Journal of Scientific Agriculture 2024, 8: 100-103
doi: 10.25081/jsa.2024.v8.9088
https://updatepublishing.com/journal/index.php/jsa

Review Article

ISSN: 2184-0261

Antiviral activity of red fluorescent proteins in silkworm, *Bombyx mori* L.

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Received: July 13, 2024
Revised: December 04, 2024
Accepted: December 05, 2024
Published: December 20, 2024

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ABSTRACT

Silkworm is an important economic insect in the textile industry for the production of silk. During its larval period, it suffers from various types of diseases *viz*., fungal, viral, bacterial and protozoan which hamper its growth and development and negatively impact the raw silk production. To fight these pathogens, different types of proteins have been found in the silkworm larva. Among these proteins, red fluorescent proteins (RFPs) possess antiviral activity, found in the digestive juice of the midgut of silkworm larvae. These RFPs are found to be more effective against *Bombyx mori* L. nucleopolyhedrosis virus (BmNPV), which is the causal agent of the most dreadful disease known as Grasserie. The RFP is synthesised in the presence of light after silkworms are fed on fresh mulberry leaves having absorbance peaks (280 and 605 nm) wave length.

KEYWORDS: Chlorophyllide-a, Digestive juice, Red fluorescent proteins, BmNPV, *Bombyx mori*

INTRODUCTION

The mulberry silkworm (*Bombyx mori* L.) is a beneficial insect for producing commercial silk and also operates as a model insect for producing different biomaterials in the silk gland. The role of good quality mulberry leaves rich in different nutrients is essential for the robust growth of mulberry silkworms, any deviation from quality mulberry leaves leads to poor immunity in silkworm larvae and results in the development of various threatening diseases which incur heavy losses to silkworm farmers (Islam *et al*., 2020a, b, 2022a, b; Majid & Islam, 2022; Qadir *et al*., 2022; Islam, 2023; Islam *et al*., 2023a, b). During its larval period, *B. mori* faces the threat of many fungal, bacterial, viral and protozoan diseases and among these diseases, viral diseases cause massive loss (Samson, 1985, 1988; Baig *et al*., 1989; Savanurmath *et al*., 1994). Suresh *et al*. (2007) investigated that the quality of mulberry leaves greatly impacts the concentration of RFPs produced in the larvae. The larvae fed on nitrogen and potassium treated mulberry leaves were found to contain increased levels of RFPs in the digestive juice and further NPV+RFP combination greatly reduced the mortality of larva compared to the NPV+BSA combination. RFPs are a group of midgut proteins which emit red fluorescence when bind with a tetrapyrrole moiety of mulberry leaves in the presence of chlorophyllase (Hayashiya *et al*., 1976; Funakoshi & Aizawa, 1989), also association of chlorophyllide with chlorophyllide-binding protein produced polycalin, a kind of fluorescent protein (Mauchamp *et al*., 2006).

RED FLUORESCENT PROTEINS (RFPS) AND ITS VIRAL ACTIVITY IN SILKWORM

The silkworms are infected by four viral diseases *viz*., nuclear polyhedrosis, cytoplasmic polyhedrosis, infectious flacherie, and densonucleosis. The nuclear-polyhedrosis virus (BmNPV) infects many silkworm body tissues and multiplies in the nucleus producing inclusion bodies known as polyhedra which occludes virus particles containing double stranded DNA (dsDNA). The cytoplasmic polyhedrosis virus (BmCPV) infects the mid gut epithelium cells thereby multiplying in the cytoplasm of columnar cells and form inclusion bodies occluded with virus particles, it contains double stranded RNA (dsRNA). The infectious flacherie virus (BmIFV) infects the midgut epithelium cells and multiplies in the cytoplasm of goblet cells but doesnot form inclusion bodies, it contains single stranded RNA (ssRNA). The densonucleosis virus (BmDNV) infects the midgut epithelium cells and multiplies in the nucleus of columnar cells of silkworm larva; it contains single stranded DNA (ssDNA) (Watanabe, 1986). The viral disease *viz*., Nuclear polyhedrosis (BmNPV) which is also known by different names like Grasserie, Milky disease, Jaundice, Hanging disease or Fatty degeneration (Steinhaus, 1949, 1963; Bergold, 1963) is epidemic in nature and causes havoc in sericulture industry (Horie & Watanabe, 1980; Attathom & Sinchaisri, 1987; Narasimhanna, 1988; Chishti & Schof, 1991; Basavarajappa, 1996; Singh, 1997). The nuclear polyhedrosis virus often resides in dormant state in silkworm body and gets activated under stressful conditions

