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Resistência a mosca branca (*Bemisia tabaci*) em plantas transgênicas expressando siRNA do gene de uma *v-ATPase*

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Whitefly (*Bemisia tabaci*) resistance in transgenic plants expressing siRNA from a *v-ATPase* gene

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Dedicated in loving memory of my mother, Safiya Mohammad, whose beautiful heart taught me what it means to be kind and patient; whose life was a lesson on love, courage and compassion; whose DNA shaped my being and whose words and wisdom lead to ultimate enlightenment.

Mi yetti ma moduujo am

The significance of our lives and our fragile planet is determined by our own wisdom and courage; we are the custodians of life's meaning. Knowledge is preferable to ignorance and it is better by far to embrace the hard truth than a reassuring fable.

Carl Sagan

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lysosomes via the mannose-6-phosphate receptor (MPR) (F), which binds newly synthesized cathepsins in the trans-Golgi network (TGN) and delivers them to late endosomes (G), where they are released for delivery to lysosomes (E). v-ATPases in secretory vesicles generate both a pH gradient and an internally positive membrane potential that is used to drive the uptake of neurotransmitters, such as glutamate and noradrenaline (H). These trafficking pathways are also exploited by pathogens. Enveloped viruses, such as influenza virus, and toxins, such as diphtheria and anthrax toxin, enter cells via acidic endosomes (I). Low pH facilitates the entry of the viral mRNA or cytotoxic portions of the toxin molecules through pores that are formed in the endosomal membrane (I). Recent evidence suggests a role for the V_0 domain in the fusion of synaptic vesicles at the presynaptic terminal, which is consistent with earlier studies of homotypic vacuole fusion in yeast (J). These trafficking pathways are also exploited by pathogens. t-SNARE; target SNARE; v-SNARE, vesicle membrane SNARE. Scheme and texts of figure reproduced from Forgac, 2007.

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PREFACE

This thesis is structured such that its first section, written in Portuguese language, presents an extended summary of the work comprising of introduction and results. The second part, introduces the foundation of the concepts used in this research followed by justification, hypothesis and objectives. This is followed by description of the general methods used in the study. In subsequent pages, findings of the thesis are presented in detail followed by discussion. As an addendum, a summary of a preliminary work on soybean done before the thesis is presented. Relevant publications and indices are attached at the end of the thesis.

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RESUMO

RNA de interferência (RNAi) é um processo bioquímico potente e específico de silenciamento de genes, que ocorre em uma variedade de organismos como mamíferos, fungos e plantas. O mecanismo atua a nível pós-transcricional, e pode resultar na degradação ou não tradução de RNAs mensageiros (mRNA). Esse mecanismo foi utilizado com o objetivo de silenciar o gene *v-ATPase* da mosca branca (*Bemisia tabaci*), que é considerada uma importante peste da agricultura em regiões tropicais e sub-tropicais em todo o mundo. Nesse estudo, um plasmídeo contendo um cassete de interferência para um fragmento de *v-ATPase* foi desenvolvido e utilizado para transformar cotilédones de alface (*Lactuca sativa*). Um total de 25 linhagens transgênicas foram geradas, das quais sete foram avaliadas para estudos moleculares. Análise de progênie confirmou a presença do inserto na geração T_1 das sete linhagens assim como a análise de Northern, que permitiu a detecção de siRNAs correspondentes ao gene de *v-ATPase*. Um estudo de silenciamento da *v-ATPase* foi feito por meio de um bioensaio no qual plantas das diferentes linhagens foram submetidas à presença de 20 moscas brancas, e a taxa de mortalidade, bem como a alteração de desenvolvimento em diferentes estágios do ciclo de vida da mosca foram avaliados, durante um período de 32 dias. A análise da mortalidade de insetos que se alimentaram em plantas transgênicas demonstrou que, em três dias de alimentação, uma queda de aproximadamente 75% nessa população pôde ser observada quando comparado ao controle (p<0,05). Alterações significativas no ciclo de desenvolvimento de insetos se alimentando em plantas transgênicas também foram observadas (p<0,05). Dessa forma, dados apresentados nesse trabalho funcionam como prova de conceito no desenvolvimento de tolerância à mosca branca mediado por RNAi.

Palavraschaves: Alface, *Bemisia tabaci,* bioensaio, controle de insetos, transgênicos, RNAi e *v-ATPase*

ABSTRACT

RNA interference (RNAi) is a potent biochemical phenomenon that targets and silences specific genes in different life forms like mammals, fungi and plants. The process of silencing takes place at post-transcriptional level leading to the degradation of messenger RNAs (mRNAs) or inhibition of their translation. RNAi has been demonstrated to be useful in silencing *v-ATPase* gene of whitefly (*Bemisia tabaci*), an important agricultural pest in the tropics and sub-tropic regions of the world. Here, a plasmid containing an interferent cassette designed to generate siRNA molecules that target *v-ATPase* gene transcript was cloned in a vector, which was used to transform cut-pieces of cotyledons from germinating lettuce (*Lactuca sativa*). A total of 25 transgenic lines were generated, of which seven were selected for further molecular analysis. Progeny analysis confirmed that these lines have passed the inserted character to the next generation (T_1) in a Mendelian segeragation. Northern blot analysis detected siRNAs corresponding to the *v-ATPase* insert. The lines were used to perform a bioassay in order to evaluate the silencing effect of *v-ATPase*. Plants from these lines were infested with 20 whiteflies and the mortality as well as alterations in growth and development of different stages of the whitefly life cycle was monitored over a period of 32 days. Analysis of mortality showed that within three days of feeding, insects on transgenic plants showed a mortality rate of about 75% higher than those on control plants ($p < 0.05$). Significant alterations in the development of the insects on transgenic plants were also observed ($p < 0.05$). Data presented in this work may serve as proof of concept in the development of plants with tolerance to whitefly via RNAi.

Keywords: *Bemisia tabaci,* bioassay, insect control, Lettuce, RNAi, transgenic crops and *v-ATPase*.

ABBREVIATIONS

Ago-1 – Argonaute-1

Ago-2 – Argonaute-2

Ago-3 – Argonaute-3

BAP – 6-Benzylaminopurine

BLAST - Basic Local Alignment Search Tool

CHS - chalcone synthase

cDNA – complementary DNA

CP – Coat protein

CTAB - cetyltrimethylammonium bromide

CTNBio –*Comissão Técnica Nacional de Biossegurança*

Cyt P450 – Cytochrome P 450

C3PO - Component 3 Promoter Of RISC

dNTP - deoxyriboNucleotide TriPhosphate

dsRNA – double-stranded RNA

EDTA – Ethylenediaminetetra acetic acid

EHA105- Elizabeth Hood Agrobacterium 105

ELISA –Enzyme Linked Immuno Assay

EMBRAPA – *Empresa Brasileira de Pesquisa Agropecuária*

EST – Expressed Sequence Tag

HIGS- Host Induced Gene Silencing

LB – Luria Bertani

LEG- Laboratorio de Engénharia Genética Aplicada a Agricultura Tropical

MEGA5 – Molecular Evolutionary Genetics Analysis 5

MPR - mannose-6-phosphate receptor

mRNA - messenger RNA

MS – <u>M</u>urashige and <u>S</u>koog

NAA – Naphthalene acetic acid

NCBI – <u>N</u>ational <u>C</u>enter for <u>B</u>iotechnology <u>I</u>nformation

NSF - *N*-ethylmaleimide-sensitive factor

Nt – Non-transgenic

PDR- <u>P</u>arasite <u>D</u>erived <u>R</u>esistance

PCR - <u>P</u>olymerase <u>C</u>hain <u>R</u>eaction

PVP – Polyvinylpyrrolidone

RACE - <u>R</u>apid <u>A</u>mplification of cDNA <u>E</u>nds

RdRp - RNA-dependent RNA polymerase

RISC - RNA-induced silencing complex

RLC - RISC-loading complex

RNAi – RNA interference

rpm – revolutions per minute

RT - Reverse Transcription

TGN - trans-Golgi network

shRNA – small hairpin RNA

sid-1 - systemic RNA interference deficient-1

siRNA – short interfering RNA

TAE – Tris Acetate EDTA

TE - Transposable Element

Tm – Melting Temperature

TRAX - translin-associated factor-X

1. INTRODUÇÃO

Os avanços científicos no século 20 possibilitaram o desenvolvimento de diversas técnicas moleculares, que permitiram, não apenas o entendimento dos processos bioquímicos em vários organismos, mas também a manipulação dos organismos de forma precisa e a utilização de recursos naturais. Dentre essas técnicas, o mecanismo de RNA de interferência (RNAi), tem demonstrado grande aplicação na medicina, indústria e agricultura (Aragao et al., 2014; Bumcrot et al., 2006; Rondinone, 2006; Tang and Galili, 2004). Em plantas, o fenômeno de RNAi foi descoberto através do estudo de resistência a vírus (Asad et al., 2003). Baseado no mecanismo natural de RNAi nos vegetais, cientistas desenvolveram plantas geneticamente modificadas, as quais foram capazes de reprimir a expressão de genes endógenos e ácidos nucleicos invasivos, oriundos de vírus, fungos e outras pragas. A aplicação prática desse mecanismo permitiu não apenas o desenvolvimento de variedades melhoradas, mas também, aumentou o conhecimento do mecanismo de silenciamento gênico mediado por RNAi, o que representa um grande avanço no conhecimento da função gênica. O mecanismo de RNAi foi observado em vírus, fungos, plantas e animais, indicando assim que se trata de um processo evolutivo altamente estável (para uma revisão ver Ibrahim e Aragão, 2015).

1.1 Importância econômica da mosca branca na agricultura

A mosca branca (*Bemisia tabaci*) é um inseto pertencente à família *Aleyrodidea*, que possui mais de 1.550 espécies identificadas (Byrne, 1991). O inseto é responsável por causar grandes danos à agricultura por ser adaptado a diversos hospedeiros e possuir desenvolvimento rápido. Esse inseto alimenta na parte abaxial das folhas, sugando o floema, introduzindo sua saliva tóxica no tecido vegetal, assim reduzindo a pressão de turgescência das plantas e causando perdas em produção de até 50% (Byrne, 1991). Danos por ninfas e adultos chegam a causar destruição total de plantas atacadas.

As moscas brancas podem transmitir pelo menos 111 espécies de vírus e colonizam mais de 600 espécies de plantas (Li et al., 2011). Entre os agentes virais

transmitidos por esse vetor, 90% pertencem ao gênero *Begomovirus*, 6% ao gênero *Crinivirus, e* os 4% restantes pertencem aos gêneros *Closterovirus, Ipomovirus* e *Carlavirus.* Como exemplo de vírus transmitidos por *B. tabaci* citam, african cassava mosaic virus, bean golden mosaic virus, bean dwarf mosaic virus, tomato yellow leaf curl virus, tomato mottle virus, e outras (De Barro et al., 2011; Li et al., 2011; Oliveira et al., 2001).

Danos causados pela mosca branca podem ser minimizados por manejo cultural e controle biológico. A aplicação inicial de pesticidas tem controlado o inseto, mas o desenvolvimento de resistência é um fator limitante. No entanto vale ressaltar que, os agrotóxicos mais utilizados contêm neonicotinoides que podem ter efeitos detrimentos aos insetos benéficos. A natureza invasora da mosca branca, e sua alta taxa de reprodução, fazem com que o inseto seja altamente resistente a diversos inseticidas. No Brasil, a aplicação de inseticidas por produtores de diferentes culturas que chegava a ser realizada 16 vezes por ciclo de cultivo, hoje pode atingir 40 aplicações (Peloso, M.J., Embrapa Arroz e Feijão, comunicação pessoal). A presença de 1 a 3 insetos por planta numa lavora, é suficiente para causar perda total de um cultivo, devido a transmissão de vírus pela mosca branca. O nível de dano causado à agricultura pela mosca branca e a manifestação de mecanismos resistência desse inseto à inseticidas, evidenciam a necessidade do desenvolvimento de estratégias mais eficazes de controle desse vetor.

1.2 ATPase vacuolar

O controle do pH nos compartimentos intracelulares em células eucarióticas é um fenômeno altamente coordenado mediado por uma enzima chamada v-ATPase. A atividade dessa enzima afeta diversos processos celulares como o transporte através da membrana, processamento e transporte de neurotransmissores, bem como a regulação da entrada de microrganismos como vírus (Beyenbach e Wieczorek, 2006). Apesar de diferentes especialistas descreverem a v-ATPase a partir de diferentes perspectivas, todas elas têm uma estrutura única composta de grandes domínios designadas V_0 e V_1, que possuem 13 subunidades (Wieczorek et al., 2000). Estudos por difração de raios-X permitiram a caracterização da estrutura de ATPase em *Manduca sexta* e *Chlostridium fervidus*. ATPases estão presentes

nas membranas de compartimentos intracelulares, tais como lipossomas, vacúolos, vesículas revestidas, grânulos secretores e no complexo de Golgi. A sua função enzimática é mediada por bombeamento de H$^+$ (juntamente com a hidrólise de ATP) para o lúmen das organelas, causando a acidificação da mesma. V-APTases também são encontradas nas membranas plasmáticas de muitos tipos de células animais e estão envolvidas na homeostase do pH e energização de membrana. A enzima também tem sido relatada em muitas células epiteliais para transporte de íons. A subunidade A do domínio V_1 é o sítio catalítico, responsável pela hidrólise de ATP. A supressão dessa subunidade é letal (Baum et al., 2007; Upadhyay et al., 2011).

1.3 RNAi e o controle de insetos

Antes da descoberta do mecanismo de RNAi, pesquisadores enfrentavam grandes entraves no estudo da função de genes específicos aos insetos (Garbutt, 2011). Com a disponibilidade de mais dados genômicas e avanços em tecnologias de sequenciamento de DNA com custo reduzido da aplicação do RNAi na pesquisa de insetos, houve aumento no número de estudos baseados nas análises da função do gene em vários insetos não-modelos (Mito et al., 2011).

No caso da mosca branca, tem-se poucos relatos envolvendo RNAi. Com a disponibilidade de uma série de dados de transcriptoma sobre esse inseto na base de dados do NCBI, a maquinaria de RNAi da mosca branca poderá ser ilustrada em breve. Proteínas importantes dessa maquinaria como Dicers2, R2D2, Argonuata2 e Sid1 já foram identificadas na mosca branca assim como suas expressões em diferentes fases de desenvolvimento (Upadhyay et al., 2013).

