is grown on several million hectares in Europe and is indispensable in many crop rotations. The main winter oilseed rape growing countries in Europe are France, Germany, Poland and UK with a total cropping area exceeding 4 m ha (Richardson, 2008). Oilseed rape is attacked by a number of invertebrate pests of the order Coleoptera, such as flea beetles (*Psylliodes* ssp., *Phyllotreta* ssp.), stem weevils and seed pod weevils (*Ceutorhynchus* ssp.) and pollen beetle (*Meligethes ssp.*) (Williams, 2004). Although the genus *Meligethes* Stephens, 1830 (Coleoptera: Nitidulidae, Meligethinae) comprises globally more than 600 pollen eating species (Audisio et al., 2005), *Meligethes aeneus* F. is by far the most destructive species attacking oilseed rape crops throughout Europe (Williams, 2004). Adult beetles emerging from overwintering sites feed almost exclusively

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on pollen and cause damage during the early (green to yellow) stages, whereas feeding larvae cause bud abscission resulting in podless stalks. Pollen beetle infestations exceeding economic damage thresholds can for example result in yield reductions as high as 70% in spring oilseed rape (Hansen, 2003; Wegorek and Zamoyska, 2008).

The main method of crop protection against pollen beetle infestations is the large scale application of chemical insecticides, and a recent survey revealed that in many European countries 100% of the crop area received treatments against pollen beetle (Richardson, 2008). Over the last two decades pollen beetle control has mainly relied on the pyrethroid class of insecticides, e.g. *alpha*-cypermethrin, bifenthrin, cypermethrin, deltamethrin, *lambda*-cyhalothrin, *tau*-fluvalinate and *zeta*-cypermethrin (Heimbach et al., 2006). Pyrethroid insecticides are neurotoxic and bind to voltage-gated sodium channels in the insect central nervous system providing fast knock-down of pests at low application rates (Khambay and Jewess, 2005). This intensive use of pyrethroids has led to the development of resistance in pollen beetle, which was first reported in 1999 in North Eastern France (Hansen, 2003; Thieme et al., 2010; Slater et al., 2011). Concerted large scale pollen beetle pyrethroid resistance monitoring campaigns between 2007 and 2011 revealed that resistance has become widespread and is at high levels in several European countries including France, Germany, UK, Poland, Czech Republic, Denmark and Sweden (Slater et al., 2011; Zimmer and Nauen, 2011a).

Insecticide resistance most commonly evolves by two main mechanisms; increased levels of detoxification enzymes resulting in metabolic resistance, and target-site mutations resulting in lower binding affinity of the respective insecticides (Feyereisen, 1995; Li et al., 2007; Heckel, 2012). Pyrethroid resistance can be conferred by both mechanisms, but target-site insensitivity caused by mutations in the voltage-gated sodium channel, known as knock-down resistance (*kdr*), is a common mechanism in many pests (Williamson et al., 1996; Khambay and Jewess, 2005; Rinkevich et al., 2013). Target-site resistance to pyrethroids was first described in *Musca domestica* and linked to two mutations in the housefly voltage-gated sodium channel gene (*Vssc1*) leading to amino acid changes at positions L1014F (*kdr*) and M918T (*s-kdr*) in domain II of the channel protein (Williamson et al., 1996). Subsequently, many more mutations in voltage-gated sodium channels conferring pyrethroid target-site resistance have been described, but L1014F remains the most common mutation described in almost 20 different pest species (Rinkevich et al., 2013), including in pollen beetle (Nauen et al., 2012). However the lack of *kdr* in a large number of highly resistant pollen beetle populations collected in European countries other than Denmark and Sweden suggests enhanced metabolic detoxification of pyrethroids may be the main driver of resistance (Nauen et al., 2012). A recent study has provided several lines of evidence that support this assumption (Zimmer and Nauen, 2011b). Firstly, the authors were able to show that the efficacy of pyrethroids can be synergized by the cytochrome P450 (P450) inhibitor piperonyl butoxide (PBO), but not S,S,S-tributyl phosphorotrithioate (DEF, an esterase inhibitor) or diethyl maleate (DEM, a glutathione depleter affecting glutathione S-transferases). The synergistic effect of PBO against resistant pollen beetle populations was also shown by other authors to be primarily correlated with oxidative metabolism (Philipou et al., 2011). Furthermore, microsomal preparations from resistant strains showed a significantly increased rate of deltamethrin degradation in the presence of NADPH which was inhibited by PBO and other well-known P450-inhibitors such as tebuconazole and 1-aminobenzotriazole (Zimmer and Nauen, 2011b). Finally, LC-MS/MS analyses of 4-hydroxy deltamethrin formation in microsomes from several pollen beetle strains correlated with the level of pyrethroid resistance. Some pyrethroids such as bifenthrin and *tau*-fluvalinate were shown to be less affected by resistance, but shown to competitively inhibit the formation of 4-hydroxy deltamethrin, which correlates to some extent with the cross-resistance profile observed in resistant strains (Zimmer and Nauen, 2011a; Heimbach and Müller, 2013). Although all these findings strongly suggest that pyrethroid (cross-) resistance in pollen beetle is based on oxidative degradation by P450s, the specific enzyme(s) involved has (have) not been identified. Individual members of the arthropod cytochrome P450 gene superfamily are well known for their involvement in the detoxification of synthetic insecticides as well as toxic secondary plant metabolites (Li et al., 2007; Feyereisen, 2005, 2011; Schuler, 2011). Recent progress in genome and transcriptome sequencing has facilitated the discovery and functional characterization of several insect P450ś involved in pyrethroid resistance (Chiu et al., 2008; Zhu et al., 2010; Stevenson et al., 2011; Mitchell et al., 2012; Joußen et al., 2012; Riveron et al., 2013). However in Coleoptera only a single P450 from the red flour beetle, *Tribolium castaneum*, has been identified and functionally characterized for its involvement in pyrethroid resistance (Zhu et al., 2010). In this study the authors exploited microarray technology and reverse genetic approaches to demonstrate that a single brain-specific P450, CYP6BQ9, is overexpressed 200-fold in a deltamethrin resistant strain of *T. castaneum* and confers high levels of deltamethrin resistance. CYP6BQ9 was shown to metabolize deltamethrin to its 4-hydroxy derivative *in vitro*, similar to CYP6M2 of *Anopheles gambiae* and members of the CYP9J subfamily of *Aedes aegypti* (Stevenson et al., 2011, 2012).

The formation of 4-hydroxy deltamethrin by microsomal preparations has recently also been described to be correlated with pyrethroid resistance in pollen beetle (Zimmer and Nauen, 2011b). Therefore the aim of this study was to identify the P450(s) involved in pyrethroid resistance in pollen beetle populations sampled across Europe. Due to the lack of available genomic and transcriptomic data for pollen beetle P450 genes, a PCR approach employing degenerate primers based on conserved helix I and heme binding regions was used to identify P450 genes involved in pyrethroid resistance. We isolated a full-length cDNA clone of a single P450, CYP6BQ23 which is several hundred fold overexpressed in pyrethroid resistant pollen beetle adults and larvae. qRT PCR data shows that the overexpression of this P450 is significantly correlated with pyrethroid resistance. We characterized the substrate profile of recombinant CYP6BQ23 expressed in insect Sf9 cells as well as its ability to hydroxylate deltamethrin and *tau*-fluvalinate. Finally a protein homology model of CYP6BQ23 was generated and subsequent docking simulations used to provide insights into the possible orientation of pyrethroid substrates in the active site.

2. Material and methods

2.1. Insect populations and bioassays

European pollen beetle (*Meligethes aeneus* F.) populations were collected in oilseed rape fields between April and June in 2009 and 2010, except adults of strain 91-11 from Ukraine which were collected in 2011. All beetles were shipped to the authors' laboratory and maintained at 4 °C until testing for pyrethroid resistance (within 24 h after arrival). Each sample was subjected to morphometric analysis to check for *M. aeneus*, based on markers described by Scherney (1953), Fritsche (1955) and Freude et al. (1999). No other *Meligethes* ssp. were found in those samples used in this study. In May 2011 pollen beetle 2nd instar larvae were collected in winter oilseed rape fields from three different regions in Germany. Larvae can easily be collected in the late flowering stage of winter

oilseed rape (BBCH 67-69) by tapping the plants into a close-meshed insect net. Larvae were also stored at 4 °C until testing (within 24 h after sampling). From each adult and larval strain collected, a sample was flash frozen in liquid nitrogen and stored at −80 °C for subsequent molecular analysis. Pyrethroid resistance levels in adults were recently assayed using *lambda*-cyhalothrin as a reference pyrethroid insecticide in an adult vial test (Zimmer and Nauen, 2011a). Briefly: Ten beetles each were incubated at room temperature in 30 ml glass vials coated on the inner surface with 5–6 different concentrations of *lambda*-cyhalothrin dissolved in acetone (concentration range between 0.6 ng cm^{-2} and 375 ng cm^{-2}). Beetles were scored for mortality after 24 h and each concentration was replicated thrice as recently described (Zimmer and Nauen, 2011a). Second instar larvae of pollen beetle strains 40-11, 59-11 and 72-11 were recently tested and shown to be resistant to pyrethroids (Zimmer et al., 2013).

