RNA INTERFERENCE TO INVESTIGATE THE FUNCTION OF *TARGET OF RAPAMYCIN* (*TOR*) GENE IN *Bemisia tabaci* (GENNADIUS)

Thesis

Submitted to Punjab Agricultural University
In partial fulfillment of the requirements
For the degree of

MASTER OF SCIENCE
in
ENTOMOLOGY
(Minor Subject: Plant Pathology)

By

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(L-2017-A-50-M)

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CERTIFICATE-I

This is to certify that the thesis entitled **"RNA interference to investigate the function of *target of rapamycin* (*TOR*) gene in *Bemisia tabaci* (Gennadius)"** submitted for the degree of **Master of Science,** in the subject of **Entomology** (Minor subject: **Plant Pathology**) of the Punjab Agricultural University, Ludhiana, is a bonafide research work carried out by **Heena (L-2017-A-50-M)** under my supervision and that no part of this thesis has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

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CERTIFICATE – II

This is to certify that the thesis entitled, '**RNA interference to investigate the function of *Target of Rapamycin (TOR)* gene in *Bemisia tabaci* (Gennadius)**' submitted by **Ms. Heena** (Admn. No. **L-2017-A-50-M**) to the Punjab Agricultural University, Ludhiana, in partial fulfillment of the requirements for the degree of **M.Sc.** in the subject of **Entomology** (Minor subject: **Plant Pathology**) has been approved by the Student's Advisory Committee along with External Examiner after an oral examination on the same.

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I may not have mentioned all; nevertheless, thoughts remain with me. It's my honest conviction and sincerest prayer that the Almighty God bless you all abundantly.

Date : ***(Heena)***

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ABSTRACT

The *target of rapamycin* gene was amplified in ten overlapping fragments from whitefly. The full length of *TOR* gene resulted in 7311 bp nucleotides and 2437 amino acids. *TOR* gene showed 98.66 per cent homology with predicted sequence of *Bemisia tabaci* serine/threonine-protein kinase mTOR. Ninety-five nucleotides were found to be different from the predicted sequence of *TOR* gene in *B. tabaci*, but only two amino acid differed. Amino acid at position 1448 i.e. asparagine replaced threonine and at position 1768, arginine replaced tryptophan in sequenced *TOR* gene as compared to predicted *TOR* amino acid. Phylogenetic analysis of amino acid sequence revealed that *TOR* gene of *B. tabaci*, *Acrythosiphum pisum* and *Nilaparvata lugens* were more closely related to each other and grouped in one cluster. dsRNA corresponding to *TOR* (@1.0, 0.5 and 0.1 µg/µl) and *gfp* (@1.0, 0.5 and 0.1 µg/µl) gene were synthesized and fed to the whitefly adults to record the adverse effect on biology of whitefly. Continuous feeding of dsRNAs to whitefly adults showed that maximum mortality (47.75 %) occurred after 48 hours of feeding, when whiteflies were fed with *dstor* @ 0.1 µg/µl, which was statistically at par with all the treatments of *dstor* and significantly higher than *dsgfp* treatments and control. The bioassay after feeding of whiteflies for 48 hours on artificial diet was conducted three times in June-July, July-August and August-September, 2019. The adult mortality after 48 hours of feeding was significantly higher in *dstor* fed whiteflies than control and *dsgfp* treatments. The maximum mortality (46.66, 41.66 and 26.25 %) were recorded in *dstor* @ 1.0, 0.5 and 2.0 µg/µl in all the experiments, respectively and the minimum mortality (16.66, 10.00 and 7.5%) was recorded in *dsgfp* @1.0 µg/µl, 1.0 µg/µl and control, respectively. Significant effect of silencing of *TOR* gene was recorded on fecundity of *B. tabaci* fed on *dstor* in two experiments, which was statistically lower than *dsgfp* and control. Minimum egg laying (41.25 and 30.33 per female) were recorded in *dstor* @ 1.0 µg/µl, however maximum egg laying were 70.00 and 64.77 per female in *dsgfp* @ 0.1 µg/µl in both the experiments. Nymphal mortality increased after silencing *TOR*. It was significantly higher when whiteflies were fed with *dstor* @ 2.0, 1.0, 0.5 and 0.1 µg/µl as compared to all the concentrations of *dsgfp* and control in all the three experiments. The major significant difference with respect to nymphal mortality was observed only during first instar, however nymphal mortality in second and third instars were statistically non-significant. No significant effect of silencing of *TOR* was observed in case of nyphal duration, pupal mortality, pupal duration and adult emergence. Upon feeding of *dstor* to adult whiteflies for 48hours, RNAi experiment showed that 55.34, 57.21 and 63.36 per cent expression of *TOR* gene in *dstor* @ 1.0, 0.5, 0.1 ug/µl, respectively as compared to *dsgfp* @ 1.0 ug/µl (100 %). After 96 hours, feeding mRNA level of *TOR* gene was 13.77, 16.52, 24.07 and 28.75 per cent, when whitefly adults were fed at *dstor* @2.0, 1.0, 0.5 and 0.1 ug/µl as compared with *dsgfp* @ 1.0 ug/µl (100 %). These qPCR experiments confirm the partial silencing of *TOR* gene in whiteflies when fed with *dstor*.

Keywords: *Bemisia tabaci*, RNA interference, gene silencing, *target of rapamycin*, fecundity, mortality

Signature of Major Advisor **Signature of the student**

ਖੋਜ ਗ੍ਰੰਥ ਦਾ ਸਿਰਲੇਖ	:	ਬੈਮੀਸੀਆ ਟੈਬੇਸਾਈ ਵਿੱਚ ਆਰ ਐੱਨ ਏ ਇੰਨਟਰਫੈਰੇਂਸ ਰਾਹੀਂ *TOR* ਜੀਨ ਦੇ ਕੰਮ ਦਾ ਅਧਿਐਨ
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ਮੁੱਖ ਸਲਾਹਕਾਰ ਦਾ ਨਾਮ **ਅਤੇ ਅਹੁੱਦਾ**		ਡਾ ਵਿਕਾਸ ਜਿੰਦਲ ਸੀਨੀਅਰ ਐਨਟੋਮੋਲੋਜਿਸਟ
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ਸਾਰ ਅੰਸ਼

ਚਿੱਟੀ ਮੱਖੀ ਵਿੱਚੋਂ ਟਾਰਗੈਟ ਆਫ਼ ਰਾਪਾਮਾਇਸੀਨ (*TOR*) ਜੀਨ ਨੂੰ ਦੱਸ ਟੁਕੜਿਆਂ ਵਿੱਚ ਵਧਾਇਆ ਗਿਆ। *TOR* ਜੀਨ ਦੀ ਪੂਰੀ ਲੰਬਾਈ 7311 ਬੇਸ ਪੇਅਰ ਨਿਊਕਲੀਓਟਾਇਡਜ਼ ਅਤੇ 2437 ਐਮੀਨੋ ਐਸਿਡ ਪਾਏ ਗਏ। *TOR* ਜੀਨ ਦੇ ਨਿਊਕਲੀਓਟਾਈਡਜ਼ ਬੈਮੀਸੀਆ ਟੈਬੇਸਾਈ ਦੇ ਉਪਲਬਧ ਸੀਰੀਨ/ਥ੍ਰਿਓਨਾਇਨ-ਪਰੋਟੀਨ ਕਾਈਨੇਜ ਦੇ ਨਾਲ 98.66 ਪ੍ਰਤੀਸ਼ਤ ਸਮਾਨਤਾ ਪਾਈ ਗਈ। *ਬੀ. ਟੈਬੇਸਾਇ* ਵਿੱਚ *TOR* ਜੀਨ ਦੇ ਅਨੁਮਾਨਿਤ ਲੜੀ ਤੋਂ 95 ਨਿਊਕਲੀਓਟਾਈਡ ਵੱਖਰੇ ਪਾਏ ਗਏ ਸਨ। ਅਮੀਨੋ ਐਸਿਡ ਦੀ ਸਥਿਤੀ 1448 ਜਿੱਥੇ ਐਸਪਾਰਾਜੀਨ, ਥ੍ਰਿਓਨਾਈਨ ਦੁਆਰਾ ਬਦਲਿਆ ਗਿਆ ਅਤੇ 1768 ਸਥਿਤੀ ਤੇ ਆਰਜੀਨਾਇਨ; ਟ੍ਰਿਪਟੋਫੈਨ ਦੁਆਰਾ ਉਪਲੱਬਧ *TOR* ਜੀਨ ਦੇ ਮੁਕਾਬਲੇ ਬਦਲੇ ਹੋਏ ਪਾਏ ਗਏ ਸਨ। *TOR* ਜੀਨ ਦੇ ਅਮੀਨੋ ਐਸਿਡ ਦੇ ਕ੍ਰਮ ਦੇ ਫਾਈਲੋਜੈਨੇਟਿਕ ਟਰੀ ਤੋਂ ਦੇਖਿਆ ਕਿ *ਬੀ. ਟੈਬੇਸਾਇ*; *ਐਕ੍ਰੀਥੋਸਾਈਫਮ ਪਾਇਸਮ* ਅਤੇ *ਨੀਲਾਪ੍ਰਵਤਾ ਲੁਗਨਜ* ਇੱਕ ਦੂਜੇ ਨਾਲ ਕਾਫੀ ਸੰਬੰਧਿਤ ਹਨ ਅਤੇ ਇੱਕ ਸਮੂਹ ਵਿੱਚ ਪਾਏ ਗਏ ਸਨ। *dstor* (@ 1.0, 0.5 ਅਤੇ 0.1 µg/µl) ਅਤੇ *dsgfp* (@ 1.0, 0.5 ਅਤੇ 0.1 µg/µl) ਨੂੰ ਬਣਾਇਆ ਗਿਆ ਅਤੇ ਚਿੱਟੀ ਮੱਖੀ ਦੇ ਬਾਲਗਾ ਨੂੰ ਖੁਆਇਆ ਗਿਆ। ਬਨੋਟੀ ਭੋਜਨ ਖੁਆਉਣ ਉਪਰੰਤ, ਚਿੱਟੀ ਮੱਖੀ ਦੇ ਜੀਵਨ ਕਾਲ ਤੇ ਉਸ ਤੇ ਪੈਣ ਵਾਲੇ ਮਾੜੇ ਅਸਰ ਨੂੰ ਰਿਕਾਰਡ ਕੀਤਾ ਗਿਆ। ਲਗਾਤਾਰ dsRNAs ਖੁਆਉਣ ਤੋਂ ਬਾਅਦ ਇਹ ਪਤਾ ਲੱਗਿਆ ਕਿ ਵੱਧ ਤੋਂ ਵੱਧ ਮੌਤ (47.75%) ਖਾਣੇ ਦੇ 48 ਘੰਟਿਆ ਬਾਅਦ ਵਾਪਰੀ ਜਦੋਂ ਚਿੱਟੀ ਮੱਖੀ ਨੂੰ *dstor* @ 1.0 µg/µl ਖੁਆਇਆ ਜਾਂਦਾ ਸੀ, ਜੋ ਕਿ *dstor* ਦੇ ਸਾਰੇ ਟ੍ਰੀਟਮੈਂਟ ਦੇ ਬਰਾਬਰ ਸੀ ਅਤੇ *dsgfp* ਅਤੇ ਕੰਟਰੋਲ ਨਾਲੋਂ ਕਾਫੀ ਜ਼ਿਆਦਾ ਸੀ। 48 ਘੰਟਿਆਂ ਲਈ ਬਨਾਉਟੀ ਭੋਜਨ ਖਵਾਉਣ ਤੋਂ ਬਾਅਦ ਚਿੱਟੀ ਮੱਖੀ ਦੇ ਜੀਵਨ ਕਾਲ ਦੇ ਪ੍ਰਭਾਵ ਦਾ ਅਧਿਐਨ ਕੀਤਾ ਗਿਆ। ਇਹ ਪ੍ਰਯੋਗ ਤਿੰਨ ਵਾਰ ਜੂਨ-ਜੁਲਾਈ, ਜੁਲਾਈ-ਅਗਸਤ ਅਤੇ ਅਗਸਤ-ਸਤੰਬਰ 2019 ਵਿੱਚ ਕੀਤਾ ਗਿਆ। 48 ਘੰਟਿਆਂ ਬਾਅਦ ਬਾਲਗ ਦੀ ਮੌਤ ਦਰ (46.66, 41.66 ਅਤੇ 26.25 ਪ੍ਰਤੀਸ਼ਤ) *dstor* (@ 1.0, 0.5 ਅਤੇ 0.1 µg/µl) ਵਿੱਚ ਸਭ ਤੋਂ ਵੱਧ ਪਾਈ ਗਈ ਜੋ ਕਿ *dsgfp* ਅਤੇ ਕੰਟਰੋਲ ਨਾਲੋਂ ਵੀ ਵੱਧ ਸੀ। ਦੋ ਪ੍ਰਯੋਗਾਂ ਤੋਂ ਪਤਾ ਲੱਗਿਆ ਕਿ *TOR* ਜੀਨ ਦੀ ਸਾਇਲੈਸਸਿੰਗ ਦਾ *ਬੀ. ਟੈਬੇਸਾਈ* ਦੀ ਅੰਡੇ ਦੇਣ ਦੀ ਸਮਰੱਥਾ ਤੇ ਬਹੁਤ ਬੁਰਾ ਪ੍ਰਭਾਵ ਪਿਆ ਜੋ ਕਿ *dsgfp* ਅਤੇ ਕੰਟਰੋਲ ਨਾਲੋਂ ਘੱਟ ਸੀ। ਸਭ ਤੋਂ ਵੱਧ ਅੰਡੇ ਦੇਣ ਦੀ ਸਮਰੱਥਾ 70.00 ਅਤੇ 64.77 ਪ੍ਰਤੀ ਮਾਦਾ *dsgfp* (@0.1 µg/µl ਵਿੱਚ ਸੀ। *TOR* ਜੀਨ ਦੀ ਸਾਇਲੈਂਸਿੰਗ ਨਾਲ ਪਹਿਲੇ ਇੰਸਟਾਰ ਵਿੱਚ ਮੌਤ ਦਰ ਜ਼ਿਆਦਾ ਦੇਖਿਆ ਗਿਆ। ਇਹ ਮੌਤ ਦਰ *dsgfp* ਦੀ ਸਾਰੀਆਂ ਮਾਤਰਾਵਾਂ ਅਤੇ ਕੰਟਰੋਲ ਨਾਲੋਂ ਵੱਧ ਸੀ । ਬੱਚਿਆਂ ਦਾ ਮੌਤ ਦਰ ਪਹਿਲੇ ਇੰਨਸਟਾਰ ਤੋਂ ਇਲਾਵਾ ਬਾਕੀਆਂ ਵਿੱਚ ਕੋਈ ਅੰਤਰ ਨਹੀਂ ਦੇਖਿਆ ਗਿਆ। *TOR* ਜੀਨ ਸਾਇਲੈਂਸਿੰਗ ਨਾਲ ਚਿੱਟੀ ਮੱਖੀ ਦੇ ਬੱਚਿਆਂ ਦਾ ਜੀਵਨ ਕਾਲ, ਕੋਆ ਦਾ ਜੀਵਨ ਕਾਲ, ਕੋਆ ਮੌਤ ਦਰ ਅਤੇ ਬਾਲਗ ਦੇ ਨਿਕਲਣ ਤੇ ਕੋਈ ਅਸਰ ਨਹੀਂ ਦੇਖਿਆ ਗਿਆ। 48 ਘੰਟਿਆਂ ਲਈ ਚਿੱਟੀ ਮੱਖੀ ਨੂੰ *dstor* ਖਵਾਉਣ ਤੇ ਇਹ ਦੇਖਿਆ ਗਿਆ ਕਿ *TOR* ਜੀਨ ਦਾ dstor @ 1.0, 0.5 ਅਤੇ 0.1 µg/µl ਤੇ 55.34, 57.21 ਅਤੇ 63.36 ਪ੍ਰਤੀਸ਼ਤ ਪ੍ਰਗਟਾਵਾ ਹੈ, ਜੋ ਕਿ *dstor* @ 1.0 µg/µl (100 ਪ੍ਰਤੀਸ਼ਤ) ਦੇ ਮੁਕਾਬਲੇ ਘੱਟ ਹੈ। 96 ਘੰਟਿਆਂ ਲਈ ਚਿੱਟੀ ਮੱਖੀ ਨੂੰ *dstor* ਖਵਾਉਣ ਤੇ ਇਹ ਦੇਖਿਆ ਗਿਆ ਕਿ *TOR* ਜੀਨ *dstor* @ 2.0, 1.0, 0.5 ਅਤੇ 0.1 µg/µl ਤੇ 13.77, 16.52, 24.07 ਅਤੇ 28.75 ਪ੍ਰਤੀਸ਼ਤ ਪ੍ਰਗਟਾਵਾ ਹੈ ਜੋ ਕਿ *dsgfp* @ 1.0 µg/µl ਦੇ ਮੁਕਾਬਲੇ ਘੱਟ ਹੈ। ਇਸ qPCR ਵਿਸ਼ਲੇਸ਼ਣ ਤੋਂ ਇਹ ਪਤਾ ਲੱਗਾ ਕਿ *ਬੈ. ਟੈਬੇਸਾਈ* ਨੂੰ *dstor* ਖੁਆਉਣ ਤੇ *TOR* ਜੀਨ ਦੀ ਸਾਇਲੈਂਸਿੰਗ ਹੋਈ ਹੈ ।

ਮੁੱਖ ਸ਼ਬਦ: *ਬੈਮੀਸੀਆ ਟੈਬੇਸਾਇ*, RNA ਇੰਟਰਫੈਰੇਂਸ, ਜੀਨ ਸਾਇਲੈਂਸਿੰਗ, ਟਾਰਗੇਟ ਆਫ ਰਾਪਾਮਾਈਸੀਨ, ਅੰਡੇ ਦੇਣ ਦੀ ਸਮਰੱਥਾ, ਮੌਤ ਦਰ ।

ਮੁੱਖ ਸਲਾਹਕਾਰ ਦੇ ਹਸਤਾਖਰ **ਵਿੱਦਿਆਰਥੀ ਦੇ ਹਸਤਾਖਰ**

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

CHAPTER I

INTRODUCTION

Every year billions of US dollars are lost due to the decrease in crop yield caused by the attack of insect herbivores. The annual yield loss due to insect pest are recorded as 16.8 and 10.8 per cent at India and global level, respectively (Dhaliwal *et al* 2015). The utilization of chemical insecticides expanded remarkably with the commencement of the Green Revolution, bringing about generous yield for quite a while, however boomeranged with a various ecological havoc, as recorded by Rachel Carson in the book 'Silent Spring' (1962). The use of conventional pesticides is placed at last in the IPM program, yet farmers still use them as the main retreat for getting speedy outcomes. However, pesticides turn out to be highly hazardous when overused, bringing about the decay of plant and soil health, pest resurgence (Ghosal and Chatterjee 2018), insecticide resistance (Kranthi *et al* 2019), loss of beneficial fauna like honey bees (Mejias *et al* 2019), pest outbreaks and pesticide poisoning (Abrol and Shankar 2014). Insects have the capability to adapt the pesticide application posing a greater problem to world agriculture. To combat these several problems, efforts are being made to develop eco-friendly pest management tactics.

Cotton (*Gossypium hirsutum* L.) is amongst the most vital fibre crops of textile industry in the world. It plays an important role in country's economy by being a part of employment generation as well as foreign exchange. In Indian agriculture, 122.38 lakh hectare land is cultivated with cotton with production level of 36.1 million bales of seed cotton (Anonymous, 2019). Cotton is being encountered by diversity of insects from germination to harvesting which are responsible for huge yield losses. In India, the annual yield loss in cotton due to insect pests attack is estimated about 30 per cent (Dhaliwal *et al* 2015). Pink bollworm *Pectinophora gossypiella* (Saunders), whitefly *Bemisia tabaci* (Gennadius), american bollworm *Helicoverpa armigera* (Hubner), aphid *Aphis gossypii* (Glover), tobacco caterpillar *Spodoptera litura* (Fabricius) and spotted bollworm *Earias vittella* (Fabricius), leafhopper *Amarasca biguttula biguttula* (Ishida) and thrips *Thrips tabaci* (Linderman) are the most important pests of cotton (Rajendran *et al* 2018). Bt cotton was introduced in India in the years 2002 and 2006 as Bollgard I and Bollgard II, respectively against the lepidopteran pests. Transgenic cotton which is considered as a promising way for pest management, stands undermined with the field evolved resistance to Cry1Ac and Cry2Ab toxins in Bollgard II specifically by pink bollworm. Because of the wide spread adoption of GM crops expressing the insecticidal toxins against lepidopteran insects, another category of insects, called as sucking insect pests have come up as a pest of economic importance (Lu *et al* 2010). Sucking pests include whitefly, aphid, jassid and mealybug cause serious damage to crop growth but no Cry toxin is proved to be effective against these pest

(Dutt 2007).

Cotton whitefly has emerged as a pest of serious concerns by being a cosmopolitan and polyphagous pest feeding on almost 900 host species causing enormous damage to plants both directly by feeding on plant phloem, honey dew contamination, leading to fungal growth on plants which reduces photosynthetic activity and indirectly by transmitting more than 100 plant viruses particularly begmoviruses (Jones 2003, Czosnek *et al* 2017, Kanakala and Ghanim 2018). Numerous biological attributes including wide host range, multivoltine, capacity to migrate, high temperature tolerance, vector of various diseases, ability to develop resistance to various chemistries have added to trouble in creating robust and ecologically effective management strategy (Naranjo 2011). Therefore, management of whitefly in efficient way is of greater concern in modern agriculture scenario.

RNA interference is a reverse genetic tool involving the site-specific degradation of mRNA by the action of exogenously delivered double stranded RNA. Its potential in crop pest management has been reported in many insects because of its site-specific nature. It is an evolutionary conserved process in eukaryotes and was first recorded in *Caenorhabditis elegans* by targeting *unc-22* gene in a systematic and specific way (Fire *et al* 1998). dsRNA shares a complete homology with the target mRNA and upon its delivery into the organism, it gets disintegrate into shorter fragments called short interfering RNA/microRNA which disrupts the target mRNA translation to protein. This technique is known as 'Post Transcriptional Gene Silencing' which helps in identifying the functional role of different genes thus it comes under the functional genomics studies. Utilising this technique, functions of various genes have been elucidated in different groups of insects like Coleoptera, Blattodea, Orthoptera, Lepidoptera, Diptera, Hymenoptera, Orthoptera and Hemiptera (Amdam *et al* 2003, Bucher *et al* 2002, Dzitoyeva *et al* 2001, Grover *et al* 2019, Kanakala *et al* 2019, Martin *et al* 2006, Mutti *et al* 2006, Nandetty *et al* 2015, Poreddy *et al* 2017, Rajagopal *et al* 2002, Tian *et al* 2009, Tomoyasu and Denell 2004).

This technique is widely studied in different insects by artificially synthesising dsRNA and delivering it into the insect body, causing silencing of target genes which produces lethal phenotypes and mortality of insects. In brown planthopper *Nilaparvata lugens*, RNAi mediated silencing of *Nlaub* and *Nlsid*-1 (midgut genes) provided a promising evidence of controlling insects in the crop fields (Zha *et al* 2011). Reduction in the larval chitin content in African malaria mosquito (*Anopheles gambiae*) occurred by the suppression of *AgCHS1* and *AgCHS2* chitin synthase genes and it also resulted in high vulnerability to diflubenzuron (Zhang *et al* 2010). RNAi has been exploited to sterilise or reduce the fecundity of male fruit flies (Ali *et al* 2017) and mosquitoes (Whyard *et al* 2015, Thailayil *et al* 2011) and could be an effective means of generating sterile males without affecting the other characteristics of physiology.

