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Review

Red fluorescent gut proteins in the mulberry silkworm with immunomodulatory properties

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ABSTRACT

The silkworm, *Bombyx mori,* has been exploited not only as an economic insect in the silk industry but also as a research model to understand various biological and physiological phenomena. For instance, while there is a concern of bacterial and viral infections leading to substantial losses for the silk producer, *B. mori* serves as a research model to attain valuable information regarding the mechanism of such infection and consequent resistance. Over the past several decades, many kinds of red fluorescent proteins (RFPs) have been identified in the gut components and/or in the juice of *B. mori* and were suggested to play a central role in the silkworm immunity. However, the actual mechanism behind the formation of those RFPs and their downstream processing to become bioactive modulators is unclear. Our lab showed that the bioactive RFPs originate due to the formation of a macromolecule complex from the binding of chlorophyllide derived from the daily diet of herbivorous *B. mori* with their midgut protein. Herein, we give a detailed overview of the RFPs and their metabolites possessing antiviral/ antimicrobial capabilities and immunomodulatory properties with a particular emphasis on *B. mori*-derived 252 kDa red fluorescent protein. Our summary could serve as useful information for the researchers working on characterizing chemical modulators derived from gut epithelium.

KEYWORDS: *Bacillus thuringiensis* insecticidal protein, chlorophyllide, insect gut components, innate immunity, red fluorescent protein, silkworm

1. INTRODUCTION

The silkworm, *Bombyx mori* has been domesticated for silk production for over 4000 years. The productivity in terms of silk quality and yield has remarkably improved over time owing to the highly sophisticated knowledge of silkworm biology particularly regarding disease resistance [1]. Accordingly, besides being an insect of economic importance, *B. mori* is also used as the insect lepidopteran model for research in entomology, molecular biology and biotechnology, providing a rich background of information on its basic biology [2]. *B. mori* was among the first eukaryotic organisms to serve as a model system for cloning genes and studying the regulation of their expression and it is also the first lepidopteran insect from which transposon-mediated germline transformants were obtained [3]. After whole genome sequencing [4], *B. mori* have kept pace with the other insect models, such as the fruit fly in acquiring knowledge about innate immunity and other resistance mechanisms.

Insects like *B. mori* are continuously exposed to potentially pathogenic microbes and viruses. While the mammals have adaptive immune response acquired over the long life span, *B. mori* have

developed faster defense response. Therefore, *B. mori* serves as an ideal model to examine the immunomodulatory factors that are readily released against pathogenic microbes and viruses. Such information could be used for developing antiviral and/or antimicrobial drugs.

In 1962, antiviral factors were reported to be present in the digestive juice of the silkworm with activity against *Bombyx mori* nucleopolyhedrosis virus (BmNPV) [5]. However, the components responsible for this immunity were unclear. As the primary route of infection is through the food, the gut components and/or their juice was thought to harbor antiviral and antimicrobial mechanism/ substances. In 1969, much detailed report about red fluorescent proteins (RFPs) in the midgut of the silkworm was done [6, 7]. These proteins had antiviral properties and the red fluorescence was suggested to be derived from the binding between insect midgut proteins and chlorophyllide-a (ChlD), the prosthetic group of the natural pigment chlorophyll. It is important to note here that the daily diet of the herbivorous *B. mori* mostly includes the natural Chlorophylls and hence, ChlD is supplied on a regular basis. Moreover, it was also suggested that the anti-BmNPV activity was attributed to chlorophyllid α rather than the protein since no bioactivity could be observed without the red fluorescence that is thought to be derived from chlorophyll [8]. The molecular size of RFP was first suggested to be large since the protein was eluted at void volume in Sephadex G-200 column chromatography [6]. However, various molecular-size proteins were found to have red fluorescence (Figure 1), such as 65-kDa RFP [9], 24-kDa serine protease [10], 30-kDa lipase RFP [11], and 302-kDa lipocalin [12]. Thus, it is reasonable to assume that RFPs with various molecular sizes exist in the digestive juice and/or midgut tracts of insect larvae. Though diversity exists in molecular size, activity, and characteristics, all these reported RFPs were suggested to be derived from the binding between ChlD and insect midgut proteins [13].

Antimicrobial RFPs were also reported to be present in the silkworms [14]. However, a large void remained regarding the mechanism of chlorophyllide binding and its chemical structure after binding. Also, the actual midgut protein(s), which forms RFP and its characteristics, were not clear except for the above-cloned 302-kDa lipocalin RFP from midgut searched from the deduced amino acid sequence database [12]. In 2004, we found, characterized and localized a *Bacillus thuringiensis* Cry1A toxin-binding 252-kDa protein (P252) from the midgut epithelia of *B. mori* [15-17]. Subsequently, we revealed that P252 plays a central role in antimicrobial and antiviral activity in *B. mori* by binding with ChlD to form a red fluorescent complex through pigment–protein-binding reaction [18]. P252 was also shown to bind strongly to both *Bacillus thuringiensis* Cry1A insecticidal protein on the midgut membrane and ChlD to retain the significant antimicrobial activity [19].

A closer look at the insect defense mechanisms could help us to develop new therapeutic agents for clinical applications [20, 21]. Several antiviral compounds purified from insects have been identified as small molecular polypeptides with the molecular size ranging from 8-30 kDa [22-24]. Insects have a robust immune system and are

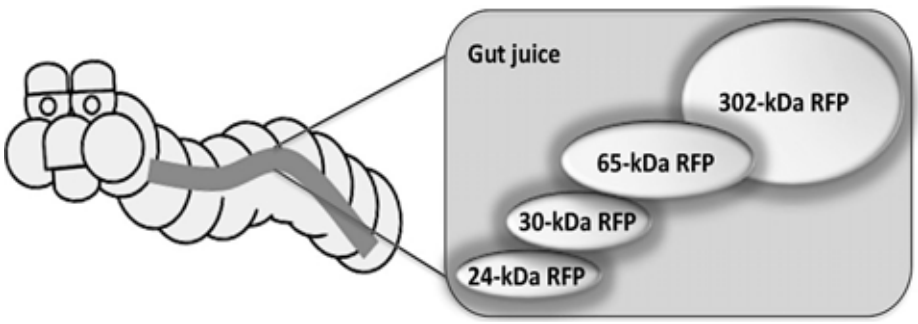


Figure 1. Red fluorescence proteins of various molecular sizes [9-12] in the gut juice of silkworm *Bombyx mori*.

known to have developed resistance to viruses, bacteria and the microbe-based pesticide *Bacillus thuringiensis* in a similar pattern, through extensive exposure. A proper understanding of host-pathogen interaction and host immunity-suppressing anti-defensive effectors from pathogen could aid in resistance control. Considering the increasing importance of RFPs and chemical modulators in immune defense mechanisms, here we give a detailed overview of the recent progress in red fluorescent gut proteins in the silkworm having immunomodulatory properties and discuss their prospects. This summary of the accumulated information of silkworm's immune response to the pathogens not only helps in improving the quality of silkworm, but also contributes in developing pathogen-killing agents that are important to human health and welfare.