2.2. Chemicals

All chemicals and solvents used in this study were of analytical grade unless otherwise stated. Technical *lambda*-cyhalothrin and NAPDH were obtained from Sigma Aldrich. Deltamethrin was a Bayer CropScience internal analytical standard (purity >99%). Fluorescent artificial P450 substrates such as BFC, 7-benzyloxy-4-trifluoromethyl coumarin; MFC, 7-methoxy-4-trifluoromethyl coumarin; EFC, 7-ethoxy-4-trifluoromethyl coumarin; BOMFC, 7-benzyloxymethoxy-4-trifluoromethyl coumarin; BOMCC, 7-benzyloxymethoxy-3-cyano coumarin; PC, 7-n-pentoxy coumarin; EC, 7-ethoxy coumarin; BOMR, 7-benzyloxymethoxy resorufin; ER, 7-ethoxyresorufin; BR, 7-benzyloxyresorufin; MR, 7-methoxyresorufin; PR, 7-n-pentoxyresorufin, and all consumables for recombinant expression were purchased from Life Technologies.

2.3. DNA/RNA extraction and cDNA synthesis

Adults and 2nd instar larvae of individual pollen beetle samples were flash frozen in liquid nitrogen and kept at −80 °C prior to nucleic acids extraction. Nucleic acids were extracted from 15 to 20 pooled insects using either Agencourt DNAdvance kit (Beckman Coulter) for DNA extraction or TRIzol reagent (Invitrogen, CA, USA) followed by Agencourt RNAdvance Tissue kit (Beckman Coulter) for RNA extraction. Pools of insects were placed into pre-cooled (−80 °C) 1.5 ml Eppendorf vials including two 3 mm tungsten beads (Qiagen, Germany). Tubes containing samples and beads were deep frozen in liquid nitrogen and afterwards subjected to a Retsch TissueLyser (Qiagen) for 1 min at a frequency of 18 Hz for sample disruption. For total RNA extraction tubes were briefly centrifuged to collect the resulted powdery content at the bottom and 0.5 ml TRIzol reagent was added. Subsequently the tubes containing TRIzol, sample and beads were subjected again to Retsch TissueLyser and homogenized for 1 min at a frequency of 20 Hz and subsequently briefly centrifuged to collect the sediment. After 3 min incubation at room temperature 0.1 ml chloroform was added to each sample and mixed on a Vortex for 15 s. After 5 min incubation at room temperature the samples were centrifuged at 4 °C and 12,000 g for 15 min. The aqueous phase was transferred to a 96 deep well plate and processed on a Biomek NXp liquid handling platform (Beckman Coulter) according to Agencourt RNAdvance Tissue kit protocol. DNase I (Ambion, TX, USA) digestion to remove any genomic DNA was included in the purification process on the liquid handling platform. The quality and quantity of RNA was determined by spectrophotometry (NanoQuant Infinite 200, Tecan, Switzerland) and by running 1 μg RNA per sample on a 1.5% agarose gel using a denaturing RNA loading dye (Thermo-Fermentas, MS, USA). Intact RNA is indicated by a single bright band without any smear because of a specific 28S RNA as known from other insect species (Winnebeck et al., 2009). The RNA content of all samples was normalized to 500 ng μL^{-1} on a Biomek 3000 liquid handling platform (Beckman Coulter) and 2.5 μg total RNA was used in 20 μL reactions for cDNA synthesis using Superscript III (Invitrogen) and random hexamers equally mixed (v/v) with oligo dT primers (Invitrogen) according to the manufacturer's instructions.

For DNA extractions, the powdery content of the tubes was suspended in lysis buffer provided with Agencourt DNAdvance kit and processed on a Biomek NXp liquid handling platform according to Agencourt DNAdvance kit protocol. After purification the samples were treated with RNase A (Ambion) in Tris–HCl buffer pH 8.0, and re-purified using Agencourt AMPure reagent (Beckman Coulter). The DNA was quantified by spectrophotometry; quality was assessed by running an aliquot on a 1.5% agarose gel and samples were diluted to 2.5 ng μL^{-1}.

2.4. Isolation of P450 and reference genes

A degenerate primer PCR strategy was used to amplify CYP4 and CYP6 family specific regions of P450 genes as well as coding sequence regions of α-tubulin and actin. For this approach the same CYP specific primer pairs and PCR cycling conditions were used which recently resulted in the identification of eleven P450 genes including *CYP6CM1* in the cotton whitefly, *Bemisia tabaci* (Karunker et al., 2008). For α-tubulin and actin degenerate primer pairs (Table S1) were designed based on highly conserved regions among insect species determined by multiple alignment of several amino acid sequences (GenBank accession numbers for tubulin: AAF54067, AAF54433, EEZ99348, NP_001036884, XP_001120096, XP_001870369; and for actin: AAA28318, BAA74592, NP_001165844, XP_966960, XP_976003). Degenerate PCR was conducted in 25 μL reaction mixtures containing 1 μL cDNA (20 ng), 10 μM of each degenerate primer and 12.5 μL DreamTaq PCR Master Mix (Thermo-Fermentas) containing Taq polymerase, 2× PCR buffer and 4 mM $MgCl_2$ (2 mM final concentration). For actin and tubulin a nested PCR strategy was used, repeating the PCR with an inner primer pair using 0.5 μL of the primary PCR as a template. Samples were separated on a 1.2% (w/v) Agarose/TAE gel and PCR products of the expected size (440bp and 390 bp for CYP4 and CYP6 sequences, and ~600bp and ~890 bp for actin and tubulin, respectively) were extracted from the gel. The nucleic acid was isolated from the gel slices by Wizard SV Gel kit (Promega, WI, USA) and cloned into pSC-A-amp/kan vector using the Strataclone PCR cloning kit (Agilent Technologies, CA, USA). The resulting plasmids were purified from Minipreps using the GeneJET plasmid kit (Thermo-Fermentas), and sequenced with an automated DNA sequencer ABI model 3700 using the ABI BigDye Terminator Cycle Sequencing kit and M13 primers. Degenerate PCR was carried out on cDNA extracted from both pyrethroid resistant and susceptible pollen beetle populations. Amino acid sequence graphics were produced using Geneious v5.5 (www.geneious.com).

2.5. Real-time qRT-PCR and determination of P450 gene copy number

Real-time qRT-PCR was performed on a CFX-96 real time cycler (Bio-Rad Laboratories, CA, USA). Primer pairs were designed using the Primer3 program (Rozen and Skaletsky, 2000) to amplify a fragment of 90–150 bp in size for each gene (Table S2). Reaction mixtures (20 μL) contained 4 μL cDNA (5 ng), 10 μL of iQ SYBR Green Supermix (Bio-Rad Laboratories) and 0.25 μM of each primer. Thermocycling conditions were 3 min at 95 °C followed by 40

cycles of 95 °C for 15 s, 57 °C for 15 s and 72 °C for 20 s. A final melt-curve step was included post-PCR (ramping from 65°C to 95 °C by 0.5 °C every 5 s) to check for nonspecific amplification. Data analysis was performed with Bio-Rad CFX Manager 3.0 built in gene expression analysis module. Two reference genes, i.e. α-tubulin (GenBank KC840056.1) and actin (GenBank KC840045.1), were used for normalization according to the ΔΔCt method since this method allows the analysis of relative changes in gene expression to a (susceptible) reference population (Pfaffl, 2001). Both reference genes were stably expressed in all strains as they had acceptable M-values and a low covariance, α-tubulin (0.671; CV 0.2409) and actin (0.614; CV 0.2236). For each primer pair used, a standard curve was made using triplicate dilutions covering a 1000-fold range (20–0.02 ng), primer pairs were only used that had an $R^2 \geq 0.99$ and a PCR efficiency > 90% (see Table S2). PCR efficiency of individual targets was taken into account for gene expression analysis. Four independent biological replicates containing 15–20 beetles each were run in triplicate in each qRT-PCR experiment. Cq's of all samples were in the range of the standard curve and therefore no specific assay to detect the limit of quantification was developed. Non template controls and non-reverse transcription controls (containing an equivalent amount of RNA instead of cDNA) did not exceed the thresholds. A similar experimental design with gDNA as a template (10 ng per reaction) was used to determine the gene copy number of *CYP6BQ23* in different pollen beetle strains.

