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Putative receptor for Israeli acute paralysis virus of the honey bee (Apis mellifera)

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Putative receptor for Israeli acute paralysis virus of the honey bee (Apis mellifera)

by

Shunji Li

A thesis submitted to the graduate faculty in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Entomology

Program of Study Committee:
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Iowa State University
Ames, Iowa
2017

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ABSTRACT

The honey bee facilitates approximately one third of crop production through provision of pollination services. However, managed honey bees are challenged by numerous factors that contribute to high levels of colony loss. Honey bee viruses are of major concern for honey bee health, and Israeli acute paralysis virus (IAPV) is a common bee virus with acute negative health impacts that has been associated with colony loss. IAPV is primarily transmitted via contaminated food and feces, and is taken up through the midgut, triggering systematic infection. As a member of the family Dicistroviridae, IAPV is presumed to utilize receptor-mediated endocytosis for uptake into gut cells but the molecular mechanisms of this process remain elusive. In this study, we identified a putative receptor for IAPV in honey bee midgut epithelial cells. The putative receptor, aspartic protease [Apis mellifera], was identified by far-western blot with honey bee brush border membrane vesicles (BBMV) as the prey proteins and IAPV virions and IAPV virus-like particles (VLPs) as the bait. The aspartic protease [Apis mellifera] is predicted to harbor an N-terminal endoplasmic reticulum (ER) secretion sequence and one N-linked and three O-linked glycosylation sites indicating ER secretion and post-translational modification. The absence of a transmembrane domain further suggests that this protein has a glycophaspatidylinositol (GPI)-anchor. These data provide an important first step toward elucidating the mechanisms underlying virus entry into honey bee gut epithelial cells. Our results provide fundamental knowledge that may be useful in studying and mitigating the effects of IAPV-mediated colony losses of honey bees.
CHAPTER 1. INTRODUCTION

Colony loss has been a major concern in apiculture

The European honey bee, *Apis mellifera* L., is the most important managed bee in the world (Levin 1983). The honey bee was originally distributed in Europe and Asia and was brought to North America as a result of deliberate human transport (Crane 2013). The most important contribution the honey bee has brought to humanity is pollination of agricultural crops (Klein et al. 2007). The value that honey bees contribute to US agricultural pollination has been estimated at 28 – 112.8 billion USD (Allsopp et al. 2008). Many crops depend heavily on honey bee pollination while other crops, if not pollinated by honey bees, would have reduced yields (Gallai et al. 2009). Honey bees also provide other valuable hive products such as honey, pollen, beeswax and royal jelly, which are important materials not only for agriculture and the food supply, but also for modern industry.

Driven by the expanding population, global agricultural production increased by 140% from 1961 to 2006 (Aizen et al. 2009). It is estimated that food demand will increase between 59% and 98% by 2050 (Valin et al. 2014), and an unprecedented increase in crop production would be required to continue to feed the human population at its current level. Therefore, in order to meet this demand, pollination provided by honey bees and other pollinators, which is critical for crop production and food supply, is of major concern (Aizen & Harder 2009). Although honey bees are economically, agriculturally, and socially important, honey bees have suffered from high recent levels of colony loss in the US and other areas in the world (Oldroyd et al. 2007).
There has been a steep decline in the number of honey bee colonies since 1954, according to USDA periodic surveys since 1943 (USDA-NASS, 1967). There was a major population drop in 1982, following the invasion of a devastating ectoparasite, the Varroa mite (Meixner 2010). A moderate level of overwintering colony mortality is expected due to cold conditions and fluctuations in food availability (Winston 1991). However, abnormally high losses (51.9%) were observed during the 2006 winter national survey, co-occurring with the unusual discovery of colonies devoid of adult worker bees (VanEngelsdorp et al 2007). The term “Colony Collapse Disorder” (CCD) was coined to describe this syndrome, which is characterized by a combination of sudden loss of worker bees, low worker to brood ratio, and high levels of infection with hive pests (Evans et al 2009). Today, although CCD is no longer commonly seen, high levels of annual colony loss continue. The annual rate of loss is around 30%, with up to 70% in some regions of the US, most notably the upper Midwest. The factors underlying these high levels of colony loss are of major concern and are the subject of extensive scientific research (Hayes Jr et al 2008; VanEngelsdorp et al 2010, 2011).

Environmental changes contribute to honey bee declines

The consensus from the honey bee research community is that colony loss is not caused by a single factor, but rather, is due to multiple interacting stressors (Cox-Foster et al 2007). It is likely that colony losses are related to various anthropogenic factors including global warming, globalization of honey bee populations, and habitat loss due to urbanization and the expansion of agriculture (Aizen et al 2009). Many of these factors have contributed to substantial changes in floral foraging resources available to
bees (Hegland et al 2009). A decline in honey bee food resources can lead to nutritional stress and negatively impact overall health and stress resistance of the bee. The honey bee diet consists of nectar (converted to honey), which provides the main source of carbohydrates, and pollen (stored as bee bread), which provide bees with protein, minerals, lipids, and vitamins (Haydak 1970). Intensive agriculture resulting from human population explosion, and economic development have led to dramatic changes in the landscape. There is a significant positive correlation between open land ratio and colony survival, and honey yields from 2003 to 2007 (Naug 2009).

Honey bees are generalist pollinators, visiting a wide range of plants, including crops, horticultural plants, weeds, and native plants. As such, honey bees are vulnerable to agrochemicals on both cultivated plants and in non-target plants within and around areas of cultivation (Mullin et al 2010). Exposure of honey bees to agrochemicals directly or indirectly can lead to a variety of honey bee health deficits and behavioral alterations (Di Prisco et al 2013; Yang et al 2008). There has been much recent concern about the potential effects of neonicotinoid insecticides on honey bee health. A recent study found that after periodic sublethal treatments of clothianidin, a common neonicotinoid used extensively in corn and soybeans, life span and hygienic activities of worker bees were reduced (Tsvetkov et al 2017). Neonicotinoid insecticides are also known to cause cognitive impairment (Palmer et al 2013) and can severely affect queen reproduction (Williams et al 2015) and queen replacement (Tsvetkov et al 2017; Williams et al 2015). Direct contact of bees with neonicotinoid-containing dust generated during seed planting may cause bee kills, and neonicotinoid residues in colonies are associated with reduced colony growth and reduced population size.
(Woodcock et al 2017). However, the effects of neonicotinoids vary widely across studies and geographic regions (Woodcock et al 2017). Fungicide exposure can also reduce the proper growth and development of honey bee adults via suppression of beneficial fungi in the bee bread (Orantes-Bermejo et al 2010; Škerl et al 2009); bee bread is the primary protein source for bee larvae, and beneficial fungi may protect the hives from microbial diseases (e.g. chalkbrood disease; Yoder et al 2013). Given these interactions between pesticides and beneficial microbes, it is not surprising that pesticides are also associated with increased honey bee pathogen loads (Pettis et al 2012). This underscores both the negative impact of pesticides and their interaction with nutrition and pathogens, in their complex effects on honey bee health.

Poor honey bee nutrition can be a direct or indirect causative factor in colony loss. Honey bees acquire natural carbohydrates from nectar or honeydew, convert them to honey, and store them in sealed honeycomb cells (Winston 1991). Adult worker bees have low glycogen reserves in their body, and mainly use consumed sugars to fuel their daily activities, the major sugar resource being stored honey in colonies (Huang 2010). Proteins, which the honey bees obtain mainly from pollen which is then stored as bee bread in honeycomb cells, make up of over 60% of the dry mass of honey bees (Haydak 1953). During late summer, when colony populations contract in preparation for winter, protein levels in the hemolymph of the last generation of worker bees are remarkably high. This, along with increased levels of lipid deposition in the fat body, provide them with the internal fuel necessary to survive the winter (Winston 1991). Malnourished worker bees are less likely to survive the winter, contributing to depopulation during the winter, and thus low worker nutritional state prior to
overwintering can contribute to overwintering mortality in weak colonies (Brodischneider & Crailsheim 2010). Severe nourishment deprivation can also lead adult bees to cannibalize brood, greatly reducing the number of larvae in the colonies (Schmickl & Crailsheim 2001). Starvation and nutrient deprivation may also affect colony division of labor leading to an increase in colony foraging effort and precocious foraging behavior (Toth & Robinson 2005). If severe or prolonged, these conditions can limit colony growth and may even lead to colony collapse in combination with other stressors under extreme circumstances (Perry et al 2015).

Pathogens are significant stressors of honey bee health

Besides habitat loss, pesticides, and nutrition, pathogens represent another important factor contributing to colony loss (Cox-Foster et al 2007; Ratnieks et al 2010). Honey bees are naturally infected by a wide range of pathogens such as fungi, bacteria, protozoans, and viruses. Honey bees are the natural host of the fungi *Ascosphaera apis* (Spiltoir 1955) and *Aspergillus* spp., which cause chalkbrood and stonebrood diseases respectively (Snowdon & Cliver 1966). Chalkbrood is a chronic disease that is specific to honey bees and bumble bees (Maxfield-Taylor et al 2015), whereas stonebrood is a facultative pathogen that infects honey bees and other insects (Ashworth et al 1971; Trienenset al 2010). American foulbrood (AFB) and European foulbrood (EFB) are two bacterial diseases described in honey bees, caused by two Gram-positive bacteria, *Paenibacillus* spp. (Genersch et al 2006) and *Melissococcus plutonius* (Forsgren 2010), respectively. AFB is often considered as destructive to bee colonies, whereas the
negative effects of EFB are mainly seen if a colony is weak or suffering from other health problems (Forsgren 2010).

The fungi *Nosema apis* and *Nosema ceranae*, have also been associated with poor honey bee health (Bailey 1955; Higes et al 2006). *Nosema apis* (Dissociodihaplophasida: Nosematidae) was first described in 1909 when the spores’ ability to cause adult honey bee disease was demonstrated (Calkins 1909). *Nosema ceranae*, first isolated from *A. cerana* in China (Fries et al 1996), is now found in *A. mellifera* and is more pathogenic with more aggressive infections than *N. apis* (Paxton et al 2007, Huang & Solter 2013). A metagenomics survey also showed *N. ceranae* positively correlated with CCD (Cox-Foster et al 2007). *Nosema ceranae* can also cause nutritional stress on honey bees (Naug & Gibbs 2009), resulting in increased levels of starvation which again underscores the impact of *N. ceranae* on honey bee health.

The single largest cause for concern in honey bee health is the Varroa mite, *Varroa destructor* (Acari: Mesostigmata: Varroidae). This mite is a major pest of honey bees, and is an obligate parasite that attacks multiple developmental stages and different honey bee castes. The whole life cycle of the mite occurs in the hive and in the bodies of their hosts (Rosenkranz et al 2010). In the last developmental stage of the brood (workers or drone larvae), adult female mites enter the brood cells before the cells are capped. These mites feed on hemolymph of the pre-pupae and pupae. After the cells are sealed, female mites lay their eggs, producing up to 10 progeny per brood cell. The new born progeny and the female mite feed on the hemolymph of the brood. Thus, the brood cell is the place where all reproductive activities of *Varroa* mites occur.
Only after the adult bee emerges from the brood cell, the female mite, along with her progeny, emerges and seeks out another brood cell, initiating a new infestation cycle (Rosenkranz et al 2010). *Varroa* mites cause a decrease in healthy hemocytes, a decrease in ecdysteroid titers, reduced lipid stores (Dolezal et al 2015), and reduced overwintering survival (Amdam et al 2004). Importantly, *Varroa* mites are also strongly associated with increased virus titers (Dolezal et al 2016) and are able to vector the spread of several major honey bee viruses (Chen et al 2006).