Different delivery methods of dsRNA have been reported to transport it efficiently into the insect body like egg soaking, microinjection, oral feeding, nanocarrier and transgenic plants (Wang *et al* 2011, Arakane *et al* 2004, Tian *et al* 2009, Zheng *et al* 2018, Mao *et al* 2007). Microinjection of dsRNA into pea aphid, *Acyrthosiphon pisum*, lead to silencing of *Ap-cath-L* and *Ap-crt* that encodes cathepsin-L and calreticulin, respectively (Possamai 2007). Ingestion of dsRNA in *Bactrocera dorsalis* larvae resulted in knockdown of gut specific genes (Ali *et al* 2017).

Recently, genome of *B. tabaci* has been sequenced and number of genes have been annotated (Xie *et al* 2017). RNAi technique was used by different researchers to identify the functions of various genes in whitefly like *V-ATPase*, *AGLU*, *AQP*, *v-ATPase*, *Tre1*, *actin*, *drosophila chickadee-like gene*, *AChR-α*, *BtGATAd*¸ *BtSnap*, *actin ortholog*, *Tret and Hsp 70*, *jhe*, *ADP/ATP translocase*, *α-tubulin* and *RPL9* (Ghanim *et al* 2007, Grover *et al* 2019, Ibrahim *et al* 2017, Raza *et al* 2016, Upadhyay *et al* 2011, Vyas *et al* 2017).

Target of rapamycin (*TOR*) is serine/threonine high molecular weight kinase, which enables the cell growth, metabolism, proliferation and survival by regulating protein synthesis (Laplante and Sabatini 2012). *TOR* gene is conserved in all eukaryotic species. In *Drosophila melanogaster*, TOR pathway plays a major role in tissue development, metabolism, aging, feeding behaviour, autophagy and protein synthesis (Bateman and McNeill 2004, Patrigde *et al* 2011, Wang *et al* 2009, Tettweiler *et al* 2005, Teleman *et al* 2008). This pathway regulates anautogeny, juvenile hormone synthesis and vitellogenin synthesis in *Aedes aegypti* which were highly affected after *TOR* gene silencing (Hansen *et al* 2004, Perez *et al* 2013). A recent study in *A. aegpti* shows that juvenile hormone biosynthesis is regulated by nutrient signaling by the TOR pathway. Microinjection of dsRNA corresponding to *TOR* gene resulted in significant decrease (83 %) in mRNA level of *TOR*. Reduction of *TOR* led in lessening of JH synthesis. RNAi of *TOR* also led to decrease (50 %) in corpora allata mRNA level of JHAMT and EPOX enzymes associated with JH biosynthesis in corpora allata. Nutritional information impacts juvenile hormone level by regulating the expression of various genes associated with the JH synthesis pathway in the corpora allata. It also regulates the expression of several genes coding for proteins associated with the TOR-signalling machinery in the *A. aegpti* fat body (Perez *et al* 2013). Similarly, a regulatory interaction between TOR signaling and juvenile hormone expression was also seen in German cockroach *Blatella germanica* (Maestro *et al* 2009). TOR signalling pathway and JH biosynthesis are interrelated with vitellogenin (vg) synthesis in response to stimulation by amino acids. After feeding of insect with normal diet, concentration of amino acid increases in hemolymph, leading to the increase in the expression of TOR pathway genes and ribosomal S6K phosphorylation. Brain hormone like allatotropin works through the TOR pathway, inducing the JH biosynthesis in the corpora allata which further regulates vitellogenesis (Lu *et al* 2016). Lu *et al* (2016) also cloned

cDNA of three enzymes present in TOR pathway in *N. lugens* i.e. *S6K, TOR* and *Rheb*. In the presence of amino acids, the expression of these genes increased but silencing of these genes highly affected the vitellogenin expression. Glutamine effect on activation of TOR pathway was studied which revealed that glutamine is also involed in regulating insects fecundity. Glutamine activates the TOR pathway by ribosomal S6K phosphorylation. Silencing of glutamate synthetase has resulted in decreasing the expression of *Rheb, TOR, S6K* genes which in turn downregulate *vg* expression and fecundity in *N. lugens* (Zhai *et al* 2015). Zhuo *et al* (2017) used RNAi technique to study the function of *TOR* gene in male plant hopper. When *NlTOR* dsRNA treated males were mated with the wild type females, no offspring was produced. After the Nl*TOR* gene silencing in male brown plant hopper, the accessory gland was poorly developed which also inhibited the formation of sperm. The epithelial layer of accessory glands was entirely disintegrated and the cell organelles were collapsed. TOR pathway was also studied in *Haemaphysalis longicornis* (Umenif-Shirafiji *et al* 2012), *Bactrocera dorsalis* (Suganya *et al* 2010), *Apis mellifera* (Corona *et al* 2007) and *Drosophila melanogaster* (Kijak *et al* 2017). However, structure and role of *TOR* gene in whitefly has not been studied till now. Therefore, the present study aims to knockdown *TOR* gene in *B. tabaci* through RNAi and observe the effect of silencing on reproduction of whitefly.

Objectives

- To characterize *TOR* gene in whitefly
- To study the role of *TOR* gene in reproduction of whitefly

CHAPTER II

REVIEW OF LITERATURE

2.1 Whitefly, *Bemisia tabaci* (Gennadius) - a notorious pest

Whitefly, *Bemisia tabaci* is a disastrous cosmopolitan insect present in agricultural, ornamental and vegetable crops worldwide. *B. tabaci* is considered as a species complex of at least 44 cryptic species (Kanakala and Ghanim 2019). Among these, B biotypes of Middle East-Minor Asia 1 and the Q biotype of the Mediterranean group are the most destructive and invasive. These biotypes have different biological characteristics in terms of insecticide resistance and host preferences. Being a pest of >900 plants species, it transmits >100 plant viruses causing loss of billions of dollars in crop yield annually (Jones 2003, Sadeh *et al* 2017, Kanakal and Ghanim, 2018). It has the ability to adapt to new host species and geographical region. It has been reported from various countries like Spain, Italy, Mexico, Hawaii, Sudan, Cuba, Eygpt, Rico, Peurto, Turkey, Jordan, Portugal, France, Italy, Venezuela, Japan, India and many other countries (Thompson 2011). Except Antarctica, it is present in all the continents (Hsieh *et al* 2006). Whiteflies affect the plant growth and development both directly by feeding and indirectly by transmitting begomoviruses such as tomato yellow leaf curl virus and cotton leaf curl virus, etc. It attacks by sucking sap from phloem vessels, transmitting viral diseases and secretes honeydew which promotes growth of sooty mould which affects photosynthesis (Brown and Czosnek 2002). Increase in whitefly population in tropical and subtropical regions is largely enhanced by the indiscriminate use of pesticides and the adoption of the monoculture cropping system.

In India, nine groups of *B. tabaci* have been identified namely, Asia 1, Asia 1-India, Asia-II-1, Asia II-5, Asia II-8, Asia II-7, Asia II-11, China-3, and MEAM 1 (Ellango *et al* 2015) Genetic groups Asia -1, Asia-II-7, Asia II-8, and MEAM 1, Minor-K (genetically close to MEAM 1 (92.6 %)) were reported by Roopa *et al* (2015). In Punjab, Asia II-1 is the most prevalent cryptic species of whitefly (Jindal, unpublished). Cotton growing states have experienced five whitefly outbreaks until now. In 2015, northern India has been hit by severe whitefly attack (Kumar *et al* 2017). The most dominant approach for the control of whitefly in diverse agricultural ecosystems is the use of chemicals. Thirty-five insecticides, including six mixtures have been registered so far for whitefly management in India, even though it has developed resistance to more than 40 active ingredients of insecticides (Basit *et al* 2013). Despite the availability of a vast variety of chemistries, whitefly management still encounters several complications (Dennehy *et al* 2010). The excessive use of insecticides against whitefly has led to the emergence of many problems such as pest resurgence (Abdullah *et al* 2006), insecticide resistance (Dangelo *et al* 2018, Houndete *et al* 2010) and damaging effects on the population of biocontrol agents (Bacci *et al* 2007). Therefore, there is a need to

develop more effective management strategies as the whitefly has the ability to reshape their metabolism and adapt to the present chemicals.

2.2 RNA Interference

2.2.1 A tale of RNAi discovery

The sequences of genomes of various eukaryotic organisms have been deduced over the years however, the functions of many genes are still obscured. Strategies for crossing over the barrier between sequence and its function are clearly important. Different techniques have been utilized for suppressing the gene expression and exploitation, for instance, ribozymes and antisense oligonucleotides. RNA interference (RNAi) is an another technique which has shown significant promises to study gene functions. RNAi is an evolutionary conserved phenomenon in animals and plants in which double stranded RNA (dsRNA) initiates the target specific silencing of complementary mRNA (Fire *et al* 1998). The discovery of RNA interference (RNAi) for site specific silencing of gene by means of short interfering RNA (siRNA) has quickly made an incredible asset for functional genomic studies. Prior to the introduction of the concept of RNAi, it is also known as post-transcriptional gene silencing (PTSG) in plants (Napoli *et al* 1990) and quelling in fungi (Romano and Macino 1992).

Initially, studies related to gene inhibition were conducted by introducing homologous DNA or antisense RNA into the target organism (Rosenberg *et al* 1985, Fire *et al* 1991). In Drosophila, Rosenberg *et al* (1985) injected antisense RNA during embryonic stage which led to the inactivation of Kruppel gene. Kruppel gene is responsible for segmentation during egg development. They considered that antisense RNAs bound to the homologous RNA inside the nucleus which lead to either its degradation or prevention of its exportation to nucleus. Fire *et al* (1991) utilised antisense strategy to disrupt the gene expression of *unc 22* and *unc-54* genes present in body wall muscles of *Caenorhabditis elegans.* They estimated that disruption of gene expression was due to the binding of antisense RNA with the sense RNA leading the steric hinderance, which may block translation. In plants, Napoli *et al* (1990) observed an unexpected result of loss of purple colour pigmentation in petunia flowers. Chalcone synthase (CHS) enzyme is responsible for violet pigmentation in petunia flower. Scientist aimed at deepening the violet color of the flowers and so over expressed the chalcone synthase by introducing anti sense transgene to the flowers. It was very surprising result that all the flowers either lost the violet pigmentation or were variegated. After analysing the CHS mRNA level in flowers, it was reported that the expression level of the transcript was fifty time lesser than the wild type flowers. They named this phenomenon as ‘Co-suppression’ because both the exogenous transgene and endogenous genes were supressed. Similar kind of phenomenon was seen in fungi by Romano and Macino (1992). When *Neurospora crassa* was transformed with portions of carotenogenic genes albino-1 and albino-3, this caused the lower transcript level of endogenous *al-3* and *al-1* genes, producing

albino phenotypes. This process was named as quelling in fungi.

Fire *et al* (1998) hypothesised that the initiation of RNAi is due to double stranded RNA (dsRNA) and not due to single stranded RNA (ssRNA). They tested their hypothesis in *C. elegans* targeting *unc-22* gene and compared the ability of sense and antisense single stranded RNA with that of combination of both sense and antisense RNA to produce interfering effect. Results shown that the combination of sense and antisense RNAs which hybridised as double stranded structure produce effectual interference of unc-22 gene expression whereas sense and antisense single stranded RNA had very less interference activity individually. They come up with the conclusion that dsRNA was the major cause of gene knockdown and named this phenomenon as RNA interference (RNAi). RNAi as an important entomological research means has been utilised to elucidate the functions of various genes which are involved in physiological processes, reproduction, behavior and embryogenesis in both model and non-model insects (Belles 2010). Efficacy of RNAi depends on the type of insect species (Belles 2010, Terenius *et al* 2011), dsRNA delivery method (Scott *et al* 2013, Terenius *et al* 2011) and the gene to be knockdown by RNAi (Belles 2010).

2.2.2 RNAi mechanism

RNAi mediated silencing of gene is initiated by transporting dsRNA into the target organism. By the action of RNAse III type endonuclease enzyme called Dicer, 100-200 bp long dsRNA is shorten into 21-25 bp siRNA/miRNA. The family of multi proteins form a RISC complex called RNA-induced silencing complex which recruits the siRNA and the double stranded siRNA is cleaved and one single strand called passenger strand is degraded by the action of Argonaute protein. The other strand called guide strand directs the RISC complex to integrate with the target mRNA and degrade it by the RNAse component. This prevents the translation of mRNA into protein leading to the silencing of the gene (Siomi and Siomi 2009).

2.2.3 Delivery of dsRNA in insects

Different delivery methods of dsRNA have been employed for successful implementation of RNAi like microinjection delivery (Kennerdell and Carthew 1998), oral feeding (Timmon and Fire 1998), soaking and transfection (Tabara *et al* 1998), topical application (Wang *et al* 2011), plant mediated RNAi (Baum *et al* 2007), foliar application (Miguel and Scott 2015) and microorganism mediated delivery (Chen *et al* 2015). Through microinjection, dsRNA can be directly delivered into hemolymph or into embryo of insect. This method has advantages over other methods as the dose of double stranded RNAs and site of application are controlled. Feeding method is effective where insects exhibit robust RNAi response. This method is used to deliver dsRNAs in high-throughput way, evading the mechanical injury caused by microinjection (Scott *et al* 2013).

In insects, RNAi through soaking has been conducted in cell lines. This method has been shown to be valued as a high throughput method for studying the gene expressions at large scale (Maeda *et al* 2001). Efficacy of topical application of double stranded RNA was demonstrated in Asian corn borer, *Ostrinia furnacalis* and concluded that dsRNAs can penetrate through integument and cause stunting during larval instars and/or mortality in lepidoptern pests (Wang *et al* 2011). A break through study in RNAi was conducted by Baum *et al* (2007) who showed that silencing of the insect genes can be done when insect feed in the plants expressing dsRNAs construct. In colorado potato beetle, *Leptinotarsa decemlineata* (Say), foliar application of dsRNA targeting actin gene has shown high effectiveness (Miguel and Scott 2015). Song *et al* (2018) demonstrated that delivery of dsRNA in *Locusta migratoria* is efficient through injection instead of feeding.

2.3 RNA interference in the whitefly, *Bemisia tabaci*

With the initiation of functional genomics project in 2002, thousands of expressed sequence tags (EST) with their corresponding sequences were generated (Leshkowitz *et al* 2006). This helped in providing basic information to design experiments with the aim of understanding the specific gene expression in whitefly. Ghanim *et al* (2007) reported the effectiveness of RNAi in whitefly for the first time and targeted three different genes namely *BtCG5885, BtSnap* and *BtGATAd* present in midgut and salivary glands. Injection of dsRNA corresponding to these three genes resulted in 70 per cent reduction in the gene expression of *BtCG5885* and *BtGATAd* in salivary glands and midgut, respectively. The decrease in the gene expression level (60-75 %) of *BtSnap* was observed in midgut and salivary glands. They also knockout *Chickadee* gene in whitefly to observe the phenotypic effect of RNAi. More than 60 per cent of gene expression level was reduced in RNAi silenced ovaries. Chickadee dsRNA injected whiteflies did not survive for more than 48 hours.

Lu and Wan (2011) reported the role of heat shock proteins (*hsp*) gene in heat tolerance in both the sexes of whitefly using RT-PCR and dsRNA feeding. When observed at 37.5 to 42 °C, the mRNA expression levels of both *hsp23* and *hsp70* were found to be higher in females than in males, whereas at 44 °C, mRNA levels of *hsp70* was higher in males as compared to females. Also, the study indicated that *hsp23* or *hsp70* dsRNA fed females followed by heat shock at 44 °C for 1 hour had significantly reduced survival rate, on the other hand males survival rate was not affected significantly. The results concluded that the expression of *hsp* mRNA promotes higher survival rate of females under heat shock conditions.

Similarly, Upadhyay *et al* (2011) explored RNAi in whitefly and selected five genes for silencing *viz*., *ADP/ATP translocase, actin ortholog, alpha-tubulin, V-ATPase A subunit and ribosomal protein L9* (*RPL9*). For oral delivery of si/dsRNA, an effective and simple insect bioassay method was developed. It was observed that 29-97 per cent of mortality

occurred upon targeting these genes after six days of feeding. Among all the different genes, silencing of V-*ATPase* and *RPL9* caused higher mortality with LC50 3.08 and 11.21ug/ml, respectively. Also, it was found that siRNAs delivered through insect diet remained stable for at least 7 days when kept at room temperature. This study provided insight for the potential use of RNAi technology in whitefly control.

Cytochrome P450 monooxygenases are responsible for high resistance to neonicotinoids in *B. tabaci* B biotype (MEAM 1) and Q biotype (Mediterranean group). When dsRNAs corresponding to *P450 CYP6CM1* genes were introduced into the body of adult whiteflies of both the biotypes, it was observed that silencing effect was more effective in B biotype as compared to Q biotype. Mortality of 85.88 and 56.40 per cent was observed in B biotype and Q biotype, respectively. Additionally, the ability of detoxification of imidacloprid and nicotine was inhibited by treating the adults with dsRNA (Li *et al* 2014).

For plant-mediated RNAi, transgenic lines of tobacco were developed to express dsRNA corresponding to *V-ATPase A* mRNA. When whiteflies were fed on these plants, the transcript level of the target gene was lowered down to 62 per cent (Thakur *et al* 2014). Similarly, Malik *et al* (2016) also generated transgenic plants of tobacco, *Nicotiana tabacum* expressing the dsRNA corresponding to *aquaporin* and *sucrase* gene, *alpha glucosidase* (*AGLU*). Both the genes were involved in osmoregulation in sap sucking plants and the aim of the study was to disrupt the osmoregulation in insects so as to increase mortality. qRT-PCR results showed that after six days of feeding the transcript level of both the genes were reduced and mortality was observed more than 70 per cent. The same kind of study was conducted in lettuce (*Lactuca sativa*) in which transgenics lettuce plants expressed dsRNA corresponding to the v-ATPase gene. After five days of feeding of whiteflies on these plants, it was observed that 83.8 - 98.1 per cent mortality was occurred due to silencing of the gene. Also, a reduced number of eggs were recorded on the transgenic lines as compared to the control lines of lettuce (Ibrahim *et al* 2017). Thus, this technology paved the way for the development of whitefly resistant varieties of major food crops.

Asokan *et al* (2015) also utilized RNAi machinery in whitefly to knockdown Glutathion S-transferase of sigma class which is responsible for detoxification of plant secondary metabolites and involved in insecticide resistance. A diet containing different concentrations of dsRNA i.e. 1.0, 0.5 and 0.25 µg/µl were fed to whiteflies and Real-time quantitative analysis showed that the expression levels of *BtGST* was reduced by 77, 65 and 53 per cent and this resulted in 77, 59 and 40 per cent mortality of whitefly, respectively.

Utilization of microorganism particularly entomopathogenic fungi for the control of whiteflies shows greater potential with RNAi technology. Chen *et al* (2015) constructed a plasmid containing dsRNA specific to Toll-like receptor 7 genes of *B. tabaci* B-biotype and introduced it into *Isaria fumorosea.* qPCR results showed that the expression levels of *TLR7*

gene were lower in the whiteflies infected by *I. fumorosea* containing the recombinant plasmid. To identify the basis of resistance to abamectin in insects, Wei *et al* (2018) cloned and characterized glutamate-gated chloride channels from whitefly. The expression level of *BtGluCl* mRNA was significantly reduced by 62.9 per cent in whitefly adults on ingestion of diet containing *dsBtGluCl* and lowered down the mortality induced by abamectin.

Role of Juvenile hormone esterase in whitefly survival and reproduction was studied through RNA interference mechanism. Artificial diets (20 % sucrose solution) containing different concentrations of dsRNA corresponding to juvenile hormone esterase transcript were fed to whiteflies. qRT-PCR analyses showed that *jhe* gene expression was reduced in adult whiteflies and also had adverse effects on the biology of the next generations of whitefly. Reduced egg hatchability and shortened egg incubation period were observed when the adults were fed with *dsjhe* @ 2.5 and 1.0 µg/µl (Grover *et al* 2019).

Whitefly has gained resistance against neonicotinoid insecticide-thiamethoxam (Horowitz *et al* 2004). To validate the involvement of hydroxyacid-oxoacid transhydrogenase (HOT) in causing resistance of whitefly towards thiamethoxam, Yang *et al* (2017) silenced HOT gene and found that the expression of this gene decreased by 57 per cent and significant decrease in thiamethoxam resistance after two days of dsRNA feeding was observed.

Since *B. tabaci* is an important agricultral pest worldwide. Adoption of RNAi technique to manage this pest could help in saving billons of dollars with regard to crop yield and also decreasing the relience on chemicals. It can be believed that RNAi may be more stable and effective means of protecting the crops against insect-pests.

2.4 *Target of rapamycin*

mTOR is the mammalian/mechanistic *target of rapamycin* belonging to phosphatidylinositol kinase related (PIKK) family. It is a serine/threonine kinase that acts as a major component of eukaryotic signalling pathway. It is structurally and functionally conserved throughout the evolution from yeast to mammals and is the target of clinically used drug called rapamycin (Guertin *et al* 2006). It is a very well-studied protein complex and there are various regulators and targets of mTOR. mTOR serves as a master regulator of cell metabolism, growth, proliferation and survival. In insects, TOR pathway regulates juvenile hormone synthesis, anautogeny, ecdysteroidogenesis and vitellogenesis which were severely affected after *TOR* gene knockdown (Hansen *et al* 2004, Perez *et al* 2013, Gu *et al* 2012). It forms the catalytic subunit of two distinct complexes known as mTOR complex 1 and mTOR complex 2 (Loewith *et al* 2002). Rapamycin was first discovered in 1964 on the Easter Island of Rapa Nui and have immunosuppressive and anti-tumor properties (Eng *et al* 1984, Martel *et al* 1977, Vezina *et al* 1975). It is an intense inhibitor of cell multiplication, blocks signal transduction and forestalls phosphorylation of ribosomal S6 in cellular systems.

2.4.1 mTORC1

mTORC1 consists of 3 core components (i) mTOR (ii) a scaffold protein- RAPTOR (regulator protein associated with mTORC1), RAPTOR allow mTOR to bind and its localization on its substrate i.e lysosomal membrane (Nojima *et al* 2003, Schalm *et al* 2003) (iii) mLST8 (mammalian lethal with Sec 13 protein 8, also known as GβL) which stabilizes the kinase loop within the mTOR complex, it allows mTOR to phosphorylates its target (Yang *et al* 2013). There are also two different protein that actually have an inhibitory role in the complex (1) PRAS40 (protein rich AKT substrate of 40 KDa (Sancak *et al* 2007, Vander *et al* 2007 and Wang *et al* 2007) (2) DEPTOR (DEP domain containing mTOR interacting protein (Peterson *et al* 2009). When the activity of mTORC1 is reduced, PRAS40 and DEPTOR promotes the inhibition of mTORC1. Upon activation, mTOR phosphorylates PRAS40 and DEPTOR which reduces the binding of these protein to mTOR and thus leads to activation of mTOR (Peterson *et al* 2009, Wang *et al* 2007). mTORC1 is sensitive to rapamycin-FKBP12 complex. The effects of rapamycin on mTOR signalling are much more complex, it forms complex with FKBP12 and interact with FKBP12-rapamycin binding domain (FRB) of mTOR and thus inhibits mTORC1 functions in its kinase activity (Guertin *et al* 2007).