2.6. Rapid amplification of cDNA ends (RACE)

Five and three prime RACE was carried out using Invitrogen's 5′ RACE and 3′RACE System for Rapid Amplification of cDNA Ends and RLM-RACE kit (Ambion) following the manufacturer's protocols. For RACE purposes a cDNA pool of both pyrethroid resistant and susceptible strains was used. The details of the gene-specific primers used for RACE are listed in Table S1. Single PCR products were extracted from the gel, purified, cloned and sequenced as described above. In order to prove the assembly the full length coding sequence of *CYP6BQ23* we amplified and sequenced the CDS in different strains by nested PCR using primers *CYP6BQ23* F1, R1 and R2, respectively (Table S1). PCR reactions (20 μL) contained 1 μL cDNA (20 ng), 0.5 μM of each primer and 10 μl Phusion Flash High-Fidelity PCR Master Mix (New England Biolabs, MA, USA) were subjected to cycling conditions of: 15 s at 98 °C followed by 30 cycles of 98 °C for 10 s, 62 °C for 15 s and 72 °C for 30 s and a final extension step at 72 °C for 1 min. PCR products were purified using Agencourt AMPure reagent (Beckman Coulter) and directly sequenced using primers as detailed in Table S1.

2.7. Functional expression of CYP6BQ23 in Sf9 cells

The *M. aeneus* full length *CYP6BQ23* coding sequence of strain 79-10 (GenBank KC840055.1) and the *M. domestica* NADPH cytochrome P450 reductase (CPR) coding sequence (GenBank Q07994) were obtained by gene synthesis (Geneart, CA, USA). The *CYP6BQ23* and *CPR* sequence was inserted into the pDEST8 expression vector (Invitrogen). The PFastbac1 vector containing no foreign DNA was used to produce a control virus. The recombinant baculovirus DNA was constructed and transfected to Sf9 insect cells (Gibco) using the Bac-to-Bac baculovirus expression system (Invitrogen) according to the manufacturer's instructions. The titer of the recombinant viruses was determined following the standard protocols of the supplier. Sf9 cells were maintained in suspension culture under serum-free conditions (SF-900 II SFM, Gibco) at 27 °C containing 25 μg mL^{-1} gentamycin (Gibco). Insect cells grown to a density of 2×10^6 cells mL^{-1} were co-infected with recombinant baculoviruses containing *CYP6BQ23* and *CPR* with various MOI (multiplicity of infection) ratios to identify the optimal conditions. Control cells were co-infected with the baculovirus containing vector with no insert (ctrl-virus) and the recombinant baculovirus only expressing CPR using the same MOI ratios as described above. Ferric citrate and δ-aminolevulinic acid hydrochloride was added to a final concentration of 0.1 mM at the time of infection and 24 h after infection to compensate the low levels of endogenous heme in the insect cells. After 60 h cells were harvested and washed with PBS, and the microsomes of the membrane fraction were prepared according to standard procedures and stored at −80 °C (Phillips and Shephard, 2006). CYP6BQ23 expression and functionality was estimated by measuring CO-difference spectra in reduced samples (Omura and Sato, 1964).

2.8. Enzyme activity determination

CYP6BQ23 enzymatic activity was confirmed by its O-dealkylation and O-dearylation activity on a range of fluorescent model substrates (50 μM, 0.1 M Na-phosphate buffer pH 7.6, 0.1% DMSO) using CYP6BQ23 microsomes in a 96-well plate with the prepared microsomes of CYP6BQ23/NADPH CPR (10 μg in 50 μL assay volume, containing 1 mM NADPH; 30 min incubation at 27 °C while shaking at 800 rpm). After adding 100 μl DMSO:TRIZMA-base buffer 50% (v/v), 0.05 M, pH 10 into the wells the fluorescence was measured with a Spectra Max M2 reader (Molecular Devices) at the appropriate excitation/emission wavelength settings according to manufacturer instructions (Invitrogen). The activity of CYP6BQ23 microsomes was compared to control microsomes obtained from Sf9 cells infected with recombinant baculovirus containing only CPR. The protein content of samples was determined according to Bradford (1976) using bovine serum albumin as a reference.

2.9. Pyrethroid metabolism and UPLC MS/MS analysis

Deltamethrin metabolism was assayed by incubation of the recombinant CYP6BQ23/CPR (2 pmol P450 per assay) or ctrl-virus/CPR microsomes in 0.1 M potassium phosphate buffer with an NADPH-regenerating system (Promega; 1.3 mM NADP+, 3.3 mM glucose-6-phosphate, 3.3 mM $MgCl_2$, 0.4 U mL^{-1} glucose-6-phosphate dehydrogenase) and 12.5 μM deltamethrin (0.8–25 μM for Michaelis–Menten kinetics) at 27 °C for 1 h. The metabolism of *tau*-fluvalinate was measured under the same conditions but incubated at 27 °C for 240 min due to its slower degradation. The total assay volume was 200 μL using three replicates for each data point. Initial experiments were repeated twice with different batches of recombinant CYP6BQ23/CPR microsomes. Microsomes incubated without NADPH served as a control. The assay was quenched by the addition of ice-cold acetonitrile (to 80% final concentration), centrifuged for 10 min at 3000 g and the supernatant subsequently analysed by tandem mass spectrometry as recently described in Zhu et al. (2010). Recovery rates of deltamethrin using microsomal fractions without NADPH were normally close to 100%. Deltamethrin turnover from two independent reactions were plotted vs. controls. Michaelis–Menten kinetics were analysed using GraphPad Prism version 5 (GraphPad Software, CA, USA).

2.10. P450 modelling and substrate docking

The molecular model of CYP6BQ23 was created using the Orchestrar Suite module within the molecular modelling software SYBYLx2.0 (Certara, L.P., St. Louis, USA) based on the crystal structure of human CYP3A4 (Yano et al., 2004; PDB-ID:1TQN). This template structure was chosen because of its close homology to the

CYP6BQ23 sequence. The resulting raw model was subjected to energy minimization using the AMBER force field 2002 within SYBYLx2.0, to remove distortions and unallowed van der Waals contacts resulting from the molecular modelling process. The docking of the pyrethroids deltamethrin and *tau*-fluvalinate into the active site of CYP6BQ23 was realized using the software LeadIt Release 2.1.3 from BioSolveIT (St. Augustin, Germany). A pharmacophoric constraint was applied which allowed only positions where any heavy atom of the ligands is within a 3.5Å radius distance of the CYP6BQ23 heme iron centre. Thus, for deltamethrin in total 191 possible orientations were obtained, whereas for *tau*-fluvalinate 112 orientations were calculated. All obtained orientations were additionally assessed with the HYDE post-scoring function as recently described (Reulecke et al., 2008). These results allowed the prediction of the most likely ligand orientations of both deltamethrin and *tau*-fluvalinate for 4-hydroxylation in the active site of CYP6BQ23.

3. Results

3.1. Identification of pollen beetle P450 and reference genes

The degenerate CYP4 and CYP6 primer PCR approach resulted in ten gene fragments of approximately 440 bp and 390 bp in length, respectively. Subsequent cloning and sequencing of 40 clones of two strains (70-10 and 79-10) revealed that all fragments likely represent real P450 sequences as they all contain the conserved EXXR helix K-motif and the PXRF motif in the deduced amino acid sequence. The deduced amino acid identity between the obtained CYP6-like sequences ranged from 49.6 to 52.8% (60.6–64.1% nucleotide identity). The amino acid identity between the seven CYP4-like sequences ranged from 42.3 to 68.5% (26.6–68.8% nucleotide identity). This clearly indicates that all isolated sequences represent unique gene fragments, rather than allelic variants. The BLASTp alignment received for all sequences revealed P450s of three different coleopteran species as best hits. Most of the hits returned P450s from *T. castaneum* (red flour beetle), followed by *Dendroctonus ponderosae* (mountain pine beetle) and *Brontispa longissima* (coconut leaf beetle). All CYP6-like sequences seemed to be related to only one subfamily, CYP6B, whereas the CYP4-like sequences are related to three subfamilies, i.e. CYP4B, CYP4H and CYP4Q. The best sequence identity scores based on deduced amino acid sequences were obtained for P450s from *T. castaneum* (Table 1). PCR products of the degenerate PCR strategy for actin- and tubulin-like sequences were 509 bp and 602 bp in length respectively. After cloning and sequencing the most similar BLASTp hits for the isolated *M. aeneus* actin- and tubulin fragments were partial sequences of *beta*-actin of *Nasutitermes takasagoensis* (Isoptera: Termitidae) with 98.8% amino acid identity (GenBank BAI22849) and *alpha*-tubulin of *Teleopsis dalmanni* (Diptera: Diopsidae) showing 100% amino acid identity (GenBank AFM80094) respectively. The partial sequences for *M. aeneus* actin (GenBank KC840045.1) and *M. aeneus* tubulin (GenBank KC840056.1) were submitted to GenBank.

