2007

Cloning and Expression of Multidrug Resistance Genes in the Cabbage Looper Moth, Trichoplusia ni

Olivia D'Souza
Western University

Cloning and Expression of Multidrug Resistance Genes in the Cabbage Looper Moth, *Trichoplusia ni*

(Spine title: Multidrug Resistance Genes in *Trichoplusia ni*)

(Thesis format: Monograph)

By

Olivia D'Souza

Graduate Program in Zoology

A thesis submitted in partial fulfillment of the requirements of the degree of Master of Science.

Faculty of Graduate Studies
The University of Western Ontario
London, Ontario, Canada

Abstract

Three genes encoding multidrug resistance proteins were identified in *Trichoplusia ni* larvae, named *trnMDR1*, *trnMDR2* and *trnMDR3*. Their open reading frames were sequenced, and the translated sequences were compared to each other as well as to multidrug resistance proteins from other species. Quantification of their expression in various tissues of caterpillars fed on either artificial diet or organically-grown cabbage revealed upregulation of *trnMDR1* in Malpighian tubules, integument and fat body, *trnMDR2* in fat body and *trnMDR3* in brain in response to the plant diet. Sf9 cells stably transformed with either *trnMDR1* or *trnMDR3* were used to assay MDR protein transport of calceinAM and vinblastine, as well as to test the inhibitory effect of cyclosporin A and verapamil on MDR protein activity. No significant calceinAM transport or inhibition of MDR activity by cyclosporin A or verapamil was observed. Cell lines stably transformed with *trnMDR1* demonstrated increased resistance to vinblastine toxicity. This study suggests that tissue-specific upregulation of MDR protein expression may contribute to caterpillar defences against toxic plant secondary metabolites, and that inhibitors of MDR proteins could be exploited as synergists with pesticides that are detoxified in a similar fashion. The assay system of Sf9 cells transformed with *T. ni* MDR genes could be used to screen for such potential MDR inhibitors.

Keywords: multidrug resistance, insect, inhibitor, pesticide, expression, diet, calceinAM, verapamil, cyclosporin A, vinblastine.

Table of Contents

List of Figures

List of Tables

List of abbreviations

ABC	ATP-binding cassette
ADP	Adenosine diphosphate
aeaMDR	*Aedes aegyptii* multidrug resistance protein
AM	acetoxymethylester
ATP	adenosine triphosphate
bp	base pair
BSA	Bovine serum albumin
°C	Degrees Celsius
caeMDR	*Caenorhabditis elegans* multidrug resistance protein
calceinAM	calcein acetoxymethyester
cDNA	complementary DNA
CF-203	*Choristonuera fumiferana* 203
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP(s)	2'-deoxynucleoside 5'-triphosphate(s)
drmMDR	*Drosophila melanogaster* multidrug resistance protein
g	acceleration due to gravity
G3PDH	Glyceraldehyde 3-phosphate dehydrogenase
h	hour

hosMDR	*Homo sapiens* multidrug resistance protein
IPTG	Isopropyl β-D-1-thiogalactopyranoside
Kb	kilobase
kDa	kiloDalton
LB	Luria Bertani
MDCK	Madden-Darby Canine Kidney
MDR	Multidrug resistance
mg	milligram
min	minute
mm	millimetre
mM	millimolar
ml	millilitre
mRNA	messenger RNA
MRP	Multidrug resistance-associated protein
MT	Malpighian tubules
mumMDR	*Mus musculus* multidrug resistance protein
µg	microgram
µl	microlitre
µM	micromolar
ng	nanogram
nm	nanometre
OD_{260}	Optical density at 260 nm
ORF	Open reading frame
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PSN	Penicillin-Streptomycin-Neomycin
qPCR	quantitative PCR
RACE	Rapid amplification of cDNA ends
RNA	Ribonucleic acid
s	second(s)
Sf21	*Spodoptera frugiperda* 21
Sf9	*Spodoptera frugiperda* 9
SF900-II SFM	*Spodoptera frugiperda* 900-II serum-free medium
Tris	trishydroxymethylaminomethane
U	unit(s)
VBL	vinblastine

To my parents, Ida and Anthony D'Souza.

Acknowledgements

I would like to thank my supervisors, Cam Donly and Stan Caveney, for their constant support and advice. Supervisors can have a significant impact on a graduate student's quality of life, and I have been fortunate that mine were always encouraging and always generous with positive feedback. They helped me interpret my data and suggested alternative experiments when ones I had already done were unsuccessful. Without their improvements to my writing and presentations, the message I intended for my audience would have had very little connection to the one I actually gave.

I am grateful for the help and encouragement I have had from the technicians in our lab, Lou Ann Verellen and Wendy Cladman, and my fellow student Roselyne Labbé. Lou Ann taught me almost every technique I have used. She and Wendy provided a great deal of technical advice and many suggestions, and between them kept my project from grinding to a halt several times. In addition to her help in dissection and RNA extraction, Roselyne provided invaluable moral support and friendship, and made my time in the lab more fun than work.

I want to thank my advisory committee, Dr. Anthony Percival-Smith and Dr. Michael O'Donnell, for their advice and suggestions. Thanks also to many people at Agriculture and Agri-Food Canada, including Aldona Gaidauskas-Scott, Pat Moy, Terry Ainsworth, Jamie McNeil, Ida van Grinsven and Jacqueline Hill for their help with various parts of my project. Many thanks to Mary Dillon at the University of Western Ontario for keeping me informed about important deadlines and procedures.

Finally, special thanks to my parents, my brother and my boyfriend, for their love and caring, and their ability to make me laugh. I could not have done this without them.

1. Introduction

The phenomenon of cellular resistance to a variety of structurally and functionally diverse compounds is known as multidrug resistance (MDR) (Ambudkar et al., 2003; Ling, 1997). A genetic factor shown to contribute to such resistance is overexpression of the multidrug resistance protein (MDR protein), also known as p-glycoprotein (Ambudkar et al., 2003; Bosch and Croop, 1998). Multidrug resistance proteins were originally discovered in the context of cancer research, as a means by which cancer cells are able to become resistant to chemotherapy (Ferry and Kerr, 1994). A line of Chinese hamster ovary cells was found to be resistant to accumulation of a range of clinically significant chemotherapy drugs. This resistance was correlated with overexpression of a membrane glycoprotein which was named p-glycoprotein. The "p" stands for permeability, and the protein was given this name because it was originally thought to decrease permeability to chemotherapy drugs (Juliano and Ling, 1976).

1.1 MDR protein structure, evolution and activity

Multidrug resistance proteins are members of the ATP-Binding Cassette (ABC) superfamily of proteins (Dean et al., 2001). These proteins are located in cell and organelle membranes where they use energy from ATP hydrolysis to drive the transport of substances out of the cell or organelle against their concentration gradient (Dean et al., 2001; Higgins and Linton, 2004). The proteins are large, ranging in molecular weight from 140 to 170 kDa and 1200 to 1400 amino acids in length (Bosch and Croop, 1998).

They are highly symmetrical membrane spanning proteins, each half typically consisting of a transmembrane region composed of six alpha-helices, and a highly conserved intracellular nucleotide-binding region. The nucleotide-binding regions contain the Walker A and Walker B motifs, as well as another motif which is diagnostic for ABC proteins, sometimes called the Walker C motif (Bosch and Croop, 1998; Sharom et al., 2001; Sharom, 2006). All organisms have ABC proteins and they are well-conserved across kingdoms from prokaryotes up to multicellular plants and animals (Bosch and Croop, 1998; Sheps et al., 2004). Most organisms have multiple ABC proteins, often with overlapping substrate specificities (Bosch and Croop, 1998).

The high homology between the two halves of MDR proteins suggests that they emerged from a gene duplication event. Polypeptides approximating half of an MDR protein, consisting of a series of hydrophobic transmembrane loops followed by a hydrophilic intracellular region containing a potential nucleotide-binding domain, have also been identified (Bosch and Croop, 1998). Examples of these are a number of prokaryotic transport proteins that export virulence toxins, as well as mammalian proteins responsible for the transport of short peptides from the cytoplasm into the endoplasmic reticulum. A group of mammalian proteins that transport components of the oxidative metabolic pathway into peroxisomes, as well as proteins in the eye of *Drosophila melanogaster* that are proposed to transport pigment precursors, are also structurally similar to a single half of an MDR protein (Bosch and Croop, 1998). These proteins are proposed to function as homo- or heterodimers in cell or organelle membranes (Bosch and Croop, 1998).

Although MDR proteins were originally discovered through their overexpression in cell lines resistant to chemotherapy drugs, they are also expressed naturally in many cell types (Bosch and Croop, 1998). They are found in cell membranes, where they form a circular pore about 5 nm across, with about half the protein embedded in the lipid bilayer and half above and below it. Ten to 15 kDa of the protein's weight is from N-linked glycosylation (Bosch and Croop, 1998). The two nucleotide-binding regions interact with one another to bring about active transport. Substrates bind in sites formed by transmembrane helices 5 and 6 in the N-terminal end and 11 and 12 in the C-terminal end, and the short cytoplasmic loops between these helices (Pleban et al., 2004; Rothnie et al., 2005). MDR transporters are able to transport a variety of structurally and functionally unrelated compounds because of their unusual substrate-binding sites (Yu et al., 2003). Their transmembrane helices form large hydrophobic regions within the membrane with side openings facing the membrane milieu (Neyfakh, 2002; Rosenberg et al., 2005; Schumacher et al., 2004; Sharom, 2006). Substrates bind to the protein from within the inner leaflet of the membrane, rather than directly from the cytosol (Neyfakh, 2002). The one thing that MDR protein substrates have in common is that they are hydrophobic and also possess electric charge, or some polar functionalities (Bosch and Croop, 1998; Neyfakh, 2002). Hydrophobic repulsion, therefore, pushes them away from the cytosol and into the membrane. Rather than forming a precise network of hydrogen bonds with specific amino acid residues in the binding site, as happens in conventional enzymes and receptors, MDR protein substrates form a number of van der Waal's interactions with the surrounding hydrophobic amino acids as well as polar interactions with some charged amino acids (Neyfakh, 2002). The substrate binding pocket is large

enough to allow different substrate molecules to form interactions with different amino acids in the pocket walls (Neyfakh, 2002; Yu et al., 2003). This polyspecificity of MDR proteins for hydrophobic molecules led to the proposal of the "hydrophobic vacuum cleaner" hypothesis (Higgins and Gottesman, 1992). The few hydrophobic molecules present in the cytosol, such as vitamins and precursors in lipid metabolism, are normally tightly bound to proteins to prevent their outward diffusion. The hypothesis proposes that MDR proteins intercept and interpret any other hydrophobic molecules as xenobiotics and transport them out of the cell (Higgins and Gottesman, 1992). This may explain why individual cells often have multiple MDR proteins with overlapping substrate spectra, since there is probably very little evolutionary pressure forcing these transporters to specialise (Neyfakh, 2002). Supporting this view is the observation of lower orthology than expected between ABC genes in different species (Sheps et al., 2004). Phylogenetic analyses of ABC gene sequences from *Caenorhabditis elegans*, *Homo sapiens*, *Saccharomyces cerevisiae* and *D. melanogaster* reveal a great deal of both gene duplication and gene loss in ABC transporter evolution in these species (Sheps et al., 2004).

In its resting state, an MDR transporter exists as an open dimer, each monomer of which consists of six transmembrane domains and one ATP-binding domain (Higgins and Linton, 2004; Kerr et al., 2001; Sauna and Ambudkar, 2001). The binding of a substrate to the substrate-binding region within the cell membrane results in increased affinity of the nucleotide-binding domains for ATP (Higgins and Linton, 2004; Sauna and Ambudkar, 2001). The two nucleotide-binding regions interact with the Walker A and B motifs of one nucleotide-binding domain aligned with the Walker C motif of the

other to form two complete ATP-binding pockets (Higgins and Linton, 2004). The binding of the two ATP molecules results in a conformational change in the protein, resulting in its substrate-binding region now facing the extracellular milieu (Higgins and Linton, 2004; Kerr et al., 2001). The binding of ATP also reduces the affinity of the substrate-binding region for the substrate. It is assumed, although not proven, that at this point the substrate is released from the transporter into the extracellular region at this time (Higgins and Linton, 2004). The hydrolysis of ATP and subsequent release of ADP and Pi is required to reset the transporter at its resting state and restore the affinity of the substrate-binding region for substrates (Sauna and Ambudkar, 2001). The two bound ATP molecules are hydrolysed one by one. Hydrolysis of the first ATP molecule is sufficient for the hydrolysis of the second, but the affinity of the substrate-binding region is not recovered until the second ATP molecule is hydrolysed (Sauna and Ambudkar, 2001). After hydrolysis, the ADP co-ordinated with the Walker A motif of one nucleotide-binding domain repels the Pi co-ordinated with the Walker C motif of the other nucleotide-binding domain. This forces the two halves of the transporter apart, thus restoring its resting-state configuration as an open dimer (Higgins and Linton, 2004).

1.2 MDR protein inhibitors

A large number of inhibitors for MDR proteins have been characterised in various species, especially in the field of oncology (Idriss et al., 2000; Persidis, 2000; Qadir et al., 2005; Roe et al., 1999; Thomas and Coley, 2003). However, MDR protein inhibitor studies have also been carried out by researchers seeking ways to combat the

phenomenon of MDR in bacteria (Morrison et al., 2003; Stavri et al., 2006), fungi (Hayashi et al., 2003; Hiraga et al., 2001; 2005; Kohli et al., 2001; Kozovská and Subik, 2003; Scheutzer-Muehlbauer et al., 2003; Yamamoto et al., 2005), and protozoa (Jones and George, 2005; Leandro and Campino, 2003), all of which can be pathogenic to humans. The study of MDR in economically important insects in agriculture will be discussed in the next section.

Among the best-characterised MDR protein inhibitors are cyclosporin A and verapamil. They function as MDR protein substrates, and thus competitively inhibit the transport of other substrates (Buss et al., 2002; Callaghan and Denny, 2002; Podsialowski et al., 1998; Qadir et al., 2005; Rautio et al., 2006; Thomas and Coley, 2003). They are, however, no longer used as synergists in chemotherapy because in order to effect MDR protein inhibition they require serum concentrations high enough to be toxic to the patient (Ferry and Kerr, 1994; Thomas and Coley, 2003). The second generation of MDR inhibitors, for example, valspodar, a derivative of cyclosporin D, were derived from the first generation of competitive substrates. They also function as competitive inhibitors, but were designed to be more potent and less toxic than the first-generation inhibitors (Krishna and Mayer, 2000). However they often caused unpredictable metabolic effects. In addition to the overexpression of MDR proteins, multidrug resistant cells have also been shown to overexpress cytochrome P450 enzymes. It is thought that these enzymes share regulatory elements with MDR genes (Thomas and Coley, 2003). Several of the second-generation MDR protein inhibitors are substrates for cytochrome P450 enzymes, as well as for other transporters in the ABC family, and thus produced many non-specific physiological effects (Thomas and Coley, 2003). The third generation of MDR inhibitors

were designed to be specific for MDR proteins and not affect cytochrome P450 enzymes or other ABC transporters. They are not MDR substrates, but instead bind to the protein and change its conformation, preventing the binding of ATP (Thomas and Coley, 2003). Several third-generation MDR protein inhibitors have been studied in clinical trials as chemotherapy synergists (Ferry and Kerr, 1994; Thomas and Coley, 1993).

Outside of the oncology field, enniatin (Hiraga et al., 2005) and isonitrile (Yamamoto et al., 2005) have been identified as inhibitors for the MDR transporter Pdr5p in *S. cerevisiae* and chlorpromazine was found to modulate MDR protein activity in the fungus *Botrytis cinerea* (Hayashi et al., 2003). A number of compounds from natural sources such as the *Geranium* taxon, rosemary and grapefruit oil have been shown to inhibit bacterial multidrug resistance (Stavri et al., 2006). In the field of agriculture, a number of pesticides, such as ivermectin (Buss et al., 2002), endosulfan (Buss et al., 2002; Pivčević and Žaja, 2006), phosalone, propiconazole, diazinon (Pivčević and Žaja, 2006) and DDT (Shabbir et al., 2005) have been observed to decrease MDR protein activity. MDR protein inhibition by ivermectin and endosulfan has been demonstrated in vivo in the mosquito, *Culex pipiens* (Buss et al., 2002), while the activity on MDR protein function of the other pesticides mentioned has been shown in cell cultures.

1.3 MDR protein expression and function in invertebrates

MDR proteins are expressed at the apical surface of polarised cells (Bosch and Croop, 1998; Dean et al., 2001). In mammals, MDR proteins are expressed at secretory surfaces such as the bile canalicular membrane, intestine and colonic epithelium (Bosch

and Croop, 1998). MDR gene expression increases in the murine uterus during gestation (Arceci, 1998). MDR proteins are also associated with the maintenance of the blood-brain barrier in mammals (Bubik et al., 2006; Demuele et al., 2002).

While most scientific interest in MDR proteins and their inhibitors relates to their medical importance, for instance, in cancer chemotherapy and protozoan parasite control, MDR genes diversified during metazoan evolution in response to other pressures. Many groups of plant-feeding insects, for example, have faced a constant barrage of toxic xenobiotics in their diet for hundreds of millions of years, and in recent industrial times, a steady exposure to synthetic chemicals designed to exterminate them. It follows that MDR proteins could be useful targets in the suppression of a pest insect's defences following exposure to toxic xenobiotics. MDR protein inhibitors could act as synergistic agents in the chemical control of insect pests.

MDR protein expression has been observed in a number of insects in tissue locations analogous to mammalian secretory surfaces. Three genes coding for MDR proteins have been discovered in *D. melanogaster*, (Wu et al., 1991; Gerrard et al., 1993) and a fourth can be found in its genome in silico. Upregulation of one of the *D. melanogaster* MDR genes has been observed in response to heat shock and feeding with the toxic MDR protein substrate colchicine (Tapadia and Lakhotia, 2005). MDR proteins have been found in a moth (Murray et al., 1994) and in *Chironomus* midges (Podsialowski et al., 1998) and evidence of MDR protein activity has been observed in mosquitoes (Buss et al., 2002). Evidence of MDR protein activity has been observed in the blood-brain barrier (Murray et al., 1994) and the Malpighian tubules of tobacco hornworm larvae (Gaertner et al., 1998), and the cuticle and fat body of tobacco

budworm larvae (Lanning et al., 1996a;b). Thus, it is likely that MDR protein activity plays a role in protecting herbivorous insects from the toxins in their natural diets. Three lines of evidence suggest a role for MDR proteins in the protection of herbivores from plant toxins: the first is the ubiquity and high conservation of MDR proteins in many species; the second is the fact that a large number of toxic plant secondary metabolites are consumed by herbivores, indicating that the herbivores have some defence mechanism against them; and the third is the presence of unmetabolised plant defence compounds in herbivore feces (Sorensen and Dearing, 2006). It has been suggested that because generalist pests are exposed to a broader range of plant secondary metabolites, that their defence mechanisms would be correspondingly broad, thus positing a role for MDR proteins (Li et al., 2004). MDR protein expression has also been shown to protect *D. melanogaster* (Callaghan and Denny, 2002) and in *Chironomus riparius* (Postma et al., 1996) from cadmium poisoning in their environments. Although some pesticides, as mentioned above, have been shown to decrease MDR protein activity, in many cases it appears that MDR proteins contribute to pesticide resistance in insects. MDR activity has been linked with decreased cuticular penetration of toxins in the tobacco budworm larva *Heliothis virescens* (Lanning et al., 1996b). The African bollworm *Helicoverpa armigera* is a major agricultural pest, which has developed resistance to several classes of pesticides and MDR gene expression has been detected in resistant larvae, but not in insecticide-susceptible larvae (Srinivas et al., 2004).

