University of Crete, Biology Department
Foundation of Research & Technology
Institute of Molecular Biology & Biotechnology
MSc "Molecular & Applied Plant Biology – Green Biotechnology"

Study of transporters in *Nezara viridula:* insights for pest control and insecticide transport

Master Thesis

Olympia Driva

Biologist

Supervisor: Prof. John Vontas, AUA

Examination committee: Prof. John Vontas, AUA

Prof. Christos Delidakis, UoC

Assoc. Prof. Panagiotis Sarris, UoC

Herakleion, October 2019

Acknowledgements

I would like to use this space to thank the people that helped me during this past (very intense!) year, without them I couldn't have done it.

First of all I would like to thank Professor John Vontas for accepting me in his lab, assigning me my project, and for always supporting me. I would also like to thank Associate Professor Panagiotis Sarris and Professor Christos Delidakis for accepting to be a part of my examination committee and for their helpful notes. Mr Giannis Leivadaras's help with injections was also invaluable!

Many many thanks go also to Shane for guiding me all this months, for discussing every detail, answering every question (many times), and calming me down every time I got stressed. Jenny helped me very much with cloning tips, aligning lots of nymphs for injections, and being great company during lunches. Aris also helped a lot with rearing my insects and discussing about bioassays, insecticides, and everything I needed. George, Maria, Mary and Sofia helped with techniques and equipment I was unfamiliar with, and I thank them for all their time. Of course I could not forget to thank Zouzouni, Venetia, Eva, Mantha, and everyone already mentioned for all the fun and nice moments during breaks or experiments. You guys made my time in the lab enjoyable!

Lots of support is always needed outside of the lab too, and I want to thank my friends for listening to all my stressed rants and helping me by talking to me (and feeding me when necessary). Dimos, Alexia, Asimina, Eleni, my sisters, all helped me even though they were out of Crete (or even Greece). My friends (and drinking buddies) in Herakleion Joanna, Manolia, Nora, thank you. Special thanks go to Aggeliki and Rania: my Master's experience would have been completely different without you and I cannot thank you enough for everything you have done for me this year.

As always the best are saved for the end. I thank Eddie for being by my side all these years, for all his patience, encouragement, and love. Finally, I want to thank my parents for supporting me in every possible way in everything I do.

Abstract

Nezara viridula is an important cosmopolitan polyphagous pest of horticultural and agricultural importance that is currently controlled mainly with the use of insecticides. These small molecules are transported through barrier tissues like the midgut is by transporter proteins belonging in the ABC and SLC superfamilies. Members of these families also have core physiological roles, and have been proposed as drug targets in other species. This study therefore focused on the identification and characterization of small molecule transporters of the ABC and SLC superfamilies in terms of their capacity to act as targets and transporters of insecticides.

For the first part of this study we have developed a bioassay that allowed us to calculate the baseline oral toxicity of different insecticides. This bioassay will serve in the future as a tool to assess changes in this baseline toxicity after the manipulation of the expression of proteins that transport insecticides. We have also identified two new putative drug targets belonging in the ABC family: knocking them down with RNAi resulted in lethality or developmental arrest. For the second part of the study we have identified two ABC and one SLC transporters that might have a role in transporting insecticides, and made the first steps for their characterization using RNAi and heterologous expression systems. Finally, the possible case of horizontal gene transfer of a bacterial SLC47 to the *N. viridula* genome was examined. Overall these studies will help us better understand the insect-insecticide interaction, as well as design new pest control strategies.

Περίληψη

Η *Nezara viridula* είναι ένα σημαντικός εχθρός καλλιεργειών με παγκόσμια εξάπλωση, που αυτή τη στιγμή ελέγχεται κυρίως μέσω της χρήσης εντομοκτόνων. Τέτοια μικρά μόρια διασχίζουν ιστούς εμπόδια όπως το μεσέντερο με τη βοήθεια πρωτεϊνών που ανήκουν στις οικογένειες των ABC και SLC μεταφορέων. Μέλη αυτών των οικογενειών έχουν επίσης σημαντικούς ρόλους για τη φυσιολογία του οργανισμού, και έχουν προταθεί ως στόχοι φαρμάκων σε άλλα είδη. Η παρούσα μελέτη επομένως επικεντρώθηκε στην ταυτοποίηση και χαρακτηρισμό μεταφορέων μικρών μορίων που ανήκουν στις ABC και SLC οικογένειες ως προς την ικανότητα τους να είναι στόχοι και μεταφορείς εντομοκτόνων.

Στο πρώτο μέρος της εργασίας, αναπτύχθηκε μια βιοδοκιμή διατροφής που μας επέτρεψε τον υπολογισμό της τοξικότητας διαφορετικών εντομοκτόνων. Η βιοδοκιμή αυτή θα μπορέσει να χρησιμοποιηθεί στο μέλλον ως εργαλείο για τον υπολογισμό αλλαγών στην τοξικότητα ύστερα από τροποποίηση των επιπέδων έκφρασης πρωτεϊνών μεταφορέων εντομοκτόνων. Επίσης ταυτοποιήθηκαν δύο νέοι πιθανοί στόχοι εντομοκτόνων που ανήκουν στην οικογένεια ABC, καθώς η σίγησή τους μέσω RNAi οδήγησε σε αύξηση της θνησιμότητας και παύση της ανάπτυξης. Στο δεύτερο μέρος της εργασίας ταυτοποιήθηκαν 2 ABC και ένας SLC μεταφορείς με πιθανό ρόλο στη μεταφορά εντομοκτόνων, και έγιναν τα πρώτα βήματα για τη μελέτη τους μέσω RNAi σίγησης και ετερόλογης έκφρασης. Τέλος, εξετάστηκε η πιθανή οριζόντια γονιδιακή μεταφορά ενός βακτηριακού SLC47 γονιδίου στο γονιδίωμα της *Nezara viridula.* Συνολικά, οι μελέτες αυτές βοηθούν στην καλύτερη κατανόηση της σχέσης εντόμου-εντομοκτόνου, και το σχεδιασμό νέων στρατηγικών αντιμετώπισης των εντόμων εχθρών καλλιεργειών.

Table of contents

1 General Introduction

1.1 Nezara viridula is an agricultural pest

Nezara viridula is an important polyphagous cosmopolitan pest of agricultural and horticultural crops. It is known to inhabit tropical and subtropical regions. Furthermore, it threatens temperate regions of Europe, Asia, Africa, Australia, and America, and the range it occupies is constantly expanding. It belongs it the family Pentatomidae (Order Hemiptera) and is a hemimetabolous insect. It is highly polyphagous as it feeds on 43 plant families, showing preference for Fabaceae, Asteraceae, Brassicaceae, Poaceae, and Solanaceae. Like all stink bugs, they are active through the warmer parts of the year, and rely on many overlapping host plants for their survival, dispersing to new hosts as they start to produce seeds or fruit. They have piercing and sucking mouthparts and therefore they cause damage as they feed on pods, seeds, or fruits which reduces their yield, quality, and commercial value. For example, bean pods attacked by stink bugs shrivel, and fruit develop black spots or growths. In addition to physical damage, these pests also transmit common plant pathogens including the bacterium *Pantoea agglomerans* and the fungal pathogen *Nematospora coryli* (Martin, 2016; Esquivel *et al.*, 2018).

Figure 1.1. Life stages of Nezara viridula. Top row: Cream colored hard eggs are layed in tight-packed single layer clusters. First instar nymphs are also usually clustered together after hatching. Middle row: Second, third, and fourth instar nymphs. The nymphs get bigger and their color patterns change with each ecdysis. Bottom row: The fifth and final nymphal stage. Two adults mating. Adults are usually green, but other color morphs have been observed such as yellow, orange, blue and black. Photos by Herb Pilcher, USDA Agricultural Research Service, Bugwood.org.

1.2 Pest control

The use of transgenic plants expressing Cry toxins derived from *Bacillus Thurungensis* (Bt) has increased substantially in recent years in the Americas. Despite reports of resistance, this strategy has been largely effective in protecting plants and controlling of lepidopteran "chewing" pests, leading to the subsequent decrease in foliar-applied insecticides. An unintended biproduct of this phenomenon is the elevation of the pest status of hemipteran pests (such as *N.* viridula) as these insects do not feed on leaves and thus avoid the *Bt* produced by most transgenic crops. (Greene *et al.*, 2018). Current and future strategies for the control of *N. viridula* are discussed below.

1.2.1 Current Strategies for *N. viridula* management

At the moment, *N.viridula* is mainly controlled through biological and synthetic means. Biological ways of control have been moderately successful and include the use of the pest's natural enemies, mainly egg parasitoids like *Trissolcus basalis* (Liljesthröm, Cingolani and Rabinovich, 2013). Further examples of control include manipulation of planting dates, density, tillage operations, water, nutrients, adjacent habitats, crop rotation, and harvest timing (Greene *et al.*, 2018). Other than that, attract-and-kill strategies take advantage of the pest's behavior: a highly attractive crop or lure is grown around the actual crop and the insects are drawn towards it and away of the crop of interest. Subsequent site-specific insecticide application or parasitoid release controls the pest population. These crops are called trap crops and are usually planted earlier than the crop of interest. The results of this strategy are varied, but the populations are usually successfully reduced as long as the stink bugs are eliminated before they complete their development or before the other crop matures (Tillman *et al.*, 2015; Esquivel *et al.*, 2018).

The best and most widely employed way to control *N.viridula* however remains the use of synthetic insecticides. These are generally small molecules (under 1kDa) that target key proteins in the insect, mostly in the muscles and the central nervous system. There are no selective insecticides available for stink bugs. As a result broad spectrum compounds are instead used such as organophosphates (IRAC group 1B), pyrethroids (IRAC group 3A), and neonicotinoids (IRAC group 4A). These are respectively inhibitors of achetylcholinesterase, modulators of sodium channels, and competitive modulators of the nicotinic acetylcholine receptor. Insecticides are usually sprayed on the plants, but neoconicotinoids in particular act as systemic insecticides. They can be translocated to all parts of the plant after their application to seeds, roots or leaves (Simon-Delso *et al.*, 2015).

1.2.2 Future Outlook

Efficient management of *N. viridula* and other stink bugs or sap-feeding insects in general will require measures beyond the conventional strategies mentioned above. Several techniques have already promising results in other types of insects: cultivation of GM crops like Bt plants and releases of sterile populations in the field using SIT (Sterile Insect Technique) or gene drives have been among the most promising ones. These approaches however have disadvantages. Developing a GM crop that only targets stink bugs can be difficult in terms of the delivery method of the insecticidal agent expressed by the plant. Because stink bug are piercing-sucking insects, expression of an insecticidal agent in a tissue like the leaves will not

affect them. Additionally, while a solution like that will limit the use of synthetic insecticidal compounds, and GM crops have been found safe for human health if consumed, the general public is still reluctant to accept them (Lucht, 2015). As for using programs for rearing and releasing sterile insects in the field, they can often be cost prohibitive. Moreover the sterilized adults in the case of stink bugs will still feed on the crops and cause major economic injury, unlike the case with the programs designed for mosquitos. Also, as stink bugs mate multiple times the possibility that females will still mate with a wild-type non-sterile male is still high (Greene *et al.*, 2018).

A new promising approach for the control of insect pests is through the use of RNAi. The goal of this approach is to trigger the insect's innate RNAi response pathways upon exposure to dsRNA or siRNA in order to silence specific genes essential for the survival or reproductive fitness of the insect. Some of the advantages of the technique are that it can be used against a big number of targets based on their function and not their structural and physicochemical properties (in contrast to insecticides like the ones described above). The method also allows for species specificity as it is sequence-based (Zotti and Smagghe, 2015). The last point is specifically helpful if we want to avoid unwanted effects on beneficial insects. On the other hand this specificity can be a disadvantage, as it might allow us to only target one insect at a time. The variability of the efficiency of the technique in different insect orders and the way of delivery are the major challenges for the commercial application of RNAi-based pest control. So far, non-transformative (like spraying of dsRNA) and transformative methods (transgenic or transplastomic plants expressing dsRNA) have been proposed, each with their advantages and disadvantages (Joga *et al.*, 2016). A main problem is the destruction of dsRNA or siRNA by plant or insect nucleases. To combat the first, the expression of dsRNA in the chloroplasts looks hopeful (Zhang *et al.*, 2015). As for overcoming the insect response, novel delivery methods that protect small RNAs like liposome encapsulation have been proposed (Joga *et al.*, 2016). Some cases of successful or promising control of sucking Hemiptera pests have finally been reported (Yu *et al.*, 2016; Eakteiman *et al.*, 2018).

1.3 Gut transporters affect insecticide pharmacokinetics

Insecticidal compounds can be delivered to insects via different routes. For pests like stink bugs, a very important one is the oral route. When an insecticide or other xenobiotic is ingested by an insect, it has to pass through the midgut in order to reach its target tissue. The midgut therefore acts as a barrier-tissue, limiting the insecticide's dispersion inside the insect body and reducing the amount that can reach its target. The midgut is not involved only in the absorption of insecticides but also their excretion and metabolism which regulates their accumulation in target tissues. This means that the midgut physiology greatly impacts the pharmacokinetics of insecticides, and ultimately their efficacy (Denecke *et al.*, 2018).

Xenobiotics are thought to cross the midgut epithelium in a paracellular or in an intracellular manner. In the latter case they can passively diffuse through the epithelial cells or they can move in or out of them with the help of transporter proteins. The importance of the contribution of gut transporters to the absorption, distribution, metabolism and elimination (ADME) of drugs has been well acknowledged in pharmaceuticals (CITATIONS). Less well understood is the role of these proteins in determining the absorption of insecticides into the body although some initial studies have been highlighted in the literature during the past few years (Dermauw and Van Leeuwen, 2014; Denecke *et al.*, 2018) . Furthermore, the endogenous roles of these protein also make them attractive targets for pest control as they are often used as pharmaceutical drug targets (Rask-Andersen *et al.*, 2013). Thus, the focus of this thesis will on describing these transporters in *N. viridula* both in terms of their capacity to act as targets and transporters of pesticides.

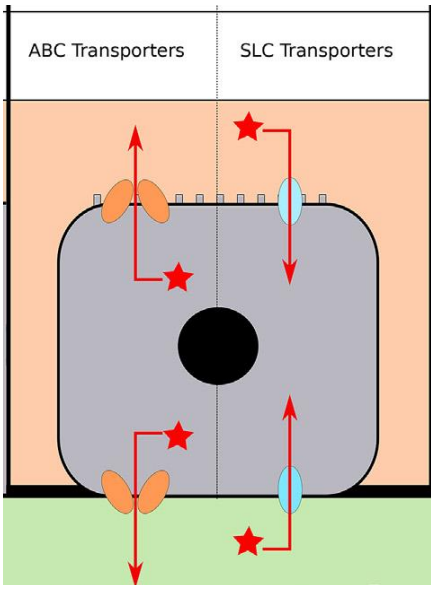


Figure 1.2. Cellular localization of transporters affects the direction of transport. This can determine their role on the distribution of insecticides or endogenous compounds within the body. The sketch is from Denecke *et. al.* 2018.