2. Mode of pathogen infection and insect defense response

About 67% of all animal species in the world are insects, and their survival success was attributed to their efficient immune system [25]. Insects are regularly exposed to both pathogenic and beneficial microbes and viruses that enter the body *via* food through the gut and *via* air through the tracheal system.

The *Bombyx mori* nucleopolyhedrovirus (BmNPV) is a major pathogen that infects the domestic silkworm and is known to cause severe losses in sericulture. The host range of BmNPV is restricted to *B. mori* larvae [26]. The virus life cycle involves two distinct forms of the virus: occlusion-derived virus (ODV) and the budded virus (BV), both forms have a different role during pathogenesis [27, 28]. The virus particles are embedded in the protein matrix that is dissolved in the alkaline environment of the host midgut. The primary infection begins when the envelopes of ODV fuse to the columnar epithelial cell membrane of the host intestine and then the ODV are taken into the cell in endosomes. In the cell nucleus, the BV particles bud out from the basolateral side and spread the infection through glycoprotein gp64, which is the major component of the envelope fusion that is essential for the infection of neighboring host cells and tissues [29]. Cells of the insect tracheal system are the important targets of BV, and their infection is critical for the rapid spread of the virus because they provide access to larval tissues surrounded by basal laminar barriers [30]. By the end of the infection cycle, most of the insect's tissues are infected and new occlusion bodies are produced, which spread into the surrounding environment for subsequent infection. Despite this accumulated knowledge, no concrete therapeutic agents are known to effectively control BmNPV infection because of the lack of understanding about the mechanism by which the silkworm recruits immune cells to the infective foci and clear them [31]. It is important to note here that only a few strains are reported to have developed resistance to BmNPV infection [32, 33]. Insects seemingly lack any adaptive immune responses that operate analogously to the well-documented antibody or histocompatibility adaptive immune responses as in vertebrates [34]. But it has evolved many different ways to defend themselves against pathogens like fungi, bacteria, nematodes and viruses. Many insects show developmental resistance to baculovirus infection with decreasing susceptibilities at older larval stages [35, 36]. The immunity plays an important role in the interaction between the host and pathogen [37] as a part of survival strategy including physical blockades such as peritrophic matrix [38], epithelial barriers, protease cascades leading to coagulation and melanization, cellular responses such as phagocytosis and encapsulation [39] and also the production of certain antimicrobial peptides [40]. After being challenged by the low pH environment, digestive enzymes and antibacterial lysozymes, microbes enter the hemocoel by passing through the peritrophic membrane and *via* the epithelial cell barriers that are localized under the cuticle in the gut and trachea. Upon infection, the few successful invading microbes trigger the physiological response like the antimicrobial protein synthesis and the protolytic cascade [41]. In contrast to vertebrates having the acquired immunity with 'immunological memory', insects resist against invading microbes by innate immunity, which is characterized by non-specific immune reactions against foreign materials. Upon infection, the coordinated cellular and humoral responses confer resistance to the insects against microbes [42].

Here we give a brief overview of the well-known component of innate immunity from pathogen recognition to the generation of effector molecules (Figure 2). Firstly, pathogen infection leads to the recognition of pathogen-associated molecular patterns (PAMPs) that use the pattern recognition receptors (PRRs) whose function is to detect and eradicate the pathogens. The recognition then leads to three different steps including phagocytosis, protolytic cascade and antimicrobial protein synthesis. The protolytic cascade then induces the production of signaling effectors either through the epithelial cell reaction or systemic response. The released effector molecules such as lysozymes, lectins, antibacterial proteins and antifungal proteins are known to confer the resistance to the host against the invading pathogen.

3. Lipocalin and innate immunity

Current profiling techniques have been revealing the importance of pathogen recognition factors in an innate immune response like the iron-withholding strategy [43]. Iron is an essential source that governs different physiological processes including oxygen transport, gene regulation and DNA biosynthesis [44]. Given that iron plays an instrumental role in the wellbeing of organisms and yet provides an open source of nutrients to the invading pathogen, it is natural that immunologists focus their attention on the host proteins that may regulate both intracellular and intercellular iron level [45]. Pathogen infection invariably triggers a particular event of the stiff competition for iron between the host and pathogen. When free metal like iron is at a low level in the host, it serves as a stimulus for bacteria to produce siderophores that aid in the acquisition of host iron from iron-binding proteins. The iron-loaded siderophore is subsequently transported into the bacteria. Such a strategy allows the proliferation of bacteria in the host cell, which favors biofilm formation (gray arrows). For countering iron piracy, the host produces lipocalin that is dependent on the Toll-like receptor (TLR) signaling induced by pathogen-associated molecular patterns (PAMPs) on the microbial cell wall, leading to the upregulation of lipocalin gene expression and hence increases translation of lipocalin. The lipocalin has a particular

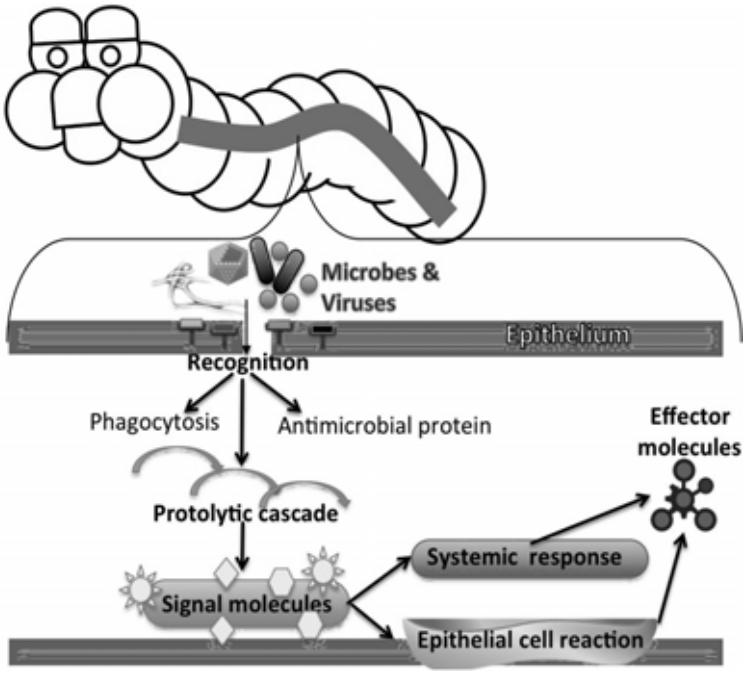


Figure 2. Schematic representation of gut epithelium-mediated immune response in insects (Modified from Hoffmann, J. A., Reichchart, J. M. and Ezekowitz, R. A. 1999, Science, 284, 1313; and Gillepe, J. P., Kanost, M. R. and Trenczeck, T. 1997, Ann. Rev. Entomol., 42, 611).

structure with varying strands of antiparallel beta-barrel with a repeated + 1 topology enclosing an internal ligand-binding site (Figure 3A).