A demonstração de que mRNAs específicos de tecidos de diferentes genes da mosca branca podem ser preferencialmente silenciados ou suas expressões reduzidas em até 70%, abriu o caminho para novas pesquisas utilizando o mecanismo de RNAi em insetos (Ghanim et al., 2007). O desenvolvimento de método de administração de siRNA e dsRNA na mosca branca por via oral, tem demonstrado ser eficiente no controle do inseto (Upadhyay et al., 2011). Este trabalho, representa a primeira tentativa de silenciamento do gene *ATPase* na

mosca branca, cuja transcrição foi significativamente reduzida quando os insetos foram alimentados com uma dieta artificial contendo dsRNA que tinham como alvo esse gene. Uma das tentativas mais recentes no silenciamento dos genes de mosca branca via RNAi envolve o desenvolvimento de um método baseado em alimentação utilizando dsRNAs que têm como alvo genes da via de biossíntese da ecdisona, um hormônio importante na regulação do crescimento e desenvolvimento de insetos (Luan et al., 2013). Embora o efeito de silenciamento ter sido baixo em adultos, este trabalho demonstrou que o silenciamento levou a uma redução de sobrevivência e atraso no desenvolvimento da mosca durante as fases de ninfas. Um trabalho recente envolvendo *B. tabaci* e um gene que codifica para uma v-ATPase, demonstrou a resistência ao inseto em plantas de tabaco. Foi possível observar reduções tanto na população de insetos quanto no nível de transcrição do gene endógeno (Thakur et al., 2014).

Estes experimentos com mosca branca vieram após a demonstração de que a ingestão de RNAs fornecidos em uma dieta artificial, pode induzir interferência de RNA em coleópteros como *Diabrotica* sp. (Baum et al., 2007; Gordon and Waterhouse, 2007; Price and Gatehouse, 2008). No trabalho de Baum et al., (2007) por exemplo, foi demonstrado que milho transgênico desenvolvido para expressar dsRNA contra o gene de *v-ATPase* de *Diabrotica* sp apresentou supressão do gene no inseto e a estratégia provocou uma redução de danos nas raízes da planta. Em algodão e *Arabidopsis thaliana*, dsRNAs contra Cyt P450, uma enzima de desintoxicação, protegeu as plantas contra lagartas através de silenciamento (Mao et al., 2007). Diante disso, o objetivo deste trabalho foi silenciar o gene *v-ATPase* de *Bemisia tabaci* através de expressão de siRNAs em plantas de alface geneticamente modificadas.

1.4 Resumo de Resultados

Inicialmente, um vetor dotado da presença de uma sequência parcial do gene *v-ATPase* foi desenvolvido e utilizado para transformar plantas de alface (*Lactuca sativa*) via *Agrobacterium tumefaciens*. Foram geradas 25 linhagens de plantas transgênicas, e um teste de progênie de sete dessas linhagens demonstrou uma segregação do tipo 3:1 para o transgene de interesse. Através da utilização de um

sistema de monitoramento da taxa de mortalidade de mosca branca em plantas, um total de 12 plantas transgênicas foram submetidas ao bioensaio com moscas recém emergidas. A mortalidade desses insetos sugadores, bem como o ciclo metamórfico completo de 32 dias foram avaliadas. Plantas não-transgênicas e plantas transgênicas - expressando somente o gene *bar* - foram utilizadas como controles nesse experimento.

Resultados desse ensaio revelaram que a mortalidade de moscas se alimentando em plantas de alface transgênicas foi superior e estatisticamente significativa pelo teste de Tukey ($P<0.05$), quando comparada a plantas controles. A mortalidade consequente da alimentação em plantas transgênicas foi observada a partir dos três primeiros dias de ensaio. Uma redução populacional nesse grupo de insetos, evidenciando uma taxa de mortalidade da ordem de 75% foi observada.

Por volta do décimo dia de observação, a população ficou próxima a zero nas plantas transgênicas, enquanto que nas plantas não-transgênicas ainda se observava uma população na ordem de 5%. Acompanhamento do ciclo das moscas para monitorar a emergência de ovos, ninfas, pupas e adultos, demonstrou-se que, enquanto as moscas em plantas não-transgênicas depositaram entre 227 e 231, com a emergência de entre 107 e 125 ninfas no inicio de um novo ciclo, em plantas transgênicas foi observado entre 24 e 66 ovos e entre 13 e 52 ninfas no início do novo ciclo. Essas diferenças foram mantidas ao longo do ciclo. Novos adultos começaram a surgir a partir do 18ª dia em plantas não-transgênicas, enquanto que em plantas transgênicas só foram observados no 22ª dia, numa taxa 78% menor que o controle. Análise de Northern blot de plantas T_1 dessas linhagens detectou as moléculas de siRNAs nas sete linhagens estudadas. Pela primeira vez, a técnica de RNAi foi utilizada nesse trabalho para desenvolver plantas transgênicas com resistência a mosca branca utilizando um sistema eficiente na avaliação de mortalidade em uma cultura. Este trabalho poderá ser usado como prova de conceito para o desenvolvimento de tolerância à mosca branca via RNAi.

Numa primeira tentativa de utilizar a técnica desenvolvida nesse trabalho em outra espécie, plantas de soja foram transformadas com um vetor contendo as sequencias silenciadoras de *v-ATPase*. Resultados preliminar mostraram que as

plantas T_2 geradas nessa transformação apresentaram um nível de tolerância a mosca branca.

2. INTRODUCTION

2.1 RNA interference (RNAi)

The scientific advances of the 20th century have led to the development of several techniques from the studies of a vast array of molecular phenomenon for the improvement of quality of life and optimal utilization of natural resources. Among these, post-transcriptional gene silencing (PTGS), or RNA interference (RNAi), has found great applications in medicine, industries and agriculture (Aragao et al., 2014; Bumcrot et al., 2006; Rondinone, 2006; Tang and Galili, 2004). RNAi evolved as a natural cellular defense mechanism in eukaryotes against viruses, genomic confinement of retrotransposons, and as a cellular strategy for post-transcriptional regulation of gene expression. The term RNA interference was coined by Fire et al (1998), following a series of experiments with *Caenorhabditis elegans* in which injecting double-stranded RNAs (dsRNA) into the nematode, led to post-transcriptional specific genes silencing. This silencing was shown to spread over a wide section of the nematode following injection of dsRNA into its extracellular abdominal cavity. Similar effect was observed when *C. elegans* was fed with *Escherichia coli* that transcribed recombinant dsRNA or indeed when the nematode was immersed in preparations containing dsRNA (Fire et al., 1998). This work by Fire et al., (1998) gained the authors the award of 2006 Nobel prize for medicine and physiology.

2.1.1 RNAi in plants

In plants, RNAi was first observed in the study of resistance against viruses (Angell and Baulcombe, 1997). By taking advantage of this intrinsic mechanism, genetically modified plants capable of suppressing the expression of endogenous genes and invasive nucleic acids from virus, fungi, bacteria and pests, have been developed. Today, the practical applications of RNAi have not only allowed for the development of improved crop varieties, but also led to better comprehension of the mechanisms involved in gene silencing mediated by RNAi, symbolized by important milestones in understanding biological functions of genes. This has resulted in the development of technologies used to silence specific genes leading to the creation of knock-out phenotypes in transgenic plants through manipulation of sequence

specific RNA hairpins, and by infection with recombinant RNA viruses harboring sequences of target genes in susceptible organisms. This potential was first demonstrated in 1986 when workers reported that plants could be genetically engineered to exhibit resistance against viral diseases (Abel et al., 1986). By introducing a chimeric gene containing the *coat protein* (CP) gene of TMV into cells of *Nicotiana tabacum* via *Agrobacterium tumefaciens*, it was possible to generate plants that expressed CP, and when inoculated with TMV, these plants showed 10–60% decrease in symptoms. Similarly, transgenic plants transformed to express a complementary RNA sequence of the coat protein gene of TMV were protected when challenged with the virus as evidenced by the accumulation of antisense RNA (Powell et al., 1989). These pioneer experiments were conducted at a time when the mechanism of resistance induced by RNAi was not fully understood. However, by 1990, Napoli et al. (1990) helped in elucidating the mechanism by introducing the gene for chalcone synthase in petunia. Instead of being over-expressed (thereby increasing pigmentation in flowers, as expected), the introduced gene actually blocked the synthesis of anthocyanin and led to the generation of plants with white flowers. Similar observation was later to be made in *Neurospora crassa* (Romano and Macino, 1992) by transforming the fungus to super-express the *albino-1* gene (*al*-1), involved in carotenoid biosynthesis, which normally confers an orange color to fungus. However, the introduction of an extra copy of the *al*-1 gene produced colonies with the albino phenotype. In plants, small RNAs arising from RNAi have been implicated in the control of cell division, leaf and meristematic patterning, environmental responses, heterochromatin maintenance, embryogenesis and development of meristem, leaves, anthers and vascular system (Garbutt, 2005; Palatnik et al., 2003; Vazquez et al., 2004)

2.1.2 Mechanism of RNAi

The mechanism of RNAi has been well documented and involves several steps (Figure 1). The key molecules involved in the process are small RNA molecules, classified into small interfering RNAs (siRNA) and microRNA (miRNA). Initial event in RNAi silencing involves endonuclease RNase III known as Dicer, which processes double stranded RNA generating small RNA molecules that range

in size from 20–30 nucleotides, and whose target is the degradation of their complementary mRNAs (Abdoulhamid et al., 2010; Czech and Hannon, 2011). The siRNAs are processed by Dicer-like enzymes (DCL2, DCL3, and DCL4) from a long double strand of RNA (Figure 1). When dsRNAs are processed, siRNAs are assembled unto a multicomponent nuclease known as RNA induced silencing complex (RISC) (Hammond et al., 2000). RISC was first identified by fractionating an extract of specific nuclease from *Drosophila melanogaster* (Hammond et al., 2001). As a member of the argonaute family, it is responsible for directing and cleaving specific sequence of RNA in the cell (Czech and Hannon, 2011; Martinez and Tuschl, 2004). This is achieved by cleaving the target mRNA at complementary region of ten nucleotides upstream of a $5^{'}$ residue (Figure 1). A helicase in the RISC complex unwinds the siRNA duplex, pairing it with the antisense strand of the target mRNA, which, on its part, has a high degree of complementarity with the siRNA sequence. The cleavage leads to gene silencing by preventing protein synthesis machinery from reading the mRNA, resulting in its degradation (Tolia and Joshua-Tor, 2007). siRNAs are classified into primary and secondary siRNAs. While the primary siRNAs are generated through the activity of Dicer, secondary siRNAs arise from an alternative pathway (Figure 1), which involves the activity of RNA dependent RNA polymerase (Pak and Fire, 2007). It appears that secondary siRNAs regulate gene expression involving signal transduction where they initiate the process of RNAi in the absence of the original signal for RNAi.

MircoRNAs (miRNAs) are endogenous RNA molecules and play important regulatory role during mRNA cleavage and repression of translation. They constitute one of the most abundant classes of regulatory molecules in multicellular organisms (Aukerman and Sakai, 2003). For the formation of the primary miRNA, a transcript of a primary micro-RNA (pre-miRNA) synthesized from the introns of the RNA polymerase II enzyme gene is processed in the nucleus by a protein complex containing a ribonuclease specific to the double-strand producing an intermediary hairpin with 70-80 nucleotides (Figure 1). This pre-miRNA is then transported to the cytoplasm where it is cleaved by Dicer. Following separation of the duplex strands, single stranded miRNA is incorporated into RISC forming the complex that inhibits translation or induces the degradation of target mRNA (Abdoulhamid et al., 2010).
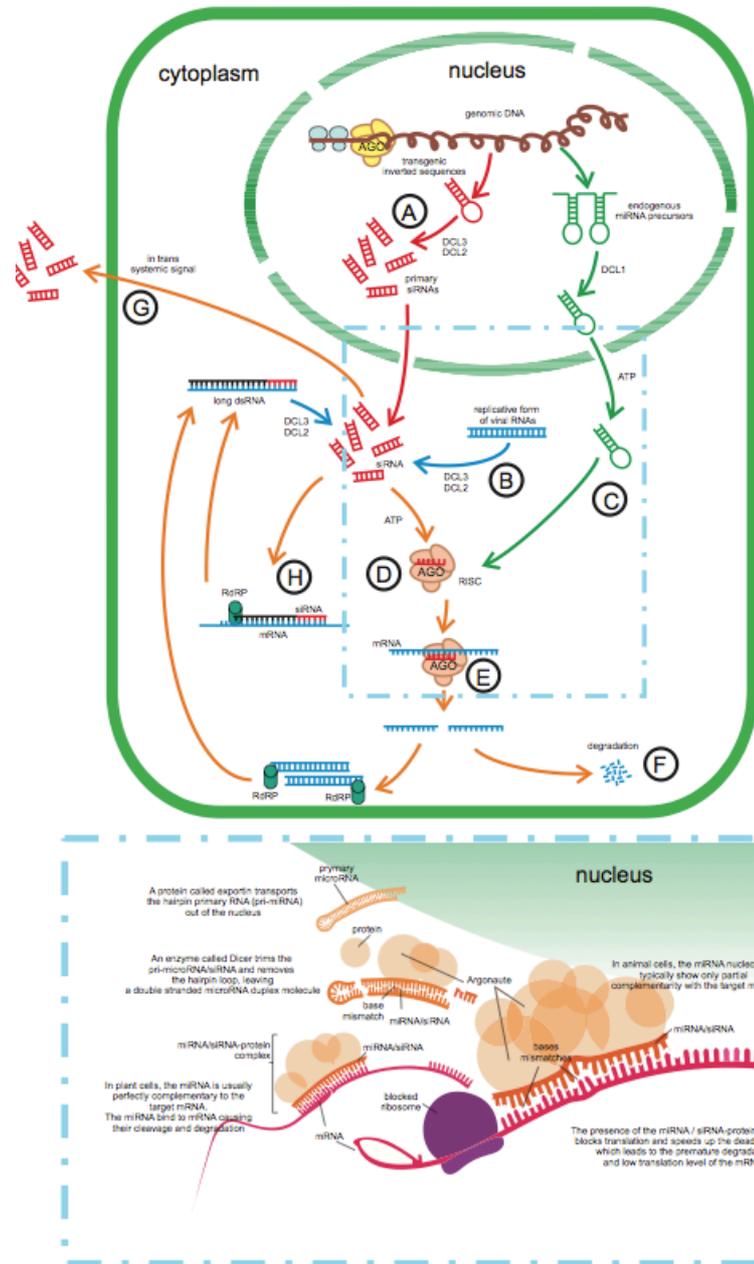
Figure 1. Gene silencing pathway. Dicer-like proteins processing transcripts containing inverted sequences (A), derived from viral RNA replication (B) and precursors of miRNA exported from the nucleus (C). Formation of siRNAs/RISC complex (D) directed to target RNA (E), which is subsequently, degraded (F). G: Systemic silencing: H: Amplification by RdRP. Figure and texts reproduced from Aragao et al., 2014

2.2 RNAi in insects

In insects, RNAi was first elucidated in *D. melanogaster* (Jaendling and McFarlane, 2010)*.* The process is initiated when siRNAs are loaded to RISC via RISC-loading complex (RLC) with the aid of Dicer-2 (Dcr-2) and a partner protein