2.4.2 mTORC2

The main difference in mTORC2 from mTORC1 is the presence of RICTOR instead of RAPTOR. RICTOR is the rapamycin insensitive companion of mTOR. RICTOR may block the FRBK12-rapamycin binding site thus exhibiting insensitivity to acute rapamycin treatment (Jacinto *et al* 2004, Sarbassov *et al* 2004) but extended rapamycin treatment may make it sensitive (Lamming *et al* 2012, Sarbassov *et al* 2006). As RAPTOR, RICTOR also allow mTOR to actually binds to its substrate. Other components are mLST8 having same function as in mTORC1 and also having inhibitory protein called DEPTOR (Peterson *et al* 2009). It also has two regulatory protein called PROTOR ½ (Pearce *et al* 2007, Thedieck *et al* 2007, Woo *et al* 2007) and mSIN1 (Frias *et al* 2006, Yang *et al* 2006). The two complexes are similar in some ways but different in terms of its regulation and its ability to bind to certain targets.

2.4.3 TOR pathway

2.4.3.1 Factors activating/deactivating mTOR

mTOR is typically regulated at the interface of lysosomal membrane by several major extracellular and intracellular cues mainly growth factors (insulin/IGF), energy status (ATP), stress, oxygen, DNA damage and amino acids (Long *et al* 2005, Sancak *et al* 2007). Lysosome acts as a recruiting platform for mTOR and also participates in amino acids sensing (Sancak 2008, Sancak 2010). Previous studies in *Drosophila melanogaster* have revealed that TOR signally pathway is controlled by the fluctuation in the amino acid level (Zhang *et al*

2000) as well as insulin signaling pathway (Lizcano *et al* 2003, Miron *et al* 2003). mTOR pathway modulates several major processes including mRNA translation (Richter *et al* 2005), ribosome biogenesis (Hannan *et al* 2003), nutrient metabolism (Peng *et al* 2002) and autophagy (Meijer and Codogna 2004).

2.4.3.1.1 Extracellular and intracellular cues in mTOR activation

Tuberous Sclerosis Complex 1 and Tuberous Sclerosis Complex 2 are the major upstream regulator of mTOR. They negatively regulate mTOR by converting activated Rheb-GTP to its inactive GDP bound state. TSC is activated and inhibited by several proteins (Long *et al* 2005, Sancak *et al* 2007). Rheb generally activate mTOR. AMPK-activated protein kinase activates TSC2 by phosphorylating it when energy status is low (Inoki *et al* 2003). Several other factors like AMP, LKB2 activate AMPK.

The other major regulator of mTOR is Akt which is activated in presence of growth factors like insulin and Ras signalling pathway (Inoki *et al* 2002, Potter *et al* 2002) inactivating TSC and thus activating mTOR (Sancak *et al* 2007, Vander Haar *et al* 2007 and Wang *et al* 2007). Certain other regulators are also present like ERK1/2 which inhibits TSC. ERK1/2 is actually regulated by RAS (Ma *et al* 2005). P53 is the activator of TSC2 and is traditionally known to be activated by certain cellular structure such as DNA damage (Feng *et al* 2007).

2.4.3.1.2 Amino acids in mTOR activation

Under enough availability of nutrient, TOR is involved in positive growth of body. There is also regulation at RAG site. RAG is known to be inhibited by GATOR1 whereas GATOR1 is inhibited by GATOR 2. CASTOR is the sensor of arginine (Chantranupong 2016, Saxton *et al* 2016). GATOR 2 itself is inhibited by CASTOR 1 which is inhibited by Arginine eventually suggesting that arginine activates GATOR 2 which activates Rag and ultimately mTOR. Earlier it was suggested that CASTOR1 experience change in conformation during its binding with arginine. However, the recent study reveals that CASTOR1 structure, both in arginine bound state and arginine free state has near identical structural conformation indicating that it does not suffer much conformational change during its arginine-binding process (Zhou *et al* 2018). Another amino acid called leucine inhibits Sestrin. Sestrin is another inhibitor of GATOR 2 (Saxton *et al* 2016, Wolfson 2016). So, leucine is the activator of mTOR through its ability to inhibit Sestrin. Thus, arginine and leucine both activates mTOR. Recently, in *Nilaparvata lugens*, it was experimentally demonstrated that glutamine activates TOR pathway by positively acting on AKT and inhibiting the 5' AMP-activated protein kinase AMPK phosphorylation activity in the pest. Thus, Glutamine also activates the TOR pathway in vivo. Initiation of moulting of larval instars is mediated by the presence of amino acids sensitive pathway in *Manduca sexta*. When the TOR inhibitor rapamycin is applied on the larval stages, the growth of prothoracic glands

suppressed with relative to the whole body which is further accompanied by the suppression of ecdysone production. Increased level of rapamycin also effects the growth rate negatively revealing that the TOR signalling in combination of nutrients plays a role in systemic growth (Karen *et al* 2012).

2.4.3.2 Effects of mTOR activation/deactivation

In response to favourable growth conditions like the availability of nutrients and deficit of rapamycin, TOR participates in several metabolic processes like mRNA translation, protein synthesis and DNA transcription at anabolic level and also inhibits various catabolic activities like autophagy and mRNA degradation (Diaz-Troya *et al* 2008)

2.4.3.2.1 Protein synthesis

mTOR is majorly involved in protein synthesis by activating P70S6k (S6K) by its phosphorylation. P70S6K activates eIF4B (eukaryotic translation initiation factor 4F complex) which is involved in elongation of protein chain or polypeptide strand within the ribosome. P70S6K can also directly involve in protein translation by phosphorylation of ribosomal S6 unit (Holz *et al* 2005). P70S6K also inhibits eEF2K (eukaryotic elongation factor 2 kinase) which negatively regulates protein formation, thus allowing eEF2 (positively regulate protein synthesis) to form protein in ribosome. Action of S6K is initiated by *TOR* facilitated phosphorylation, which eventually increases translation of mRNA transcripts (Kimball and Jefferson 2004).

2.4.3.2.2 Pyrmidine synthesis, lipid formation and glucose metabolism

mTOR also promotes nucleotide synthesis for the DNA replication in the cell. Activated S6K phosphorylates CAD (carbamoyl-phosphate synthetase 2, aspartate transcarbamylase and dihydrotase) which is involved in and indispensable for pyrimidine synthesis (Ben-Sahra *et al* 2013, Robitaille *et al* 2013). mTOR promotes lipid formation by activating SREBP (sterol responsive element binding protein) through S6K (Duvel *et al* 2010) or by inhibiting Lipin 1(Peterson *et al* 2011) which inhibits SREBP. mTOR is also involved in glucose metabolism thus regulating the growth of organism. mTOR increases the translation of transcription factor HIFI alpha which promotes glucose metabolism (Duvel *et al* 2010).

2.4.3.2.3 Regulation of protein turnover

The replacement of older protein with the formation of newer protein for maintaining the level of protein formation and degradation is called as protein turnover. After the formation of polypeptide chains, prevention of the degradation of these proteins becomes the main purpose of the cell. For the prevention of autophagy, mTORC1 inhibits ulK1 (which is known as ATG-Autophagy regulating gene in insects) by phosphorylating it, as it is involved in autophagy (Kim *et al* 2011). When prolonged mTOR activation occurs, it triggers compensatory hike in protein turnover for balancing the increased protein synthesis rate.

mTOR phosphorylates TFEB, transcription factor EB which drives the expression of genes for lysosomal biogenesis (Martina *et al* 2012, Settembre *et al* 2012).

2.4.4 Role of TOR pathway in insects

2.4.4.1 Autophagy

Autophagy is a physiological process involved in degradation of cellular content including protein and organelles for the maintenance of homeostasis in the cell. It is also called the self-cannibalism process and type II programmed cell death occur in response to unfavourable conditions like nutritional stress, hormone stimulation and starvation. Autophagy is generally of three kinds in cell i) Macroautphagy ii) Microautophagy iii) Chaperone-Mediated Autophagy. It involves the degradation of bigger cellular components like protein aggregates and damaged organelles. All types of degradation of substrates occurs in lysosome whether those substrates be proteins, lipid droplets or organelles. Autophagy occurs by the sequestering of the waste material in the autophagosomes, a double membrane vesicle, which transfer the cargo into lysosome for degradation and then recycling occurs. However, increased autophagy can lead to autophagic programmed cell death. *Target of rapamycin* pathway is one of the important pathways which regulate this biological process. TOR pathway is activated in the presence of nutrients, when nutrients are available, Class I phosphatidylinositol-3-kinase (PI3K) signalling pathway activates TOR and then TOR inhibits autophagy by phosphorylation of ATG1 (autophagy regulated genes). In this same way, when nutrients are in restricted status, TOR pathway is inactivated and autophagy is stimulated. However, AMPK inactivates TOR and bring on autophagy even under availability of nutrients in response to calcium signalling in mammals**.** During metaphorphosis in Drosophila, starvation in this period induces autophagy by inhibiting PI3K/ mTOR pathway (Tracy *et al* 2013, Rusten *et al* 2004).

Application of botanical pesticides in lepidopterans lead to the induction of autophagy. The molecular mechanism behind the autophagy and apoptosis of SL-1 cells after the application of azadirachtin is the blocking of the activation of signalling pathways including downstream *TOR*, PI3K and AKT in *Spodotera litura* (Shao *et al* 2016). Another study reported the activation of mitophagy and nucleophagy followed by autophagy in Sf9 cells of *Spodoptera furgiperda* upon the application of spinosad is mediated by the inhibition of PI3K/AKT/TOR pathway (Yang *et al* 2017). The physiological-biochemical approach involved in study of autophagy in lepidopterans has recognised several signals which are required for the activation and regulation of autophagy. Also, ecdysone 20E is involved in triggering autophagy in the fat body and suggested that the onset of autophagy can be averted by the application of ligatures behind Weismann gland (Priester *et al* 1979, Dean 1978). A single beat of 20E infused in the hemocoel is adequate to initiate autophagy in *B. mori* fat body (Owa *et al* 2008). A new era emerging in the last fifteen years is basically focused on

the molecular perspectives of autophagy and is chiefly initiated after the genome sequencing of *B. mori.* Several autophagy related genes (ATG) were identified in Lepidoptera for example, ATG1, ATG2, ATG3, ATG4, ATG5, ATG6, ATG7, ATG9, ATG11, ATG12, ATG13, ATG16, ATG18 (Tian *et al* 2013, Zhang *et al* 2009, Owa *et al* 2008, Casati *et al* 2012). Tian and his collegues clearly showed that ATG genes are upregulated by injecting 20E titre in the larva of *B. mori* and subsequently inhibited *target of rapamycin* complex 1 (Tian *et al* 2013). 20E is eventually leading to the increase in the autophagy by two distinctive ways, firstly by acting on ecdysone receptor to actuate ATG genes transcription and secondly by inhibiting the TORC1/PI3K pathway, permitting triggering of downstream ATG13/ATG11 complex and starting autophagosome arrangement. Similarly, in fat bodies of Drosophila, TOR signalling is inhibited by 20E initiated autophagy (Rusten *et al* 2004). Also, autophagy in Drosophila can be spiked by injecting the larva with rapamycin, a TOR inhibitor (Tian *et al* 2013).

In many insects, cells and tissues which are deprived of nutrients show autophagic response (Khoa and Takeda 2012, Scott *et al* 2004). *TOR* is the key mediator which negatively regulate autophagy (Table 1) and is activated by PI3K signalling pathway which effects the repression of autophagy regulated genes mainly ATG1. In Drosophila, it has been shown that autophagy in the fat body is regulated by *TOR* and PI3K pathways under nutritional stress and presence of ecdysone (Rusten *et al* 2004). TOR signalling pathway plays vital role in the induction autophagy of cell in response to the nutrients availability and energy metabolism.

Table 1. Autophagy in different insects due to the involvement of *TOR* gene

Insects	**Target part in insect body where autophagy is seen**	**References**
Spodoptera litura	Ovarian cells	Shao *et al* (2016)
Spodoptera furgiperda	Sf 9 cells	Veeran *et al* (2019)
Drosophila melanogaster	Midgut cells, salivary gland, fat body in larval stage	Lee and Baehrecke (2001), Jones and Bowen (1993), Lee *et al* (2002), Butterworth and LaTendresse (1973)
Bombyx mori	Fat body	Tian *et al* (2013)

2.4.4.2 Ageing

Decreased activity of TOR pathway in conjunction with Insulin/Igf like signalling pathway is (IIS) responsible for extending the life span of *C. elegans* and Drosophila (Vellai

et al 2003, Kapahi *et al* 2004). Regulation of various genes like *Dtor, Ds6K, dTsc1, dTsc2* for reducing the activity of TOR pathway increases the lifespan and also treatment of rapamycin inhibit the activity of *TOR* leading to extend lifespan. Rapamycin induces autophagy and also decreases the protein translation. Both these results of rapamycin might be the cause in the extending the lifespan because S6 activation and hindering of autophagy obstruct the expansion in life span (Bjedov *et al* 2010). Dietary restriction is the decrease in food consumption that leads to starvation or malnutrition, moreover it leads to increasing lifespan and reducing fecundity in several organisms (Mair and Dillin 2008). Over expression of 4EBP, downstream *TOR* effector can expand lifespan and is also necessary for giving response to dietary restriction on food having yeast extract.

2.4.4.3 Vitellogenesis

Vitellogenesis is the process of formation of yolk protein in the fat body through amino acid signalling which is then passed into haemolymph and eventually gets deposited in the oocytes of developing eggs (Raikhel and Dhadialla 1992**).** Various studies have been reported which shows the important role of TOR pathway in vitellogenesis and other important functions (Table 2)**.** In female mosquitoes, amino acids derived from the blood meal, play an important role in enabling 20 hydroxyecdysone to stimulate Vg expression (Hansen *et al* 2004). *Target of rapamycin* (TOR) signalling pathway in female mosquitoes deduces amino acid nutritional information to activate yolk protein precursor genes in the fat body. Without the infusion of blood meal in mosquitoes, 20E alone is not enough to activate egg development (Lea 1982). This involves the presence of amino acids and a signal transporter pathway. Knockdown of two important proteins involved in TOR signalling pathway named as *TOR* and upstream protein TSC2 confirmed the role of *TOR* signalling in vitellogenesis process in *A. agepti*. Knockdown of *TOR* gene also inhibited the egg development in *A. agepti* (Hansen *et al* 2004). Thus, TOR pathway signalling in response to the presence of amino acids in blood meal is necessary for the production and expression of yolk protein precursors. In oriental fruit fly, *Bactrocera dorsalis*, importance of TOR pathway/S6K in yolk protein synthesis was reported (Suganya *et al* 2010, 2011). Similarly, involvement of downstream target S6K was also confirmed through RNAi in adult females *A. agepti*. Expression of *Aa*TOR is somewhat varied in fat body after blood feeding (Hansen *et al* 2005). Incongruence to this report, *TOR* expression in *Haemaphysalis longicornis* is hiked in fat body after 2 days of feeding and then decreased on 4^{th} day after feeding. The regulatory region present on Vg gene contains GATA factors which are responsible for stage and tissue specific increase in Vg expression (Kokoza *et al* 2001). In anautogenous *A. agepti*, GATA (AaGATAa) factor is identified and considered to act as transcriptional initiator of Vg and is regulated by TOR signalling pathway (Park *et al* 2006). TOR nutritional signalling pathway is also involved in the secretion and translation of early trypsin protein in the midgut of *A.*

Table 2. RNA interference technique used in different insects elucidate the function of *TOR* genes and the other related genes present in TOR pathway

Insect	Gene	Effect	References
Tribolium castaneum	*TcTOR*	1. Growth rate reduced 2. Arrested development at larval pupal transition and at pupal stage and did not molt into adults 3. Reduction in body size elytra, wings, legs and mandibles	Lin *et al* (2019)
Aedes aegypti	*i) AaS6K, AaGATAa, AaTOR, AaRHeb*	1. Reduction in vitellogenin expression 2. Inhibition of vitellogenin gene activation and egg development 3. Reduction in number of eggs deposited 4. Reduction in size of ovary 5. Reduced early trypsin protein levels	Hansen *et al* (2005), Park *et al* (2006), Hansen *et al* (2004), Brandon *et al* (2008), Roy and Raikhel (2012)
Haemaphysalis longicornis	*HITOR*	1. Inhibition of S6K phosphorylation in fat bodies 2. Vitellogenin expression decreased Ovaries had white colored oocytes as compared to brown and closed in control	Umenif-Shirafiji *et al* (2012)
Blatella germanica	*BGTOR*	1. Inhibition of JH synthesis in adult female CA 2. Reduction in mRNA level of 3-hydroxy-3-methylglutanyl coenzyme-A-synthase I, HMG-CoA synthase-2 and HMG-CoA reductase 3. Reduction in vitellogenin mRNA and ovaries did not grow	Maestro *et al* (2009)
Bactocera dorsalis	*BdTOR*	Reduction in ovary size and yolk protein synthesis	Suganya *et al* (2010)
Nilaparvata lugens	*NlTOR*	1. Effect on male reproductive system particularly accessory glands and cell organelles 2 Reduced fecundity in females	Zhou *et al* (2017), Zhai *et al* (2015)
Drosophila melanogaster	*TOR*	1. Decrease in locomotive activity rhythm period by 0.5 hour 2. Total activity of flies decreased	Kijak *et al* (2017)

agepti (Bradon *et al* 2008). An upstream activator of *TOR* pathway, Rheb GTPase is essential for AA mediated activation of *TOR* in fat body of *A. agepti.* In *N. lugens*, TOR pathway regulates the fecundity by mediating vitellogenin (Vg) expression. Combined analysis of iTRAQ (isobaric tags for relative and absolute quantification) and DGE (tag based digital gene expression) data in *N. lugens* at protein and transcript levels after glutamate synthetase RNAi revealed that 52 pathways were overlapped, and TOR was also one of them (Zhai *et al* 2015).

2.4.4.4 Ecdysteroids production

TOR signalling pathway plays a crucial role in ecdysteroids production and metamorphosis in combination with nutrient sensing in prothoracic gland (PG). Layalle *et al* (2008) reported that slight TOR complex 1 inhibition in the PG triggered prolonged larval development without affecting the growth rate which resulted in overgrowth phenotypes. This showed that signalling in PGs is necessary for ecdysteroids production in larval-pupal transition. Also, there was a decrease in the expression of *phantom* (*phm*) and *disembodied* (*dib*) genes which are involved in ecdysteroid biosynthesis. Additionally, when larvae feed on 20E, developmental delay could be rescued. In the same way, activated TOR signalling can also rescue developmental delay under nutrition stress by restoring ecdysteroid production. These results indicate that TOR signalling pathway also participates in controlling ecdysteroids production in the prothoracic glands in reaction to environmental signals.

Ecdysteroids, consequently, regulate TOR signalling throughout body. Ecdysteroids control systemic growth in feeding *Drosophila* larvae by inhibiting TOR pathway and producing insulin like peptides (ILPs) in its fat body. Similarly, in *B. mori* ecdysteroid induces an ILP in adult fat body during its development. Therfore, ecdysteroids play important role to inhibit/stimulate TOR pathway in same tissue depending upon the developmental stage (Okamoto *et al* 2009).

2.4.4.5 Organ and body growth

TOR signalling pathway and insulin signalling pathway in combination regulates the growth of body and organs. This was demonstrated by Linn *et al* (2019) by silencing *TOR* gene in *T. castenum.* Growth rate was decreased in TcTOR silenced insects. Also the size and weight of pupae and pupal organ like mandibles, gin traps, wings and legs were reduced. During the larval development, TOR and Insulin like receptor contribute in the formation of ovarian niches and germline stem cells in *Drosophila melanogaster* by affecting the cell numbers and differentiation both. This also helps in the formation of terminal filaments (part of somatic niche) and controls the proliferation of somatic ovary, somatic patterning and cell behaviour (Gancz and Gilboa 2013). It reveals that TOR along with Insulin signalling sense the extracellular cues that control the adult niches and stem cells. TOR also regulates growth, proper proliferation and maintenance of GSC and early cyst formation (Drummond-Barbosa

and Spradling 2001, LaFever and Drummond-Barbosa 2005, LaFever *et al* 2010, Richard *et al* 2005, Sun *et al* 2010). In *C. elegans* these pathways promote germ cell proliferation, repress precocious germ cell differentiation and participate in the establishment of a fairly sized progenitor pool (Korta *et al* 2012, Michaelson *et al* 2010).

Earlier it was found that only yeast has two *TOR* genes but in 2010, it was reported that *Bombyx mori* has two paralogues genes, BmTor1 and BmTor2, existing as inverted repeats in the silkworm genome. Surprisingly both the *Bm*Tor genes have similar gene expression profiles in silk glands, fat body, midgut and other organs and are regulated in a similar manner by starvation and moulting hormone 20-hydroecdysone(20E). Also, the expression is more during the 4^{th} moulting and the larval pupal transition stages in the fat body usually when feeding is ceased and the levels of 20E is high. These genes are located on chromosome 3 and are 9259 nucleotides away from each other (Zhou *et al* 2010, Kamimura *et al* 1997).

A recent study shows that JH biosynthesis is in part regulated by nutrient signalling via the TOR pathway (Perez *et al* 2013). Nutritional information impacts JH levels by regulating the expression of genes associated with the JH synthesis pathway in the corpora allata. A similar regulatory interplay between TOR signalling and JH expression was also found in the German cockroach, *Blatella germanica*. Silencing *BgTOR* gene in cockroach led to the reduction in the production of juvenile hormone in corpora allata. This also led to the reduction in the expression of HMG-CoA synthase-2, HMG-CoA reductase and 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) synthase-1 (Maestro *et al* 2009). In the mosquito fat body, JH regulates the expression of several genes coding for proteins associated with the TOR-signaling machinery (Roy *et al* 2007).

CHAPTER III

MATERIAL AND METHODS

The studies on 'RNA interference to investigate the function of *Target of rapamycin (TOR)* gene in *Bemisia tabaci* (Gennadius)' were conducted during *kharif*, 2019 at Insect Molecular Biology Laboratory and Entomological Research Farm, Department of Entomology, Punjab Agricultural University, Ludhiana.

3.1 Maintenance of *B. tabaci* culture in screen house

The culture of *B. tabaci* cryptic species Asia II-1 was maintained on cotton plants, variety RCH 773 in screen house conditions at Entomological Research Farm, Punjab Agricultural University, Ludhiana. Relevant agronomic practices were followed for optimum growth of plants. Soil and farm yard manure were mixed in equal proportion to fill the earthen pots and pre-sowing watering was done to facilitate good germination. Cotton seeds were soaked for 4-5 hrs before sowing. Sowing was done in the evening and the pots were placed outside the screen house in sunlight till germination occurred. After germination, the plants were shifted in the screen house having two compartments, one containing the previously maintained culture of whitefly for its rearing and other for performing the bioassays. Thinning of plants was done to maintain 2 plants per pots. For maintaining the continuous availability of whitefly, the culture was maintained on brinjal, potato and tomato plants during winter season also (Plate 1).