Transporters (distinct from other transmembrane proteins such as aquaporins and channels) belong to two superfamilies: ABC (ATP-binding cassette) and SLC (Solute Carrier) transporters. ABCs actively transport compounds out of the cell by using ATP as a source of energy, while SLCs generally import molecules to the cell without requiring ATP. Instead they make use of electrochemical gradients or use secondary energy sources functioning as antiport or symport systems. Both ABCs and SLCs can be positioned either in the apical or the basolateral side of the epithelial cell. As Figure 1.2 showcases, the direction of transport and the localization of proteins from both families is predicted to determine their effect on the distribution of insecticides or endogenous compounds within the body (Ming and Thakker, 2010). Studying the expression, localization, and the mechanism of function of these transporters will therefore enable us to better understand the insect-insecticide interaction, and to design successful new pest control strategies and compounds.

1.4 The ABC transporter family

Proteins of the ABC superfamily are present in all domains of life. The family is known to act on a wide range of substrates and specific members are known to be polyspecific, transporting substrates such as sugars, lipids, amino acids and peptides, xenobiotics or drugs. Some of them are not transporters but channels, receptors, or part of the translational machinery of the cell (Dermauw and Van Leeuwen, 2014). Most of our knowledge about them comes from studies in bacterial and human proteins. Their role in multidrug resistance in humans has been well established (Estudante *et al.*, 2013) as well as their involvement in basic physiological processes like eye pigmentation in *Drosophila melanogaster* (Ewart *et al.*, 1994). Although their function and role in insecticide transport and resistance has been the subject of several recent studies, more are needed to better understand them in insects.

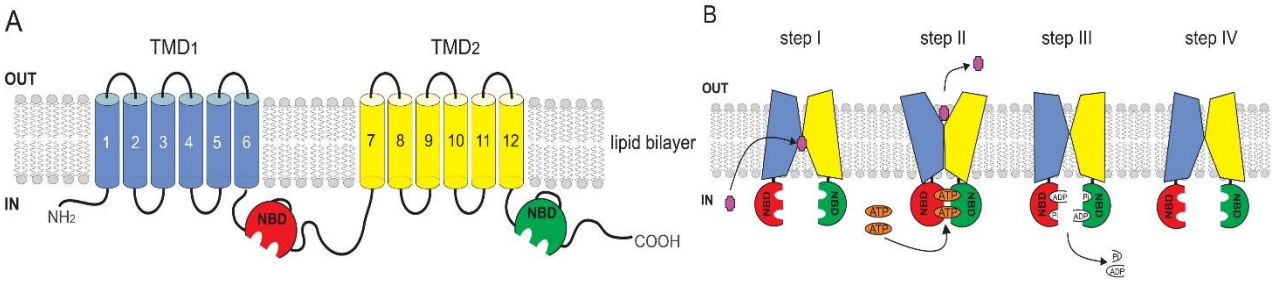


Figure 1.3. Structure of an ABC FT and the ATP-switch model. A) An ABC FT has two TMDS (shown in blue and yellow) each containing 6 TM regions, and 2 cytoplasmic NBDs (shown in red and green). B) A transport round starts when a substrate (purple) binds on the TMDs causing a conformational change to the NBDs that allows the ATP (orange) to bind to them. This causes further conformational changes in the TMDs that rotate and open towards the outer side of the cell, resulting in substrate transport. Following that the ATP gets hydrolyzed allowing the disassociation of the two NBDs. When the ADP and phosphate get released in the next step, the initial transporter conformation is restored and a new transport round can begin. Diagram is from Dermauw and Van Leeuwen, 2014.

Based on studies in bacteria, mammals, and *C. elegans* some general features about their overall structure can be ascertained. ABC proteins' main characteristic is the presence of two cytoplasmic nucleotide-binding domains (NBDs) and two transmembrane domains (TMDs). ABCs can be full transporters (FT) if all these components are in the same polypeptide chain, or half transporters (HT). HTs have only one of each domain in each polypeptide chain and need to homo- or hetero-dimerize in order to become functional. Dimerization allows for the correct orientation of NBDs that can then bind and hydrolyze ATP to effectively transport the protein's substrate. The NBD is itself modular and contains several conserved motifs: a Walker A motif that participates in binding ATP, a Walker B motif that catalyzes its hydrolysis, a D-loop involved in NBD dimerization, a Q-loop that is also involved in ATP hydrolysis, an H motif that stabilizes the protein when it is not loaded with a substrate, and an a-helical region containing the ABC signature sequence LSGGQ. The TMDs each contain 6 transmembrane helices that adopt different conformations allowing substrate specific translocation (Merzendorfer, 2014).

The ABC protein superfamily can be further divided into nine subfamilies (ABCA-ABCI) according to the sequence of their NBDs. Of these subfamilies, ABCA does not have any members in yeast, while ABCH proteins are present only in arthropods and a few other metazoans. The ABCI subfamily is only present in plants (Kretzschmar *et al.*, 2011). Proteins belonging to ABCA, ABCB, ABCC, ABCD, ABCG and ABCH subfamilies are all half or full transporters, while members of the ABCE and ABCF subfamilies are soluble cytoplasmic

proteins. Members of the ABCB, ABCC and ABCG subfamilies have been shown to play role in drug transport and resistance in humans and insects and resistance (Dermauw and Van Leeuwen, 2014; Merzendorfer, 2014). Although an increasing number of arthropod genomes have been sequenced, ABC transporters have been identified in only a few species, and a very small amount of them have been functionally characterized. To this date, data on ABCs are available on 28 arthropod species listed on Table 1-1. The species with the least ABCs is the parasitic salmon louse *Lepeoptheirus salmonis* with 33 transporters, and the species with the most ABCs is the mite *Tetranychus urticae* with 104 transporters.

Four ABC genes were chosen for this study, belonging in the subfamilies ABCB, ABCC, ABCE, and ABCH. Further information is therefore given for these specific subfamilies.

Species	**Order**	**# of ABCs**	**Reference**
Acyrthosiphon pisum	Homoptera	71	(Wang *et al.*, 2014)
Anopheles aegypti	Diptera	69	(Lu, Xu and Cui, 2016)
Anopheles gambiae	Diptera	44/52/55	(Roth *et al.*, 2003; Liu *et al.*, 2011; Lu *et al.*, 2016)
Anopheles sinesins	Diptera	61	(He *et al.*, 2019)
Apis mellifera	Hymenoptera	43	(Liu *et al.*, 2011)
Bactrocera dorsalis	Diptera	47	(Xiao *et al.*, 2018)
Bemisia tabaci	Hemiptera	55	(Tian *et al.*, 2017)
Bombyx mori	Lepidoptera	51/53	(Liu *et al.*, 2011; Xie *et al.*, 2012)
Caligus rogercresseyi	Siphonostomatoida	57*	(Valenzuela-Muñoz *et al.,* 2015)
Chrysomela populi	Coleoptera	65*	(Strauss *et al.*, 2014)
Culex pipiens	Diptera	70	(Lu, Xu and Cui, 2016)
Danaus plexippus	Lepidoptera	62	(Qi *et al.*, 2016)
Daphnia pulex	Cladocera	64	(Sturm *et al.*, 2009)
Diaphorina citri	Hemiptera	44	(Liu *et al.*, 2019)
Drosophila melanogaster	Diptera	56	(Dean and Annilo, 2005; Liu *et al.*, 2011)
Heliconius melpomene	Lepidoptera	62	(Qi *et al.*, 2016)
Helicoverpa armigera	Lepidoptera	54	(Bretschneider *et al.*, 2016; Liu *et al.*, 2019)
Helicoverpa zea	Lepidoptera	54	(Pearce *et al.*, 2017)
Laodelphax striatellus	Hemiptera	40*	(Sun *et al.*, 2017)
Lepeoptheirus salmonis	Siphonostomatoida	33	(Carmona-Antoñanzas *et al.*, 2015)
Locusta migratoria	Orthoptera	65	(Wang *et al.*, 2014)
Lygus hesperus	Hemiptera	65*	(Hull *et al.*, 2014)
Manduca sexta	Lepidoptera	51	(Qi *et al.*, 2016)
Pediculus humanus	Phthiraptera	40	(Lee *et al.*, 2010)
Plutella xylotela	Lepidoptera	82	(Qi *et al.*, 2016)
Tetranychus urticae	Trombidiformes	104	(Dermauw *et al.*, 2013)
Tigriopus japonicus	Harpacticoida	46	(Jeong *et al.*, 2014)
Tribolium castaneum	Coleoptera	68/73	(Liu *et al.*, 2011; Broehan *et al.*, 2013)

Table 1-1 A list of the arthropod species that have had their ABC transporters identified. The number of ABCs is predicted based on genomic data, except on the listings marked with an asterisk where it is based on transcriptomic data. The species are listed alphabetically.

1.4.1 ABCB and ABCC proteins are involved in xenobiotic pharmacokinetics

The ABCB subfamily members can be either FTs or HTs. In humans, they been associated with multi-drug resistance of cancer cells, iron homeostasis, liver-specific transport of lipids, and antigen processing. More specifically, HTs in humans play a role in iron homeostasis, protection against ischemia and oxidative stress, and resistance to chemotherapeutic drugs. There is evidence that insect ABCB HTs are orthologous to the human proteins suggesting they share similar functions and RNAi studies point towards this direction as well (e.g. in Metzendorf, Wu and Lind, 2009). As for FTs, in humans they are involved in the transport of hydrophobic substrates and multi-drug resistance, while insect ABCB FTs have a well-documented role in modifying insecticide toxicity. The latter is showcased in many studies that quantify mRNA or proteins after exposure to insecticides, as well as in synergism studies that use known ABC inhibitors (Epis *et al.*, 2015; Valenzuela-Muñoz, Sturm and Gallardo-Escárate, 2015; Sun *et al.*, 2017).

The most well studied member of this group is the human HsABCB1 or P-glycoprotein (P-gp), an efflux pump for xenobiotic compounds that is a FT. Its preferred substrates are hydrophobic, amphipathic or cationic molecules (Li, Jaimes and Aller, 2014). It is expressed in key barrier tissues like the intestinal epithelium and the blood-brain barrier. Close homologues to it have been found in insects, and have been implicated in insecticide transport and insecticide resistance using techniques like RNAi, CRISPR/Cas9, and heterologous expression in cell lines (Xu *et al.*, 2016; Denecke, Fusetto and Batterham, 2017).

The ABCC subfamily also contains both FTs and HTs, while some of its members have an additional TMD. In humans they are involved in ion transport, signal transduction, drug elimination, and toxin secretion. Because they are associated with a very broad range of substrates they were named multidrug-resistance associated proteins (MRPs), and further categorized as “long” or “short” MRPs depending on the presence or absence of an extra TMD (TMD0). They have also been shown to act in synergy with detoxifying enzymes like GSTs and UGTs (Morrow *et al.*, 1998). Many studies conducted with Lepidoptera connect ABCC proteins with resistance to Cry toxins produced by Bt-crops (Heckel, 2012; Park *et al.*, 2014; Guo *et al.*, 2019).

One of the most well studied member of this group is the human MRP1, a “long” MRP that effluxes a variety of compounds and prevents their accumulation inside enterocytes. It transports organic anions, such as drugs conjugated to glutathione (Estudante *et al.*, 2013). Insect homologues to this protein have been reported to be upregulated after exposure to certain insecticides in different species (Sun *et al.*, 2017; Kim *et al.*, 2018; Liu *et al.*, 2019).

It is of interest to note that alongside the continuous increase in reports of positive correlations between insecticide transport or resistance and ABC expression, there are also a significant number of neutral or negative reports. These variations can be explained if we keep in mind that ABC transporters of different species respond in a different way against the same insecticides, and that ABC transporters of the same species are not necessarily able to interact with every different insecticide due to their structure. Furthermore, differences in insecticide response can even be found between sexes or life-stages in a certain species for a certain compound. These observations underline the heterogeneity that can be found in ABC transporter role in insecticide transport and pharmacokinetics (Porretta *et al.*, 2016).

1.4.2 ABCE and ABCH proteins perform important physiological functions

Other ABC subfamilies contain transporters that play essential physiological roles that do not involve drug transport. The ABCE subfamily does not contain transporters, but cytoplasmic proteins that have 2 NBDs and no TMDs. Eukaryotes usually contain only one ABCE gene that is highly conserved and has ubiquitous expression. In humans it was first annotated as an RNase L inhibitor. It has been shown to be essential for the initiation of translation and ribosome biogenesis and recycling, while it may also have a role in innate immunity. Mutants for ABCE in insects, other animals and plants are reported to be either lethal or induce very slow growth compared to the wild type. These growth defects can in most cases be explained by ineffective translation. Some phenotypes however are linked with compromised regulatory networks that are still uncharacterized (Navarro-Quiles, Mateo-Bonmatí and Micol, 2018; Wu *et al.*, 2019).

The ABCH subfamily contains HTs and have the reverse domain organization with the NBD connecting to the N-terminal side of the TMD. ABCH transporters have only been found in arthropods, some teleosts, the purple sea urchin *Strongylocentrotus purpuratus,* and the slime mould *Dictyostelium discoideum* (Guo *et al.*, 2015). Studies in *D. melanogaster* and *Locusta migratoria* suggest a role in the formation of the cuticle lipid barrier, while RNAi experiments in *T. castaneum* and *Plutella xylostella* resulted in lethal phenotypes (Broehan *et al.*, 2013; Guo *et al.*, 2015; Yu *et al.*, 2017; Zuber *et al.*, 2018). As a result, ABCH has been suggested by these researchers as a possible RNAi target for pest control.

1.5 SLC proteins are involved in pharmacokinetics

The SLC superfamily is one of the biggest in humans, with 66 families and more than 400 members and a classification and nomenclature is established for it. Although the superfamily is not monophyletic, members of individual families are related phylogenetically. Experimental validation of many transporters in humans have also suggested that transporters within the same family often (but not always) transport similar substrates. The superfamily is represented in all domains of life and contains passive transporters, ion-coupled transporters (symporters) and exchangers (antiporters) that are located in cell and organelle membranes (Figure 1.4). They participate in the uptake of endogenous nutrients, vitamins, lipids, hormones and xenobiotics (including drugs), as well as in ion transport. Many SLC proteins are involved in drug pharmacokinetics and greatly affect drug bioavailability (Hediger *et al.*, 2013; Estudante *et al.*, 2016).