1.4 Statement of Intent

The cabbage looper moth *Trichoplusia ni* Hubner (Lepidoptera: Noctuidae) is a common pest on a number of crops, especially those of the crucifer family such as cabbage, mustard and horseradish. These plants contain a number of potentially toxic chemicals such as the glucosinolates, a class of organic anions responsible for their pungent taste. In order to protect themselves from the toxic compounds in their diets, insects need efficient systems to detoxify or eliminate these substances. Based on previous observations of MDR protein activity in other lepidopteran larvae, it is likely that *T. ni* larvae also use MDR proteins to eliminate toxins. Since feeding on toxic substances has been shown to upregulate the expression of certain MDR genes, we propose that *T. ni* larvae fed on a plant diet will show higher MDR gene expression than those fed on a wheat germ-based artificial diet. This project, therefore, has three main objectives. The first is to identify and sequence MDR cDNAs in larval *T. ni*. The second is to quantify MDR gene expression in larval tissues important for the extrusion of toxic chemicals, and to compare MDR gene expression in artificial diet- and plant-fed *T. ni* larvae. The third is to generate an in vitro assay system by stably transforming Sf9 cells with *T. ni* MDR genes, and use this system to identify and characterise potential MDR inhibitors. The long-term goal of projects such as this one is to identify MDR inhibitors that may function as synergists with pesticides.

2. Methods

2.1 Insect rearing

Insects were reared at 24°C with a 16 h:8 h light:dark cycle at ambient humidity on either artificial diet (containing wheat germ, casein, cellulose, alginic acid, cholesterol, sucrose, Vanderzant vitamin mix, Wesson salt mix, ascorbic acid, sorbic acid, streptomycin, benomyl, wheat germ oil, formalin, propionic acid, phosphoric acid and agar) or pieces of washed organically-grown cabbage leaves ad libitum. Eggs were allowed to hatch in a lidded container. First- and second-instar larvae were transferred into individual plastic cups. Larvae remained in individual cups until fifth instar when they were harvested.

2.2 Degenerate PCR

Sequences of three Chinese hamster and three *Drosophila mdr* genes were aligned and three degenerate primers were designed from regions of high homology between these sequences (Fig 1). The primers were MDR1, 5′-GCNYTNGCNTTYTGGTAYGG-3′; MDR2, 5′-GGNGTNGTNGGNCARGARCCNGT-3′ and MDR3, 5′-GCDATNCKYTGYTTYTG-3′. PCR amplifications using MDR1 and MDR3 on a *T. ni* head cDNA library yielded a 700 bp DNA fragment from which two distinct clones were identified. These clones represented partial sequences of *trnMDR1* and *trnMDR2*. PCR amplification using MDR2 and MDR3 yielded a 200 bp fragment representing a partial

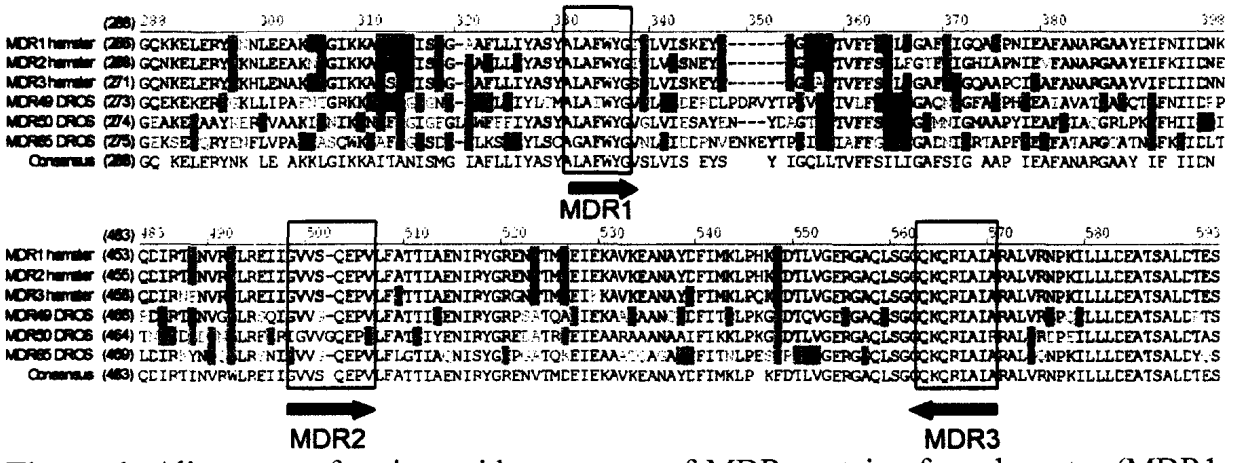

Figure 1. Alignment of amino acid sequences of MDR proteins from hamster (MDR1, MDR2, MDR3) and fruitfly (MDR49, MDR50, MDR65). Degenerate primers were designed in regions of high homology marked by boxes. (M. Rheault, unpublished data).

sequence of *trnMDR3* (M. Rheault, unpublished data).

2.3 RACE-PCR

Rapid amplification of cDNA ends was used to extend the known sequences of *trnMDR1*, *trnMDR2* and *trnMDR3* up to the 5′ and 3′ ends of their ORFs.

2.3.1 3′ RACE

Template for 3′ RACE was produced by reverse transcription of DNased *T. ni* head polyA mRNA using the FirstChoice RLM-RACE kit to generate full-length cDNA molecules with a 3′ RACE adapter 5′-GCCAGCACAGAATTAATACGACTCACTCACTATAGGT_{12}VN-3′ ligated to the 3′ end of each molecule (Ambion). Nested PCR amplifications were performed using primary and nested primers complementary to the 3′ RACE adapter (Table 1) and primary and nested primers designed to be complementary to the 3′ end of the known sequence (Table 1). PCRs contained 1X PCR Buffer Minus Mg (Invitrogen); 0.4 μM of each primer; 2.5 mM $MgCl_2$ (Invitrogen); 0.2 mM of each dNTP; 2.5 U of Platinum Taq DNA Polymerase (Invitrogen) and 1 μl of template, which was diet-fed *T. ni* whole head first-strand cDNA for the primary reactions. Cycling began with a 3 min incubation at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 55-60°C and 2 min at 72°C. The final step was a 7 min incubation at 72°C. PCR products were run on 1.2% w/v agarose gels. The bands were cut out and extracted from the gel using the QIAquick Gel

Extraction kit (Qiagen) and eluted in 1mM Tris. Purified DNA was ligated overnight at 4°C into the vector pGemTEasy (Promega) in the presence of 3 U of T4 DNA Ligase in 1X Rapid Ligation Buffer (Promega). Competent *E. coli* cells were transformed with the ligation mixes either by electroporation (MRF'ec XL1Blue cells, Stratagene) or by heat shock (DH5α cells, Invitrogen) and plated on LB plates containing 100 μg/ml ampicillin and 0.8 mg/plate of X-gal (Invitrogen). Plates on which MRF'ec cells were plated also contained 9.5 mg/plate of IPTG (Invitrogen). Clones containing an insert were selected by blue-white selection were picked with a sterile pipette tip and stored in 100 μl each of Luria Bertani (LB) medium. Fifty μl of LB medium from each clone were grown overnight in 5 ml LB medium containing ampicillin (LB Amp) and plasmid DNA was purified using the QIAquick 8 Miniprep kit (Qiagen). Plasmid preparations were eluted in 100 μl each of 1mM Tris and sequenced by a dedicated core sequencing facility.

2.3.2 5′ RACE

Template for 5′RACE was a *T. ni* head cDNA library ligated in the vector pBluescript-SK (Stratagene). Nested PCR amplifications were performed using primary and nested primers designed to be complementary to the 5′ end of the known sequences (Table 1) and the vector primers M13r (primary) and $T3^{+}$ (nested) (Table 1). The composition and cycling conditions of the PCRs were as described previously for 3′ RACE. PCR products were gel purified, ligated in pGemTEasy, the plasmids extracted and sequenced as described previously.

Table 1. Primers used in RACE-PCR

Name	Sequence	Notes
3′ Outer	GCTGATGGCGATGAATGAACACTG	3′ primary from Ambion Race kit
3′ Inner	CGCGGATCCGAACACTGCGTTTGCTGGCTTTGATG	3′ nested from Ambion Race kit
M13r	AGCGGATAACAATTTCACACAGG	5′ primary for pBS-SK
T3+	AATTAACCCTCACTAAAGGG	5′ nested for pBS-SK
700#4-3	GACGAAATTCTGGAATCAGTGAGA	Primary for 3′ Race of *trnMDR1*
700#4-4	CTCTGGGATCACGAATTTCATAGT	Nested for 3′ Race of *trnMDR1*
700#4-1	CGAGAGCCATGATCACTGAACTTA	Primary for 5′ Race of *trnMDR1*
700#4-2	TATTCCGTCTTCGAGCTTCACAAC	Nested for 5′ Race of *trnMDR1*
700#6-3	TACCTCCGCTCTTGATACACACAG	Primary for 3′ Race of *trnMDR2*
700#6-4	TGGTGCAAGAAGCGTTAGACAGAG	Nested for 3′ Race of *trnMDR2*
700#6-5	CCGATGACCATGGGAACAGACAC	Primary for 5′ Race of *trnMDR2*
700#6-6	ATCAGCGGCTAGACGGGCAGAC	Nested for 5′ Race of *trnMDR2*
200#1-1	CGCTACGATCGTTCTGACAGACTT	Primary for 3′ Race of *trnMDR3*
200#1-2	CCTGCCTGACTGTACGATTCCATC	Nested for 3′ Race of *trnMDR3*
200#1-4	CCGGAAGGTCACCATGCACGAAAT	Primary for 5′ Race of *trnMDR3*
200#1-5	GCCGCGAAAGCTGCTAATATACAC	Nested for 5′ Race of *trnMDR3*

2.4 Cloning of full-length MDR genes

2.4.1 Cloning of full-length MDR genes in pGemT Easy

2.4.1.1 *trnMDR1*

The strategy by which the ORF of *trnMDR1* was cloned in pGemT Easy is represented by Fig 2.

2.4.1.1.1 PCR amplification of the *trnMDR1* ORF in two overlapping sections

To assemble a full-length copy of *trnMDR1*, the complete ORF sequence was first amplified in two overlapping amplicons in nested PCRs. Primer pairs 700#4-16 and 700#4-10 (primary) and 700#4-17 and 700#4-11 (nested) (Table 2) were used to amplify a 2950 bp sequence corresponding to the 5′ end of the ORF. Primer pairs 700#4-3 and 700#4-18 (primary) and 700#4-4 and 700#4-19 (nested) (Table 2) were used to amplify a 2350 bp sequence corresponding to the 3′ end of the ORF. The ORF of *trnMDR1* contains a single *Sca*I restriction site, and the overlapping amplicons were designed such that the *Sca*I site was in the overlap. PCR amplifications contained 2 µl of first-strand gut cDNA from artificial diet-fed fifth-instar *T. ni*, 0.4 U of Phusion High-Fidelity DNA Polymerase (NEB), 200 µM of each dNTP and 0.5 µM of each primer in 1X Phusion HF Buffer (NEB). The cDNA template was produced by reverse-transcribing 0.5 µg of gut RNA, using SuperScript II H- Reverse Transcriptase (Invitrogen) according to the

manufacturer's instructions. Cycling began with 30 s at 98°C; followed by 35 cycles consisting of 5 s at 98°C, 15 s at 58°C and 2 min at 72°C; and the last step was a 7 min extension period at 72°C. Half the volume (10 µl) of the nested PCR products was run on a 1.2% w/v agarose gel and the bands of the expected sizes were cut out and eluted using the QIAquick Gel Extraction kit (Qiagen). These products were then used as template for further PCR reactions using Easy-A High Fidelity PCR Cloning Enzyme (Stratagene) to produce A-tailed products for cloning into pGemTEasy. The primer pairs used were the ones from the previously mentioned nested reactions. The reactions contained 3 µl of either the gel extract or the nested PCR product, 200 µM of each dNTP, 0.5 µM of each primer and 2.5 U of Easy-A High Fidelity PCR Cloning Enzyme (Stratagene) in 1X Easy-A Buffer (Stratagene). The Easy-A amplification consisted of a 40 s pre-incubation period of 95°C followed by 15 cycles of 30 s at 95°C, 30 s at 60°C and 3 min at 72°C. The last step was a 7 min extension at 72°C. PCR products were analysed on a gel, and bands of the expected sizes were cut out and eluted as previously described. Gel extracts were cloned in pGemT Easy (Promega), used to transform XL1Blue MRF'ec cells (3′ end sequence) or DH5α cells (5′ end sequence) and plated as previously described. Clones appearing positive by blue white selection were picked with a sterile pipette tip and stored in 100 µl each of LB medium. Clones were screened for inserts by PCR with gene-specific primers 700#4-17 and 700#4-2 for the 5′ end sequence, and 700#4-19 and 700#4-6 for the 3′ end sequence (Table 2). The PCR amplifications used *Taq* DNA Polymerase (Sigma) according to the protocol described previously. PCR-positive clones were grown overnight and plasmid DNA was purified using the GenElute Plasmid Miniprep Kit (Sigma).

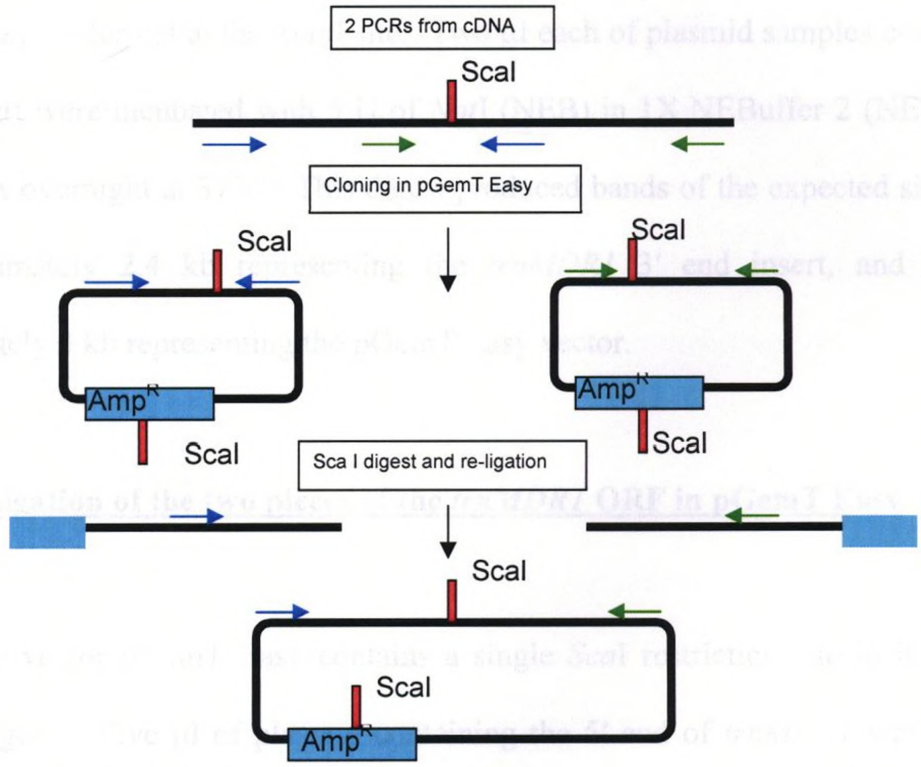

Figure 2. Cloning strategy for *trnMDR1*. Pairs of arrows of the same colour represent primer pairs. The blue box represents the ampicillin resistance gene in pGemT Easy. The red boxes mark the *Sca*I restriction sites, one in the *trnMDR1* insert and the other in the ampicillin resistance gene.

The plasmid samples were tested by restriction digests to confirm that they contained inserts of the expected lengths. Two µl each of plasmid sample containing the 5′ end insert were incubated with 5 U of *Not*I (NEB) and 10 U of *Xmn*I (NEB) in 1X NEBuffer 2 (NEB) and 100 µg/ml bovine serum albumin (BSA) overnight at 37°C. This digest produced bands of the expected sizes: a band of approximately 3 kb representing the *trnMDR1* 5′ end insert, and bands of approximately 1kb and 2 kb representing the pGemT Easy vector cut at the *Xmn*I site. Two µl each of plasmid samples containing the 3′ end insert were incubated with 5 U of *Not*I (NEB) in 1X NEBuffer 2 (NEB) and 100 µg/ml BSA overnight at 37°C. This digest produced bands of the expected sizes: a band of approximately 2.4 kb representing the *trnMDR1* 3′ end insert, and a band of approximately 3 kb representing the pGemT Easy vector.

2.4.1.1.2 Ligation of the two pieces of the *trnMDR1* ORF in pGemT Easy

The vector pGemT Easy contains a single *Sca*I restriction site in its ampicillin resistance gene. Five µl of plasmid containing the 5′ end of *trnMDR1* were incubated with 50 U of *Sca*I (NEB) in 1X NEBuffer *Sca*I (NEB) overnight at 37°C. Five µl of plasmid containing the 3′ end of *trnMDR1* were incubated with 50 U of *Sca*I (NEB) and 20 U of *Nco*I (NEB) in 1X NEBuffer *Sca*I (NEB) overnight at 37°C. The *Nco*I was added to remove residual uncut vector. The digests were analysed on gels and the inserts were gel purified. Gel extracts were concentrated by precipitation of the DNA using sodium acetate and ethanol and resuspension in smaller volumes of TE buffer. Ninety ng of the resuspended 5′ end piece and 140 ng of the resuspended 3′ end piece were

ligated together using Ready-To-Go T4 DNA ligase (Amersham) according to the manufacturer's instructions. The ligation was used to chemically transform DH5α cells which were plated on LB plates containing ampicillin (C. Donly, personal communication). It was expected that all clones that were able to grow on ampicillin-containing plates would be ligated such that the *trnMDR1* ORF was formed, since only in this circumstance would the ampicillin resistance gene be intact. Clones from these plates were picked and PCR-screened using *Taq* DNA polymerase (Sigma) as previously described with an annealing temperature of 55°C and an extension time of 3 minutes, using the internal primer pair 700#4-5 and 700#4-11 (Table 2). This primer pair was expected to amplify a 2.3 kb section of the *trnMDR1* ORF spanning the *Sca*I site. Five PCR-positive clones were grown overnight and plasmid purified as previously described. Two restriction digests were done of each plasmid sample to ascertain that they contained the complete ORF. Each plasmid was incubated overnight at 37°C with 5 U of *Nco*I and *Eco*RI (NEB) in NEBuffer 4 or NEBuffer *Eco*RI respectively (NEB). The sizes of the expected digestion products were a ~4 kb band representing the insert and a ~3 kb band representing the vector in the *Eco*RI digest and a ~ 7 kb band representing the linearised vector containing the insert in the *Nco*I digest. The digestion products were run on a gel and visualised by staining with ethidium bromide. One plasmid had bands of the expected sizes.