3.2 RNA extraction from whiteflies and cDNA synthesis

Total RNA was isolated from 100 adult whiteflies using RNA isozol (Takara) following manufacturer's protocol. To avoid the contamination, all the glasswares, centrifuge tubes, microtips, ligation and PCR tubes were given the treatment with 0.1 % di-ethylpyrocarbonate (DEPC) treated water. These were soaked in DEPC solution for overnight and washed with distilled water and then placed in oven to remove the traces of DEPC treated water followed by autoclaving at 121 ℃ and 15 psi for 30 minutes.

Whiteflies were collected with the help of aspirator and anesthetized. Whitefly tissue was homogenised in 100 µl of isozol reagent. Homogenised sample was then added with 400 µl of RNA isozol and incubated at room temperature for 5 minutes. Centrifugation of the sample was done @ 13000 rpm (4 °C) for 5 minutes followed by collection of supernatants in the new micro-centrifuge tube. Chloroform (250 µl) was added to it and the sample was mixed properly and incubated at room temperature for five minutes. Again, the sample was centrifugated @ 13000 rpm (4 °C) for 5 minutes. An aqueous phase appeared on the top was collected into the new micro-centrifuge tube followed by the addition of chilled isopropanol and incubated overnight at 20 °C. The sample was centrifugated @ 13000 rpm (4 °C) for 5 minutes. RNA pellet was seen at the bottom of the tube and washing of the pellet was done with 75 per cent ethanol. The pellet was subsequently dissolved in 20 µl of nuclease free

Plate 1. Growing of cotton and brinjal plants and maintenance of culture of *Bemisia tabaci* on these plants under screen house conditions

water and stored at -20 ℃ for further use.

3.3 Gel electrophoresis

The integrity of RNA was checked on 1 % agarose gel and purity was determined at OD260/280 absorbance ratio.

3.3.1 Preparation of Tris-Acetic Acid-EDTA (Tris) buffer (50X stock)

For preparing 50X TAE buffer, 242 g tris base, 57.1 ml glacial acetic acid and 100 ml ethylene diamine tetra acetic acid (EDTA) (pH 8.0) were dissolved in 500 ml of distilled water. Then the final volume was made to 1000 ml.

3.3.2 Preparation of agarose gel (1.5 %)

Agarose gel was prepared by dissolving 1.5 g of agarose (Agarose, LE, Analytical grade, Promega Cooperation) in 100 ml of TAE buffer and heated for 2 minutes. The viscous liquid was then supplemented with 3.5 µl of Red Safe, Nucleic Acid Staining Solution (Intron Biotechnology) for proper visualisation of amplified bands under UV light and poured into the gel casting plate having combs. After the gel was solidified, it was placed in electrophoresis chamber containing 1X TAE buffer.

3.3.3 Preparation of RNA sample for gel run

RNA sample was prepared by adding 2 µl of RNA, 1 µl of 6X Bromophenol dye and 3 µl of distilled water. Denaturation of RNA sample was done by heating the sample at 70 ℃ for 10 minutes and immediately chilled on ice. The sample was then loaded into the well of gel and also the DNA ladder used to determine the size of amplified product was added in the subsequent well. The gel was run at the supply of constant voltage of 80 V until the dye reached 2/3 of the gel. The visualisation of the gel was done using UV-Gel Documentation system (Alpha Innotech) and image was captured. RNA purity was determined on Biospectrometer (Eppendorf)

3.4 cDNA synthesis

First strand complementary DNA (cDNA) was synthesised from 1µg RNA using Superscript III Reverse transcriptase kit (Invitrogen) following manufacturer's procedure. Following components were added in nuclease free micro centrifuge tube

Component	Amount (µl)
oligo dt (50uM)	1
Total RNA	1
dNTP Mix	1
Distilled water	11

Sample was heated to 65 ℃ for 5 minutes and incubation was done for 1 minutes on ice. The mixture was briefly centrifuged to collect the contents at the bottom. Following

components were further added to the mixture.

Component	Amount (µl)
5X First Strand Buffer	4
0.1M DTT	1
dNTP Mix	1

Mixing of the sample was done by pipetting up and down followed by incubation at 50 °C for 30-60 minutes. The reaction was finally inactivated by heating it at 70 °C for 15 minutes. cDNA was then diluted (1:10) for PCR amplification of *TOR* gene with site specific primers.

3.5 Primer designing

The predicted sequence of mTOR gene in whitefly was downloaded from NCBI (GenBank accession no. XM_019060193.1) and total of nineteen forward and reverse primers were designed (Table 3) using Primer 3 software.

Table 3. List of primers used for *TOR* gene amplification

Primer	Primer sequence 5'-3'	Length
BtTOR F1	catcgcaaaatgtctaatactctgatg	27
BtTOR F2	catgttagagcgaggacctcaaa	23
BtTOR R1	ccttttaagggcaggtactgtgac	24
BtTOR F3	actcggaacaaggctgctaa	20
BtTOR R3	atagccacgttcccactgag	20
BtTOR R5	*tagcagttgcctgctgaatg*	20
BtTOR F6	aggcactcatgcttcgactt	20
BtTOR R6	ggctgcgatccaaagattta	20
BtTOR F7	cgagccgtaagacttgaagc	20
BtTOR R7	tgtacgggacgcatttgata	20
BtTOR F8	cttgctgcgtgttttgactc	20
BtTOR R8	tgccccacttctgatttacc	20
BtTOR F9	caagaacgatggcatgagaa	20
BtTOR R9	caaaccaaagggtgagcaat	20
BtTORF10	tactgttcctgcggtggag	20
BtTORR10	cagggacatatgagccaggt	20
BtTOR F15	tgcttgtcaatatgggttcttc	22
BtTOR R15	ggagttcagagcaagattcg	20
BtTOR R17	aaattgctcggccaagatag	20

The primers were custom synthesized from Integrated DNA Technologies, Inc (IDT). The stock solution (100 µM) of each primer was made by dissolving the primer in distilled

water. The working solution was made to 10µM.

3.6 Amplification of *TOR* gene and purification of the amplicons

Different combinations of forward and reverse primers in different PCR reactions were used to amplify partial cDNA sequence of *TOR* gene (Fig 1.) using Phusion High-Fidelity DNA Polymerase kit (Thermo Fisher Scientific). PCR reaction mixture contains following components:

Components	**Amount (µl)**
Double Distilled water	15.5
2X Phusion HF PCR Master Mix with HF Buffer	25.0
cDNA	4.5
Primer F	2.5
Primer R	2.5
Total volume	50.0

Initially BtTOR-F1 and BtTOR-R5 primers were used to amplify full length *TOR* gene. The PCR profile consisted of initial denaturation at 98 °C for 30s (1cycle), followed by 35 cycles of denaturation at 98 °C for 10s, annealing at 55 °C for 30s and extension at 72 °C for 3.5 minutes and final extension at 72 °C for 10 minutes and the product was stored at 4 °C for further use. This PCR product was further used as template for nested PCR to amplify other *TOR* gene fragments. Different combinations of forward and reverse primer set were used to amplify cDNA of *TOR* gene. Primers set a) BtTOR-F1 & BtTOR-R3, b) BtTOR-F3 & BtTOR-R1, c) BtTOR-F6 & BtTOR-R6, d) BtTOR-F7 & BtTOR-R7, e) BtTOR-R15 & BtTOR-F15, f) BtTOR-F8 & BtTOR-R8, g) BtTOR-F9 & BtTOR-R9, h) BtTOR-F10 & BtTOR-R10, i) BtTOR-F2 & BtTOR-R5 and j) BtTOR-F2 & BtTOR-R17 were used for amplification of fragments of *TOR* gene (Fig 1). The last fragment was amplified using primers BtTOR F2 and BtTOR R17 for which whitefly cDNA was used. The amplified PCR product were run on agarose gel (1.5 %) along with 1kb DNA ladder (SMOBIO) at a constant voltage of 80V for 1 hour. The amplified bands were visualised under UV rays in UV-Gel Documentation system and were cut and purified by using NucleoSpin Gel and PCR purification Kit (Macherey Nagel).

For excising the bands, the gel block was placed on the open UV trans illuminator. The visible amplified gel bands of desired sizes were sliced out with the help of clean and sterile scalper blade and then placed in a microcentrifuge tube. The gel was purified by using NucleoSpin Gel and PCR purification Kit (Macherey Nagel) following manufacturer's protocol. The weight of the gel was determined and if it weighed X units, 2X units of NTI buffer was added and the sample was incubated for 10 minutes at 50 °C. The sample was

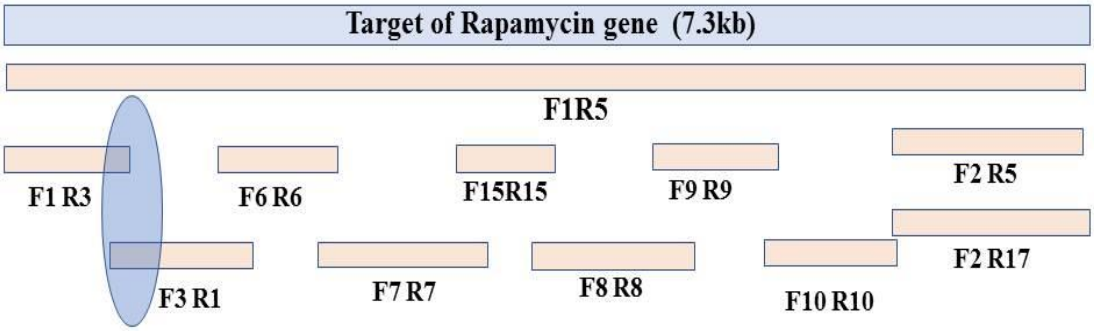

Fig 1. Different overlapping fragments to amplify *TOR* gene

vortexed 2-3 times in between incubation until it was completely dissolved followed by loading the sample into NucleoSpin Gel and PCR Clean Up column. The sample was then centrifuged for 30s at 11,000xg. The DNA bound to the base of the silica membrane was then washed by adding 700 ml Buffer NT3 to the column. The flow through was discarded and the column was placed back to the collection tube. For complete removal of Buffer NT3, the sample was centrifuged for 1min at 11,000 xg. For elution, the column was placed in new microcentrifuge tube and 20 µl elution buffer was added and incubated for 1 minute at room temperature followed by centrifugation for 1min at 11,000 xg.

3.7 Cloning of purified fragments of *TOR* gene

Initially we tried to clone all the fragments of *TOR* gene. But only three fragments were successfully cloned. Later purified products (7 fragments) and 3 cloned plasmids were send for custom sequencing.

3.7.1 Ligation of the DNA fragment into pMD20 vector

The purified genes of interest were then ligated into pMD20 cloning vector to construct a recombinant plasmid using Mighty TA cloning kit (Takara). The ligation mixture consist of following components:

Components	Amount (µl)
PCR cloning vector pMD20-T	0.5
Ligation Mighty Mix	5
Gene of interest (Purified PCR product)	2
Double distilled water	2.5
Total	10

The ligation mixture was prepared in microcentrifuge tube and then incubated at 4 °C for overnight.

3.7.2 Transformation of recombinant plasmid into *Escherichia coli* DH-5alpha cells

3.7.2.1 Making of Luria Bertani (LB) liquid and LB agar medium plates:

For preparing LB liquid medium, 12.5 g of LB (Luria-Bertani Hi Veg Broth Miller,

Granulated, Hi Media Laboratory Pvt Ltd) was dissolved in 500ml of distilled water. In the similar way, another LB liquid medium of 500 ml was prepared and 100 ml of the media was poured into five different flasks separately. To each, 2g agar (Agar Powder for Bacteriology, Laboratory Chemical Pvt Ltd) was added. Both the media were subjected to autoclaving at 121 °C at 15psi for 30 minutes.

3.7.2.2 Spreading of X-Gal and IPTG on LB-ampicillin plates

After autoclaving LB agar medium, it was allowed to cool down. Further, 100 µl of ampicillin was added per 100ml of LB agar medium. The purpose of adding ampicillin is to select the *E. coli* cells containing plasmids. PCR cloning vector pMD-20 T has the selectable marker i.e. ampicillin resistance gene AmpR which allows only the cells which have taken up the plasmids and transformed to grow in the presence of ampicillin. The media was then poured into petri dishes and allowed to solidify.

After solidification of medium, 10 µl of IPTG solution (isopropyl-β-D-thiogalactopyranoside) and 50 µl of chromogenic substrate i.e. X-Gal solution (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) were poured and spread on the medium with the help of clean and sterile L-shaped cell spreader. The plates were left in the laminar with open lid for drying. In blue-white cloning, IPTG along with X-gal helps in the detection of the *E. coli* cells containing recombinant plasmids. IPTG is accountable for the induction of expression of LacZ gene which produces beta-galactosidase enzyme due to alpha complementation process which is responsible for metabolising lactose into galactose and glucose. pMD-20T vector contains a short site for LacZ gene and multiple cloning site is present into this segment. When the gene of interest is inserted into the MCS (by producing nicks in the site with the help of restriction enzymes), beta-galactosidase enzyme is not produced and vice-versa. The clones having nonrecombinant plasmids produces beta galactosidase enzyme. This enzyme makes X-Gal to hydrolyse into 5-bromo-4-chloro-indoxyl, which instinctively dimerises to produce blue colour pigment named as 5,5'-dibromo-4,4'-dichloro-indigo. Therefore, the recombinant clones produce white colour and non-recombinant clones blue colour, thus helps in screening of the desired recombinant clones.

3.7.2.3 Transformation of ligation mixture into *E. coli* DH-5α cells

The transformation of ligated product was done following NEB high efficiency transformation protocol. Fifty microlitre of *E. coli* competent cells were thawed on ice for 10 minutes and afterwards 10 µl of ligation mixture was added into it. The mixture was mixed gently by pipetting up and down and placed on ice for 30 minutes. Heat shock was given to the cells at 42 °C for 30 seconds. The mixture was then immediately placed on ice for 10 minutes. During the cell recovery step, the transformed cells were cultured in 950 µl of Super Optimal broth (SOC) medium with shaking at 150 rpm for 1hour at 37 °C. SOC media is

used to increase the transformation efficiency. After growing of the cells in SOC medium, the cells were extracted from the SOC media by centrifugation at 10000 rpm for 1 minute. The supernatant was discarded and 100 µl of media containing the pellet of cells were plated on LB-ampicillin agar plates with X-Gal and IPTG spread on it. After spreading, the plates were allowed to dry and then incubatednovernight at 37 °C for growth of cells. The cultured plates were examined the following day for colony formation.

3.7.2.4 Screening of recombinant plasmids (blue-white screening) from the cloned cells

The recombinant clones having white colour were picked with the help of autoclaved micropipette tips and inoculated into 3ml LB-ampicillin broth. For the growth of the cells, the tubes were placed in incubator for shaking (150 rpm) at 37 °C for 16 hrs. The plasmids were isolated from the broth culture following 'alkaline lysis method' given by Birboin and Doly (1979). One and a half millilitre of broth culture was centrifuged (10,000 rpm, 1 min) in 1.5ml micro-centrifuge microcentrifuge tubes. Bacterial pellet was resuspended in 100 µl suspension buffer i.e. P1 solution by continuous tapping/vortexing. PII solution (cell lysis solution) was added double the amount of P1 solution i.e. 200 µl and the tube content was mixed properly by inverting 5 times. In this case the partial lysis of the cells was done to allow only the plasmid DNA to come out of the cells. 150 µl of PIII solution (Neutralising solution) was added into the tube and tube were inverted for mixing and then placed at -20 °C for 10 minutes. To collect the plasmid DNA, the tube was centrifuged (12000 rpm, 5min) and the supernatant was collected in new tube followed by precipitation of DNA by the addition of 450 µl of chilled isopropanol. The supernatant contained the plasmid DNA as the PIII solution removed the protein in the form of pellet. The tubes were inverted 5 times and then placed at -20 °C for 10-15 min. The final recovery of the DNA pellet was done by centrifugation (12000 rpm, 2 min), followed by the removal of supernatant by vacuum and the pellet was washed down with 500 µl of 70 per cent ethanol. Ethanol was removed and the pellet was dried in open laminar flow and then dissolved in 50 µl TE and RNAse A solution. Thereafter, the plasmid DNA was stored at -20 °C until further use.

3.7.2.5 Analysis of recombinant clones

The recombinant clones were confirmed through PCR reaction using specific primers. The PCR profile consisted of initial denaturation at 98 °C for 30s (1cycle), followed by 35 cycles of denaturation at 98 °C for 10s, annealing at 55 °C for 30s and extension at 72 °C for 3.5 minutes and final extension at 72 °C for 10 minutes and the product was stored at 4 °C for further use. The size of the insert DNA fragment was determined on agarose gel electrophoresis (1 %) along 1kb DNA ladder (SMOBIO).

3.8 Nucleotide sequencing of purified and cloned DNA

The plasmid DNA which were confirmed for having DNA insert and purified DNA

samples were submitted to "M/S Xcelris, Ahmedabad" for nucleotide sequencing with concentration of 20 ng/µl in 40 µl. The DNA insert cloned in plasmid vector and all the purified DNA samples (total 10 overlapping fragments) were custom sequenced in forward and reverse directions using universal primers (M13-forward and T7 reverse) and specific primers, respectively. The DNA inserts and the sequence from purified DNA product were edited by using CLC sequence viewer programme. Atleast 4 replications of each fragment were used for sequence editing. For the confirmation of *TOR* sequence, the nucleotide sequence was confirmed through "BLAST tool" of "National Centre for Biotechnology Information" (NCBI) (available at www.ncbi.nlm.nih.gov/ Blast).

3.9 Phylogenetic relationship of *TOR* gene from whitefly with other insects

The full length of amino acid sequence of *TOR* gene was aligned and comparison was done with the sequence of following insects (GenBank accession numbers are given in parentheses): *Nilaparvata. lugens* (JQ793898.1), *Pediculus humanus corporis* (XP_002426707), *Acrythosiphon pisum* (XP_001948118.2), *Bombyx mori isoform 1* 2 (NP_001171774.1), *Bombyx mori isoform 2* (NP_001171774.1), *Danaus plexippus* (EHJ65030.1), *Aedes aegypti* (AAR97336.1), *Ceratis capitata* (XP_004531450.1), *Drosophila melanogaster* (CCB63108), *Blatella germanica (ACH47049), Solenopsis invicta* (EFZ20258), *Megachile rotundata* (XP_003699312.1), *Bombus terrestris* (XP_003399034.1), *Apis mellifera* (XP_625130.2) and *Apis cerana* (XP_016909071.1). Phylogenetic tree was constructed for different insect based on amino acid sequence of *target of rapamycin (TOR)* gene by Maximum Likelihood method based on the JTT matrix-based model using MEGA 7 software (Jones *et al* 1992, Kumar *et al* 2016).

3.10 Double stranded RNA (dsRNA) synthesis

3.10.1 Amplification of *TOR* and *gfp* genes using the designed primer sets one containing T7 RNA polymerase promoter sequence

For the synthesis of ssRNA, firstly the amplification of template DNA of 200-250 nucleotide sequence was required. For this, a set of forward and reverse primers having T7 RNA polymerase promoter site corresponding to *TOR* and *gfp* genes were designed using Primer 3 software (Table 4). Here, *gfp* gene served as control gene. Combinations of the primer sets (Table 5) having specific primers and primers with T7 promoter were used to amplify the target amplicon of *TOR* gene using PCR product as template (generated by PCR amplification using primers BtTOR-F1 and BtTOR-R5 (Fig 1). Similarly, amplification of partial gene was done using *gfp* plasmid as template and primer sets with and without T7 polymerase were used.

Table 4. List of primers used for dsRNA synthesis

Primers	Sequence 5'→3'	Length (bp)
BtTOR-T7-F11	ggatcctaatacgactcactataggaatctttggatcgcagccta	45
BtTOR-T7-R11	ggatcctaatacgactcactataggcatgcgtgcactctgttctt	45
BtTOR-F11	aatctttggatcgcagccta	20
BtTOR-R11	catgcgtgcactctgttctt	20
GFP2-F	gtgcaggagaggaccatctt	20
GFP2-R	ttgtcggccatgatgtatac	20
GFP-T7-F	gaattgtaatacgactcactatagggtgcaggagaggaccatctt	45
GFP-T7-R	gaattgtaatacgactcactataggttgtcggccatgatgtatac	45

Table 5. Primer combination for amplification of target gene sequence

Target amplicon	Primer set
Amplicon 1st for Forward *TOR* seq	BtTOR-T7-F AND BtTOR-R11
Amplicon 2nd for Reverse *TOR* seq	BtTOR-T7-R AND BtTOR-F11
Amplicon 1st for Forward *gfp* seq	GFP2-T7-F AND GFP2-R
Amplicon 2nd for Reverse *gfp* seq	GFP2-T7-R AND GFP2-F

The PCR amplification was done using GeNei PCR kit by following the programme as 94 °C for 5 min (Initial denaturation), 94 °C for 1 min, 52 °C for 1min, 72 °C for 30 sec (35 cycles), 72 °C for 10 min (final extension) for both the genes. Composition of PCR reaction mix is given below

Components	Amount (µl)
Double Distilled water	24.0
dNTPs 1mM	12.5
10X Buffer	5.0
Taq Polymerase	2.30
Template	3.0
Primer F 10 uM	1.50
Primer R 10 uM	1.50
Total volume	50

The integrity of the amplified DNA products was checked by resolving the bands on agarose gel (1 %) subjected to horizontal gel electrophoresis and the size was verified with

the marker (100 bp DNA gene ruler ladder) (as explained in Section 3.3.2). The ampilified PCR products were purified using NucleoSpin Gel and PCR purification Kit (Macherey Nagel) following manufacturer's protocol (Section 3.6). The purified products were then used as template for ssRNA synthesis using Invitrogen MEGAscript RNAi kit (Thermo Fisher Scientific).

3.10.2 ssRNA synthesis

Both the forward and reverse strand of ssRNA corresponding to *TOR* and *gfp* were synthesised using Invitrogen MEGAscript RNAi kit (Thermo Fisher Scientific) following manufacturer's protocol. Following reaction mixture was prepared adding specific template to each. Composition of reaction mix to synthesize ssRNA is given below:

Component	Amount (µL)
Template DNA (150ng)	2
10X T7 Reaction Buffer	2
ATP Solution	2
CTP Solution	2
GTP Solution	2
UTP Solution	2
T7 Enzyme Mix	2
Nuclease-free Water	6
Total volume	20

All the components were placed on ice to avoid degradation except 10X buffer and nuclease free water. DEPC water treated tips were used and the working space was wiped out with RNAse ZAP. The reaction was assembled at room temperature. It was mixed properly and incubated at 37 °C for 16 hours.

3.10.3 Annealing of ssRNA

After incubation, the ssRNAs (sense and antisense strands) were pooled to prepare dsRNA of both the genes. Both the strands were then allowed to get annealed by placing the mixture in hot water (75 °C) which was placed at room temperature and allowed to cool slowly upto room temperature. The integrity of dsRNA was analysed by subjecting it to horizontal agarose gel (1 %) electrophoresis and concentration was measured using Biospectrometer, (Eppendorf).