Lipocalin is a family of proteins that share consensus regions of sequence homology and operates by transporting small hydrophobic molecules including steroids, bilins, retinoids, and lipids [44]. When the bioactive lipocalins are expressed at high levels, they bind bacterial siderophore and prevent them from pirating host iron (Figure 3B). In this way, bacterial growth is inhibited. The invading pathogens respond to low iron concentration by forming siderophores to grab the iron and the host responds to this loss of iron by creating innate immune protein molecules (Figure 3C) [46, 47].

Also, lipocalin proteins play a central role in immune system activation in mammals and are known to be involved in inflammation and detoxification processes [48]. In recent years, additional defense mechanisms other than that mentioned above have been gaining attention particularly with those associated with gut components.

4. Gut epithelium and toxin resistance mechanisms

Gut components are known to be associated with the disease resistance and the current pieces of evidence still validate the 2000-year-old-quote, 'All diseases begins in the gut' of Hippocrates (Founder of medicine as a rational science), partially.

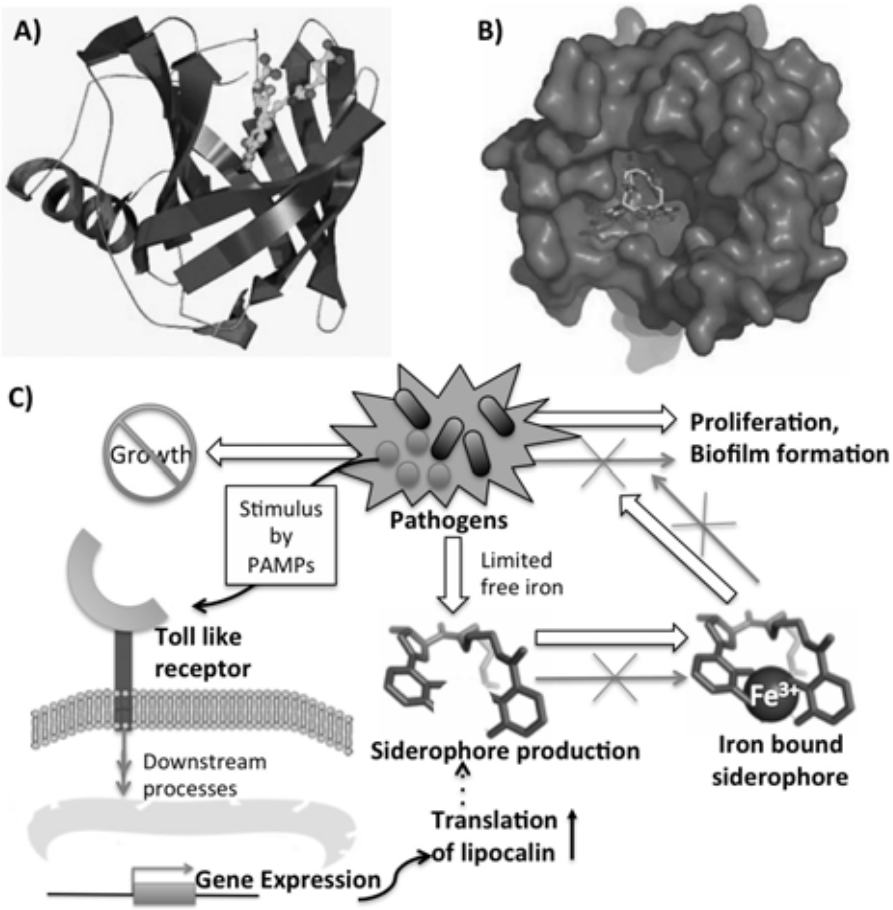
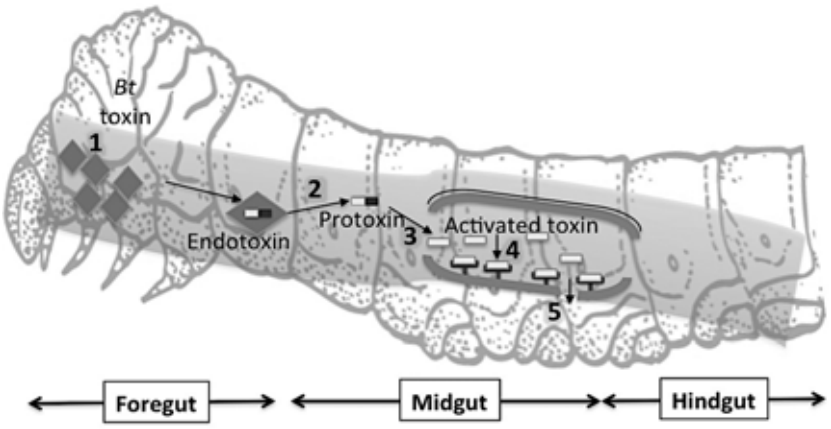


Figure 3. A) The structure of beta-barrel lipocalin. **B)** Lipocalin protein sequestering siderophores. **C)** The implication of host lipocalin in countering iron piracy by bacteria. The host response to bacteria leading to lipocalin production is shown in outlined white arrows (Modified from Ong, S. T., Ho, J. Z. S., Ho, B. and Ding, J. L. 2006, Immunobiology, 211, 295); PAMPs: Pathogen associated molecular patterns.

The gut epithelium and their microbes form an important protective barrier to the insects like silkworm against the invading pathogens from the external environment. Impairment in this intertwined protective barrier by microenvironment alterations and physical disruption of epithelial cells lead to diseased states. Pathogens are known to possess specific strategies to cross the gut barrier like the production of toxins capable of physically disrupting cells in the gut epithelium [49-52].