**Viruses are key players in honey bee colony loss**

*Varroa* mites have raised global concern not only because they directly weaken honey bees but also because of their role in transmitting honey bee viruses (Di Prisco et al 2011; Gisder et al 2009). The feeding behavior of *Varroa* mites facilitates virus infection by weakening the host immune response and/or by mechanical transmission of the viruses on piercing of the epidermis (DeGrandi-Hoffman & Chen 2015; Chen et al 2006). *Varroa* mites are commonly associated with deformed wing virus (DWV), and it has been suggested that a mutualism between the mite and the virus may contribute to immunosuppression in host bees (Di Prisco et al 2016). Significant relationships were also found between *Varroa* mite infestation and levels of Kashmir bee virus (KBV) (Chen et al 2004; Shen et al 2005b), and Israeli acute paralysis virus (IAPV; Di Prisco et al 2011). The *Varroa* mite may also mediate horizontal transmission for Sacbrood virus (SBV) among adult bees, as SBV RNA was detected in whole mites and in mite saliva (Shen et al 2005a). There is a strong correlation between levels of honey bee colony loss and the presence of honey bee viruses (Chen & Siede 2007).
To date, some twenty-three honey bee viruses have been described worldwide (Chen & Siede 2007; Genersch et al 2010). Except for *Apis mellifera* filamentous virus (AmFV), which is a double-stranded DNA virus (Bailey et al 1981), the rest are single-stranded RNA viruses (Chen et al 2007). Viruses commonly found in honey bees include: Deformed wing virus (DWV), Black queen cell virus (BQCV), Acute bee paralysis virus (ABPV), Israeli acute paralysis virus (IAPV), Kashmir bee virus (KBV), Sacbrood virus (SBV), and Chronic bee paralysis virus (CBPV).

Symptoms of viral infection vary according to family and genus. DWV, with the widest host range, is known to cause deformed wings at high virus loads, which is the most well-defined disease symptom (De Miranda & Genersch 2010). Typical symptoms also include shrunken, decreased body size and discoloration in adult bees. Several paralytic viruses such as ABPV and IAPV exhibit the primary symptom of whole body paralysis (Genersch et al 2010). Rather than attacking pupae and adults, some viruses cause diseases in brood. SBV, the most widely distributed virus found all over the world (Allen & Ball 1996; Bradbear 1988; Ellis & Munn 2005), results in elongated brood development time and disrupts the metabolic rate of both workers and drones (Bailey & Fernando 1972). If heavily infected, brood are often not capped, and dead larvae turn black and brittle (Bailey & Fernando 1972). Black queen cell virus, as indicated by its name, causes the developing queen larvae and pupae to blacken and infected larvae die rapidly (Chen et al 2005). This disease is more prevalent in the spring and summer, and can greatly limit queen rearing and colony reproduction (Chen et al 2005).
**Virus transmission and immunity**

Viruses of honey bees can be transmitted horizontally (food-borne, venereal, vector-borne), and vertically (maternal transmission) (Chen et al 2006). Six viruses (ABPV, BQCV, CBPV, DWV, KBV, and SBV) were detected in pollen, and KBV and SBV have been found in brood food, honey, pollen, and royal jelly (Chen et al 2006). Oral infection via contaminated food is suggested by the presence of viruses in digestive tissues, such as the gut, salivary glands, and hypopharyngeal glands, where viral titers were higher than other non-digestive tissues (Chen et al 2006). Moreover, viruses were detected from the feces of queen bees, which additionally supports a food-borne horizontal viral transmission. Viruses were found in reproductive tissues indicating venereal transmission, and viruses found in *Varroa* mite saliva provide evidence of vector-borne transmission. Vertical transmission of viruses is supported by the detection of DWV in all developmental stages of honey bees (Chen et al 2006), and the detection of viruses in ovaries and eggs that had been surface-sterilized.

Honey bees use RNA interference (RNAi) as their primary innate immune antiviral defense (Evans et al 2006; Chejanovsky et al 2014). This defense is triggered by the detection of exogenous double-stranded RNA (dsRNA) generated as an intermediate during viral replication (Wang et al 2006) and by hairpin RNA stimulators (Taxman et al 2010). The honey bee genome carries the genes of the core elements of the RNAi pathway, and a homologue of the systemic RNA interference defective protein (SID-1), which is crucial to systemically spread RNAi among cells (Aronstein et al 2006). Deep-sequencing showed honey bee workers harbored abundant siRNA that mapped
to many honey bee virus genomes associated with colony collapse (Chejanovsky et al 2014).

**Israeli acute paralysis virus threatens honey bee health**

**Basic features of IAPV**

This thesis is focused on Israeli acute paralysis virus (IAPV), due to concerns about IAPV as a cause of honey bee mortality, colony loss, and claimed links between IAPV and CCD (Anderson & East 2008; Hou et al 2014). A high incidence of small interfering RNA targeting IAPV was found in CCD symptomatic bees (Chejanovsky et al 2014), suggesting a possible connection between this virus and CCD.

IAPV was originally isolated from heavy loss colonies in Alon Hagalil, Israel, with a combination of symptoms similar to those of Acute bee paralysis virus (ABPV) (Maori et al 2007a). IAPV has also been widely reported including in the US (Chen & Evans 2007), Australia (Stokstad 2007), France (Blanchard et al 2008), China (Yan et al 2009), Poland (Pohorecka et al 2011), Italy (Formato et al 2011), Turkey (Özkırım & Schiesser 2013), and Argentina (Reynaldi et al 2011), suggesting a worldwide distribution. Injection of IAPV viral RNA has the potential to result in extremely high and rapid adult honey bee mortality (Maori et al 2007a). The full-length genome is 9,487 nt (excluding the poly-A tail). The genome carries two open reading frames (ORFs), separated by a 184-nt-long intergenic region. ORF 1 at the 5’ end encodes 1900 amino acids, which are helicase, protease, and RNA-dependent-RNA polymerase (RdRp), involved in RNA replication and protein processing. ORF 2 at the 3’ end encodes 908 amino acids, which
is a polyprotein. The viral protease cleaves this polyprotein to generate the capsid proteins, VP1, VP2, VP3, and VP4. There are internal ribosome entry sites (IRES) at the N-terminus of each ORF, indicating the unique translation pattern of this virus (Wilson et al 2000). These characteristics are typical for the family Dicistriviridae (Bonning 2009; Maori et al 2007a; see Fig. 1).

Figure 1 Schematic of the IAPV genome and encoded proteins

The polyprotein of IAPV is cleaved to produce four capsid proteins during virion assembly (Maori et al 2007a). Gel electrophoresis of IAPV showed four proteins of approximately 17, 26, 33, and 35 kDa and three minor proteins of about 19, 30, and 46 kDa. Edman degradation assays provided the N-terminal sequences of these proteins, indicating the cleavage sites in the polyprotein used to generate the capsid proteins. It is worth noting that the \(^{398}\text{GWSKP}^{402}\) motif in IAPV, corresponding to the VP2/VP4 cleavage site, was similar to the G[F/W]SKP motif, residing in residues 300 to 400 in the structural polyproteins of other dicistroviruses (e.g., ALPV, RhPV, DCV, CrPV, BQCV,
ABPV, KBV) (van Munster et al 2002), indicating this cleavage site is conserved. Although the cleavage sites were identified, the three minor proteins may result from alternate protein processing (Maori et al 2007a; Fig. 1).

Analysis of the sequences of RdRp of IAPV and other related viruses showed IAPV phylogenetically clustered with KBV and ABPV (Maori et al 2007a). The untranslated region (UTR) however is distinct with only weak homology to the UTRs of the other two viruses (Maori et al 2007a). In addition, phylogenetic analysis based on RdRp revealed 3 groups of IAPV: group 1 contained isolates from western US and Australia; group 2 referred to as the Israeli isolate; group 3 contained isolates from eastern US, and Canada (Palacios et al 2008). The IAPV strain reported by Maori et al (2007a) did not cluster with any other isolates (Palacios et al 2008).

Effects of IAPV on honey bees

Like most dicistroviruses, IAPV infection is commonly persistent in bee hives and lacks severe symptoms (Chen et al 2014; Bonning & Miller 2010). However, high titers of IAPV infecting individual pupae or adults can be virulent. Heavily-infected bees are often characterized by darkening and loss of hair on their thorax and abdomen. These symptoms are accompanied by weight loss (Hunter et al 2010), paralysis, and trembling. Paralysis is also a prominent symptom of infection of another dicistrovirus, Cricket paralysis virus. IAPV may disrupt mitochondrial function, which could lead to disruption of energy-related processes (Chen et al 2014). IAPV also affects bee behavior by reducing their sucrose responsiveness (Li et al 2013).
Proteomic analysis (Michaud et al 2014) revealed that viral proteins were identified at 12 and 36 h after viral injection. Gene ontology (GO) analysis showed a significant 36 h enrichment of proteins related to translation functions, mRNA binding, metabolic activities, indicating a significant disruption of regular cellular functions in infected bees. Also, the expression of proteasomal proteins, unfolded-protein-binding components, and mRNA 3’-UTR binding proteins was down-regulated (Michaud et al 2014).

Partial IAPV sequences have been discovered to be integrated into the honey bee genome (Maori et al 2007b). Translation and expression of these sequences were confirmed by northern blot and western blot (Maori et al 2007b). Reciprocal exchange of sequences between IAPV and the honey bee also involves partial honey bee sequences embedded in an IAPV-associated defective RNA. When injected with IAPV, the majority of bees died of viral infection based on the presence of both positive and negative strand RNA. IAPV sequences were not integrated into the genomes of these bees. The survivors developed to healthy adults and all carried the aforementioned viral sequence (residing in the structural protein) (Maori et al 2007b). These evidences suggested bees carrying partial IAPV sequences may be resistant to IAPV infection. Recent research indicates that integration of viral sequences into the host genome provides an addition mechanism of antiviral immunity by boosting RNAi-mediated protection (Goic et al, 2013), and the aforementioned study suggests this may also be occurring with IAPV.
Transmission of IAPV

IAPV can be transmitted vertically and horizontally among bees and colonies. Vertical transmission is supported by the detection of IAPV in queen ovaries, and in semen and spermathecae in drones. The presence of IAPV in sexual organs also suggests that IAPV may be sexually transmitted (Chen et al 2014). Horizontal transmission includes both food- and vector-borne routes. Food-borne transmission of IAPV is suggested by IAPV-positive digestive tracts and feces of queen bees, and food supplies. IAPV can be detected in all developmental stages and all colony castes (Chen et al 2014). Varroa destructor, which feeds on bee hemolymph is a vector of IAPV (Di Prisco et al 2011; Chen et al 2014). Not only do the mites transmit IAPV but they are also IAPV replication-competent, as shown by detection of IAPV negative strand in the mites (Di Prisco et al 2011) and therefore can amplify the titer of mite-ingested virus. IAPV is detected in mite salivary glands, further supporting the capacity of Varroa to vector IAPV (Chen et al 2006). Moreover, the expression levels of immune-related genes were decreased as a result of mite infestation, indicating that mite infestation weakens bee immunity and thus elevates virus infection and replication (Di Prisco et al 2011).

Mechanisms of viral entry

Receptor-mediated viral entry

Viruses have to enter host cells, where they hijack the host cell machinery to replicate (Dimitrov 2004). Several routes are used for virus entry, depending on the
capsid structure of the viruses. For example, 1) membrane fusion, used by most enveloped viruses [e.g., HIV (Stein et al 1987), herpes simplex virus (Montgomery et al 1996)]; 2) endocytosis used by some positive-sense, single-stranded RNA viruses [e.g., poliovirus (Blanchard et al 2006), Hepatitis C virus (Meertens et al 2006)]. Membrane fusion and endocytosis are often dependent on a cellular receptor, bound by the virus as the first step of viral attachment (Dimitrov 2004).