3.2. Expression of P450 genes in different strains of M. aeneus

The gene expression level of each P450 gene was checked by qRT-PCR on mRNA isolated from a number of pollen beetle strains collected from different regions in Europe. Two of the strains were completely susceptible (s) to pyrethroids (70-10 and 127-10) and all others were recently described as highly resistant (*r*) to pyrethroids showing resistance ratios >100-fold (Zimmer and Nauen, 2011a). Only a single P450, *CYP6BQ23* was significantly overexpressed in all pyrethroid resistant strains, but not in the two susceptible strains (Table 2). In strain 127-10 (s) some P450 genes are significantly down-regulated, but no difference is seen in *CYP6BQ23* expression compared to the original pyrethroid susceptible reference strain 70-10. A more detailed secondary screening was carried out to correlate the extent of pyrethroid resistance (based on *in vivo* bioassay results) and the expression level of *CYP6BQ23* mRNA in the same strains (Table 3). In total 13 pollen beetle strains from 8 different European countries were re-analysed and we obtained a significant correlation between the recently calculated resistance ratio based on LC_{50}-values for *lambda*-cyhalothrin and fold change in expression level (ΔΔCq) for *CYP6BQ23* ($r = 0.79$, $F = 18.7$, p-value $= 0.0012$). The remarkably high expression level of *CYP6BQ23* in pyrethroid resistant strains of *M. aeneus* was confirmed using a second primer set (Table S3). In conclusion our results suggest a significant link between overexpression of *CYP6BQ23* and pyrethroid resistance level irrespective of the geographic origin of the strain. In a subsequent analysis we also correlated the expression level of *CYP6BQ23* in a different set of strains recently analysed for pyrethroid resistance based on adult vial tests and the formation of 4-hydroxy deltamethrin in respective microsomal preparations (Zimmer and Nauen, 2011b). Once again we obtained a clear correlation between *in vivo* bioassays, *in vitro* deltamethrin metabolism and fold change in *CYP6BQ23* expression (Fig. 1A and B). Finally we investigated the expression level of *CYP6BQ23* in pollen beetle adults and larvae of individual strains collected in Germany and known to be resistant to pyrethroids (Zimmer et al., 2013). Again *CYP6BQ23* expression was significantly elevated in all three strains in both adults (ΔΔCq 175–365) and 2nd instar larvae (ΔΔCq 131–251) (Fig. 2).

3.3. Gene copy number

Quantitative PCR was used to compare *CYP6BQ23* gene copy number in six pollen beetle strains, i.e. 70-10 (s), 25-10 (r), 79-10

Table 1
Sequence identity calculated from BLASTp alignments of *M. aeneus* partial (helix I to heme binding region) CYP4, CYP6 and CYP6BQ23 deduced amino acid sequences to their most similar cytochrome P450 in *T. castaneum*.

P450 family[a]	Most similar P450 in *T. castaneum* (GenBank accession no.)	E-value	% Pairwise AA identity	Assigned *M. aeneus* name (GenBank accession no.)
CYP6	CYP6BQ10 (NP_001164249)	1e-166	53.0	CYP6BQ23 (KC840055.1)
	CYP6BK5 (EFA12633)	1.91e-50	59.2	CYP6-like 1 (KC840047.1)
	CYP6BQ13 (EEZ99338)	8.75e-51	63.1	CYP6-like 2 (KC840046.1)
CYP4	CYP4BN1 (NP_001123993)	1.52e-51	59.3	CYP4-like 1 (KC840051.1)
	CYP4H10 (NP_001107836)	3.07e-53	56.5	CYP4-like 2 (KC840054.1)
	CYP4Q6 (XP_970404)	4.04e-50	57.1	CYP4-like 3 (KC840048.1)
	CYP4Q9 (NP_001107850)	2.59e-62	64.4	CYP4-like 4 (KC840053.1)
	CYP4BN1 (NP_001123993)	2.19e-53	59.4	CYP4-like 5 (KC840049.1)
	CYP4BN1 (NP_001123993)	4.04e-54	62.8	CYP4-like 6 (KC840052.1)
	CYP4Q2 (NP_001107846)	4.12e-57	61.3	CYP4-like 7 (KC840050.1)

[a] CYP family was assigned based on BLASTp hits (except for CYP6BQ23).

Table 2
Fold change in expression of ten P450 genes in pyrethroid resistant (r) and susceptible (s) strains of *Meligethes aeneus*.

Strain		ΔΔCq (fold) ± SEM CYP6BQ23	CYP6-like 1	CYP6-like 2	CYP4-like 1	CYP4-like 2	CYP4-like 3	CYP4-like 4	CYP4-like 5	CYP4-like 6	CYP4-like 7
70-10[a]	s	1 ± 0.501	1 ± 0.122	1 ± 0.218	1 ± 0.362	1 ± 0.119	1 ± 0.272	1 ± 0.163	1 ± 0.249	1 ± 0.229	1 ± 0.163
127-10	s	1.45 ± 0.224	0.382 ± 0.08	0.159 ± 0.029*	0.455 ± 0.2	0.204 ± 0.013*	0.469 ± 0.166	0.102 ± 0.016**	2.26 ± 0.583*	0.52 ± 0.063	0.185 ± 0.004**
8-10	r	491 ± 169***	1.44 ± 0.173	0.503 ± 0.04	1.07 ± 0.669	0.687 ± 0.181	0.625 ± 0.073	0.495 ± 0.06	1.67 ± 0.403	1.02 ± 0.023	0.678 ± 0.042
57-10	r	548 ± 82.0***	0.874 ± 0.052	0.632 ± 0.039	1.18 ± 0.526	1.10 ± 0.247	0.705 ± 0.096	0.503 ± 0.04	2.42 ± 0.682	1.22 ± 0.122	0.743 ± 0.045
68-10	r	337 ± 87.0***	0.513 ± 0.105	0.725 ± 0.253	0.307 ± 0.042	1.10 ± 0.302	0.69 ± 0.133	1.19 ± 0.064	1.80 ± 0.288	1.08 ± 0.205	0.501 ± 0.171
79-10	r	938 ± 62.6***	1.33 ± 0.22	0.911 ± 0.127	2.29 ± 1.33	0.81 ± 0.119	1.30 ± 0.104	1.49 ± 0.341	1.51 ± 0.476	0.669 ± 0.079	0.711 ± 0.051
96-10	r	493 ± 81.5***	1.58 ± 0.559	0.433 ± 0.124	4.39 ± 1.72	0.979 ± 0.115	0.834 ± 0.05	0.862 ± 0.105	3.23 ± 1.63	1.59 ± 0.5	1.06 ± 0.22
106-10	r	549 ± 117***	0.878 ± 0.288	0.584 ± 0.111	2.57 ± 1.24	0.861 ± 0.095	1.21 ± 0.382	0.682 ± 0.07	126 ± 0.325	0.742 ± 0.03	0.807 ± 0.155
120-10	r	269 ± 85.2***	2.15 ± 0.943	0.627 ± 0.187	2.33 ± 0.995	0.63 ± 0.193	1.74 ± 0.26	1.03 ± 0.064	1.19 ± 0.492	1.04 ± 0.125	1.02 ± 0.192

Significance: ***P value < 0.001; **P value < 0.01; *P value < 0.05 (all other changes in gene expression are not significantly different from reference strain 70-10).
[a] Reference strain 70-10 (Ukraine, susceptible) (according to Zimmer and Nauen, 2011a).

(r), 106-10 (r), 120-10 (r) and 127-10 (s) using genomic DNA as a template. Data were normalized using two genes: *M. aeneus* actin and tubulin as described above. Even though the exact copy number for these reference genes is unknown they have been used due to the lack of other available sequence information and because the method is based on relative (s vs. *r*) rather than absolute quantification. No significant difference in copy number between pyrethroid susceptible and resistant strains was observed (fold change in copy number range from 0.985 to 1.46 for tubulin, and 0.956 to 1.52 for actin), indicating that the *CYP6BQ23* gene is not amplified or duplicated.