Bacillus thuringiensis (*Bt*) is a pathogen having large crystalline virulent inclusions generated through sporulation [53]. These inclusions are composed of pore-forming proteins known as crystal (Cry) toxins. After getting ingested by the susceptible larvae, the toxins of *Bt* trigger the killing mechanism through a multi-step process including the formation of pores and lysis of midgut epithelial cells [54]. In recent years, there has been an exponential increase in the information regarding the possible mechanisms about how toxins bind to and disrupt the midgut epithelium. However, the subsequent events leading to the larval mortality is often overlooked and underexplored [55]. Until now, three distinct mechanisms are suggested to occur and trigger different responses by the host that ultimately led to the death of the larvae. The first mechanism indicates the direct toxemia-induced death of larvae within few hours to a day of ingestion [56]. The second mechanism suggested that the extended ingestion of *Bt* triggers the arrest of host cell development that eventually causes death *via* starvation [57]. Lastly, the most commonly cited mechanism involves the *Bt*-triggered sepsis that occurs by the growth of *Bt* in the hemocoel followed by the translocation of spores from the toxin-damaged gut into the hemolymph [55, 58]. However, the proposed mechanism of death by *Bt* is not supported by the bioactivity of toxins in cell-free preparations [59] or in transgenic plant tissues [60]. *Bt* mode of action starts right after the ingestion of the bacteria where the Cry toxins get activated by insect proteases and gut pH. The toxin then crosses the peritrophic membrane to bind to receptors and induce pore formation (Figure 4). Despite the accumulated information regarding the pore formation mechanism and the possible routes towards resistance**,** the actual factors involved is still under debate.



Bt Mode of Action	Possible mechanisms for resistance
1.Ingest Bt Toxin	Still under debate
2.Activation by proteases/gut pH	Failure to activate protoxin
3.Toxin crosses peritrophic membrane	Unable to cross peritrophic membrane
4.Active toxin binds receptors	Failure to bind receptor
5.Toxin inserts into midgut epithelium causing cell lysis and death	Rapid repair of midgut cells or failure to cause pore by conformational change

Figure 4. Bt mode of action and possible mechanisms of resistance. The numbers shown in the gut of the insect correspond to those in the table.

5. P252 – an insect midgut epithelial protein

In our lab, we have been working on the Cry toxin-binding proteins from *Bt* that are involved in the insecticidal or resistance mechanisms. In *B. mori*, many detergent-soluble brush-border membrane vesicles (BBMV) proteins like amino-peptidase (APN) was observed to bind with the Cry1A toxins (Cry1Aa, Cry1Ab, and Cry1Ac) in both ligand blots and the toxin affinity columns [61]. The difference among the toxins will be discussed later. Although the biological relevance of the Cry-toxin and midgut protein is yet to be clarified, it is possible that toxin-protein communication could serve as the vital components of the Cry1A toxin-mediated insecticidal mechanism. In 2004 while studying the toxin-protein interplay in the insecticidal and resistance mechanism, we identified a 252-kDa protein, denoted P252, which might be an important toxin-binding component of BBMV proteins. P252 was shown to bind strongly with Cry1Aa, Cry1Ab, and Cry1Ac toxins of *Bt* with 30, 180, and 20 nM K_d (dissociation constant) values, respectively [15]. The binding of P252 to Cry1A toxins were comparable to that occurring between the other detergent soluble BBM 110-kDa APN and Cry1Aa or APN-120K and Cry1Ac APN that was shown before [61]. P252 was suggested to bind with all three toxins in a specific manner because the respective unlabeled toxin significantly inhibited each labeled toxin. Since P252 did not react with either anti-APN-120K [61] or anti-Cadherin-like proteins [62], we proposed that P252 constitutes a unique Cry1A binding factor. Moreover, the investigation of K_d values showed that P252 binds to Cry1A toxins with relatively higher affinity than other toxins in the midgut epithelial cells and is implied to be occurring under physiological conditions. Taken together, the results clearly indicated a selective binding between P252 and Cry1A toxins thus complementing the existing knowledge of strongest Cry1A toxins binding to midgut epithelial membrane proteins, including APN and cadherin-like proteins. Immunofluorescence analysis using anti-P252 antiserum demonstrated the presence of P252 in BBMV of the midgut. Further studies with the anti-P252 antiserum and Cy3-labeled Cry1A toxins validated the binding with BBMV as 30% decrease in the fluorescence was observed. This observation suggested a specific role for P252 in Cry1A binding to BBMV [16]. The distribution of P252 of the *B. mori* midgut was also examined with histochemical methods. Substantial signals of FITC-labeled antibody against P252, even though not all, were evident in the apical cells, and these were coincident with Cy3-CrylAa and Cy3-CrylAc signals (Figure 5A-C) [17]. Ligand blot analysis and visualization of binding using antibodies substantiated the binding of Cry1A toxins to P252 (Figure 5D).

Based on all the above data, we inferred that P252 is an interesting protein, which might play a vital role in the insecticidal mechanism. However, the real function and activity of P252 were still unclear and thus these essential characteristics warranted further investigation of the functional significance of P252 and its actual role.

6. Chlorophyllide-binding red fluorescent proteins

Initial internal amino acid sequence analysis indicated that P252 is a novel protein. In 2006, a poly-lipocalin (polycalin), chlorophyllid α (ChlD)-binding protein (CBP), was molecular biologically deduced [12] and the protein had an isoelectric point (pI) of 5.2 with a molecular weight (MW) near 302 kDa. The *cbp* coding sequence was 8310 nucleotides in a whole genomic sequence of 45570 nucleotides. In 2008, we found that midgut protein P252 shares high sequence similarity with the CBP and were suggested to play a similar role as P252 in the midgut. Mauchamp *et al*., [12] characterized this protein by deduced amino acid sequences after they cloned a 302-kDa protein. However, binding between their CBP and ChlD was not experimentally proved, nor were the biochemical characteristics and the physiological role of this red fluorescent pigment-protein complex shown. Historically, red fluorescent proteins (RFP) are known to occur in the haemolymph and gut juices of the silkworm; especially the earlier workers have identified them to have antiviral activity against the BmNPV [6-14]. Hayashiya, K. *et al*, [8] firstly demonstrated that the RFPs' biosynthesis required the reaction between chlorophyllide-a and an unknown silkworm protein under *in vitro*