Viruses that use endocytosis to enter host cells exploit pathways used by harmless substances or nutritional components, or employ host immune effectors as viral receptors (Vossen et al 2002). Once in the host cell, the virion is often delivered to intracellular endosomes where low pH will trigger genome release from the virion (Madshus et al 1984; Pérez & Carrasco 1993), followed by viral RNA transcription and translation within the cell (see Fig. 2).

Figure 2 Schematic of clathrin-mediated endocytosis, the mechanism of cell entry for most picornaviruses. Modified from Brandenburg et al 2007.
Viruses have evolved to exploit a variety of cellular molecules as receptors to facilitate their entry into host cells. Most of these molecules are proteins, carbohydrates, and glycolipids (He et al 2000). However, binding of a cellular molecule to a virion is not sufficient for receptor functionality. Rather, a competent cellular receptor for a virus should be able to initiate the full cycle from virus recognition to the release of the viral genome and viral protein production. Thus, the receptors of viruses often determine the tissue tropism of the virus (Nomoto et al 1994). Sometimes, a single cellular molecule mediates all of these tasks, and sometimes more than one molecule is involved (He et al 1997). Some viruses can use more than one receptor, even structurally unrelated, in different situations (Krummenacher et al 1998).

Following virion-receptor binding, viral entry and virion uncoating are associated with a conversion of the virion into an Altered particle (A particle, shown in Fig. 2). In polioviruses, CryoEM showed VP4 is absent in the A particle, and this absence is evident by 5% mass loss and changes in sedimentation coefficient from 160S to 135S (Belnap et al 2000). Also, this transition is accompanied by genomic RNA release and the irreversible exposure of N-terminal extensions of VP1 in poliovirus (Belnap et al 2000). Attachment of the 160S particle (infectious virion) to a competent cellular receptor, triggers the formation of the 135S transitional particle (Butan et al 2014). Stabilization of the virus at the cell surface is mediated by externalized VP4 and the N-terminus of VP1 (Bubeck et al 2005). The rearranged VP1 inserts into the cell membrane to form a pore, allowing genomic RNA to enter the cell through the pore. There are also small RNA viruses that do not form transitional particles before genome
release, for example, Slow bee paralysis virus (SBPV) from *Iflaviridae* (Kalynych et al 2017) and IAPV (Mullapudi et al 2017).

**Receptors of small RNA viruses**

*Picornaviridae*, a sister group of *Dicistroviridae* in the Order *Picornavirales*, is a group of viruses with icosahedral, non-enveloped virions, and with single-stranded positive-sense, RNA viral genomes (Hogle et al 1985; Kitamura et al 1981). Many receptors for picornaviruses have been identified primarily due to extensive research resulting from their human and animal health importance (see Tbl. 1).

Table 1 Picornavirus receptors identified to date

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<td>Intercellular adhesion molecule 1 (ICAM-1)</td>
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<td>Coxsackievirus-adenovirus receptor (DAR)</td>
<td>He et al 2001; Myers et al 2004</td>
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<td>Nishimura et al 2009; Yamayoshi et al 2013</td>
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<td>ICAM-1</td>
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<td>Low-density lipoprotein receptor (LDL-R)</td>
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<td>Poliovirus receptor (PVR)</td>
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<tr>
<td>Theiler’s murine encephalomyelitis virus</td>
<td>P0 protein (IgSF family)</td>
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Receptors of many picornaviruses (e.g., rhinoviruses and enteroviruses) belong to the immunoglobulin super family (IgSF), which are type I transmembrane glycoproteins with five extracellular immunoglobulin (Ig) domains (Greve et al. 1989; Mendelsohn et al. 1989). The virus-recognition site is in the D1 domain, which is distal from the plasma membrane (Olson et al. 1993). These long molecules also penetrate into the canyon located in the picornavirus virion (Rossmann et al. 2000). The amino acid residues involved in receptor-virus binding are highly conserved (Aoki et al. 1994). The unique structure of the canyon is assumed to protect the virus from host immune surveillance because most of the host neutralizing antibodies cannot access the depression of the virion (Rossmann 1989; Xing et al. 2000), known as the canyon. Similar structures were found in several coxsackieviruses, polioviruses, and adenoviruses shown by cryo-electron microscopy (CryoEM) (Stewart et al. 1997; Yoder et al. 2012).

There are also cellular receptor molecules that do not bind to canyons and do not belong to the Ig family, known as the low-density lipoprotein receptor (LDL-R) and decay-accelerating factor (DAF or CD55). These molecules are receptors for some echoviruses and coxsackie B viruses (He et al. 2002; Karnauchow et al. 1998; Stuart et al. 2002). The binding sites are often located at the five-fold axis of the virions (Hewat et al. 2000), and this prominent structure is often vulnerable to host neutralizing antibodies and thus the viruses are often subject to a change in their specificity to different hosts and tissues. IAPV virions do not exhibit canyon structures but demonstrate prominent spikes in the 5-fold axis. It is predicted that these spikes mediate receptor binding (Mullapudi et al. 2017).
Endocytosis of a dicistrovirus Drosophila C virus (DCV)

The most detailed molecular analysis of dicistroviruses has been conducted with viruses of *Drosophila melanogaster*, owing to the wealth of genetic resources available for this species. The viral entry mechanism has been elucidated for Drosophila C virus (Dicistroviridae), providing fundamental information for studies of other dicistroviruses.

Drosophila C virus (DCV) (Jousset et al 1977) utilizes clathrin-mediated endocytosis for entry into the host cell (Huszar & Imler 2008). Mutations of α-adaptin and clathrin heavy chain resulted in resistance of *Drosophila* flies to viral infection and reduced production of viral antigens. A functional protein synaptotagmin was found to be involved in the endocytosis of Drosophila C virus (DCV) virions in DCV infection in *Drosophila* (Cherry & Perrimon 2004). Synaptotagmin is a vesicular trafficking protein, which contains an N-terminal transmembrane sequence and a cytoplasmic tail sequence consisting of two structurally similar calcium-phospholipid binding domains C\(_2\)A and C\(_2\)B; one of the calcium-phospholipid binding domains links to the adaptor protein 2 (AP-2) (Zhang et al 1994), which is a marker protein for endocytosis (Traub 2003). It is suggested that the formation of multimers of synaptotagmin is a critical step in the regulation of clathrin coated pit assembly (von Poser et al 2000). In *Drosophila*, synaptotagmin I, an intrinsic membrane protein of synaptic vesicles, was shown to be a receptor with high affinity for clathrin AP-2. This binding, coming into play in both endocytosis and exocytosis, is critical to maintain a proper membrane balance especially in nerve terminal (Poskanzer et al 2003).
Evidence suggests that synaptotagmin is one of the crucial elements in clathrin-mediated endocytosis trafficking DCV virions, but whether that the synaptotagmin is the receptor for DCV still needs to be examined. A receptor serves as a first viral attachment site to the host cell with conserved amino acid residues within the virus-receptor complex. We would expect that mutations made in this complex would interfere with the interaction between the receptor and the virus. Protein-protein interaction analysis studies via protein overlay assays provides a useful approach to identify binding between the molecule and the virus. The current work presents the identification of a putative receptor for IAPV using far-western blotting, and the results will serve as a reference for future research in this area.

**Thesis organization**

In Chapter 2, we describe identification and characterization of the putative honey bee midgut receptor protein for IAPV, aspartic protease [*Apis mellifera*] (Fig. 3). In Chapter 3, we develop a model for IAPV entry and discuss the potential use of this midgut receptor for management of viral disease to help protect against IAPV-mediated colony losses of honey bees. The appendix describes efforts toward *E. coli* expression of individual structural proteins of IAPV. These individual capsid proteins were intended for use as additional ligands in ligand blot analysis. The data presented may be helpful for future research on IAPV.
Figure 3 A schematic diagram based on functional motif prediction of the GPI-anchored aspartic protease [Apis mellifera], a putative gut receptor for IAPV. The C-terminus of the mature aspartic protease attaches to the carbohydrate linker (containing mannose, glucosamine, and inositol) via an ethanolamine phosphate (EtNP). A fatty acid molecule with two fatty acid chains (not shown) within the hydrophobic phosphatidyl-inositol group anchor the protein to the cell membrane. PI-PLC is the bond linking the phosphate and the fatty acid.

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CHAPTER 2. PUTATIVE RECEPTOR FOR ISRAELI ACUTE PARALYSIS VIRUS OF THE HONEY BEE (APIS MELLIFERA)

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Abstract

Israeli acute paralysis virus (IAPV) is one of several RNA viruses that threatens honey bee colony health by causing lethal and sublethal infections. IAPV has a positive sense, single stranded RNA genome and belongs to the family Dicistroviridae. A primary route of infection occurs via ingestion of virions, which are predicted to be taken up into gut epithelial cells via receptor-mediated endocytosis. Identification of the honey bee midgut receptor is thus critical to understand the mechanisms of viral entry and this knowledge can contribute to reducing virus transmission. Here, we report a putative midgut receptor for IAPV. Putative receptor proteins were identified by far-western blotting. Brush border membrane vesicles (BBMV) were prepared from dissected honey bee midguts and proteins separated and analyzed by 2-dimensional ligand blot. IAPV virions and IAPV virus-like particles (VLPs) were used as ligands with detection mediated by anti-VP1 antiserum. Peptide sequences of candidate receptor proteins were determined by liquid chromatography with mass spectrometry (LC-MS/MS), and candidate proteins were identified via sequence comparison to the translated genome of
the honey bee. Functional motifs in the candidate proteins were identified, thus
providing novel and potentially useful information on the molecular basis of IAPV entry
into honey bee cells.

Introduction

Honey bees are the single most important pollinator in agricultural systems. It is
estimated that approximately one-third of food consumed in the western world results
from honey bee pollination (Allsopp et al 2008). Although beekeepers have historically
reported moderate levels of yearly losses (averaging around 10%), a sudden and
dramatic loss was reported in 2006 and raised worldwide concern (Oldroyd 2007). In
the following years, the total colony loss estimated annually in the US was reported to
be around 25-51%, which is higher than the economically viable loss rate of 13-17%
(Hayes et al 2008; vanEngelsdorp & Meixner 2010; vanEngelsdorp et al 2012;
Neumann & Carreck 2010).

Israeli acute paralysis virus (IAPV), has been linked to colony loss of honey bees
(Cox-Foster et al, 2007; Oldroyd 2007; Chen & Siede 2007). IAPV has non-enveloped
icosahedral capsids, which protect a linear, single-stranded, positive-sense RNA
genome of 9,600 nucleotides (Maori et al 2007a). The genome has two open reading
frames, ORF 1 and ORF 2, encoding the viral nonstructural proteins and structural
proteins (capsid proteins), respectively. ORF 2 encodes a polyprotein, which is post-
translationally cleaved by the viral protease to produce functional capsid protein
subunits, VP1, VP2, VP3, and VP4. The major capsid proteins, VP1 to VP3, form the
outer surface of the capsid whereas the smallest capsid protein VP4 attaches to the
capsid on the inner surface. The mature capsid shell has a pseudo T = 3 icosahedral symmetry (Maori et al. 2007a). Although the basic structure of IAPV is known, the mechanisms by which IAPV enters and infects host cells are largely unknown.

Infection by most members in Picornavirales relies on receptor-mediated viral entry (Bergelson & Coyne 2013). Virions attach to host receptors and transcytosis of virions in a clathrin-dependent manner (Brandenburg et al. 2007), the viral genome is released from the virion in the low pH environment of the endosomes. This genome release is often preceded by structural changes in the capsid, resulting in formation of an expanded A particle induced by receptor-binding or by the low pH in endosomes (Fricks & Hogle 1990; Prchla et al. 1994). The A particles provide pores allowing for release of the genome and VP4 subunits (Mullapudi et al. 2016). After genome release, the virus then takes over the cellular machinery for virus replication.