R2D2 (Figure 2). These two proteins have been shown to activate RISC in an experiment that involves their addition to a recombinant Ago 2 and duplex siRNA, allowing for the identification of what is now known as RISC enhancer component 3 promoter of RISC (C3PO) consisting of translin and TRAX (translin-associated factor-X), required in *D. melanogaster* for *in vivo* RNAi (Jaendling and McFarlane, 2010). C3PO possesses ribonuclease activity and associates with Dcr2–R2D2 during RLC activity to form active RISC complex and serves within this complex to remove the passenger strand of the duplex siRNA, freeing the guide strand to target Ago2 to the target mRNA (Garbutt, 2005)
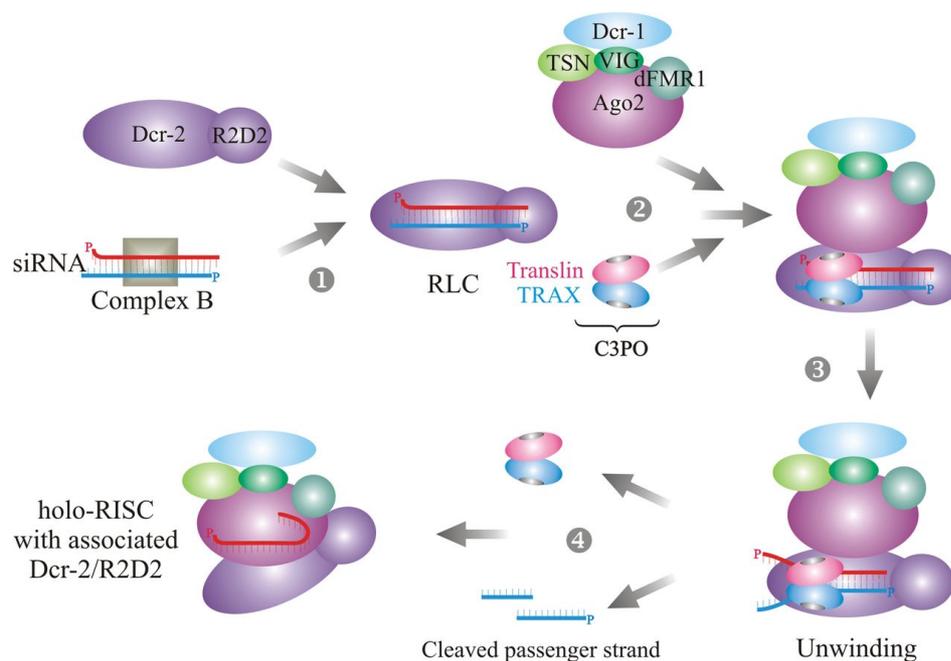
Figure 2: The mechanism of exogenous RNAi pathway and RISC assembly in *Drosophila melanogaster*. 1: Transfer of siRNA duplex from complex B to the RLC (RISC Loading Complex), consisting of Dcr-2 and R2D2. 2: C3PO (translin and TRAX) are linked with the RLC and the RISC complex {consisting of the Dcr-1, TSN (Tudor-staphylococcal nuclease), VIG (vasa intronic gene), dFMR (Drosophila FMR) and Ago2 subunits} to generate the holoRISC by a Drc-2–Ago2 interaction. 3: the passenger strand is removed/endonucleolytically cleaved from the siRNA, which is enhanced by C3PO activity. 4: the holoRISC complex can proceed to associate with target mRNAs. Scheme and texts reproduced from Jaendling and McFarlane, 2010.

2.3 Application of RNAi in agriculture

The potentials for application of RNAi in improving agronomic traits in plants are enormous. Several crops have benefitted from the techniques with some of them having attained commercialization (Aragão and Faria, 2009; Bonfim et al., 2007;

Kusaba et al., 2003; Sunilkumar et al., 2006). Application of RNAi strategies has led to improved productivity and conferred resistance and/or tolerance to many pests and diseases (Aragão *et al*., 2013). In addition, a number of crops have had their nutritional values improved and raw materials derived therefrom, for industrial use optimized (Abdoulhamid et al., 2010; Ossowski et al., 2008; Sunilkumar et al., 2006; Yin et al., 2007)

2.3.1 RNAi and crop improvement

The foundation of RNAi and crop improvement was laid through studies of mutation in plants (for a review see Ibrahim and Aragão, 2015). One such study was demonstrated using seed coat of soybean (*Glycine max*), which exploited the accumulation of anthocyanin in the control of seed coat color. A key enzyme in the biosynthetic pathway of anthocyanin is chalcone synthase (CHS) (Palmer et al., 2004). At chromosomal level, control of pigmentation is mediated by four alleles (*I*, *i*, i^k, *i*) of locus *I* (inhibitor). Of these, *I*, *i*, and i^k are dominant alleles where *I* is responsible for the phenotypic features when seeds are colorless or bear yellow coloration, i^i gives rise to pigmented husk, and i^k gives rise to seeds with patches of pigment. In contrast, *i* allele is recessive and produces seeds with brown or black pigment (Todd and Vodkin, 1993). Structural studies of *I* locus (located on chromosome 8) revealed two inverted repeat clusters on the genes *CHS1*, *CHS3*, and *CHS4* (Tuteja and Vodkin, 2008). Six other CHS coding genes (*CHS2*, *CHS5*, *CHS6*, *CHS7*, *CHS8*, and *CHS9*) are also found in soybean and varieties with colorless seeds have reduced transcript level of *CHS* (Tuteja et al., 2004). Subsequent studies reported having found large quantities of siRNAs (predominantly 22 nt), which corresponded to the regions of the *CHS* genes. These small RNA molecules are indeed specific to seed coat and arise from the transcription of *CHS*1, *CHS3*, and *CHS4* arranged in inverted repeat regions, leading to the formation of dsRNA (Tuteja et al., 2009). Similarly, when *C2-Idf* allele (*colorless2*; containing a mutated chalcone synthase gene) occurs in the homozygous state, different seed parts are colorless (pericarp, aleurone layer of the endosperm, and vegetative organs). Plants with functional heterozygous *C2* allele exhibit an intermediary phenotype, characterized by lesser pigmentation (Vedova et al., 2005). Cloning and

sequence analysis of *C2-Idf* allele showed that its structure is quite different from the normal *C2* allele as two of its three copies of the *CHS* gene lay side by side in an inverted orientation, leading to reduction in the level of its mRNA and consequently the enzyme (Dooner et al., 1991). Indeed, siRNAs have been found in plants containing C2-*Idf* allele and not in normal homozygous containing C2, indicating that the colorless phenotype is mediated by RNAi (Vedova et al., 2005).

In rice with reduced levels of glutenin, similar observation has been made (Kusaba et al., 2003). The consumption of food substances with reduced levels of glutenin is important in patients with celiac disease whose diet must not contain this protein. The phenotype with low level of glutenin arises due to RNAi-mediated inverted copies of genes near the glutenin-coding gene on *Lgc1* locus.

These early studies demonstrate the key role RNAi plays in plant growth, development and the potentials of exploiting its intrinsic mechanism to improve crop.

2.3.2 RNAi technology and control of plant pathogens and pests

Several of the biotic stresses confronted by crops, which hinder their productivity and growth, have been the subjects of research on RNAi. These include viruses, bacteria, fungus and nematodes. Other pests serve as vectors for transmission of some of the pathogens. The benefits of application of RNAi in addressing these problems are enormous. However, viruses pose the most serious threat to plants because of their ability to rapidly multiply and spread across the same or different plant species using their formidable arsenals. In addition, the versatile nature by which they are transmitted via hosts makes their control highly difficult. RNAi has been used in developing crops with resistance to both DNA and RNA plant viruses (Ibrahim and Aragão, 2015). The development of transgenic crop based on RNAi technique was first reported in wheat in which gene sequences of a polymerase from BYDV were expressed (Asad et al., 2003). This led to the silencing of the gene in the virus and plants arising therefrom were found to be immune to the virus as confirmed by ELISA. Transgenic tobacco plants expressing sense and antisense RNAs of DNA-A of CLCuV DNA A and DNA-B of CLCuV were also generated using similar approach (Yang et al., 2004) as was the case for tomato

plants with resistance against TYLCSV (Nahid et al., 2011). Other beneficiaries of the technique include *Nicotiana benthamiana* expressing the coat protein gene of SPFMV resistant to the virus (Nahid et al., 2011) and *N. benthamiana* resistant to CpCDPKV (Pooggin et al., 2003). In leguminous plants, the promoter sequence of DNA A of VMYMV, was silenced via RNAi in *Vigna* spp., resulting in resistance against viral infection (Cruz and Aragão, 2014). Similar strategy was applied using a viral *AC1* gene sequence encoding a multifunctional protein (Rep) of BGMV to generate transgenic common bean (*Phaseolus vulgaris* L.) resistant to geminivirus (Aragão and Faria, 2009; Bonfim et al., 2007). This resulted in the development of an event now known as Embrapa 5.1; the first transgenic bean line approved for commercial use following biosafety regulations set by the Brazilian Technical Commission for Biosafety (CTNBio) (Ibrahim and Aragão 2015). RNAi-engineered soybean has also been used to enhance resistance against the geminivirus MYMV (Yadav and Chattopadhyay, 2011) as was cowpea (*Vigna unguiculata*) with an intron-hairpin construct to silence the proteinase cofactor gene from CPSMV and the coat protein gene from CABMV Cowpea (Vanderschuren et al., 2009). ACMV has also been controlled by using cassava expressing *AC1* siRNA that interrupts Rep/AC1 function during viral replication (Vanderschuren et al., 2007)

2.4 Host–Induce Gene Silencing (HIGS)

The concept of parasite-derived-resistance (PDR) first gained ground following its description by Sanford and Johnston (1985). It was identified as an intrinsic strategy for controlling disease in which the expression of parasite gene in a host plant interferes with the ability of the parasite to grow and develop, leading to disease resistance. It was later to be shown that this was indeed because of host-induced gene silencing mediated by RNAi. Since its discovery, several plants have been shown to elicit such responses. For example, systemic movement of mRNA through phloem between tomato and the parasitic plant *Cuscuta pentagona* Engelm was reported (Roney et al., 2007). Experiments described by Tomilov et al., (2008) also showed that host plants transformed with constructs that generate interference hairpins could silence specific genes in parasitic plants. Roots of transgenic *Triphysaria versicolor* expressing the reporter gene *GUS* became parasitic to

transgenic lettuce expressing RNA hairpin containing a fragment of the *GUS* gene (hpGUS). Additionally, Aly et al., (2009) showed that a construct containing the binary vector pBIN-IR-M6PR inserted into tomato genome can silence M6PR gene in tubers of *Orobanche* that parasitize the roots of transgenic plants. The observation that molecules produced by host plants are responsible for silencing specific genes in parasitic plants suggests a new strategy for engineering plants resistant to parasites.

Several pathogenic fungi have been implicated in the devastation of crops worldwide leading quests for new techniques of addressing this problem. A turning point in this field was the report of genetically modified tobacco engineered to release siRNA specific to a vital gene of *Fusarium verticillioides* (Tinoco et al., 2010). The movement of silencing signals in the form of siRNAs derived from one organism, exerting their effects on another, has also been observed in nematodes where gene silencing was triggered when nematodes were fed on a diet made from transgenic plants engineered to express dsRNA (Waterhouse et al., 1998).

2.5 RNAi and the study of gene function in insects

The discovery of RNAi has made available tools for analysis gene function in insects which were previously restricted to genetic model insects like *D. melanogaster* and *Tribolium castaneum* (Garbutt, 2011). With the availability of more genome data and advances in DNA sequencing technologies with reduced cost of the application of RNAi in insect research, there has been an increase in the number of high throughput analyses of gene function in several non-model insects (Mito et al., 2011). The boost RNAi technology gave to insect research involving gene function is such that within a short period, several publications appeared on the subject. Indeed by 2010, a Web of Science search using the search query "RNA interference* and insect*" returned 380 articles (Garbutt, 2011). At the time of writing this thesis, this number was 3,404. Several reports on RNAi cover such range of insects as *Aedes aegypti* (Xi et al., 2008), *Anopheles gambiae* (Magalhaes et al., 2008) and *D. melanogaster* (Miller et al., 2008). In case of the lepidopterans, RNAi research focus on *Bombyx mori* (Hossain et al., 2008), *Manduca sexta*

(Eleftherianos et al., 2009) and *Spodoptera litura* (Chen et al., 2008) and in coleopterans like *Onthophagus taurus* and *Onthophagus binodis* (Moczek and Rose, 2009) and *Tribolium castaneum* are used (Angell and Baulcombe, 1997).

The champion beneficiaries of insect control using *Bacillus thuringiensis* toxin (Bt-toxin) are lepidopteran and coleopteran pests. Attempts to extend the technology to sap-sucking hemipterans have met with serious drawbacks (Upadhyay et al., 2013). RNAi technology has come to the rescue via different approaches including the use of host plants. The potential usefulness of insect pest control mediated by RNAi emerged with the demonstration that 2nd instar nymphs of the bug *Rhodnius prolixus* Stål fed with dsRNAs targeting the insect's gene for *nitrophorin 2* (coding for a heme-binding protein with anticoagulant activity and essential for feeding), reduced its activity in the insect's salivary gland (Araujo et al., 2006). Similarly, the activities of gut enzyme and pheromone binding protein (PBP) in adult antennae of *Epiphyrsu postvittana* Walker were interrupted when 3rd instar larvae of the insect were targeted with dsRNAs specific to their transcripts (Turner et al., 2006).

2.6 Plant mediated RNAi for the control of insect

The activation of RNAi pathway in insects may be initiated in a host plant transformed with specific gene sequence following the principle of HIGS. It has been demonstrated that silencing of essential insects genes mediated by dsRNA can interrupt feeding or lead to death in whitefly (Ghanim et al., 2007). Ingestion of dsRNAs provided in artificial diet induces RNA interference in *Diabrotica virgifera* (Baum et al., 2007). This study reported on the reduction of western corn root (WCR) damage in transgenic maize plants producing *v-ATPase* dsRNA after infestation of the plant with the WCR. Transgenic cotton and *Arabidopsis* plants engineered to express dsRNA directed against the gene coding for Cyt P450, a detoxification enzyme against gossypol in cotton bollworm, induced feeding damage in the insect (Mao et al., 2007). Similarly, the midgut genes of *Nilaparvara lugens* were knocked down using dsRNA expressed by *Oriza sativa* targeting the trancripts of three genes (Zha et al., 2011). Similar plant-mediated gene silencing approaches were reported in *M. sexta* and *N. tabacum* (Kumar et al., 2012) and in *Acyrthosiphon pisum*. These

studies formed the foundation of HIGS-mediated plant-RNAi-insect research in which transgenic plants expressing dsRNAs target and silence insect specific gene. The execution of RNAi mediated strategies for insect control via transgenic plants requires careful design and study.

2.6.1 Strategies for plant mediated RNAi control of insects

The first factor that ensures the success of strategies for the control insect using RNAi-mediated transgenic plant is the design of transformation vector (Aragão et al., 2014). The general approach involves the juxta-positioning of dsRNA coding region in-between an intron that will lead to the formation of hairpin following its acquisition and expression in transgenic plants. When the targets for control are viruses, the small nature of viral genomes might be an advantage in designing dsRNA vectors. For example, the expression vector pNW55, derived from a natural miRNA (osa-MIR528 of rice) in which an artificially inserted miRNA sequence, was designed to silence *Pds*, *Spl11*, and *Eui1/CYP714D1,* was successful (Warthmann et al., 2008). Other examples are seen in the silencing of *P69* gene of *Turnip yellow mosaic virus* and the HC-pro gene of *Turnip mosaic virus* in *Arabidopsis thaliana* by using miR159 from the plant to construct a vector that expressed artificial miRNAs (amiRNA). Plants generated from this work were reported to be resistant to both viruses (even at 15°C) (Niu et al., 2008).