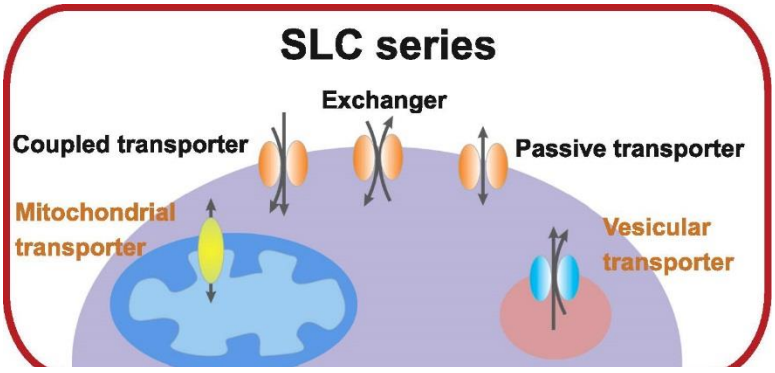


Figure 1.4. Members of the SLC superfamily have diverse structures, modes of action, and subcellular localization. Diagram is taken from Hediger *et al.* 2013.

In particular, the oligopeptide transporter PEPT1 that belongs in the SLC15 family has been the subject of much attention. In humans it is primarily expressed in the intestinal epithelium and it functions as a transporter of di- and tri-peptides. The oligopeptide uptake is dependent of an inward-directed H^+ gradient. It has a very broad substrate range, transporting almost all di- and tri-peptides with varying capacities (Ito *et al.*, 2013). Additionally, many peptidomimetic antibiotics, enzyme inhibitors and antivirals are also substrates (Spanier and Rohm, 2018). Comparative studies of orthologous proteins from different species have explored PEPT1 substrate specificity and pH and temperature dependence in order to elucidate its mode of action (Margheritis *et al.*, 2013; Romano *et al.*, 2014). The protein's localization, function, and wide substrate specificity make it a good candidate for pharmaceutical and medical applied research. More specifically, its ability to transport peptidomimetic drugs or peptide-conjugated drugs and regulate their bioavailability make it interesting to study in terms of drug delivery (Yang and Hinner, 2015; Estudante *et al.*, 2016). In principle, the bioavailability of a drug can be increased by making it a substrate for this transporter; this has been shown previously with the case of acyclovir and valacyclovir (the same compound with a valine attached; Yang, Hu and Smith, 2013).

1.6 Aim of the study

This study focused on studying transporters of small molecules important for the control of *Nezara viridula*. In particular, the aim of the study was the identification and preliminary characterization of transporters of small molecules in terms of their potential to act as insecticide targets, as well as their involvement in the transport of insecticides and prodrugs. We went with an *in silico* approach for the identification of the transporters, using proteomic and transcriptomic data already in our disposal. This would be followed by a reverse genetics approach in order to characterize the transporters. Finally, heterologous expression systems would provide us with important data on the protein function.

2 Transporters implicated in pest control

The goal for this part of the project was double. Firstly, we wanted to develop an appropriate bioassay to assess insecticide toxicity in our lab strain of *N. viridula*. This bioassay would help us acquire consistent baseline toxicity for insecticides of interest, and in the second part of the project it would serve as a tool to assess changes in the baseline susceptibility after manipulating gene expression, using only diagnostic doses. The second goal was to identify and characterize putative drug targets belonging in the ABC family. This goal was approached with the use of RNAi, a tool that has been recently shown to be effective in *N. viridula.*

2.1 Methods

2.1.1 Nezara viridula rearing

Nezara viridula eggs were imported from Bayer (Crop Science department) and maintained in the laboratory according to Bayer's rearing protocol. The population was kept in mesh cages under controlled conditions, at 23 ±1 °C with a 12:12 light:dark photoperiod. Insects were provided with water, organic beans, organic carrots, and sunflower seeds. Adults typically laid eggs on the papers and nymphs were reared in the same manner.

2.1.2 Insecticides

Commercial formulations of insecticides were used to perform toxicity bioassays. The active ingredients were Spinosad (Laser 480 SC), Abamectin (Vertimec 1.8 EC), and Fipronil (Termidor 9 SC). All of them were diluted to required concentration using dH_2O. Five concentrations were used to determine mortality.

2.1.3 Feeding bioassays

The establishment of a bioassay that will serve as a tool to assess insecticide toxicity was needed. As we are interested in gut physiology, the bioassay would need to ensure that the insect is exposed to the insecticide through feeding only and not by contact. After a literature search 3 different set-ups were tested: dipped bean bioassay, immersed bean bioassay, and bubble feeding bioassay. For each type of bioassay the set-up was optimized using a red food dye and water solution (1:10 ratio). Then a range of insecticide concentrations was tested in order to determine LC50s for them. Fresh organic green beans and 2^{nd} stage nymphs were used for all bioassays, and the insects were kept at room temperature in 24 hour dark conditions. Initial tests used 20 nymphs/dose. Final experiments used 60 nymphs/each dose, and the bioassays were replicated at least three times. A nymph was considered dead or moribund if it cannot right itself if placed on its dorsal surface. The results were statistically analyzed using the software PoloPLUS (LeOra Software).

2.1.4 dsRNA synthesis

Using midgut cDNA from nymphs as template, and the primers indicated in TableX in Appendix, regions of the selected genes were amplified (364 bp for ABCE and 301 bp for ABCH) with Taq-Polymerase (Minotech) by performing a PCR. Both the forward and the reverse primer had the T7 promoter sequence at their 5'. The conditions for the PCR were 95 °C for 5 min for an initial denaturation, followed by 35 cycles of denaturation at 95 °C for 30 sec, annealing at 57 °C for 30 sec, extension at 72 °C for 1 min, and a final step of extension at 72 °C for 5 min. The amplicon was purified using a gel extraction kit (Macherey-Nagel) after electrophoresis. The purified DNA served as a template for in vitro transcription, using the HiScribe™ T7 High Yield RNA Synthesis Kit (New England BioLabs). The resulting dsRNA was

purified using the MegaClear kit (Ambion) and diluted in dH_2O at a final concentration of 2μg/μl.

2.1.5 Injections

The dsRNA was injected into 3-4 day old 1st instar nymphs. Nymphs were lined up on their side on a chilled agar step block. After incubation on ice for 5-10 min (or until they were completely still), approximately 20 nl were injected in the abdomen of each nymph using a IM300 Microinjector (Narishige) with a mechanical micromanipulator. The nymphs were transferred to separate containers post injection based on the gene injected to them, and were maintained as usually. Two repeats of the experiment were performed, each with at least 100 nymphs injected per gene. DsRNA targeting actin was used as a positive control, and dsRNA targeting lacZ was used as a negative control. These were synthesized by Dr Maria Riga. Mortality was scored usually every other day for up to 15 days. A nymph was considered dead if it was not moving. The mortality presented at the results was corrected using Scneider-Orelli's formula. Raw data are provided in the appendix.

$$CorrectedMortality = \frac{(TargetGeneMortality - LacZMortality)}{(100 - LacZMortality)} \times 100$$

2.2 Results

2.2.1 Development of feeding bioassay

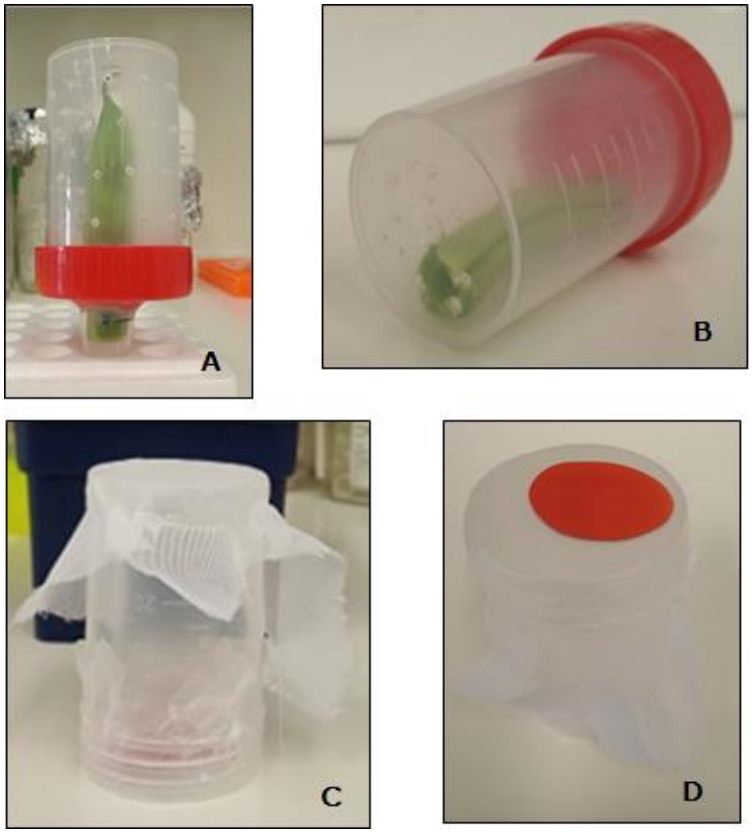


Figure 2.1 3 A) Immersed bean bioassay B) Dipped bean bioassay C) Bubble feeding bioassay side view D) Bubble feeding bioassay top view

The first step towards studying the levels of insecticide toxicity is to develop and establish an appropriate bioassay that can provide consistent baseline susceptibility data. After the standardization of the technique, it can easily be used as a tool to assess changes of this baseline toxicity. Three different set-ups (Figure 2.1) were tested and their advantages and disadvantages are presented below.

2.2.1.1 Dipped bean bioassay

This assay was modified based on the assay in Kamminga et al. 2012. Green beans were cut in 5cm pieces, dipped for 30 sec in water and red food dye solution and left to dry under the hood. After several tests, it was found that 2 hours under the hood were necessary for the beans to dry completely. One bean was then placed in polypropylene vials with holes on them for ventilation purposes, with 10 or 20 nymphs (Figure 1B). After 48 hours, red excreta were observed at the bottom of the vial, indicating that the insects were feeding successfully. In both 10 and 20 nymph vials all nymphs were on the bean feeding.

This bioassay was the fastest and easiest to set up. However, it also had disadvantages, the main ones being the difficulty in counting the alive vs dead/moribund nymphs and that high output of excreta resulted in nymphs getting trapped in them, leading to high mortality unrelated to insecticide treatments. As a result this type of assay was abandoned.

2.2.1.2 Immersed bean bioassay

This assay was based on the feeding assays in Ghosh *et al.*, 2017 and Mogilicherla *et al.*, 2018. Lean green beans are cut in 7-8 cm pieces and are immersed in a cut 15ml falcon containing 2ml of water or insecticide solution. The falcon is then sealed with Parafilm. The falcon tube is fixed on the lid of a polypropylene vial with silicone glue. 20 nymphs are put in a polypropylene vial with numerous holes for ventilation purposes, that is finally screwed on the lid (Figure 1A). After 48 hours red excreta were observed, indicating that the solution was absorbed by the bean and the insects were successfully feeding with it.

This assay has the advantage of continuous supply of insecticide so that the bean will not dry out, something very important as the assay lasts up to 6 days, and allows for easy counting of alive vs dead/moribund nymphs. There were also many disadvantages with this assay. Firstly, the high humidity that develops in the vial results in insects drowning in the vial. It was also the bioassay that lasted the longest, as it required 6 days of insecticide exposure to show results. In addition, fungi very frequently grew on the beans and killed the insects. Bean quality and size were variable and therefore this assay could not be trusted, and was used only for initial screening of the toxicity of Spinosad and Abamectin.

2.2.1.3 Bubble feeding bioassay

This assay was based on assays previously performed in our laboratory. The bottom of a polypropylene vial is cut off, and a piece of mesh is glued in its place for ventilation purposes. Nymphs are put in the vial, whose top is then covered with a stretched out piece of Parafilm. 0,5 ml of 30% sugar water with or without insecticide is placed on top of the Parafilm. Finally another stretched out piece of Parafilm is placed on top of the first one, trapping the bubble of liquid between them. The vials are kept vertically (Figure 1C-D).A population of 5, 10, and 20 nymphs was tested (using only sugar water) to examine how many days the insects can survive while on this diet. Survival was good in all populations tested, and therefore all subsequent experiments were done with 20 nymphs per vial.

This type of assay is different than the other two, as it does not involve a way of feeding that is similar to field conditions. This was at first considered a disadvantage of the bubble feeding method. However, bubble feeding removed the unpredictable bean quality as a factor that skewed the results. Other advantages of this type of assay is that it is relatively quick to setup, it requires less amount of insecticide to work, and its duration is only 4 days. Overall, using this bioassay resulted in more reliable and repeatable data. We therefore used it to determine LC50s for all insecticides.

2.2.2 LD50s for Spinosad, Abamectin, and Fipronil

Preliminary dosages were determined before commencement of tests for the selected insecticide to establish mortality ranging from 5 to 95%. We started testing with Spinosad. Spinosad belongs to the IRAC group 5 that targets the nervous system (more specifically the nicotinic acetylcholine receptor) and belongs in the spinosyns, a group of macrocylic lactones derived from the soil actinomycete *Saccharopolyspora spinosa.* It exhibits its neurotoxic action through contact or ingestion against many orders of insects (Bacci *et al.*, 2016). During this preliminary process, nymphs exposed to Spinosad did not die even at 1000 ppm and it was assumed that Spinosad is not effective against *N. viridula* when ingested. It is although important to note that due to a shortage of nymphs its effectiveness was only assessed with the immersed bean bioassay, and therefore we cannot safely conclude if it is indeed not toxic when ingested, or if it could not move up the bean.

After that, Abamectin was selected for testing. Abamectin belongs in IRAC group 6 that contains insecticides that act as allosteric modulators of the glutamate-gated chloride channel, and acts both on nerves and muscles. Chemically it belongs in the avermectins, a group of of macrocyclic lactones with nematocidal, avaricidal, and insecticidal properties that are derived from the soil actinomycete *Streptomyces avermitili* (Bai and Ogbourne, 2016). Preliminary dosages were determined using the dipped bean bioassay, but we ended up using the bubble feeding bioassay to calculate the LC_{50} for Abamectin for the reasons discussed above. It is interesting to note that the doses used did not differ much between the two methods, suggesting that both bioassay methods have similar sensitivity.