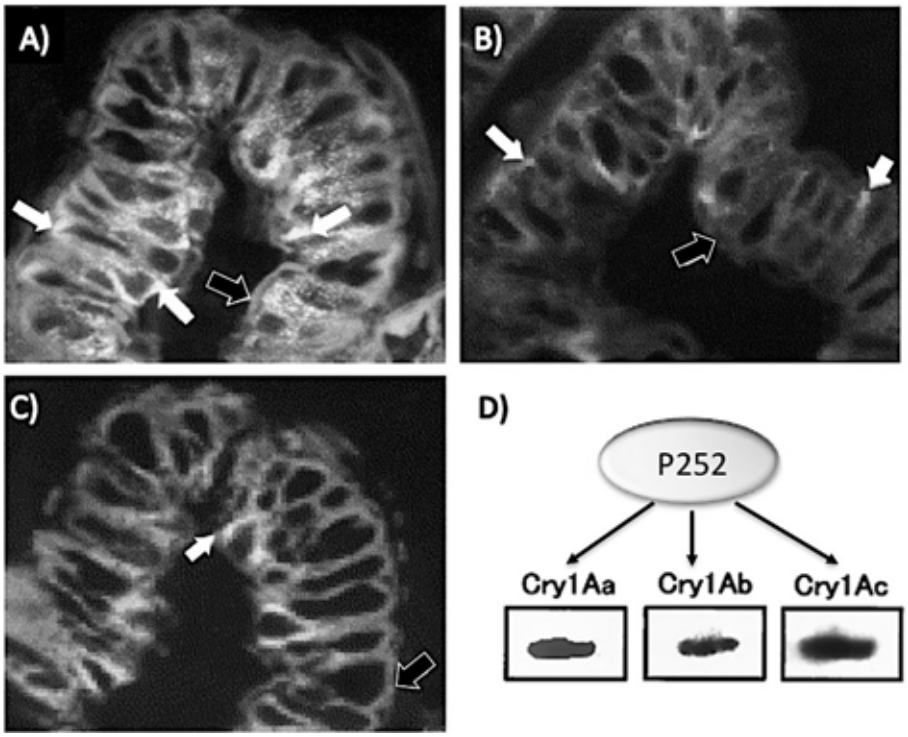


Figure 5. Fluorescence micrographs (merged) of midgut tissue labeled with **A)** Cy3-CrylAa, **B)** Cy3-CrylAab and **C)** Cy3–CrylAc and anti-P252 detected with a FITC-conjugated secondary antibody. White arrows indicate A) red fluorescence in microvillae, B and C) overlaid yellow fluorescence in the apical and/or basolateral membrane areas. The black arrows indicate microvillae without any red fluorescence (Figures combined and modified from Hossain, D. M., Hayakawa, T., Shitomi, Y., Itoh, K., Mitsui, T., Sato, S. and Hori, H. 2007, Pestic. Biochem. Physiol., 87, 30). **D)** Ligand blot of immobilized 252-kDa protein (P252) incubated with Cry1Aa, Cry1Ab, and Cry1Ac toxins.

conditions. In the synthesis process of this protein, chlorophyll-a of mulberry leaves is first converted into chlorophyllide-a under the action of light and its further synthesized in the midgut cells, and then released into midgut to form RFPs, which have two absorbance peaks at 280 and 605 nm wavelength, that is known to be specific for red fluorescent-derived pigment-protein complex [8]. Several works have focused on the study of RFPs physiological activities, which revealed it had the antiviral effect against BmNPV, but the exact mechanism about how ChlD got this activity was not proven. Encouraged by our homology studies, our lab characterized P252 as the first epithelial cell membrane protein capable of binding to ChlD and forming a red fluorescent protein complex with an absorbance peak at 664 nm. Furthermore, emission scan at 500-700 nm revealed that upon excitation at the wavelength of 495 nm, a peak observed with the ChlD alone at 670 nm shifted to 620 nm after incubation with P252. The resultant complex was termed as Bm252RFP (*B. mori*-derived 252-kDa RFP) (Figure 6A). Studies to evaluate the antimicrobial activity revealed that Bm252RFP and not P252 and ChlD had substantial antimicrobial activity (EC_{50} < 10 μM) against pathogenic microbes (*Escherichia coli*, *Serratia marcescens* and *Bacillus thuringiensis*) and a moderate activity against the beneficial yeast Saccharomyces cerevisiae (Figure 6B). Interestingly, P252 was shown to have significant antimicrobial activity even after strongly binding with Cry1Aa, Cry1Ab, and Cry1Ac toxins of *B. thuringiensis* [18]. Later Sunagar *et al*. [33, 63] reported there were multiple forms of red fluorescent proteins (RFPs) observed in the gut juice of the silkworm. Since this pattern could be attributed to the breeding variation, the gut juice of multivoltine

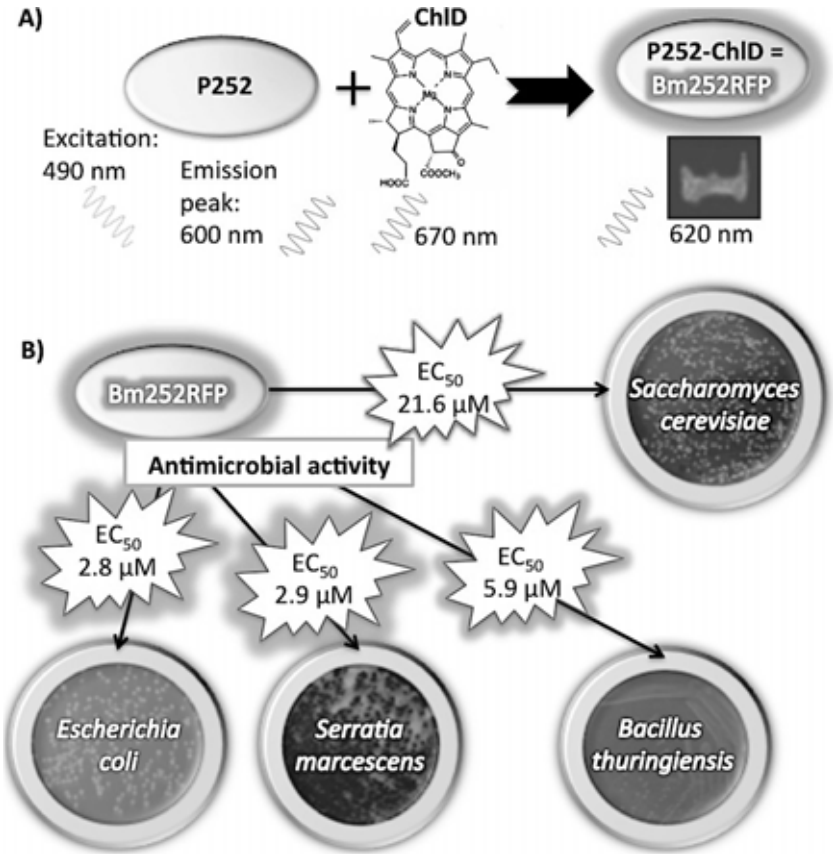


Figure 6. A) P252-ChlD complex emitting red fluorescence (620 nm) upon excitation at 490 nm. **B)** Antimicrobial activity of Bm252RFP.

and bivoltine silkworm was screened for the fluorescent protein bands using electrophoretograms and chromatographic elution. Nevertheless, only three bands were found. RFPs can be used as a biomarker to measure the degree of susceptibility of silkworm races to NPV as separate RFPs could participate in the viral resistant control. However, the chemical mechanism behind the formation of these RFPs was not characterized. Identification of such chemical modulators has the capability to open up new vistas of opportunities in photo-activated dynamic chemotherapy.