Receptor-binding is the first step of and thus critical to viral infection. Many viruses use host cellular proteins as their receptors to support their initial attachment. The canyon structure of the virion often plays an important role in host cell surface receptor binding for picornaviruses (Rossmann 1989). Multiple cellular receptors of picornaviruses have been identified, and many of them belong to the immunoglobulin superfamily (IgSF) (Mendelsohn et al. 1989). Viruses also use receptors outside of the IgSF such as the human rhinoviruses, which bind to low-density lipoprotein receptor (LDLR), very-low-density LR, and LDLR-related protein at the cell membrane (Rankl et al. 2008). In addition, receptors for polioviruses are produced both as membrane-bound proteins and secreted forms (Koike et al. 1990).
Dicistroviruses, may differ in their viral entry mechanisms compared to polioviruses, because many dicistroviruses lack the flexible canyons found in most picornaviruses (Tate et al 1996; Mullapudi et al 2016). The prominent spikes located between the icosahedral 5-fold and 3-fold axes of symmetry of the IAPV virion are hypothesized to be involved in receptor-binding (Mullapudi et al 2016). The entry mechanisms of dicistroviruses have been studied in Drosophila C virus (DCV), revealing that DCV enters cells through clathrin-mediated endocytosis (Cherry & Perrimon 2004). However, how dicistroviruses attach to host cellular receptors and trigger endocytosis is unclear. The goal of the present study is to explore the interaction between honey bee gut proteins and IAPV virions to pinpoint putative IAPV-binding gut proteins in vitro, which could potentially serve as receptors in the IAPV infection cycle, thus providing novel information relevant to understanding how IAPV infects its honey bee host.

**Materials and Methods**

**Insects**

Hive worker bees (for midgut dissection) were obtained from healthy colonies from the research apiary at Iowa State University and kept in cages (~100 honey bee per cage) in an incubator at 32 °C and 50% relative humidity before use for gut preparations (described below). Frames of pupae were obtained from at least 3 different healthy colonies from the same research apiaries. Pupae (for IAPV amplification) were isolated as soon as they were obtained from the apiaries for generating viruses through a pupal injection and purification procedure (described below, Carrillo-Tripp et al, 2016).
**Preparation of honey bee brush border membrane vesicles (BBMV)**

Honey bees were chilled on ice to induce short-term anesthesia. Midguts were dissected by dragging out the stinger, which connects to the rectum along with the midgut. The midgut dissociates from the crop when pulled out with the stinger. Rectums were removed and the midguts were washed twice in cold PBS and then stored in cold Buffer A (0.3 M mannitol, 5 mM EGTA, 17 mM Tris-HCl [pH 7.5]) with 1× cocktail protease inhibitor (Thermo Fisher Scientific). Midguts were stored at -80 °C or used immediately. Honey bee BBMV were prepared as described previously (Linz et al, 2015). The final pellet was re-suspended with cold Buffer A with 1:2 water dilutions. Protein concentration was determined by Bradford assay (Kruger 1994). Aminopeptidase activity was measured for the homogenate and final BBMV for calculation of relative enrichment. Honey bee BBMV were snap-frozen with liquid nitrogen and stored at -80 °C.

**Production of IAPV-rich viral stock**

An IAPV-rich viral stock (referred to as IAPV virions below) was prepared as previously described (Carrillo-Tripp et al, 2016). The IAPV stock was comprised of 95.6% IAPV, 2.98% BQCV, 1.33% SBV, <=1% DWV, with an IAPV titer of 2.44×10⁷ genome equivalent/100 ng RNA. Briefly, white-eye or pink-eye stage honey bee pupae were isolated from healthy-looking combs and incubated in 28 °C overnight. One µL of IAPV inoculum diluted 100-fold was injected into the abdomen of pupae using a micro-injector (Harvard Apparatus Model PLI-100 Pico Injector). Injected pupae were
incubated in petri dishes at 28°C for 5 days. Injected pupae were then homogenized and PEG-8000 precipitation carried out. Viral RNA was extracted from the viral stocks using Trizol and a DNase I treatment was performed. RNA concentration was determined by NanoDrop (Thermo Fisher Scientific). Viral composition was determined using iTaq™ Universal SYBR® Green One-Step Kit (BioRad) following an absolute quantification based on 74 ng of total RNA per sample. Each sample was amplified with technical duplicates in a CFX 384 Thermocycler (BioRad) as described previously (Carrillo-Tripp et al 2016): Reverse transcription (50 °C, 25min); PCR [95 °C, 5min] - 40×95 °C, 5 s, 58 °C, 30 s]; melting curve (95 °C, 30 s, 55 °C, 30 s); stepwise 0.5 °C increase from 55 – 95 °C. The universal reference standard plasmid containing several viral RNA fragments (ABPV, BQCV, CBPV, DWV, IAPV, LSV, KBV, and SBV) was used as a reference (Carrillo-Tripp et al 2016). The final copy number was calculated by extrapolation to the standard curve based on 10-fold serial dilution of the reference.

**Production of IAPV virus-like particles**

IAPV virus-like particles (VLP) were produced in Sf9 cells in suspension using the recombinant baculovirus ORF2-5TFS-3C that expresses the IAPV structural proteins (Ren et al, 2014). Sf9 cells in suspension culture in logarithmic phase of growth were infected with an MOI of 5 and incubated at 28 °C for 48 h before harvest. Cells were spun down at 3,000 rpm for 15 min and the pellet lysed with 1/20 of original cell culture volume of PBS 1% Triton X-100 at 4 °C 1 h with occasional shaking. The lysate was cleared by centrifugation at 4,500 rpm 45 min at 4 °C. The supernatant was loaded onto a 30% sucrose cushion and centrifuged at 100,000 g 100 min at 4 °C. The final
pellet was recovered with PBS with 3,500 units of Benzonase (Sigma) and was incubated at room temperature for 30 min before being loaded on to a 30% - 60% sucrose gradient. The IAPV-VLPs were harvested and concentrated to 200 µL using Microcon centrifugal units 10k (Millipore). The sucrose gradient fractions were loaded (10 µL per well) in a 10% polyacrylamide gel. VLP were detected by western blot with rabbit anti-VP1 antiserum (which recognizes the peptide sequence GGRRYKFFNTTPLK present in the VP1 of IAPV, KBV, and ABPV; Carrillo-Tripp et al 2016) at a dilution of 1:2,000 and a secondary antibody goat anti rabbit IgG (1:5,000 dilution). Enriched IAPV virions isolated from the pupal injection experiment were used as a positive control in western blots.

2-D gel electrophoresis and far-western blotting

A total of 300 µg honey bee BBMV was thawed on ice and then subjected to a 2-D-Clean-Up kit (GE Healthcare). The final pellet was re-suspended with rehydrating buffer and insoluble materials were removed by centrifuging at 12,000 g for 5 min. The supernatant was loaded on to a 7cm pH 3-10 strip gel (Bio-Rad). Focusing was performed with IPGphor (Bio-Rad) at 50 V for 10 h, 500 V for 1 h, 1 000 V for 1 h, 8,000 V for 5 h. The strip was then equilibrated with equilibration buffer A (Tris-HCl pH 8.8, urea, SDS, glycerol, bromophenol blue, DTT) for 15 min, and equilibration buffer B (Tris-HCl pH 8.8, urea, SDS, glycerol, bromophenol blue, iodoacetamide) for 15 min. The equilibrated strip was run in the second dimension with a pre-made SDS-PAGE gradient gel (Bio-Rad) at 135 V. Proteins in the SDS-PAGE gel was transferred to a nitrocellulose membrane at a constant 100 mA overnight at 4°C.
The membrane was blocked with blocking buffer (5% non-fat milk powder in PBS 0.1% Tween [pH 6.0]) at room temperature for 1 h. The membrane was then incubated in 10 mL blocking buffer at room temperature for 45 min, with 1) IAPV virions (99.9% IAPV; IAPV 8.4×10^{12} genome equivalents), or 2) IAPV inoculum (IAPV 1×10^{9} genome equivalents), or 3) IAPV-VLP purified in a sucrose cushion. The membrane was washed 2 times with rocking with blocking buffer for 5 min each at room temperature before incubation with rabbit anti-VP1 (1:100; Carrillo-Tripp et al 2016) in 10 mL blocking buffer at room temperature for 1 h. The membrane was washed 2 times as described above and then incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:5,000) in 10 mL blocking buffer at room temperature for 1 h. The membrane used as a no-ligand control was run in parallel. Membranes were incubated in HyGlo Chemiluminescent HRP detection reagent (Thermo Fisher Scientific) for 1 min, with luminescence detected on X-ray film according to standard procedures. The experiments were repeated multiple times for each ligand to ensure consistency of results across at least three replicates.

**Protein identification**

Proteins consistently bound by ligand in far western blots from at least three replicates were isolated from Coomassie blue-stained gels. Proteins were isolated with reference to probed membranes and sent to the Iowa State University Protein Facility for identification using LC-MS/MS (Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer [Thermo Fisher Scientific]). Amino acid sequence data were analyzed using Thermo Fisher Scientific's Proteome Discoverer Software and peak lists were
generated by Mascot or Sequest HT (or both) against the honey bee genome (*Apis mellifera* Amel_4.5). Candidate receptor proteins were characterized using Myhits for identification of functional motifs (http://myhits.isb-sib.ch), PSORT II (http://www.psort.org/) for predicted subcellular locations, SignalP v4.1 Server for endoplasmic reticulum (ER) secretion signals (http://www.cbs.dtu.dk/services/SignalP/), TMHMM v2.0 for transmembrane domains (http://www.cbs.dtu.dk/services/TMHMM/), NetNGlyc and NetOGlyc for glycosylation sites (http://www.cbs.dtu.dk/services/NetNGlyc/ and http://www.cbs.dtu.dk/services/NetOGlyc/) (Zhang et al, 2013).

**Results**

**Enrichment of honey bee midgut proteins**

Honey bee brush border membrane vesicles (BBMV) were prepared from honey bee midguts using the Wolfersberger method (with minor alterations; Cioff & Wolfersberger 1983; Linz et al 2015), which had previously been applied to lepidopterans, dipterans, and hemipterans. The specific activity of the marker enzyme, aminopeptidase, was increased approximately 2-fold in the honey bee BBMV preparation relative to the midgut homogenate. BBMV proteins of around 37-kDa, 20-kDa and lower were enriched compared to the homogenate (Fig S1).
IAPV-injected pupae yield high levels of IAPV particles

Managed apiaries often harbor moderate background levels of viruses, which vary from time to time and from region to region. RT-qPCR results showed that pupae from 4 out of 6 colonies yielded >99.8% IAPV with <0.1% other honey bee viruses in 3 of these colonies. One additional colony yielded 97.8% IAPV with 2.2% DWV. Pupae from the sixth colony yielded 49.69% IAPV and 50.31% DWV. The IAPV stock used in the present study for far-western blotting consisted of IAPV virions (99.9%) with an IAPV titer of 4.2×10^{12} genome equivalents (g.e.)/µL and IAPV virions (95.6%), which was used as inoculum for the pupal injection (see Materials and Methods) with IAPV titration 2.44×10^{7} g.e./100 ng RNA (Carrillo-Tripp et al, 2016).

Baculovirus-expressed IAPV virus-like particles

Western blotting of sucrose gradient fractions showed VLPs were concentrated in the 40% to 55% sucrose fractions (Fig. 1), similar to previous results (Ren et al 2014).