Compound	n	Slope ± SE	LC_{30} ppm (95% CI)	LC_{50} ppm (95% CI)	LC_{70} ppm (95% CI)
Spinosad	100				>1000
Abamectin	898	3.606±0.337	0.239 (0.163 - 0.309)	0.334 (0.239 - 0.409)	0.466 (0.376 - 0.574)
Fipronil		In progress	In progress	In progress	In progress

Finally, initial tests were performed for Fipronil. Fipronil belongs in the IRAC category 2B, acts on the nervous system, and is a blocker of GABA-gated chloride channel. It has a similar toxicity profile and physicochemical properties with neonicotinoids. Fipronil is a phenyl-pyrazole that can enter plant tissues and translocate to all its parts and is a broad acting insecticide with high oral toxicity (Simon-Delso *et al.*, 2015). Preliminary dosage tests suggest that Fipronil needs to be about 10 times less concentrated than Abamectin in order to have the same toxic effect. Bioassays to determine the LC_{50} remain to be performed.

2.2.3 RNAi of putative drug targets

A list of all the ABC proteins of *N. viridula* was made by Shane Denecke based on transcriptomic data, suggesting that there are 78 of them in our insect. These were further divided into the ABCA-ABCH subfamilies according to their sequence. A phylogenetic tree

based on data from *Drosophila melanogaster, Homo sapiens, Bemisia tabaci* and *Nezara viridula* was also constructed by Shane Denecke. Both the list and the tree are provided in the Appendix. With these tools in our hands we could select potential drug targets from the ABC family. As outlined in the Introduction the ABCE and ABCH proteins perform basic physiological roles, and we hypothesized that knocking down members of these subfamilies would be detrimental to the insect survival and/or growth.

The ABCE subfamily has two members in *N. viridula* as we can see in Figure 2.2. Out of these, Nv_DN107193_c0_g2 had a clear 1:1 relationship with the ABCE gene in humans and *Drosophila* and was selected for knock-down. The other member of the ABCE subfamily had a short length, and was not deemed a good candidate to study. The ABCH subfamily has thirteen members in *N. viridula* as we can see in Figure 2. Based on gene expression and length from the trancriptomic data, and phylogenetic relationships with the Snu gene in *Drosophila* (for which literature data exist) we chose Nv_ DN106392_c1_g2 to knockdown.

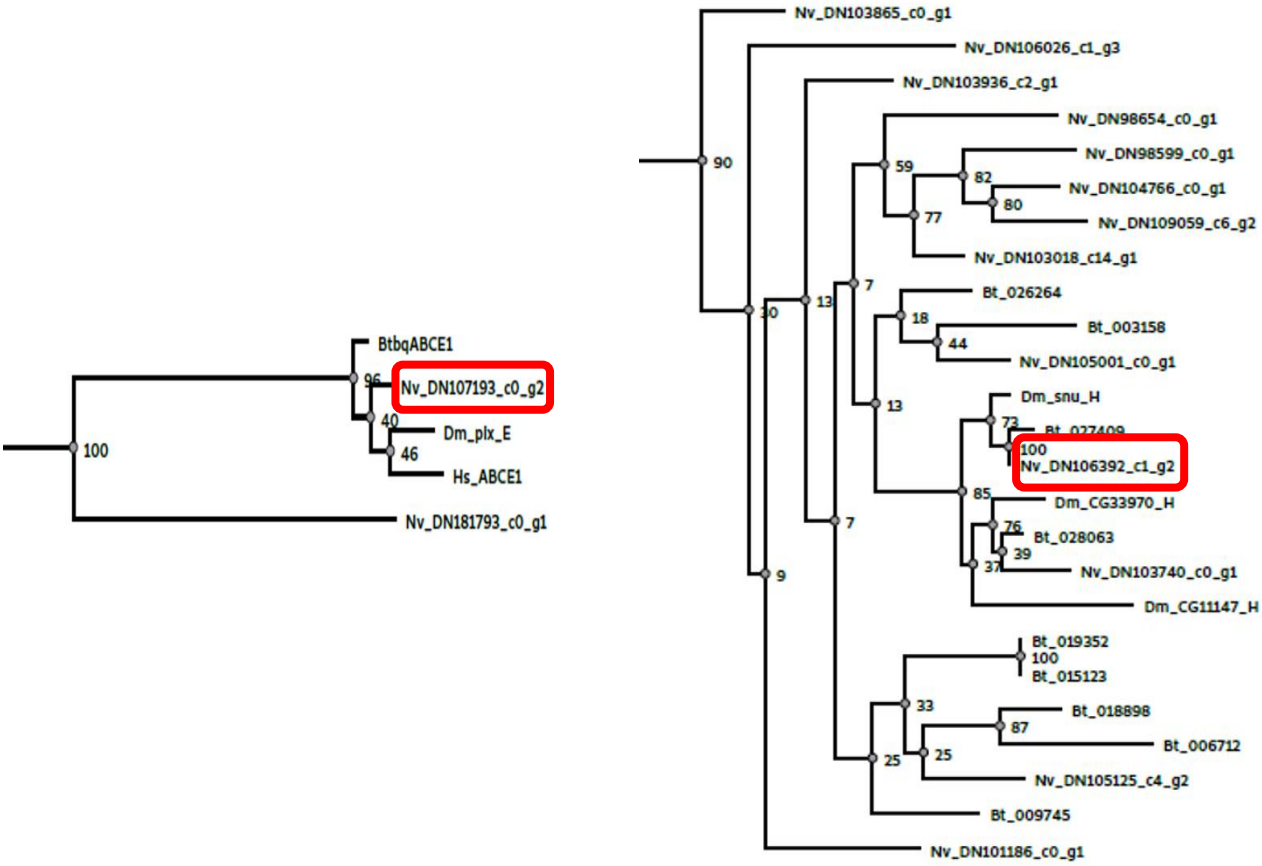


Figure 2.2 Phylogenetic tree for ABCE subfamily (left) and ABCH subfamily (right).Circled in red are the genes that were chosen to be studied as putative drug targets. Bt = Bemicia tabaci, Dm = Drosophila melanogaster, Hs = Homo sapiens, Nv = Nezara viridula.

Figure 2.3 presents the corrected mortality that resulted after injecting dsRNA designed for these two genes and actin in 1st instar nymphs. Two independent rounds of injections were performed. Actin and ABCH knock-down (KD) resulted in 100% mortality in both rounds of

injections, and the pattern of mortality was very similar between them. This result indicates that ABCH is essential for survival, and could serve as a potential drug target. Knocking-down the ABCE gene resulted in the same pattern of mortality over the days post-injection in the two repeats of the experiment. The percentage of mortality was however variable. This indicates difference in the penetrance of the effect, something that is reported in the literature in cases of other genes as well (e.g. Lu *et al.*, 2017).

Besides mortality, the phenotype of the injected nymphs was also assessed. The phenotypes for each KD were repeated in both rounds of injection, and were specific for each gene. Nymphs injected with dsRNA targeting ABCH could not moult effectively and in their majority died before reaching the 3rd nymphal stage. Dead nymphs also had a desiccated appearance. Nymphs injected with dsRNA targeting ABCE showcased the same phenotype in both rounds of injections, even though the mortality levels were very different. These nymphs were growing very slowly, remaining in the 2nd nymphal instar while all other nymphs had moulted. They were also less energetic and moved more slowly. These phenotypes are similar to those reported in the literature after KDs of homologous genes in other insects.

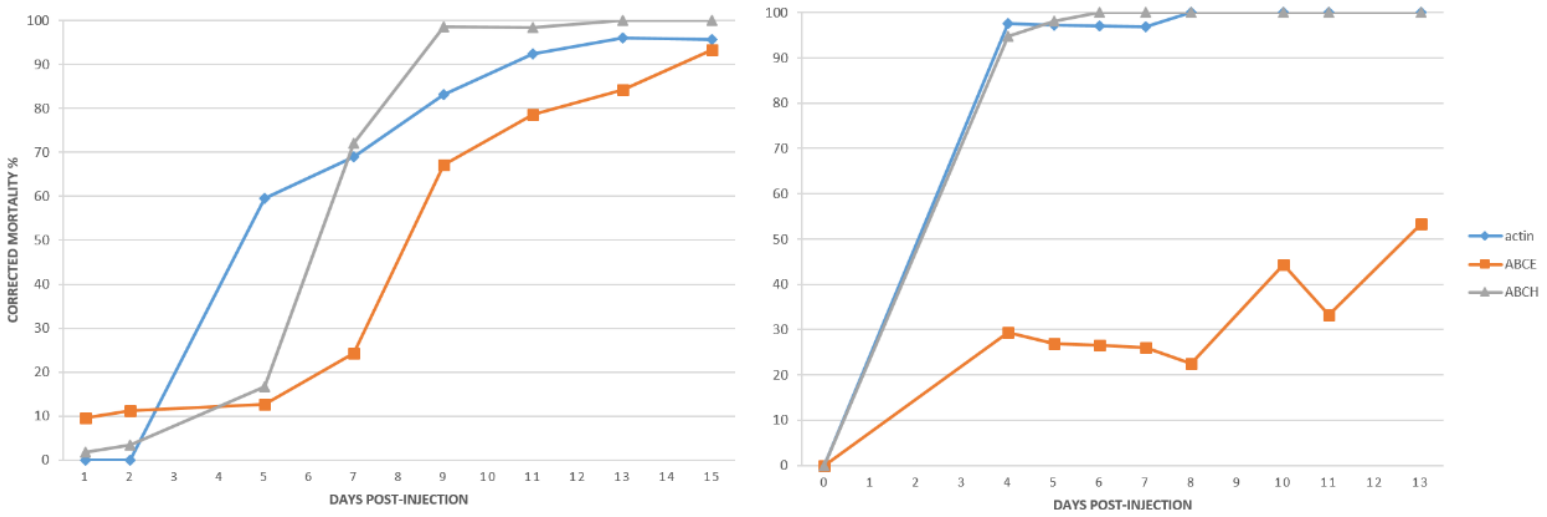


Figure 2. 4 Mortality after injection of dsRNA in 1st instar nymphs, for 15 days post-injection. Presented are two independent rounds of injections, the first in the left and the second in the right. Differences in respond time were observed between the two rounds. Actin and ABCH follow the same mortality pattern for both rounds, and KD of both results in mortality >98%. ABCE KD started affecting nymph mortality earlier for the second round of injections, but killed significantly less insects. All mortality values are corrected based on a population injected with dsRNA targeting lacZ.

2.3 Discussion

The status of *Nezara viridula* as an emerging horticultural and agricultural pest drives the need to study its response to current pest management strategies, as well as investigate new ways to control it. As the current strategies mainly contain the use of small insecticidal molecules, we developed an assay to measure and set the baseline oral toxicity for a selection of such molecules. In addition, we explored the potential of ABC transporters in terms of their capacity to act as targets of small molecules, as the majority of drug and drug-like molecules are in their majority transporters (Van den Broeck, 2015).

As we are interested in oral toxicity, we needed a bioassay that would ensure the delivery of insecticides through feeding and not contact. Most bioassays for Hemiptera described in

literature and the IRAC website are based on contact with residues of insecticides. An exception is this (Tillman, 2006) paper, but the procedure described in it was lengthy and we did not try it. We then based ourselves on methods for oral dsRNA delivery described in the literature, as well as previous knowledge from our bioassays in other insects performed in our lab, and developed the bubble feeding bioassay described in the results. This assay provided data for baseline susceptibility of our lab strain to insecticides in terms of their ingestion activity. As the insecticides tested belong in different chemical classes and have different modes of actions, they will hopefully provide interesting results for the part of the project that is described in the next chapter.

By knocking down the ABC transporters we selected, we provided another example of the efficacy of RNAi by injection of dsRNA in the hemocoel in a Hempiteran species. In addition, we shed some light in the function of representatives of the ABCE and ABCH subfamilies that are not extensively studied in the literature. Finally, we revealed two putative drug targets in *Nezara viridula.*

RNAi is a useful tool when working with non-model organisms, allowing the knock down of expression of genes of interest. RNAi has been used for insects with variable results in terms of efficiency: the robustness of the technique is best in Coleoptera, while Lepidoptera and Hemiptera show variable responses (Terenius *et al.*, 2011; Christiaens and Smagghe, 2014; Zhang *et al.*, 2017). In our lab we have shown that dsRNA microinjections in nymphs effectively knocks down genes in *Nezara viridula* (submitted paper by Riga *et al.* 2019; this thesis). RNAi results can vary in its efficacy and penetrance not only in different species, but also in different genes. These differences are not fully understood, but can be explained by the gene expression pattern, the time-course of the silencing, the turnover of the dsRNA target, the number of paralogs that might exist and substitute the function of the knocked-down gene, or native mRNA secondary structures that inhibit the function of the insect RNAi machinery (Zhang *et al.*, 2017; Knorr *et al.*, 2018). RNAi has many applications in research, and one of them is the identification of putative new drug targets. This is because the knock-down caused by dsRNA mimics the effect of an antagonistic drug, revealing the potential of the protein as drug target, without having the actual drug already in our disposal (Van den Broeck, 2015).

In our case, ABCH knockdown provided repeated results between two rounds of experiments. The results were in accordance with analogous experiments performed in other insects (Broehan *et al.*, 2013; Guo *et al.*, 2015; Yu *et al.*, 2017; Zuber *et al.*, 2018) and showcased that it is an essential gene for survival. The phenotype of the injected insects also suggest its involvement in cuticle formation. In order to confirm that the selected ABCH indeed transports lipids towards the cuticle in *N. viridula* we could use a lipid-staining assay after the manipulation of the gene like the one described in Yu *et al.* 2017. On the other hand, ABCE knockdown provided us with variable results in terms of lethality and repeated results in terms of phenotype: all surviving nymphs presented an arrest in their development. This is in similarity with the literature: knock outs or high level knockdowns of the gene result in lethal phenotype, while hypomorphic alleles result in slow growth (Navarro-Quiles, Mateo-Bonmatí and Micol, 2018). These similarities were expected as ABCE genes are the most conserved in the ABC family.

In order for a protein to be considered as a target for insecticides, it needs to be essential for the survival of the organism. If its depletion leads to lethality or developmental arrest, we can assume that its interaction with an insecticide will also have the same effects, resulting ultimately to the suppression of the pest population. Additionally a protein might be a good

target if it is central in determining the reproductive success of the organism, or if is involved in the detoxification of other harmful substances (Eakteiman *et al.*, 2018). In this sense, both ABCH and ABCE that we studied in *N. viridula* are good putative drug targets as when their expression is lowered the result is mortality or growth suppression respectively. However, when choosing drug targets we also have to keep in mind that essential genes might not always be the best choice as they tend to be highly conserved among different species. This is because the insecticide that targets them might also have side effects in other non-target organisms like mammals or beneficial insects. This is a reason why proteins like actin are not good drug targets despite their essentiality. Finally, another factor to consider when proposing a new target is its druggability: the ability to modulate the protein's activity through the use of drug-like compounds (Abi Hussein *et al.*, 2017). Computational methods and machine learning are often used to calculate the druggability of a protein, but this calculations are frequently hindered by the lack of structural data and are therefore difficult to execute for non-model insects like *N. viridula.*

3 Transporters implicated in insecticide transport

The goal for this part of the project was the identification and preliminary characterization of transporter proteins that are implicated in insecticide and prodrug transport. Transporters from the ABC family were selected for studying, as well as a member the SLC family. Finally, a possible case of horizontal gene of a bacterial SLC into the *N. viridula* genome was investigated.