3.4. CYP6BQ23 cDNA characterization

The partial sequence of ~390 bp of *CYP6BQ23* obtained by degenerate PCR and represented by 10 clones of the resistant strain 79-10 was subjected to 3′ and 5′ RACE to obtain the complete cDNA sequence. The resulting CYP6BQ23 cDNA sequence (GenBank KC840055.1) contains an open reading frame (ORF) of 1566 bp encoding a putative P450 protein containing 522 amino acid residues (Fig. 3). Its calculated molecular weight is 59,460 Da and the predicted isoelectric point is 8.2. A BLASTp search indicated that CYP6BQ23 shows highest pairwise amino acid similarity with *T. castaneum* CYP6BQ10 (GenBank NP_001164249), i.e. 53% identity. The amino acid alignment revealed that the encoded protein contains a hydrophobic N-terminal domain that likely acts as a transmembrane anchor as is typical for microsomal monooxygenases. Furthermore the deduced amino acid sequence contains a number of conserved domains characteristic for P450 proteins such as the WxxxR motif (helix C), the oxygen binding motif of helix I (A/GGxE/DTT/S), the helix K motif (ExLR), the PxRF motif located after helix K and the heme-binding "signature" motif (PFxxGxxxCxG), as well as 6 predicted substrate recognition sites (SRS). Variation in the sequence of *CYP6BQ23* in several pollen beetle strains was examined by both direct sequencing and by cloning and sequencing a ~2040 bp fragment containing the full length ORF encoding for *CYP6BQ23* and most of the 5′ and 3′ UTR. The obtained fragments showed a 98.7% and 99.6% pairwise identity at nucleotide and amino acid level, respectively. In total seven non-synonymous SNP's were detected resulting in amino acid changes at positions 96, 156, 160, 174, 251, 420 and 443, but no obvious link to pyrethroid resistant or susceptible phenotypes could be found (Fig. 3; Table S4).

3.5. Functional expression of CYP6BQ23 and metabolism studies

CYP6BQ23 was recombinantly co-expressed with cytochrome P450 reductase (CPR) of *M. domestica* in Sf9 cells using a baculovirus expression system. The reduced CO-difference spectrum of CYP6BQ23 microsomes showed a distinct peak at 450.3 nm. The prepared microsomes contained a P450 concentration of 193 pmol/mg protein (data not shown). The functional activity of CYP6BQ23 was tested with a broad range of fluorescent model substrates and revealed that the recombinantly expressed protein is catalytically active (Fig. 4). The specific activity of CYP6BQ23 was highest with the artificial substrate BOMFC, i.e. 50.5 ± 0.13 pmol/min/mg protein (equal to 0.52 ± pmol/min/pmol P450 ± 0.023). The results seem to suggest a preference of CYP6BQ23 for bulkier molecules such as BOMFC, BFC and BOMR, whereas well-known standard substrates such as 7-ethoxy coumarin were not significantly metabolized (Fig. 4).

The metabolism of deltamethrin was measured by LC-MS/MS detection and quantification of its metabolite 4-hydroxy deltamethrin. The detection limit under the analytical conditions chosen for both deltamethrin and its 4-hydroxy metabolite was

Table 3
Relation between *CYP6BQ23* expression and pyrethroid resistance in *Meligethes aeneus*.

Strain	Country	LC_{50}, ng cm^{-2} (field rate %)[b]	95% FL[c]	RR[d]	MCq_E[e]	$_{\Delta\Delta}Cq$[f]	Expression SEM	*P*-value
70-10[a]	Ukraine	0.1 (0.1)	0.1–0.1	1	27.52	1	0.164	N/A
91-11	Ukraine	Not tested	–	–	27.32	1.09	0.192	>0.05
127-10	Finland	0.8 (1.1)	0.4–1.4	8	26.76	1.63	3.87	>0.05
84-09	Austria	0.8 (1.1)	0.6–1.2	8	25.18	4.54	0.677	<0.001
67-09	Austria	1.0 (1.4)	0.8–1.4	10	24.39	15.6	5.15	<0.01
128-10	Finland	1.2 (1.6)	0.4–2.8	12	25.51	10.5	0.190	<0.01
107-10	Czechia	3.7 (5)	2.3–6.1	37	21.89	107	7.21	<0.0001
8-10	France	15.5 (20.6)	4.9–45	155	18.59	634	107	<0.0001
120-10	Sweden	16.2 (21.6)	11.9–21.9	162	19.66	491	37.6	<0.0001
96-10	Poland	23.1 (30.8)	8.8–57.5	231	18.17	750	8.74	<0.0001
106-10	Czechia	36.9 (49.2)	18.8–69.4	369	18.49	804	54.9	<0.0001
68-10	Germany	38.3 (51.1)	13.1–109	383	19.75	325	14.8	<0.0001
82-10	Germany	50.6 (67.4)	25.5–93.8	506	18.68	614	37.5	<0.0001

[a] Susceptible reference strain.
[b] Rounded LC_{50} values for *lambda*-cyhalothrin in an adult vial test (data taken from Zimmer and Nauen, 2011a, b); the manufacturer recommended field rate for lambda-cyhalothrin is 7.5 g ha^{-1}.
[c] 95% fiducial limits.
[d] Resistance ratio (LC_{50} value of strain × divided by LC_{50} value of strain 70-10).
[e] MCq_E = Mean Efficiency Corrected Cq.
[f] $_{\Delta\Delta}Cq$ = fold change in expression.

0.1 ng ml^{-1}, so even very low metabolic rates could be detected. CYP6BQ23 microsomes incubated with deltamethrin in the absence of NADPH (even for prolonged times) did not show any metabolic activity as no 4-hydroxy deltamethrin was detected (Fig. 5), whereas incubations in the presence of NADPH resulted in the formation of 4-hydroxy deltamethrin (0.49 ± 0.039 pmol/min/pmol P450). Based on protein content Sf9 microsomes containing CYP6B23/CPR metabolized deltamethrin at a rate of approx. 60 pmol/min/mg, whereas virus-control (+CPR) Sf9 microsomes show a low turnover of 2.05 ± 0.14 pmol/min/mg protein, i.e. 30-fold lower efficiency. This result confirms the metabolism of deltamethrin by recombinantly expressed CYP6BQ23.

The rate of deltamethrin hydroxylation by recombinantly expressed CYP6BQ23 is time-dependent and followed Michaelis–Menten kinetics in response to deltamethrin concentration resulting in a K_m value of 9.5 ± 1.4 μM and a catalytic activity K_{cat} of 0.92 ± 0.06 pmol/min/pmol P450 (Fig. 6).

In a second set of experiments utilizing Michaelis–Menten kinetics we were able to show that both *tau*-fluvalinate and *lambda*-cyhalothrin competitively inhibit the formation of 4-hydroxy deltamethrin when co-incubated with different concentrations of deltamethrin, and as a result K_m-values change dramatically (Fig. 7). The finding that *tau*-fluvalinate is likely to compete with deltamethrin binding to the catalytic site of recombinantly expressed CYP6BQ23 is supported by the fact that we were able to detect 4-hydroxy *tau*-fluvalinate, when incubating CYP6BQ23 microsomes with *tau*-fluvalinate. Analysis of samples subjected to ESI-TOF high resolution MS/MS clearly revealed the presence of 4-hydroxy *tau*-fluvalinate and several characteristic fragments (Fig. 8), providing confirmation that CYP6BQ23 is capable of metabolizing both deltamethrin and *tau*-fluvalinate by 4-hydroxylation of the pyrethroid alcohol moiety.

3.6. CYP6BQ23 modelling and substrate docking

The results obtained from microsomal incubations containing recombinantly expressed CYP6BQ23 are supported by molecular modelling studies. A protein model of CYP6BQ23 based on the crystal structure of CYP3A4 was generated and *in silico* docking studies with both deltamethrin and *tau*-fluvalinate helped to rationalize the experimental findings with regard to their hydroxylation of the phenoxybenzyl 4′ site (Fig. 9). The predicted binding site of CYP6BQ23 easily accommodates both pyrethroids and Arg112 and Phe128 of SRS1 are predicted to be most important in relation to the correct orientation of both substrates. However docking simulations support 4-hydroxylation as a major mechanism of deltamethrin as well as *tau*-fluvalinate metabolism, albeit the catalytic reactivity is potentially greater with deltamethrin as it docks closer to the oxygen coordinated heme center of the active site of CYP6BQ23 (Fig. 9B).