![Figure 1 Baculovirus-expressed IAPV-VLP detected by western blot. IAPV-VLP enriched from a 30% sucrose cushion was applied to a 30 - 60% sucrose gradient (5% increment increase; 60% fraction not shown). IAPV virions used as a positive control are shown on the right. Particles were detected in the 40 – 50% sucrose fractions with anti-VP1 antiserum (25 kDa).](image-url)
IAPV bound to multiple BBMV proteins

Silver-stained two-dimensional (2-D) SDS-PAGE showed several major protein clusters around pH 5-7 (Fig. 2), which is consistent with the acidic environment of the honey bee midgut (Pohorecka 2004). To identify midgut membrane proteins that interact with IAPV, BBMV proteins prepared from honey bee midguts were separated on a 2-D ligand blot and overlaid with 1) IAPV virions (either 99.9% or 95.6%), 2) IAPV-VLP, or 3) antiserum only. IAPV virions (either 99.9% or 95.6%) bound three BBMV proteins, 37-, 32-, and 60-kDa, and the 37-kDa protein showed the strongest binding (Fig. 2). The most intensely bound spot was around 37-kDa, the no-ligand control blot showed weak binding to a protein of around 32-kDa (Fig. 2). The protein isoelectric point (pI) of these three proteins clustered around pH 5 – 6 (Fig. 2). While 3 proteins were identified in blots incubated with IAPV virions, only 1 protein was identified in blots incubated with IAPV-VLP (Fig. 2), which aligned with the 32-kDa protein corresponding to the blots incubated with IAPV virions (Fig. 2). Non-specific binding appeared at the edges of the IAPV virion (99.9%) blot.
Aspartic protease *Apis mellifera* as a putative receptor for IAPV

The three aforementioned spots were isolated from a reference Coomassie blue-stained gel and sent for LC-MS/MS analysis (Tbl. S1). Both the 37-kDa protein and the 32-kDa protein had high similarity to lysosomal aspartic protease *Apis mellifera* with coverage scores of 51.27% and 41.03% respectively. Other proteins identified by LC-MS/MS were ruled out because of low identities (Tbl. S1). The 60-kDa protein sequence had similarity to 60-kDa heat shock protein *Apis mellifera* with a coverage score of 54.21%. Lysosomal aspartic protease (accession number XP_392857.2, heretofore
referred to as aspartic protease), despite its previous naming as lysosomal, was predicted by Protein Subcellular Localization Prediction Tool (PSORT II) to be localized as (probability of) 66.7% extracellular, 22.2% vacuolar, and 11.1% mitochondrial. The amino acid sequence possessed an aspartyl peptidase domain, as the name suggests. We found that the probability of a transmembrane helix was low (p < 0.2). The protein had 1 potential (0.7008, threshold 0.5; Jury agreement 9/9) N-linked glycosylation in the position 120NGTD, and 3 potential O-linked glycosylation sites in strand 112, 114, and 115. These 3 characteristics together indicate that the aspartic protease [Apis mellifera] has a glycophasphatidylinisitol (GPI) anchor. Other candidate proteins and adjacent proteins identified were unlikely to function as receptors because they lacked characteristics of extracellular proteins (Tbl. S2).

Our investigations cast doubt on the lysosomal designation of lysosomal aspartic protease [Apis mellifera] (assembly accession GCF_000002195.4). BLAST analysis of this protein sequence against all related species, resulted in hits to lysosomal aspartic proteases from other species with high identity. However, aspartic protease [Apis mellifera] has additional sequence at the N-terminus (Fig. 3A). Based on analysis with SignalP v4.0, this additional N-terminal sequence has an endoplasmic reticulum (ER) secretion signal. This result indicates that the aspartic protease [Apis mellifera] is unlikely to be localized in lysosomes (Fig. 3B). This result also lends confidence to the possibility that this aspartic protease could be involved in receptor-mediated activity, rather than intracellular lysosomal functions.
Figure 3 (A) N-terminal amino acid alignment of aspartic protease \textit{Apis mellifera} with lysosomal aspartic proteases from related species, \textit{Habropoda laboriosa} and \textit{Eufriesea mexicana}, showing the aspartic protease \textit{Apis mellifera} has an additional N-terminal sequence. The aspartic proteases from both species have high similarity to aspartic protease \textit{Apis mellifera} (82% and 78%, respectively). (B) Secretion signal plots of the corresponding species. The additional N-terminal sequence of the aspartic protease \textit{Apis mellifera} appears to be the endoplasmic reticulum (ER) secretion signal (upper left), which is absent in the other two lysosomal aspartic proteases of \textit{H. laboriosa} (upper right) and \textit{E. mexicana} (lower left). This result indicates the aspartic protease \textit{Apis mellifera} is not lysosomal. S-score: signal peptide score, which shows the position of signal peptides within the mature part of the protein. C-score: raw cleavage site score, trained to be high immediately after the signal peptide. Y-score: combined cleavage site score, a combination of Y-, C-, and S-score provides a better cleavage site prediction than a raw C-score. Only the combination of a sharp S-score slope and a high C-score indicates a real cleavage site, as indicated by the Y-score.

**Discussion**

IAPV in the family Dicistroviridae, is presumed to enter host cells during infection using a receptor-mediated endocytosis pathway, which has been described in
Picornaviridae, a sister group of Dicistroviridae, and as described for Drosophila C virus. Our results show that IAPV virions were able to bind the honey bee midgut protein aspartic protease in vitro, indicating an affinity between the virions and this honey bee midgut protein. The post-translationally modified aspartic protease is predicted to be a gut surface protein because it has features characteristic of GPI-anchored proteins. GPI-anchored proteins have previously been reported to be insect gut receptors exploited by Bacillus thuringiensis toxins (Fernandez et al 2006; Simpson RM, Newcomb 2000) and are also utilized by some plant viruses in arthropod-mediated viral transmission (Liu et al 2010; Linz et al 2015).

The roles of secreted aspartic proteases in host-pathogen interactions

Aspartic proteases are a family of proteases characterized by one or more aspartate residues used for catalysis of their substrates (Tang & Wong 1987). Originally, these proteases were known to function solely in executing proteolysis within the acidic environment of cellular lysosomes (Blum et al, 1991; reviewed in Müller et al, 2012). However, accumulating evidence shows more specific and regulatory functions of these proteases that are associated with many cellular processes when pathogens are encountered. Over-expression of an extracellular aspartic protease (47-kDa CDR1) in Arabidopsis can provide disease resistance, indicating it might be part of the basal host defense complex (Xia et al 2004). Cathepsin L, in the aspartic protease family, was found to participate in the proteolytic processing of paramyxovirus fusion proteins, converting the precursor protein F0 to the active F1 + F2 disulfide-linked heterodimer (Pager & Dutch, 2005).
GPI-anchored secreted aspartic proteases were studied in fungi, especially in *Candida* spp. In human cells, the virulent yeast *C. albicans* uses its GPI-anchored secreted aspartic proteases to degrade host tissue barriers, destroy host defending molecules, and digest nutrition supplies. These contribute to different host niches (Lermann & Morschhaüser, 2008). In our case, the putative receptor for IAPV could be a secreted protein tethered by a GPI anchor, but more experiments are needed to confirm functionality.

**Aspartic protease [Apis mellifera] as a putative receptor for IAPV**

A weak cross-reaction was detected between the anti-VP1 antiserum and the putative receptor, aspartic protease [*Apis mellifera*] (Fig. 2). This suggests a weak affinity between the molecule and the antiserum raised to a partial IAPV VP1 sequence. Integration of partial IAPV sequences into the honey bee genome has been detected (Maori et al. 2007b). The integrated sequence was within the C-terminus of the polyprotein, corresponding to the VP1 region (Maori et al. 2007b). The authors demonstrated that the integrated sequence was expressed and the product was detected by western blot (Maori et al. 2007b). Further experiments are needed to explain whether the cross reaction observed in our experiments was caused by the integration and expression of VP1 sequences from the honey bee genome. The aspartic protease is predicted to be 42 kDa, while VP1 is 25 kDa.

Whether this aspartic protease [*Apis mellifera*] is a functional receptor for IAPV entry requires further study. Cell lines that are not susceptible to IAPV and related dicistroviruses such as KBV and ABPV (e.g. Sf9 originating from *Spodoptera frugiperda*)
ovarian tissue) can be used for baculovirus expression of the recombinant aspartic protease \([Apis mellifera]\). Labeled IAPV virions will be observed within the cell if the recombinant aspartic protease \([Apis mellifera]\) functions sufficiently (without additional bee factors) to mediate endocytosis of IAPV virions. The presence of the GPI anchor of the aspartic protease can also be tested by lipid raft enrichment and a PI-PLC treatment followed by western blot against this protein in both fractions. A competition assay between IAPV virions and other aspartic-protease \([Apis mellifera]\)-binding peptides in vivo can also assist in understanding the function of this protein and address the specificity of IAPV binding in bee-feeding experiments (as described by Liu et al 2010). Because the virus-receptor binding is transient, the time intervals between competitors and IAPV virions should be taken into account for in vivo competition assays.

**KBV and ABPV may use the same receptor**

Far-western blotting used in the present study relies on an antiserum that recognizes a partial VP1 sequence common to three dicistroviruses. Here, the primary antibody used in the 2-D ligand blot was raised to a conserved amino acid sequence of VP1 of KBV, IAPV, and ABPV (GGRRYKFFNTTPLK) (Carrillo-Tripp et al 2016). IAPV virions used as a ligand were obtained by IAPV-rich inoculum injection of a cohort of pupae. Although we confirmed a proportion of 99.9% IAPV, with KBV and ABPV less than 0.01%, there is still a possibility that the binding signal did not result exclusively from interaction between IAPV and its putative receptor. To address this issue, we used baculovirus-expressed IAPV VLP, and showed that these VLP also bound the aspartic protease (Fig 2). IAPV individual structural proteins can also facilitate receptor-
identification, but we were unable to obtain sufficient amounts for use of individual structural proteins in ligand blot experiments (Appendix).

KBV and ABPV are phylogenetically closely related to IAPV based on their RdRp sequences (Reddy et al. 2014). Sequence analysis revealed that IAPV differed from the two mainly in the 5’ un-translated region (UTR). KBV and ABPV also have low identity in the 5’ UTR (42%) to each other (De Miranda et al. 2004). However, the sequences of the structural polyproteins of IAPV, KBV, and ABPV are highly conserved. Because the amino acid sequences in receptor-ligands are often conserved (Stuart et al. 2002), in this regard, IAPV, KBV, and ABPV may share the same midgut receptor for their entry due to the presence of conserved receptor-binding sequences. In vitro experiments showed that when a honey bee cell line (AmE-711) was transfected with a virus mixture (SBV, IAPV, DWV, and BQCV, but predominantly SBV), IAPV took over rapidly in the first 36 h post-transfection (Carrillo-Tripp et al. 2016). When KBV was present in a virus mixture of KBV-SBV-IAPV-BQCV-DWV (KBV-predominant), KBV outcompeted IAPV in cell infection at 24 hpi (Carrillo-Tripp et al. 2016). Given the high similarity between IAPV and KBV, receptor-based antiviral strategies may help mitigate the impact of IAPV, KBV and ABPV.

**Viral infection in honey bees is dynamic and facilitated by multiple stressors**

Viral infection dynamics are complex resulting from highly variable background pathogen levels in samples from the field and from colonies. The production of IAPV virions by pupal injection in the present study showed that even though pupae come from the same colony but were obtained on different dates, the final IAPV composition
after IAPV inoculum injection was variable. Pupae obtained from a bee colony in late October, 2016, yielded half DWV and half IAPV when they were subjected to the same IAPV inoculum (present study). Pupae from the same bee colony collected in early November gave 99.9% IAPV. Carrillo-Tripp et al (2016) injected caged bees (harboring DWV, IAPV, BQCV, and >50% SBV) with an SBV-rich viral mixture (97.94% SBV, IAPV+DWV+BQCV<2%), but IAPV titers exceeded all others by 36 h post-injection (Carrillo-Tripp et al 2016).