However, in cases where the target organisms are higher pathogens, this specificity constitutes a problem in attempts to confer wide and reliable resistance in both *cis* and *trans* approaches. In order to circumvent this, the choice of appropriate nucleotide sequence is extremely important because the sequence used will determine possible off-target effects in the target organism and in other insects. In both feeding and *in vivo* experiments, most sequences used range in sizes between 300 and 560 bp (Garbutt, 2011). Other factors include the concentration of dsRNA because exceeding optimal concentration does not result in more silencing. An ideal concentration for attaining lethal effect in silencing of *v-ATPase* gene in western corn rootworm larvae has been shown to be around 52 ng/cm^2 (Baum et al., 2007). The silencing effect may be transient or stable and this is also determined by life stage of

the target organism. Their intrinsic RNAi machinery often determines the stability of the dsRNA after uptake by the insects. Key component of this is their possession of SID-1, a dsRNA-selective dsRNA-gated channel responsible for uptake and systemic spreading of RNAi in many species including insects (Gu and Knipple, 2013). It appears that insects that lack SID-1 are not susceptible to RNAi although this is not always the case. Based on life cycle, insects at younger stages often show larger silencing effects than older stages although the latter are easier to handle (Garbutt, 2011).

2.7 Whitefly

Whiteflies are arthropods belonging to the family of Aleyrodoidea with more than 1,550 species identified (Byrne, 1991). In general, whiteflies may be classified based on their morphological features or ecological niche. The banded winged whitefly (*Trialeurodes abutilonea* Haldeman) has pale yellow colour with a tinge of green on its thorax. They have two irregular zigzag smoky-grey lines transversely across each wing. Greenhouse whitefly (*Trialeurodes vaporariorum* Westwood) characteristically has 4-5 leg segments and two to three antennal segments. It may be transparent or opaque, ranging in color from light green to yellow, light brown through dark brown and black. It secretes powdery white wax after its crawlers settle and begin feeding.

2.7.1 *Bemisia tabaci* Gennadius

Sweet potato whitefly or silverleaf whitefly (*Bemisia tabaci* Gennadius) is the most common whitefly in the tropical, subtropical, and other less temperate regions (De Barro et al., 2011). The characterization of silverleaf whitefly into A and B-types arose following the identification of a viruliferous form of the insect in 1980, which was therefore identified as B-type (Fan and Petitt, 1998). Although the B-type is regarded as the most common, the species name *Bemisia tabaci* is actually a complex species comprising of at least 24 morphologically different species (De Barro et al., 2011).

An adult *B. tabaci* is 0.8 mm in length. At rest, it holds its solid white wings roof like over its pale yellow body. It inhabits and feeds on the undersurfaces of leaves. Its snow-white color is attributed to the secretion of wax on its body and wings. It is the most common and destructive whitefly in Brazil and many other tropical regions, causing damage to many agricultural crops throughout the world (Byrne and Bellows, 1991; Oliveira et al., 2001).

Whitefly shares a modified form of hemimetabolous metamorphosis, in that the immature stages begin life as mobile individuals, but soon attach to host plants. Female whiteflies are diploid emerging from fertilized eggs while the males are haploid emerging from unfertilized eggs. A newly emerged adult is about 0.8 mm in length. Typically, females deposit 50 to 400 eggs, which are about 0.10 mm - 0.25 mm on the abaxial region of leaves as they feed. The eggs are initially whitish but turn to brown 5 to 7 days before hatching. The nymphs (also known as crawlers) arising from eggs grow in size from 0.3 mm to 0.6 mm. The first instar is a greenish flat bodied and motile nymph that moves around until it finds a suitable space on a leaf, where it settles and proceeds to other instar stages before finally emerging as an adult. The nymph feeds by stabbing the plant to consume juices. Although the stage before adult is called a pupa, it shares little in common with the pupal stage of holometabolous insects. At pupal stage, red eyes are visible with thickening of body, which turns yellow as it becomes adult while the wing remains white. The cycle often lasts for about 28-30 days (Figure 3) (Byrne, 1991).

2.7. 2 Whitefly and Agriculture

Whiteflies feed on the undersides of plant leaves and tap into their phloem, introducing toxic saliva and decreasing the plants' overall turgor pressure. The damage caused by this feeding alone, can lead to more than 50% reduction in yield (Byrne, 1991). Feeding damage by both nymphs and adults results in accumulation of honeydew on leaves, and subsequent growth of molds (Brown and Czosnek, 2002). This leads to plant breakdown, chlorotic spots, yellowing, blanching of vegetative structures, leaf shedding, and irregular ripening in tomatoes or other abnormalities of fruit structures. In the tropics and subtropics, whiteflies have become one of the most serious crop protection problems because they carry and

transmit diseases with losses estimated in hundreds of millions of dollars especially due to viral transmission. As early as the 1990's, in the US alone, 90% of winter vegetable crop suffered an estimated loss of $500 million in crop damage due to silverleaf whitefly while losses in other crops and ornamental plants reached an excess of $1 billion (Brown, 1995).

Figure 3: The Life cycle of whitefly. The eggs are initially whitish but turn to brown 5 to 7 days before hatching. The first instar is a greenish flat bodied and motile nymph that moves around until it finds a suitable space on the leaf, where it settles and proceeds to other instar stages before emerging as an adult. Figure reproduced from ucanr.edu/blogs

Whiteflies transmit several viruses, among which 90% belong to the genera Begomovirus, 6% Crinivirus and the remaining 4% are Closterovirus, Ipomovirus and Carlavirus. *Bemisia tabaci* cause diseases like *African cassava mosaic, Bean golden mosaic, Bean dwarf mosaic, Bean calico mosaic, Tomato yellow leaf curl, Tomato mottle*, and others. *B tabaci* is known to attack up to 600 plant species (Li et al., 2011).

Traditionally, damage caused by *B. tabaci* is prevented through cultural and biological control (Byrne, 1991). Initial application of pesticide may work but soon resistance emerges. The commonly used pesticides contain neonicotinoids, derived from nicotine, which binds to nicotinic acetylcholine receptors and paralyses central nervous system of insects. Besides the environmental risks in the use of these insecticides, whiteflies have developed resistance against them (Ahmad et al., 2002). The invasive nature of whitefly and its ability to rapidly reproduce, have conferred on the insect the ability to easily develop resistance against the many insecticides often applied for its control. In Brazil, where the B-type first emerged in 1995, by 2001, losses had reached over 5 billion USD (Oliveira et al., 2001). Local farmers in the country, who traditionally applied insecticides 16 times per cycle, now resort to applying 40 times. Field experience has shown that, 1 to 3 whitefly per plant is sufficient to cause total loss of field due to virus (Personal communication, Aragão, 2014). Indeed, not even the adoption of the so-called "sanitary gap" (*vazio sanitário* in Portuguese), a practice of suspending growing of whitefly-susceptible crops within a season, has helped matters. The emergence of this resistance underscores the need to develop new methods of controlling sucking pests like whitefly.

2.8 Vacuolar ATPase (v-ATPase)

Vacuolar ATPases are a class of ubiquitous proton pumps that utilize ATP hydrolysis to maintain vacuolar acidic pH (Nishi and Forgac, 2002). The maintenance of this pH generates an electrochemical potential, which is used for the accumulation of positively charged substrates like calcium and basic amino acids. Structurally, ATPases posses 13 distinct subunits designated $A_3:B_3:C:D:E:F:G_y:H_z:a:d:c:c:c''$ subdivided in two components: V_0 and V_1. V_0 is membrane bound and contains ion channel while V_1 is extrinsic and is the location of ATP hydrolysis (Figure 4) (Forgac, 2007; Rizzo et al., 2003). Although v-ATPases share the similar bi-domain architecture and rotational mechanism with ATP synthase (whose components are designated F_0 and F_1), they are larger and have more subunits than ATP synthase (Muench et al., 2009).
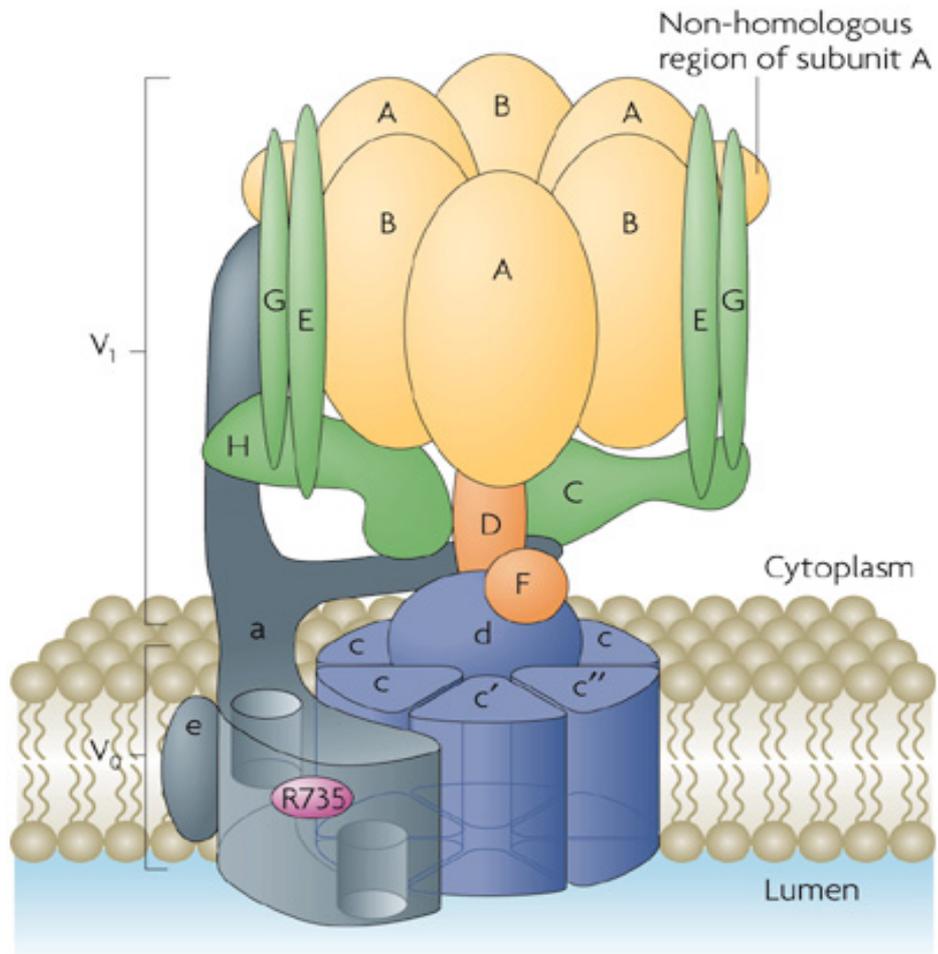
Figure 4. Structure of vacuolar (v-) ATPase. The enzyme comprises of a peripheral domain V_1 (yellow and orange), responsible for ATP hydrolysis, and an integral domain V_0 (blue and grey), involved in proton translocation across the membrane. The core of the V_1 domain is composed of a hexameric arrangement of alternating A and B subunits, which participate in ATP binding and hydrolysis. The V_0 domain includes a ring of proteolipid subunits (c, c' and c") that are adjacent to subunits a and e. The two main domains are connected by a central stalk, composed of subunits D and F of V_1 and subunit d of V_0, and multiple peripheral stalks, composed of subunits C, E, G, H and the N-terminal domain of subunit a. Subunit a has two hemi-channels and a crucial buried Arg residue (R735), which are required for proton translocation across the membrane. Texts and scheme reproduced from Forgac, (2007)

ATPase essentially mediates a highly coordinated control of pH of intracellular compartments in eukaryotic cells. Its activities affect such diverse cellular processes as intracellular membrane transport, processing and transport of neurotransmitters as well as regulation of entry of microorganisms like viruses (Figure 5) (Beyenbach and Wieczorek, 2006; Forgac, 2007). The enzyme functions by pumping H^+ into the

lumen of organelles, leading to their acidification. The v-ATPase enzyme has also been reported in numerous ion-transporting insect epithelia. The singular activity of acidifying lumens of almost all organelles of eukaryotes by v-ATPase makes it nature's most versatile proton pump (Nishi and Forgac, 2002).

Although different specialists describe v-ATPase from different perspectives, one thing common in all v-ATPases is their unique structure comprising of V_0 and V_1 domains that have been characterized at molecular level (Figure 4). The application of spherical harmonics using X-ray scattering has allowed for the characterization of the structure of ATPase in *M sexta* and *Chlostridium fervidus* where it is found essentially in endomembranes and plasma membranes (Wieczorek et al., 2000). As a member of an evolutionarily conserved family of enzymes with diverse functions in eukaryotes, ATPase is present in the membranes of intracellular compartments, like vacuoles, lysosomes, coated vesicles, secretary granules, and the trans-Golgi network.
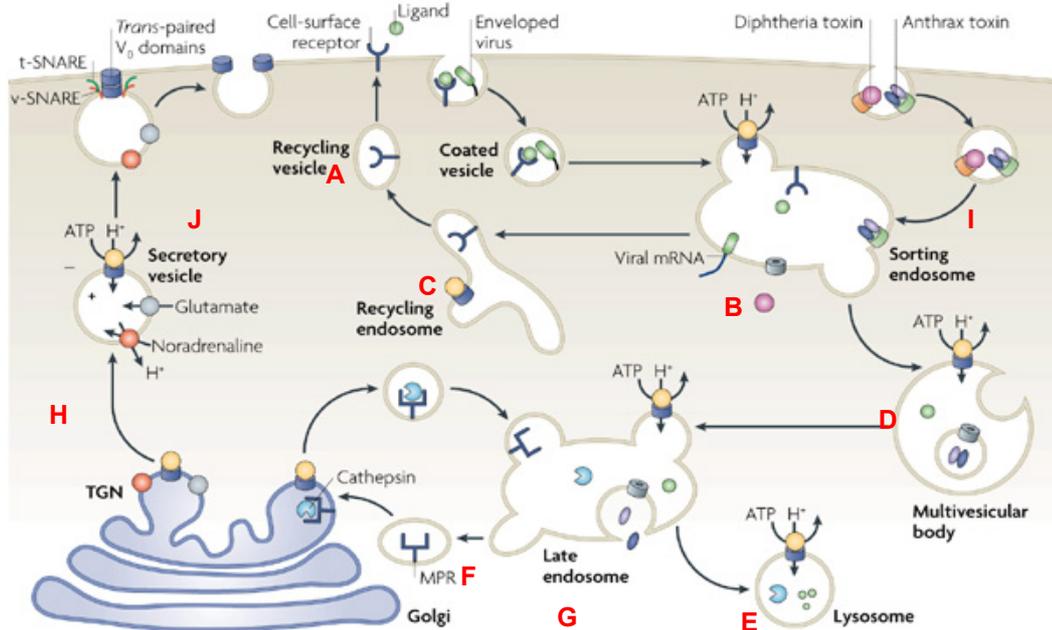
Figure 5   Functions of v-ATPase. Extracellular ligands can be internalized from the plasma membrane by receptor-mediated endocytosis and trafficked to the sorting (also known as early) endosome (A). Acidification of sorting endosomes by vacuolar (v-) ATPases causes the dissociation of ligand–receptor complexes (B), which facilitates the recycling of unoccupied receptors to the cell surfaces (C) and the targeting of the released ligands to lysosomes for degradation via multivesicular bodies (D). Formation of multivesicular bodies (also known as endosomal carrier vesicles) is also dependent on a lumenal acidic pH. Lysosomal degradation is carried out by cathepsins, which require a low pH for activity (E). Cathepsins are delivered to lysosomes via the mannose-6-phosphate receptor (MPR) (F), which binds newly synthesized cathepsins in the trans-Golgi network (TGN) and delivers them to late endosomes (G), where they are released for delivery to lysosomes (E). v-ATPases in secretory vesicles generate both a pH gradient and an internally positive membrane potential that is used to drive the uptake of neurotransmitters, such as glutamate and noradrenaline (H). These trafficking pathways are also exploited by pathogens. Enveloped viruses, such as influenza virus, and toxins, such as diphtheria and anthrax toxin, enter cells via acidic endosomes (I). Low pH facilitates the entry of the viral mRNA or cytotoxic portions of the toxin molecules through pores that are formed in the endosomal membrane (I). Recent evidence suggests a role for the V_0 domain in the fusion of synaptic vesicles at the presynaptic terminal, which is consistent with earlier studies of homotypic vacuole fusion in yeast (J). These trafficking pathways are also exploited by pathogens. t-SNARE; target SNARE; v-SNARE, vesicle membrane SNARE. Scheme and texts of figure reproduced from Forgac, 2007.