3.11 Silencing of *Target of rapamycin* (*TOR*) gene in whitefly

3.11.1 Preparation of leaf cages for feeding of dsRNA to whitefly

The studies related to RNA interference of *TOR* gene in whitefly were conducted using leaf cages in which the whiteflies were fed with dsRNA and then those whiteflies were

released on the leaves in the confined area of leaf cage. For preparing the leaf cages, 50 ml plastic conical tubes were cut into smaller cylinders with the help of sharp knife. The measurements of the cylinder was 2.5 cm length and diameter of 3cm. Five circular holes were cut out from the sides of the cylinder at an equal distance. Four holes were of same size (0.75 cm dia) and the fifth one was of little bigger size (1cm dia). Smaller holes were covered with muslin cloth using glue to facilitate aeration and the bigger hole was left opened for releasing the whiteflies into it. Afterwards this hole was plugged with cotton swab. A fine transparent sheet was pasted on one side of the tube.

3.11.2 Preparation of dsRNA contained artificial diet

Artificial diet containing dsRNA, sucrose and nuclease free water was prepared. 50 per cent sucrose stock solution was prepared which was used for preparing the final diet. Different concentrations of dsRNA corresponding to *TOR* gene *viz* 2.0, 1.0, 0.5, 0.1 µg/µl were fed to whiteflies. Artificial diets (200 µl) containing above mentioned concentrations of dsRNA were prepared in 20 % sucrose solution. In the similar way, diet containing dsRNA corresponding to *gfp* gene was also prepared and the concentrations of dsRNA were 1.0, 0.5, 0.1 µg/µl. The control was 20 per cent sucrose solution only. All the steps were performed in aseptic environment conditions.

3.11.3 Bioassays

Bioassay was conducted using one day old *B. tabaci*. Whitefly culture was maintained in screen house on cotton potted plants at 28 °C. Bioassays were also conducted in same conditions. Cotton plants having a large population of red eyed pupa were placed in separate screen house while the already feeding whiteflies were removed from the plants. Whiteflies emerging next day from pupa (one day old whiteflies) were used for the experiments.

Two hundred micro litres of previously prepared artificial diet containing dsRNA corresponding to *TOR* and *gfp* gene was placed on a finely stretched parafilm on the one side of cage. The diet was sandwiched by placing an another stretched parafilm over it such that the diet can be properly spread in-between the parafilm (Plate 2). Similarly, 200 µl of sucrose solution was sandwiched into two parafilm layers which served as control. Ten pairs of one day old whiteflies were collected from the cotton plants with the help of aspirator and released into the cage of each treatment through bigger hole. Subsequently the hole was covered with cotton swab. All the cages were prepared in the similar way and were placed in incubator at 28 °C to allow whiteflies to feed on the diet. The bioassay experiment was conducted in two sets i.e. continuous feeding on artificial diet and 48 hours feeding on dsRNA and later shifted on cotton leaves.

3.11.3.1 Continuous feeding on artificial diet bioassay

In first set, ten pairs of one day old whiteflies were allowed to feed continuously on

dsRNA mediated artificial diet. The concentrations of dstor and ds*gfp* were 1.0, 0.5, 0.1 µg/µl and 4 replications were used in all the treatments. Adult mortality was recorded at two days interval till maximum mortality occurred and cumulative mortality was calculated.

3.11.3.2 Feeding of whiteflies for 48 hours on artificial diet

In second set, the whiteflies were allowed to feed on dsRNA diet in parafilm for 48 hours in the leaf cages of all the treatments and later shifted on cotton leaves in potted plants in screen house. The parafilm sheet was pricked with needle to remove the sandwiched diet from it and then soaked with tissue paper. The parafilm sheets were carefully removed maintaining the whiteflies inside the cage and the cage was immediately placed over the lower surface of the leaf. The upper leaf area immediately above the cage was given support with foam pasted on sunmica sheet. The cage and foam were clipped together with hairpin having a loop in it (Plate 3). The detailed observations were recorded daily on different biological parameters under microscope (Plate 4). The experiment was conducted three times during June-July (4 replications), July-August (4 replications) and August-September (3 replications). In third experiment, one higher concentration of 2.0 µg/µl was also included to check the effect of higher concentration of *dstor* on biology of whitefly.

3.11.3.2.1 Adult mortality: The live and dead adult whiteflies were recorded through transparent plastic sheet after 48 hours of feeding on dsRNA mediated artificial diet. The per cent mortality was calculated.

3.11.3.2.2 Fecundity: The whiteflies were allowed to feed and oviposit on the leaf surface for 72 hours after shifting to leaves. After 72 hours of oviposition, whitefly adults were shifted to another new leaf and allowed to oviposit. The total number of eggs and nymphal instars were recorded from the older and new leaves which is noted as total fecundity. Total number of live females after feeding on dsRNA for 48hours were recorded before shifting to newer leaves and fecundity was calculated.

3.11.3.2.3 Duration of various life stages of *B. tabaci*: The area under the leaf cage where oviposition by dsRNA fed whiteflies took place was marked. Twenty nymphs were marked and observed daily under stereomicroscope binocular (40X) to study biology of whitefly. The duration of nymphal instars and pupae were noted and the means were calculated. There are three nymphal instar of whiteflies. First nymphal instars are tiny crawlers, translucent, coccoid type, having reddish eyes and a pair of clearly visible yellow abdominal mycetomes. Crawlers crawl for 2-4 hours after hatching. Second instar nymphs are larger in size as compared to first instar nymphs and are more transparent and sessile. Third instar nymphs have slightly oval and bigger size. Pupae has prominent red coloured eyes with yellowish white body. The time period between the appearance of first nymphal instar to the appearance of red eyed pupae was consider as the total nymphal duration. Also, the time period between the appearance of pupa and emergence of adults was considered as pupal duration.

3.11.3.2.4 Mortality of nymphal and pupal stages: Number of live individuals at first, second and third nymphal instars and pupal stage were counted daily under stereomicroscope binocular (40X). Per cent mortality was calculated for all the nymphal instars, total nymphal mortality and pupal mortality.

3.11.3.2.5 Adult emergence: Number of adult emerging from from pupae were recorded and per cent adult emergence was calculated.

3.12 Statistical analysis

The data from bioassays was analysed by using Completely Randomized Design (CRD) with the help of CPCS1 statistical software. The per cent mortality viz., adult mortality, nymphal mortality and pupal mortality data were transformed using arc sine root transformation.

3.13 qRT-PCR analysis

The experiment was conducted in two sets. In one set, concentrations used for both *dstor* were @ 1.0, 0.5, 0.1 ug/µl and feeding was allowed for 48hours. In other set, concentrations of *dstor* were @ 2.0, 1.0, 0.5, 0.1 ug/µl and feeding was allowed for 96 hours. In the same way, whiteflies were also fed with *dsgfp* @1.0 µg/µl as control for both the sets. Feeding method was same as explained under section 3.10.3. After feeding for specified period, cages were placed in deep freezer for 5 mins to immobilise the whiteflies. There were two replications in each experiment.

3.13.1 RNA isolation and cDNA synthesis

Total RNA from immobiliaed whiteflies was isolated using Nucleo Spin RNA kit (Macherey Nagel) as per manufacturer's protocol for each treatment separately. The quality and quantity of total RNA was quantified on gel electrophoresis and Biospectrometer. Total RNA (500 ng) was used for cDNA synthesis using Superscript III Reverse transcriptase kit following manufacturer's procedure (see section 3.2). PCR reaction was performed using *Bt*TOR qPCR primers to validate the cDNA (Table 6). PCR reaction mixture is same as give in section 3.10.1. The PCR programme consists initial denaturation at 94 °C for 5 minutes (1cycle), followed by 35 cycles of denaturation at 94 °C for 1 minute, annealing at 52 °C for 1 minute and extension at 72 °C for 30 seconds and final extension at 72 °C for 10 minutes of 35 cycle. The amplification of cDNA is observed on gel electrophoresis.

Table 6. List of primers used for qRT-PCR

Primers	**Sequence 5'→3'**	**Length (bp)**
BtTOR-Q-F1	gttgtcaaaccgtacaaccaatac	24
BtTOR-Q-R1	agcataccaagtagccgaatg	21
hsp90-F	atcgccaaatctggaactaaagc	19
hsp90-R	gtgttttgagacgactgtgacggtg	22

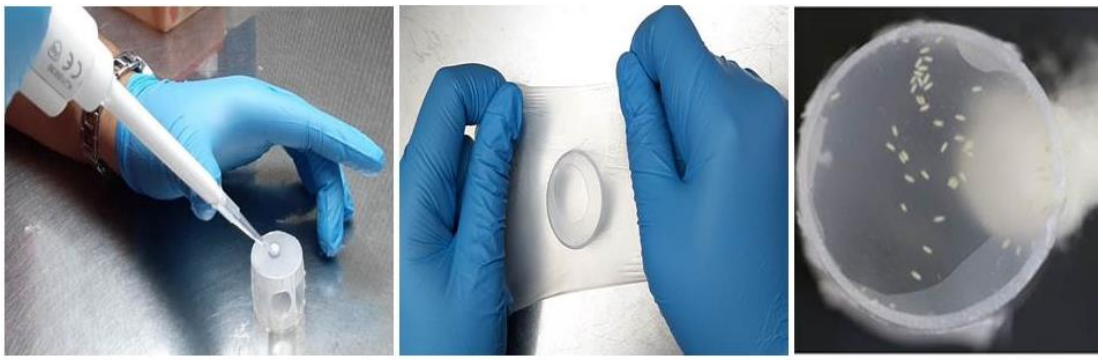

Plate 2. Preparation of leaf cages for feeding of whitefly adults on dsRNA mediated artificial diet (a) Pouring of dsRNA mediated artificial diet on parafilm sheet, (b) Sandwiching the dsRNA diet between two parafilms, (c) whiteflies feeding on dsRNA- mediated artificial diet (200µl volume) sandwiched between two parafilm layers over one end of plastic cage

Plate 3. (a) Fixing of cage containing dsRNA fed whiteflies on the lower surface of leaf with the help of clip and cushion on the backside (b) Cages containing dsRNA fed whiteflies attached to cotton leaves

Plate 4. Recording of observations regarding biology of whitefly under stereomicroscope binocular using 40X

3.13.2 Gene expression analysis through qRT-PCR

The qRT-PCR was conducted in duplicates for 2 biological replicate using PowerUp SYBR Green Supermix (Applied Biosystems) on Roche light cycler PCR machine. The 15µl reaction mixture contained of 1µl of cDNA (with 1:10 dilution factor), 10 µl of SYBR Green, 1µl of forward and reverse 10 µM primers, and 2 µl of nuclease free water. The cycling profile consists of 40 cycles each consisting of an initial holding at 94 °C for 3 min, denaturation at 94 °C for 10s and annealing at 55 °C for 30s followed by a melt curve analysis. Relative gene expression of *TOR* gene was analysed using delta delta CT method (Livak and Schmittgen 2001). Heat shock protein (hsp) was used as internal control gene to normalise the level of mRNA. Expression of *TOR* gene was calculated by comparing the normalized mRNA level of *TOR* gene in whiteflies fed on different concentrations of dsRNA with respect to *dsgfp* @ 1.0 µl/µg fed whiteflies.

CHAPTER IV

RESULTS AND DISCUSSION

The results of the current study are discussed and presented under the following heading:

4.1 Amplification of *target of rapamycin* (*TOR*) gene from whitefly

4.2 Sequence analysis of all the amplified DNA fragments of *TOR* gene

4.3 Phylogenetic relationship of *TOR* gene

4.4 Synthesis of double stranded RNA

4.5 Effects of dsRNA feeding on the biology of whitefly

4.6 Estimation of down regulation of *TOR* gene in whitefly after RNAi

4.1 Amplification of *target of rapamycin* (*TOR*) gene fragments from whitefly

4.1.1 Total RNA isolation from *B. tabaci* adults

The good quality of total RNA was isolated from adult whiteflies which was confirmed by agarose gel (2.0 %) electrophoresis (Plate 5). Two bands corresponding to 18S and 28S rRNA genes were observed. cDNA was synthesized from tRNA.

4.1.2 PCR amplification of *target of rapamycin* gene fragments from *B. tabaci*

Ten gene fragments with overlapping sequence were analysed on agarose gel (1 %) and amplification was observed for all the fragments as desired amplicon size (Table 7, Plate 6).

Table 7. Primer sets used to amplify different overlapping fragments of *TOR* gene

Primer	Length (bp)
BtTOR-F1 & BtTOR-R3	813
BtTOR-F3 & BtTOR-R1	1443
BtTOR-F6 & BtTOR-R6	1122
BtTOR-F7 & BtTOR-R7	1104
BtTOR-F15 & BtTOR-R15	700
BtTOR-F8 & BtTOR-R8	1256
BtTOR-F9 & BtTOR-R9	1219
BtTOR-F10 & BtTOR-R10	800
BtTOR-F2 & BtTOR-R5	1417
BtTOR-F2 & BtTOR-R17	1511

4.2 Sequence analysis of all the amplified DNA fragments of *TOR* gene

4.2.1 Sequencing of cDNA of *TOR* gene fragments

The amplified *TOR* fragments (three) were cloned into pMD-T 20 PCR cloning

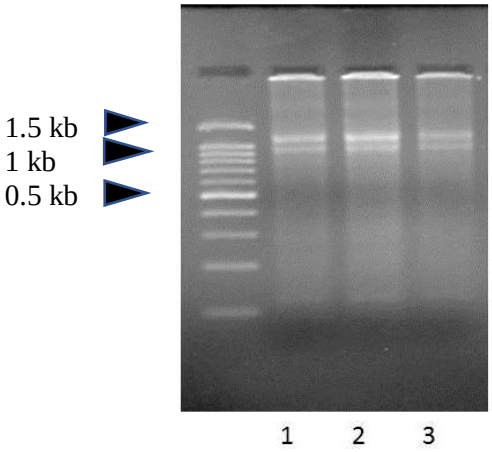

Plate 5. Total RNA isolated from adult whitefly individuals resolved on agarose gel (2%). Lane 1-3: RNA isolated in three replicates

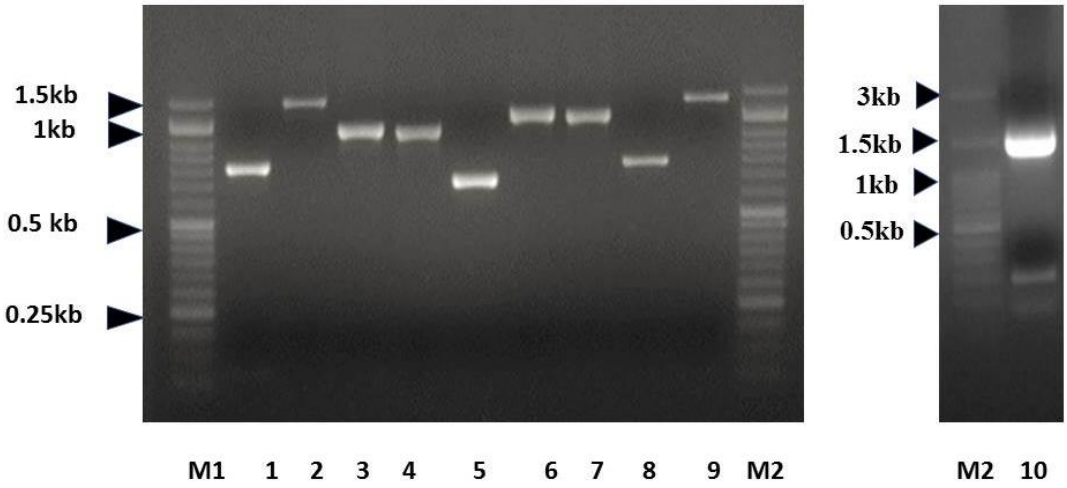

Plate 6. Amplification of different regions of *TOR* gene M1: 50bp DNA ladder (SMOBIO), M2: 1kb DNA ladder (SMOBIO). Lane 1 to 10 cDNA corresponding to overlapping fragments of *TOR* gene

vector. On the basis of blue/white screening, recombinant clones were selected. The insert DNA was confirmed through PCR reactions using specific primers. The results showed that three fragments were successfully cloned into pMD-T 20 PCR cloning vector (F1R3, F6R6 and F8R8) (Plate 7). The purified product of seven DNA fragments and three recombinant plasmids were custom sequenced through the services provided by "M/S Xcelris Labs, Ahmedabad". The raw data of all the sequences were edited for any misread bases by comparing with their respective chromatograms files of original sequence. All the fragments of *TOR* gene sequence were aligned with their reverse complementary sequence and other replications using CLC sequence viewer programme. The full-length contig of *TOR* gene is 7311 bp and 2437 amino acids. The full nucleotide sequence is given below.

atgtctaatactctgatgcagcagtttgtgtcggggctaaagtcccggcacccggaaactcggaacaaggctgctaaggacctttaccttacgtcaaaacagagttacgagaagtcccaccggaggagctcaattcattccttgatgactttaaccggcacattttcgaaatggtttccagttctgatgtcaatgagaagaaaggagggatccttgccatagtttgcctaatcggagcagatgtaggaatcgtcaacaaccgtataagcaggtttgccaactatctgcgtaatttattaccctcacacgacattggtgtaatggagcttgccgctaaaactgttggaaaacttgctttggtatcgggtacttactcagctgaatattttgaatttgaggtgaagaaagcatttgagtggcttggcggagaccggcatgaaggcaaaagacatgctgcggtgctcgttttgaaagaattggcgtgctctgtcccaacatacttttttcagcaagtacagatgtttttgatcttatattcaatgccgttcgtgacccaaaacccgtcatcagagaaggagcagttgaggcattacgtgcagtccttgtggtaacagctcaaagagaaacagcgaaacaaactcagaaaccacagtggtataagcaatgttatgatgaagcaatccacagctttgaagaaatacctggacaaaaaagtgtgaatagagatgaaaaaatccatggatccatcctagttttcaacgaactactacgatgcagcaatgctcagtgggaacgtggctatgaggcactcatgcttcgacttcagtttcagaattcaaatgagccctcgagcagtcttccaaggttgacacttaaatcatctctgattgcctctcgccaaaaaggaaccgttggtgagcggctaacatccttatcagtgtcatcgacaaatgcacctgagccccatccattgtatgaaagtgccgcatgccgctccttgatgctggaaagatttgatgacgtgtgctacatggtgcttcaacagcgcaattcaagatcctcttacatccagcacattctcttcacaattcttccacgactcgcagctttaaataaggaaaaatttgtcgaacagcatttgagtggcactttagcctacttgctttctacgctgaggagtcgtgaacgggacagagcatctgcttttagtactatcggactcattgccgttgctgttgaagacaatatcaaaccacatttgccaaaaataatggaattgattcgatcatatcttcctatgaaggacatgagtagtaagaaaagaggcaacttagaacctgctttattcgtgtgtattacactgctaggacacgctgttggcacacatatcaaagatgatttgaaagatattcttgatccaatgcttgcaactggactaagtcctgctcttacaactgctttgcgtgaacttgctgtcacagtacctgcccttaaaaggaatatttcagacggccttctagacatgctgtccatggtgttgatgaaccaacctcttcgccaccctggaatgccttctaacatgtcttccatatcaaattcaggaatgagtctgtctgatacgcagcaaaatgtgtcaagtatcaaactagccctgcgtactctcggcagctttaattttgatggtcactctttgctacagtttgtacgaagatgtgctgatcattttctgaaaagcaatgatcgagccgtaagacttgaagcagtttgtacatgcgcgagacttctgcgcttggcattagaaaatcgcaactcactgattgtcacaacaaccgtatctgatgttctggctaaactgcttgttgtaggaatcactgacactgatgccgatgttagatattgggtgcttaaatctttggatcgtagcctagatactcatcttgcccaggcagaaaatttaagtgctttgttgattgctgtcaatgatgaagtgtttgacattcgcgagttagcagtgcaaacaatagctcggctcagtctactgaaccccgcatatgtcatgccttcacttcgcaaaatactcattcagttttgacagaggttgaacacagtggcttggggcgcaacaaagaacagagtgcacgcatgatagatcatcttgtcgtcaatgcgcctcgacttgtgaagccatacatggagccaattttaaaagttcttgtaccaaaattaaaagaaccagaatccaatccaagtgttgttatcagtgtcctgaccgccattggcgatgtagcagaggtgaatggcacaacaattgagttgtggatgccagaactcctgccaattctgctcgaaatgctcggagatgcaagtagcccagaaaaaagaggtgttgctctttgggcccttggtcagctaatcagtgcaactggttttgttgtcaaaccgtacaaccaatacccttcacttctagatctactgatcaatttttgaagacagaacagcagccgctaattcggcgagaaaccattcggctacttggtatgcttggtgctttagatccatacaaacacaagatgaatttaggccaaattgattctcaagcagatgtcacagccctcatgtccatgtctgatgctaattcggaaaatgaaactagtcatgaattaacaacaagtgaaatgcttgtcaatatgggttcttcatctttagtagattactatcctgccattgca*a*ttgccactttaatgcgtattattcgcgatccaactttggcccaacaccacacaatggttgtccaggctgtaacttttatttttcaagtcattgggtatcaaatgtgtcccgtacattgctcaggtcatgcctagttttctcaacgtcattcgcactgcggatattaatttcagagaattcttgttccaacaactcggccaactcatcatgatagtcaaacagcatatccgcaattatttggacgatatttttgccctgatcaaggaattatgggttgtcaacagccctttgcagagcacactcattaccctagttgagaacatcgctgactcccttggtgctgagtttaagaactatctgccacagcttatgcctcacttgctgcgtgttttgactcatgacacaagtaaagaacgtgctgtcactgtgaaacttctccaagctctacccatgttccgaaataatcttgatgaatatctccatctcattcttccacctatcgtccggttgtttgattcttcagactgccctataagtgtttgtaaggtcgccatggagacagtagatgatctctccatcactttagacttttctgattatgcctcaaaaataattcatccattggtgcgcagcctcgaatcttgctctgaactccgagacactgcaatggacactatgtgtgctctaatcttacagcttgggcgaaaattctgtatttttgtacctttagttagtagtgttgtcacaaaacatagaattcactgcccacgctatgattcactcaccactagaatattaaccgagtcgactgaagaagactatttgttgcataagcaacgtataatgaagaacaaaatacgagaaaaagccctaatgtcatctgacactataaccacaattaaaagaatgcacacgtctgttgtcaatttgcaaagagcctggacagcca

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ctcattcagcaggcaactgctaatgaaaatttgtgtcaatgctacattggttggtgtccgttctgg**taa**

The sequence of *TOR* gene was confirmed by using "NCBI BLAST tool". The full-length sequence of *TOR* gene having 7311 bp showed 98.66 per cent homology with predicted *B. tabaci* serine/threonine-protein kinase mTOR (LOC109043116), mRNA (GenBank Accession Number XM_019060193.1) (Table 8). The multiple alignment of two sequences revealed ninety-five nucleotides were different in *B. tabaci*, however the amino acid encoding that nucleotide remains the same except for two. In *B. tabaci* of *TOR* gene, amino acid at position 1448, Asparagine replaced Threonine and at position 1768, Arginine replaced Tryptophan as compared to predicted *B. tabaci TOR* amino acid sequence (Plate 8).