2.4.1.2 *trnMDR2*

The complete ORF sequence of *trnMDR2* was amplified using nested PCR with the primer pairs 700#6 up1 and 700#6 down1 (primary) and 700#6 up2 and 700#6 down2 (nested) (Table 2). The template for the primary PCR was single-stranded cDNA made by reverse transcribing polyA selected *T. ni* head RNA (C. Donly, personal communication). The primary reaction contained 1 µl of cDNA, and the nested reaction contained 1 µl of primary reaction product. Amplifications were performed with Phusion High Fidelity DNA Polymerase as described for the *trnMDR1* ORF, with an annealing temperature of 65°C. The nested PCR product of approximately 4 kb was gel purified as described previously. The fragment was A-tailed by incubating for 30 min at 72°C with 2.5 mM $MgCl_2$, 200 µM dATP and 5 U of Taq DNA Polymerase (Sigma) in 1X PCR Buffer (Sigma) and ligated into pGemT Easy as described previously. This ligation was used to transform MRF'ec XL1 blue cells by electroporation. The transformed cells were plated on LB Amp plates containing X-Gal and IPTG, and positive clones were identified by blue-white selection. Positive clones were PCR screened using the internal primer pair 700#6-5 and 700#6-9 (Table 2) which were expected to form a product of 215 bp. The PCR was conducted using the same reaction concentrations as previously described with *Taq* DNA Polymerase (Sigma) with an annealing temperature of 55°C and an extension time of 40 s. The expected PCR product was formed in five clones. Overnight liquid cultures of these clones were grown in 50 ml LB Amp. Plasmid DNA was extracted from 40 ml of each culture using the Bio-Rad Midiprep kit. The plasmids were further tested by digestion with *Not*I. Fifteen µl of each plasmid were incubated

Table 2. Primers used for cloning of full-length ORFs of *T. ni* MDR genes.

Name	Sequence
700#4-2	TATTCCGTCTTCGAGCTTCACAAC
700#4-4	CTCTGGGATCACGAATTTCATAGT
700#4-5	ACGCGGGTCTGAATCAGGACTTTG
700#4-6	ACATTCCGCAAAGAGTACGCTAAG
700#4-10	AAGGGACGATGCTATGACAAAGTTG
700#4-11	CCCGAAGACGATGCCTCTCCAGTG
700#4-16	TTTCGAGGAGTGGCCGTCCAGTG
700#4-17	CAGTGGACCGCGTCGAGGCCTC
700#4-18	GTGTGACTGGTGTAATAGTTTTAGAC
700#4-19	GACTAGCCTCCGAATACTAATACTA
700#6 up 1	GCGCCATATACAGCCAACCTCATT
700#6 down 1	CGATTGACCGTGAAAAGCTGATAG
700#6 up 2	GGCGTAAACGTCCAAAAGCATAC
700#6 down 2	GGGATTCTACGCCTGCAACTACAG
200#1-5	GCCGCGAAAGCTGCTAATATACAC
200#1-7	GACGTAACCTAGCCCGCTCTCGCA
200#1-8	AGCCCGCTCTCGCACCTTCTAAAT
200#1-9	GGCGACGAGTGTAGTGTAAGATCT
200#1-10	GTCCCTCTCCCAAAACCGCAATAG

overnight at 37°C with 25 U of *Not*I (NEB) in 1X NEBuffer 3 (NEB) and 100 µg/ml of BSA. The digestion products were separated on a gel and visualised as described previously. The gel confirmed the presence of bands of the expected sizes – approximately 4 kb for the insert and 3 kb for the vector – in four of the five plasmids.

2.4.1.3 *trnMDR3*

The ORF sequence of *trnMDR3* was amplified using nested PCRs with primer pairs 200#1-7 and 200#1-10 (primary) and 200#1-8 and 200#1-9 (nested) (Table 2). The template for the primary PCR was first-strand cDNA from plant-fed *T. ni* fat body. Primary PCRs contained 1.5 µl of cDNA and nested PCRs contained 3 µl of the primary reaction. Amplifications were performed with Phusion High-Fidelity DNA Polymerase (NEB) as described for the ORF of *trnMDR1*, except the annealing temperature was 42°C for the first 10 cycles and 56°C for the next 25 cycles. The products were separated on a gel and a band of ~4 kb was cut out and gel extracted as described previously. The gel extract was A-tailed and ligated into pGemT Easy as described previously. The ligation was used to transform MRF'ec XL1 Blue cells by electroporation. The transformed cells were plated on LB Amp agar plates containing X-Gal and IPTG, and positive clones were identified by blue-white selection. Plasmid DNA was purified from sixteen white clones as previously described. Two µl of each plasmid were incubated overnight at 37°C with 5 U of *Not*I (NEB) in 1X NEBuffer 3 (NEB) and 100 µg/ml BSA. The digestion products were analysed by gel electrophoresis. Eight plasmids were seen to contain the expected

~4 kb band representing the ORF sequence of *trnMDR3* and the ~ 3 kb band representing the linearised vector pGemT Easy.

2.4.2 Cloning of full-length MDR genes in pIZ/V5-His

2.4.2.1 Determination of orientation of MDR ORFs in pGemT Easy

The orientation of the full-length *T. ni* MDR inserts in pGemT Easy were determined by digestion with the restriction enzyme *Sac*II. The vector pGemT Easy has a single *Sac*II restriction site at position 49, and the ORFs of *trnMDR1*, *trnMDR2* and *trnMDR3* have a single *Sac*II restriction site each at positions 90, 7 and 3464 respectively. Therefore if the inserts were ligated in the vector in the forward (5′ → 3′) orientation, the *Sac*II digest should produce bands of ~ 7kb and ~ 100 bp from the *trnMDR1* plasmid preparations; ~7 kb and ~50bp from the *trnMDR2* plasmid preparations and ~900 bp and ~6500 bp from the *trnMDR3* plasmid preparations.

Two µl of plasmid for each clone with the full-length ORF of *trnMDR1*, two with the full-length ORF of *trnMDR2* and three with the full length ORF of *trnMDR3* were incubated overnight at 37°C with 10 U of SacII (NEB) in 1X NEBuffer 4 (NEB) and the digestion products analysed on a gel. All three of the *trnMDR1* plasmids; both of the *trnMDR2* plasmids and two of the three *trnMDR3* plasmids had digestion products of sizes indicating forward orientation in pGemT Easy.

2.4.2.2 PCR mutagenesis to create an *Age*I site to clone *T. ni* MDR genes into pIZ/V5-His

The primer pGemTEasy+AgeI (5′- CGCGAATTCACCGGTGATT -3′) was designed in the polylinker region of the vector pGemT Easy immediately downstream of the insert. The primer has a two base pair mismatch that changes the *Spe*I restriction site in the pGemT Easy polylinker region to an *Age*I restriction site. *Age*I was chosen because none of the three MDRs in this study have an *Age*I site in their ORFs. The full-length ORFs of all three genes were PCR amplified using this primer to add an *Age*I restriction site at the 3′ end. The template for each of these PCRs was 1 µl of a 1:10 dilution of one plasmid preparation of each gene ligated in pGemT Easy in forward orientation. The primer pair used in all three cases was pGemTEasy+AgeI and T7 (5′-TAATACGACTCACTATAGGG-3′). The primer T7 is a downstream-facing primer designed in the T7 promoter region of pGemT Easy. Amplifications were performed using Phusion High-Fidelity DNA Polymerase as previously described.

2.5 Quantitative PCR

Quantitative PCR (qPCR) using the TaqMan system (Roche) was used to quantify the expression of *trnMDR1*, *trnMDR2* and *trnMDR3* in the gut, Malpighian tubules, integument, brain, muscle and fat body of late fifth-instar *T. ni* larvae raised on either artificial diet or organically-grown cabbage.

2.5.1 Template preparation

Tissues were dissected in isotonic saline (10.7 mM NaCl, 25.8 mM KCl, 29 mM $CaCl_2$, 20 mM $MgCl_2.6H_2O$, 90 mM D-glucose, 5 mM HEPES) and stored at 4°C in RNA*later* (Ambion) until use. All tissues from artificial diet-fed animals as well as brain, muscle and fat body tissue from plant-fed animals were homogenized in 1.5 ml microfuge tubes with glass pestles. Total RNA was extracted using TRIzol Reagent (Life Technologies) treated with RNase Out (Invitrogen) and DNased using DNaseI Amplification Grade (Invitrogen) to remove genomic DNA contamination. Digested fragments of genomic DNA were removed using Chromaspin 100 DEPC-H_20 columns (Clontech). Total RNA was extracted from gut, Malpighian tubule and integument tissue from plant-fed insects using the RNeasy Mini Kit (Qiagen) and DNased using the RNase-Free DNase Set (Qiagen) according to the manufacturer's instructions. The concentrations of all DNase-treated RNA samples were determined from OD_{260} values read in a Bio-Rad SmartSpec Plus spectrophotometer.

Aliquots of 0.2-0.5 μg from each RNA sample were treated with RNase Out Ribonuclease Inhibitor (Invitrogen) and then reverse-transcribed into cDNA using SuperScript II H- Reverse Transcriptase (Invitrogen). The primer used in the reverse transcription reactions was the 3′ RACE Adapter (Ambion). This primer was chosen because its poly-dT sequence should promote the reverse transcription of mRNA molecules with polyA tails over other RNA molecules. The integrity of the cDNA samples was tested by PCR amplifying the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (G3PDH) using the primers G3PDH5, (5′-

AACAACATTTATCTCTACACTGCTA-3′) and G3PDH8, (5′-CTTGTTTCTACATAAATTTATTCC-3′) in order to check that the RNA had been successfully reverse-transcribed. These two primers bind on either side of a 1.3kb long intron in *T. ni* genomic DNA (Malutan, 1999), and thus were also used to check for genomic DNA contamination. They form a PCR product of 1.1 kb from a cDNA template, however the product formed from genomic DNA is 2.4 kb. The 1.1 kb product was formed from all cDNA samples indicating successful reverse-transcription as well as lack of genomic DNA contamination.

2.5.2 Primer and probe design

The 3′ ends of the ORF sequences of *trnMDR1*, *trnMDR2* and *trnMDR3* were aligned. Upstream- and downstream-facing primers for qPCR were designed in regions of low homology in areas similarly distant from the 3′ end of each ORF (Table 3). This was done so that the amplification of one gene relative to another would not be favoured by different amounts of full-length cDNA in the template. The primers were designed to produce amplicons of approximately 500 bp. Oligonucleotide probes containing the fluorescent dye DFAM and the quencher dye DTAM were designed approximately in the middle of each amplicon (Table 3).

Sequences of the housekeeping genes *G3PDH*, *actin* and *S5* ribosomal protein in *T. ni* were acquired from GenBank (*actin* accession numbers AY646104, AY646105, AY646106, AY646107; *S5* accession number AY837869) or from data acquired previously in our lab (*G3PDH*, Malutan 1999). Primer pairs were designed to produce

approximately a 100bp amplicon for *G3PDH* (C. Donly, personal communication) and 500 bp amplicons for *actin* and *S5* (Table 3). Probes containing DTAM and DFAM were also designed for these three genes (Table 3).

2.5.3 Preparation of standards

Standards for qPCR were prepared by PCR amplifying with Platinum Taq (Invitrogen) the qPCR amplicons of the three MDR genes of interest as well as those of *actin*, *S5* and *G3PDH* using the primers described previously. Templates were 1 µl of diet-fed *T. ni* whole head cDNA for *G3PDH*, *actin* and *S5*, and 1 µl of diet-fed *T. ni* gut cDNA for *trnMDR1*, *trnMDR2* and *trnMDR3*. Amplifications contained 0.4 µM of each primer, 2.5 mM $MgCl_2$ (Invitrogen), 0.2 mM of each dNTP and 2.5 U of Platinum *Taq* DNA Polymerase (Invitrogen) in 1X PCR Buffer Minus Mg (Invitrogen). Cycling began with a 3 min incubation at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 60°C and 30 s at 72°C. The final step was a 7 min incubation at 72°C. The PCR products were cloned in pGemTEasy (Promega) and plasmid DNA was purified as described previously. The plasmid inserts were sequenced to confirm that none of them contained restriction sites for the enzyme *Nco*I. The pGemTEasy vector contains a single *Nco*I site in its polylinker region. Two to five µl of each plasmid DNA sample were digested with 5 U NcoI (NEB) in 1X NEBuffer 4 (NEB) overnight at 37°C to linearise them. The linearised plasmid preparations were run on 1.2% w/v agarose gels and eluted using the QIAquick Gel Extraction kit (Qiagen). The DNA concentrations were determined from OD_{260} values

Table 3. Primers and probes used in qPCR

Name	Type of oligo	Sequence
For *trnMDR1*		
700#4 FOR2	Primer	GCCCGGATGAAGACGA
700#4 REV	Primer	CTTAGCGTACTCTTTGCGGAAT
700#4 probe	Probe	[DFAM]ACTAAACCAACTCGCCACTCGTAGCTC[DTAM]
For *trnMDR2*		
700#6 FOR	Primer	GGATTGGGTACGAGGACAGA
700#6 REV3	Primer	GCCACACGACCCTCAAT
700#6 probe	Probe	[DFAM]CCGGCATCTGTCTGTCTCTGTACTTC[DTAM]
For trnMDR3		
200#1 FOR	Primer	GAAGATAGGACCGAAGTGATT
200#1 REV	Primer	GCCTTACAAGCCTCTACTAAC
200#1 probe	Probe	[DFAM]TCAAGCCATATACTGCCAACCATCA[DTAM]
For G3PDH		
G3PDH 10	Primer	ATCAAGTACATCCAGACCA
G3PDH 11	Primer	AGCAATATCCAGAACTGTG
G3PDH 12	Probe	[DFAM]TAACAACATTTATCTCTACACTGCTATC[DTAM]
For S5		
S5 FOR	Primer	GTCGACAGCATGCCTTTA
S5 REV	Primer	CACATCCACAGCCTGAC
S5 probe	Probe	[DFAM]CATCATCAGAGAGTTTGTCAAGCGCT[DTAM]
For Actin		
trnActin 6	Primer	ACACGGTGCCCATCTA
trnActin 8	Primer	GAGGGAGGCGAGGAT
Actin 2 probe	Probe	[DFAM]AGAGAGCTTCAGGGCAACGGA[DTAM]

read in a Bio-Rad SmartSpec Plus spectrophotometer. Serial dilutions of the linearised plasmids were used as standards.

2.5.4 Quantification of "housekeeping" gene expression relative to standard curves

Gene transcripts were quantified using a Roche 2.0 LightCycler system. Each reaction contained 5 µl of 1:10 diluted cDNA or 5 µl of water for negative controls or 5 µl of an appropriately diluted standard; final primer concentrations of 0.5 µM each and final probe concentrations of 0.05 µM in 1 X LightCycler TaqMan Master Mix (Roche). Reactions were prepared in a cooler box at 4°C and protected from light. The cycling protocol began with a 10 min pre-incubation at 95°C. This was followed by as many amplification cycles as necessary with a 10 s denaturation step at 95°C, a 30 s annealing step at either 58°C or 60°C and a 5 s extension step at 72°C. The final step was a 30 s cooling period at 40°C.

Serial dilutions of the standards were used to generate standard curves to quantify expression of all six genes. The slopes of each standard curve were used to calculate the efficiency (E) of the PCR amplification of each gene using the formula:

$$E = 10^{-1/slope}$$

The expression of *G3PDH*, *S5* and *actin* in each tissue was averaged to provide a value, termed the housekeeping gene expression, for each tissue. The expression of *trnMDR1*, *trnMDR2* and *trnMDR3* in each tissue was divided by the corresponding housekeeping gene expression to provide a unitless value representing its expression relative to the three housekeeping genes. Expression of each gene in each tissue was

compared between artificial diet-fed and plant-fed animals using the unpaired one-tailed Student's t-test, with $P < 0.05$.

2.6 Stable Transformation of Sf9 cells with *T. ni* MDR genes

2.6.1 Cloning of *T. ni* MDR genes in pIZ/V5-His

Amplified products for each of the three *T. ni* MDR ORFs were precipitated in sodium acetate and ethanol and recovered by centrifugation before being incubated overnight with 5 U of *Age*I (NEB) in 1X NEBuffer I (NEB) at 37°C. Three μg of the vector pIZ/V5-His were also incubated with 5 U of *Age*I (NEB) under the same conditions. The enzyme was then heat-inactivated by incubating for 20 minutes at 65°C and the DNA recovered by precipitation with sodium acetate and ethanol. All samples were then incubated overnight at 37°C with 10 U of *Not*I (NEB) in 1X NEBuffer 3 (NEB) and 100 μg/ml BSA (NEB). The next day, the samples were analysed on a gel as previously described. The bands of the expected sizes (~4 kb for the MDRs and ~3 kb for pIZ/V5-His) were cut out of the gel and the DNA extracted as previously described. One μl of each extract was assessed by gel electrophoresis beside a known quantity of λ Hind III digest (Sigma) in order to estimate their concentrations and check their purity. The concentrations were estimated to be ~200 ng/μl for the MDR samples and ~100 ng/μl for the pIZ/V5-His.

The DNA fragments were then ligated into pIZ/V5-His. One μl of each MDR sample and 0.5 μl of the pIZ/V5-His sample were used in each ligation reaction for a

4:1.5 insert:vector ratio. The ligation reactions took place overnight at 4°C in 1X Rapid Ligation Buffer (Promega) in the presence of 3 U of T4 DNA Ligase (Promega). Control ligations with no MDR insert, with and without T4 DNA Ligase were also performed. The control containing ligase was to check for vector re-ligation, and the one without ligase checked for undigested vector.

DH5α cells were transformed with 5 µl each of these ligations by heat shocking, and plated on low-salt LB Zeocin plates. Sixteen clones from each ligation were picked and PCR-screened. The primer pairs used for the PCR screens were 700#4-4 and 700#4-11 (Table 2) for *trnMDR1*, 700#6-5 and 700#6-9 (Table 2) for *trnMDR2* and 200#1-5 and 200#1-9 (Table 2) for *trnMDR3*. PCR amplifications used the same component concentrations and cycling conditions as previously described for *Taq* DNA Polymerase (Invitrogen) with an extension time of 1 min. Four clones of *trnMDR1* named 1-5, 1-7, 1-10 and 1-13 and two of *trnMDR3* named 3-1 and 3-2 were identified as PCR positive. Plasmid was prepared from these clones using the Bio-Rad Midiprep kit, and the plasmids were sequenced to confirm that they contained the complete expected MDR ORF sequence. The plasmids were filter sterilised by centrifuging for 20 minutes at 10,000 *g* through a Whatman Centrifuge Filter with a 0.2 µm pore size. Their concentrations were determined using a Bio-Rad SmartSpec Plus spectrophotometer.

2.6.2 Transformation of Sf9 cells *T. ni* MDR genes in pIZ/V5-His

Sf9 cells were stably transformed with plasmids containing *T. ni MDR* genes using the InsectSelect System (Invitrogen). Sixty mm petri dishes were seeded with

$1.5*10^6$ Sf9 cells in the serum-free medium Sf-900 II SFM (Invitrogen) containing Penicillin-Streptomycin-Neomycin (PSN) Antibiotic Mixture (Invitrogen) at 0.1 X (5 ng penicillin, 5 ng streptomycin, 10 ng neomycin/ ml) and left to attach overnight at 27°C. Prior to transformation all medium was removed from the cells and transfection mixtures containing 1 ml of Sf-900 II SFM (Invitrogen) containing no antibiotics, 20 µl of Cellfectin Reagent (Invitrogen) and 5 µg of plasmid were added to the cells. The cells were incubated with the transfection mixes for 4 hours at 27°C with very gentle side-to-side rocking. Two ml of Sf-900 II SFM (Invitrogen) containing PSN Antibiotic Mixture (Invitrogen) at 0.1 X were added to each Petri dish. For the rest of this thesis, the word "medium" refers to Sf-900 II SFM (Invitrogen) with 0.1 X PSN Antibiotic Mixture (Invitrogen). The Petri dishes were incubated for 48 hours at 27°C in sealed plastic bags with moist paper towels. The cells were then passaged every 3-4 days, whenever they became 85- 95% confluent. They were passaged into 60 mm Petri dishes at 1:3 for the first two passages and then at 1:5 – 1.5:4.5 in T-25 flasks for subsequent passages. From the second passage onwards, Zeocin (Invitrogen) was added at 200 µg/ml to select for stably transformed cells. Prior to passaging, medium containing Zeocin was removed from the cells and 4 ml of fresh medium was added. Newly passaged Petri dishes or flasks of cells were left at least 2 h at 27°C before the addition of Zeocin (Invitrogen).