2.9 Engineering plants for the control of whitefly

Early studies on insects RNAi centered on studies in *Drosophila melanogaster* mutants (Garbutt, 2011). With the availability of a number of transcriptomic data on whitefly in the NCBI database, RNAi machinery of whitefly may soon be fully deciphered. Indeed, important components of RNAi machinery like Dicers 2, R2D2, Argonaute2 and Sid1 have already been identified in whitefly and their expression at different developmental stages described (Upadhyay et al., 2011). The demonstration that tissue-specific mRNAs of different genes of whitefly may be preferentially silenced or expression thereof reduced by up to 70%, has paved ways for further research in RNAi-mediated control of whitefly (Ghanim et al., 2007). A method for oral route delivery of dsRNA and siRNA into whitefly, which effectively controlled the insect by silencing and knocking down the expression of important genes, has been described (Upadhyay et al., 2011). This represents the first attempt at silencing *ATPase* gene whose transcript was shown to be significantly reduced when dsRNA were fed to the insect in an artificial diet. One of the more recent attempts at silencing genes in whitefly via RNAi entails the development of a high throughput method based on feeding using dsRNAs that target genes within the molting hormone, ecdysone (an important component in regulating growth and development in insects) synthesis and signaling pathway (Luan et al., 2013). Although the silencing effect reported was low in adult whiteflies, it reduced survival and delayed development of nymphs. Recently, silencing of *v-ATPase* gene coding for subunit A of the v-ATPase was reported by Thakur et al., (2014), who used tobacco plants engineered to silence the gene in whitefly. They demonstrated this through both reductions in insect population and in the transcript level of the gene.

These experiments gained impetus following earlier reports that ingestion of dsRNAs provided in an artificial diet induces RNA interference in coleopterans such as *Diabrotica* sp. (Baum et al., 2007; Gordon and Waterhouse, 2007; Price and Gatehouse, 2008; Upadhyay et al., 2011). The choice of *v-ATPase* gene was apparently informed by the demonstration that transgenic corn engineered to express dsRNAs against the gene in western corn rootworm showed suppression of mRNA in the insect and reduction in feeding damage (Baum et al., 2007). Similarly, RNAi was reported in cotton and *Arabidopsis* expressing dsRNA directed against Cyt

P450, a detoxification enzyme in insects against gossypol, an alellochemical with which plants normally protect themselves from attack by insects. Since insects deploy Cyt P450 to avoid the toxic effect of gossypol, it follows that silencing of the gene in cotton bollworm would reduce its tolerance to gossypol. Indeed this was shown by the high mortality and feeding damage (Mao et al., 2007).

The focus of this thesis is the deployment of these approaches in silencing *v-ATPase* gene in whitefly using lettuce as a model. The choice of this crop was inspired, not only by its nature and economic importance, but because it serves as one of the primary targets of attack by the insect. The Laboratory for Genetic Engineering for Tropical Agriculture (LEG) at EMBRAPA Center for Genetic Resources and Biotechnology (CENARGEN), where this work was conducted, has a well established protocol for transformation of lettuce (Nunes et al., 2009). The bulk of this thesis is based on the study using lettuce. However, proof of concept attempts at developing this technique was also earlier made using soybean (*Glycine max*), a summary of which is presented at the end of this thesis.

3. JUSTIFICATION

The emerging shift in evolutionary pressure on agricultural crops by insect pests that have come to be controlled using Bt toxins, has led to a growing demand for alternative strategies of controlling multiple insect pests. In particular, this shift has emboldened hemipterans like aphid, mealy bugs, mite and whitefly, which now pose even more serious risks in agricultural production. With reports on the emergence resistance against Bt toxins in a number of coleopterans, the need for new alternatives for addressing insect pests has never been so urgent. *Bemisia tabaci* is a devastating pest all over the world, causing great damage in crops. Besides feeding damage that results in accumulation of toxic substances in plants, whiteflies transmit several viruses in more than 600 plant species. The invasive nature of whitefly and its rapid reproduction cycle have conferred on it the ability to easily develop resistance against the many insecticides often applied for its control. This underscores the need to develop new methods of controlling sucking pests like whitefly. With the demonstration that whitefly can be controlled via RNAi strategy through preferential silencing of genes by dsRNA and siRNA, *v-ATPase* gene was identified as a candidate gene of choice. Recently, the gene coding for subunit A of v-ATPase has been shown to be suppressed in the model plant tobacco. This thesis reports on the silencing of *v-ATPase* in whitefly by lettuce engineered to express a siRNA specific to the gene.

4. HYPOTHESIS

Transgenic lettuce plants engineered to express siRNA bearing sequence homology with *v-ATPase* will silence the gene in *Bemisia tabaci* upon feeding and lead to more whitefly-tolerant plants.

5. OBJECTIVES

The objective of this work was to silence *v-ATPase* gene in whitefly using siRNA expressed by transgenic lettuce lines.

Specifically:

1. Establish whitefly culture for use in experiments.
2. Construction of transformation vector.
3. Generate transgenic lettuce.
4. Detect siRNAs in different lines of transgenic lettuce.
5. Analyze off-target effect of the siRNAs arising from the transgenic lettuce
6. Develop a system for whitefly feeding experiment using lettuce.
7. Perform bioassay on the effect of feeding emerging whiteflies on transgenic lettuce.
8. Monitor the eggs to adult conversion of whiteflies feeding on transgenic lettuce expressing *v-ATPase* siRNA.

6. MATERIALS AND METHODS

6.1 Insect culture

Whiteflies were kindly provided by Dr Josias Correa de Faria of EMBRAPA Arroz e Feijão, Brazil, from a known non-viruliferous colony maintained in green house. The insects were released and maintained on potted cotton plants in EMBRAPA CENARGEN.

6.2 Construction of dsRNA vectors

A search in the NCBI database of *v-ATPase* sequence of the v-ATPase was performed from which six related sequences from *Helicoperva armigera, Bombyx mori, Culex quinquefascitatus, Ostrinia furnacalis, Manduca sexta* and *Spodoptera littralis* were identified and aligned using MEGA5 program. Conserved region of the alignment was used to design primers that would amplify the expected *v-ATPase* region. These primers were used to amplify a fragment of *v-ATPase* from genomic DNA of *B. tabaci*. Cloning of the fragment in *E coli,* followed by sequencing and analysis using NCBI nucleotide database revealed the identity of the gene, which corresponded to 576 bp partial sequence of the gene. A new set of primers was designed to contain restriction sites of *Xba*I and *Spe*I on one end and *Sac*I and *Kpn*I sites on the other and then used to clone interfering fragments of the gene in pSIU vector (Tinoco et al., 2010) separated by intron to ensure the formation of hairpin following expression. This led to the construction of a vector named pBtATPase. For *Agrobacterium tumefaciens* transformation, the vector pBtATPaseC3300 was constructed by digesting pBtATPase and GS54365-5pCAMBIA33000-Construct5 with *Hind*III and *EcoR*I releasing the *v-ATPase* cassette and a larger fragment respectively. The resulting fragments were ligated to form pBtATPaseC3300, which was used to transform *A. tumefaciens*.

6.3 DNA sequencing, sequence manipulation, cloning and primer design

Purified nucleic acids were sent to Macrogen (Korea) for sequencing. Sequence data were analysed using BLAST (Basic Local Alignment Search Tool)

searches and MEGA5 (http://www.megasoftware.net), a molecular genetics sequence alignment program, was used to align sequences of interest.

Primers used were designed using PrimerQuest tool available on http://www.idtdna.com/site, and chosen according to the following design parameters: 1. Melting temperatures between 55 $^{\circ}$C and 63 $^{\circ}$C, 2. Size of the primers was between 18 and 27 bases 3. GC content was between 20-80%. Oligos were synthesized by Macrogen (Korea).

Table 1 Primers used for cloning and detection of DNA sequences

Primer	Sequence	Use
ATPaF	5' GAGGGTGACATGGCCACCATCCAGGT 3'	Identification, cloning and purification of a 647 bp partial sequence of *v-ATPase*
ATPaR1	5'GACRTCRGAGTTGGAGTACTTGGACAG 3'	
ATPSX	5'CTGACTTCTAGAGCTCGCATCCGAAAGCGCCGGGAATG 3'	Cloning and detection of 576 bp partial sequence of *v-ATPase*
ATPSK	5'ACGTACGGGTACCACTAGTCGGCGACCCTGTACAGCGAAC3'	
ATPase51F	5'CACACTGGGAAAGAGAGCGT 3'	Cloning and detection of 407 bp partial sequence of *v-ATPase*
ATPase457R	5'AGGGCATCAGCGATAAGAGC 3'	
Bar F	5'AAACCCACGTCATGCCAGTT 3'	Cloning and detection of 408 bp partial sequence of Bar
BarR	5'CATCGAGACAAGCACGGTCA 3'	

6.4 Agarose gel electrophoresis

Agarose gel electrophoresis was used to check the integrity of DNA and RNA and to verify the size of PCR products as well as purification of PCR products for downstream use. Typically a 1% agarose gel was prepared by weighing 1 g UltraPureTM Agarose (Invitrogen) in a 100 ml conical flask and 100 ml of Tris-acetate-EDTA (TAE) buffer. The mixture was heated in a microwave oven to melt the powder. A volume of 3 µL of SYBR® DNA gel stain (Invitrogen; S33102) was added

and mixed into the agarose solution by swirling the conical flask. The solution was then poured into a gel block and a gel comb inserted. Following gellification, the comb was removed and the gel placed in a gel tank (BioRad) containing TAE buffer. Samples were mixed with loading buffer (New England Biolabs, composition: 2.5% Ficoll-400 11 mM EDTA 3.3 mM Tris-HCL (pH 8.0) 0.017% SDS 0.015% bromophenol blue) and loaded into the gel (in a total volume of between 5-50 μl). Typically, 3 μg of 1kb GeneRulerTM (Fermentas, SM0311) was used as marker. Gels were run at 80V for 60-120 minutes and observed under a UV transilluminator (BioRad).

6.5 Bacterial transformation

Throughout bacterial culturing, sterile technique was followed. Competent *E. coli* cells were used to prepare mini concentration of the vector pBtATPaseC3300. The bacteria was cultured in Petri dish (15 x 90 mm) containing 25 mL of Luria-Bertani (LB) liquid medium which was composed of 10 g/L trypton, 5 g/L yeast extract and 10 g/L sodium chloride at pH 7.0. The medium was supplemented with kanamycin at 100 mg/L concentrations, and incubated at $37 \pm 2°C$ for 48 h. Twelve individual colonies were selected and used for PCR using the primer pair ATPXS (5'CTGACTTCTAGAGCTCGCATCCGAAAGCGCCGGGAATG 3') and ATPSK (5'ACGTACGGGTACCACTAGTCGGCGACCCTGTACAGCGAAC3'). An individual colony was selected and plasmid isolated therefrom used to transfect EHA105 strain of *A. tumefaciens.* The bacteria were stored at - $80°C$ in a 50% glycerol and LB media stock.

6.6 Plant tissue culture and genetic transformation

Sterile procedures were followed throughout. Transformation was carried out using modified protocol of (Dias et al., 2006). Seeds of lettuce (cultivar Verônica) were surface sterilized by immersion in 0.01 % of Tween 20 followed by 2.5 % of NaOCl for 7 minutes. The seeds were then rinsed three times in sterile distil water and cultured in Petri dish containing 20 mL 1/2 MS (Murashige and Skoog, 1962) media and 0.8% of agar. The Petri dishes containing the seeds were then sealed

with cling film and cultured for 2-3 days in the dark 25 ± 2 °C, until their germination. At the same time, frozen *Agrobacterium* was activated on LB media containing kanamycin and rifampycin for 48 hours. For co-culture, colonies were scrapped and dispensed into co-culture media (MS salts containing 0.5 M acetoseringone and 3 % glucose pH 5.2), which had been filter-sterilized. The bacterial suspension was homogenized to an O D$_{.600}$ of 0.5 – 1.0 measured with spectrophotometer (Spectrometry Genegys 8) and then used to co-culture with cut pieces of lettuce cotyledon for 15 min. The explants were dried on autoclaved filter paper and incubated in an MS media containing 3% sucrose, 0.05 mg/L de Naphthaleneacetc acid (NAA) and 0.2 mg/L of 6-benzylaminopurine (BAP) at 25 ± 2 ^{o}C for 48 hours. The bacteria was eliminated by washing the explants in sterile distilled water containing 250mg/L timetin and dried on sterile filter paper.

The explants were then subcultured to the same media with addition of 4mg/L of ammonium glufosinate. Proliferating callus arising from the explants were subcultured into the same media every week. Emerging shoots were individualized and cultured on MS media containing 3% sucrose, 0.5mg/L kinetin, 0.5 mg/L zeatin and 4 mg/L ammonium glufosinate. The explants were subcultured on weekly basis until they were fully grown from where they were transferred to a rooting media containing MS and 4 mg/L ammonium glufosinate. With the development of full roots, explants were acclimatized in plastic cups containing 50:50 ratio of soil and vermiculate in a humidity chamber made of plastic and rubber band. When fully hardened, the plants were transferred to large pots. With the emergence of seeds, these were collected and planted in plastic cups for further screening and analysis.