3.1 Methods

3.1.1 dsRNA synthesis

Using midgut cDNA from nymphs as template, and the primers indicated in TableX in Appendix, regions of the selected genes were amplified (363 bp for MDR-like and 309 bp for MRP-like) with Taq-Polymerase (Minotech) by performing a PCR. Both the forward and the reverse primer had the T7 promoter sequence at their 5'. The conditions for the PCR were 95 °C for 5 min for an initial denaturation, followed by 35 cycles of denaturation at 95 °C for 30 sec, annealing at 57 °C for 30 sec, extension at 72 °C for 1 min, and a final step of extension at 72 °C for 5 min. The amplicon was purified using a gel extraction kit (Macherey-Nagel) after electrophoresis. The purified DNA served as a template for in vitro transcription, using the HiScribe™ T7 High Yield RNA Synthesis Kit (New England BioLabs). The resulting dsRNA was purified using the MegaClear kit (Ambion) and diluted in dH_2O at a final concentration of 2μg/μl.

3.1.2 Cloning of SLC15

The putative ortholog of SLC15 was identified by BLAST, using the *Drosophila melanogaster* sequence as a query and the *N. viridula* transcriptome as a subject. Then primers were designed in order to amplify with PCR the whole sequence. The PCR used nymphal midgut cDNA as a template and Phusion polymerase under the following conditions: 95 °C for 5 min for an initial denaturation, followed by 35 cycles of denaturation at 95 °C for 30 sec, annealing at 64 °C for 30 sec, extension at 72 °C for 1 min, and a final step of extension at 72 °C for 5 min. The PCR product was purified using a gel extraction kit (Macherey-Nagel) after electrophoresis, A-tailing using Minotech protocol was performed, and was then cloned into pGEM-Teasy vector according to the manufacturer's protocol (Promega Corporations) using DH5a competent cells. The cells were cultured at 37 °C for 16 hours using LB plates in the presence of 100 μg/ml Ampicillin, 0.5mM IPTG and 50mg/ml Xgal allowing for blue-white selection. Single white colonies were picked and subsequently cultured in liquid medium containing Ampicillin. After performing a miniprep using the Nucleospin Plasmid kit by Macherey Nagel, the plasmids were screened for the insert by digestion with NotI (Minotech) and BamHI (Minotech) according to the supplier's directions. Two positive clones were identified, and stored at -20 °C for future use.

Starting from this recombined plasmid we could then subclone the SLC15 gene onto two other expression vectors: the pGemHE vector that would allow heterologous expression of our gene inside a *Xenopus laviae* oocyte, and the pEIA vector that would allow heterologous expression of our gene in insect cell lines. The recombined plasmid and the new empty plasmid (pGemHE or pEIA) were digested and the insert gel-purified as previously described. Cloning was performed as already described, but without the use of IPTG and Xgal for blue-white selection. Resulting colonies were first screened with colony PCR using primers for the gene and Taq-Polymerase (Minotech) in the following conditions: 95 °C for 5 min for an initial denaturation, followed by 35 cycles of denaturation at 95 °C for 30 sec, annealing at 54 °C for 30 sec, extension at 72 °C for 2 min, and a final step of extension at 72 °C for 5 min. Positive

clones were cultured overnight in liquid medium, and digested as described after a miniprep. One positive clone was sent for sequencing (Cemia) that was performed using M13 universal primer as well as the sequencing primers listed in the primer Table in Appendix.

3.1.3 Semi-quantitative PCR for SLC47

A sequence similar to a bacterial SLC47 was found in our transcriptomic data. Primers were designed to amplify a 300 bp region of the predicted gene, listed in the primer Table in Appendix. Semi-quantitative PCR was performed using Taq-polymerase (Minotech) as the enzyme and genomic DNA obtained from M1 and M4 regions of the midgut of adult *N. viridula* insects. The genomic DNA was extracted by Evi Boulasiki. The conditions of the PCR were: 95 °C for 5 min for an initial denaturation, followed by 28 cycles of denaturation at 95 °C for 30 sec, annealing at 54 °C for 30 sec, extension at 72 °C for 1 min, or by 35 cycles of denaturation at 95 °C for 30 sec, annealing at 54 °C for 30 sec, extension at 72 °C for 1 min. Four biological and two technical repeats were performed for each midgut region. Actin was also amplified using the primers in the primer Table in Appendix under the same conditions, in order to serve as a control.

3.2 Results

3.2.1 Identification of members of the ABC family with a putative role in insecticide transport

As stated in the introduction, many members of the ABC family have been implemented in the transport of xenobiotics or their level of expression has been reported altered upon exposure to insecticides. Involved in these functions are members of the ABCB, ABCC, and ABCG subfamilies, with Mdrs and MRPs (belonging to ABCB and ABCC subfamilies accordingly) being the most studied proteins across different species. Thus when choosing possible targets to knock-down and study, we decided to look for homologues of these proteins.

The phylogenetic analysis presented in the Appendix shows that there many nodes of expansions in these two subfamilies, and finding 1:1 evolutionary relationships between the genes of interest in studied species and *N. viridula* is very difficult. As a result we chose our target genes based on their upregulation in the midgut against the carcass, since this is our tissue of interest. We also took under consideration the length of each protein as suggested in our proteomic data, taking care to choose FTs. Figure 3.1 provides a detailed view of the part of the tree were we can find the proteins that we chose to study highlighted in red.

For the selected genes, primers for the synthesis of dsRNA have been synthesized and used. Microinjections of this dsRNA will be performed in the following months.

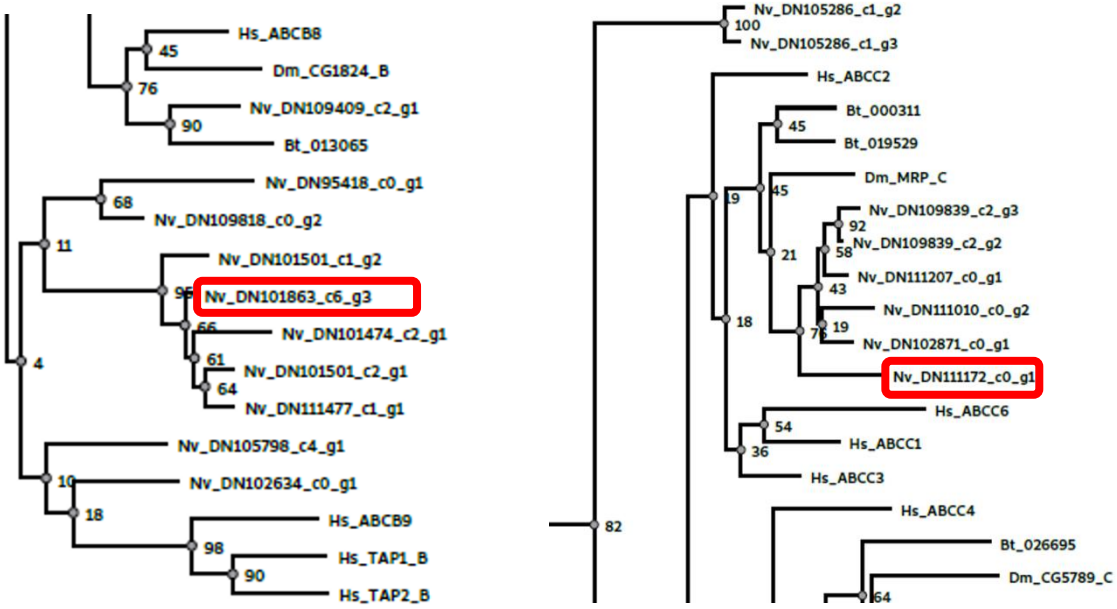


Figure 3. 5 Details of the phylogenetic tree for the ABCB subfamily (left) and ABCC subfamily (right). Circled in red are the genes that were chosen to be studied. Bt = Bemicia tabaci, Dm = Drosophila melanogaster, Hs = Homo sapiens, Nv = Nezara viridula.

3.2.2 Cloning of SLC15 for heterologous expression

In humans, the prodrug approach entails the conjugation of a drug with known substrates of specific transporters in order to enhance their bioavailability by improving their uptake through barrier tissues. We wanted to take advantage of this practice and study its possible application on enhancing insecticide bioavailability for sucking pests like *N. viridula.* We selected to study SLC15 as it has been studied in humans and fish, it is expressed in the midgut, and there is a list of known substrates for it. The first step for the study was the cloning of the gene in appropriate vectors that would allow its heterologous expression in different systems.

We report the successful cloning of the gene in the pGemHE vector. Digestion with appropriate enzymes showed an insertion of the correct size in the vector as shown in Figure 2. Plasmids were sent for sequencing in order to detect for any mutations resulting in stop codons, or differences compared to the predicted sequence. The first 1,7 kb of the gene have been sequenced and the consensus sequence can be found in the Appendix. We did not detect any stop codon mutations, but it seems that we have cloned an isoform with different splicing that the predicted one.

Cloning into the pEIA vector has been unsuccessful so far.

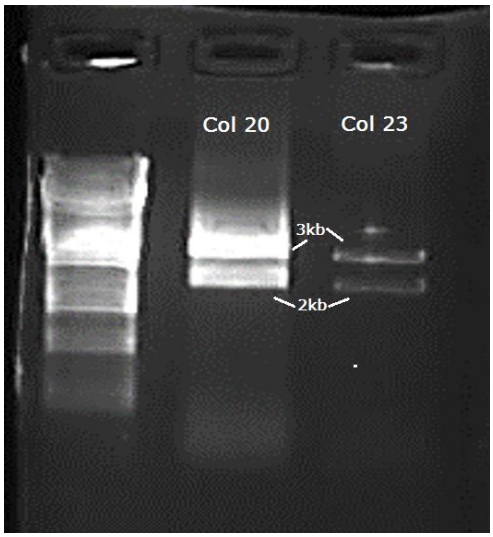


Figure 3. 6 Digestion of pGemHE plasmids from two colonies (colony 20 and 23) to verify that the vector contains NvSLC15.. Plasmids were isolated with a mini prep and then digested with BamHI and NotI in the presence of BSA for 2 hours. On the left there is the ladder. The expected bands are 3kb (pGemHE) and 2kb (NvSLC15).

3.2.3 There isn't horizontal gene transfer of SLC47 into the *N. viridula* genome

While analyzing transcriptomic data, we detected a sequence that is identical to a bacterial SLC47. That meant that the gene was either horizontally transferred from endosymbiotic bacteria into the *N. viridula* genome and is being translated by the insect, or that there was significant bacterial contamination in our proteomic samples. To clarify which was the case, we compared the amount of DNA amplified from genomic DNA sourced from migdut sections known to contain different amount of symbiotic bacteria. If they showed the same levels of amplification it would mean that there is the same amount of copies of the gene in the genome, pointing towards a horizontal gene transfer (HGT). If however it showed different levels of amplification it would mean that there is a different amount of gene copies between the samples, suggesting bacterial contamination. As a control we also amplified actin using the same genomic DNA templates, a gene that is known to have a single copy. We used genomic DNA from the M1 midgut region that is known to be relatively bacteria-free, and the M4 midgut region that is known to be bacteria-rich.

As we can clearly see in Figure 3, DNA amplification in the different midgut regions yielded clear differences in the amount of produced DNA both at 28 and 35 cycles of reactions, despite the standardization of total amount of template DNA. Interestingly, SLC47 amplification is already very high in M4 at 28 cycles, when the actin amplicon is barely visible in M1. Lastly, even with standardized amounts of template DNA, actin in M4 is not amplified as much as in M1, suggesting that a big portion of the template DNA's origin is from bacteria and not the insect tissue. Overall this data indicate that the SLC47 transporter detected in our transcriptomic data was a result of bacterial contamination and not a case of HGT.