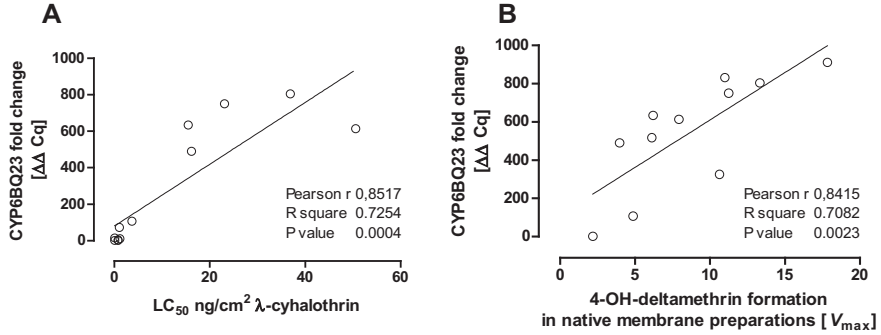

Fig. 1. Pearson correlation between *CYP6BQ23* fold change in expression level and pyrethroid resistance in different pollen beetle populations (A) *in vivo* based on LC_{50}-values for *lambda*-cyhalothrin obtained in an adult vial test (24 h), and (B) *in vitro* based on 4-OH-deltamethrin formation in microsomal preparations. The level of *CYP6BQ23* transcripts was determined in this study in pollen beetle populations frozen at −80 °C and recently characterized for pyrethroid resistance in another study (Zimmer and Nauen, 2011b).

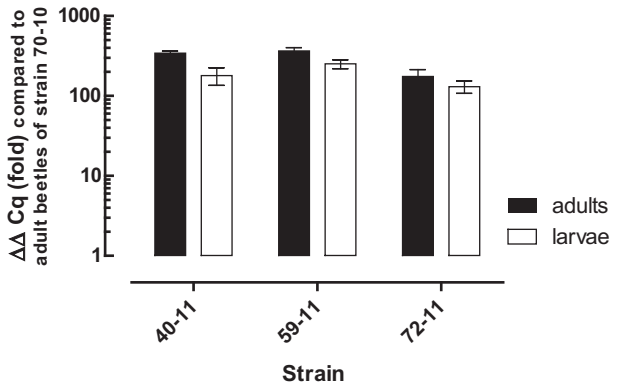

Fig. 2. *CYP6BQ23* fold change expression in adults and larvae of pyrethroid resistant pollen beetle (collected in Germany in 2011) compared to a highly susceptible strain 70-10 (Ukraine, data not shown). Data are mean values ± SEM ($n = 4$).

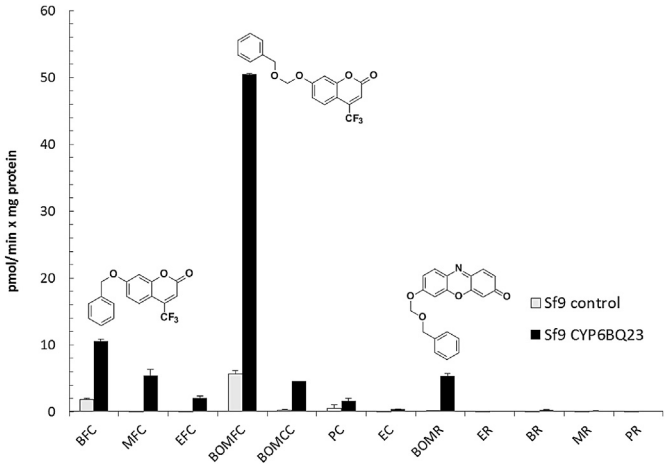

Fig. 4. Metabolism (O-dealkylation/dearylation) of different artificial coumarin and resorufin substrates by functionally expressed CYP6BQ23. Data are mean values ± SD ($n = 4$). Abbreviations: BFC, 7-benzyloxy-4-trifluoromethyl coumarin; MFC, 7-methoxy-4-trifluoromethyl coumarin; EFC, 7-ethoxy-4-trifluoromethyl coumarin; BOMFC, 7-benzyloxymethoxy-4-trifluoromethyl coumarin; BOMCC, 7-benzyloxymethoxy-3-cyano coumarin; PC, 7-n-pentoxy coumarin; EC, 7-ethoxy coumarin; BOMR, 7-benzyloxymethoxy resorufin; ER, 7-ethoxyresorufin; BR, 7-benzyloxyresorufin; MR, 7-methoxyresorufin; PR, 7-n-pentoxyresorufin.

4. Discussion

The main aim of this study was to identify the molecular mechanism(s) driving metabolic resistance to pyrethroid insecticides in *M. aeneus*, a destructive coleopteran pest in European oilseed rape production. As a consequence of increased resistance problems with pyrethroids, insecticides with different modes of action were tested (Schroeder et al., 2009), and subsequently introduced for control, such as the neonicotinoid thiacloprid, which acts agonistically on insect nicotinic acetylcholine receptors (Jeschke and Nauen, 2008). Several resistance monitoring projects revealed the spread of pyrethroid resistance in pollen beetle all over Europe (Slater et al., 2011; Zimmer and Nauen, 2011a). However, it was only recently that elevated levels of microsomal monooxygenase activity coupled with increased deltamethrin metabolism was described as a mechanism of resistance which significantly correlates with pyrethroid resistance in pollen beetle collected from a wide geographic range (Zimmer and Nauen, 2011b). Pyrethroid resistance in pollen beetle frequently exceeds resistance ratios of 500-fold based on pyrethroid contact bioassays (Zimmer and Nauen, 2011a), so the metabolic mechanism

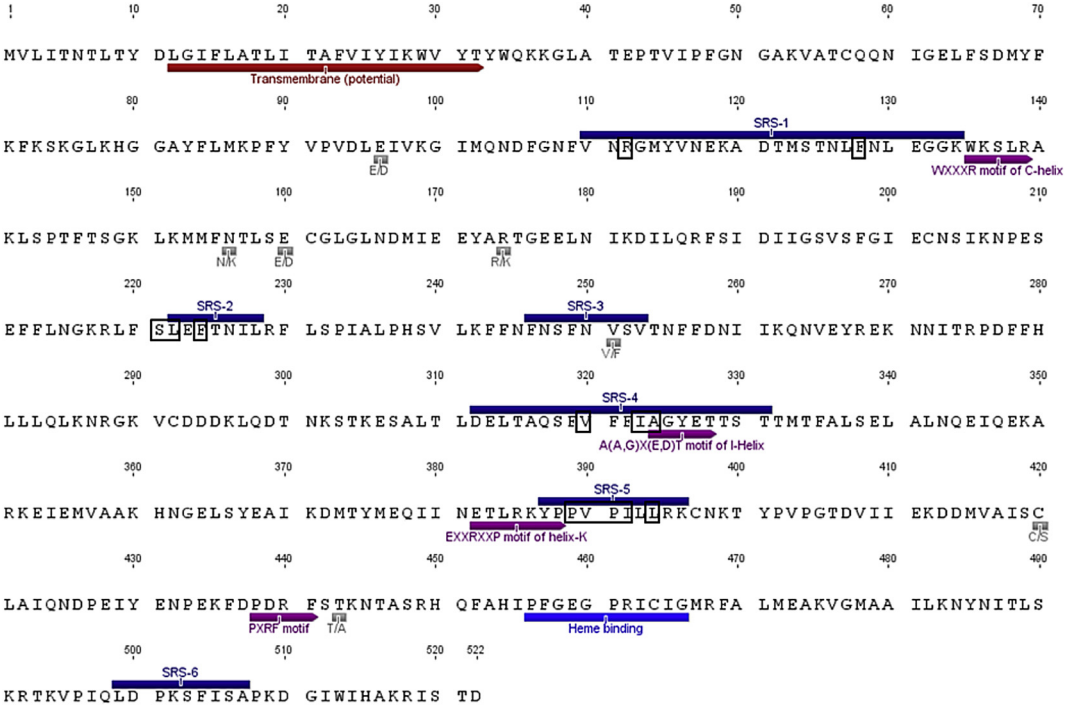

Fig. 3. Deduced amino acid sequence of *Meligethes aeneus* CYP6BQ23 (GenBank KC840055.1). Amino acid substitutions are indicated by a grey box below the sequence. The predicted transmembrane region is indicated by an arrow (position 12). Conserved domains common to cytochrome P450s such as the helix C motif (position 135), the helix I motif (position 324), the helix K motif (position 382), the PXRF motif (position 438) and the heme binding motif (position 456) as well as proposed substrate recognition sites (SRS) are indicated. Boxed amino acid residues constitute the binding site of the CYP6BQ23 protein model, and are defined by a distance of ≤3.5Å from any atom of both deltamethrin and tau-fluvalinate substrates docked to the active site.