6.7 Immunochromatographic lateral flow analysis for detection of *bar* gene

Regenerated plants were used in preliminary screening analysis using the immunochromatographic method of TraitChek™ (Romer Labs) based on the manufacture's recommendation. Cut pieces of leaves were macerated in 1.5 mL tubes containing 300 μL of AgraStrip buffer as described by the manufacturer. Strips were inserted to detect the expression of PAT protein.

6.8 DNA extraction and PCR

Total DNA was extracted from approximately 100 insects using a slightly modified version of the method described by Calderón-Cortés et al., (2010). The insects were grounded in a mortar with pestle containing liquid Nitrogen until a fine powder was formed, which was transferred to 1.5 mL tube and homogenized in 1mL pre-warmed extraction buffer containing 20 mM EDTA (pH 8), 100 mM Tris-HCl (pH 7.5), 1.4 M NaCl, 2% CTAB, 4% PVP and 2% β-mercaptoethanol. This was incubated at 60 ^{o}C for 30 min with occasional mixing. Next, 2 µL of 1mg/mL of RNase was added and incubated at 37 ^{o}C for 15 min. Equal volume of chlorophane was then added and the mixture emulsified by gentle inversion. This was centrifuged at 13,000 xg for 15 min and the top aqueous layer collected, precipitated with twice volume of cold absolute ethanol and incubated for 20 minutes. The resulting DNA pellets were washed with 70% ethanol and suspended in H_2O. This DNA was used for cloning and identification of the *v-ATPase* fragment.

For DNA isolation from leaves, a modified version of Doyle and Doyle, (1987) method was used. Cut leaf discs were collected and homogenized in CTAB containing 1% PVP at 65 ^{o}C. This was treated with pure chlorophorm and centrifuged at 13,000 x g for 5 min. The supernatant was collected in fresh tubes and precipitated with 2.5 volume of cold ethanol. The pellet was washed in 70% ethanol and suspended in H_2O. Before use in PCR, lettuce DNA samples were heated at 95 ^{o}C for 5 min. All nucleic acids were quantified using ND-1000 NanoDrop Spectrophotemeter (BioRad).

PCR reactions were performed using Taq DNA Polymerase (Invitrogen) unless otherwise stated. Reagents were thawed on ice block and reactions set up in 0.2 ml thin-walled PCR tubes in a total volume of 20 µL. Typically, PCR reactions prepared contained the following components:

Table 2. PCR reaction mixture

Component	Volume (μL)	Final concentration
10X PCR buffer	2	1X
50mM Magnesium Chloride	0.6	1.5 mM
10mM dNTPs	1	0.2 mM
Forward primer	0.5	10 pM
Reverse primer	0.5	10 pM
Taq DNA Polymerase (5U/uL)	0.5	2.5 units
DNA	1-5	Up to 100ng
Autoclaved H$_2$O	-	-
Total	20	

After set-up, PCR reactions were placed in MyCycler^{TM} (BioRad) thermal cycler with a heated lid. A typical thermal profile for amplification was as follows:

- Initial denaturation step of 95 ^{o}C for 5 minutes
- 39 cycles of 95 ^{o}C for 1 minute, 58 ^{o}C for 1 minute and 72 ^{o}C for 1 minute with a final extension step of 72 ^{o}C for 7 minutes.

When required, PCR products were purified for downstream applications using a Promega Wizard$^®$ SV Gel and PCR clean-up system (A9282) as recommended by the manufacturer. A negative (no-template) control, containing the PCR reagents, was set up alongside the reactions.

6.9 Progeny analysis

Seeds of first generation (T$_1$) of self-pollinated plants were germinated and analyzed for the presence of *v-ATPase-LEG* by PCR as described above. Pearson's Chi-square (χ^2) was used to determine whether or not the observed segregation ratio was consistent with a Mendelian ratio (3: 1), at 95% level of confidence. Where necessary, Yate's correction factor was used for the same purpose.

6.10 Analysis of siRNAs

Total RNA from lettuce was isolated using miRNeasy mini kit from Quiagen (217004) as recommended by the manufacturer. siRNA analysis was carried out as described by Bonfim et al., (2007). Hybridization was performed using a DNA probe corresponding to the *v-ATPase* PCR fragment amplified using the primer pair ATPXS (5'CTGACTTCTAGAGCTCGCATCCGAAAGCGCCGGGAATG 3') and ATPSK (5'ACGTACGGGTACCACTAGTCGGCGACCCTGTACAGCGAAC3'). (Table 1), labeled with α^{32}P dCTP using a random primer DNA labeling kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Hybridization and post hybridization washes were conducted as described (Yoo et al., 2004). Three oligomers (18, 24, and 39 nucleotides) were used as molecular size markers. The bands were visualized with a fluorescent image analyzer (FLA-3000; FUJIFILM). Three independent autoradiographs, corresponding to three biological replicates (individual plants) were used.

6.11 Whitefly toxicity assay

Initially, four different systems of monitoring the survival of whiteflies were developed. These were designated A, B C and D. Systems A and B were made from glass and C and D were made of transparent plastic. System D was selected for its superiority in maintaining a normal whitefly cycle. The toxicity test was performed using 20 emerging young whiteflies released on 4-week-old potted plants in plastic cups, which were then transferred into plastic jars with cover. The covers were perforated at the top and the hole sealed with cotton wool to allow for air circulation. The population of the flies was then monitored on daily basis. Number of whiteflies per plant was recorded over a period of 2 weeks until all the whiteflies have died off. During the third week after release, emerging eggs, crawlers (nymphs), pupae and adults were recorded on the leaves by visualizing individual leaves of each plant with stereomicroscope. Transgenic lines 1, 3, 4, 6, 19, 25 and 31 were used in this analysis. Two types of controls were used; transgenic plants expressing *bar* gene, and non-transgenic plants. Emergence of eggs, crawlers (nymphs, all instar stages), pupa and adults was monitored along a cycle of 28-32 days. The set-up was kept in

a 16 h photoperiod and 25±2 ^{o}C in system D described above (Figure 19). Plants were irrigated by means of hypodermic syringe and removed for counting of eggs, crawlers, pupa and adults after every 4 days starting 11 days after release of the insects. Day 11 after inoculation was considered day 1 for egg to adult analysis. For each line, 12 biological repetitions were used and their mean values used for Tukey analysis using Prism software, version 5.0.

6.12 *In silico* analysis of off-target effects

The siRNA-based sequence used in this work was subjected to *in silico* analysis using algorithms available at siRNA at Whitehead, DEQOR, siDESIGN, DSIR and SiDRM in order to evaluate its possible off-target effects in *Homo sapiens* and *Rattus norvegicus*.

7. RESULTS

7.1 Development of *v-ATPase* dsRNA/siRNA vector

The search for the gene sequence of *v-ATPase* gene on the NCBI database, based on six related sequences from *H. armigera, B. mori, C. quinquefascitatus, O. furnacalis, M. sexta* and *S. littralis* and alignment using MEGA5 program allowed for the designing of primers that were used to identify its partial sequence which was 647bp and designated *v-ATPase A-LEG* (Figure 6). The conserved region among these species was used in designing primers.

Figure 6. Alignment of *ATPase* gene sequences from different insect species. Primers used for cloning and identification of the fragment were designed based on aligned regions on positions 916 to 745 and 307 regions. Figure generated from MEGA5

The target sequence was generated by amplifying *B. tabaci* DNA with the primers ATPaF1/ATPaR1 (Table 1). Cloning of the fragment in pGEM®-T Easy vector (Promega) allowed for the addition of the restriction sites *Xba* I, *Sac* I, *Spe* I and *Kpn* I to generate a new fragment that was 576 bp. This fragment was then cloned in sense and antisense orientations in the vector pSIU (Tinoco *et al.,* 2010), generating pBtATPase with the fragment interspersed by the intron 3 (370 bp) from the malate synthase gene of *Arabidopsis thaliana* (ms-i3) (Figure 7). pBtATPase was initially designed for use in the transformation of soybean. The interfering cassette from pBtATPase was removed with *Hind*III and *EcoR*I from the vector pBtATPase and cloned into the sites for *Hind* III and *EcoR* I in the vector ppCAMBIA33000-Construct5 (GS54365-5), an intermediary vector constructed by Epoch Life Science, Inc. (Missouri City, TX, USA), which contains the gene *bar* from *Arabidopsis thaliana* to form the final vector (Figure 8). The resulting vector pBtATPaseC3300 (Figure 9) was used to transform cut pieces of lettuce cotyledons using *A. tumefaciens* strain of

EHA105, which inserted its T-DNA.
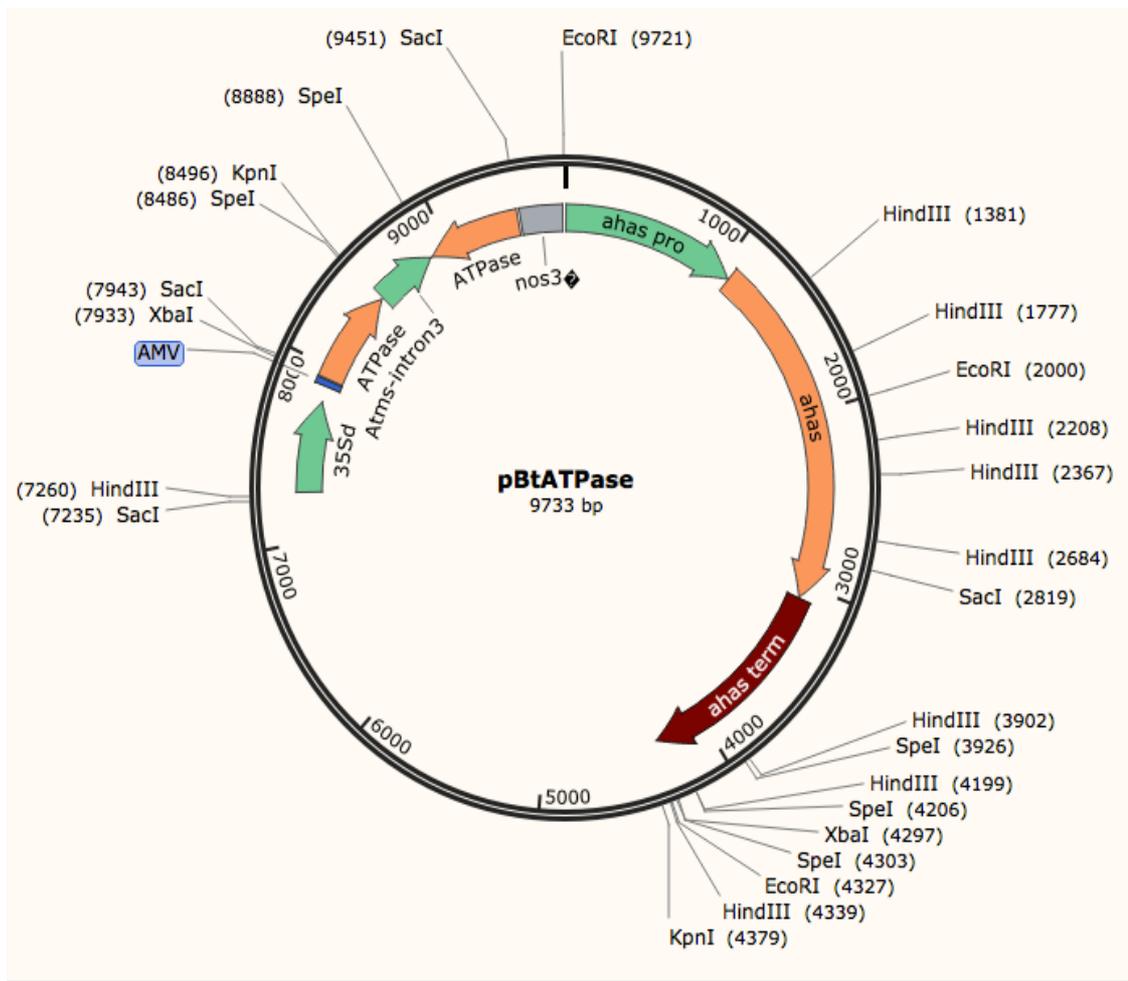
Figure 7. Engineering pBtATPaseC3300 vector. A: pBtATPase constructed from pSIU. Figure generated using SnapGene software.
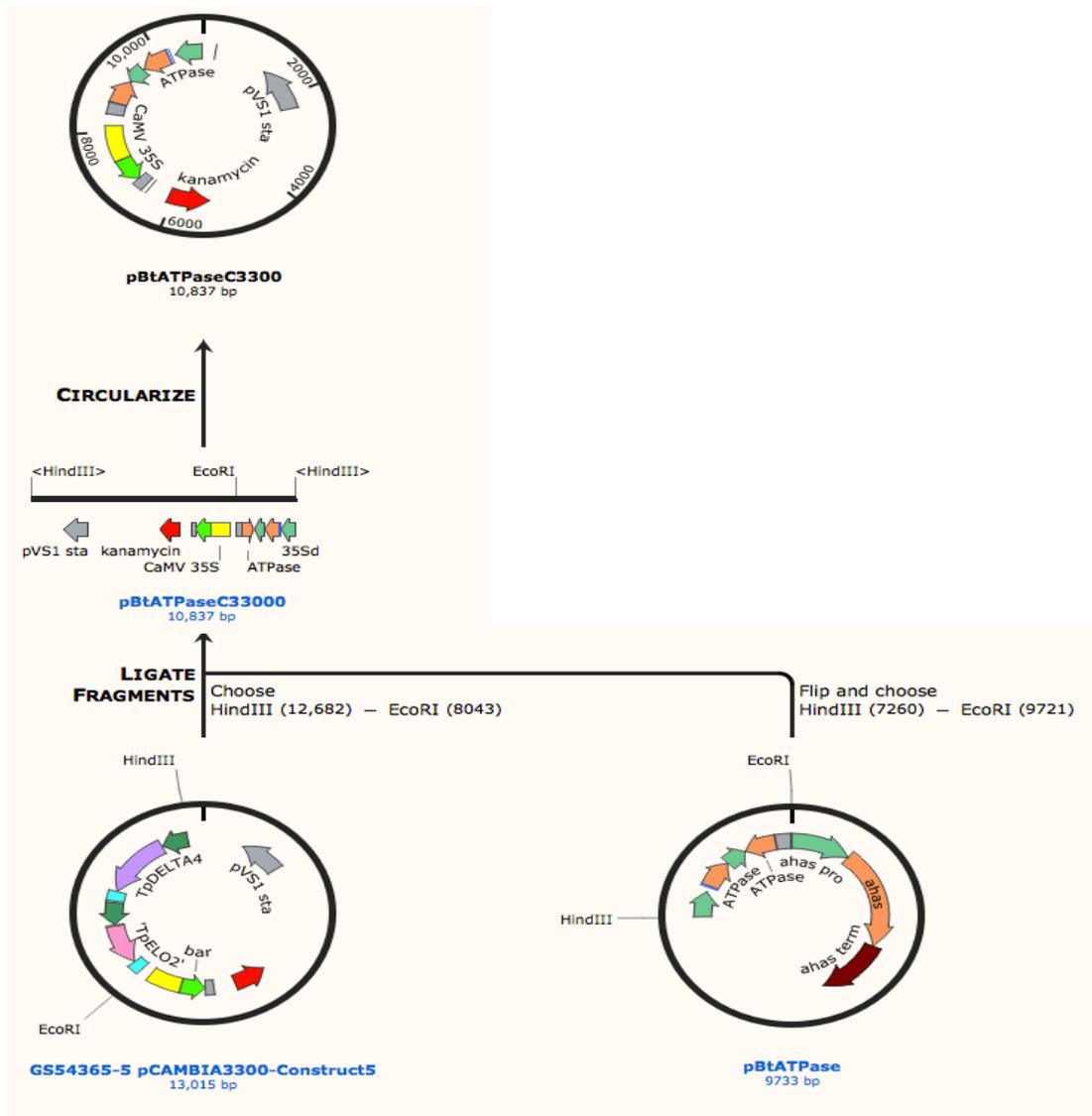
Figure 8. Engineering pBtATPaseC3300 vector. History of construction; digestion of pBtATPase and a pCAMBIA intermediary vector with *BamH*I and *EcoR*I released the v-ATPase cassette bearing the dsRNA coding region separated by an intron and the selectable marker regions of bar and kanamycin respectively. Figure generated using SnapGene software
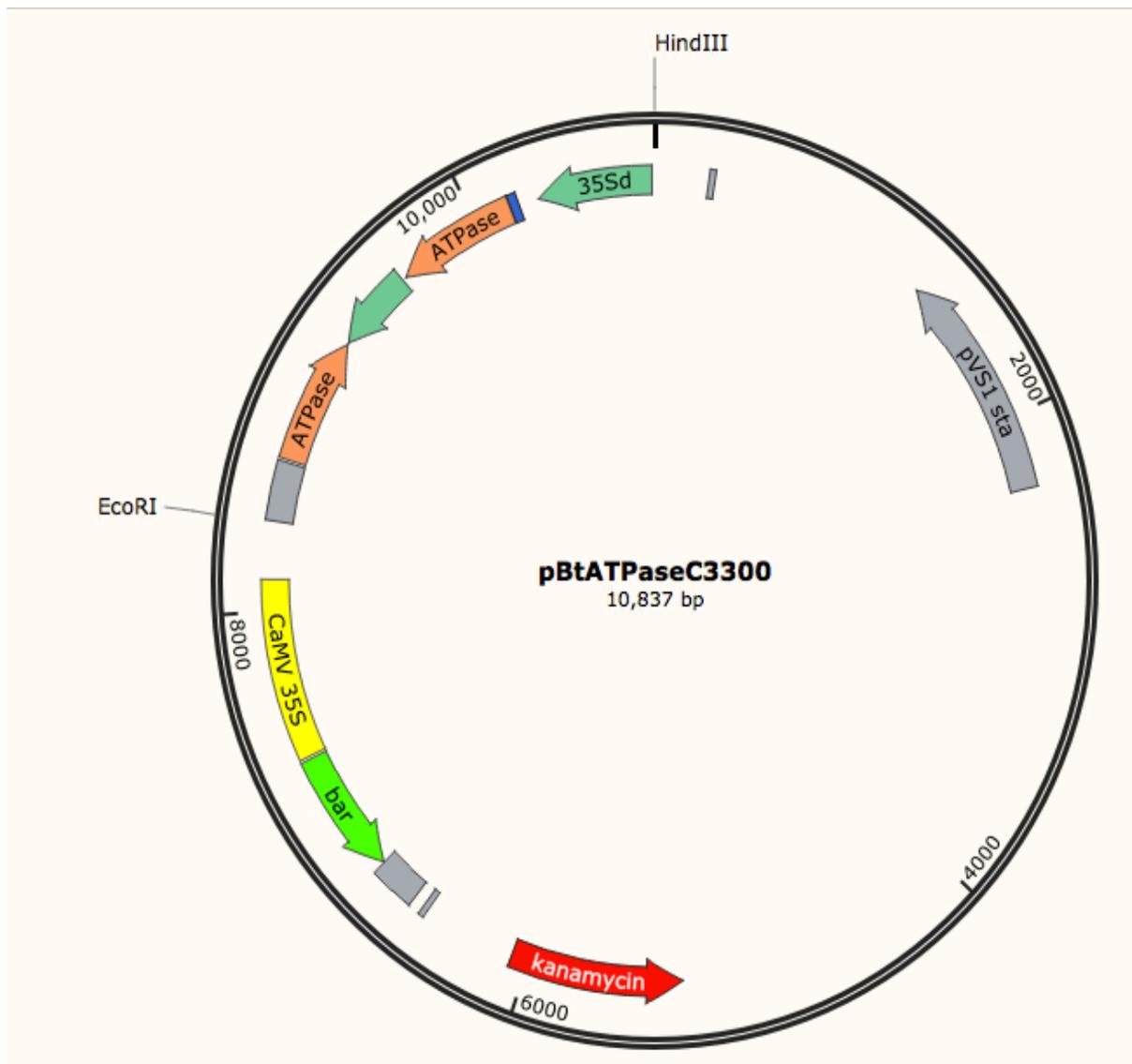
Figure 9. Engineering pBtATPaseC3300 vector. pBtATPaseC3300. Figure generated using SnapGene software