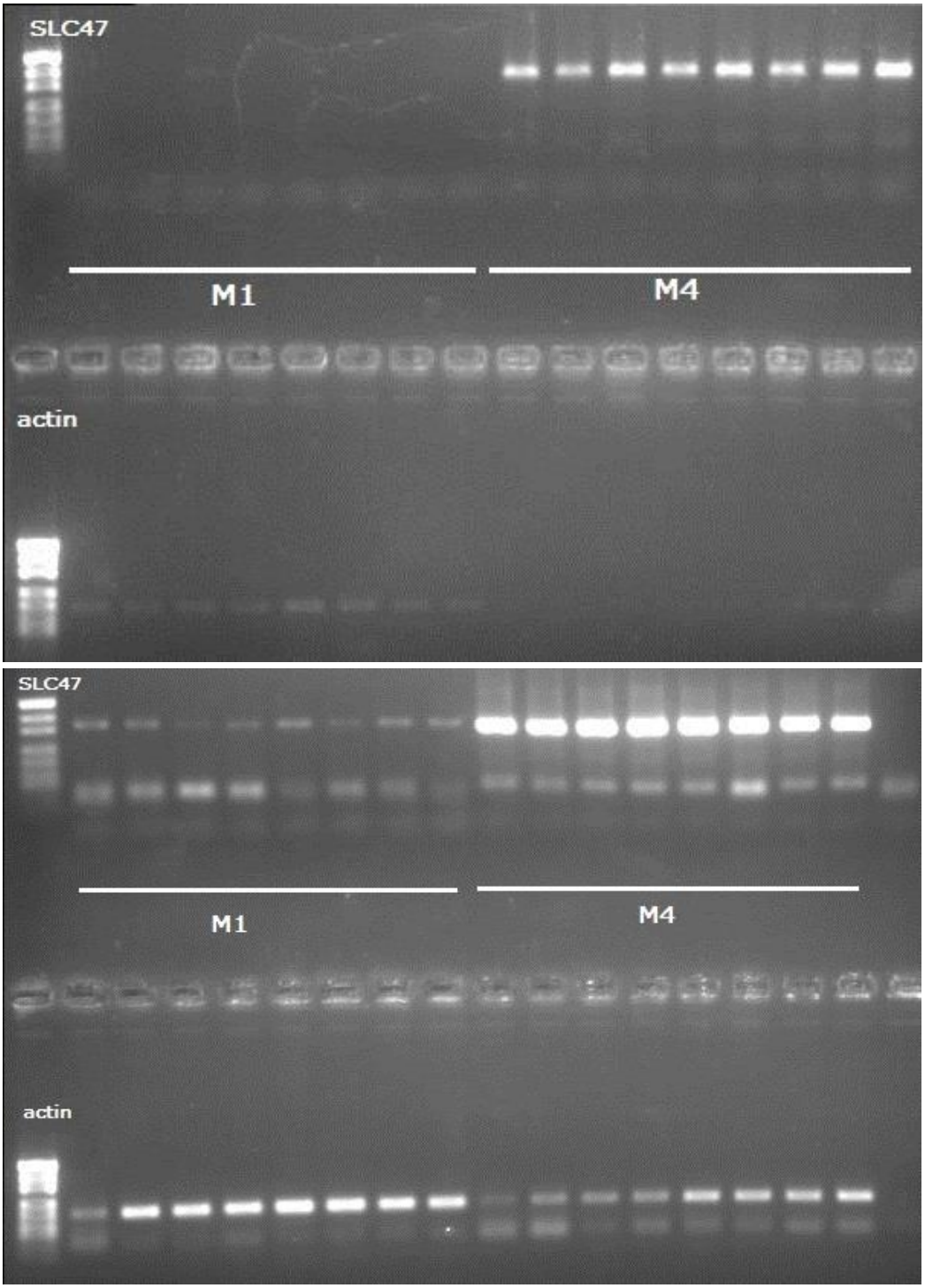


Figure 3. 7 Semi-quantitative PCR for the detection of SLC47 in the genome of N. viridula. Top photo: 28 PCR cycles. Bottom photo: 35 PCR cycles. For each photo: top row shows the amplification of SLC47 and bottom row shows the amplification of actin. On the first lane of each row is the ladder. The negative control is in the last lane of 35 cycles photo. For each gene genomic DNA from M1 and M4 midgut sections was used as a template as noted on the photo. 8 reactions were performed for each midgut section: 4 biological samples (2 from males and 2 from females) with 2 technical replicates each were tested for each section. Total DNA amount was standardized for every sample. The differences in amplification levels of SLC47 between M1 and M4 suggest that SLC47 is not incorporated in the N. viridula genome, but is a result of bacterial contamination in samples.

3.3 Discussion - Future plans

The importance of epithelial membrane transporters in drug and pesticide absorption, distribution, metabolism, and elimination has been highlighted the past years. Studying the function of these proteins in terms of transporting insecticides towards or away from target tissues will provide very useful information that will help us design better control strategies. However, the identification and molecular characterization of transporters has proved difficult compared to other kinds of proteins mainly due to their localization, hydrophobicity, and low abundance. In addition, transporters are under-represented in structural databases because of difficulties in crystallization and computational modeling. When studying non-model organisms like *N. viridula* the lack of an annotated genome and other molecular tools add to the difficulties of performing studies.

For this project, we were based on transcriptomic data in order to identify transporters of interest belonging in the ABC and SLC superfamilies based on their homology to well-studied species. We then made the first steps towards their characterization. In the following months we plan to approach this task using RNAi as an *in vivo* reverse genetics tool, as well two different heterologous expression systems: *Xenopus* oocytes and insect cell lines.

The last two tools have been previously used to study transporters as they allow localization of the expressed protein in the plasma membrane. *Xenopus* oocytes have been extensively used for electrophysiology studies as they present several advantages: they are big and easy to handle and maintain, they reliably express injected cRNA or cDNA, and they provide robust data (Terhag, Cavara and Hollmann, 2010; Marchant, 2018). Their most important disadvantages are the endogenous expression of transporters or other proteins that might interfere with the function of the transporter under study, and that they only be used for low- or medium- throughput screenings (Terhag, Cavara and Hollmann, 2010). As for heterologous expression in an insect cell line, it can be the first step towards exploring transporter activity by monitoring in a high-throughput manner the *in vitro* cellular influx or efflux of reference substrates in cell systems that express them. Fluorescent dye uptake competition assays can also be set-up in order to identify potential new substrates for the studied transporters. This approach also has some problems, like passive dye diffusion through plasma membrane and quenching, and endogenous substances interfering with the substrate transport (Fardel *et al.*, 2015)

As far as the ABC transporters are concerned, we decided to target 2 genes, an Mdr-like and an MRP-like homologue upregulated in the midgut, and knock them down using RNAi. DsRNA has been synthesized targeting these genes. During the next months we plan to inject nymphs with it as described in the previous chapter. This time however we are not interested in the mortality caused by the KD, but in documenting changes in the baseline toxicity against different insecticides that has already been calculated. To achieve this, 2 days post-injection (when all nymphs will have passed on to the 2nd nymphal instar) changes in baseline toxicity will be tested using the bubble feeding bioassay by exposing the injected nymphs to the LC_{30} and LC_{70} doses already calculated for Abamectin and Fipronil. A decrease in toxicity would suggest that the transporter we knocked-down was involved in the transport of the insecticide towards the target tissue. An increase in toxicity on the other hand would suggest that the transporter we knocked-down was involved in the transport of the insecticide away from the target tissue to be excreted. Zero changes in toxicity would mean that the transporter does

not affect the transport of that specific insecticide. Differences in the response between the two insecticides will provide us with hints about the kind of substrates that the selected transporters prefer. We hope that these results will help us explore the involvement of ABC transporters in insecticide transport, and understand better the insect-insecticide interaction.

As for the SLC superfamily, we focused our attention on SLC15, for which we have in our hands an extensive list of di- and tri-peptide substrates for a bacterial homologue of this gene (Ito *et al.*, 2013), as well as a list of fluorescent dyes that act as substrates (Fardel *et al.*, 2015). Comparative studies in fish have also revealed details about its function and the physicochemical parameters that affect it (e.g. in Margheritis *et al.*, 2013 and Romano *et al.*, 2014). Taking these under consideration, we proceeded with cloning the *N. viridula* gene in vectors that will allow us to investigate its function in insects. Alongside NvSLC15 we are also cloning the homologous gene from *Helicoverpa armigera* (Lepidoptera), *C. elegans,* and *Homo sapiens* in order to compare them with one another (data not shown). During the next months we will perform electrophysiological studies in *Xenopus* oocytes, as well as fluorescent dye competition assays in an insect cell line. For the latter, after co-transfection of the appropriate vector containing GFP and the vector containing SLC15, cells of the Sf9 insect cell line will be incubated in the presence of either i) a dipeptide conjugated with the fluorescent dye AMCA that is a known substrate of SLC15 or ii) both the AMCA-dipeptide conjugate and a compound that might be a substrate of SLC15. Differences in dye uptake of the cells between the two experimental will show if the known substrate and the putative substrate compete to bind to the carrier.

Furthermore, this solute carrier has already been explored as a target for carrier-mediated increase of bioavailability in specific tissues through the use of prodrugs in mammals (Yang, Hu and Smith, 2013). It is therefore interesting to learn more about insect SLC15 homologues, as they could be exploited when designing pro-insecticide strategies. Designing completely new compounds as substrates of specific transporters can be very difficult, but with the pro-insecticide approach we could increase the efficiency of currently used compounds simply by conjugating them with a known substrate of this specific transporter. At the same time non-target toxicity will be lowered, and the insecticide will be targeted more precisely to the target tissue. Of course, as with every other approach, there are challenges towards the actualization of this idea: the structure of the conjugate might not be compatible with the reaction sites of the carrier, the added ligand might result in unwanted biological activity, or the approach might prove to be cost-prohibitive. Overall however, this is a very promising approach as it takes advantage of the insect membrane transporters, and drives the efficient site-targeted distribution of insecticides while minimizing their undesirable distribution in the insect.

4 References

Abi Hussein, H. *et al.* (2017) 'Global vision of druggability issues: applications and perspectives', *Drug Discovery Today*. Elsevier Ltd, pp. 404–415. doi: 10.1016/j.drudis.2016.11.021.

Bacci, L. *et al.* (2016) 'A review of Spinosyns, a derivative of biological acting substances as a class of insecticides with a broad range of action against many insect pests', *Journal of Entomological and Acarological Research*, 48(1), p. 40. doi: 10.4081/jear.2016.5653.

Bai, S. H. and Ogbourne, S. (2016) 'Eco-toxicological effects of the avermectin family with a focus on abamectin and ivermectin', *Chemosphere*. Pergamon, 154, pp. 204–214. doi: 10.1016/J.CHEMOSPHERE.2016.03.113.

Bretschneider, A., Heckel, D. G. and Vogel, H. (2016) 'Know your ABCs: Characterization and gene expression dynamics of ABC transporters in the polyphagous herbivore Helicoverpa armigera', *Insect Biochemistry and Molecular Biology*. Pergamon, 72, pp. 1–9. doi: 10.1016/J.IBMB.2016.03.001.

Van den Broeck, W. M. M. (2015) 'Drug Targets, Target Identification, Validation, and Screening', in *The Practice of Medicinal Chemistry: Fourth Edition*. Elsevier Inc., pp. 45–70. doi: 10.1016/B978-0-12-417205-0.00003-1.

Broehan, G. *et al.* (2013) 'Functional analysis of the ATP-binding cassette (ABC) transporter gene family of Tribolium castaneum.', *BMC genomics*, 14, p. 6. doi: 10.1186/1471-2164-14-6.

Carmona-Antoñanzas, G. *et al.* (2015) 'A Survey of the ATP-Binding Cassette (ABC) Gene Superfamily in the Salmon Louse (Lepeophtheirus salmonis).', *PloS one*. Public Library of Science, 10(9), p. e0137394. doi: 10.1371/journal.pone.0137394.

Christiaens, O. and Smagghe, G. (2014) 'The challenge of RNAi-mediated control of hemipterans', *Current Opinion in Insect Science*. Elsevier Inc., pp. 15–21. doi: 10.1016/j.cois.2014.09.012.

Dean, M. and Annilo, T. (2005) 'EVOLUTION OF THE ATP-BINDING CASSETTE (ABC) TRANSPORTER SUPERFAMILY IN VERTEBRATES', *Annual Review of Genomics and Human Genetics*, 6(1), pp. 123–142. doi: 10.1146/annurev.genom.6.080604.162122.

Denecke, S. *et al.* (2018) 'How do oral insecticidal compounds cross the insect midgut epithelium?', *Insect Biochemistry and Molecular Biology*. Elsevier Ltd, pp. 22–35. doi: 10.1016/j.ibmb.2018.10.005.

Denecke, S., Fusetto, R. and Batterham, P. (2017) 'Describing the role of Drosophila melanogaster ABC transporters in insecticide biology using CRISPR-Cas9 knockouts', *Insect Biochemistry and Molecular Biology*, 91, pp. 1–9. doi: 10.1016/j.ibmb.2017.09.017.

Dermauw, W. *et al.* (2013) 'A burst of ABC genes in the genome of the polyphagous spider mite Tetranychus urticae', *BMC Genomics*, 14(1), p. 317. doi: 10.1186/1471-2164-14-317.

Dermauw, W. and Van Leeuwen, T. (2014) 'The ABC gene family in arthropods: comparative genomics and role in insecticide transport and resistance.', *Insect biochemistry and molecular biology*, 45, pp. 89–110. doi: 10.1016/j.ibmb.2013.11.001.

Eakteiman, G. *et al.* (2018) 'Targeting detoxification genes by phloem-mediated RNAi: A new approach for controlling phloem-feeding insect pests', *Insect Biochemistry and Molecular Biology*, 100. doi: 10.1016/j.ibmb.2018.05.008.

Epis, S. *et al.* (2015) 'Temporal dynamics of the ABC transporter response to insecticide

treatment: insights from the malaria vector Anopheles stephensi', *Scientific Reports*, 4(1), p. 7435. doi: 10.1038/srep07435.

Esquivel, J. F. *et al.* (2018) 'Nezara viridula (L.)', in *Invasive Stink Bugs and Related Species (Pentatomoidea)*. Boca Raton : Taylor & Francis, 2017.: CRC Press, pp. 351–424. doi: 10.1201/9781315371221-7.

Estudante, M. *et al.* (2013) 'Intestinal drug transporters: An overview', *Advanced Drug Delivery Reviews*, 65(10), pp. 1340–1356. doi: 10.1016/j.addr.2012.09.042.

Estudante, M. *et al.* (2016) 'Insights into solute carriers: physiological functions and implications in disease and pharmacokinetics', *MedChemComm*. The Royal Society of Chemistry, 7(8), pp. 1462–1478. doi: 10.1039/C6MD00188B.

Ewart, G. D. *et al.* (1994) 'Mutational analysis of the traffic ATPase (ABC) transporters involved in uptake of eye pigment precursors in Drosophila melanogaster. Implications for structure-function relationships.', *The Journal of biological chemistry*, 269(14), pp. 10370–7.

Fardel, O. *et al.* (2015) 'Nature and uses of fluorescent dyes for drug transporter studies', *Expert Opinion on Drug Metabolism & Toxicology*. Informa Healthcare, 11(8), pp. 1233–1251. doi: 10.1517/17425255.2015.1053462.

Ghosh, S. K. B. *et al.* (2017) 'Double strand RNA delivery system for plant-sap-feeding insects', *PLOS ONE*. Edited by D. Doucet, 12(2), p. e0171861. doi: 10.1371/journal.pone.0171861.

Greene, J. K. *et al.* (2018) 'General Insect Management', in *Invasive Stink Bugs and Related Species (Pentatomoidea)*. Boca Raton : Taylor & Francis, 2017.: CRC Press, pp. 729–774. doi: 10.1201/9781315371221-16.

Guo, Z. *et al.* (2015) 'The novel ABC transporter ABCH1 is a potential target for RNAi-based insect pest control and resistance management.', *Scientific reports*. Nature Publishing Group, 5, p. 13728. doi: 10.1038/srep13728.

Guo, Z. *et al.* (2019) 'CRISPR/Cas9-mediated knockout of both the PxABCC2 and PxABCC3 genes confers high-level resistance to Bacillus thuringiensis Cry1Ac toxin in the diamondback moth, Plutella xylostella (L.)', *Insect Biochemistry and Molecular Biology*, 107, pp. 31–38. doi: 10.1016/j.ibmb.2019.01.009.

He, Q. *et al.* (2019) 'ATP-Binding Cassette (ABC) Transporter Genes Involved in Pyrethroid Resistance in the Malaria Vector Anopheles sinensis: Genome-Wide Identification, Characteristics, Phylogenetics, and Expression Profile', *International Journal of Molecular Sciences*. Multidisciplinary Digital Publishing Institute, 20(6), p. 1409. doi: 10.3390/ijms20061409.