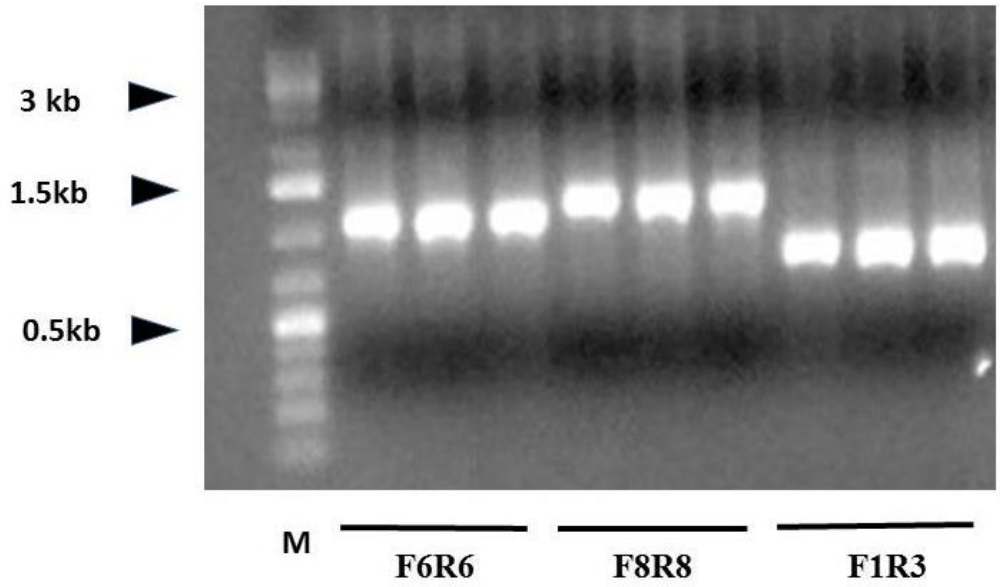

Plate 7. PCR amplification of recombinant plasmid DNA cloned with *TOR* gene, with specific primers (three replications of each segment). M: 1kb DNA ladder

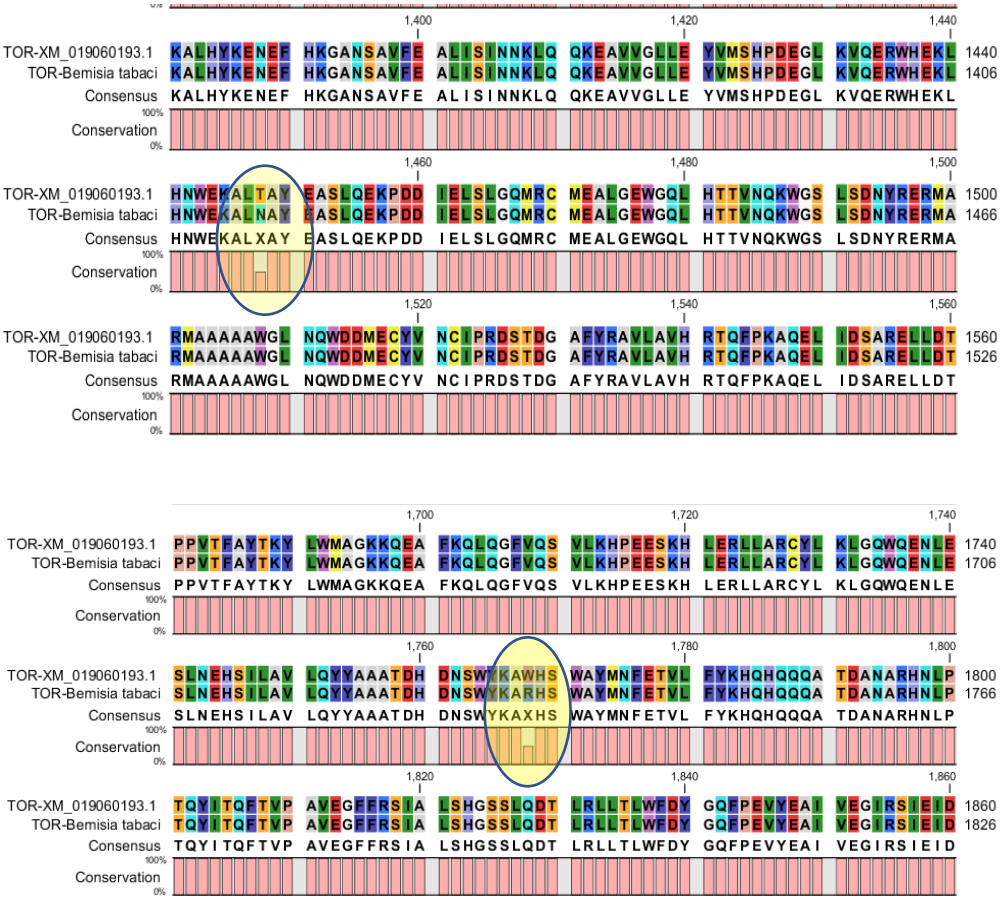

Plate 8. Amino acid sequence alignment of predicted (XM_019050193.1) and sequenced *TOR* from *B. tabaci*. Encircled shown change in amino acid of *TOR*

Table 8. Sequence homology of *target of rapamycin* (*TOR*) gene from *B. tabaci* with the available sequence in GenBank databases

Gene sequence	Description	Max score	Query coverage	Identity	GenBank Accession number
TOR	predicted *Bemisia tabaci* serine/threonine-protein kinase mTOR (LOC109043116), mRNA	12959	100 %	98.66 %	XM_019060193.1

Various studies on *target of rapamycin* gene has been conducted in different insects: *Drosophila (*Kijak *et al* 2017), *Blatella germanica* (Maestro *et al* 2009), *Nilaparvata lugens* (Zhou *et al* 2017), *Aedes agepti* (Hansen *et al* 2004, Roy *et al* 2012), *Bombyx mori* (Zhou *et al* 2010, Kamimura *et al* 1997). Suganya *et al* (2010) also obtained a full-length sequence of *BdTOR* in *Bactrocera dorsalis* having open reading frame of 7380 bp, encoding a protein of 2460 amino acids. Umemiya-Shirafuji *et al* (2012) cloned and sequenced *TOR* gene of *Haemaphysalis longicornis HlTOR* and reported that gene contained an ORF of 7572 bp, which encodes 2523 amino acids polypetide chain having a conserved domains: ataxia telangiectasia mutated (ATM), FAT (FKBP12-rapamycin-associated protein (FRAP), FBP (rapamycin/FK506-binding protein (FKBP12)-binding domain), FATC (FAT carboxyl terminal domain) transformation/transcription domain-associated protein (TRRAP) and (FKBP12)-binding domain), Kinase (phosphoinositide 3-kinase catalytic domain). It was reported that *Bombyx mori* has two paralogues *TOR* genes, BmTor1 and BmTor2, existing as inverted repeats in the silkworm genome (Zhou *et al* 2010). Maestro *et al* (2009) obtained a sequence of *TOR* gene in *B. germanica* having an ORF of 7921 bp. In *N. lugens,* Zhou *et al* (2017) revealed 7347 bp of *TOR* gene which encoded 2448 amino acids. More particularly, it has FAT, FATC, FRB and PI3/PI4 conserved protein domains. Also, 10 HEAT repeats were predicted at N-terminal which is also present in *A. aegpti* (Hansen *et al* 2004).

4.3 Phylogenetic relationship of *TOR* gene

The full length of amino sequence of *TOR* gene was aligned with the amino acid sequence of following insects: *Nilaparvata lugens*, *Pediculus humanus corporis*, *Acyrthosiphon pisum*, *Bombyx mori* isoform *1*, *Bombyx mori* isoform *2*, *Danaus plexippus*, *Aedes aegypti*, *Ceratitis capitata*, *Drosophila melanogaster*, *Blatella germanica*, *Solenopsis invicta*, *Megachile rotundata*, *Bombus terrestris*, *Apis mellifera* and *Apis cerana.* To explain the evolutionary relationship of *TOR* gene in *B. tabaci*, phylogenetic tree was constructed for different insect based on amino acid sequence of *target of rapamycin (TOR)* gene by maximum likelihood method based on the JTT matrix-based model using MEGA 7 programme. There were a total of 2311 positions in the final dataset. Evolutionary analyses were conducted in MEGA7. The phylogenetic tree attained indicates that hemipterans i.e. *B.*

tabaci, *A. pisum* and *N. lugens* were more closely related to each other than those of other insects (Fig 2).

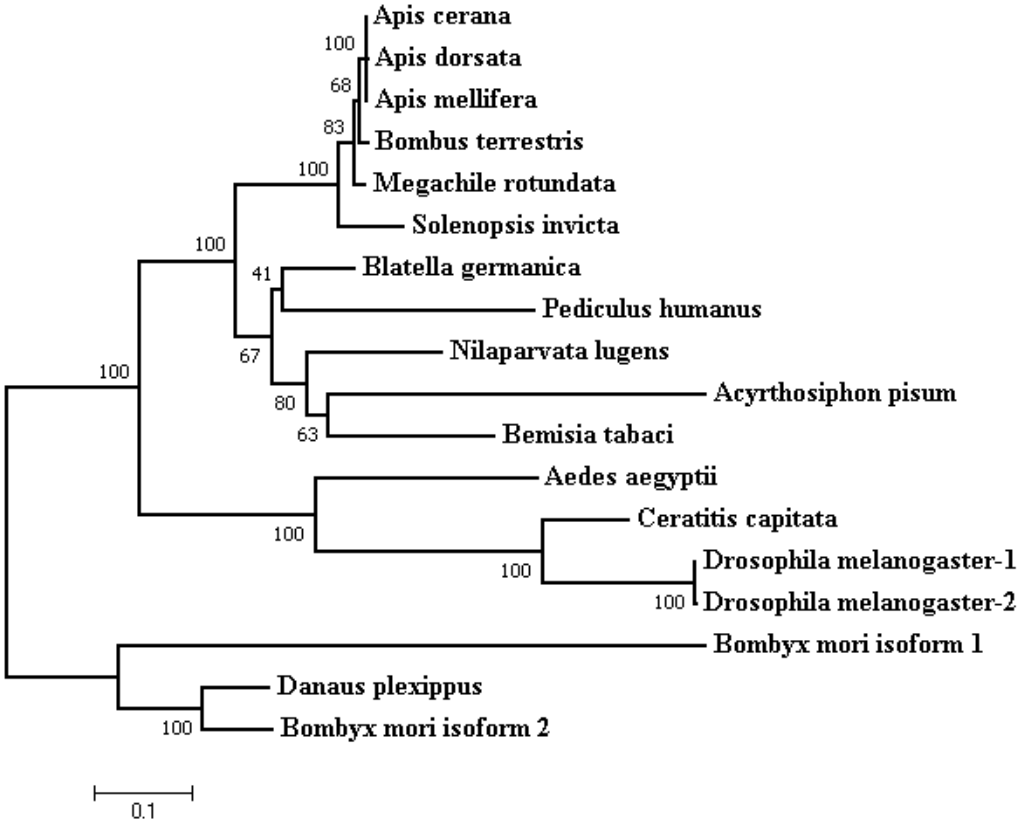

Fig 2. Phylogenetic tree constructed for different insect based on amino acid sequence of *target of rapamycin (TOR)* gene by maximum likelihood method based on the JTT matrix-based model

The amino acid sequence of BdTOR showed 79 per cent homology with dTOR of Drosophila *TOR* (Suganya *et al* 2010). BgTOR amino acid sequence showed maximum identity to *A. mellifera* (80 %) than other *TOR* proteins *Homo sapiens* (61 %), *D. melanogaster* (59 %), *Tribolium castaneum* (67 %) or (Maestro *et al* 2008). *H. longicornis* amino acid sequence showed 64.00, 61.00, 57.00, 57.00 and 55.00 per cent homology with the amino acid acid of *B. germanica*, *A. mellifera*, *A. agepti*, *B. mori* and *D. melanogaster* respectively (Umemiya-Shirafuji *et al* 2012). The *NlTOR* amino acid sequence was found to be highly conserved with full length of amino acid sequence *TOR* of *D. melanogaste*r having 56 % identity (Zhuo *et al* 2017). In our study, *TOR* gene of *B. tabaci* is more closely related to that of *N. lugens* and *A. pisum*.

4.4 Synthesis of double stranded RNA (dsRNA)

4.4.1 Amplification of *TOR* and *gfp* gene amplicons with particular primer sets

TOR and *gfp* gene fragments with primer sets with and without having T7 promoter sequence (5'-GAATTGTAATACGACTCACTATAGG-3') in each primer were PCR amplified and gel purified. Good quality amplification was observed on gel and the amplified

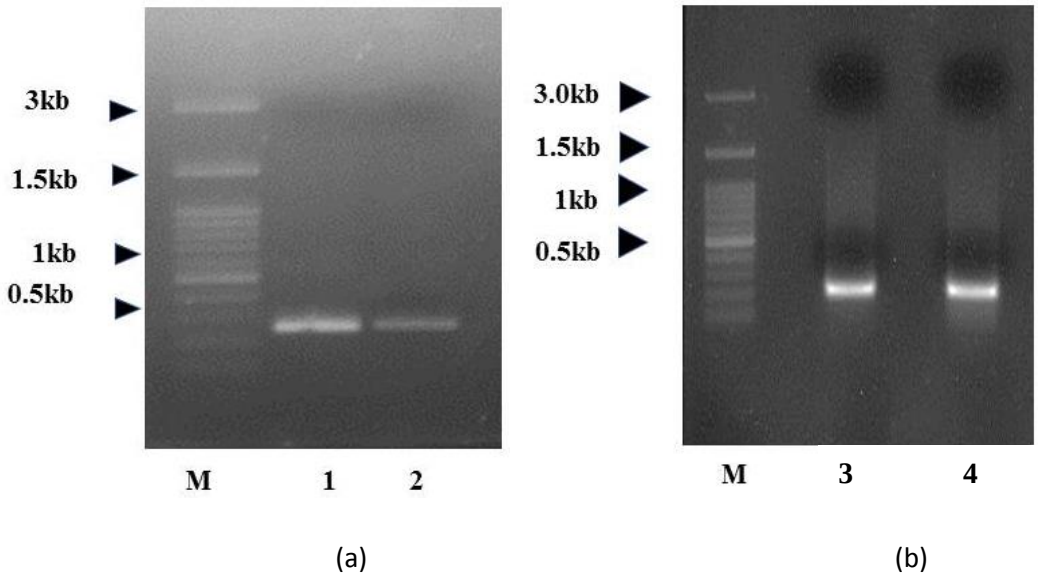

Plate 9. PCR amplification of *TOR* and *gfp* gene fragments with primer sets containing T7 promoter sequence. (a) Lane 1: BtTOR-F-T7 (~ 264bp), Lane 2: BtTOR-R-T7 (~ 264bp). (b) Lane 1: *GFP* -F-T7 (~ 200bp), Lane 2: *gfp*-R-T7 (~ 200bp). M: 100bp DNA ladder (SMOBIO)

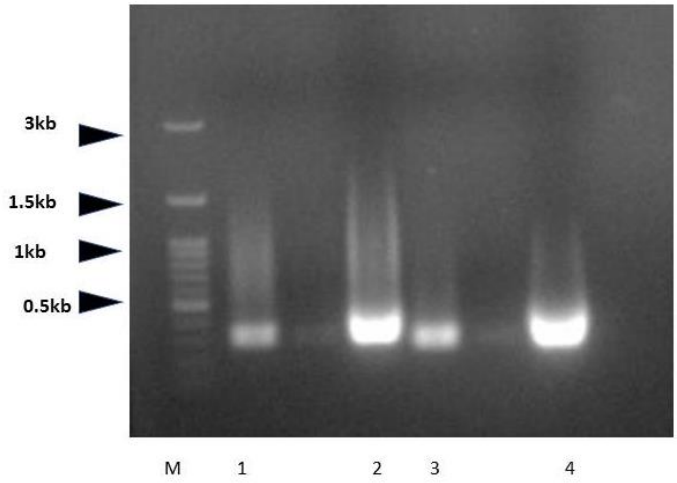

Plate 10. dsRNA synthesis corresponding to *TOR* and *gfp* gene fragments resolved on agarose gel (1.5%). M: 100bp DNA Ladder (SMOBIO). Lane 1: dsRNA corresponding to *TOR* gene (dilution factor-1:10), Lane 2: dsRNA corresponding to *TOR* gene, Lane 3: dsRNA corresponding to *gfp* gene (dilution factor- 1:10), Lane 4: dsRNA corresponding to *gfp* gene

products were considered appropriate for synthesis of ssRNA, corresponding to *TOR* and *gfp* genes fragments (Plate 9 a and b).

4.4.2 dsRNA synthesis

The amplified products were then used as templates for ssRNA synthesis using MEGAscript RNAi kit (Thermo Fisher Scientific). Forward and reverse ssRNAs were pooled together to anneal the RNA for dsRNA synthesis. The quality and quantity of dsRNA was checked on agarose gel (1.5 %) electrophoresis and Biospectrometer. The concentration of dsRNA was 1200 and 2100 ng/µl for *dstor* and *dsgfp*, respectively and the size of *dstor* and *dsgfp* were 265 and 200 bp, respectively as desired (Plate 10).

Ghanim *et al* (2007), Asokan *et al* (2015) and Upadhyay *et al* (2011) also used this similar process for the synthesis of ssRNA. Saleh *et al* (2006) reported the length of dsRNA which should be efficient for causing gene silencing. The length of dsRNA required for efficient RNAi varies from insect to insect. In S2 cells, dsRNA of minimum 211 bp is optimum for RNAi. Bolognesi *et al* (2012) reported that the length of dsRNA has effect on biological activity of corn rootworms. They found that 240 bp had more toxic effect than any other shorter length of dsRNA against root cornworms. Agarwal *et al* (2004) investigated the effect of two different sizes of dsRNA at different concentration and reported that decrease in the length of dsRNA did not enhanced the silencing effect. In *Manduca sexta, Bactericera cockerelli* and *Acyrthosiphon pisum*, it has been reported that dsRNAs length ranging from 21 to 27 nucleotides cause gene suppression. These shorter molecules are known as short interfering RNAs (siRNA) (Kumar *et al* 2012, Wurinyanghan *et al* 2011, Mutti *et al* 2006). However, (Miller *et al* 2012) reported that for achieving successful interference, a least of 70 nucleotides are needed for cellular uptake.

4.5 Effect of feeding dsNRA on the biology of *B. tabaci*

Artificial diet was prepared by incorporating different concentrations of dsRNA corresponding to *TOR* and *gfp* gene in 20 per cent sucrose solution *viz*., 0.1, 0.5, 1.0 and 2.0 µg/µl. In experiment 1 (June-July 2019) and experiment 2 (July-August 2019), the concentrations of dsRNA fed to whitefly were *viz*. 0.1, 0.5 and 1.0 µg/µl. During experiment 3 (August-September 2019), one more treatment was added by increasing the concentration to 2 µg/µl. Different parameters were studied to check the overall effect of dsRNA feeding on whitefly. This includes adult mortality, fecundity, mortality of three nymphal instars and pupal stage, duration of nymphal and pupal stage and adult emergence.

4.5.1 Continuous feeding on artificial diet bioassay

After 2 days of feeding, the adult mortality was found to be significantly higher (35.00, 43.75 and 47.75 %) in all the treatments of *dstor* fed whiteflies (@ 1.0, 0.5 and 0.1 µg/µl), respectively, as compared to all the treatments of *dsgfp* and control which were statistically at par to each other (Table 9). Similar results were recorded after 4 and 6 days of

feeding. After 8 days of feeding, mortality was found in whiteflies fed with *dstor* @ 0.5 µg/µl followed by *dstor* 0.1 µg/µl which are statistically better than *dstor* @ 1.0 µg/µl and control. After ten days of feeding, mortality in all the treatments of *dstor*, *dsgfp* and control were statistically at par with each other.

Table 9. Adult mortality of *Bemisia tabaci* fed on different concentrations of dsRNA corresponding to *Target of rapamycin* (*TOR*) gene in leaf cages

Treatment	Concentration of dsRNA (µg/µl)	Adult mortality (%)				
		(After 2 days)	(After 4 days)	(After 6 days)	(After 8 days)	(After 10 days)
dstor	1.0	35.00 (36.04)	41.25 (39.75)	63.75 (53.45)	75.00 (60.08)	90.00
	0.5	43.75 (41.29)	52.50 (46.54)	71.25 (62.14)	93.75 (75.67)	100.00
	0.1	47.75 (43.47)	67.50 (59.17)	85.00 (70.27)	93.75 (79.66)	100.00
dsgfp	1.0	11.25 (19.08)	28.75 (32.01)	51.25 (45.72)	87.50 (74.96)	88.75
	0.5	22.50 (27.16)	37.50 (37.24)	51.25 (45.72)	73.75 (60.20)	78.75
	0.1	13.75 (21.02)	21.25 (26.90)	23.75 (28.40)	78.75 (63.06)	97.50
Control (Sucrose solution 20 %)		13.75 (21.54)	26.25 (30.69)	40.00 (38.93)	53.75 (47.28)	97.50
CD (p=0.05)		(11.67)	(17.93)	(20.69)	(15.63)	NS

dstor: dsRNA corresponding *TOR* gene, *dsgfp*: dsRNA corresponding to green fluorescent protein gene as control gene, Number of replications: 4, Figures in parentheses are arc sine root percentage transformed values

4.5.2 Bioassay after feeding of whiteflies for 48 hours on artificial diet

4.5.2.1 Adult mortality after 48 hours of feeding

In June-July, maximum mortality was recorded in whitefly adults fed with *dstor* @ 1.0 µg/µl (46.66 %) which was statistically at par with other treatment of *dstor* @0.5 and 0.1 µg/µl (41.66 and 43.33 %) and significantly higher than all the treatments of *dsgfp* and control (Table 10). Minimum mortality (16.66 %) was recorded at *dsgfp* @ 1.0 µg/µl.

During July-August, maximum mortality was recorded in whiteflies fed with *dstor* @ 0.5 and 0.1 µg/µl (41.66 %) which was statistically at par with *dstor* @ 1.0 µg/µl (35.00) but significantly higher than all the treatments of *dsgfp* and control. Minimum mortality was recorded at *dsgfp* @ 1.0 µg/µl (10.00 %) Mortality in all *dsgfp* treatments and control (sucrose 20 %) was statistically similar.

In Aug-Sept, maximum mortality (26.25 %) was found in whiteflies fed with *dstor* @ 2.0 µg/µl which was statistically at par with dstor @ 1.0 µg/µl (18.75 %), but significantly

higher than all the treatments of *dstor* (@ 0.5 and 0.1 µg/µl), *dsgfp* and control. Minimum mortality was recorded at control (7.5 %). All the experiments showed, the adult mortality was significantly higher when *dstor* was fed to whiteflies.