7.3 Generation of transgenic plants

A total of 6,400 cotyledons were subjected to transformation via *Agrobacterium* from which twenty five (25) independent lines of genetically modified lettuce plants were obtained (Table 3). Most of the lines generated were successfully acclimatized and grew to produce seeds (Figure 10 and Table 3). Some either died during acclimatization, aborted their seeds during flowering, met with laboratory accident or lost in green house (Table 3). The plants were screened by lateral flow tests, which allowed for the detection of PAT protein (Figure 11).
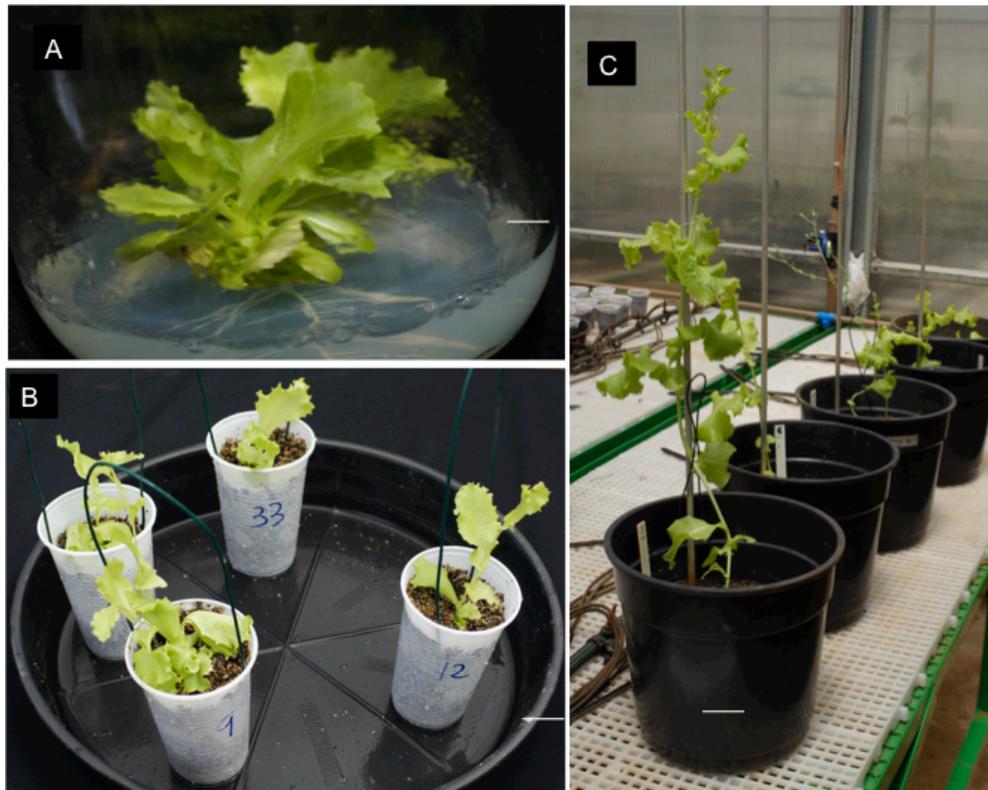
Figure 10. Generation of transgenic lettuce (*Lactuca sativa*) transformed to express *v-ATPase* siRNA. *In vitro* regeneration and rooting on selection media (A) allowed the acclimatization of the plants (B) which were subsequently transferred to vases for seed formation (C).

Confirmatory PCR analysis in T_0 and T_1 using the primers ATPSK/ATPXS led to the detection of the 576 bp probe corresponding to the *v-ATPase* region. This was further confirmed when the primer pair BarF/BarR was used, which allowed for the detection of a 408 bp probe which detected the presence of *bar* gene (Figure 12).

The trait was passed to T_1 generation in a Mendelian fashion in most of the first lines to produce seeds. Based on this analysis, PCR positive individuals from lines 1, 3, 4, 6, 19, 25 and 31 were selected for bioassay and further molecular analysis.
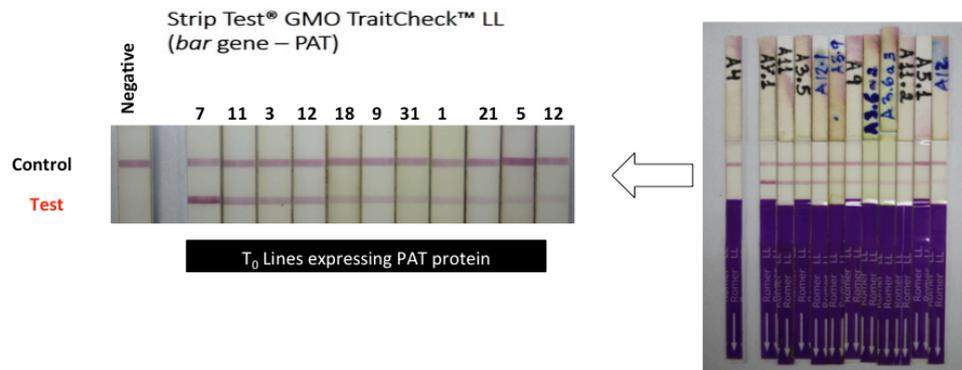
Figure 11. Lateral flow test for the detection of *bar* gene in transgenic lettuce plants. Up to 25 transgenic lines were generated. Numbers on the left panel denote line numbers as presented in Table 3.
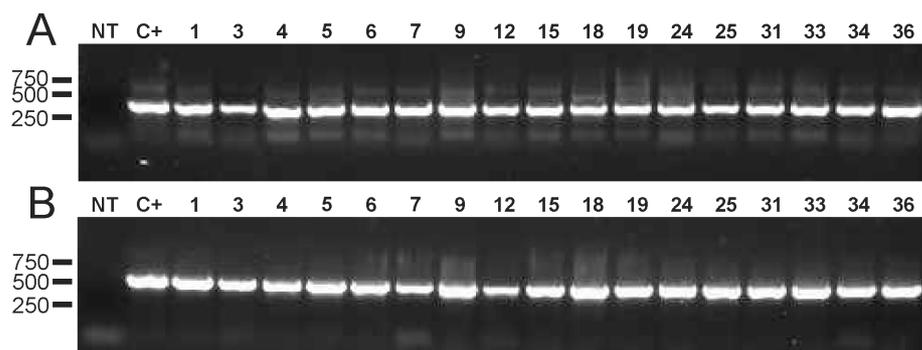
Figure 12. PCR analysis of transgenic lettuce transformed with pBtATPaseC3300 vector. PCR of T_0 lines 1, 3, 4, 5, 6, 9, 12, 15,18,19, 24, 25, 31, 33, 34 and 36 using the primer pair BarF/BarR allowed for the detection of a 408 bp probe corresponding to the bar region (A) while the primer pair ATPSK/ATPXS led to the detection of the 576 bp probe corresponding to the *v-ATPase* region (B).

Table 3 Summary of 25 transgenic lettuce lines generated by transformation with *A. tumefaciens.*

Line	Analysis			Remark/observation
	FlowStrip	**T_0 PCR**	**T_1 PCR**	
1	+	+	+	T_1 used in bioassay. Mendelian segregation observed
2	+	?	?	Died on acclimatization
3	+	+	+	T_1 used in bioassay. Mendelian segregation observed
4	+	+	+	T_1 used in bioassay. Mendelian segregation observed
5	+	+	?	Line lost in screen house No 42C of Embrapa Cenargen
6	+	+	+	T_1 used in bioassay. Mendelian segregation observed
7	+	+	+	Only 7 T_1 seeds were produced and when planted, only 2 germinated, of which 1 was PCR positive to *ATPase*
8	+	?	?	Died by contamination *in vitro*
9	+	+	+	T_1 germinated
10	+	?	?	Failed to root and died
11	?	?	?	Failed to root
12	+	+	+	T_1 germinated
13	+	?	?	Died on acclimatization
14	+	+	?	Seeds collected
15	+	?	?	Aborted flower
18	+	+	+	Seeds generated
19	+	+	+	T_1 used in bioassay. Mendelian segregation observed
22	+	+	+	No Mendelian segregation observed
24	+	+	+	Seeds produced
25	+	+	+	T_1 used in bioassay. Mendelian segregation observed
30	+			Aborted flower
31	+	+	+	T_1 used in bioassay. Mendelian segregation observed
33	+	+	?	Seeds being collected
34	+	+	?	Seeds collected
36	+	+	?	Seeds collected

+ Positive by FlowStrip test or PCR - Negative by FlowStrip test or PCR

7.3.1 Progeny analysis

Pearson's Chi-squared test and Yate's correction for continuity were used to analyze the segregation pattern of the insert in 7 of the T_1 lines used in the bioassay (Table 4). The result showed that they all inherited the insert in a Mendelian fashion.