2.7 qPCR test for *T. ni* MDR mRNA expression in stably transformed Sf9 cells

Zeocin-containing medium was removed from 95-100% confluent cells and 4 ml of fresh medium were added to each flask. Cells were scraped off the surface of the flask

with a cell-scraper (Sarstedt). One hundred µl of medium containing cells was added to 10 µl of 0.4% Trypan Blue (Sigma) and mixed by pipetting up and down. A drop of this mixture was placed on a haemocytometer under a coverslip. The number of cells in a millilitre of fluid was counted and the viability of the cell line was calculated. This procedure was done for each of the six transformed cell lines as well as the untransformed (parental) cell line. The volume of medium containing $2.5*10^6$ cells from each cell line was transferred into a 12 ml culture tube (Falcon) and centrifuged in a Beckman Tabletop Centrifuge for 5 minutes at 300 *g* to pellet the cells. RNA was then extracted from each cell pellet using the RNeasy Mini Kit (Qiagen) with the RNase-Free DNase kit (Qiagen) according to the manufacturer's instructions.

Five µg of each RNA sample were reverse-transcribed into first-strand cDNA using SuperScript II H- Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. The mRNA expression level of the MDR gene with which each cell line had been transformed was determined using qPCR with the TaqMan system as previously described in order to confirm *T. ni* MDR expression in the cell lines. Untransformed (parental) Sf9 cells were also tested for *trnMDR1* and *trnMDR3* expression as a control.

2.8 MDR protein activity assays

The Vybrant Multidrug Resistance Assay kit (Invitrogen) was used to quantify MDR protein activity in stably transformed Sf9 cells. Cells were always 95-100% confluent when assayed. Old medium was removed from the cells and fresh medium

added as described previously. Cells were stained with Trypan Blue (Sigma) and the number of cells per millilitre of medium was counted and percent viability of the cells determined as described previously. Cells used in assays were always 85-95% viable. The method followed uses some of the modifications made by Rautio et al. (2002) to the original protocol developed by Tiberghien and Loor (1996).

2.8.1 Preliminary assays

Preliminary assays were conducted to compare MDR activity of stably transformed cells with untransformed cells. Fifty µl of sterile phosphate-buffered saline (PBS) (pH 7.4) were added to each well of a black, flat-bottomed 96-well plate (VWR). One hundred µl of medium containing 70,000 cells were added to each well and mixed by pipetting up and down. The plate was incubated at 27°C for 15 minutes. During this incubation, a sufficient quantity of calceinAM solutions ranging in concentration from 0.5 µM to 4 µM was made by diluting a stock solution of 1 mM calceinAM in anhydrous dimethyl sulphoxide (provided in the kit) with sterile PBS. Following the incubation, 50 µl of varying concentrations of calceinAM were added to each well and mixed by pipetting up and down. The plate was again incubated at 27°C for 15, 30 or 60 minutes. The plate was centrifuged for 5 minutes at 200 *g* in an Eppendorf 5804 centrifuge to pellet the cells. The supernatant was discarded and the cells were resuspended in 200 µl per well of cold medium. This was repeated two more times. The plate was then read in a MicroFX Plate Fluorometer at an excitation wavelength of 485 nm and emission wavelength of 510 nm.

Calcein retention was calculated by the formula:

$$\text{Calcein retention}_{Par} = \frac{\text{Fluorescence of treated parental cells}}{\text{Fluorescence of untreated parental cells}} \times 100$$

$$\text{Calcein retention}_{MDR} = \frac{\text{Fluorescence of treated transformed cells}}{\text{Fluorescence of untreated parental cells}} \times 100$$

Where Calcein retention$_{Par}$ is the calcein retention of untransformed Sf9 cells and Calcein retention$_{MDR}$ is the calcein retention of Sf9 cells stably transformed with *T. ni* MDRs.

2.8.2 MDR protein activity assays in the presence of inhibitors

Solutions of the MDR activity inhibitors verapamil and cyclosporin A ranging in concentration from 0.05 μM to 200 μM were made by diluting stock solutions (in absolute ethanol) in PBS. A range of inhibitor concentrations and calceinAM concentrations was used for preliminary assays. Based on the preliminary results, the final concentration of 50 μM of both inhibitors and 1 μM of calceinAM was used in all subsequent assays. Fifty μl of each 200 μM inhibitor solution were added to each well of a black, flat-bottomed 96-well plate. Control wells containing only PBS with no inhibitor were also set up for each cell line. One hundred μl of medium containing 70,000 cells were then added to each well and the plate was incubated at 27°C for 15 min. Fifty μl of 4 μM calcein AM were then added to each well and mixed by pipetting up and down.

Blank wells containing no calceinAM were also set up. The plate was incubated for 1 h at 27°C. The plate was centrifuged for 5 min at 200 *g* in an Eppendorf 5804 centrifuge to pellet the cells. The supernatant was discarded and the cells were resuspended in 200 µl per well of cold medium. This was repeated two more times. The fluorescence of each well was then read in a MicroFX Plate Fluorometer at an excitation wavelength of 485 nm and emission wavelength of 510 nm.

2.8.3 Survival of MDR protein-expressing Sf9 cells in the presence of a cytotoxic substrate

The cytotoxic MDR protein substrate vinblastine was used to confirm the presence of functional MDR transporters in the stably transformed cell lines. $2.25*10^5$ cells of the transformed cell lines 1-7, 1-13 and 3-1 as well as untransformed parental cells were seeded in three wells each of three 12-well plates with 2 ml medium. The cells were left to settle overnight. The next morning the cells in each well were approximately 50% confluent. Four µM and 0.4 µM solutions of vinblastine were made up by diluting the 11 mM stock solution (in sterile distilled water) in PBS. The medium was removed and 375 µl of conditioned medium was replaced in each well. One hundred and twenty-five µl of 4 µM vinblastine was added to each well of the first plate, for a final vinblastine concentration of 1 µM. One hundred and twenty-five µl of 0.4 µM vinblastine was added to each well of the second plate, for a final vinblastine concentration of 0.1 µM. One hundred and twenty-five µl of PBS was added to each well of the third plate as a control. The plates were stored in sealed plastic bags and incubated

at 27°C. The cells in each well were photographed after 24 h and 48 h of incubation using a CoolSnap digital camera attached to a Zeiss Axiovert 35 microscope. The images were acquired using MetaView software. After the 48 h incubation photographs were taken, the cells were scraped, stained with Trypan blue and counted using a haemocytometer as previously described.

3. Results

3.1. Cloning and sequencing

A 4.3 kb nucleotide sequence was identified encoding the ORF of *trnMDR1*, a 5.4 kb sequence for that of *trnMDR2* and a 4.1 kb sequence for that of *trnMDR3*. The appropriate reading frame in which the sequences could be translated was identified using BLASTX. Start and stop codons were identified for all three sequences. The DNA sequences were translated and compared with one another (Fig 3) as well as with amino acid sequences of MDR proteins from other species (Table 4). The predicted amino acid sequences of the genes show 30-40% total sequence identity to each other as well as to other vertebrate and invertebrate MDR amino acid sequences (Table 4). The amino acid sequences contained motifs expected in MDR proteins, including nucleotide-binding regions, and membrane-spanning regions typical of ABC proteins (Fig 4). There was strong sequence similarity between the putative nucleotide-binding regions of the three *T. ni* MDRs, and lower similarity in putative membrane-spanning regions (Fig 3).

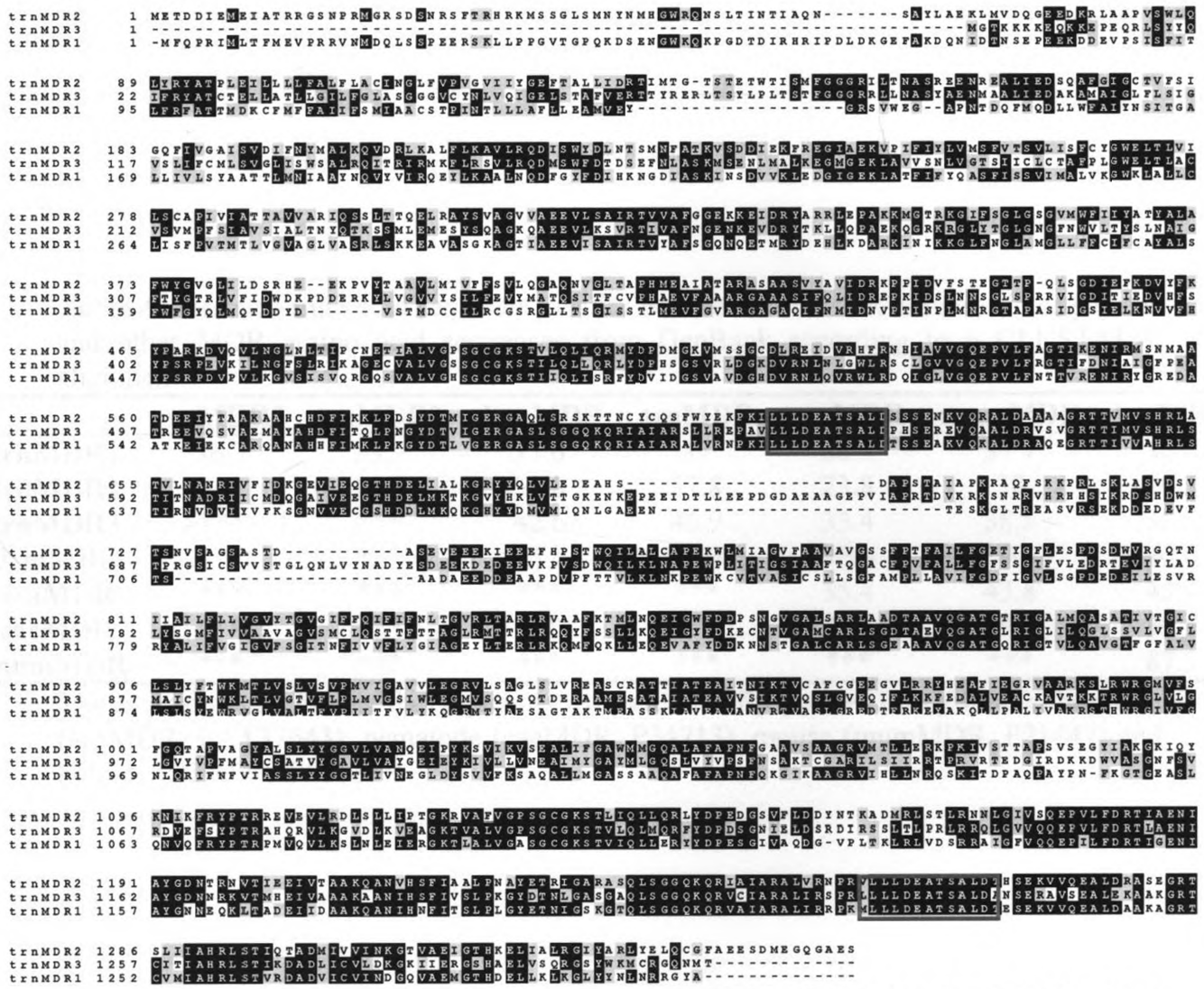

Figure 3. Amino acid sequence alignment of trnMDR1, trnMDR2 and trnMDR3. The alignment was performed using CLUSTALX (1.81) and shaded using BOXSHADE (3.21). Identical residues are shaded black and similar residues are shaded grey. Red boxes are drawn around putative nucleotide-binding regions.

Table 4. Percent amino acid sequence identity between *T. ni* MDR amino acid sequences and other MDR amino acid sequences from GenBank according to a CLUSTALW alignment.

	trnMDR2	**trnMDR3**	**drmMDR**	**aeaMDR**	**caeMDR**	**mumMDR**	**hosMDR**
trnMDR1	36.3	33.9	37.6	37	30.4	37.1	36.8
trnMDR2	***	30.1	33.8	37.8	23.8	30	29.3
trnMDR3	***	***	42.6	46.9	33.4	38.5	38.5
drmMDR	***	***	***	56.3	35.3	39.9	40.6
aeaMDR	***	***	***	***	35.4	43.8	43.7
caeMDR	***	***	***	***	***	39.7	39.5
mumMDR	***	***	***	***	***	***	87.3

The accession numbers of the sequences used are: fruitfly (drmMDR, Q00449), mosquito (aeaMDR, EAT37643), nematode (caeMDR, P34713), mouse (mumMDR, P21447) and human (hosMDR, P08183).

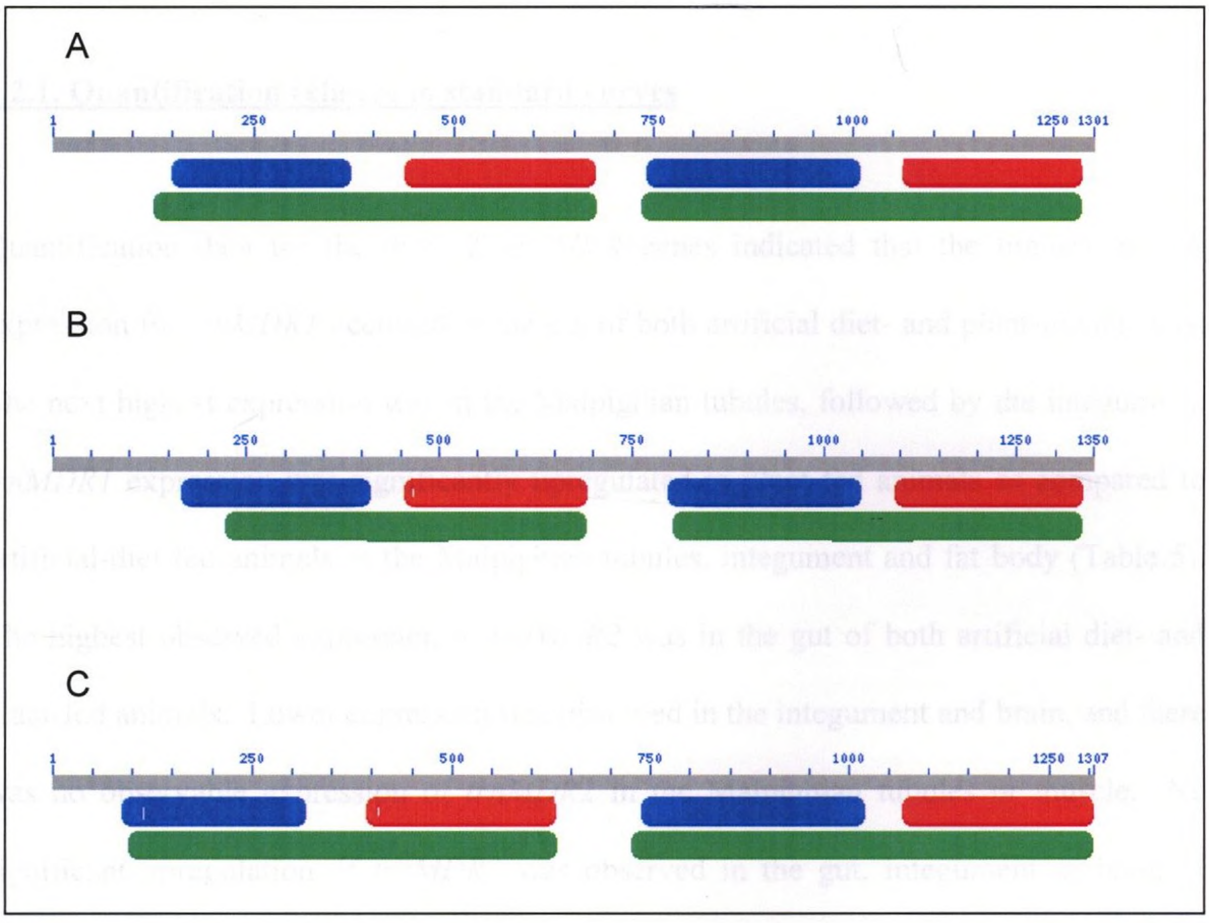

Figure 4. Identification of conserved domains in the translated sequences of trnMDR1 (A), trnMDR (B) and trnMDR3 (C). The blue domains represent transmembrane regions of ABC proteins, the red domains represent ATP-binding regions, and the green domains represent the combined ATP-binding and substrate-binding regions of ABC proteins.

3.2. MDR gene expression

3.2.1. Quantification relative to standard curves

Quantification data for the three *T. ni MDR* genes indicated that the highest mRNA expression for *trnMDR1* occurred in the gut of both artificial diet- and plant-fed animals. The next highest expression was in the Malpighian tubules, followed by the integument. *trnMDR1* expression was significantly upregulated in plant-fed animals as compared to artificial-diet fed animals in the Malpighian tubules, integument and fat body (Table 5). The highest observed expression of *trnMDR2* was in the gut of both artificial diet- and plant-fed animals. Lower expression was observed in the integument and brain, and there was no observable expression of *trnMDR2* in the Malpighian tubules or muscle. No significant upregulation of *trnMDR2* was observed in the gut, integument or brain of plant-fed animals compared to artificial diet-fed animals. However, *trnMDR2* was expressed in the fat body of plant-fed animals, but not in artificial diet-fed ones (Table 5).

Higher expression of *trnMDR3* was observed in five of the six tissues of plant-fed animals as compared to artificial diet-fed animals, however none of these trends in expression were statistically significant. *trnMDR3* expression was higher in the muscle tissue of artificial diet-fed animals than in that of plant-fed animals. No significant differences in *trnMDR3* expression were observed in the six tissues (Table 5).

Table 5. Expression (in ng/μl of cDNA) of *trnMDR1*, *trnMDR2* and trnMDR3 relative to standard curves in various tissues of larval *T. ni* under two dietary regimes.

	trnMDR1		***trnMDR2***		***trnMDR3***	
	diet	plant	diet	plant	diet	plant
Gut	$1.34*10^{-4}$	$9.84*10^{-5}$	$1.52*10^{-5}$	$1.32*10^{-5}$	$9.38*10^{-7}$	$8.02*10^{-6}$
MT	**$1.04*10^{-6}$**	**$1.07*10^{-5}$**	$<10^{-9}$	$<10^{-9}$	$1.20*10^{-6}$	$2.98*10^{-5}$
Integument	**$3.06*10^{-7}$**	**$3.87*10^{-6}$**	$2.34*10^{-7}$	$7.44*10^{-7}$	$9.38*10^{-7}$	$3.10*10^{-5}$
Brain	$3.65*10^{-7}$	$2.43*10^{-7}$	$2.10*10^{-6}$	$1.73*10^{-6}$	$5.58*10^{-6}$	$1.56*10^{-4}$
Muscle	$1.55*10^{-8}$	$1.35*10^{-7}$	$<10^{-9}$	$<10^{-9}$	$1.43*10^{-5}$	$3.50*10^{-6}$
Fat body	**$8.44*10^{-9}$**	**$2.68*10^{-6}$**	**$<10^{-9}$**	**$1.61*10^{-5}$**	$9.60*10^{-7}$	$2.99*10^{-4}$

Each number represents the means of 3-4 values. Pairs of values significantly different according to Unpaired Student's t-test ($p<0.05$) are shown in red.