of host (Aruga, 1994) and outbreaks by causing huge losses to silkworm farmers. More than 60% of the total cocoon loss occurs by this disease and there is no perfect solution for prevention and treatment of this disease and the effective measure to control this disease is the use of disease resistant varieties produced by genetic modification of silkworms (Hu *et al.*, 2023). Insects were studied for humoral and cellular immune systems and in cellular immunity, phagocytosis and encapsulation were found to be working (Ratcliffe *et al.*, 1985; Kimbrell, 1991; Sethuraman *et al.*, 1993). Humoral responses are related to the production of antibacterial proteins namely cercopins, and attacins (Boman & Hultmark, 1987) that are produced against infection (Abraham *et al.*, 1995). The RFPs were reported to have anti nucleopolyhedrovirus activity and light has an important role in the synthesis of this protein (Sunagar *et al.*, 2011) (Table 1). The light helps in the activation of the functional RFPs against NPV and the formation of pigment protein complex (Hayashiya *et al.*, 1969). The maximum susceptibility of *B. mori* to NPV may be due to less or no formation of RFPs in the larvae reared in dark conditions (Hayashiya *et al.*, 1968). Yao *et al.* (2009) stated that the silkworm midgut acts as an innate immunity and is a natural barrier to fight the silkworm pathogens. Many researchers have reported the presence of antiviral substances in the midgut juice (Suzuki, 1936; Aizawa, 1962) and the presence of RFPs in midgut was found to inactivate the nucleopolyhedrovirus (NPV) (Hayashiya *et al.*, 1969, 1976; Funakoshi & Aizawa, 1989). Hayashiya *et al.* (1968) and Nishida *et al.* (1973) found RFPs in the digestive fluid of anterior part of the midgut and were absent in the haemolymph part of silkworms. The presence of RFPs containing chlorophyll-like substances having antiviral activity was also reported in faecal matter of healthy silkworm larvae (Hiraki *et al.*, 1997, 2000). Comparatively, the Indian multivoltine silkworm races like Pure Mysore and Nistari were found to be more resistant to NPV than bivoltine races like NB4D2 (Acharya *et al.*, 2002) and this resistance is controlled by many genes producing proteases and lipases in gut juice of silkworm (Ponnuvel *et al.*, 2003; Nakazawa *et al.*, 2004). Goldsmith *et al.* (2005) observed that the polygenic control of inheritance of resistance to NPV makes it complicated to understand. The RFP has an antiviral activity (Sethuraman *et al.*, 1993) and it is absent in those insects which are reared on artificial diets without fresh leaves (Matsubara & Hayashiya, 1969; Hou & Chiu, 1986).

BIOSYNTHESIS AND MECHANISM OF ACTION OF RFPS IN SILKWORM LARVA

The biosynthesis of this protein needs chlorophyll-a and chlorophyllase for its formation and when chlorophyllide-a reacts with an insect membrane protein, it leads to the formation of the final product (RFP) (Hayashiya, 1978; Pandian *et al.*, 2008) (Figure 1). Few proteins were reported in the silkworm gut juice which have baculovirus resistance *viz.*, Bmlipase-1, serine protease, BmNOX and NADPH oxidoreductase (Nakazawa *et al.*, 2004; Selot *et al.*, 2007). Bmlipase-1 was transferred into susceptible silkworms by the use of transgenic methods (Jiang *et al.*, 2012) and after this survival rate of transgenic silkworms increased by 33% after infected by BmNPV thereby

Table 1: Antiviral activity of individual RFP bands (NPV resistant Pure Mysore) race under light and dark conditions (Sunagar *et al.*, 2011)

S. No.	Treated groups	No. of silkworms treated	% survival under light	% survival under dark
1.	BSA (+ve control)	30×3	100	90
2.	BSA+NPV	30×3	0	0
3.	Phosphate buffer treated	30×3	100	86
4.	NPV treated (-ve control)	30×3	0	0
5.	RFPPM1+NPV	30×3	97	0
6.	RFPPM2+NPV	30×3	88	0
7.	RFPPM3+NPV	30×3	0	0
8.	RFPPM4+NPV	30×3	0	0
9.	RFPPM5+NPV	30×3	0	0
10.	RFPPM6+NPV	30×3	0	0
11.	RFPPM7+NPV	30×3	0	0
12.	RFPPM8+NPV	30×3	0	0
13.	RFPPM9+NPV	30×3	0	0
14.	RFPPM10+NPV	30×3	86	0
15.	RFPPM11+NPV	30×3	0	0