IAPV specific dsRNA has the potential to protect honey bees from IAPV infection in both small-scale lab experiments (Maori et al 2009) and large-scale colony experiments (Hunter et al 2010). This treatment is based on the RNAi-based defenses of the honey bee, which have been described in cell lines and in whole insects (Carrillo-Tripp et al, 2016). However, honey bees are challenged with many stressors such as commercial pesticides and Varroa mites (Whitehorn et al 2012; Henry et al 2012; Di Prisco et al 2011; Yang & Cox-Foster 2007), therefore, the effects of RNAi based viral defenses may be reduced in the long run as a result of stress. Our study allows for another possibility that IAPV infection may be blocked by receptor-binding peptides or proteins, which may serve as a new therapy for viral disease of honey bees.

Future directions

The cell surface receptors for a particular virus determine the tropism and pathogenesis of the virus for a given host (Evans & Almond, 1998). IAPV is not only transmitted orally but also by the Varroa mite that feeds on hemolymph. As IAPV replicates in other non-digestive tissues (Chen et al 2014), identifying IAPV receptors
from other tissues apart from the midgut may help toward disruption of Varroa-associated IAPV spread within colonies.

With increasing concern over the global decline in non-Apis pollinators, the identification of specific virus receptors in honey bees will help us to assess potential for interspecies pathogen transmission originating from apiaries to the pollinator community. Due to their close phylogenetic relationship to honey bees, bumble bees (Bombus spp.) are likely to be among the most vulnerable of wild bee species to honey bee disease. Several honey bee viruses are infectious to bumble bees, including DWV (Genersch et al 2006), BQCV (Peng et al 2011), the KBV/ABPV/IAPV complex (Meeus et al 2014a) and several others (McMahon et al 2015; Cox-Foster et al 2007; Dolezal et al 2016). Viruses that infect both the honey bee and the bumble bee may use receptor molecules that are conserved in honey bees and bumble bees.

Honey bees are generalists that visit a variety of flowers regardless of whether specific flower species are suitable for them to pollinate (Winston 1991). Therefore, because most aparaviruses are transmitted per os, it is important to consider the potential pathogen-spillover caused by shared floral resources between managed honey bees and wild bee species. Indeed, some honey bee viruses have been detected in species outside of the family Apidae (Levitt et al, 2013, Dolezal et al 2016). Alfalfa leaf cutting bees (Megachilidae) were shown to carry SBV and, less frequently, ABPV, and co-infection of the two viruses lowered reproductive capacity (Melathopoulos et al 2017). This reemphasizes the role played by the generalist honey bee in pathogen-spillover among pollinators.
Identification of the putative IAPV receptor in the present study may help to mitigate honey bee colony losses by using receptor antagonists that compete with IAPV virions for the aspartic protease \textit{Apis mellifera}. This competition would block the binding of IAPV to its receptor and prevent viral infection. A similar strategy was used to block entry of Pea enation mosaic virus (PEMV) (Liu et al 2010), human rotavirus (Civra et al 2015) and Epstein-Barr virus (EBV) (by use of neutralizing antibodies (Kanekiyo et al 2015)). In the Human rotavirus study, a 20-aa peptide derived from the competitor, Human milk lacadherin, interacts directly with the cellular receptor $\alpha 2\beta 1$ integrin, hindering the rotavirus attachment process (Civra et al 2015). Peptides that potentially interact with the putative receptor can be isolated by screening a Phage Display library as described previously (Liu et al 2010). Given that KBV, ABPV, and IAPV share highly conserved structural protein sequences, the receptor antagonist could have a broad impact to block viruses within the KBV/ABPV/IAPV complex.

Two \textit{Varroa}-pathogenic viruses that do not infect honey bees have been described (Levin et al 2016), that could provide novel tools for mite control. Given that IAPV infects bees both orally and via the \textit{Varroa} mite, a receptor blocking therapy used in conjunction with mite control strategies could mitigate colony losses in honey bees mediated by multiple viruses.

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Supplementary Data

Figure S1 One-dimensional gel electrophoresis profile of honey bee BBMV and homogenate. Lanes were loaded with different protein amounts as indicated. The position of Mr markers is shown.
Figure S2 Coomassie-stained BBMV (left panel) and silver-stained BBMV (right panel). Numbers in yellow indicate isolation of adjacent BBMV-bound proteins for LC-MS/MS. Numbers in red indicate BBMV-bound proteins identified by 2-D ligand blots (details see Result in Chapter 2).
Table S1 BBMV proteins bound by IAPV ligands and identified by LC-MS/MS.

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<tr>
<th>Position*</th>
<th>Accession</th>
<th>Description</th>
<th>Coverage (5% cut off)</th>
<th>Molecular mass</th>
<th>Calculated isoelectric point</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>XP_392899.2</td>
<td>PREDICTED: 60 kDa heat shock protein, mitochondrial-like [Apis mellifera]</td>
<td>54.21</td>
<td>60.4</td>
<td>5.83</td>
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<td>1A</td>
<td>NP_001172075.1</td>
<td>actin related protein 1 [Apis mellifera]</td>
<td>9.04-10.90</td>
<td>41.8</td>
<td>5.48</td>
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<tr>
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<td>XP_006564892.1</td>
<td>PREDICTED: ATP synthase subunit beta, mitochondrial [Apis mellifera]</td>
<td>63.37</td>
<td>55.1</td>
<td>5.41</td>
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<tr>
<td>1</td>
<td>XP_006572722.1</td>
<td>PREDICTED: putative 2-hydroxyacid dehydrogenase HI_1556 [Apis mellifera]</td>
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<td>34.3</td>
<td>6.64</td>
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<tr>
<td>2</td>
<td>XP_392857.2</td>
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<td>6.32</td>
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<tr>
<td>2</td>
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<td>PREDICTED: NADH dehydrogenase [ubiquinone] flavoprotein 1, mitochondrial [Apis mellifera]</td>
<td>15.53</td>
<td>52.6</td>
<td>8.34</td>
</tr>
<tr>
<td>2</td>
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<td>PREDICTED: 14-3-3 protein zeta isoform X3 [Apis mellifera]</td>
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<td>28.1</td>
<td>4.98</td>
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<tr>
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<td>PREDICTED: arginine kinase isoform X1 [Apis mellifera]</td>
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<td>pyruvate dehydrogenase E1 component subunit beta, mitochondrial [Apis mellifera]</td>
<td>9.15</td>
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</table>

* Position refers to Fig. 2 in Chapter 2 and Fig. S2. Adjacent proteins were listed as “A”
Table S2 Candidate receptors and their features

<table>
<thead>
<tr>
<th>Description</th>
<th>N-terminal secretion signal</th>
<th>Glycosylation signals</th>
<th>Transmembrane domain</th>
<th>Subcellular location (% probability)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PREDICTED: lysosomal aspartic protease [Apis mellifera]</td>
<td>+</td>
<td>N + O +</td>
<td>-</td>
<td>66.6% EC 22.2% VA 11.1% MI</td>
</tr>
<tr>
<td>PREDICTED: 60 kDa heat shock protein, mitochondrial-like [Apis mellifera]</td>
<td>-</td>
<td>N + O +</td>
<td>-</td>
<td>52.2% CP 17.4% CK 17.4% NC 4.3% VC 4.3% PO 4.3% EC</td>
</tr>
<tr>
<td>actin related protein 1 [Apis mellifera]</td>
<td>-</td>
<td>N + O -</td>
<td>-</td>
<td>44.4% CK 33.3% CP 22.2% NC</td>
</tr>
<tr>
<td>pyruvate dehydrogenase E1 component subunit beta, mitochondrial [Apis mellifera]</td>
<td>-</td>
<td>N + O +</td>
<td>-</td>
<td>78.3% MI 8.7% CP 8.7% NC 4.3% VC or secretory system</td>
</tr>
<tr>
<td>PREDICTED: 14-3-3 protein zeta isoform X3 [Apis mellifera]</td>
<td>-</td>
<td>N + O +</td>
<td>-</td>
<td>39.1% NC 34.8% CP 13.0% MI 4.3% CK 4.3% vesicles of secretory system</td>
</tr>
<tr>
<td>PREDICTED: arginine kinase isoform X1 [Apis mellifera]</td>
<td>-</td>
<td>N + O +</td>
<td>-</td>
<td>60.9% CP 17.4% MI 13.0% NC 8.7% VA</td>
</tr>
</tbody>
</table>

Abbreviations: EC, extracellular; VA, vacuolar; MI, mitochondrial; CK, cytoskeletal; CP, cytoplasmic; PO, peroxisomal; NC, nuclear; GO, golgi. +, present; - not present.
CHAPTER 3. CONCLUSIONS AND DISCUSSION

Conclusions

In summary, in this thesis, we identified a putative receptor protein for the honey bee virus-of-concern, Israeli acute paralysis virus (IAPV). To do this, we generated an immunoblot for honey bee midgut brush border membrane vesicles (BBMV), on the basis that this tissue is likely a primary site for Israeli acute paralysis virus (IAPV) entry. Honey bee brush border membrane protein vesicles were enriched and proteins then separated by 2-dimensional electrophoresis. Using IAPV virions and IAPV virus-like particles (VLPs) as ligands, both showed high affinity for a midgut protein, aspartic protease \[ \text{Apis mellifera} \], which was weakly recognized by the anti-VP1 antibody. This protein has an additional N-terminal sequence when aligned with aspartic proteases with high identities from other species. This sequence is predicted to have an endoplasmic reticulum (ER) secretion signal. The protein also bears an N-linked (attachment of oligosaccharides through a nitrogen atom on an amino acid residue) and three O-linked (through oxygen atom) glycosylation sites, indicating that this protein is secreted rather than being intracellular. In addition, this protein is not predicted to contain a transmembrane domain. Based on these findings, this protein has the aspartic protease motif and is predicted to be post-translationally modified when mature, is glycosylphosphatidylinositol (GPI) anchored on the surface of the midgut cell, and hypothesized to bind to the IAPV virion. Although our data present compelling evidence for a putative receptor, this hypothesis needs to be tested with further experiments. For example, if this aspartic protease indeed functions as a virus receptor, a non-IAPV-susceptible cell line infected with a baculovirus vector expressing the receptor protein...
on the cell surface should allow IAPV virions to enter these cells. Future experiments should aim to test functionality in this way to lend more direct support for the functionality of aspartic protease as a virus receptor.

Two-dimensional far-western blotting facilitates receptor protein identification by presenting a pool of midgut membrane-bound vesicle proteins to the ligand. This enrichment of brush border membrane receptor proteins is critical for identification of receptors for viruses or toxins in the gut. Similar strategies have been applied in the successful identification of midgut receptors of many *Bacillus thuringiensis* (Bt) toxins in mosquitoes (Chen et al 2007) and in Lepidoptera (Spodoptera spp.; Knight et al 1994), and plant virus receptors in aphids (Bandla et al 1998; Liu et al 2010). In another example, Tomato spotted wilt virus (TSWV) bound to a 94-kDa aphid protein but evidence that the protein was a gut receptor for the first attachment of TSWV was lacking (Kikkert et al 1998). To maximize the chances that a gut receptor protein is identified, high quality BBMV is a prerequisite —a series of washes and precipitation processes should not reduce the enrichment of the vesicles, which will be tested by marker enzyme activities in final BBMV products. In addition, transient interaction between viruses and gut proteins may be affected by several wash steps in far-western blotting and by ligand incubation time; we thus reduced the detergent concentration to 1% and the incubation time from overnight to 1 hour, compared to the method used by Linz et al (2015). We also adjusted pH and salt concentration to mimic the natural gut environment in bees (Pohorecka 2004).
Receptor-mediated endocytosis has been exploited for diverse disease-control strategies

A valuable approach in anti-viral therapy development is the production of antibodies that protect hosts from virus challenge via receptor competition. Optimally designed compounds that compete with viral receptors upon attachment have been applied in influenza virus antiviral therapies. Monoclonal antibodies raised against the virus surface glycoprotein hemagglutinin (HA) can neutralize virus infection (Simmons et al 2007). Specifically, human immunoglobulin A (IgA) was shown to reduce viral titer in vitro by binding to newly synthesized virus particles via the HA glycoprotein within epithelial cells (Mazanec et al 1995), indicating that the antiviral therapies are not limited to the cell surface. It is worth noting that compounds that bind to the receptor or the virus itself are equally valuable. The synthesized compound polyacrylamide-based sialylglycopolymer PAA-YDS contains sialic acid, which is the cell receptor for influenza virus, and can inhibit viral infection resulting in increased survival of mice (Gambaryan et al 2002).