3.2.2. Quantification relative to endogenous "housekeeping" controls

The expression levels of the three *MDR* genes of interest expressed relative to the combined expression of the three housekeeping genes demonstrated trends similar to those observed in the absolute quantification data. With the exceptions of *trnMDR1* expression in the gut and *trnMDR3* expression in the brain of plant-fed animals and the muscle of artificial diet-fed animals, expression of all three *MDR* genes was observed to be less than 5% of the expression of the averaged housekeeping genes (Fig 5). Highest relative expression of *trnMDR1* and *trnMDR2* was seen in the gut (Fig 5A, B). Twenty-five-fold higher relative expression of *trnMDR3* was observed in the brain of plant-fed animals than artificial diet-fed animals, but 170-fold higher relative expression was observed in the muscle of artificial diet-fed animals than plant-fed animals (Fig 5C).

3.2.3. "Housekeeping" gene expression

Higher housekeeping gene expression was observed in plant-fed animals than artificial diet-fed animals, with the exception of *actin* expression in brain tissue (Fig 6C). Housekeeping gene expression varied between the six tissues, but not to a significant degree. Expression of *actin* in the gut and Malpighian tubules is not shown (Fig 6C). In general, the highest housekeeping gene expression was recorded for *S5*, followed by *G3PDH* and then *actin* (Fig 6).

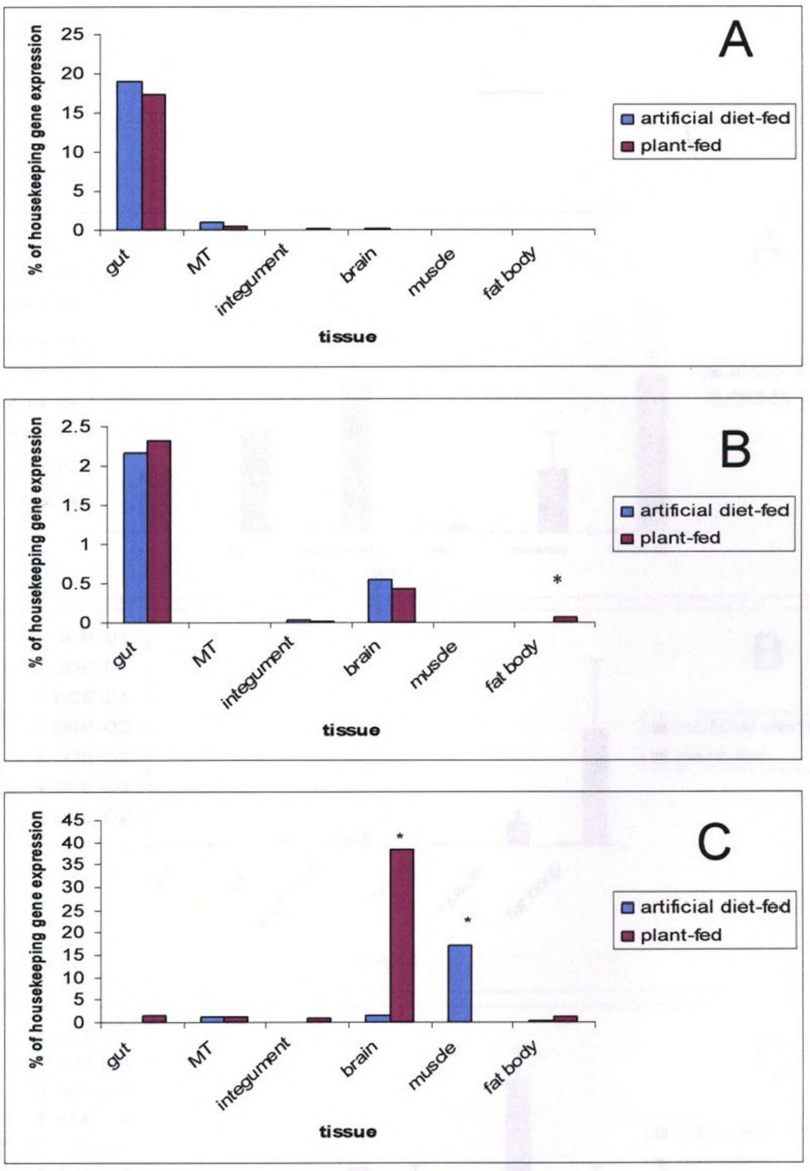

Figure 5. Expression of *trnMDR1* (A), *trnMDR2* (B) and *trnMDR3* (C) mRNA in artificial diet- and plant-fed tissue in terms of percentage of housekeeping gene expression. *T. ni* MDR mRNA expression in integument, brain, muscle and fat body is shown in terms of combined expression of *G3PDH*, *S5* and *actin* mRNA. Note that *T. ni* MDR expression in gut and MT is shown in terms of combined *G3PDH* and *S5* expression only. Pairs of bars with a >10-fold difference in mRNA expression are marked with an asterisk.

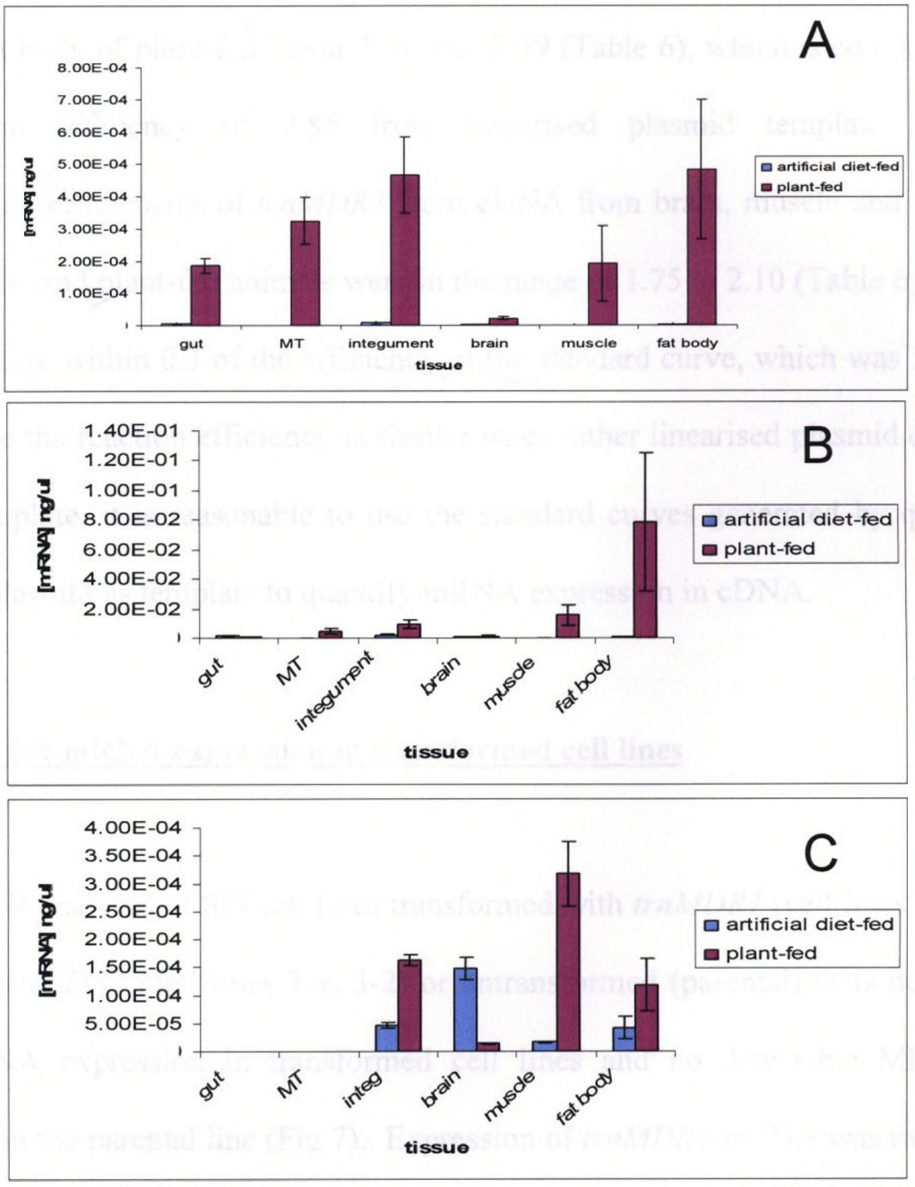

Figure 6. Quantification of three housekeeping genes: *G3PDH* (A), *S5* (B), and *actin* (C) relative to standard curves in artificial diet- and plant-fed *T. ni* tissues. Each bar represents the average of 4-5 values ± s.e.m. Note that *actin* mRNA expression in gut and MT is not shown.

3.2.4 qPCR amplification efficiency

It was observed that the efficiency of amplification of *trnMDR2* in the cDNA from the fat body of plant-fed larval *T. ni* was 2.99 (Table 6), which is comparable to its amplification efficiency of 2.85 from linearised plasmid template (Table 7). Amplification efficiencies of *trnMDR3* from cDNA from brain, muscle and fat body of artificial diet- and plant-fed animals were in the range of 1.75 to 2.10 (Table 6). All these efficiencies are within 0.3 of the efficiency of the standard curve, which was 1.86 (Table 7). Because the reaction efficiency is similar when either linearised plasmid or cDNA is used as template, it is reasonable to use the standard curves generated by qPCR using linearised plasmid as template to quantify mRNA expression in cDNA.

3.3 *T. ni* MDR mRNA expression in transformed cell lines

qPCR analyses of Sf9 cell lines transformed with *trnMDR1* (cell lines 1-5, 1-7, 1-10, 1-13), *trnMDR3* (cell lines 3-1, 3-2) or untransformed (parental) cells demonstrated MDR mRNA expression in transformed cell lines and no detectable MDR mRNA expression in the parental line (Fig 7). Expression of *trnMDR1* mRNA was two orders of magnitude higher than that of *trnMDR3* mRNA (Fig 7).

Table 6. Efficiencies of qPCR amplifications using serial dilutions of cDNA

Gene	Template	Efficiency
trnMDR2	Plant-fed fat body	2.99
trnMDR3	Diet-fed brain	1.86
trnMDR3	Plant-fed brain	2.10
trnMDR3	Diet-fed muscle	2.10
trnMDR3	Plant-fed muscle	2.10
trnMDR3	Diet-fed fat body	1.75
trnMDR3	Plant-fed fat body	1.97

Each number represents the quantification efficiency of the respective qPCR amplicon for each gene, using serial dilutions of first-strand cDNA as template. Three replicates each of three dilutions of cDNA were used to calculate each efficiency.

Table 7. Efficiencies of qPCR amplifications used to generate standard curves

Template	Efficiency
trnMDR1	2.28
trnMDR2	2.85
trnMDR3	1.86
G3PDH	2.28
S5	2.28
actin	1.93

Each number represents the amplification efficiency of the respective qPCR amplicon for each gene, using serial dilutions of linearised plasmid as template. Three replicates each of five dilutions were used for each standard curve.

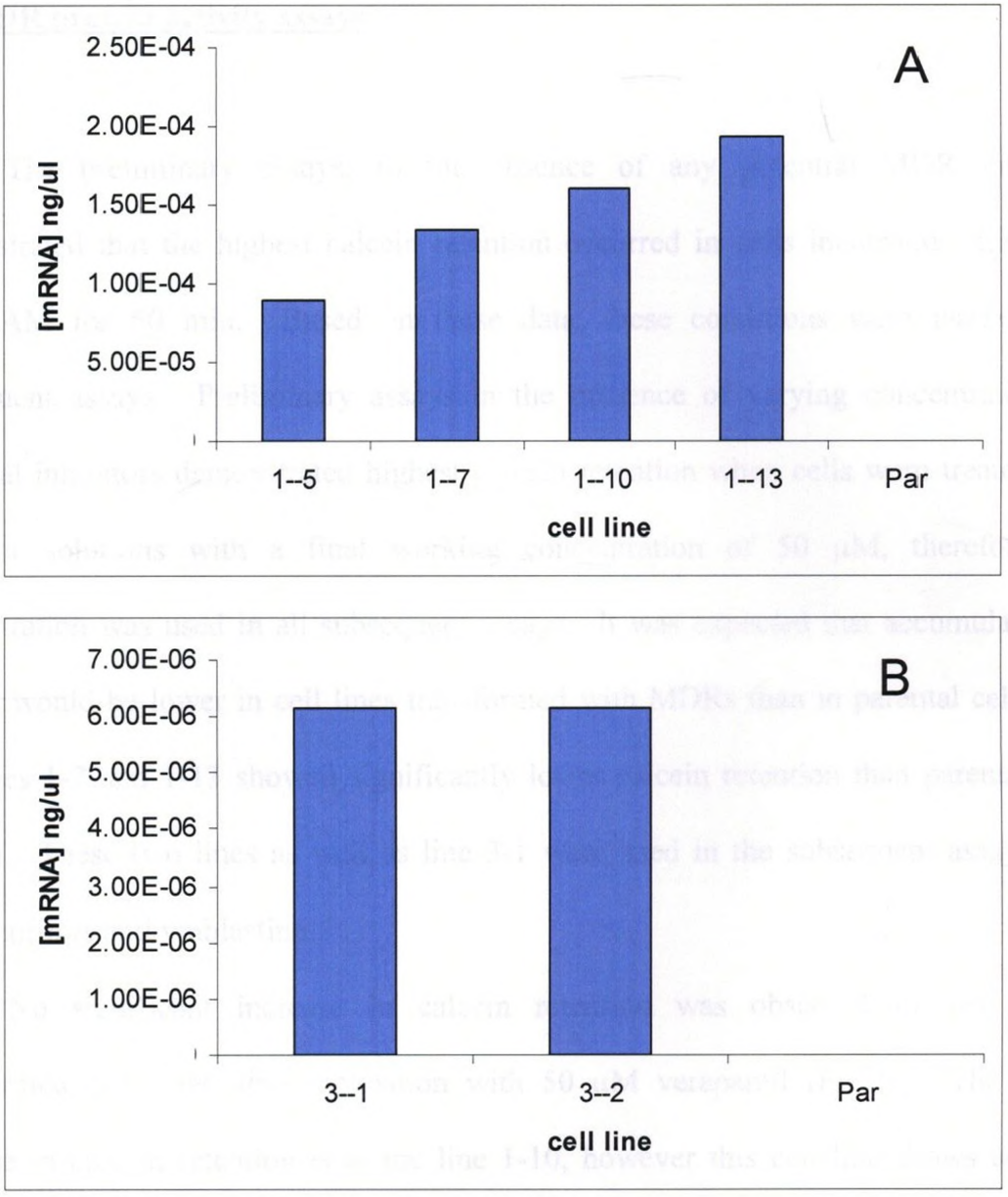

Figure 7. Quantification of *trnMDR1* (A) and *trnMDR3* (B) mRNA expression in transformed Sf9 cell lines and parental (Par) Sf9 cells relative to standard curves. Each bar represents the average of two values.

3.4 MDR protein activity assays

The preliminary assays, in the absence of any potential MDR inhibitor, demonstrated that the highest calcein retention occurred in cells incubated with 1 μM calceinAM for 60 min. Based on these data, these conditions were used for all subsequent assays. Preliminary assays in the presence of varying concentrations of potential inhibitors demonstrated highest calcein retention when cells were treated with inhibitor solutions with a final working concentration of 50 μM, therefore this concentration was used in all subsequent assays. It was expected that accumulation of calcein would be lower in cell lines transformed with MDRs than in parental cells. The cell lines 1-7 and 1-13 showed significantly lower calcein retention than parental cells (Fig 8). These two lines as well as line 3-1 were used in the subsequent assays with cyclosporin A and vinblastine.

No significant increase in calcein retention was observed in any of the transformed cell lines after incubation with 50 μM verapamil (Fig 9). The largest increase in calcein retention is in the line 1-10, however this cell line shows as much calcein retention as uninhibited parental cells even in the absence of verapamil (Fig 9).

Preliminary results showed increased calcein accumulation in the cell line 1-7 after incubation with 50 μM cyclosporin A (Fig 10). However subsequent repeats of this experiment using only three transformed cell lines demonstrated no significant change in calcein retention as a result of incubation with 50 μM cyclosporin A (Fig 11).

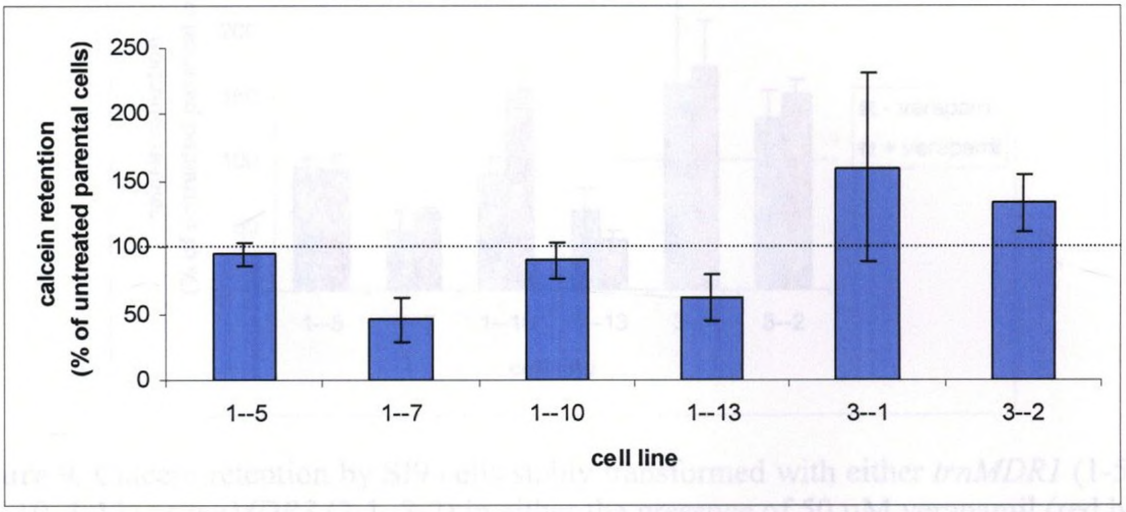

Figure 8. Calcein retention of the six Sf9 cell lines stably transformed with either *trnMDR1* (1-5, 1-7, 1-10, 1-13) or *trnMDR3* (3-1, 3-2) expressed in terms of percent of calcein retention by untransformed Sf9 cells. Each bar represents the average of 4-6 values ± s.e.m.