Table 10. Adult mortality of *Bemisia tabaci* fed on different concentrations of dsRNA corresponding to *Target of rapamycin* (*TOR*) gene for 48 hours

Treatment	Concentration of dsRNA (µg/µl)	Adult mortality (%)		
		June-July*	July-Aug*	Aug-Sept**
dstor	2.0	-	-	26.25 (30.75)
	1.0	46.66 (42.97)	35.00 (36.25)	18.75 (25.61)
	0.5	41.66 (40.15)	41.66 (40.16)	13.75 (21.25)
	0.1	43.33 (41.05)	41.66 (40.16)	15.00 (21.97)
dsgfp	1.0	16.66 (24.03)	10.00 (18.04)	10.00 (18.13)
	0.5	28.33 (32.00)	18.33 (24.98)	8.75 (16.75)
	0.1	30.00 (32.74)	21.66 (27.69)	10.00 (18.13)
Control (Sucrose solution 20 %)		23.33 (28.65)	16.66 (23.73)	7.5 (15.67)
CD (p=0.05)		(11.24)	(8.02)	(6.79)

dstor: dsRNA corresponding *TOR* gene, *dsgfp*: dsRNA corresponding to green fluorescent protein gene as control gene. *Number of replications: 3, **Number of replications: 4, Figures in parentheses are arc sine root percentage transformed values
Note: 2 µg/µl is only added in during Aug-Sept

TOR serves as a master regulator of cell metabolism, growth, proliferation and survival. Adulty mortality recorded in our study might be due the involvement of *TOR* in important growth signalling pathways (Laplante and Sabatini, 2012). *TOR* gene silencing resulted in mortality of adult whiteflies when fed with dsRNA, similar kind of results were stated in other insects by using RNAi. *dsNlTOR* treated males of BPH had significantly higher mortality as compared to dsGFP fed males. In *B. tabaci*, knockdown of juvenile hormone esterase (*jhe*) also caused mortality (63 %) of adult whiteflies after 2 days of feeding dsRNA corresponding to *jhe* (Grover *et al* 2019). Perez *et al* (2013) showed that JH biosynthesis is in part regulated by nutrient signalling via the TOR pathway.

Using RNAi, targeting various genes in different insects also caused mortality of insects. Surakasi *et al* (2011) reported that feeding of *Spodoptera exigua* (Hübner) larvae with dsRNA against β1 integrin subunit (βSe1) caused significant mortality. Zhu *et al* (2011) knockdown *sec23, COP B,* actin, vATPase A and *vATPase B* by utilising RNAi for managing *Leptinotarsa decemlineata* and reported significant mortalities. In *A. pisum* also, silencing of *vATPase* gene led to its mortality (Sadeghi *et al* 2009). Knockdown of trehalose phosphate synthase gene in *N. lugens* resulted in significant decrease in mRNA levels of TPS gene,

enzymatic activity and also led to mortality/lethality due to decreased development (Chen *et al* 2010). Twenty per cent *Bactrocera dorsalis* (Hendel) adults were killed upon ingestion of dsRNA corresponding to *Rab 11* (Li *et al* 2011). dsRNA corresponding to *ATPase* and *actin* were fed and injected, respectively to *Bactericera cockerelli* (Sulc), which provoke RNAi response and caused mortality of potato/tomato psyllid (Wuriyanghan *et al* 2011).

Whyard *et al* (2009) demonstrated that species-specific dsRNA against E-subunit of *vATPase* incorporated in artificial diet or placed on surface of solid food resulted in 50-75 per cent mortality of *A. pisum*, *M. sexta* and *T. castaneum*. Upadhyay *et al* (2011) targeted five genes viz., ADP/ATP translocase, ribosomal protein L9 (RPL9), α-tubulin, actin ortholog and V-ATPase A subunit. dsRNA against these genes were fed to adult whiteflies which resulted in 29-97 per cent mortality after 6 days of feeding. Asokan *et al* (2015) also utilized RNAi machinery in whitefly to knockdown *Glutathion S-transferase* of sigma class which is responsible for detoxification of plant secondary metabolites and involved in insecticide resistance. Diets containing different concentrations of dsRNA i.e. 1.0, 0.5 and 0.25 µg/µl were fed to whiteflies. Real-time quantitative analysis showed that the expression levels of *BtGST* was reduced by 77, 65 and 53 per cent and this resulted in 77, 59 and 40 per cent mortality of whitefly. Role of Juvenile hormone in whitefly survival and reproduction was studied through RNA interference mechanism. Artificial diets (20 % sucrose solution) containing different concentrations of dsRNA corresponding to juvenile hormone transcript were fed to whiteflies. Mutti *et al* 2006 utilised RNAi technique in *A. pisum* to investigate the importance of C002 protein and the results indicated that knockdown of this gene led significant mortality and decreased survival. Plant mediated expression of dsRNA corresponding to ecdysone receptor and acetylcholinesterase using tobacco rattle virus plasmid significantly induced mortality in adult whiteflies (Malik *et al* 2016). The same kind of study was conducted in lettuce (*Lactuca sativa*) in which transgenics lettuce plants expressed dsRNA corresponding to the v-ATPase gene. After five days of feeding of whiteflies on these plants, it was observed that 83.8 - 98.1 per cent mortality was occurred due to silencing of the gene (Ibrahim *et al* 2017). In *Diaphorina citri*, adult and nyphmal mortality was observed upon artificial feeding of dsRNA corresponding to *boule* gene.

4.6.2.2 Fecundity

In Aug-Sept, minimum number of eggs (41.25 eggs/female) were laid when whiteflies were fed with *dstor* @ 1.0 µg/µl, which was statistically at par with *dstor* @ 2.0 and 0.5 µg/µl and significantly lower (70 eggs/female) than *dstor* @ 0.1 µg/µl and all the treatments of *dsgfp* and control (Table 11). Maximum number of eggs (70.00) were laid when whiteflies were fed at *dsgfp* @0.1 µg/µl which was significantly at par with control.

In another experiment conducted in October, minimum number of eggs were laid when whiteflies were fed at *dstor* @ 1.0 µg/µl (30.33) which was statistically at par with all

the concentrations of *dstor* and significantly lower than all the treatments of *dsgfp* and control. Maximum number of eggs (64.77 eggs/female) were laid at *dsgfp* @ 0.1 µg/µl. Number of eggs laid in all the treatments of *dsgfp* and control were statistically at par with each other.

Table 11. Fecundity of *Bemisia tabaci* fed on different concentrations of dsRNA corresponding to *target of rapamycin* (*TOR*) gene

Treatment	Concentration of dsRNA (µg/µl)	Eggs laid (No of eggs laid per female)	
		Aug-Sept	October
dstor	2.0	44.25	33.81
	1.0	41.25	30.33
	0.5	45.00	31.57
	0.1	59.00	33.10
dsgfp	1.0	58.25	51.93
	0.5	52.75	63.22
	0.1	70.00	64.77
Control (Sucrose solution 20 %)		62.75	55.78
CD (p=0.05)		11.24	16.63

dstor: dsRNA corresponding *TOR* gene *dsgfp*: dsRNA corresponding to green fluorescent protein gene as control gene. Number of replications: 4

In *N. lugens,* knockdown of *NlTOR* gene in female planthoppers inhibited the ovary development and oogenesis. Regulation of fecundity in *N. lugens* were demonstrated by (Zhai *et al* 2015). mRNA of *TOR* was significantly decreased by 59, 68 and 66 percent at 24h, 48h and 72h, respectively. Similarly, the Vg mRNA level was also reduced by 47, 52 and 59 per cent upon dsTOR injection 24h, 48h and 72h, respectively. Less than 100 progenies were observed in dsTOR treated adults suggesting that reduced fecundity is probably due to lower Vg expression. Glutamine effect on activation of *TOR* pathway was studied which revealed that glutamine is also involed in regulating insect fecundity. Glutamine activates the TOR pathway by ribosomal S6K phosphorylation. Silencing of glutamate synthetase has resulted in decreasing the expression of *Rheb, TOR, S6K* genes which in turn downregulate *vg* expression and fecundity in *N. lugens*. Also, it has been reported that in male BPH, *NlTOR* had complex functions in male fertility. The impact of dsNlTOR was found to be more tissue specific on accessory gland's development in males and accessory gland were poorly developed and lost the function of creating seminal fluid (Zhou *et al* 2017, Zhai *et al* 2015). Similar kind of effect could have happened in male whiteflies which might have led to failure in the delivery of sperms to females. Also, in female whiteflies, the production of vitellegenin might be

affected by *TOR* gene silencing decrease fecundity.

Vitellogenesis is an important process in insect reproduction. *TOR* plays an important role in nutrient signalling pathway. Hansen *et al* (2004) reported that upon injecting dsTOR to female mosquitoes, vitellogenin expression was reduced which led to the decrease in number of eggs laid and smaller ovaries were observed. There is a direct relationship between phosphorylation of S6K through *TOR* and amino acid signalling in fat bodies and ovaries of *A. aegypti*. Silencing of *AaS6K* effectively blocked egg development in mosquito after blood meal (Hansen *et al* 2004). In *B. germanica*, mRNA level of vitellogenein was low and ovaries did not grow upon silencing of *BgTOR* gene (Maestro *et al* 2009). Suganya *et al* (2010) reported the reduction in ovary size and yolk protein synthesis in *Bactrocera dorsalis* when BdTOR was targeted. GATA transcription factor is involved in controlling the expression of vitellogenin under nutritional signalling (Park *et al* 2006). Previous study in Drosophila has shown a link between nutrient signally and insulin-dependent growth and revealed that two insulin- like peptides are essential for female fertility (Ikeya *et al* 2002). In *A. aegypti* insulin-like peptide 3 is an important regulator of egg production (Gulia-Nuss *et al* 2011). Roy *et al* (2007) reported that presence or absence of TOR pathway components greatly influences the sensing of nutritional signals by the fat body when mosquito feeds on a blood meal. It decides that whether a blood meal results in immediate or delayed YPP synthesis in mosquito. TOR pathway is directly related to vitellogenesis and silencing of *TOR* gene might have affected ribosomal protein S6K which is involved in production of vitellogenin. This could be the possible reason for lower fecundity in *dstor* treated whitefly females.

In addition silencing of some other genes also affected the fecundity through RNAi in different insects. Injection of dsRNA corresponding to *chickadee* gene in *B. tabaci* resulted in disruption of actin network for egg development and whiteflies could not last for more than 48 hours after injection. Therefore, *chickadee* knockdown might be lethal for egg laying and whitefly development (Ghanim *et al* 2007). Grover *et al* (2019) reported the reduced fecundity in *B. tabaci* females. *dsjhe* @ 2.5 µg/µl fed whitefly females laid 4.83 eggs/female which was lower as compared to *dsgfp* i.e. 22 eggs/female. Attardo *et al* (2008) reported that silencing of *gmmmgp*, a milk gland protein in *Glossina moritans* Westwood resulted in lowering down of fecundity and females were unsuccessful to oviposit. Effect on egg production was seen by silencing a small GTPase Rab11 and fatty acid elongase *Noa* in *B. dorsalis* through RNAi (Li *et al* 2011). Decrease in vitellogenin expression level and inhibition of fecundity in females (around 99 % reduction in oviposition) were reported by Wang *et al* (2013) by targeting 3hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) gene using RNAi. In *N. lugens*, microinjection of dsRNA corresponding to ribosomal protein L5 led to the reduction in development of ovary and number of eggs laid per female (Zhu *et*

al 2017). Similarly, Lu *et al* (2018) microinjected insulin-like peptides and DNA/tRNA methyltransferases reduced ovarian growth and maturation of oocyte in *N. lugens.* Will *et al* (2017) microinjection dsRNA against heat shock protein 83 in *A. pisum* which resulted in decrease in lifespan and fecundity. Transgenic lettuce expressing dsRNA corresponding to v-ATPase were constructed by Ibrahim *et al* (2017) against *B. tabaci*. A reduced number of eggs were recorded on the transgenic lines as compared to the control lines of lettuce. Yu and Killiny (2018) targeted *transformer 2* gene through RNAi in *D. Citri* and reported that female of citrus pysliid laid fewer eggs.

4.5.2.3 Total nymphal mortality

The first experiment conducted during June-July revealed the maximum nymphal mortality at *dstor* @ 1.0 µg/µl (56.67 %) which was statistically at par with *dstor* @ 0.5 and 0.1 µg/µl (53.33 and 41.67 %) (Table 12). Mortality at all the treatments of *dstor* were significantly higher as compared to all the treatments of *dsgfp* and control. Minimum mortality was recorded at *dsgfp* @0.1 µg/µl (28.33 %).

During June-July, maximum nymphal mortality was recorded at *dstor* @ 1.0 µg/µl (57.16 %) which was statistically at par with *dstor* @ 0.5 and 0.1 µg/µl (42.16 and 53.83 %). These were significantly higher as compared to all the treatments of *dsgfp* and control. Minimum mortality was recorded at *dsgfp* @0.1 µg/µl (17.16 %).

Table 12. Nymphal mortality of next generation of *Bemisia tabaci* fed on different concentrations of dsRNA corresponding to *target of rapamycin* (TOR) gene

Treatment	Concentration of dsRNA (µg/µl)	Nymphal mortality (%)		
		June-July*	July-August*	Aug-Sept**
dstor	2.0	-	-	62.50 (52.26)
	1.0	56.67 (49.02)	57.16 (49.14)	56.25 (48.60)
	0.5	53.33 (46.90)	42.16 (40.28)	51.25 (45.98)
	0.1	41.67 (40.16)	53.83 (47.19)	61.25 (52.03)
dsgfp	1.0	30.00 (33.14)	27.16 (31.26)	25.00 (29.93)
	0.5	25.00 (29.91)	25.50 (30.24)	23.75 (28.89)
	0.1	28.33 (32.12)	17.16 (21.49)	22.50 (28.12)
Control (Sucrose solution 20 %)		31.66 (34.13)	22.16 (27.37)	16.25 (22.59)
CD (p=0.05)		(10.18)	(14.62)	(11.36)

dstor: dsRNA corresponding *TOR* gene, *dsgfp*: dsRNA corresponding to green fluorescent protein gene as control gene. *Number of replications: 3, **Number of replications: 4, Figures in parentheses are arc sine √percentage transformed values
Note: 2 µg/µl is only added in experiment conducted during Aug-Sept

In Aug-Sept, maximum nymphal mortality was recorded at *dstor* @ 2.0 µg/µl (62.50 %) which was statistically at par with *dstor* @ 1.0, 0.5 and 0.1 µg/µl (48.60, 45.98 and 52.03 %). Mortality in all the *dstor* treatments were significantly higher as compared to all the treatments of *dsgfp* and control. Minimum mortality (16.25 %) was recorded in control treatment.

4.5.2.4 Nymphal instars mortality

During July-Aug, the maximum first instar nymphal mortality (42.16 %) was recorded in whiteflies fed with *dstor* @ 0.1 µg/µl and minimum (7.16 %) in control was recorded. In all the treatment of *dstor* fed whiteflies, first instar nymphal mortality was statistically at par to each other and significantly higher than all the treatments of *dsgfp* and control (Table 13). The maximum second instar nymphal mortality (15.05 %) was recorded in whiteflies fed with *dstor* @ 0.1 µg/µl and minimum mortality (4.54 %) in *dsgfp* @ 0.1 µg/µl was recorded. The maximum third instar nymphal mortality (5.02 %) was recorded in whiteflies fed with *dstor* @ 1.0 µg/µl and minimum mortality (0.50 %) in *dstor* @ 0.5 and 0.1 µg/µl and *dsgfp* @ 0.1 µg/µl was recorded. However, no significant difference was observed in the treatments of *dstor*, *dsgfp* and control with respect to second and third nymphal instar mortality.

Table 13. Mortality of nymphal instars of next generation of *Bemisia tabaci* fed on different concentrations of dsRNA corresponding to *Target of rapamycin* (*TOR*) gene (July-August 2019)

Treatment	Concentration of dsRNA (µg/µl)	Nymphal instar mortality (%)		
		First instar	Second instar	Third instar
dstor	1.0	37.16 (37.40)	8.60	5.02
	0.5	38.83 (37.90)	4.20	0.50
	0.1	42.16 (40.44)	5.84	0.50
	1.0	15.50 (22.99)	15.05	0.50
dsgfp	0.5	15.50 (20.24)	7.22	2.58
	0.1	12.16 (18.04)	4.54	0.50
Control (Sucrose solution 20 %)		7.16 (15.33)	5.70	2.58
CD (p=0.05)		(16.93)	NS	NS

dstor: dsRNA corresponding *TOR* gene, *dsgfp*: dsRNA corresponding to green fluorescent protein gene as control gene, Number of replications: 3, Figures in parentheses are arc sine √percentage transformed values

In experiment conducted during Aug-Sept, the maximum first instar nymphal mortality (49.25 %) was recorded in whiteflies fed with *dstor* @ 2.00 µg/µl (Table 14). The minimum mortality (5.50 %) was recorded when whiteflies fed on *dsgfp* @ 0.5 µg/µl, which was statistically at par with dstor @ 0.1 µg/µl and control. The maximum second instar nymphal mortality (25.94 %) was recorded in whiteflies fed with *dstor* @ 2.0 µg/µl and

minimum (3.62 %) was in *dsgfp* @ 0.5 µg/µl. The maximum third instar nymphal mortality (20.85 %) was recorded in whiteflies fed with *dstor* @ 1.0 µg/µl and minimum (3.00 %) in *dstor* @ 0.1 µg/µl. No significant difference was observed in the treatments of *dstor*, *dsgfp* and control with respect to second and third nymphal instar mortality. Both experiments data revealed that effect of silencing of *TOR* gene in *B. tabaci* is upto the first nymphal significantly higher mortality.

Table 14. Mortality of nymphal instars of next generation of *Bemisia tabaci* fed on different concentrations of dsRNA corresponding to *Target of rapamycin (TOR)* gene (August-September 2019)

Treatment	Concentration of dsRNA (µg/µl)	Nymphal instar mortality (%)		
		First instar	Second instar	Third instar
dstor	2.0	49.25 (44.60)	25.94	8.62
	1.0	23.00 (26.93)	15.76	20.85
	0.5	41.75 (40.13)	6.63	19.19
	0.1	48.00 (43.85)	20.18	13.00
dsgfp	1.0	29.52 (32.37)	5.89	3.00
	0.5	5.50 (9.77)	3.62	12.98
	0.1	13.45 (19.33)	14.94	3.62
Control (Sucrose solution 20 %)		8.00 (16.22)	13.12	3.19
CD (p=0.05)		(14.91)	NS	NS

dstor: dsRNA corresponding *TOR* gene, *dsgfp*: dsRNA corresponding to green fluorescent protein gene as control gene, Number of replications: 4, Figures in parentheses are arc sine √percentage transformed values

Zhou *et al* (2017) found that survival of nymphal instars of *N. lugens* decreased with the treatment of *dsNlTOR* as compared to dsGFP treated instars. Spatio-temporal NlTOR gene expression suggested that this gene might be important for BPH's development. The nymphal mortality could have also occured due to the persistence of *dstor* in the initial developmental stages of whitefly. In larval instars of Drosophila, fat bodies act as amino acid sensor and those AAs are sensed by TOR signalling pathway. Initiation of moulting of larval instars is mediated by the presence of amino acids sensitive pathway in *Manduca sexta*. When the TOR inhibitor rapamycin is applied on the larval stages, the growth of prothoracic glands suppressed with relative to the whole body which is further accompanied by the suppression of ecdysone production in *M. sexta*. Increased level of rapamycin also effects the growth rate negatively revealing that the TOR signalling plays a role in systemic growth (Karen *et al* 2012).

RNAi technique has been used in different insects in which targeting a particular gene resulted in nymphal/larval mortality. Ramaseshadri *et al* (2013) used oral deliverly method of

dsRNA for RNAi in *Diabrotica virgifera virgifera* Le Conte (WCR) larvae. They targeted Snf7 which is a vacuolar sorting protein responsible for organization of transmembrane proteins. RNAi on this gene resulted in decrease in mRNA and protein levels leading to autophagy and mortality of larvae. dsRNA feeding corresponding to *OnCht* gene to European corn borer larvae revealed that *OnCht* gene plays an crucial role in maintaining chitin content of peritrophic membrane of midgut which eventually regulates its growth and development (Khajuria *et al* 2010).

4.5.2.5 Pupal mortality

In June-July, the maximum pupal mortality (20.15 %) was recorded in whiteflies fed with *dstor* @ 1.0 µg/µl and minimum (11.61 %) in *dsgfp* @ 1.0 µg/µl and control (Table 15). During July-August, maximum mortality was recorded in whiteflies fed with *dstor* @ 0.1 µg/µl (10.50 %) and minimum mortality (0.05 %) was recorded in control. During August-September, pupal mortality ranges from 2.43 to 13.83 per cent. However, no significant differences were observed in all the treatments across all the experiments with respect to pupal mortality.

Table 15. Mortality in pupal stage of next generation of *Bemisia tabaci* fed on different concentrations of dsRNA corresponding to *Target of rapamycin (TOR)* gene

Treatment	Concentration of dsRNA (µg/µl)	Pupal mortality (%)		
		June-July*	July-Aug*	Aug-Sept**
dstor	2.0	-	-	2.42
	1.0	20.15	6.38	3.83
	0.5	18.19	4.42	5.91
	0.1	14.92	10.50	5.25
dsgfp	1.0	11.61	3.26	10.63
	0.5	14.92	3.83	13.83
	0.1	14.39	4.20	3.83
Control (Sucrose solution 20 %)		11.61	0.50	0.50
CD (p=0.05)		NS	NS	NS

dstor: dsRNA corresponding *TOR* gene, *dsgfp*: dsRNA corresponding to green fluorescent protein gene as control gene. *Number of replications: 3, **Number of replications: 4, Figures in parentheses are arc sine √percentage transformed values
Note: 2 µg/µl is only added in during Aug-Sept

4.5.2.6 Nymphal duration

In June-July, the duration of nymphal stage varied from 9.33 to 10.83 days with minimum in *dsgfp* @ 1.0 µg/µl and maximum in *dstor* @ 1.0 µg/µl and all treatments were at par with each other (Table 16). During July-August, the nymphal duration ranged from 9.12 to 10.88 days, showed no significant difference between all treatments. Similarly, no significant difference was observed in nymphal duration between all treatments in experiment

conducted during August- September, which ranged from 9.72 to 11.5 days.

Table 16. Duration of nymphal stage of next generation of *Bemisia tabaci* fed on different concentrations of dsRNA corresponding to *Target of rapamycin* (TOR) gene

Treatment	Concentration of dsRNA (µg/µl)	Mean (±SEM) duration of nymphal stage (days)		
		June-July*	July-Aug*	Aug-Sept**
dstor	2.0	-	-	11.16±0.48
	1.0	10.83±0.30	10.12±0.36	10.47±0.52
	0.5	10.19±0.54	10.55±0.52	10.73±0.28
	0.1	10.35±0.67	9.67±0.36	10.11±0.26
dsgfp	1.0	9.33±0.67	10.88±0.75	10.62±0.58
	0.5	9.99±0.40	10.73±0.37	9.72±0.27
	0.1	10.27±0.62	9.12±0.24	11.11±0.16
Control (Sucrose solution 20 %)		10.46±0.20	10.28±0.17	11.50±0.85
CD (p=0.05)		NS	NS	NS

dstor: dsRNA corresponding *TOR* gene, *dsgfp*: dsRNA corresponding to green fluorescent protein gene as control gene. *Number of replications: 3, **Number of replications: 4
Note: 2 µg/µl is only added in during Aug-Sept

4.5.2.7 Pupal duration

During June-July 2019, all treatments were at par with each other with the duration of pupal stage varying from 3.57 to 4.57 days with minimum in *dsgfp* @ 1.0 µg/µl and

Table 17. Duration of pupal stage of next generation of *Bemisia tabaci* fed on different concentrations of dsRNA corresponding to *Target of rapamycin* (TOR) gene

Treatment	Concentration of dsRNA (µg/µl)	Mean (±SEM) duration of pupal stage (days)		
		June-July*	July-Aug*	Aug-Sept**
dstor	2.0	-	-	3.3±0.15
	1.0	3.69±0.25	3.54±0.03	3.13±0.05
	0.5	4.57±0.18	3.40±0.18	3.18±0.10
	0.1	3.75±0.14	3.35±0.15	3.05±0.27
dsgfp	1.0	3.57±0.30	3.61±0.07	3.12±0.13
	0.5	4.28±0.25	3.46±0.13	2.95±0.27
	0.1	3.95±0.19	3.67±0.10	2.73±0.05
Control (Sucrose solution 20 %)		3.69±0.29	3.58±0.08	2.79±0.15
CD (p=0.05)		NS	NS	NS

dstor: dsRNA corresponding *TOR* gene, *dsgfp*: dsRNA corresponding to green fluorescent protein gene as control gene. *Number of replications: 3, **Number of replications: 4
Note: 2 µg/µl is only added in during Aug-Sept

maximum in *dstor* @ 0.5 µg/µl (Table 17). Experiment conducted in July-August, the pupal duration ranged from 3.35 to 3.67 days, showed no significant difference between all treatments. Similarly, no significant difference was observed in pupal duration between all treatments of experiment conducted during August- September, which ranged from 2.73 to 3.3 days.