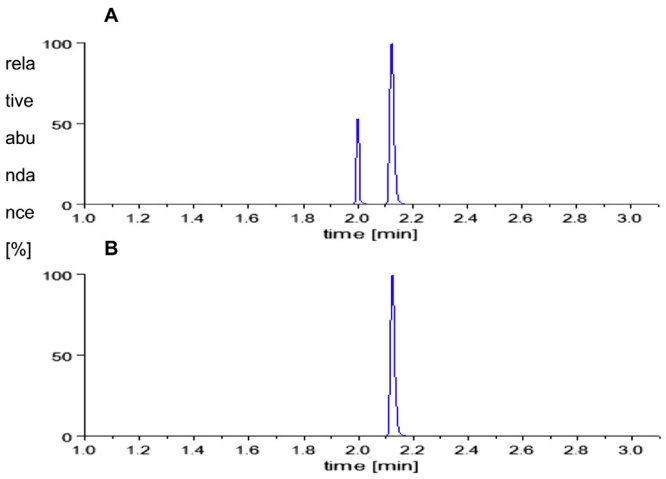

Fig. 5. UPLC-MS analysis of deltamethrin and its 4-hydroxy metabolite after incubation with microsomes isolated from Sf9-cells recombinantly expressing CYP6BQ23: A) Incubation of 2.5 nmol deltamethrin for 60 min in the presence of NADPH; B) incubation of 2.5 nmol deltamethrin for 240 min in the absence of NADPH.

expressed by pollen beetles seems well able to confer strong levels of resistance. Similar cases of P450-mediated resistance have recently been described in other insect pests including *B. tabaci* (Karunker et al., 2008, 2009; Nauen et al., 2013), *Myzus persicae* (Puinean et al., 2010), *Nilaparvata lugens* (Bass et al., 2011), *T. castaneum* (Zhu et al., 2010), *M. domestica* (Scott, 1999) and *Trialeurodes vaporariorum* (Karatolos et al., 2012). In these cases a single P450 of the CYP6 family was shown to be associated with resistance to insecticides belonging to different chemical classes, and in some cases functional evidence was provided that the candidate gene identified encodes a P450 protein which metabolizes the insecticide of interest, e.g. CYP6CM1 of *B. tabaci* was shown to metabolize imidacloprid and CYP6BQ9 of *T. castaneum* detoxifies deltamethrin (Karunker et al., 2009; Zhu et al., 2010).

Out of the ten P450 partial gene sequences identified in this study only one, CYP6BQ23 could be correlated with high levels of pyrethroid resistance in *M. aeneus*. Despite this finding we cannot exclude the possibility that other CYP6BQ enzymes are overexpressed in resistant strains in addition to CYP6BQ23 but were not identified by the degenerate primers used in our PCR approach. Due to the lack of transcriptomic data for *M. aeneus* we resorted to an approach recently used to successfully identify CYP6CM1 as the major P450 conferring neonicotinoid resistance in *B. tabaci*

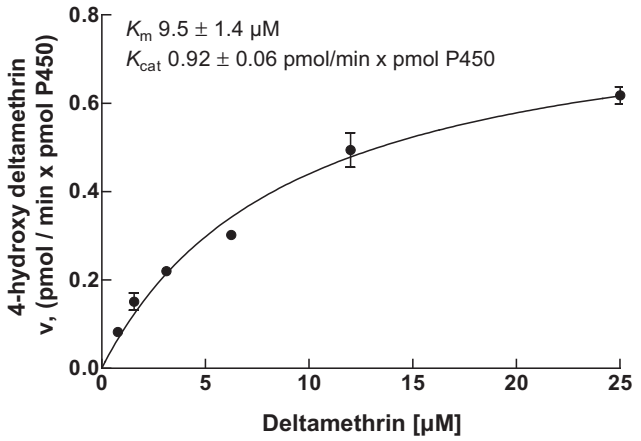

Fig. 6. Michaelis–Menten kinetics of deltamethrin hydroxylation by recombinantly expressed CYP6BQ23 analysed by non-linear regression. Data points are mean values ± SEM ($n = 3$).

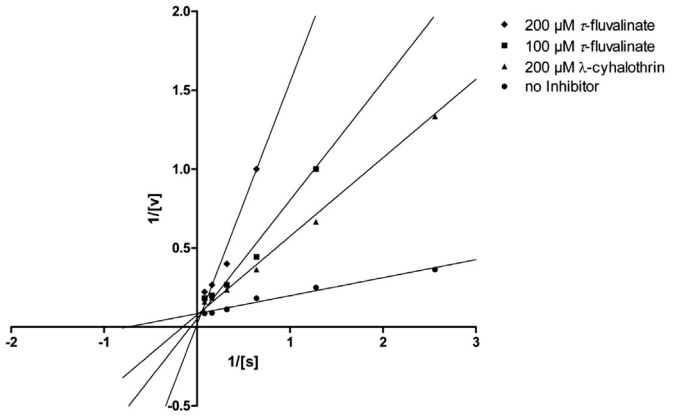

Fig. 7. Lineweaver–Burk plot showing the competitive inhibition of CYP6BQ23-catalysed deltamethrin hydroxylation by different concentrations of *tau*-fluvalinate and *lambda*-cyhalothrin (1 h incubation at 27 °C). [s] = µM deltamethrin.

(Karunker et al., 2008). We are aware that this approach has its limitations and we may have missed additional P450s that may also contribute to pyrethroid resistance. However, based on pairwise amino acid similarity CYP6BQ10 of *T. castaneum* shows the highest identity to CYP6BQ23, followed by CYP6BQ9 recently described as a brain-specific P450 conferring deltamethrin resistance (Zhu et al., 2010). Similar to recombinantly expressed CYP6BQ23, CYP6BQ9 was shown to metabolize deltamethrin to 4-hydroxy deltamethrin. Considering deltamethrin metabolism in insects, the phenoxybenzyl 4′ site is also described in other studies to be the major site of hydroxylation in deltamethrin metabolism, for example CYP6M2 shows 4-hydroxylation as a main route of deltamethrin detoxification in the malaria vector *A. gambiae*, whereas hydroxymethyl deltamethrin was described as a minor metabolite (Stevenson et al., 2011). The CYP6M2 K_m and K_{cat} values of 2.0 µM and 1.2 min^{-1} reported for deltamethrin are similar to those values obtained for CYP6BQ23. CYP6P9 of *Anopheles funestus* was also shown to metabolize deltamethrin as well as other pyrethroids such as permethrin and bifenthrin as measured by substrate depletion (Riveron et al., 2013). However, no primary metabolites were included as standards or elucidated by structural analysis, so it remains unclear whether the observed substrate depletion of highly hydrophobic pyrethroids provided by CYP6P9 is due to sequestration or indeed metabolism. Furthermore a series of CYP9J P450s of *A. aegypti* were also shown to metabolize pyrethroids with CYP9J32 showing the strongest detoxification of deltamethrin and again kinetic parameters were in a similar range to those we described in this paper for CYP6BQ23 (Stevenson et al., 2012). CYP6BQ23 was shown to hydroxylate the phenoxybenzyl 4′ site of deltamethrin and it would be interesting in future studies to examine its capability to sequentially metabolize deltamethrin and other pyrethroids as recently shown for CYP6M2 (Stevenson et al., 2011). Other P450s such as CYP6Z8 of *A. aegypti* were recently shown to play a pivotal role in processing deltamethrin metabolites resulting from esterase mediated metabolism, i.e. phenoxybenzyl-alcohol and -aldehyde (Chandor-Proust et al., 2013). Apart from CYP6BQ23 there is only one additional P450 from an agricultural pest which was shown to metabolize pyrethroids when functionally expressed: CYP337B3 in Australian *Helicoverpa armigera* which resulted from an equal crossing-over of two parental P450s and was recently shown to 4-hydroxylate fenvalerate when functionally expressed in Ha2302 cells (Jouβen et al., 2012).

Only a few P450s have been shown to metabolize *tau*-fluvalinate, a pyrethroid not used for vector control due to its lower overall efficacy and limited knockdown properties compared to