Knowledge of virus receptor-binding has potential value for controlling or mitigating honey bee virus-mediated colony losses. For example, a receptor-competing peptide, GBP3.1, isolated using a Phage Display Library screen, was shown to significantly reduce Pea enation mosaic virus (PEMV) acquisition in pea aphids (Liu et al 2010), thus potentially disrupting PEMV transmission in the field. This peptide binds to the alanyl aminopeptidase N (APN), which is a midgut cellular receptor for PEMV entry, according to far-western blot and in vivo results (Linz et al 2015). This reduced viral acquisition is effective within a specific time period (Liu et al 2010). These findings
indicate that if a functional IAPV receptor is identified for honey bees, competing molecules could be administered to honey bee colonies to reduce uptake of IAPV, and related viruses, and combat the negative impacts of virus infection on bee health.

One important consideration for the use of receptor-competing molecules as anti-viral therapy for honey bees is temperature. Extended exposure of virions to 37°C and above can induce the changes in viral protein structure interfering with receptor-binding (McDermott et al 2000). For example, at high temperatures poliovirus virion structures are altered, leading to failure to bind to cells (Li et al 1994). IAPV virions also undergo physical changes induced by high temperature (Mullapudi et al 2017), but it is not yet known whether receptor-binding of IAPV is affected by this change. However, it is intriguing that colony IAPV loads are often lower during the hottest months where bees are exposed to over 37°C (June - August) of the year (Chen et al 2014). Thus, it may be important to consider temperature when using anti-viral therapies that involve receptor-competing molecules, which may be more effectively applied during cooler times, e.g. late summer through winter.

To sum up, IAPV, a member of the family Discistroviridae, is presumed to enter honey bee gut cells via receptor-mediated endocytosis and we have identified aspartic protease as a putative receptor to mediate virus entry. Future research may focus on antiviral therapies targeting the binding and endocytosis of IAPV.
Future research

Our current understanding of receptor-mediated endocytosis has encouraged a body of research in disease-control and in agricultural pest control, as briefly discussed above. These achievements provide insights into virus-mediated colony loss in honey bees. In Chapter 2, we identified a putative receptor for IAPV from honey bee gut epithelial cells using far-western blotting. However, whether this candidate protein is the functional receptor for IAPV still needs to be examined. We suggest that it would be fruitful for future research to include (but not be limited to) the following areas of investigation, which can further substantiate whether the putative receptor is indeed an IAPV receptor, as well as move further towards using this information to develop anti-viral therapies for honey bees.

In vitro demonstration of receptor function

We have identified a putative receptor for IAPV, and the sequence for this protein can be obtained from the GenBank honey bee genome database (accession number GCA_000002195.1). As an important immediate next step, Sf9 cells, known to have no susceptibility to IAPV or other bee viruses, will be infected with a baculovirus vector for expression of the putative receptor on the cell surface. Then, a baculovirus-expressed IAPV-VLP with a fluorescent protein will be added to the cells. IAPV internalization and cell permeability will be visualized under a fluorescence microscope if the aspartic protease alone is sufficient for IAPV entry. Besides fluorescence visualization, another approach is to add IAPV virions (see Production of IAPV virions in Chapter 2). After 1-hour of incubation followed by several washing steps, RNA will be extracted from Sf9
cells and compared to the initial viral RNA inoculum. An increase of IAPV RNA levels in the cells would support the function of the putative receptor. A third approach is to use a VP1-GFP fusion protein to demonstrate receptor-mediated entry into the Sf9 cells, similar to the work of Linz et al. (2015)

**Screening for receptor-binding peptides that interfere with virus-receptor binding**

Ligands, such as viruses and receptor-bound molecules, bind to receptors until receptor saturation (Latek et al. 2009). Therefore, competition for the receptors between virions and receptor-binding peptides has the potential to block the attachment of the virus and thus disrupt virus infection. A phage display library will be used to select receptor-binding peptides. After several rounds of feeding honey bees on the phage display library and eluting bound phage from the dissected gut tissue, bound phages from each round of enrichment will be amplified in *E. coli* and the encoded peptide sequences determined. These peptides will be tested for their ability to bind to the IAPV receptor and for potential to compete with IAPV for receptor binding.

**Identifying the IAPV capsid motif(s) that interact with the receptor**

The IAPV capsid motif(s) that directly interact with the receptor are expected to be conserved (as discussed in Chapter 1) among IAPV-related viruses. Identification of IAPV receptor-interacting motifs will provide insight for research focusing on KBV and ABPV. Thus, the next step would be identifying the capsid motif(s) of IAPV that interacts with the gut receptor. Capsid protein sequences synthesized with a tag, would be screened for binding to the recombinant, baculovirus-expressed receptor. Bound capsid
protein sequences would be detected by immunoassay, with affinity assessed by immunoblotting. Baculovirus vectors expressing the IAPV-VLP can be genetically-engineered with these motifs mutated, to test with mutation of candidate binding sequences prevents receptor binding for further confirmation.

References


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APPENDIX. EXPRESSION OF INDIVIDUAL IAPV STRUCTURAL PROTEINS IN E. COLI

Introduction

In the present study, we attempted to express individual IAPV viral proteins (VP) using *E. coli*-expression for use in ligand blots to understand their interaction with honey bee midgut proteins. However, as the amounts of recombinant capsid proteins were insufficient for use in ligand blot experiments, details of the work are provided here for future reference.

Virus particles enclose viral genomes to protect them from the environment and also mediate virus entry into host cells, introducing the viral genome into the host cell (Rossmann & Johnson 1989). Because viral genomes are small, the virus has to use a limited number of proteins to form a three-dimensional coat (Crick & Watson 1957). This requires every subunit to occupy a position in a regular manner (Crick & Watson 1957).

The role of the virus particles in binding to a host cell is mediated by capsid protein(s) that interact directly with host cellular receptors (Haywood 1994). According to different patterns of viral structure, some capsid subunits localize to the surface of the virion for host cell binding while others localize inside the virion to interact with the viral genome (Crick & Watson 1957). It is predicted that the spikes on the IAPV virion, which are formed by the C-terminal VP1 and the CD loop of VP3, interact with cell receptors (Mullapudi et al 2017).
**Materials and Methods**

**Plasmids, enzymes, and strains**

Commercial plasmids used to express individual viral proteins were pBAD/His B (Thermo Fisher Scientific), pMAL-c2X (NEB), and pGex-4T1 (GE Healthcare). Competent cells JM109 and BL21 were prepared by Mix&Go kit following the manufacturer’s instructions (Zymo research). Restriction enzymes SacI, XhoI, BamHI, EcoRI, PstI and their related buffers were from Promega. The fluorescent protein iLOV (Chapman et al 2008) in a plasmid pGex-4T1 was obtained from Addgene. Rabbit anti-iLOV was kindly provided by Dr. Christie Faulkner (University of Edinburgh, United Kingdom). Individual viral protein sequences were amplified from an IAPV construct pJAZZ-IJJ5 (Carrillo-Tripp et al 2016).

**Cloning of IAPV individual viral structural capsid proteins**

Because IAPV ORF 2 encodes a polyprotein upon translation, and each individual viral protein is produced on cleavage by the viral protease, we referred to published genomic sequences of related dicistroviruses (de Miranda et al 2004; Leat et al 2000; Maori et al 2007a; Ren et al 2014; Govan et al 2000) to identify potential cleavage sites within ORF 2. Then, primers for VP1, VP2-4, and VP3 were designed based on the IAPV genome sequence (Maori et al 2007; Figure 1). To facilitate expression, VPs were fused to the green fluorescent protein iLOV by extension PCR using a proline-rich linker (PP) as a bridge (Tbl. 1). Briefly, the sequence of PP plus partial iLOV sequence were added to the reverse primer of each VP. This long primer and a regular forward primer of each VP were used to amplify each VP from the
construct IJJ5. The long forward primer containing partial PP and a regular reverse primer of iLOV were used to amplify iLOV from the pGex-iLOV. Thus, the overlapping region contained the partial PP and partial iLOV. PCR products were checked by agarose gel (1%) in TAE electrophoresis and DNAs were purified from the gels by Gel Purification Kit (Qiagen). Finally, the PCR products of iLOV and VP were mixed in a single PCR tube to generate a fusion product using a desirable annealing temperature and extension time.

![Diagram of structural proteins]

Figure 1 Construct IJJ5 showing positions of IAPV individual viral protein clones. Numbers show the position of cleavage sites in the construct IJJ5 (Maori et al. 2007a; Ren et al. 2014; Carrillo-Tripp, unpublished)

After double digestion of pBAD/His B vectors (pBAD/His B, pGex-4T1, or pMAL-c2X) and the fusion products, each VP-PP-iLOV (VP-iLOV for short) was ligated in a 10 μL volume (1 u ligase (Thermo Fisher Scientific), 1 μL 10x ligase buffer, vector 50 ng, fragment added to 10 μL) at 4°C overnight. SacI + EcoRI, XhoI + EcoRI, and SacI + EcoRI were used for VP1-, VP2-, VP3-iLOV cloning, respectively.

In case pBAD/His B would not give the desired expression, other vectors were also tried. VP1 was cloned onto pMAL-c2X using EcoRI and Psfl. VP3 was cloned into pGex-4T1 using BamHI and EcoRI. Competent cells JM109 were transformed...
with the positive plasmids. Positive colonies were confirmed by colony PCR, double
digestion, and sequencing.

<table>
<thead>
<tr>
<th>Cleavage site</th>
<th>Primers</th>
<th>Length of nucleotides (bp)</th>
<th>Predicted MW (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VP2-4</td>
<td>F: ATGGATACGGGTAATAAAGAAACTAACGATCC R: GTGGCATCTATTTTTTGATGG GGGGACGACGCTCCCCCGTCACCAGGGGCT GATCCGGGGCCCAACCACCACCACCCCTCCAC CCCCCAATCTCCACTCCGGTAGGAATAGAGA</td>
<td>1197</td>
<td>44</td>
</tr>
<tr>
<td>VP3</td>
<td>F: GGATCTGAACAAGCTCA R: GTCTTAATGTAGAACTTCAA GGGGACGACGCTCCCCCGTCACCAGGGGCT GATCCGGGGCCCAACCACCACCACCCCTCCAC CCCCCAATCTCCACTCCGGTAGGAATAGAGA</td>
<td>903</td>
<td>33</td>
</tr>
<tr>
<td>VP1</td>
<td>F: GATAAACACCACCTGACTTACAAC R: CAAACATGCTATATAAAGCGGGGACGACGCTCCCCCGTCACCAGGGGCTGATCCGGGGCCCAACCACCACCACCCCTCCAC CACTCCGGTAGGAATAGAGA</td>
<td>336</td>
<td>12</td>
</tr>
<tr>
<td>iLOV</td>
<td>F: AGTCCCACTCCCCGTAGGAATAGAGAAGAATTT CGTCATCA R: TACATGATCCTTCCATCGAGCT</td>
<td>iLOV 330 proline-rich linker 84</td>
<td>iLOV 11 proline-rich linker 2.8</td>
</tr>
</tbody>
</table>

* The C-terminus of VP1 was predicted according to Ren et al 2014, so the Mr is different from intact VP1 (25 kDa). Dashed-underlined sequence is the proline-rich linker. Underlined sequence is partial iLOV sequence used as overlapping region for extension. Restriction enzyme sequences not included.