7. Bioactive chemical modulators from chlorophyll

Chlorophyllide (ChlD) are the derivatives of chlorophylls (Chl), the green pigments that is the known daily diet component of herbivorous insects [64]. In nature, chlorophyll degradation occurs while 1) leaf undergoes senescence; 2) fruit ripens and 3) as a response to biotic and abiotic stresses [65-67]. In recent years, the linear tetrapyrrole structure has gained increasing attention in the Chl degradation [68]. Despite the well-established mechanism recognized regarding Chl degradation in plants [69], the actual mechanism of Chl degradation inside the insects that feed the plants is underexplored. Several reports showed the possible chemical structures produced as a result of Chl degradation in aquatic grazers [70], mussels [71] and aphids [72]. Among the separate reports available in different insects, a report regarding the qualitative analysis of Chl degradation products was shown in *B. mori* [73]. In 1975, Thornberg suggested that the derivative of Chl pigment form complexes in the protein and generate photoactive bio products [74]. Since lepidopteran larvae primarily feed on plant leaves, such reaction between the Chl catabolites and the protein could

occur in the gut juice, which in-turn lead to the biosynthesis of photoactive pigment-protein complexes like the red fluorescent proteins [75]. It is interesting to note here that most of the lepidopteran insects encompass a peritrophic membrane lining in their midgut and proteomic analysis implied the presence of several large fluorescent lipocalins (termed polycalin) and their isoforms [76]. In general, polycalins are expected to be anchored by glycosylphosphatidylinositol (GPI) to the BBM; however, they were also found as soluble proteins having multiple lipocalin domains in the guts of some lepidopterans like *Helicoverpa armigera*, *B. mori*, and *Manduca sexta*, [77-80]. As mentioned before, in *B. mori*, polycalin binds to and reacts with ChlD (III in figure 7), producing a Bm252RFP with broad-spectrum anti-microbial activity [18] and is suggested to aid the insect's enhanced resistance [81]. Boland and colleagues previously showed the similarities between the early events of Chl (I) degradation in plants during senescence [82] leading to the generation of the catabolites like pheophorbide (Phe), (IV) and pyropheophorbide (Pph) (V) [66].

Suzuki *et al*. showed that the methylester of pheophorbide (IV) is converted to C-13^{2-} carboxylpyropheophorbide through two different mechanisms to generate pyropheophorbide (V) [83]. The wide-spectrum of metabolites observed in lepidopteran larvae could be the result of the co-ordinated response of both the enzymes and the alkaline environment of the digestive tract [84]. In accordance to the biochemistry observed in plants, early phase of Chl a/b degradation (I) involves the loss of the central Mg^{2+} (metal-chelating substance) and the removal of phytol side chain to generate pheophorbides (Phe a/b) (IV) through different intermediates (III) [85, 86].

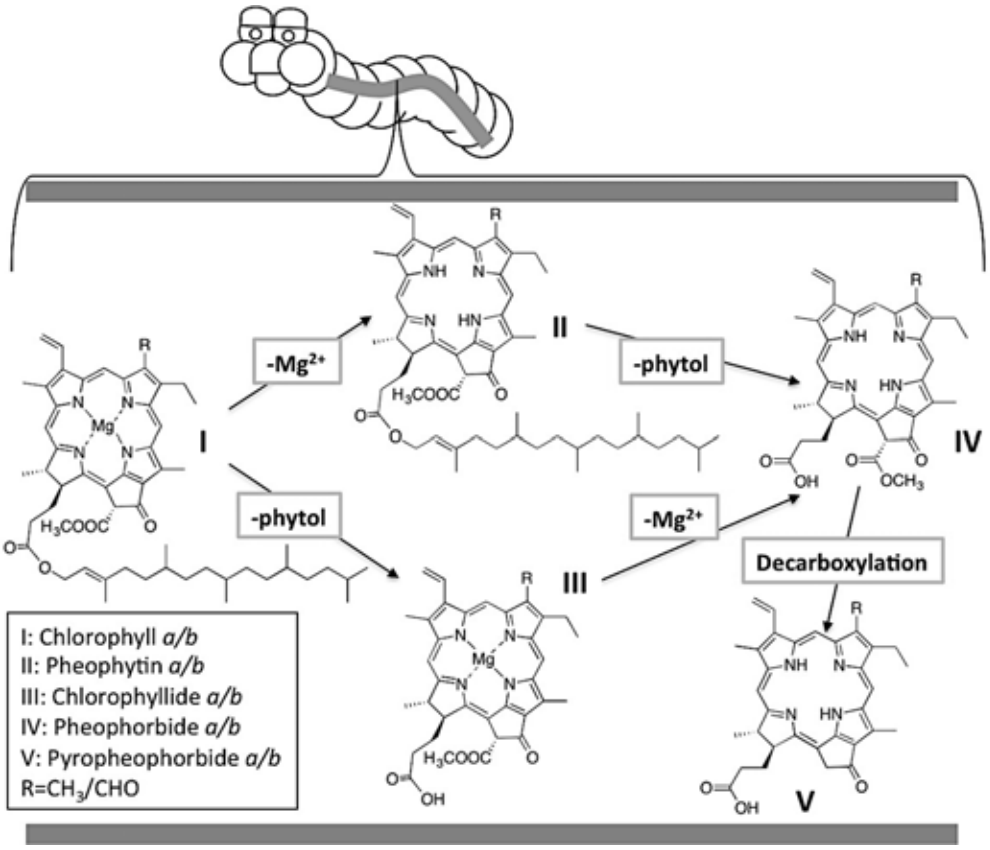


Figure 7. Scheme of chlorophyll degradation in the insect gut (Modified from Badgaa, A., Buchler, R., Wielsch, N., Walde, M., Heintzmann, R., Pauchet, Y., Svatos, A., Ploss, K. and Boland, W. 2015, J. Chem. Ecol., 41, 965).