Heckel, D. G. (2012) 'Learning the ABCs of Bt: ABC transporters and insect resistance to Bacillus thuringiensis provide clues to a crucial step in toxin mode of action', *Pesticide Biochemistry and Physiology*. Academic Press, 104(2), pp. 103–110. doi: 10.1016/J.PESTBP.2012.05.007.

Hediger, M. A. *et al.* (2013) 'The ABCs of membrane transporters in health and disease (SLC series): Introduction', *Molecular Aspects of Medicine*, 34(2–3), pp. 95–107. doi: 10.1016/j.mam.2012.12.009.

Hull, J. J. *et al.* (2014) 'Transcriptome-Based Identification of ABC Transporters in the Western Tarnished Plant Bug Lygus hesperus', *PLoS ONE*. Edited by Y. Zhang, 9(11), p. e113046. doi: 10.1371/journal.pone.0113046.

Ito, K. *et al.* (2013) 'Analysing the substrate multispecificity of a proton-coupled oligopeptide transporter using a dipeptide library', *Nature Communications*. Nature Publishing Group, 4. doi: 10.1038/ncomms3502.

Jeong, C.-B. *et al.* (2014) 'Genome-wide identification of whole ATP-binding cassette (ABC) transporters in the intertidal copepod Tigriopus japonicus', *BMC Genomics*, 15(1), p. 651. doi: 10.1186/1471-2164-15-651.

Joga, M. R. *et al.* (2016) 'RNAi efficiency, systemic properties, and novel delivery methods for pest insect control: What we know so far', *Frontiers in Physiology*. Frontiers Media S.A. doi: 10.3389/fphys.2016.00553.

Kamminga, K. L. *et al.* (2012) 'Effects of the Insect Growth Regulators Novaluron and Diflubenzuron on the Brown Marmorated Stink Bug', *Plant Health Progress*. Scientific Societies, 13(1), p. 2.

Kim, J. H. *et al.* (2018) 'RNA interference validation of detoxification genes involved in ivermectin tolerance in Drosophila melanogaster', *Insect Molecular Biology*. John Wiley & Sons, Ltd (10.1111), 27(5), pp. 651–660. doi: 10.1111/imb.12512.

Knorr, E. *et al.* (2018) 'Gene silencing in Tribolium castaneum as a tool for the targeted identification of candidate RNAi targets in crop pests', *Scientific Reports*. Nature Publishing Group, 8(1), p. 2061. doi: 10.1038/s41598-018-20416-y.

Kretzschmar, T. *et al.* (2011) 'Functions of ABC transporters in plants', *Essays In Biochemistry*, 50(1), pp. 145–160. doi: 10.1042/bse0500145.

Lee, S. H. *et al.* (2010) 'Decreased detoxification genes and genome size make the human body louse an efficient model to study xenobiotic metabolism', *Insect Molecular Biology*. John Wiley & Sons, Ltd (10.1111), 19(5), pp. 599–615. doi: 10.1111/j.1365-2583.2010.01024.x.

Li, J., Jaimes, K. F. and Aller, S. G. (2014) 'Refined structures of mouse P-glycoprotein.', *Protein science : a publication of the Protein Society*, 23(1), pp. 34–46. doi: 10.1002/pro.2387.

Liljesthröm, G. G., Cingolani, M. F. and Rabinovich, J. E. (2013) 'The functional and numerical responses of Trissolcus basalis (Hymenoptera: Platygastridae) parasitizing Nezara viridula (Hemiptera: Pentatomidae) eggs in the field.', *Bulletin of entomological research*, 103(4), pp. 441–50. doi: 10.1017/S0007485313000023.

Liu, S. *et al.* (2011) 'Genome-wide identification and characterization of ATP-binding cassette transporters in the silkworm, Bombyx mori', *BMC Genomics*. BioMed Central, 12(1), p. 491. doi: 10.1186/1471-2164-12-491.

Liu, X.-Q. *et al.* (2019) 'Genome-wide identification of ATP-binding cassette transporters and expression profiles in the Asian citrus psyllid, Diaphorina citri, exposed to imidacloprid', *Comparative Biochemistry and Physiology Part D: Genomics and Proteomics*. Elsevier, 30, pp. 305–311. doi: 10.1016/J.CBD.2019.04.003.

Lu, H., Xu, Y. and Cui, F. (2016) 'Phylogenetic analysis of the ATP-binding cassette transporter family in three mosquito species', *Pesticide Biochemistry and Physiology*, 132, pp. 118–124. doi: 10.1016/j.pestbp.2015.11.006.

Lu, Y. *et al.* (2017) 'Establishment of molecular genetic approaches to study gene expression and function in an invasive hemipteran, Halyomorpha halys', *EvoDevo*. BioMed Central, 8(1), p. 15. doi: 10.1186/s13227-017-0078-6.

Lucht, J. (2015) 'Public Acceptance of Plant Biotechnology and GM Crops', *Viruses*, 7(8), pp. 4254–4281. doi: 10.3390/v7082819.

Marchant, J. S. (2018) 'Heterologous protein expression in the xenopus oocyte', *Cold Spring Harbor Protocols*. Cold Spring Harbor Laboratory Press, 2018(4), pp. 253–257. doi: 10.1101/pdb.prot096990.

Margheritis, E. *et al.* (2013) 'Characterization of the transport of lysine-containing dipeptides by PepT1 orthologs expressed in Xenopus laevis oocytes', *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*. Pergamon, 164(3), pp. 520–528. doi: 10.1016/J.CBPA.2012.12.016.

Martin, N. A. (2016) *Green vegetable bug - Nezara viridula. Interesting Insects and other Invertebrates. New Zealand Arthropods*. Landcare Research. Available at: https://nzacfactsheets.landcareresearch.co.nz/factsheet/InterestingInsects/Green-vegetable-bug---Nezara-viridula.html (Accessed: 21 September 2019).

Merzendorfer, H. (2014) 'ABC Transporters and Their Role in Protecting Insects from Pesticides and Their Metabolites', *Advances in Insect Physiology*. Academic Press, 46, pp. 1–72. doi: 10.1016/B978-0-12-417010-0.00001-X.

Metzendorf, C., Wu, W. and Lind, M. I. (2009) 'Overexpression of Drosophila mitoferrin in l(2)mbn cells results in dysregulation of Fer1HCH expression', *Biochemical Journal*, 421(3), pp. 463–471. doi: 10.1042/BJ20082231.

Ming, X. and Thakker, D. R. (2010) 'Role of basolateral efflux transporter MRP4 in the intestinal absorption of the antiviral drug adefovir dipivoxil', *Biochemical Pharmacology*, 79(3), pp. 455–462. doi: 10.1016/j.bcp.2009.08.029.

Mogilicherla, K., Howell, J. L. and Palli, S. R. (2018) 'Improving RNAi in the Brown Marmorated Stink Bug: Identification of target genes and reference genes for RT-qPCR', *Scientific Reports*. Nature Publishing Group, 8(1). doi: 10.1038/s41598-018-22035-z.

Morrow, C. S. *et al.* (1998) 'Coordinated action of glutathione S-transferases (GSTs) and multidrug resistance protein 1 (MRP1) in antineoplastic drug detoxification. Mechanism of GST A1-1- and MRP1-associated resistance to chlorambucil in MCF7 breast carcinoma cells.', *The Journal of biological chemistry*, 273(32), pp. 20114–20. doi: 10.1074/jbc.273.32.20114.

Navarro-Quiles, C., Mateo-Bonmatí, E. and Micol, J. L. (2018) 'ABCE Proteins: From Molecules to Development', *Frontiers in Plant Science*, 9, p. 1125. doi: 10.3389/fpls.2018.01125.

Park, Y. *et al.* (2014) 'ABCC transporters mediate insect resistance to multiple Bt toxins revealed by bulk segregant analysis', *BMC Biology*, 12(1), p. 46. doi: 10.1186/1741-7007-12-46.

Pearce, S. L. *et al.* (2017) 'Genomic innovations, transcriptional plasticity and gene loss underlying the evolution and divergence of two highly polyphagous and invasive Helicoverpa pest species.', *BMC biology*. BioMed Central, 15(1), p. 63. doi: 10.1186/s12915-017-0402-6.

Porretta, D. *et al.* (2016) 'How heterogeneous is the involvement of ABC transporters against insecticides?', *Acta Tropica*. Elsevier, 157, pp. 131–135. doi: 10.1016/J.ACTATROPICA.2016.02.002.

Qi, W. *et al.* (2016) 'Characterization and expression profiling of ATP-binding cassette transporter genes in the diamondback moth, Plutella xylostella (L.)', *BMC Genomics*. doi: 10.1186/s12864-016-3096-1.

Rask-Andersen, M. *et al.* (2013) 'Solute carriers as drug targets: Current use, clinical trials and prospective', *Molecular Aspects of Medicine*, pp. 702–710. doi: 10.1016/j.mam.2012.07.015.

Romano, A. *et al.* (2014) 'Teleost fish models in membrane transport research: the PEPT1(SLC15A1) H+-oligopeptide transporter as a case study.', *The Journal of physiology*. Wiley-Blackwell, 592(5), pp. 881–97. doi: 10.1113/jphysiol.2013.259622.

Roth, C. W. *et al.* (2003) 'Identification of the Anopheles gambiae ATP-binding cassette transporter superfamily genes.', *Molecules and cells*, 15(2), pp. 150–8.

Simon-Delso, N. *et al.* (2015) 'Systemic insecticides (neonicotinoids and fipronil): trends, uses, mode of action and metabolites.', *Environmental science and pollution research international*. Springer, 22(1), pp. 5–34. doi: 10.1007/s11356-014-3470-y.

Spanier, B. and Rohm, F. (2018) 'Proton Coupled Oligopeptide Transporter 1 (PepT1) Function, Regulation, and Influence on the Intestinal Homeostasis', in *Comprehensive Physiology*. Hoboken, NJ, USA: John Wiley & Sons, Inc., pp. 843–869. doi: 10.1002/cphy.c170038.

Strauss, A. S. *et al.* (2014) 'Tissue-Specific Transcript Profiling for ABC Transporters in the Sequestering Larvae of the Phytophagous Leaf Beetle Chrysomela populi', *PLoS ONE*. Edited by G. Szakacs, 9(6), p. e98637. doi: 10.1371/journal.pone.0098637.

Sturm, A., Cunningham, P. and Dean, M. (2009) 'The ABC transporter gene family of Daphnia pulex', *BMC Genomics*, 10(1), p. 170. doi: 10.1186/1471-2164-10-170.

Sun, H. *et al.* (2017) 'Multiple ATP-binding cassette transporters are involved in insecticide resistance in the small brown planthopper, *Laodelphax striatellus*', *Insect Molecular Biology*. John Wiley & Sons, Ltd (10.1111), 26(3), pp. 343–355. doi: 10.1111/imb.12299.

Terenius, O. *et al.* (2011) 'RNA interference in Lepidoptera: An overview of successful and unsuccessful studies and implications for experimental design', *Journal of Insect Physiology*, pp. 231–245. doi: 10.1016/j.jinsphys.2010.11.006.

Terhag, J., Cavara, N. A. and Hollmann, M. (2010) 'Cave Canalem: how endogenous ion channels may interfere with heterologous expression in Xenopus oocytes.', *Methods (San Diego, Calif.)*, 51(1), pp. 66–74. doi: 10.1016/j.ymeth.2010.01.034.

Tian, L. *et al.* (2017) 'Genome-wide analysis of ATP-binding cassette (ABC) transporters in the sweetpotato whitefly, Bemisia tabaci', *BMC Genomics*, 18(1), p. 330. doi: 10.1186/s12864-017-3706-6.

Tillman, P. G. (2006) 'Susceptibility of Pest <I>Nezara viridula</I> (Heteroptera: Pentatomidae) and Parasitoid <I>Trichopoda pennipes</I> (Diptera: Tachinidae) to Selected Insecticides', *Journal of Economic Entomology*, 99(3), pp. 648–657. doi: 10.1603/0022-0493-99.3.648.

Tillman, P. G. *et al.* (2015) 'Trap Cropping Systems and a Physical Barrier for Suppression of Stink Bugs (Hemiptera: Pentatomidae) in Cotton', *Journal of Economic Entomology*. Narnia, 108(5), pp. 2324–2334. doi: 10.1093/jee/tov217.

Valenzuela-Muñoz, V., Sturm, A. and Gallardo-Escárate, C. (2015) 'Transcriptomic insights on the ABC transporter gene family in the salmon louse Caligus rogercresseyi', *Parasites & Vectors*, 8(1), p. 209. doi: 10.1186/s13071-015-0801-x.

Wang, Xianhui *et al.* (2014) 'The locust genome provides insight into swarm formation and

long-distance flight', *Nature Communications*, 5(1), p. 2957. doi: 10.1038/ncomms3957.

Wu, C. *et al.* (2019) 'Insect ATP-Binding Cassette (ABC) Transporters: Roles in Xenobiotic Detoxification and Bt Insecticidal Activity', *International Journal of Molecular Sciences*, 20(11), p. 2829. doi: 10.3390/ijms20112829.

Xiao, L.-F. *et al.* (2018) 'Genome-wide identification, phylogenetic analysis, and expression profiles of ATP-binding cassette transporter genes in the oriental fruit fly, Bactrocera dorsalis (Hendel) (Diptera: Tephritidae)', *Comparative Biochemistry and Physiology Part D: Genomics and Proteomics*, 25, pp. 1–8. doi: 10.1016/j.cbd.2017.10.001.

Xie, X. *et al.* (2012) 'Genome-wide analysis of the ATP-binding cassette (ABC) transporter gene family in the silkworm, Bombyx mori', *Molecular Biology Reports*. Springer Netherlands, 39(7), pp. 7281–7291. doi: 10.1007/s11033-012-1558-3.

Xu, Z. *et al.* (2016) 'Analysis of the relationship between P-glycoprotein and abamectin resistance in Tetranychus cinnabarinus (Boisduval)', *Pesticide Biochemistry and Physiology*, 129, pp. 75–82. doi: 10.1016/j.pestbp.2015.10.021.

Yang, B., Hu, Y. and Smith, D. E. (2013) 'Impact of peptide transporter 1 on the intestinal absorption and pharmacokinetics of valacyclovir after oral dose escalation in wild-type and PepT1 knockout mice.', *Drug metabolism and disposition: the biological fate of chemicals*, 41(10), pp. 1867–74. doi: 10.1124/dmd.113.052597.