Expression of individual IAPV capsid proteins

Colonies showing the correct insertions were picked to inoculate 5 mL LB medium. Cells were grown on a shaker (220 rpm) overnight at 37°C and then these cells were used to re-inoculate 50 mL LB media. Cultures were grown to OD\_660 0.5 then 0.06 mM IPTG (for pGex4T1) or 0.02% arabinose was added into the medium. The cultures were shaken (220 rpm) at 25°C overnight.
Cells were harvested by centrifugation (3,000 rpm 15 min) and lysed in PBS pH 7.4, 1×Cocktail protease (Sigma) by sonication on ice for 15 s with rest for 15 s up to 6 times. The lysates were collected and boiled (95°C 5 min) with 1× loading buffer (50 mM Tris-Cl [pH 6.8], 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol). Samples were checked by 12% polyacrylamide gel electrophoresis.

Western blotting was used to check for expression of VPs. For methodology, see Materials and Methods in Chapter 2. iLOV antiserum was used with the dilution ratio of 1:1,000.

**VP1 recovery from inclusion bodies of *E. coli***

VP1-iLOV was amplified from pBAD-VP1-iLOV and cloned into pMAL-c2X (NEB) by changing both restriction enzymes from *Sac*I+*EcoRI* to *EcoRI*+*PstI* (5’ to 3’). Positive clones were confirmed by sequencing (not shown). BL21 cells were transformed with positive plasmids for expression following the manufacturer’s instructions, with minor adjustments: the recombinant MBP-VP1-iLOV was grown with 0.05 mM IPTG added when OD$_{660}$ reached 0.5, and the culture was centrifuged at 220 rpm at 25°C or 20°C overnight. Cells were harvested from a 1 L culture and lysed by sonicating in lysis buffer (PBS-CMF, 2 mM EDTA, 10 mM DTT and 1× cocktail protease [Sigma]). After centrifuging at 15,000 rpm for 25 min, the pellet was resuspended by sonicating in PBS 10 mM DTT, 0.1% SDS, 10% glycerol. The product was then centrifuged and the pellet was again suspended by sonicking in PBS 10 mM DTT, 2 M urea. Centrifugation was performed to obtain a pellet, which was resuspended in PBS 10 mM DTT, 8 M urea. The product was centrifuged and the supernatant was dialysed sequentially against 6
M, 5 M, 4 M, 3 M, and 2 M urea in PBS with 10 mM DTT for no more than 2 h for each step. The 2 M urea product was added to amylose beads (NEB) following manufacturer’s instructions. Each fraction was confirmed on acrylamide gels and the gross amount of final product was determined by Bradford assay.

Results

Cloning of individual viral structural proteins

PCR amplification of coding sequences for individual viral proteins showed approximately 2,000 bp, 900 bp, and 300 bp bands, indicating VP2, VP3, and VP1 were amplified from the construct IJJ5 (Fig. 2A). Fusion of the VP, proline-rich linker (PP), and the fluorescent protein iLOV sequences was achieved by extension PCR (Fig. 2B). The expected size of VP1-, VP2-, and VP3-PP-iLOV was approximately 700, 1,600, and 1,200 bp, respectively. The specificity of VP1 and the linker plus iLOV amplification was not very high, resulting in non-specific amplification (Fig. 2B). The same situation occurred for VP2 (Fig. 2B)

![PCR amplification of coding sequences for VP1, VP2, and VP3 from the construct IJJ5, and the fluorescent protein from a construct containing full-length iLOV DNA sequence (A) and extension PCR of VPs, proline-rich linker (PP), and iLOV (B).](image-url)
Positive clones were confirmed by double digestion (Fig. 3) and sequencing (not shown). Two colonies were picked from an LB plate of each VP, and the results showed that insertion of VP1, VP2, and VP3-PP-iLOV (700, 1, 600, and 1,200 bp, respectively) into the vectors was successful. Positive clones were isolated and VP-iLOV cloned into other vectors (pMAL-c2X and pGex-4T1) by changing the restriction enzymes of these clones (not shown).

Figure 3 Positive clones of VP-iLOV by double digestion. EV, empty vector pBAD/His B sized 4,100 bp. EV-, empty vector not double digested. EV+, empty vector double digested. The expected approximate insertion of VP1, VP2, and VP3 clone were 700 bp, 1,600 bp, and 1,200 bp, respectively.

Expression of individual viral proteins  

Individual viral coat proteins, VP1, VP2, and VP3, were expressed in *E. coli* under standard conditions. VP1 was observed from a Coomassie blue-stained gel (Fig. 4; arrow). However, VP3 and VP2 were not detectable at their expected sizes (Fig. 4). Western blot showed VP1 and VP2 were detected by iLOV antiserum at the expected
molecular mass of VP1 and VP2, respectively (Fig. 5), whereas VP3 was not detectable (Fig. 5).

![Western blot of VP1-, VP2- and VP3-iLOV expression using the vector pBAD/His B in E. coli strain BL21. The expected molecular mass of VP1-, VP2-, and VP3-iLOV are 25, 61, and 59 kDa, respectively. Five mL of cells were grown and harvested. Cell lysates were boiled and loaded with 1× loading buffer with 20 μL each well. Samples were loaded in biological replicates. EV, empty vector,](image)

![Coomassie-stained gel to assess VP1-, VP2-, and VP3-iLOV expression using pBAD/His B. Samples were loaded in biological replicates. VP1-, VP2-, and VP3-iLOV were expected to be 26 kDa, 71 kDa, and 59 kDa, respectively. The arrow showed bands where the protein of interest VP1-iLOV may be present.](image)

Figure 4 Coomassie-stained gel to assess VP1-, VP2-, and VP3-iLOV expression using pBAD/His B. Samples were loaded in biological replicates. VP1-, VP2-, and VP3-iLOV were expected to be 26 kDa, 71 kDa, and 59 kDa, respectively. The arrow showed bands where the protein of interest VP1-iLOV may be present.

Figure 5 Western blot of VP1-, VP2- and VP3-iLOV expression using the vector pBAD/His B in *E. coli* strain BL21. The expected molecular mass of VP1-, VP2-, and VP3-iLOV are 25, 61, and 59 kDa, respectively. Five mL of cells were grown and harvested. Cell lysates were boiled and loaded with 1× loading buffer with 20 μL each well. Samples were loaded in biological replicates. EV, empty vector,
expressing only the N-terminal 6x His tag. The arrow shows the expected VP1- and VP2-iLOV band but VP2 with low amount. No bands indicate VP3-iLOV was expressed. Rabbit anti-iLOV was used as a primary antibody (1 μg/mL), and goat anti rabbit IgG (1:5,000) was used as a secondary antibody.

Recovery of VP1-iLOV from inclusion bodies of *E. coli*

The protein of interest MBP-VP1-iLOV was most soluble under low temperature (20°C) and low IPTG concentration (0.05 mM; not shown), whereas with other combinations of temperature and IPTG concentration, we were not able to identify a prominent band corresponding to the expected molecular mass. On the other hand, even though it seemed that the fusion protein was expressed at 20°C and with 0.05 mM IPTG, the amount of the protein was not sufficient for a ligand blot. After different concentrations of urea were added to gradually denature and then re-fold the protein of interest, most proteins were detected bound to the amyloid beads (Fig. 6A lane 7). The affinity was high between the protein of interest and the beads (Fig. 6A lane 8), but still non-specific binding occurred. Proteins were eluted by maltose and collected as the final product and the molecular mass was the same as expected (Fig. 6A lane 9). Bradford assay showed that the amount of proteins obtained from a 1 L cell culture was 6.49 mg/mL (in 500 μL). A protein with expected molecular mass was detected by western blot (Fig. 6B).
Figure 6 Coomassie-stained (A) and western blot (B) of MBP-VP1-iLOV fusion protein recovered from inclusion bodies of *E. coli* and purified using amylose beads. *E. coli* cells were harvested and sonicated to obtain the (1) lysate and SDS was added to the pellet before sonicating and centrifuging to obtain the (2) pellet; 2 M urea was added to the pellet before sonicating and centrifuging to obtain the (3) pellet; 8 M urea was added to the pellet before sonicating and centrifuging to obtain the (4) pellet and (5) supernatant; the supernatant was dialysed against a series of dilution of urea (8 M, 6 M, 5 M, 4 M, 3 M, 2 M each for 2 hours) and the final product was added to amylose beads; flow through (6) and beads (7) were checked; the beads were washed three times and the wash (8) was checked; the beads were finally washed with elution buffer and the first fraction (9) was checked.

**Discussion**

Individual IAPV viral proteins may be expressed in *E. coli* strain BL21 using commercial vectors (pBAD/His B, pMAL-c2X, and pGex-4T1), but if high temperature (above 25°C) fermentation is applied, these viral proteins may not be purified from the soluble cell fraction (VP1-iLOV was recovered from inclusion bodies in the present study).

High level expression of recombinant proteins in *E. coli* often leads to aggregation of these proteins in inclusion bodies of *E. coli* (Bowden et al 1991). The
accumulation of heterologous proteins greatly exceeds the regular protein-producing rate in *E. coli* cells, thus challenging the host protein translation and folding system (Baneyx & Mujacic; Singh et al. 2015). Evidence showed that inclusion bodies of heterologous proteins expressed in *E. coli* were free from cellular proteolytic degradation (Villaverde & Carrió 2003; Kane & Hartley 1988). Thus, the primary component of inclusion bodies of *E. coli* are the folded intermediates, truncated, or elongated derivatives of recombinant proteins (Rinas et al. 1993). Some host cell ribosomal subunits and outer membrane proteins were also found in inclusion bodies but their detection was argued to be the result of co-precipitated contamination (Kane & Hartley 1988). These data suggest that IAPV viral proteins with high purity level may be obtained by solubilization of inclusion bodies and refolding.

The *E. coli* expression system is commonly used to obtain high amounts of proteins of interest in a relatively short time. Depending on the nature and downstream applications of the proteins of interest, other expression systems, such as cell-free systems, insect-cell-expression systems, and mammalian-cell-expression systems are also options. Cell-free expression systems are preferred for proteins of high molecular mass (Mr), high probability of degradation, and with toxicity to host cells (reviewed in Endo & Sawasaki 2006). Eukaryotic cells offer post-translational modifications and appropriate folding, which are good for complex proteins harboring many post-translational sites and with biological activities (reviewed in Geisse et al. 1996). In the present study, we used the *E. coli* expression system to obtain IAPV individual viral proteins for use as ligands in 2-D ligand blots. As the size of each VP is small, the translation and elongation of these proteins is not expected to disrupt the balance of
translation and folding kinetics in *E. coli*. In addition, the VPs have few post-translational modifications. CryoEM and program predictions both revealed that IAPV viral proteins are made up of regular helices and strands (Mullapudi et al 2017), which are easily processed by *E. coli*.

The acquisition of IAPV individual VPs with sufficient quantity and purity for use in ligand blot analysis may not facilitate identification of a functional receptor in the honey bee midgut. Rather, the receptor-recognized structure of the IAPV virion may involve more than one individual VP. In addition, if the binding requires the native conformation of the tissue protein and the viral protein, receptor binding may not occur (Hall 2004). Thus, using IAPV particles or virions in ligand blot analysis is preferable to the use of individual, recombinant capsid proteins.

**References**