Subsequently, the pheophorbides (IV) metabolize into pyropheophorbides (Pph a/b) (V) as stable end products through hydrolysis and decarboxylation and gets accumulated in the gut fluids and feces (Figure 7).

The above-mentioned Chl (I) degradation pathway was shown to be predominantly alike in lepidopteran insects; however, succeeding studies have also indicated a complex pattern of Chl (I) degradation in gut milieu [65]. In particular, a food-dependent, co-operative binding of free Chl and their derivatives preferentially to native proteins in the gut fluid and not to heat-inactivated samples, has gained attention [86]. These metabolic effectors are suggested to play an essential role in maintaining cellular homeostasis. Nevertheless, the actual protein in the gut milieu and the chemical compound(s) obtained after the conversion of ChlD (III) to form RFP and the reason behind its bio reactivity are unknown.

8. RFP-derived reactive oxygen species in insect immunity

Antiviral activity and fluorescence characteristics of different ChlD-derived RFP's is suggested to be through the photosensitization of the chromophore which ultimately leads to the opening of the porphyrin ring [8]. As discussed in previous sections, new antimicrobial ChlD (II)-derived Bm252RFP was characterized in our lab [18] with activity against *E. coli*, *Serratia marcescens*, *B. thuringiensis* and *Saccharomyces cerevisiae*. Photosensitized porphyrin ring structures are known to have both antimicrobial and antiviral activities [87, 88]. Accordingly, our lab showed for the first time about the characterization of a novel chemical structure derived by the binding of ChlD with our midgut protein P252 to form RFP. In 2009, we identified and isolated [87] the unique ring-opened ChlD (ROC) as the chemical compound responsible for the typical spectral characteristics, red fluorescence and antimicrobial activity against wide range of microbes. Subsequently, we explored [87] the mechanism behind such potent antimicrobial activity of Bm252RFP and its active metabolite ROC that was shown to be the factor behind such activity. A close look at the structure of ROC suggested the possibility of evolution of reactive oxygen species (ROS) radicals such as hydroxyl ion, hydrogen peroxide and superoxide through photooxidation.

Subsequently, radical formation was demonstrated as the mechanism behind the antimicrobial activity of Bm252RFP and ROC [87]. Photosensitized chlorophyllide structure was suggested to form reactive oxygen species (ROS). ROS is comprised of superoxide radicals ($O_2^{\cdot}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^{\cdot}$) that originate from electron transfers (one-, two-, or three electrons, respectively) to dioxygen (O_2). Such partially reduced oxygen species are the predominant toxic byproducts of physiologically important O_2-consuming redox processes and are known to cause oxidative damage when ROS production exceeds the capacity of ROS-scavenging reactions. The presence and absence of Fenton catalysts like iron ions or peroxidase decide the toxicity to ROS and when toxic causes extremely reactive $OH^{\cdot}$ radicals in the presence of H_2O_2 and $O_2^{\cdot}$ [89, 90]. In nature, ROS are known to be formed either as by-products of mitochondrial respiration or as a component of the defense response against pathogens [91, 92]. Like vertebrates, insect hemocytes that are functionally similar to vertebrate neutrophils and macrophages also undergo a respiratory burst that involves the generation of the $O_2^{\cdot}$ and H_2O_2 using nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and superoxide dismutase (SOD), respectively [93, 94]. However, the actual mechanism by which ROS are generated in these cells is still under debate.

In our lab, our photosensitized Bm252RFP was checked [87] for the presence of ROS using specific radical assays. In addition, Bm252RFP and/or ROC had an electron paramagnetic resonance signal at (g = 2.0035) corresponding to the ROS radicals, and not native P252 or ChlD. The radical formed was stable until 6 h in Bm252RFP and/or ROC. Scavenger studies done to check the role of ROS in the mechanism of antimicrobial activity revealed that only 21% of Bm252RFP antimicrobial activity was lost with catalase and superoxide dismutase that are scavengers of hydrogen peroxide and superoxide, respectively [87]. However, when mannitol, the scavenger for hydroxyl ion radical was used, 63% of the inhibitory activity of Bm252RFP was lost.

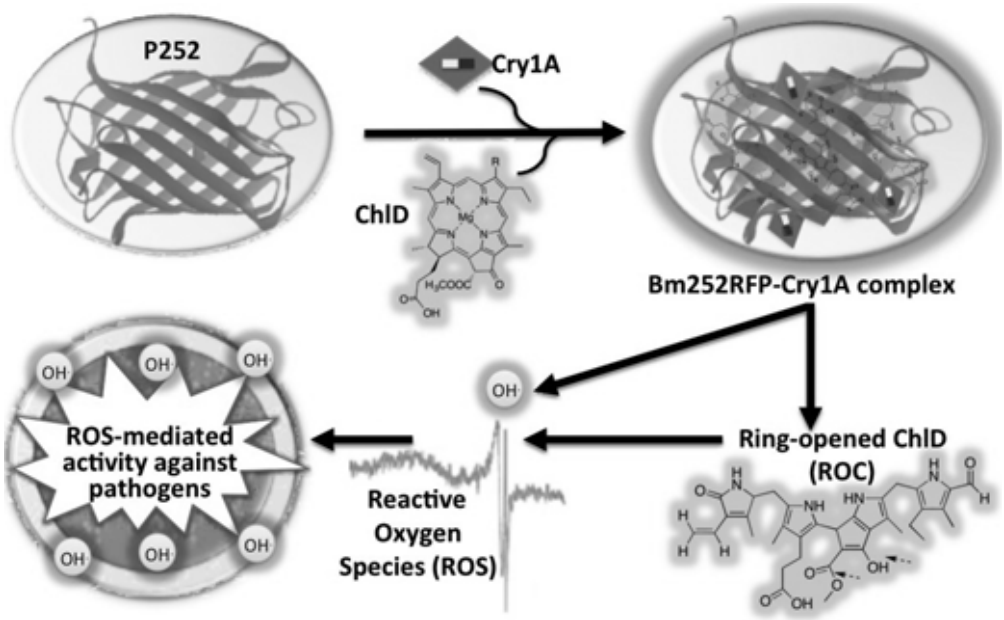


Figure 8. Scheme of the formation of a macromolecule (Bm252RFP-Cry1A) complex and purification and characterization of a novel bioactive metabolite (Ring-opened ChlD, ROC), which was shown to have activity against pathogens *via* the generation of hydroxyl radical ($OH^{\cdot}$).