Yang, N. J. and Hinner, M. J. (2015) 'Getting Across the Cell Membrane: An Overview for Small Molecules, Peptides, and Proteins', in *Methods in molecular biology (Clifton, N.J.)*, pp. 29–53. doi: 10.1007/978-1-4939-2272-7_3.

Yu, X.-D. *et al.* (2016) 'RNAi-mediated plant protection against aphids', *Pest Management Science*, 72(6), pp. 1090–1098. doi: 10.1002/ps.4258.

Yu, Z. *et al.* (2017) 'The ABC transporter ABCH-9C is needed for cuticle barrier construction in Locusta migratoria', *Insect Biochemistry and Molecular Biology*. Elsevier Ltd, 87, pp. 90–99. doi: 10.1016/j.ibmb.2017.06.005.

Zhang, J. *et al.* (2015) 'Full crop protection from an insect pest by expression of long double-stranded RNAs in plastids', *Science*. American Association for the Advancement of Science, 347(6225), pp. 991–994. doi: 10.1126/science.1261680.

Zhang, J. *et al.* (2017) 'Next-Generation Insect-Resistant Plants: RNAi-Mediated Crop Protection', *Trends in Biotechnology*. Elsevier Current Trends, 35(9), pp. 871–882. doi: 10.1016/J.TIBTECH.2017.04.009.

Zotti, M. J. and Smagghe, G. (2015) 'RNAi Technology for Insect Management and Protection of Beneficial Insects from Diseases: Lessons, Challenges and Risk Assessments', *Neotropical Entomology*. Kluwer Academic Publishers, pp. 197–213. doi: 10.1007/s13744-015-0291-8.

Zuber, R. *et al.* (2018) 'The ABC transporter Snu and the extracellular protein Snsl cooperate in the formation of the lipid-based inward and outward barrier in the skin of Drosophila', *European Journal of Cell Biology*, 97(2), pp. 90–101. doi: 10.1016/j.ejcb.2017.12.003.

5 Appendix

Table 1: List of primers used in this study

Primer pair sequence	Use
F: TAATACGACTCACTATAGGGAGAACTAAAGATGGCAACAAGACCG R: TAATACGACTCACTATAGGGAGAAGCCGACGACTCCACTTTC	dsRNA synthesis targeting ABCH
F: TAATACGACTCACTATAGGGAGATGGTTCTGGTGATTTGCCTCAG R: TAATACGACTCACTATAGGGAGAGTGCTCGACAACGAATCCAGTC	dsRNA synthesis targeting ABCE
F: CGACGAACTGGCATATCACG R: ACGAACCTAGCTTTACAGACTTTG	RT-qPCR in ABCH KD nymphs
F: CCAGAAAGGTTCTCTATGGACCTG R: TAAGGGTACTGTTCAAGCTCTGTTG	RT-qPCR in ABCE KD nymphs
F: TAATACGACTCACTATAGGGAGACCTCCCGATAGCTTCGTTCC R: TAATACGACTCACTATAGGGAGAGACATTGGACATTGAGCCCG	dsRNA synthesis targeting MDR-like
F: TAATACGACTCACTATAGGGAGATCCTACATGGCTATCAACGGC R: TAATACGACTCACTATAGGGAGAAGCGTCAATGTGAGGGGTTC	dsRNA synthesis targeting MRP-like
F: ATCAGTGGGAAGGGTTAGCG R: TGGCGATACTATCAGTGCGAC	Amplification of SLC47 from gDNA
F: GTACGGATCCATGACAGCTGAAAGTCAA R: GTACGAATTCTTATATCTTAGTTTGTTCTTCTAGTAC	Cloning SLC15 ORF. F contains BamHI restriction site, R contains EcoRI restriction site
F: GGTTTTCCCAGTCACGAC	M13 universal primer for sequencing
F: TCCTCAACAAGAACGAGAGC	Sequencing of cloned SLC15
F: GCTGATATAAAGGTTCTGATGAGTC	Sequencing of cloned SLC15
F: GTGGATCAAACCAACTTTCAATAC	Sequencing of cloned SLC15

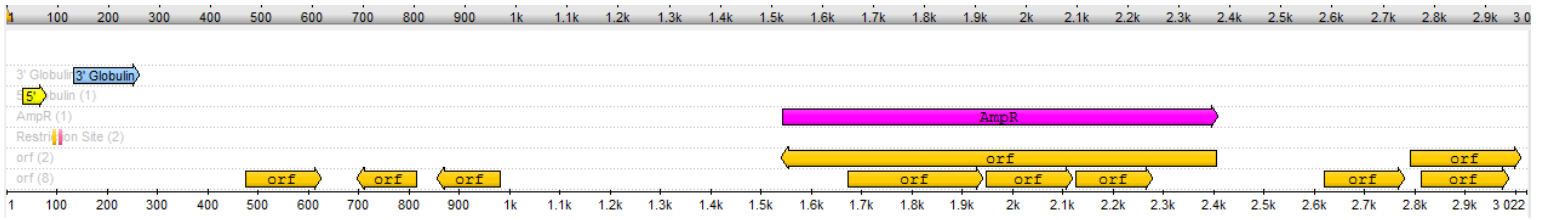
Table 2: Putative members of the ABC family in *Nezara viridula*

Number	Name	Subfamily	Length (aa)	Midgut/carcass expression ratio
1	DN111233_c1_g1	A	1408	18.89364921
2	DN109820_c1_g2	A	2125	1.887051515
3	DN105237_c7_g1	A	1605	1.832432681
4	DN107484_c4_g2	A	2029	0.001793386
5	DN101863_c6_g3	B	1079	12.1295103
6	DN99780_c0_g2	B	694	1.74909522
7	DN109409_c2_g1	B	672	0.921806994
8	DN107282_c0_g1	B	834	1.491383133
9	DN102739_c0_g1	B	769	0.708235271
10	DN109818_c0_g2	B	375	0.239600728
11	DN111477_c1_g1	B	746	0.137992393

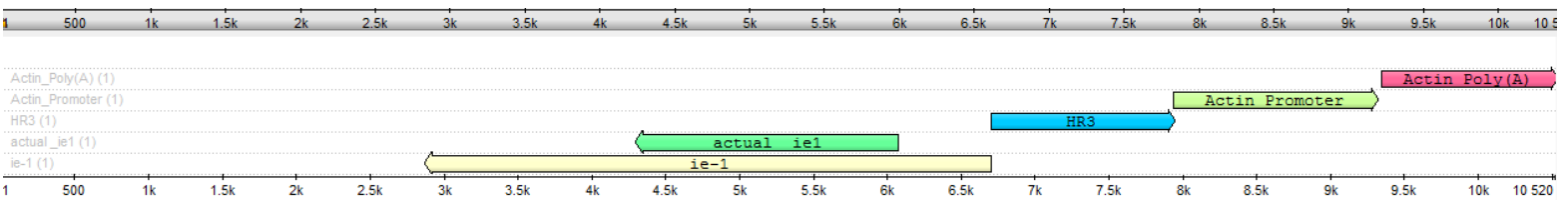
12	DN101501_c2_g1	B	849	0.02246959
13	DN105798_c4_g1	B	1331	0.020172078
14	DN95418_c0_g1	B	588	0.013849142
15	DN101474_c2_g1	B	1254	0.00817349
16	DN101501_c1_g2	B	342	0.006822431
17	DN102634_c0_g1	B	1347	0.00304952
18	DN105538_c6_g1	B	537	18.34504741
19	DN108357_c0_g2	B-C	402	2.265949955
20	DN110952_c1_g1	B-C	412	9.005888266
21	DN111172_c0_g1	C	1496	4.958448226
22	DN106506_c3_g1	C	524	9.206022803
23	DN105286_c1_g3	C	662	9.56592435
24	DN111207_c0_g1	C	784	3.666610304
25	DN109839_c2_g2	C	946	4.447023273
26	DN111207_c1_g2	C	601	3.606927085
27	DN111361_c3_g1	C	1254	3.484224227
28	DN110421_c0_g1	C	1238	3.677672092
29	DN111478_c1_g1	C	1487	2.89372991
30	DN105286_c1_g2	C	717	3.372237141
31	DN102871_c0_g1	C	1505	1.394891331
32	DN109839_c2_g3	C	791	4.078201083
33	DN110097_c0_g1	C	1347	5.045308242
34	DN111010_c0_g2	C	1507	0.24222091
35	DN108950_c0_g1	D	680	1.988433581
36	DN102204_c0_g1	D	656	1.045584168
37	DN110224_c3_g2	DEF	271	11.7026532
38	DN109758_c2_g1	DEF	372	5.402177726
39	DN106064_c2_g1	DEF	941	7.954315713
40	DN101968_c5_g1	DEF	266	9.783765333
41	DN181793_c0_g1	E	266	3.026374987
42	DN107193_c0_g2	E	608	1.048115364
43	DN107796_c3_g2	F	714	0.955039883
44	DN107419_c0_g1	F	1023	0.949833977
45	DN104065_c0_g1	F	597	0.845143173
46	DN22030_c0_g1	G	802	8.308659411
47	DN33017_c0_g1	G	346	3.121637846
48	DN178072_c0_g1	G	802	2.64379371
49	DN110268_c3_g3	G	615	3.245046985
50	DN100783_c0_g1	G	610	1.633815324
51	DN108144_c5_g1	G	678	1.092622203
52	DN108901_c1_g1	G	621	0.872148586
53	DN102643_c3_g1	G	750	1.285801157
54	DN107219_c1_g1	G	579	0.973336739
55	DN99157_c0_g1	G	588	0.361863558
56	DN105287_c4_g3	G	578	0.454144527
57	DN110146_c0_g1	G	612	0.518510877
58	DN109821_c3_g2	G	615	0.080124109
59	DN105481_c1_g1	G	610	0.146443985
60	DN102249_c1_g1	G	689	0.101378267
61	DN110886_c2_g3	G	635	0.481831706

62	DN104020_c6_g2	G	600	0.303823478
63	DN97607_c0_g1	G	670	0.007909617
64	DN107667_c0_g1	G	673	0.007274403
65	DN108588_c0_g1	G	637	0.002606786
66	DN105125_c4_g2	H	697	5.693165099
67	DN104766_c0_g1	H	672	0.115427134
68	DN101186_c0_g1	H	682	0.341332402
69	DN105001_c0_g1	H	683	0.654849115
70	DN106026_c1_g3	H	687	2.450238603
71	DN103018_c14_g1	H	692	0.03625682
72	DN106392_c1_g2	H	804	0.614563068
73	DN103740_c0_g1	H	757	0.027322291
74	DN103865_c0_g1	H	678	0.404607543
75	DN103936_c2_g1	H	678	0.098334807
76	DN98654_c0_g1	H	674	0.002351981
77	DN98599_c0_g1	H	666	0
78	DN109059_c6_g2	H	684	0

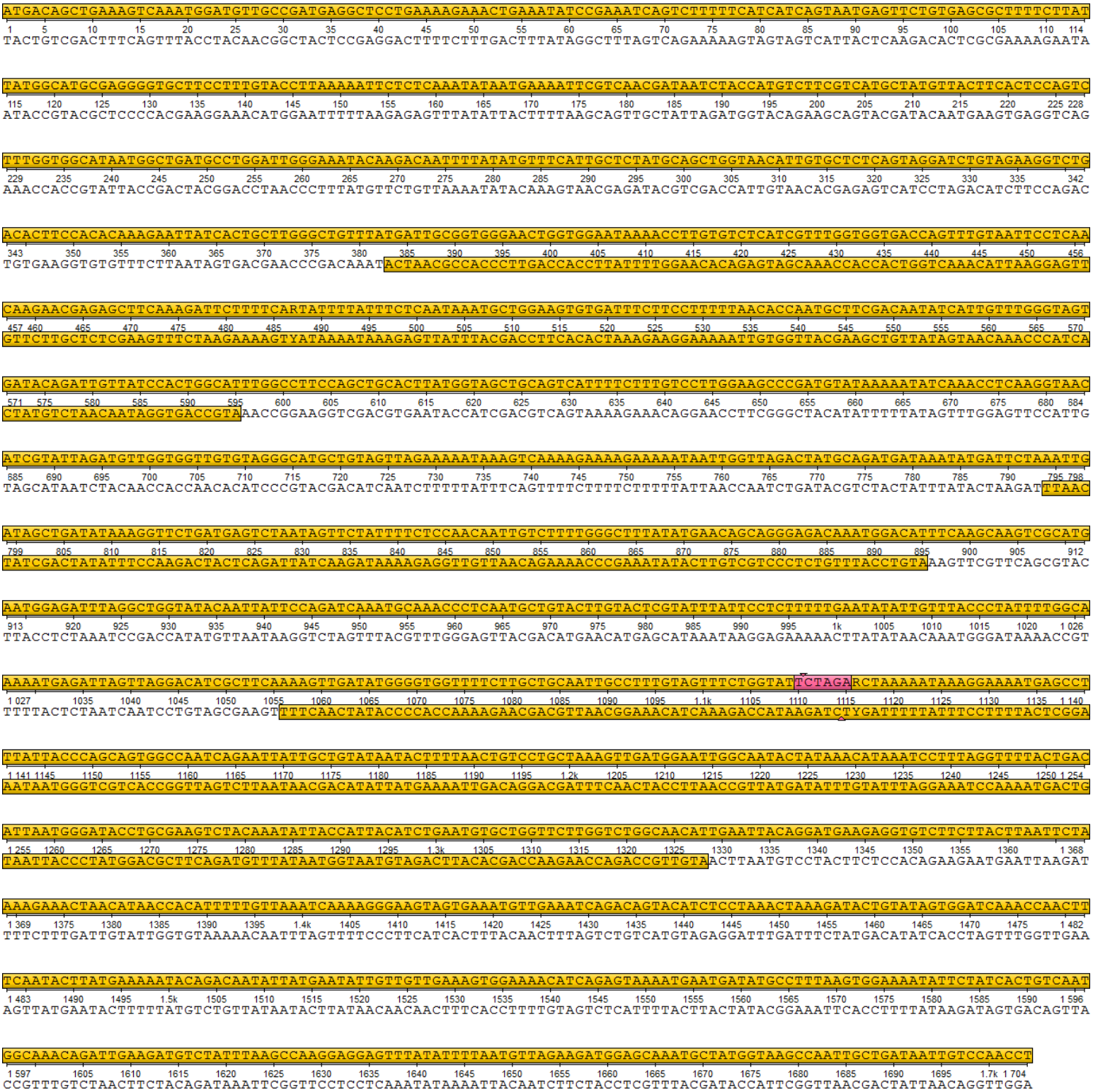
pGemHE plasmid linear map



pEIA plasmid linear map



NvSLC15 sequence



Phylogenetic tree of ABC family