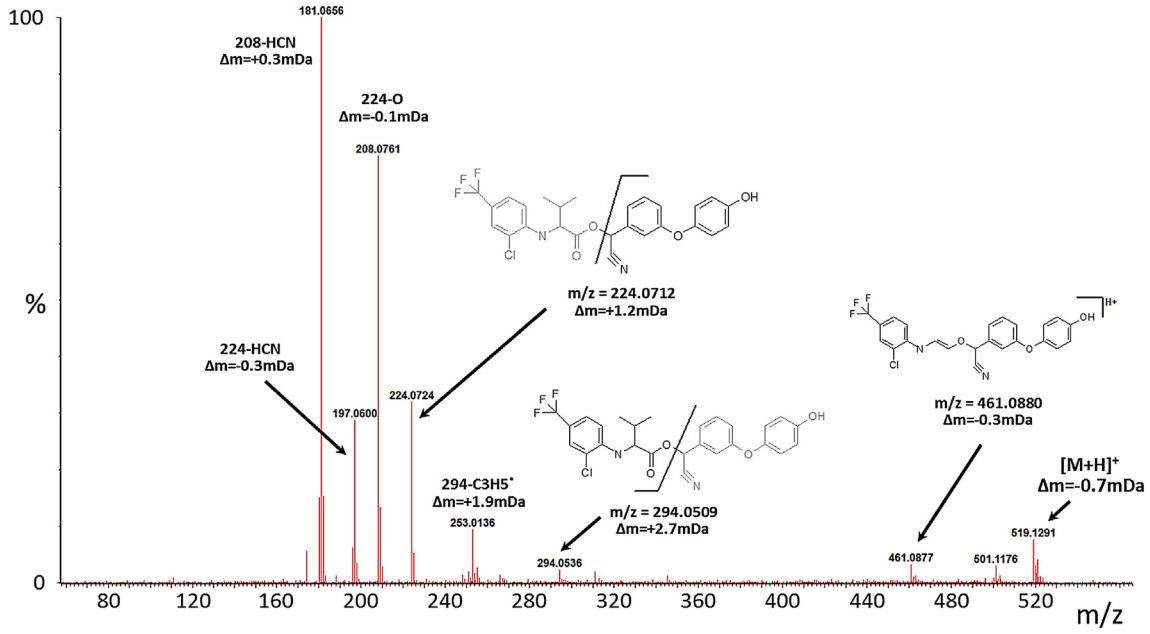

Fig. 8. ESI-TOF high resolution MS/MS-spectrum of 4-hydroxy *tau*-fluvalinate resulting from incubations of *tau*-fluvalinate with recombinantly expressed CYP6BQ23. The molecular ion $[M + H]^+$ is detected at *m/z* 519.13. Δm is the mass difference in Milli-Dalton of the detected fragment vs. its theoretical molecular mass.

other pyrethroids (Khambay and Jewess, 2005). Our study represents the first description of 4-hydroxylation of *tau*-fluvalinate by a functionally expressed P450 derived from a major agricultural pest. However 4-hydroxylation was recently described by CYP9Qs from *Apis mellifera* in order to investigate the selectivity of *tau*-fluvalinate as it is particularly used for *Varroa* mite control in bee hives without affecting honeybees (Mao et al., 2011). The metabolic fate of *tau*-fluvalinate is well understood in vertebrates such as rats and P450 mediated formation of 4-hydroxy *tau*-fluvalinate is an important initial step in its degradation (Quistad et al., 1983). It is interesting that our CYP6BQ23 modelling coupled with substrate docking simulations for both deltamethrin and *tau*-fluvalinate suggests to some extent a lower catalytic activity towards the latter due to the increased distance of the phenoxybenzyl 4′ site to the heme iron. It has been shown that *tau*-fluvalinate is less affected by microsomal oxidation in pollen beetles expressing high resistance to *lambda*-cyhalothrin, however, cross-resistance between these insecticides has been described, albeit at a somewhat lower level than between deltamethrin and *tau*-fluvalinate (Zimmer and Nauen, 2011b). It would be interesting therefore to look for additional P450s in *M. aeneus* that might be more specific for *tau*-fluvalinate, as increasing resistance to this pyrethroid was recently reported in some German populations (Heimbach and Müller, 2013). In relation to this it has been recently shown that Danish and Swedish *M. aeneus* populations survive field rate applications of *tau*-fluvalinate by expressing *kdr*-like target-site resistance in combination with elevated levels of oxidative detoxification (Nauen et al., 2012).

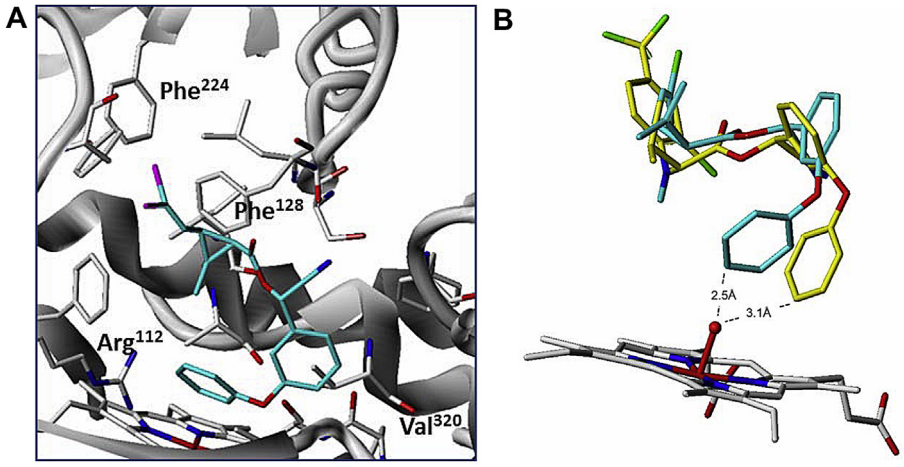

Fig. 9. (A) Binding site model showing the predicted substrate-binding mode of deltamethrin in *Meligethes aeneus* CYP6BQ23 protein. The predicted structure of CYP6BQ23 is based on the crystal structure of human CYP3A4 (PDB-ID:1TQN). Amino acid residues shown constitute the binding site defined by a distance of ≤3.5Å from any atom of deltamethrin shown in light blue elemental stick format on the ribbon backbone. Amino acids predicted to be most critical for the observed substrate orientation and docking are R112/F128 (SRS1), F224 (SRS2) and V320 (SRS3). (B) Docking models of CYP6BQ23 with both deltamethrin and tau-fluvalinate in light blue and yellow elemental stick format, respectively. For the sake of clarity the amino acid residues forming the active site within a distance of ≤3.5Å from any atom of both substrates are not shown, but the predicted binding mode and best-ranked docking for 4-hydroxylation with the distance to O coordinated to Fe indicated. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The metabolism profile of fluorescent model substrates by CYP6BQ23 suggests a preference for bulkier substrates being O-dearylated rather than smaller ones being O-dealkylated. CYP6BQ23 shows the highest activity with BOMFC, followed by BFC and BOMR. However, it is not yet clear if these substrates could serve as non-pyrethroid probes to monitor for the presence of elevated P450 activity conferring pyrethroid resistance. BOMR was recently also shown to be an excellent substrate for functionally expressed CYP6BQ9 which metabolizes deltamethrin in *T. castaneum* (Zhu et al., 2010). Stevenson et al. (2012) also investigated the metabolism of a number of fluorescent substrates by *A. aegypti* and *A. gambiae* P450s and concluded that non-pyrethroid metabolizing CYP6Z2 showed a marked preference for smaller probe substrates which was in contrast to pyrethroid-metabolizing CYP6P3 and CYP6M2 which preferred bulkier substrates. Recently BFC was also demonstrated to be a preferred substrate for CYP6Z8 of *A. aegypti* (Chandor-Proust et al., 2013).

CYP6BQ23 is several hundred folds overexpressed suggesting a fairly high concentration of this P450 in microsomes of pollen beetles which are resistant to pyrethroids. This may be a mechanism of compensating for the somewhat lower catalytic rate of deltamethrin detoxification of this P450 compared to other insect P450s especially those from mosquitoes. The molecular mechanisms explaining the evolutionary origin of CYP6BQ23 and those driving the constitutive overexpression of CYP6BQ23 in resistant *M. aeneus* are not known, but we show that overexpression does not result from gene amplification, a mechanism shown to drive overexpression of CYP6CY3 in neonicotinoid resistant *M. persicae* (Puinean et al., 2010). Similarly tandemly duplicated CYP6P9 P450s in *A. funestus* were shown to drive pyrethroid resistance (Riveron et al., 2013). An interesting case of gene-duplication and parallel evolution of *cis*-acting genomic changes by insertion of the retrotransposon *accord* resulting in the upregulation of *Drosophila melanogaster Cyp6g1* expression, which confers resistance to DDT, has recently been reviewed (ffrench-Constant, 2013). Another *cis*-regulatory motif was recently described that enhances the expression of CYP9M10 in larvae of *Culex quinquefasciatus* and is associated with pyrethroid resistance. In this case the regulatory element was only present upstream of *CYP9M10* in resistant strains where it was shown to drive 10-times higher expression of a fluorescent reporter gene (Wilding et al., 2012). It is possible that the CYP6BQ23 mediated resistance mechanism in *M. aeneus* evolved independently in different geographic areas since the dispersal rate of pollen beetles is limited by the fact that they normally overwinter close to the sites where they feed during spring/summer time (Williams, 2004). To examine this possibility and to check for overexpression of P450 genes other than CYP6BQ23 it would be useful to carry out a transcriptomic analysis of pollen beetle populations from different areas. Such an analysis may also disclose other P450s that together with CYP6BQ23 contribute to the sequential detoxification of pyrethroids in pollen beetle populations throughout Europe. In combination with the present work such studies will provide a better understanding of how to manage insecticide resistance in *M. aeneus* and so contribute to sustainable oilseed production in Europe.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ibmb.2013.11.008.

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