Microscopic studies of *E. coli* stained with propidium iodide showed the disintegration of DNA, which resembled the mechanism of hydroxyl ion activity. Bm252RFP had 26% inhibitory effect on midgut juice microbes, while ROC had 46% effect [87]. ROC is spontaneously released from Bm252RFP after 2 h under incubation with midgut juice or *E. coli* cells and the released ROC was stable for just 1 h. However, with continuous consumption of ChlD, antimicrobial ROC could be consistently formed under midgut conditions to provide immunity to the insects with specificity against only microbes and not the host (Figure 8).

9. CONCLUSION

Insects develop resistance against their killing factors like microbes and viruses with extensive exposure. *Bt* Cry1A toxins are widely used as biopesticides and Cry1A toxin-mediated pore formation leading to cell death is considered as the insecticidal mechanism. While searching for Cry1A-binding proteins related to insecticidal mechanism, a novel 252-kDa protein (P252) with high binding affinity to Cry1A was isolated and characterized [15] from midgut membrane of silkworm *B. mori,* in our lab. Homology study to assign its activity had revealed that P252 shared sequence similarity to a ChlD-binding protein (CBP). Historically, red fluorescent proteins (RFP) are thought to originate from the midgut CBP(s) to form a complex with typical spectral characteristics resulted by the opening of porphyrin ring in ChlD. Based on sequence similarity and midgut localization, P252 was incubated with ChlD and it indeed bound to form a complex, that displayed typical CBP spectral characteristics with red fluorescence, termed Bm252RFP with antimicrobial activity against a wide range of microbes but with no insecticidal activity. While exploring the mechanism behind the antimicrobial activity, our lab purified a novel chemical structure with reactive oxygen species termed ring-opened chlorophyllide (ROC), which is responsible for Bm252RFP spectral characteristics and antimicrobial activity. Radical scavenger assays, EPR analysis and microscopic studies confirmed hydroxyl ion as the primary reactive oxygen species. ROC had the inhibitory effect on midgut juice microbes and was released from Bm252RFP under midgut conditions. Bm252RFP also retained the Cry1A-binding characteristic of P252 and formed

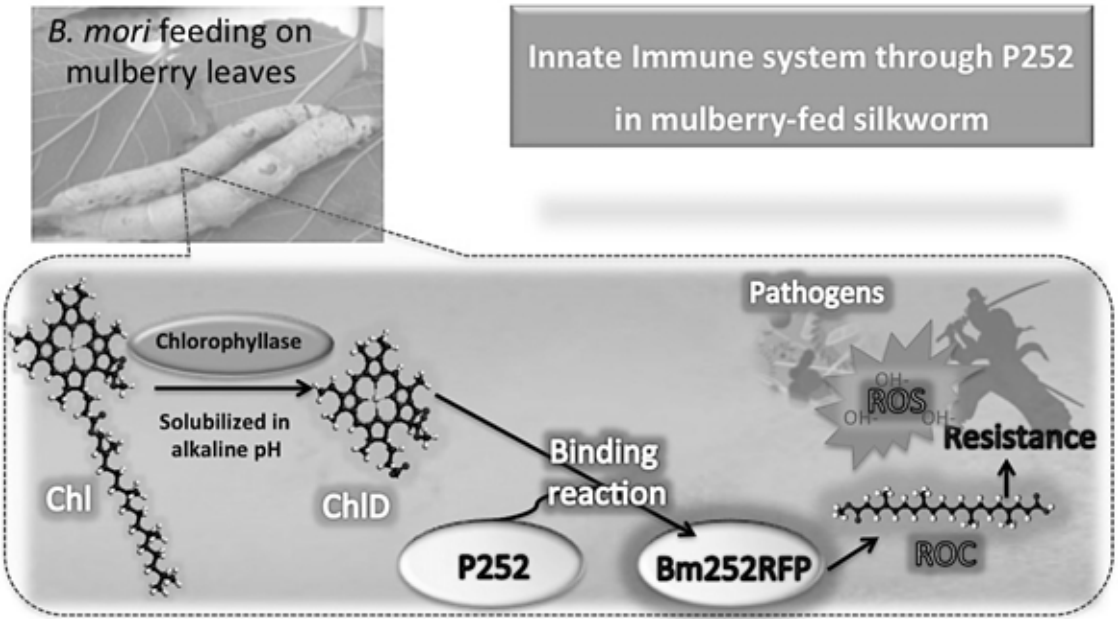


Figure 9. A novel immune system in silkworm through a 252-kDa chlorophyllide-binding protein in the midgut of a silkworm. Chl: Chlorophyll, ChlD: Chlorphyllide; ROC: Ring-opened ChlD; Bm252RFP: *B. mori*-derived 252 kDa RFP; ROS: Reactive oxygen species.

Bm252RFP-Cry1A complexes. Effect of binding on the respective protein activities was studied and only Bm252RFP-Cry1Ab complex retained complete antimicrobial activity and also reduced the toxicity of Cry1Ab against *B. mori* and this might attribute to a novel resistance mechanism. Polycalin with aminopeptidase activity has been reported recently to be present in *Helicoverpa armigera* larvae [80]. It is also interesting to point out that 252-kDa protein reacting with antiserum raised against *B. mori* P252 was found in BBMV prepared from *Aedes aegypti* mosquito larvae, (Seangduen M. *et al*., unpublished data in our lab). If these are the cases in many other insects, it may be implied that 252-kDa protein as well as other RFP(s) could be a common protein in antimicrobial systems in insects. Bm252RFP was observed to have reactive oxygen species (ROS) and was shown to be the reason behind the antimicrobial activity of Bm252RFP. Interestingly; ROS radicals are known to modulate the immunity of *Anopheles gambiae* against bacteria and *plasmodium*. However, the exact mechanism of ROS formation is yet to be ascertained [94]. Interestingly, the blood meal used in this study contains hemoglobin, which has similar porphyrin ring structure like ChlD. Therefore, the formation of ROS by the binding of porphyrin ring structures to P252 might be the mechanism of ROS formation. Bm252RFP was specifically active against microbes and no killing effect was observed against insects even with excess concentrations. Based on this result we believe that expression of various antioxidant enzymes in *B. mori* in midgut suppresses the ROS radicals on exposure. Thus ROS formation could be kept under control to modulate the immunity as described in [93]. Thus, we propose a novel immune system by the ROS formation through P252, which widens the research on the insecticidal mechanism (Figure 9). Detailed *in vivo* studies will give more insights into the insect immune mechanism that could be used for mimicking bioactive molecules for therapeutic purposes.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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