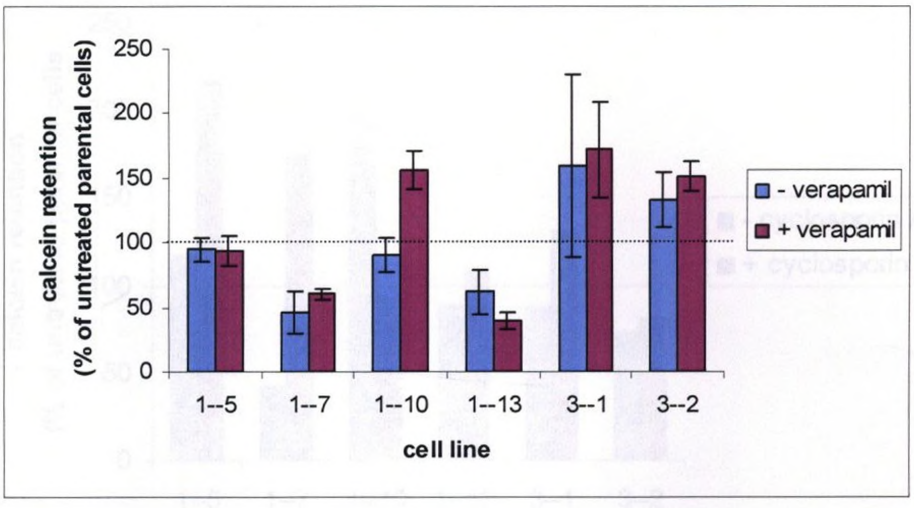

Figure 9. Calcein retention by Sf9 cells stably transformed with either *trnMDR1* (1-5, 1-7, 1-10, 1-13) or *trnMDR3* (3-1, 3-2) in either the presence of 50 μM verapamil (red bars) or absence of verapamil (blue bars). Data are expressed in terms of percent of calcein retention by untransformed Sf9 cells in the absence of verapamil. Each bar represents the average of 4-6 values ± s.e.m.

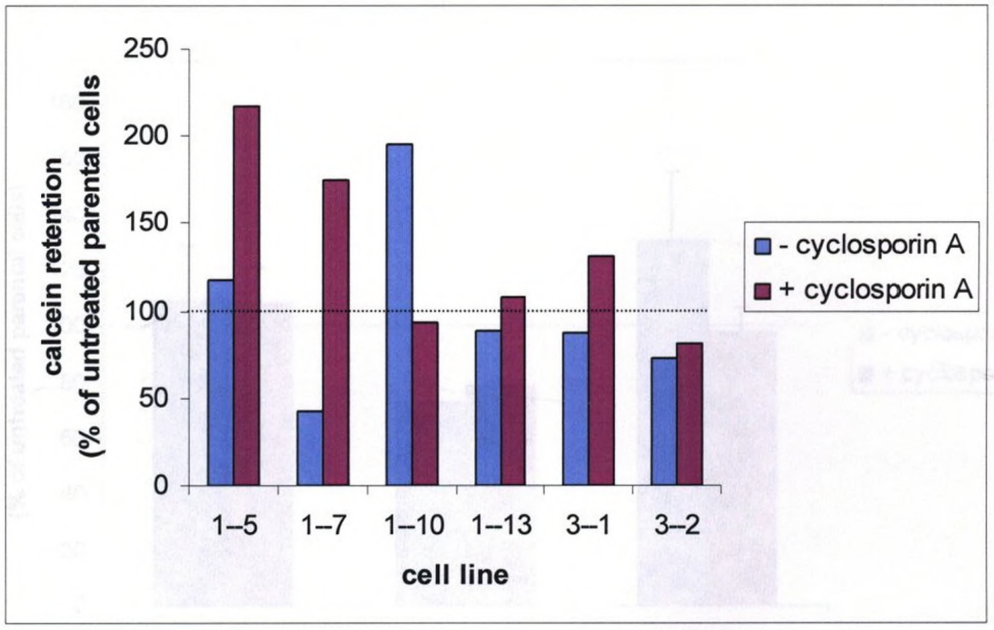

Figure 10. Calcein retention by Sf9 cells stably transformed with either *trnMDR1* (1-5, 1-7, 1-10, 1-13) or *trnMDR3* (3-1, 3-2) in either the presence of 50 μM cyclosporin A (red bars) or absence of cyclosporin A (blue bars). Data are expressed in terms of percent of calcein retention by untransformed Sf9 cells in the absence of cyclosporin A.

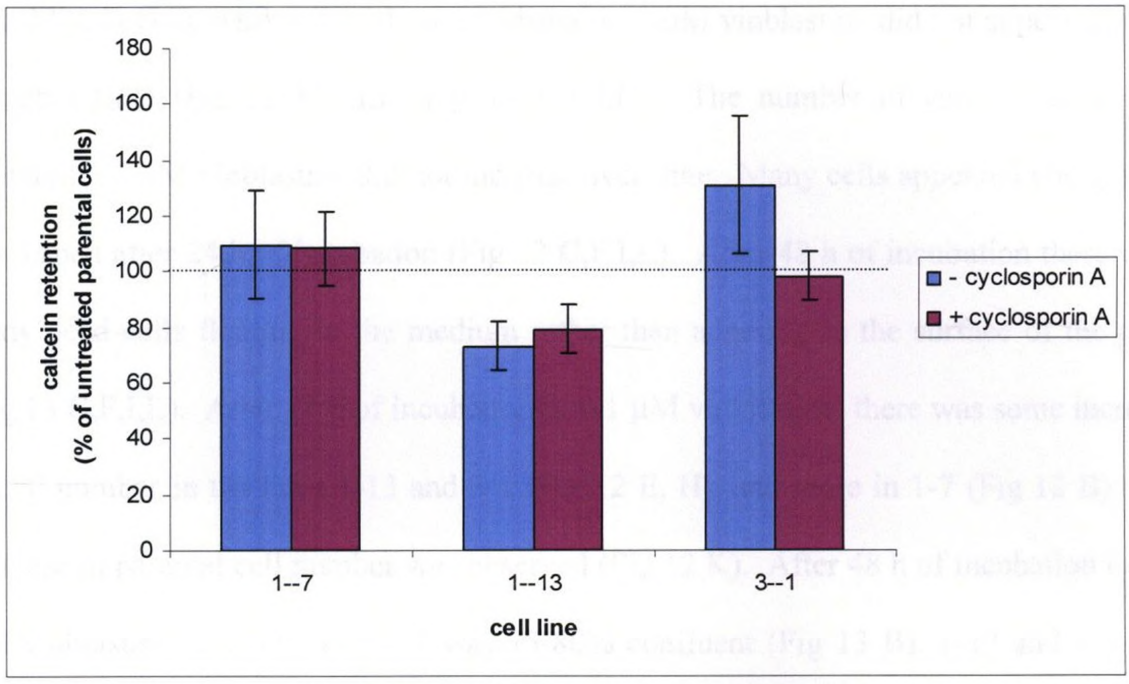

Figure 11. Calcein retention by Sf9 cells stably transformed with either *trnMDR1* (1-7, 1-13) or *trnMDR3* (3-1) in either the presence of 50 μM cyclosporin A (red bars) or absence of cyclosporin A (blue bars). Data are expressed in terms of percent of calcein retention by untransformed Sf9 cells in the absence of cyclosporin A. Each bar represents the average of 13 values ± s.e.m.

3.5 Vinblastine cytotoxicity assay

All cells incubated in PBS appeared healthy after 24 h (Fig 12 A,D,G,J) and 48 h (Fig 13 A,D,G,J), while all of those incubated in 1 μM vinblastine did not appear healthy at either time (Fig 12 C,F,I,L; Fig 13 C,F,I,L). The number of cells in each well containing 1 μM vinblastine did not increase over time. Many cells appeared enlarged or misshapen after 24 h of incubation (Fig 12 C,F,I,L). After 48 h of incubation there were many dead cells floating in the medium rather than adhering to the surface of the plate (Fig 13 C,F,I,L). After 24 h of incubation in 0.1 μM vinblastine, there was some increase in cell number in the lines 1-13 and 3-1 (Fig 12 E, H), and more in 1-7 (Fig 12 B). No increase in parental cell number was observed (Fig 12 K). After 48 h of incubation in 0.1 μM vinblastine, the cell line 1-7 was 75-80% confluent (Fig 13 B). 1-13 and 3-1 was about 60% confluent although some clumping of the cells was observed (Fig 13 E, H). No increase in cell number was observed in the parental cells, and many cells appeared enlarged (Fig 13 K).

Quantitative observation in terms of cell counts after 48h of incubation revealed the highest number of cells in the wells containing no vinblastine and lowest in 1 μM vinblastine in all four cell lines (Table 8). Among the cells incubated in 0.1 μM vinblastine, the highest number of cells was observed in 1-7, the next highest in 1-13 and 3-1 and the lowest number of cells in the parental cell line (Table 8). This trend was repeated when the cell counts were normalised by dividing the average cell count of each cell line incubated in vinblastine by the corresponding average cell count of the same line with no vinblastine. Cells incubated in 1 μM vinblastine all had around 20% of the cell

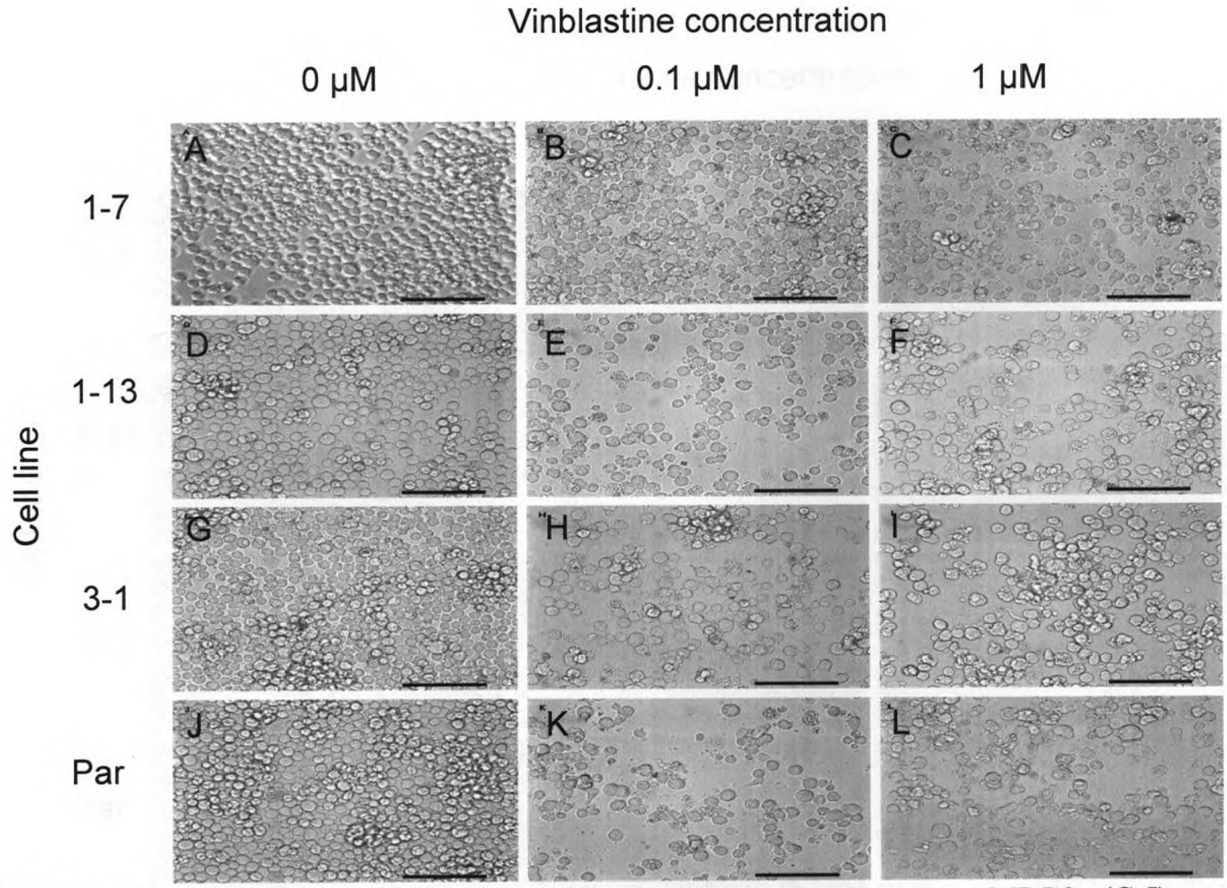

Figure 12. Sf9 cells transformed with *trnMDR1* (A-F) or *trnMDR3* (G-I) or untransformed Sf9 cells (J-L) incubated for 24 h with either PBS (A,D,G,J), or 0.1 μM vinblastine (B,E,H,K) or 1 μM vinblastine (C,F,I,L). Scale bars represent 125 μm.

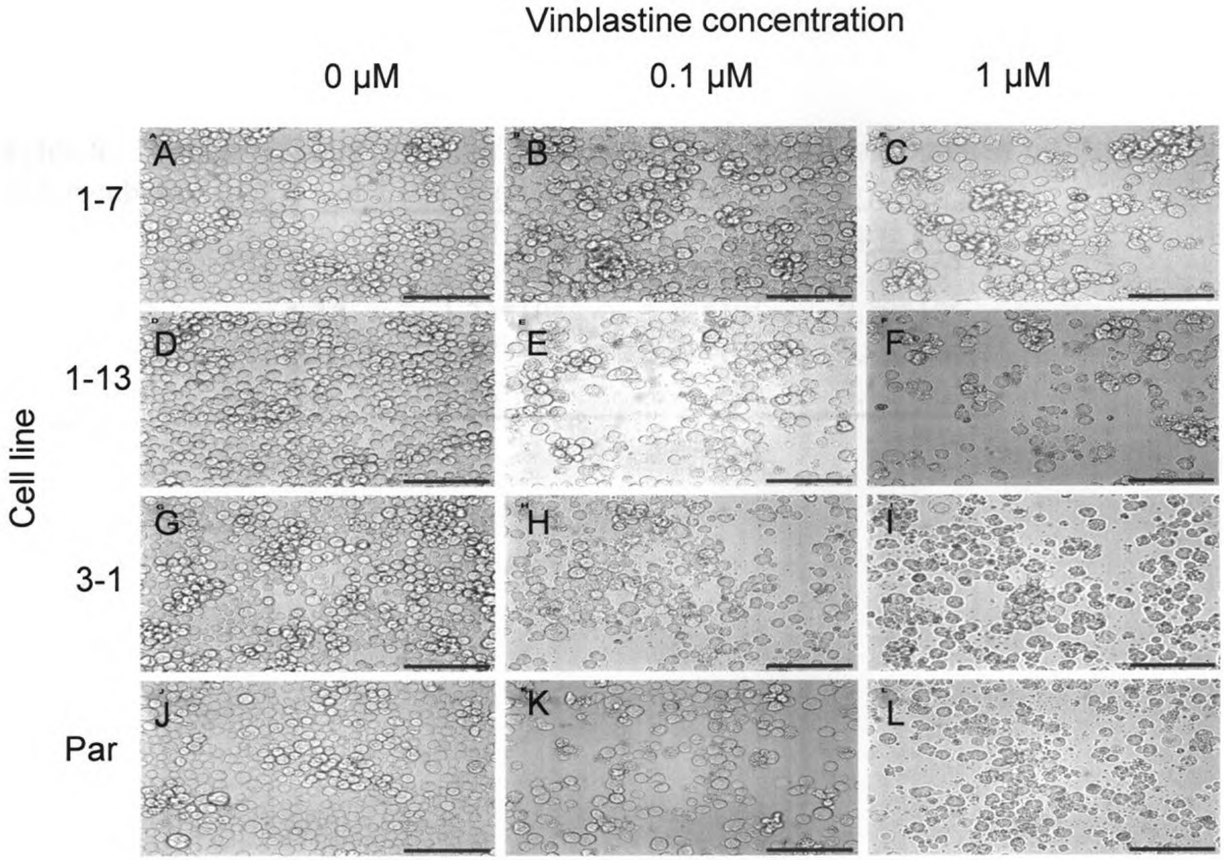

Figure 13. Sf9 cells transformed with *trnMDR1* (A-F) or *trnMDR3* (G-I) or untransformed Sf9 cells (J-L) incubated for 48 h with either PBS (A,D,G,J), or 0.1 µM vinblastine (B,E,H,K) or 1 µM vinblastine (C,F,I,L). Scale bars represent 125 µm.

Table 8. Number of cells per ml of medium of transformed and parental cell lines after 48 h incubation in 0, 0.1 or 1 μM vinblastine (VBL).

	0 μM VBL	**0.1 μM VBL**	**1 μM VBL**
1-7	$1.84*10^6$	$9.97*10^5$	$4.55*10^5$
1-13	$1.33*10^6$	$5.72*10^5$	$2.79*10^5$
3-1	$2.37*10^6$	$6.01*10^5$	$3.45*10^5$
Par	$1.35*10^6$	$3.78*10^5$	$3.12*10^5$

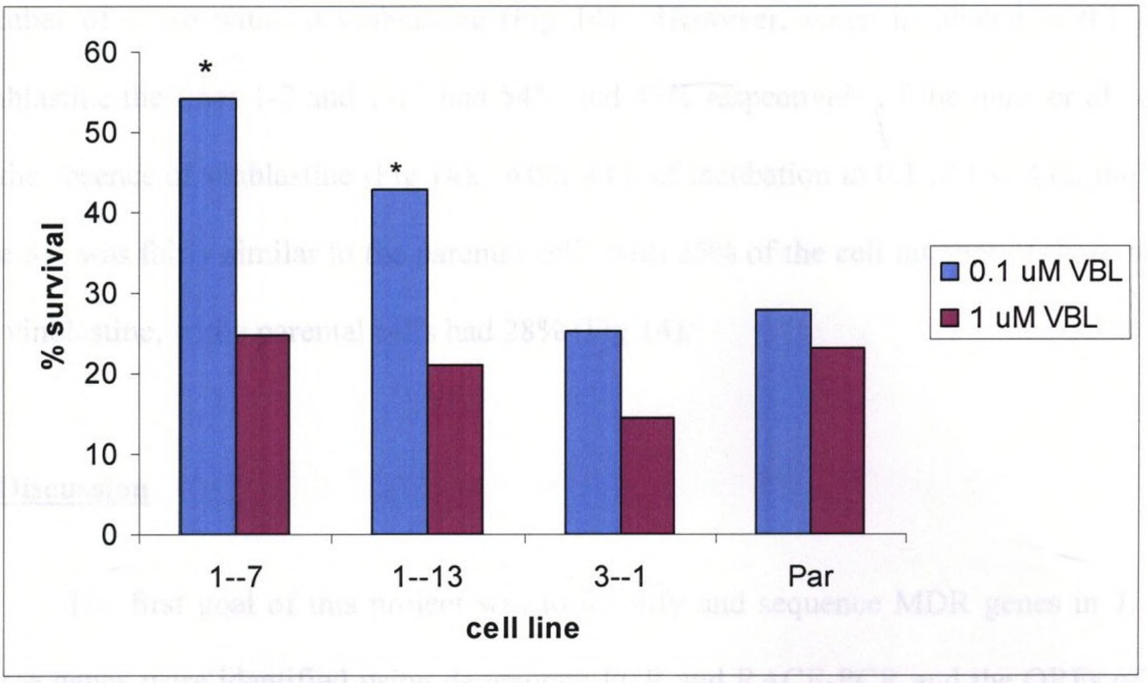

Figure 14. Percentage of Sf9 cell lines transformed with *trnMDR1* (1-7, 1-13) or *trnMDR3* (3-1) or untransformed cells (Par) alive after 48 h incubation in 0.1 μM or 1 μM vinblastine. The number of cells/ml of medium incubated in vinblastine were normalised to the same cell lines incubated for 48 h in the absence of vinblastine to calculate percent survival. Bars marked with an asterisk represent a cell number >30% of the number of untreated cells.

number of those with no vinblastine (Fig 14). However, when incubated in 0.1 μM vinblastine the lines 1-7 and 1-13 had 54% and 43% respectively of the number of cells in the absence of vinblastine (Fig 14). After 48 h of incubation in 0.1 μM vinblastine the line 3-1 was fairly similar to the parental cells with 25% of the cell number of those with no vinblastine, while parental cells had 28% (Fig 14).