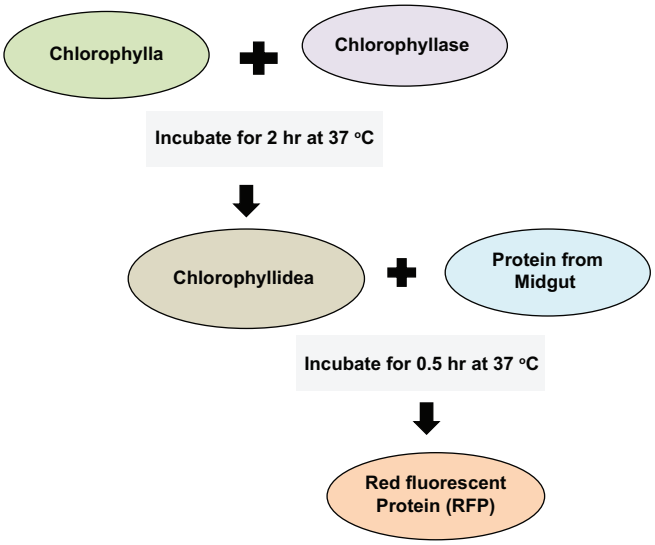

Figure 1: Biosynthesis of Red Fluorescent protein (RFP) (Pandian *et al.*, 2008)

confirming the anti-BmNPV activity of this protein. There are four hypotheses through which RFPs neutralize NPV (a) Encapsulation/inhibiting the transport of polyhedra by the use of RFPs by blocking the entry of viral particles to receptor cells in the midgut of insects (b) Functional RFPs may hydrolyze the carbohydrate/protein portion of the polyhedra by impairing the recognition of the host cells (c) The tetrapyrrole attached with RFPs may bring about the photodynamic inactivation of NPVs and (d) RFPs may show nuclease activity and cause degradation of viral DNA (Hiraki *et al.*, 2000; Park *et al.*, 2003; Marmaras & Lampropoulou, 2009). Matti *et al.* (2010) revealed that silkworm excretory red fluorescent protein (SE-RFP) has antimicrobial activity against many pathogenic bacterial and fungal strains and recorded highest activity (lowest minimum inhibitory concentration and largest zone of inhibition) in the case of *Staphylococcus aureus* and *Candida albicans* respectively (Table 2).

Table 2: Antibacterial Activity of Purified SE-RFP, Zone of inhibition (mm)[a]

Microorganism	SE-RFP (25 μg)	SE-RFP (25 μg)	SE-RFP (100 μg)	Positive control[b]	Negative control[c]
Gram+^{ve} bacteria					
S. aureus	8±0.38	17±0.23	21±0.25	28±0.18	5±0.0
K.pneumoniae	9±0.26	15±0.45	19±0.43	22±0.66	5±0.0
MR*SA*	8±0.62	13±0.42	18±0.68	24±0.13	5±0.0
P.mirabilus	6±0.05	6±0.27	12±0.35	20±0.07	5±0.0
B.subtilis	6±0.21	7±0.33	9±0.19	26±0.53	5±0.0
Gram –^{ve} bacteria					
E. coli	7±0.34	12±0.47	19±0.42	25±0.28	5±0.0
*S.paratyphi*B	9±0.48	11±0.55	17±0.18	28±0.40	5±0.0
S. typhi	8±0.43	9±0.20	10±0.64	18±0.44	5±0.0
*S.paratyphi*A	6±0.59	7±0.78	14±0.16	22±0.15	5±0.0
S.pyogenes	7±0.32	8±0.37	10±0.53	31±0.08	5±0.0
Fungi					
C. albicans	9±0.17	11±0.23	16±0.38	28±0.52	5±0.0
A. flavus	8±0.29	9±0.34	11±0.66	23±0.35	5±0.0
A.niger	6±0.64	8±0.58	9±0.18	29±0.47	5±0.0

[a]Values are the diameter of zone of inhibition (mean of three replicates) in millimeter±standard deviation. [b]Standard antibiotics viz., amoxicillin (25 mg) and nystatin (25 mg) were used as positive controls against bacteria and fungi, respectively 5 mm is the diameter of bore prepared. [c]No inhibition zones were observed for negative control (2 mMTris–HCl buffer, pH 7.4).

CONCLUSION

The mulberry silkworms are very prone to different kinds of silkworm pathogens due to its continuous domestic nature for thousands of years. Proper prevention and treatment strategies are necessary to maintain the healthy growth of larvae to harness good silk production. The antiviral activity of RFPs present in the digestive juice of *B. mori* as reported by many researchers is well documented. The precursors for the formation of functional RFPs are present in the fresh mulberry leaves after they conjugate with the protein part in the gut of silkworm larva. Also the concentration of RFPs in the digestive juice of silkworms is majorly governed by the type of silkworm race as it is well known that multivoltine races contain more RFPs than bivoltine races and that may be the reason behind more resistance of multivoltine races against NPV compared to bivoltines. As suggested by some researchers RFP patterns may be used as biomarkers to study the relative resistance of silkworm races against nuclear polyhedrosis virus in the future. More research needs to be done in this area to fully understand the different aspects associated with the RFPs for developing an efficient system of disease control system in silkworm larvae to boost the overall economy of silkworm farmers worldwide.

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