4.5.2.8 Adult emergence

Adult emergence of progeny of whiteflies failed to differ significantly among different treatments. The emergence was found 100 per cent in all the treatments across experiments conducted during June-July and July-August. However, during Aug-Sept, adult emergence ranged from 97.22 to 100 per cent and differed non-significantly with respect to adult emergence (Table 18).

Table 18. Adult emergence of progeny of whiteflies fed on different concentrations of dsRNA corresponding to *Target of rapamycin* (TOR) gene

Treatment	Concentration of dsRNA (µg/µl)	Per cent adult emergence		
		June-July	July-Aug*	Aug-Sept**
dstor	2.0	-	-	100.00
	1.0	100.00	100.00	97.22
	0.5	100.00	100.00	100.00
	0.1	100.00	100.00	100.00
dsgfp	1.0	100.00	100.00	100.00
	0.5	100.00	100.00	100.00
	0.1	100.00	100.00	100.00
Control (Sucrose solution 20 %)		100.00	100.00	100.00
CD (p=0.05)		NS	NS	NS

dstor: dsRNA corresponding *TOR* gene, *dsgfp*: dsRNA corresponding to green fluorescent protein gene as control gene. *Number of replications: 3, **Number of replications: 4
Note: 2 µg/µl is only added in during Aug-Sept

Silencing of *TOR* in adult whiteflies is effective till first instar nymphs but ineffective at later developmental stages. It might be possible that the persistence of dsRNA mediated diet effects only for initial development phases of whitefly progeny and parental RNAi is established till first instar nymphs only.

4.6 Estimation of down regulation of *TOR* gene in whitefly after RNAi

qRT-PCR experiment was conducted for *TOR* gene expression analysis in *B. tabaci.* Adult whiteflies were fed with the different concentrations of dsRNA corresponding to *TOR* and *gfp* gene. The validity of cDNA was checked by running normal PCR using BtTOR

primers designed for qPCR for each treatment (Plate 11).

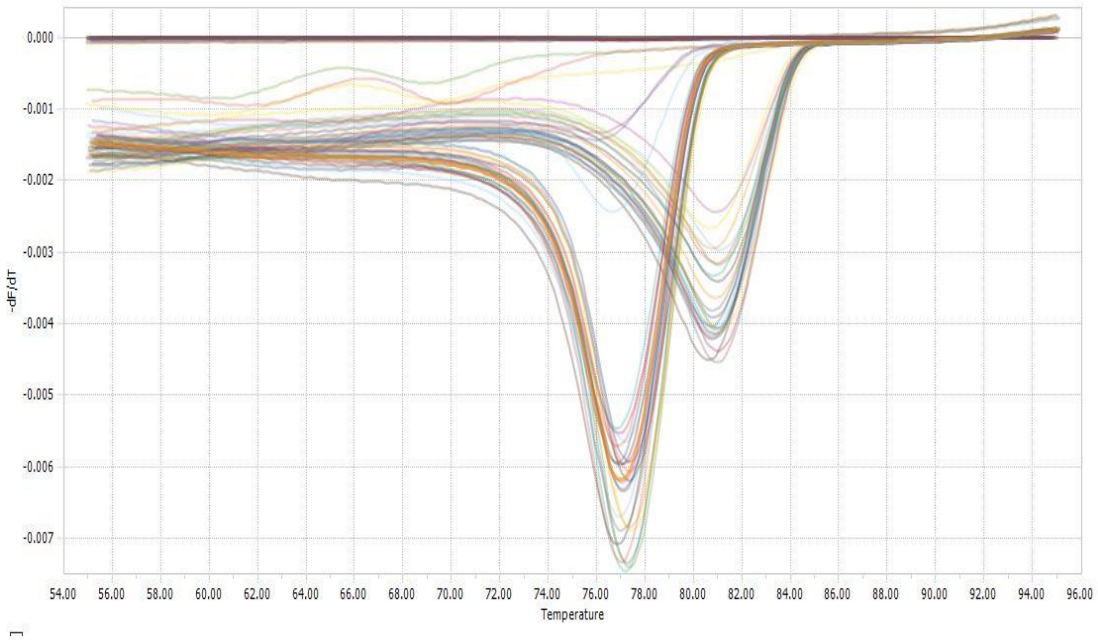

Fig 3. Melt curve analysis obtained from qPCR study after feeding dsRNA for 48 h to adult whiteflies

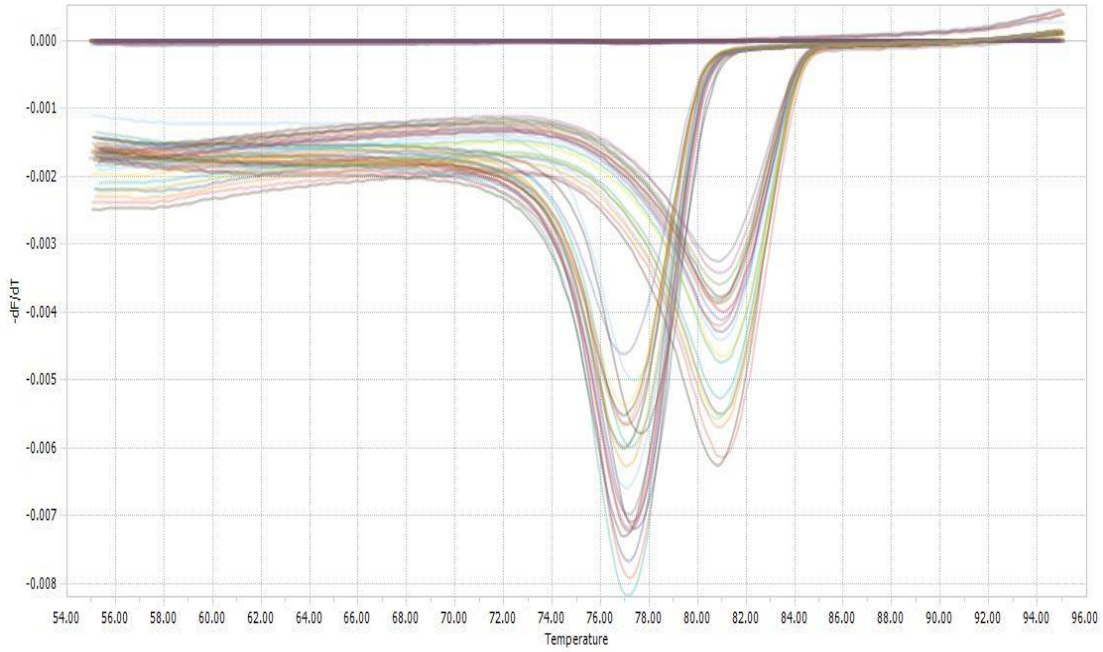

Fig 4. Melt curve analysis obtained from qPCR study after feeding dsRNA for 96 h to adult whiteflies

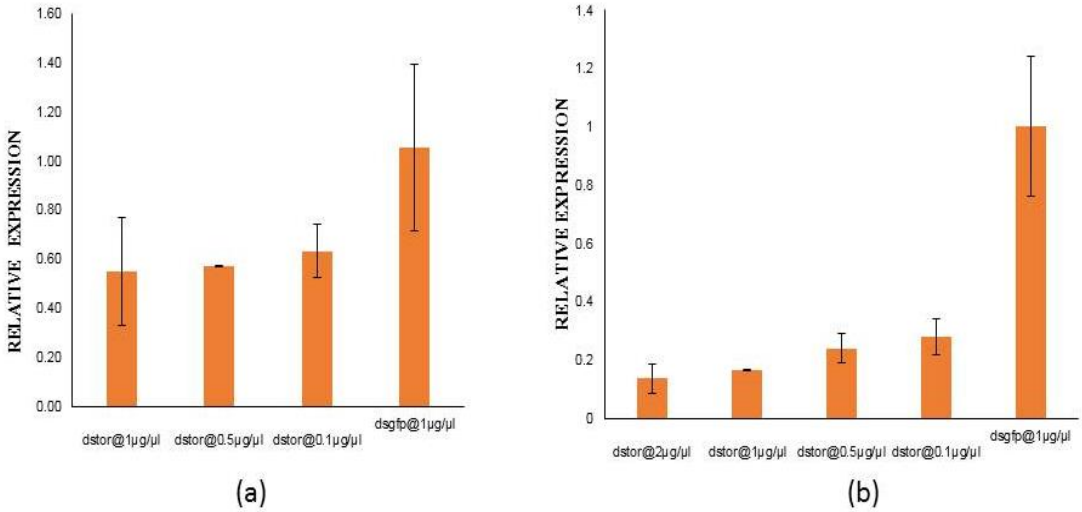

Fig 5. Knockdown of *TOR* gene in *B. tabaci* fed with different concentrations of dsRNA a) Expression of *TOR* gene after 48 hrs of dsRNA feeding to b) Expression of *TOR* gene after 96 hrs of dsRNA feeding

Relative gene expression of *TOR* gene was analysed using delta delta CT method. The melting curve analysis is also done to assess the specificity of amplification (Fig 3 and 4). In first set, 55.34, 57.21 and 63.36 per cent expression of *TOR* gene when adults whiteflies were fed on *dstor* @ 1.0, 0.5, 0.1 ug/µl as compared to whiteflies fed with *dsgfp* @ 1.0 ug/µl (Fig 5). In second set, it was found that when the whiteflies were fed with higher concentration of dstor and for 96 hours, the expression level of *TOR* mRNA decreased compared to *dsgfp*. Expression of *TOR* gene was 13.77, 16.52, 24.07 and 28.75 per cent when whitefly adults were fed at *dstor* @ 2.0, 1.0, 0.5 and 0.1 ug/µl, respectively, compared with *dsgfp* fed whiteflies. These results confirmed the silencing of *TOR* gene in whitefly when *dstor* was delivered to whiteflies through artificial diet sandwiched between parafilm layers.

Gene silencing through dsRNA mediated artificial diet feeding method was reported to be effective in *B. tabaci* (Upadhyay *et al* 2011, Grover *et al* 2019). After two days of feeding *dsjhe* @ 2.5 and 1.0 µg/µl to whitefly adults showed significant knockdown of *juvenile hormone esterase* mRNA levels (Grover *et al* 2019). Vyas *et al* (2017) silenced the gut specific genes of whitefly viz, Acetylcholine receptor subunit α, α-glucosidase 1, Aquaporin 1, Heat shock protein 70, Trehalase 1 and Trehalase transporter 1 involved in neurotransmission, osmoregulation, thermotolerance, sugar metabolism and sugar transport and reported 40-70 per cent reduction in genes expression. Ghanim *et al* (2007) reported the suppression of *BtCG5885*, *BtSnap* and *BtGATAd* genes expression upto an extent of 70 per cent in whitefly. The decrease in the expression level of *AchE* in *Scirpophaga incertulas* (Walker) was determined at 12 DAT and 15 DAT and it was reported to be downregulated by 2 and 1.4 fold respectively, when compared to control (Kola *et al* 2019). Therefore, dsRNA induced silencing of target gene is effective for studying the gene functions.

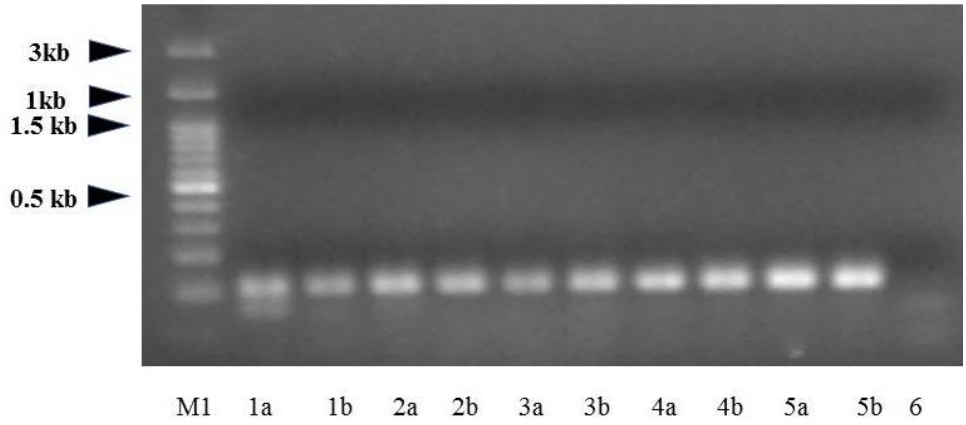

Plate 11. Amplification of cDNA corresponding to RNA isolated from whiteflies adults fed with different concentrations of dsRNA using Bt*TOR* qPCR primers
M1: 100bp DNA Ladder (SMOBIO), Lane 1a & 1b-*dstor* @1ug/ul, Lane 2a & 2b- *dstor* @0.5ug/ul, Lane 3a & 3b- *dstor* @0.1 ug/ul, Lane 4a & 4b- *dsgfp* @1ug/ul, Lane 5a & 5b- sucrose 20%

CHAPTER V

SUMMARY

The whitefly, *Bemisia tabaci* (Gennadius) is amongst world's topmost 100 invasive organisms. It is species complex of at least 44 cryptic species (Kanakala and Ghanim 2019). It is highly polyphagous and reported to attack more than 900 host plant species, belonging to 77 families. *B. tabaci* causes serious losses in around 60 crop plants by being a phloem sap sucking pest or vector of various viral diseases. The intensive use of insecticides to manage whitely has led to its resistance development to most of the conventional insecticides. Broader host range and adaptability, cryptic species status, viral transmission abilities and development of resistance have made the management of whitefly difficult. It has developed resistance to more than 40 active ingredients of insecticides. Insecticides also caused the problem of insecticide induced resurgence and effecting the population of various predators and parasitoids. Therefore, alternate management strategies are needed to manage this pest.

RNA interference is emerging as potent tool for insect pest management. RNAi is post transcriptional gene silencing technique in which exogenously delivered double stranded RNA having complete homology with target mRNA, diced down to short interfering RNAs (siRNA) which degrade the target gene and prevents the translation of gene into protein. In the current study, the effect of silencing of *Target of rapamycin* (*TOR*) gene in whitefly through RNAi was observed. *TOR* gene is conserved in all the eukaryotes. This gene plays an important role in cell proliferation, tissue development, protein synthesis and autophagy. *TOR* pathway also regulates ecdysteroidogeneis, vitellogenin synthesis, anautogeny and juvenile hormone synthesis in insects which were greatly affected after *TOR* gene knockdown. TOR pathway is studied in various insects *viz*., *Aedes aegpti* (Hansen *et al* 2005), *Apis mellifera* (Corona *et al* 2007), *Haemaphysalis longicornis* (Umenif-Shirafiji *et al* 2012), *Bactrocera dorsalis* (Suganya *et al* 2010), *Drosophila melanogaster* (Kijak and Pyza 2017) and *Nilaparvata lugens* (Zhou *et al* 2017, Zhai *et al* 2015).

B. tabaci culture was maintained on cotton plants (RCH 773) under screen house conditions. Hundred whitefly adults were processed for RNA isolation using trizol method. Two distinct band of 18S and 28S were visible on gel electrophoresis. 500 ng of total RNA was then used for synthesis of the cDNA using Superscript III Reverse transcriptase kit. The predicted sequence of *TOR* gene (XM_019060193.1) in *B. tabaci* was retrieved from whitefly genome database and different primers were designed to characterize full length *TOR* gene. Nineteen primer were used for amplification and ten combinations of forward and reverse primers were used for PCR reaction. All the ten fragments of *TOR* gene were custom sequenced through the services provided by "M/S Xcelris Labs, Ahmedabad". The raw nucleotide sequences of each fragment were edited using CLC sequence viewer. Ten

overlapping fragments were used to prepare full length contig of *TOR* gene which resulted in 7311 bp and 2437 amino acids. The nucleotide sequence was confirmed as *TOR* sequence of *B. tabaci* by aligning the sequence with the predicted sequence of *TOR* gene in whitefly and also by using the BLAST tool offered by National Centre for Biotechnology Information (NCBI) which showed 98.66 per cent homology with predicted *Bemisia tabaci* mTOR sequence. Ninety-five nucleotides were found be different from the predicted sequence of *TOR* gene in *B. tabaci*, however the amino acid remains the same except for two. In *B. tabaci* of *TOR* gene, amino acid at position 1448, asparagine replaced threonine and at position 1768, arginine replaced tryptophan as compared to predicted *B. tabaci* TOR amino acid sequence. Phylogenetic tree was constructed for different insect based on amino acid sequence of *target of rapamycin (TOR)* gene by maximum likelihood method based on the JTT matrix-based model. Amino acid sequence of *B. tabaci* showed maximum homology with *N. lugens*. The phylogenetic analysis revealed that *B. tabaci TOR* amino acids are grouped with *N. lugens* and *A. pisum* and other hemipteran groups.

TOR and *gfp* gene fragments with specific primers, each primer with and without having T7 promoter sequence were amplified using whitefly cDNA and mGFP plasmid, respectively. Amplified PCR product were used for dsRNA synthesis corresponding to *TOR* and *gfp* using Invitrogen MEGAscript RNAi kit (Thermo Fisher Scientific). The concentrations of *dstor* and *dsgfp* varied from 1200 and 2100 ng/ul, respectively.

dsRNA mediated artificial diet was prepared in sucrose solution so as to have the final concentration of dsRNA @ 0.1, 0.5, 1.0 and 2.0 µg/µl corresponding to *TOR* gene fragment. *dsgfp* was prepared @ 0.1, 0.5 and 1.0 µg/µl. Leaf cages were prepared with dimensions of length 2.5 cm, diameter 3 cm and diameter of side holes 0.75 cm. The artificial diet with dsRNA was sandwiched between two sheets of parafilm and ten pairs of whitefly adults (one day old) were released and permitted to feed on artificial diet of dsRNA and control. One experiment was conducted in lab in which adult whiteflies were allowed to feed continuously on dsRNA mediated artificial diet. Adult mortality was recorded at two days interval till the death occurred in all treatments.

In other set of experiment, the whiteflies were allowed to feed on dsRNA incorporated artificial diet for 48 hrs. Survival of whiteflies in leaf cages were recorded after 48 hrs of release in leaf cages. After 48 hrs of feeding on dsRNA diet, the adults were shifted on lower surface of cotton leaves in leaf cages for observing the effect of target gene silencing on egg laying, nymphal mortality, nymphal duration, pupal mortality, pupal duration and adult emergence. Three experiments were performed during June-July, July-Aug, Aug-Sept, 2019. The experiments performed during June-July and July-Aug, concentration of *dstor* used were @ 0.1, 0.5 and 1.0 µg/µl and in Aug-Sept, one more concentration was added i.e. @ 2.0 µg/µl. Experiment during October was conducted only to record the fecundity.

Adult mortality in the lab experiment when whitefly was fed continuously on dsRNA, was recorded at two days interval till maximum mortality occurred and cumulative mortality was worked out. The adult mortality after two days of feeding was found to be significantly higher (35.00, 43.75 and 47.75 %) in all the treatments of *dstor* fed whiteflies (@1.0, 0.5 and 0.1 µg/µl), respectively, as compared to all the treatments of *dsgfp* and control which were statistically at par to each other. Similar results were recorded after 4^{th} and 6^{th} day of feeding. After 10 days of feeding, no significant difference was found in *dstor*, *dsgfp* and control treatments.

In the second experiment whiteflies were fed only for 48 hours on dsRNA was conducted under screen house conditions. Adult mortality after 48 hours of feeding was maximum (46.66 %) when whitefly adults were fed with *dstor* @ 1.0 µg/µl in the June-July experiment which was statistically at par with dstor @0.5 and 0.1µg/µl and significantly higher than dsgfp and control treatments. Similarly, significantly higher mortality was recorded in dstor treatments at higher concentrations in other two experiments and minimum mortality was recorded at control (7.5 %) in Aug-Sept experiment.

Fecundity was adversely affected when whiteflies were fed with *dstor* as compared to *dsgfp* and control. The minimum eggs (41.25 and 30.33 per female) were recorded at *dstor* @ 1.0 ug/µl in both Aug-Sept and October experiments. These treatments were at par with *dstor* @ 2.0, 0.5 ug/µl and 2.0, 0.5 and 0.1 ug/µl, in Aug-Sept and October experiments, respectively. *dstor* treatments recorded significantly lower fecundity as compared to all the treatments of *dsgfp* and control. Significant higher nymphal mortality was recorded when whiteflies were fed with *dstor* compared to *dsgfp* and control. The maximum mortality of 56.67, 57.16 and 62.50 per cent was recorded at *dstor* @1.0 ug/µl, 1.0 ug/µl and 2.0 ug/µl, in all three experiments, respectively. all treatments of *dstor* were statistically higher than *dsgfp* and control in all experiments. The calcuations of instar wise mortality revealed that significant difference in nymphal mortality is due to first instar mortality. No difference was observed with respect to second and third instar nymphal mortality in all the treatments of all the experiments. The first instar nymphal mortality was maximum (42.16 and 49.25 per cent) at dsTOR @ 0.1 ug/µl and 2.0 ug/µl in two experiments which was significantly higher than all *dsgfp* and control treatments.

No effect of *TOR* gene silencing on duration of nymphal and pupal stages, pupal mortality and adult emergence was observed. Peculiar effects of *TOR* silencing are observed on adult mortality, fecundity and first instar nymphal mortality. This was confirmed by qRT-PCR experiment in which the expression of *TOR* gene in *dstor* fed whiteflies was lowered down as compared to the *dsgfp* fed whiteflies. In first set, 55.34, 57.21 and 63.36 per cent expression of *TOR* gene when adults whiteflies were fed on *dstor* @ 1.0, 0.5, 0.1 ug/µl as compared to whiteflies fed with *dsgfp* @ 1.0 ug/µl (Fig 5). In second set, it was found that

when the whiteflies were fed with higher concentration of dstor and for 96 hours, the expression level of *TOR* mRNA decreased compared to *dsgfp*. Expression of *TOR* gene was 13.77, 16.52, 24.07 and 28.75 per cent when whitefly adults were fed at *dstor* @ 2.0, 1.0, 0.5 and 0.1 ug/µl, respectively, compared with *dsgfp* fed whiteflies. These results confirmed the silencing of *TOR* gene in whitefly when *dstor* was delivered to whiteflies. Relative gene expression studies showed that the expression of *TOR* gene decreased in whiteflies when fed for 96 hours as compared to the whiteflies which were fed for 48 hours.

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