4. Discussion

The first goal of this project was to identify and sequence MDR genes in *T. ni*. Three genes were identified using degenerate PCR and RACE-PCR and the ORFs of all three were sequenced. BLASTX analyses of all three genes demonstrated significant sequence identity between each of them and MDR amino acid sequences of other species (Table 4). Putative nucleotide-binding regions were identified based on regions of highest homology with other MDR amino acid sequences as well as conserved-domain identification using CDSearch on the translated sequences (Fig 4). They also show significant similarity to each other not only in the putative nucleotide-binding regions, which are the most highly conserved areas of MDR gene sequences (Bosch and Croop, 1998), but also in the putative transmembrane regions, which are the least conserved among species (Bosch and Croop, 1998).

Four MDR sequences have been identified in the genome of the fruit fly, *D. melanogaster* (Gerrard et al., 1993; Wu et al., 1991). The *D. melanogaster* MDR amino acid sequence used for comparison in Table 4 is that of MDR49 (Wu et al., 1991). ClustalW analysis of all four *D. melanogaster* sequences along with the three *T. ni* sequences demonstrated that the *D. melanogaster* sequences were more similar to one

another, in the range of 40% identity at the amino acid level, than were the *T. ni* sequences which were in the range of 35% amino acid identity (Table 9). However phylogenetic analysis grouped *trnMDR1* with *D. melanogaster mdr50*, *trnMDR2* with *D. melanogaster mdr49*, *mdr65* and the fourth *D. melanogaster* MDR protein which is as yet unnamed in the literature, and *trnMDR3* was an outgroup (Fig 15). Of the four *D. melanogaster* MDR proteins, *trnMDR1* showed the greatest amino acid sequence identity to *mdr50* (Table 9). No functional studies of *mdr50* are available in the literature; therefore any predictions for the function of *trnMDR1* are based solely on the results of this project. *trnMDR3* showed approximately 40% sequence identity to all four *D. melanogaster* MDR proteins, but only approximately 30% identity to the other *T. ni* MDR amino acid sequences (Table 9), which resulted in its being an outgroup in the phylogenetic tree (Fig 15). *trnMDR3* could be an orthologue of any or none of the four *D. melanogaster* MDR proteins, and therefore no functional studies of *D. melanogaster* MDR proteins can be used to predict the function of *trnMDR3*. It is also possible that *trnMDR3* is closest in sequence to the ancestral insect or invertebrate MDR protein. Of the three *T. ni* MDRs, *trnMDR3* shows the highest amino acid sequence identity to the MDR proteins of other species (Table 4). It is also notable that *trnMDR3* expression is observed in all tissues studied in both artificial diet- and plant-fed *T. ni* larvae, and at higher levels than the other two MDRs. This also suggests that *trnMDR3* may be the ancestral *T. ni* MDR, from which *trnMDR1* and *trnMDR2* evolved independently to fulfill similar functions to the *D. melanogaster* MDR proteins *mdr50*, and *mdr49* or *mdr65* respectively. Independent evolution of ABC proteins to perform similar functions has been observed in other species (Neyfakh, 2002) and has probably contributed to the

Table 9. Percent amino acid sequence identity of *T. ni* and *D. melanogaster* MDR amino acid sequences

	trnMDR2	trnMDR3	mdr49	mdr50	mdr65	drmMDR
trnMDR1	36.4	34.3	37	41.8	34.3	33.7
trnMDR2	***	30.2	33.8	29.7	33.6	30.3
trnMDR3	***	***	42.7	37.8	40.4	39.8
mdr49	***	***	***	41.5	44.4	42.5
mdr50	***	***	***	***	38.8	36.9
mdr65	***	***	***	***	***	54.9

The accession numbers of the *D. melanogaster* sequences used are: mdr49 (Q00449), mdr50 (NP 52370), mdr65 (Q00748) and the fourth *D. melanogaster* MDR, called drmMDR here (NP 648040).

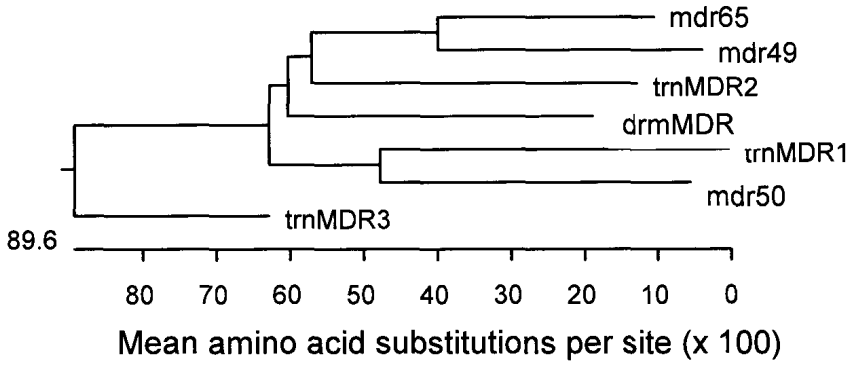

Figure 15. Rooted phylogenetic tree of amino acid sequences of *T. ni* and *D. melanogaster* MDR proteins. *D. melanogaster* MDR amino acid sequences have the same GenBank accession numbers as those listed in Table 9.

lack of orthology observed between the MDR protein families of various species (Sheps et al., 2004).

These data may reflect differences in the function of the *T. ni* MDR proteins. Functional differences in response to heat shock and colchicine feeding between *mdr49* and *mdr65* have been observed in *D. melanogaster* larvae (Tapadia and Lakhotia, 2005). Enhanced expression of *mdr49* was observed in larval brain and gut in response to both heat shock and colchicine feeding, while *mdr65* levels remained unchanged under both stress conditions (Tapadia and Lakhotia, 2005). The *T. ni* MDR protein closest in amino acid sequence to *mdr49*, *trnMDR2*, was strongly expressed in the fat body of plant-fed *T. ni* larvae, while no expression could be observed in the fat body of artificial diet-fed larvae (Table 4). The earlier study suggests that *mdr49* is the MDR transporter gene in *D. melanogaster* likely to be upregulated in response to stress, however it was present at an observable similar basal level in the brain, gut and wing imaginal discs of *D. melanogaster* larvae (Tapadia and Lakhotia, 2005). *trnMDR2* mRNA expression was detected at a low level in the brain and integument of artificial diet-fed *T. ni* larvae, at a higher level in the gut, and no detectable expression was observed in the muscle or Malpighian tubules (Table 5). It is possible that *trnMDR2* is expressed in all tissues of larval *T. ni*, but at levels below the range of detection of the qPCR system used in this project. No experiments have been done to determine conclusively whether *trnMDR2* is an orthologue of *mdr49*. However, its expression pattern suggests that it does respond to the stress of feeding on cabbage with its defensive secondary metabolites, as compared to feeding on artificial diet. The fat body of insects is analogous to the mammalian liver, and like the liver is a site for sequestration of toxins in order to remove them from the rest

of the body. Upregulation of *trnMDR2* in the fat body of larval *T. ni* in response to feeding on cabbage may indicate that this gene plays a protective function. It may be of interest in the future to observe whether *trnMDR2* is upregulated in response to feeding on other toxins.

Other significant changes in expression level of *T. ni* MDR mRNA in response to plant feeding were the upregulation with respect to the standard curve of *trnMDR1* mRNA in the Malpighian tubules, integument and fat body (Table 5); the increased relative expression of *trnMDR3* mRNA in the brain of plant fed *T. ni* larvae and the decreased relative to housekeeping gene expression of *trnMDR3* mRNA in the muscle of plant-fed *T. ni* larvae (Fig 5C). Large differences were observed between *trnMDR3* mRNA expression levels relative to the standard curve in artificial diet- and plant-fed *T. ni* larvae; however the large variation in the data prevented these differences from being significant (Table 5). However when quantification relative to housekeeping gene expression was used to analyse the *trnMDR3* mRNA expression data, these differences did appear significant, with expression in the brain of plant-fed animals demonstrating a 25-fold increase from expression in artificial diet-fed animals, and the expression in the muscle of plant-fed animals demonstrating a 170-fold decrease from that of artificial diet-fed animals (Fig 5C). It is possible that under the relatively stress-free condition of feeding on artificial diet, the *T. ni* larvae do not need to spend the energy required to produce many MDR transporters, which are large proteins, in muscle since muscle tissue is not an important surface for excretion of toxins from the body. The brain, however, is an important secretory area since the function of the blood-brain barrier is to protect the brain and nervous system from toxic substances. Therefore it is possible that the

exposure to plant defensive compounds caused an increase in expression of *trnMDR3* mRNA in the brain of plant-fed larval *T. ni*. Similarly, the upregulation of *trnMDR1* expression in the Malpighian tubules, integument and fat body of plant-fed larvae may have been a defensive response to the toxins in the plant diet.

While the changes observed in the expression of MDR mRNA in larval *T. ni* are most likely to be the result of plant-feeding, other factors may also contribute to higher MDR mRNA expression. An important point to note is that the highest expression of *trnMDR1* and *trnMDR2* was observed in the gut of insects under both dietary conditions, however no significant difference in their expression was observed in response to different diets (Table 5, Fig 5). These data suggest that although MDR proteins are responsible for removal of toxins from the gut, their expression is not dependent on the insects' diet. Since the gut is a large organ, and the largest one studied in this thesis, the lack of significant MDR mRNA upregulation in it suggests that MDR mRNA upregulation is not a major response to different dietary conditions, but a more specialised response in specific tissues.

Gut, Malpighian tubules, brain and integument were chosen for this study because they are known to be important secretory surfaces in insects. Muscle and fat body were originally intended as controls, because of the difficulty of removing all of the muscle and fat body from the integument during dissection. However fat body was observed to be an interesting tissue in its own right, showing increased expression of *trnMDR1* mRNA relative to the standard curve (Table 5) and *trnMDR3* mRNA relative to housekeeping gene expression (Fig 5C) in response to plant feeding. The increased MDR mRNA expression in Malpighian tubules, integument, fat body and brain were expected

in response to plant feeding, as a defense mechanism against plant secondary metabolites. The observation that different MDR proteins were upregulated in different tissues suggests that these results reflect differences in the function of these MDR proteins, rather than being experimental artifacts. It must be noted that all of the qPCR results presented in this thesis represent a single biological data set. All the replicates are technical rather than biological. More biological replicates, in which tissues are dissected from new batches of *T. ni* larvae and RNA extracted from these tissues, would provide a better indication of MDR mRNA expression in different tissues and under different dietary conditions. Insects raised on cabbage grew much more slowly and had higher death rates than those raised on artificial diet. Therefore time constraints prevented the acquisition of data from more biological replicates for use in this thesis. However, additional tissue collection is underway in our laboratory, and we hope to have biological replicates of all qPCR data in the near future.

The difficulties encountered in attempting to rear *T. ni* on cabbage indicate the extent to which the laboratory-raised population of *T. ni* used in this thesis appear to have lost resistance to the toxins in their natural diet. The *T. ni* population used in this study has been in culture for more than 12 years, and thus has undergone many generations without selection pressure to maintain resistance to plant toxins. *T. ni* populations from greenhouses lose resistance to *Bacillus thuringiensis* within four generations of being raised in a laboratory setting (Janmaat and Myers, 2003). It is reasonable to assume, therefore, that the effect of diet on the *T. ni* larvae in this study is not the same as would be observed on *T. ni* larvae in the wild. Another factor that may influence the level of MDR protein expression is the insects' response to the different nutritional quality of

artificial or plant diet. It may be higher protein content in cabbage, compared to the high carbohydrate content of the wheat-germ-based artificial diet, which resulted in MDR mRNA upregulation in larval *T. ni*. Although other lepidopteran larvae have been observed to select protein-biased diets when given a choice of diets (Bede et al., 2007), the *T. ni* larvae in this study have been reared for many generations on a carbohydrate-biased artificial diet, and thus may respond to cabbage-feeding as if it were excessively high in protein. It is possible, therefore, that MDR proteins were upregulated in response to feeding on cabbage in order to excrete excess nitrogenous compounds, rather than plant defense compounds.

Normalisation of the qPCR data relative to housekeeping genes takes into account the differences between each individual cDNA sample, thus correcting for differences in cDNA quantity and efficiency of reverse-transcription in each cDNA synthesis reaction. As much care as possible was taken to keep the cDNA samples identical to one another, however, as shown by the differences in housekeeping gene expression, the samples were different from one another to some extent (Fig 6). In addition, relative quantification to a number of housekeeping genes is more reliable than relative quantification to a single one, since expression of even housekeeping genes varies considerably (Vandersompele et al., 2002). Considerable variation was observed between housekeeping gene expression in the six tissues studied here, as well as between the two dietary regimes (Fig 6). Therefore, by using three housekeeping genes, I have attempted to provide a reliable normalisation factor.

The reaction efficiencies of the standard curves (Table 7) were calculated to determine how well each PCR amplification was working. The efficiency of each

reaction is a measure of the fold-increase in number of template molecules per cycle. Theoretically the number of template molecules should double with each cycle, thus the efficiency of a PCR amplification should be 2. However in practice a number of factors can prevent a PCR amplification from achieving perfect efficiency. These factors can include accessibility of the template amplicon to the primers or polymerase; rate of formation of the polymerisation complex; or formation of non-specific PCR products which slow the amplification of the intended product. The reason that the plasmids used to generate standard curves were linearised was to make the amplicons more accessible to the other components of each PCR amplification. It was observed that qPCR amplifications using linearised plasmid as template provided higher reaction efficiencies and lower crossing points than those using non-linearised plasmid as template (data not shown). In order to provide further confirmation of the expression results of the most interesting tissues, qPCR assays using serial dilutions of cDNA were performed. This was done to confirm that the efficiency of PCR amplification using cDNA as the reaction template was close to the efficiency of amplifications using linearised plasmid as template.

The calcein retention assay and the vinblastine cytotoxicity assay were used to characterise the protein function of *trnMDR1* and *trnMDR3*. The aim of both assays was to determine whether these genes could produce functional proteins in transformed Sf9 cells. The principle of the calceinAM assay is that calceinAM, being a hydrophobic compound, is able to diffuse passively through the cell membrane into the cytoplasm (Tiberghien and Loor, 1996). Once in the cytoplasm, endogenous esterases cleave off the acetoxymethylester (AM) group, leaving calcein. The calcein is no longer hydrophobic,

and is a poor substrate of MDR transporters (Tiberghien and Loor, 1996). Additionally, calcein is fluorescent; therefore measuring the fluorescence of the cells gives a measure of the amount of calcein that has accumulated. It was expected that cells transformed with MDR genes would show significantly less calcein accumulation than untransformed cells, because functional MDR proteins would transport the calceinAM out of the cell before it was cleaved by the esterases in the cytoplasm. Addition of an MDR protein inhibitor should lead to increased calcein accumulation by transformed cells. The data presented here suggest that calceinAM is a poor substrate of *T. ni* MDR proteins expressed in Sf9 cells. Only two transformed cell lines, 1-7 and 1-13 showed significantly less calcein accumulation than untransformed cells (Fig 8). The cell lines 1-5 and 1-10 showed no significant difference from the untransformed cells in their calcein retention, while the cell lines 3-1 and 3-2 showed higher calcein accumulation than did the untransformed cells (Fig 8). No previous studies of this sort have been conducted on insect MDR proteins, however similar assays using calceinAM as well as other substrates have been conducted on mammalian MDCK cells transformed with the human gene *mdr1* (Polli et al., 2001; Rautio et al., 2006). A comparison of five substrates in terms of their ability to be inhibited by a number of clinical drugs concluded that digoxin was the most sensitive MDR protein substrate, while vinblastine and calceinAM were slightly less sensitive (Rautio et al., 2006). However since this study used the human MDR1 protein, it cannot be assumed that its results are applicable to an insect MDR protein. It is unlikely that the Sf9 cells used to express the proteins are responsible for their lower than expected affinity for well-characterised MDR protein substrates, since in previous studies both human (Sarkadi et al., 1992) and fungal (Kohli et al., 2001) MDR proteins have

been expressed in Sf9 cells where they behaved as expected. The bacterial MDR protein PDR5 expressed in the lepidopteran cell lines Sf21 and CF-203, also conferred the expected drug resistance to transformed cells (Zhang et al., 2006). Thus it is likely that the unexpected results of this assay are a feature of the particular genes used to transform the Sf9 cells.

A number of reasons may explain the high calcein retention observed in the cell lines 1-5, 1-10, 3-1 and 3-2 in the absence of any MDR protein inhibitor. It is possible that although calcein is not considered an MDR protein substrate (Tiberghien and Loor, 1996), it may be transported out of the cell to some extent by the *T. ni* MDR proteins in this study. If these four cell lines transport less calcein than the other transformed lines, this could explain their low calcein retention. Calcein is an anion, and thus a more likely substrate for another group of ABC proteins, the multidrug resistance associated proteins (MRPs). MDR proteins show some overlap in substrate spectra with MRPs, although the MRP substrate spectrum is limited to organic anions and substances conjugated to glutathione or glucuronide or sulphate ions (Deeley and Cole, 1997). The *trnMDR1* and *trnMDR3* amino acid sequences are most homologous to MDR proteins in other species, (Tables 4, 9, Fig 15), and show only 11% amino acid sequence similarity with a *T. ni* MRP homologue (R. Labbé, unpublished data). Based on sequence data alone it seems unlikely that the MDR proteins in this study would behave like MRPs, however an assay of the ability of the protein products of *trnMDR1* and *trnMDR3* to transport a common MRP substrate such as methotrexate would demonstrate their ability, if any, to transport anions. If they have this ability, it would explain some of the differences in the calcein retention of the transformed cell lines. Another possibility is that the cell lines which

demonstrate high calcein retention do so as a result of increased activity of endogenous esterases. These esterases may cleave the AM group off calceinAM before MDR proteins have time to remove the calceinAM from the cytoplasm. A test to compare esterase activity in the transformed cell lines would demonstrate whether increased esterase activity is the cause of high calcein retention in 1-5, 1-10, 3-1 and 3-2. Another possibility is that calceinAM is indeed a substrate of *T. ni* MDR proteins, however the assay conditions in this study were not optimized for maximal calceinAM transport by MDR proteins. Although the conditions of this assay were modified from the original assay using human MDRs, further modifications in terms of calceinAM concentration and incubation time may be necessary. The assay was conducted with cells in SF-900 II SFM medium (Invitrogen) containing penicillin, streptomycin and neomycin. All three of these antibiotics have been reported to be substrates of fungal and bacterial mutidrug resistant cells (O'Mahoney et al., 2006; Phongpaichit et al., 2007; Zhanel et al., 2006). If they are also substrates of *T. ni* MDR proteins, they may function as competitive inhibitors of calceinAM transport. If the same assay were repeated in the absence of any antibiotics, greater calceinAM transport may be observed in the transformed cell lines.

Vinblastine is a *Vinca* alkaloid widely used in cancer chemotherapy. Its cellular effect is to block tubulin polymerization, thus preventing the formation of microtubules in the cell. This prevents the formation of the mitotic spindle structure, thus arresting cell division at metaphase (Himes et al., 1976). Vinblastine has been characterised as a substrate of MDR proteins (Polli et al., 2001). The hypothesis of this assay was that cells expressing functional MDR proteins would be able to resist the effects of vinblastine by transporting it out of the cell. The cells should be able to divide, and thus after 24 h or 48

h the number of cells should have increased. In contrast, the untransformed cells have no means of extruding vinblastine, therefore their cell division should be arrested.

The results of the experiment support the hypothesis, demonstrating that two of the three cell lines express a functional MDR protein that enables them to resist the effects of vinblastine. After 24 h of incubation in 0.1 μM vinblastine, the cell lines 1-7 and 1-13 appeared to have undergone more division than the parental line (Fig 12 B, E, K). This was confirmed by counting the cells after 48 h of incubation with or without vinblastine (Table 8). After 48 h of incubation in 0.1 μM vinblastine, the cell line 1-7 had 50% the number of cells in the untreated wells, while 1-13 had approximately 40% the number of untreated cells (Fig 14). In contrast, parental cells had only 28% of the number of untreated cells at this time, while the transformed cell line 3-1 had 25% of the number of untreated cells (Fig 14). Attempts were made to determine the viability of each cell line after incubation with vinblastine, however the dead cells which could be seen under the microscope did not maintain their integrity during the process of scraping and trypan blue staining. When Trypan-blue stained cells were observed on a haemocytometer, many flecks of Trypan-blue stained debris but very few intact dead cells were observed. Thus, it was not possible to make an accurate count of dead cells. It is possible that these cells died during metaphase arrest and the stretching of the cell membrane resulting from the prevention of cell division caused the dead cells to be too fragile to maintain their integrity during scraping and mixing.

The results of this assay indicate that the cell lines 1-7 and 1-13 express functional MDR proteins that are able to protect the cells from vinblastine toxicity. The cell line 3-1 does not appear to express a functional MDR protein, as evidenced by its susceptibility to

vinblastine (Fig 14) as well as its high calcein retention (Fig 8). qPCR analysis confirmed that the cell lines 3-1 and 3-2 expressed the mRNA product of *trnMDR3*, however their level of expression was lower than that of *trnMDR1* mRNA in the cell lines 1-5, 1-7, 1-10 and 1-13 (Fig 7). It is possible that the protein was not expressed at a high enough level for its function to be observed, or that the expressed mRNA may contain a mutation or rearrangement resulting in a lack of protein production or a non-functional protein. A competitive-inhibition assay using vinblastine and calceinAM at the same time could be used to determine whether calceinAM is transported out of transformed cells by the same pump that transports vinblastine.

The long-term goal of this project was to identify MDR protein inhibitors which could be used as synergists with pesticides. While this goal was not fully achieved in this study, the development of an assay system to characterise potential MDR protein inhibitors is an important step towards it. Optimisation of the MDR activity assays using calceinAM or vinblastine or both as substrates will enable us to better characterise the ability of cyclosporin A and verapamil to inhibit *T. ni* MDR protein function, as well as test other substances for their potential as insect MDR activity inhibitors.

References

Ambudkar, S. V., Kinchi-Safarty, C., Sauna, Z. E. and Gottesman, M. M. (2003). P-glycoprotein: from genomics to mechanism. *Oncogene* **22**, 7468-7485.

Arceci, R. J., Croop, J. M., Horwitz, S. B. and Housman, D. (1988). The gene encoding multidrug resistance is induced and expressed at high levels during pregnancy in the secretory epithelium of the uterus. *Proc. Natl. Acad. Sci. USA* **85**, 4350-4354.

Bede, J. C., McNeil, J. N. and Tobe, S. S. (2007). The role of neuropeptides in caterpillar nutritional ecology. *Peptides* **28**, 185-196.

Bosch, I. and Croop, J. M. (1998). P-glycoprotein structure and evolutionary homologies. *Cytotechnology* **27**, 1-30.

Bubik, M., Ott, M., Mahringer, A. and Fricker, G. (2006). Rapid assessment of p-glycoprotein-drug interactions at the blood-brain barrier. *Anal. Biochem.* **358**, 51-58.

Buss, D. S., McCaffery, A. R and Callaghan, A. (2002). Evidence for p-glycoprotein modification of insecticide toxicity in mosquitoes of the *Culex pipiens* complex. *Med. Vet. Entomol.* **16**, 218-222.

Callaghan, A. and Denny, N. (2002). Evidence for an interaction between p-glycoprotein and cadmium toxicity in cadmium-resistant and -susceptible strains of *Drosophila melanogaster*. *Ecotoxicol. Environ. Safety* **52**, 211-213.

Dean, M., Hamon, Y. and Chimini, G. (2001). The human ATP-binding cassette (ABC) transporter superfamily. *J. Lipid Res.* **42**, 1007-1017.

Deeley, R. G. and Cole, S. P. C. (1997). Function, evolution and structure of multidrug resistance protein (MRP). *Semin. Cancer Biol.* **8**, 193-204.

Demeule, M., Régina, A., Jodoin, J., Laplante, A., Dagenais, C., Berthelet, F., Moghrabi, A. and Béliveau, R. (2002). Drug transport to the brain: key roles for the efflux pump p-glycoprotein in the blood-brain barrier. *Vasc. Pharmacol.* **38**, 339-348.

Ferry, D. R. and Kerr, D. J. (1994). Multidrug resistance in cancer. *Br. Med. J.* **308**, 148-149.

Gaertner, L. S., Murray, C. L. and Morris, C. E. (1998). Transepithelial transport of nicotine and vinblastine in isolated malpighian tubules of the tobacco hornworm (*Manduca sexta*) suggests a p-glycoprotein-like mechanism. *J. Exp. Biol.* **201**, 2637-2645.

Gerrard, B., Stewart, C. and Dean, M. (1993). Analysis of Mdr50: a *Drosophila* p-glycoprotein/multidrug resistance gene homolog. *Genomics* **17**, 83-88.

Hayashi, K., Schoonbeek, H. and De Waard, M. A. (2003). Modulators of membrane drug transporters potentiate the activity of the DMI fungicide oxpoconazole against *Botrytis cinerea. Pest Manag. Sci.* **59**, 294-302.

Higgins, C. F. and Gottesman, M. M. (1992). Is the mutidrug transporter a flippase? *Trends Biochem. Sci.* **17**, 18-21.

Higgins, C. F. and Linton, K. J. (2004). The ATP switch model for ABC transporters. *Nature Struc. Mol. Biol.* **11**, 918-926.

Himes, R. H., Kersey, R. N., Heller-Bettinger, I. and Samson, F. E. (1976). Action of the *Vinca* alkloids vincristine, vinblastine and desacetyl vinblastine amide on microtubules *in vitro. Cancer Res.* **36**, 3798-3802.

Hiraga, K., Wanigasekera, A., Sugi, H., Hamanaka, N. and Oda, K. (2001). A novel screening for inhibitors of a pleiotropic drug resistant pump, Pdr5, in *Saccharomyces cerevisiae. Biosci. Biotechnol. Biochem.* **65**, 1589-1595.

Hiraga, K., Yamamoto, S., Fukuda, H., Hamanaka, N. and Oda, K. (2005). Enniatin has a new function as an inhibitor of Pdr5p, one of the ABC transporters in *Saccharomyces cerevisiae. Biochem. Biophys. Res. Commun.* **328**, 1119-1125.

Idriss, H., Urquidi, V. and Basavappa, S. (2000). Selective modulation of p-glycoprotein's ATPase and anion efflux regulation activities with PKC α and PKC ε in Sf9 cells. *Cancer Chemother. Pharmacol.* **46**, 287-292.

Janmaat, A. F. and Myers, J. (2003). Rapid evolution and cost of resistance to *Bacillus thuringiensis* in greenhouse populations of cabbage loopers, *Trichoplusia ni. Proc. Biol. Sci.* **270**, 2263-2270.

Jones, P. M. and George, A. M. (2005). Multidrug resistance in parasites: ABC transporters, p-glycoproteins and molecular modelling. *Int. J. Parasitol.* **35**, 555-566.

Juliano, R. L. and Ling, V. (1976). A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. *Biochim. Biophys. Acta* **455**, 152-162.

Kerr, K. M., Sauna, Z. E. and Ambudkar, S. V. (2001). Correlation between steady-state ATP-hydrolysis and vanadate-induced ADP trapping in human p-glycoprotein. *J. Biol. Chem.* **276**, 8657-8664.

Kohli, A., Gupta, V., Krishnamurthy, S., Hasnain, S. E. and Prasad, R. (2001). Specificity of drug transport mediated by *CaMDR1*: a major facilitator of *Candida albicans. J. Biosci.* **26**, 333-339.

Kozovská, Z. and Subik, J. (2003). Screening for effectors that modify multidrug resistance in yeast. *Int. J. Antimicrob. Agents* **22**, 284-290.

Krishna, R. and Mayer, L. D. (2000). Multidrug resistance (MDR) in cancer: mechanisms, reversal using modulators of MDR and the role of MDR modulators in influencing the pharmacokinetics of anti-cancer drugs. *Eur. J. Pharm. Sci.* **11**, 265-283.

Lanning, C. L., Fine, R. L., Corcoran, J. J., Ayad, H. M., Rose, R. L. and Abou-Donia, M. B. (1996a). Tobacco budworm p-glycoprotein: biochemical characterization and its involvement in pesticide resistance. *Biochim. Biophys. Acta* **291**, 155-162.

Lanning, C. L., Ayad, H. M. and Abou-Donia, M. B. (1996b). P-glycoprotein involvement in cuticular penetration of $^{[14C]}$thiodicarb in resistant tobacco budworms. *Toxicol. Lett.* **85**, 127-133.

Leandro, C. and Campino, L. (2003). Leishmaniasis: efflux pumps and chemoresistance. *Int. J. Antimicrob. Agents* **22**, 352-357.

Li, X., Baudry, J., Berenbaum, M. R. and Schuler, M. A. (2004). Structural and functional divergence of insect CYP6B proteins: from specialist to generalist cytochrome P450. *Proc. Natl. Acad. Sci. USA* **101**, 2939-2944.

Ling, V. (1997). Multidrug resistance: molecular mechanisms and clinical relevance. *Cancer Chemother. Pharmacol.* **40**, S3-S8.

Malutan, T. (1999). Molecular cloning, functional expression and pharmacological characterization of a high-affinity octopamine/tyramine transporter from the cabbage looper *Trichoplusia ni.* M. Sc. Thesis. The University of Western Ontario. 92 pages.

Morrison, S., Ward, A., Hoyle, C. J. and Henderson, P. J. F. (2003). Cloning, expression, purification and properties of a putative multidrug resistance efflux protein from *Helicobacter pylori. Int. J. Antimicrob. Agents* **22**, 242-249.

Murray, C. L., Quaglia, M., Arnason, J. T. and Morris, C. E. (1994). A putative nicotine pump at the metabolic blood-brain barrier of the tobacco hornworm. *J. Neurobiol.* **25**, 23-34.

Neyfakh, A. A. (2002). Mystery of multidrug transporters: the answer can be simple. *Mol. Microbiol.* **44**, 1123-1130.

O'Mahoney, R., Quinn, T., Drudy, D., Walsh, C., Whyte, P., Mattar, S. and Fanning, S. (2006). Antimicrobial resistance in nontyphoidal *Salmonella* from food sources in Colombia: evidence for an unusual plasmid-localized class 1 integron in serotypes *Typhimurium* and *Anatum. Microb. Drug Resist.* **12**, 269-277.

Persidis, A. (2000). Cancer multidrug resistance. *Nature Biotech.* **18**, IT18-IT20.

Phongpaichit, S., Liamthong, S., Mathew, A. G. and Chetanond, U. (2007). Prevalence of class 1 integrons in commensal *Escherichia coli* from pigs and pig farmers in Thailand. *J. Food Prot.* **70**, 292-299.

Pivčević, B. and Žaja, R. (2006). Pesticides and their binary combinations as p-glycoprotein inhibitors in NIH 3T3/MDR1 cells. *Environ. Toxicol. Pharmacol.* **22**, 268-276.

Pleban, K., Kopp, S., Csaszar, E., Peer, M., Hrebicek, T., Rizzi, A., Ecker, G. F. and Chiba, P. (2004). P-glycoprotein substrate-binding domains are located at the transmembrane domain/transmembrane domain interfaces: a combined photoaffinity labelling-protein homology modelling approach. *Mol. Pharmacol.* **67**, 365-374.

Podsiadlowski, L., Matha, V. and Vilcinskas, A. (1998). Detection of a p-glycoprotein related pump in *Chironomus* larvae and its inhibition by verapamil and cyclosporin A. *Comp. Biochem. Physiol. B* **121**, 443-450.

Polli, J. W., Wring, S. A., Humphreys, J. E., Huang, L., Morgan, J. B., Webster, L. O. and Serabjit-Singh, C. S. (2001). Rational use of in vitro p-glycoprotein assays in drug discovery. *J. Pharmacol. Exp. Ther.* **299**, 620-628.

Postma, J. F., van Nugteren, P. and Buckert-de Jong, M. B. (1996). Increased cadmium excretion in metal-adapted populations of the midge *Chironomus riparia* (Diptera). *Environ. Toxicol. Chem.* **15**, 332-339.

Qadir, M., O'Loughlin, K. L., Fricke, S. M., Williamson, N. A., Greco, W. R., Minderman, H. and Baer, M. R. (2005). Cyclosporin A is a broad-spectrum multidrug resistance modulator. *Clin. Cancer Res.* **11**, 2320-2326.

Rautio, J., Humphreys, J. E., Webster, L. O., Balakrishnan, A., Keogh, J. P., Kunta, J. R., Serabjit-Singh, C. J. and Polli, J. W. (2006). In vitro p-glycoprotein inhibition assays for assessment of clinical drug interaction potential of new drug candidates: a recommendation for probe substrates. *Drug Metab. Dispos.* **34**, 786-792.

Roe, M., Folkes, A., Ashworth, P., Brumwell, J., Chima, L., Hunjan, S., Pretswell, I., Dangerfield, W., Ryder, H. and Charlton, P. (1999). Reversal of p-glycoprotein mediated multidrug resistance by novel anthranilamide derivatives. *Bioorg. Med. Chem. Lett.* **9**, 595-600.

Rosenberg, M. F., Callaghan, R., Modok, S., Higgins, C. F. and Ford, R. C. (2005). Three-dimensional structure of p-glycoprotein: the transmembrane regions adopt an asymmetric configuration in the nucleotide-bound state. *J. Biol. Chem.* **280**, 2857-2862.

Rothnie, A., Storm, S., McMahon, R., Taylor, A., Kerr, I. D. and Callaghan, R. (2005). The coupling mechanism of p-glycoprotein involves residue L339 in the sixth membrane spanning segment. *FEBS Lett.* **579**, 3984-3990.

Sarkadi, B., Price, E. M., Boucher, R. C., Germann, U. A. and Scarborough, G. A. (1992). Expression of the human multidrug resistance cDNA in insect cells generates a high activity drug-stimulated membrane ATPase. *J. Biol. Chem.* **267**, 4854-4858.

Sauna, Z. E. and Ambudkar, S. V. (2001). Characterization of the catalytic cycle of ATP hydrolysis by human p-glycoprotein. *J. Biol. Chem.* **276**, 1153-1161.

Schuetzer-Muehlbauer, M., Willinger, B., Egner, R., Ecker, G. and Kuchler, K. (2003). Reversal of antifungal resistance mediated by ABC efflux pumps from *Candida albicans* functionally expressed in yeast. *Int. J. Antimicrob. Agents* **22**, 291-300.

Schumacher, M. A., Miller, M. C. and Brennan, R. G. (2004). Structural mechanism of the simultaneous binding of two drugs to a multidrug-binding protein. *EMBO J.* **23**, 2923-2930.

Shabbir, A., DiStasio, S., Zhao, J., Cardozo, C. P., Wolff, M. S. and Caplan, A. J. (2005). Differential effects of the organochlorine pesticide DDT and its metabolite *p,p'*-DDE on p-glycoprotein activity and expression. *Toxicol. App. Pharmacol.* **203**, 91-98.

Sharom, F. J. (2006). Shedding light on drug transport: structure and function of the p-glycoprotein multidrug transporter (ABCB1). *Biochem. Cell Biol.* **84**, 979-992.

Sharom, F. J., Liu, R., Qu, Q. and Romsicki, Y. (2001). Exploring the structure and function of the p-glycoprotein multidrug transporter using fluorescence spectroscopic tools. *Semin. Cell Dev. Biol.* **12**, 257-266.

Sheps, J. A., Ralph, S., Zhao, Z., Baillie, D. L. and Ling, V. (2004). The ABC transporter gene family of *Caenorhabditis elegans* has implications for the evolutionary dynamics of multidrug resistance in eukaryotes. *Genome Biol.* **5**, R15.

Sorensen, J. S. and Dearing, M. D. (2006). Efflux transporters as a novel herbivore countermechanism to plant chemical defenses. *J. Chem. Ecol.* **32**, 1181-1196.

Srinivas, R., Udikeri, S. S., Jayalakshmi, S. K. and Sreeramulu, K. (2004). Identification of factors responsible for insecticide resistance in *Helicoverpa armigera*. *Comp. Biochem. Physiol. C* **137**, 261-269.

Stavri, M., Piddock, L. J. V. and Gibbons, S. (2006). Bacterial efflux pump inhibitors from natural sources. *J. Antimicrob. Chemother.* In press.

Tapadia, M. G. and Lakhotia, S. C. (2005). Expression of *mdr49* and *mdr65* multidrug resistance genes in larval tissues of *Drosophila melanogaster* under normal and stress conditions. *Cell Stress Chaperones* **10**, 7-11.

Thomas, H. and Coley, H. M. (2003). Overcoming multidrug resistance in cancer: an update on the clinical strategy of inhibiting p-glycoprotein. *Cancer Control* **10**, 159-165.

Tiberghien, F. and Loor, F. (1996). Ranking of p-glycoprotein substrates and inhibitors by a calceinAM fluorometry screening assay. *Anticancer Drugs* **7**, 568-78.

Vandersompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A. and Speleman, F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology* **3**, research0034.1-0034.11.

Wu, C. T., Budding, M., Griffin, M. S. and Croop, J. M. (1991). Isolation and characterization of *Drosophila* multidrug resistance gene homologs. *Mol. Cell. Biol.* **11**, 3940-3948.

Yamamoto, S., Hiraga, K., Abiko, A., Hamanaka, N. and Oda, K. (2005). A new function of isonitrile as an inhibitor of the Pdr5p multidrug ABC transporter in *Saccharomyces cerevisiae*. *Biochem. Biophys. Res. Commun.* **330**, 622-628.

Yu, E. W., Aires, J. R. and Nikaido, H. (2003). AcrB multidrug efflux pump of *Escherichia coli*: Composite substrate-binding cavity of exceptional flexibility generates its extremely wide substrate specificity. *J. Bacteriol.* **185**, 5657-5664.

Zhanel, G. G., Wang, X., Nichol, K., Nikulin, A., Wierzbowski, A. K., Mulvey, M. and Hoban, D. J. (2006). Molecular characterisation of Canadian paediatric multidrug-resistant *Streptococcus pneumoniae* from 1998–2004. *Int. J. Antimicrob. Agents* **28**, 465-471.

Zhang, D. Y., Krell, P. J. and Feng, Q. L. (2006). Two lepidopteran cell lines stably transformed by the ABC transporter gene *pdr5* show tolerance to diacetoxyscirpenol. *In Vitro Cell Dev. Biol. Anim.* **42**, 27-32.