Table 4 Segregation analyses of self-fertilized transgenic lettuce plants in T_1 generation

Line	T_1 generation [a]		Segregation	X^2	P [b]
	Positive	Negative			
1	28	7	3:1	0.25	0.61
3	16	4	3:1	0.46*	0.6*
4	23	12	3:1	1.6	0.2
6	23	10	3:1	0.49	0.48
19	18	12	3:1	3.6	0.57
25	25	10	3:1	0.23	0.62
31	12	6	3:1	0.66	0.41

a data based on PCR analysis of T_1

b P is the probability that the observed ratios reflect the expected segregation ratio

* Yate's correction for continuity was used to analyze the segregation pattern

7.9 siRNAs of v-ATPase detected in 7 lines of transgenic lettuce plants by Northern blot analysis

Seven of the transgenic plants (T_1) were used to isolate total RNA from which Northern blot analysis was carried out to detect the transgene-derived siRNA in leaves of the 7 transgenic lines used in the bioassay and a non-transgenic plant (Nt) (Figure 13). The analysis revealed siRNA bands of expected size range in all transgenic plants with more intense signals observed in lines 1, 4 19 and 31 in comparison to other lines.
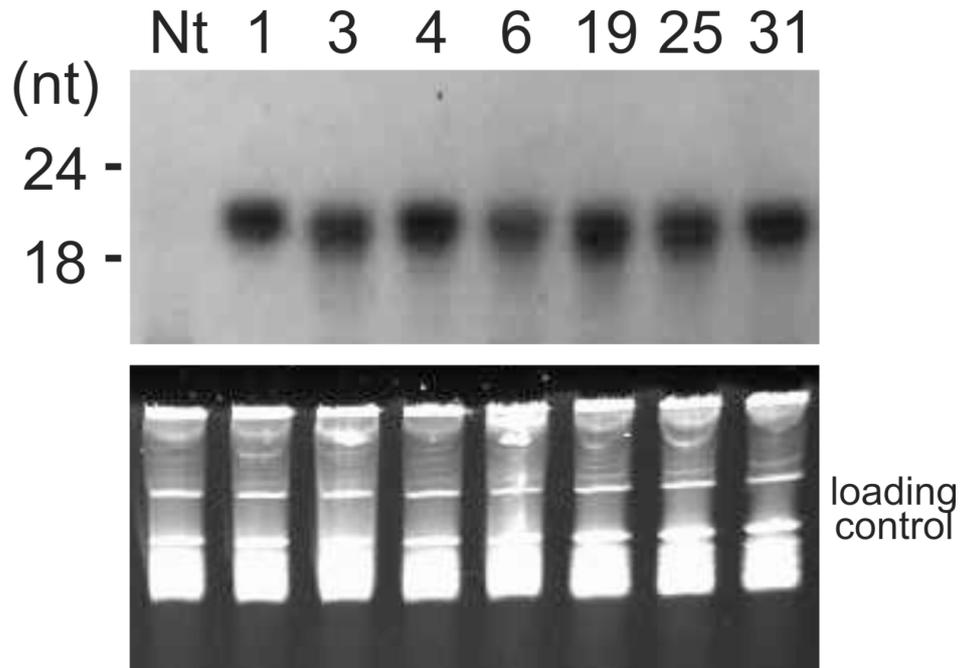
Figure 13. Northern blot analysis of small interfering (si) RNA isolated from intron-hairpin RNA-transformed lettuce plants. RNA blots were hybridized with a probe amplified using ATPXS/ATPSK primers to detect the presence of siRNA molecules corresponding to the v-ATPase fragment. SYBR-stained RNA serves as the loading control. Control is a non-transgenic plant (Nt)

7.4 Developing method for monitoring whitefly population

Because it was expected that any adult death due to silencing of *v-ATPase* would occur within the first few days after release, four different systems (designated A, B, C and D) of culturing whiteflies in tubes, conceived and designed to evaluate the mortality of the flies were tested on non-transgenic lettuce plants. This was done by releasing 50 whiteflies in bottles or tubes containing potted lettuce plants and their mortality monitored over a period of at least, 7 days. Systems A and B comprised of glass bottles with partially perforated tops covered with sieves differing in size and area of coverture. Systems C and D comprised of plastic jars with C having the top sealed with a sieve secured by rubber band while D was covered with a corresponding plastic cover with a perforation at the top, which was sealed with cotton wool. Following initial adaptation of 24 hours, the population of the flies stabilized over 4 days until death by natural cause started to occur by the 5th day. This was seen in all the four systems. This baseline study showed that system D

presented higher and more stable survival rate of the flies (Figure 14). It comprised of the use of plastic jar 15cm in depth, with an upper diameter of 9.5cm and lower diameter of 6.5cm. Based on its superior performance, system D was therefore selected for use in both whitefly toxicity assay and for pooling insects for further analyses.
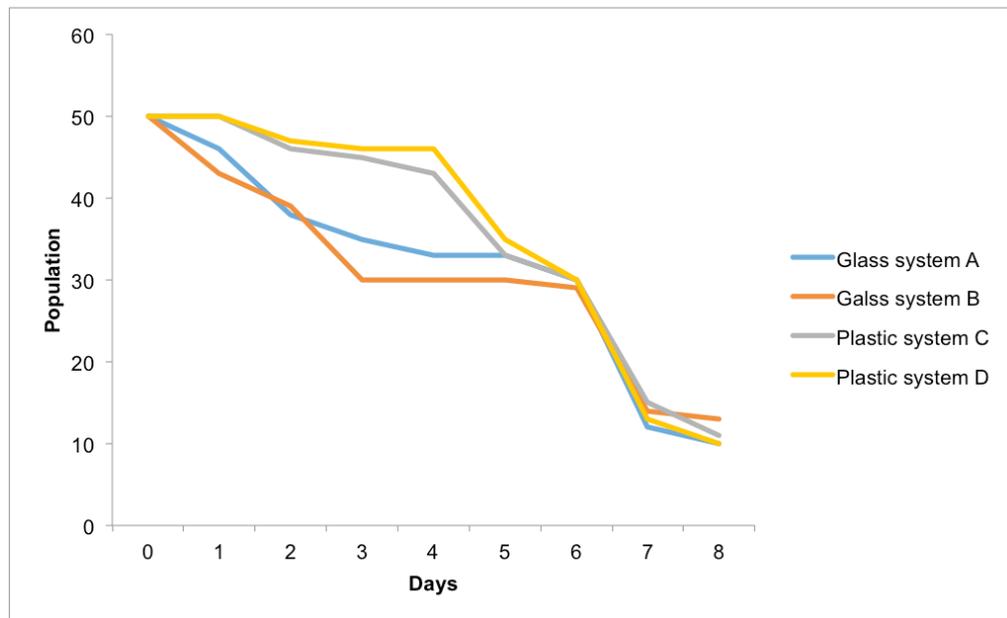
Figure 14. Base line study for monitoring whiteflies population. Mortality of fifty flies feeding on non-transgenic lettuce (*Lactuca sativa*) using 4 different systems was monitored. Systems A and B were made of glass jars with sieve and plastic coverings respectively while systems C and D were made with plastic jars both with plastic cover, with D having special perforation and cotton wool.

7.5. Transgenic lettuce plants expressing *v-ATPase* siRNA induce high mortality in whitefly

Feeding experiment in which transgenic lettuce plants expressing *v-ATPase* siRNA were challenged with newly emerged adult whiteflies and their mortality monitored over a period of 32 days was done. Within the first three days, there was a sharp decrease in the number of whiteflies feeding on all transgenic lines (Figure 15). This decrease was significantly slower in the two controls used. On day five, several flies were visible on control plants while there were only a few on transgenic lines (Figure 16). Whiteflies fed with transgenic plants presented statistically significant higher mortality rate when compared to insects fed with non-transgenic plants and transgenic plants expressing a marker gene (P< 0.05). Death by

ingestion of the siRNA from the leaves appeared to start from the first three days of feeding when more than 50% of the earlier introduced adults was dead, where as only 5% of the population was reduced in control plants, representing a 75% mortality rate (Figure 15). For example, on day three, when there were between 15.25 and 17 insects (corresponding to 76.25 – 85%) on control plants, there were between 6 and 10.92 (30-54.6%) insects on the test plants (Table 5). This may be translated to a mortality of between 56.4% and 70% in transgenic plants within the first three days. On day five, this numerical relation stood at 11.25 and 11.33 insects (56.25 – 56.65 %) in control plants and 1.92 and 3.25 (9.6 – 16.25%) in transgenic plants, corresponding to a mortality of between 83.75 and 98.08% on test plants respectively. By the tenth day, there was near zero number of whiteflies in all the transgenic plants expressing *v-ATPase* siRNA while the control plants still had a little below 5% of the original population.

Table 5: Number of whiteflies feeding on lettuce over 11 days. Numbers are mean values of 12 repetitions. Detailed table with standard deviation is presented as index

Line	Day											
	0	1	2	3	4	5	6	7	8	9	10	11
*Control-	20	19.33	18.33	17	14.08	11.25	7.83	5	3.25	2.33	1.58	0.83
*Control	20	19.25	18.17	15.25	14.58	11.33	7.67	4.33	3	2	1.2	0.67
Line 1	20	11.66	10.75	9.42	5.33	2.16	1.17	0.33	0	0	0	0
Line 3	20	11.83	9.58	6	4.42	2.08	0.33	0	0	0	0	0
Line 4	20	13	10.58	7.16	4.83	3.25	3.08	1.42	0.58	0.25	0	0
Line 6	20	12.66	10.58	8.58	5.16	3.08	1.5	0.17	0	0	0	0
Line 19	20	14.83	11.5	8.42	5.08	2.83	1.33	0.66	0	0	0	0
Line 25	20	14.24	10.92	8.75	4.33	1.92	1.33	0.83	0	0	0	0
Line 31	20	14	10.67	7.08	5.42	2.42	1.17	0.08	0	0	0	0

*Control- (non-transgenic plants), *control (plants expressing bar gene)
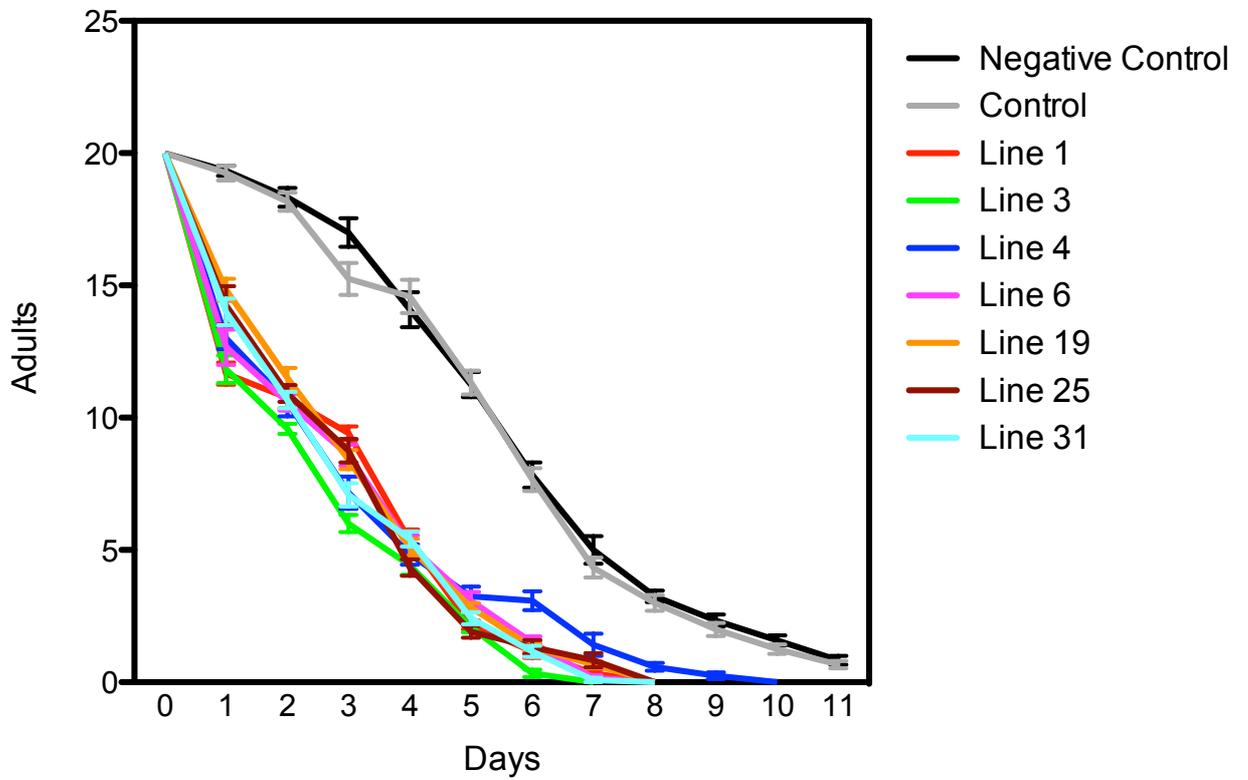
Figure 15. Toxicity of transgenic lettuce plants producing *v-ATPase* siRNAs against 20 whiteflies inoculated and monitored over 11 days. All v-ATPase transgenic plants (Lines 1, 3, 4, 6, 19, 25 and 31) induced significantly (P < 0.05) higher mortality than internal control (plants expressing bar gene) and negative control (non-transgenic plants), within the first 5 days of infestation. Analysis was done using Tukey test with Prism software version 5.0 at P < 0.05 from mean values of 12 repetitions
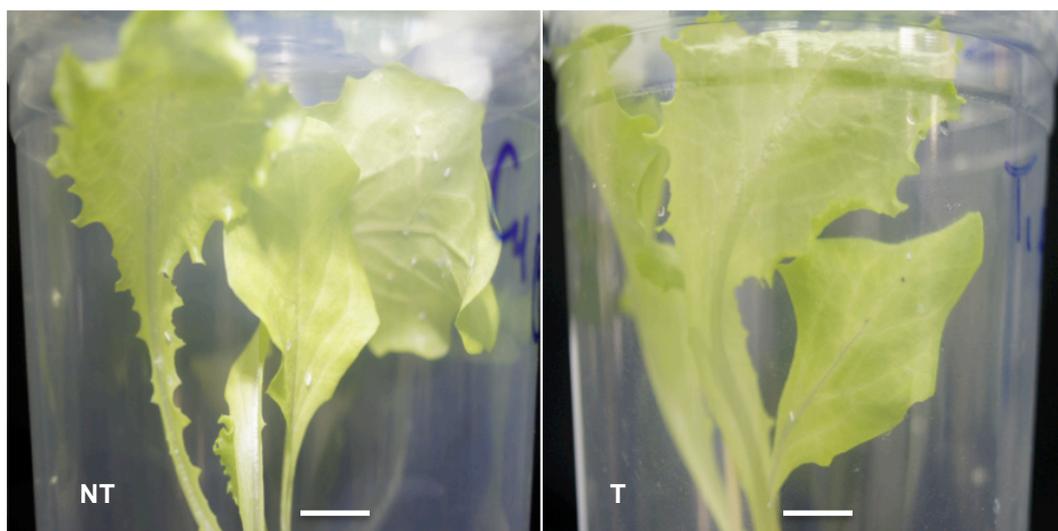
Figure 16. Monitoring the population of whiteflies feeding on non-transgenic (NT) and transgenic (T) lettuce plants. The figure shows higher number of whiteflies on NT than T on day 5 after inoculation with 20 whiteflies. Bar = 1cm

7.6. *V-ATPase* siRNAs in transgenic lettuce promote low oviposition and delay pupation in whitefly

The cycle of the whiteflies feeding on lettuce plants was evaluated to monitor the emergence of eggs, crawlers, pupa and adults. Generally, lower number of all stages was recorded in flies feeding on test plants than control plants. Monitoring Oviposition by 20 whiteflies feeding on transgenic lettuce plants showed that flies on test plants produced lower number of eggs than both controls (P < 0.05). On the 12th day (day 1 of this count) after inoculation, the 20 whiteflies deposited between 227 to 231 eggs (Figure 17) and produced between 107 to 125 crawlers (Figure 18) in control plants, the transgenic plants produced between 24 to 66 eggs and and between 13 to 52 crawlers. The number of eggs fell in all lines and control along the 32 days of analysis. However, in the control plants, this fall appeared to be sharper within the first nine days from the counting of eggs. It then slowed down and fell sharply by the 17th day, when it suddenly started to rise again. This new rise correlates with the increasing number of the emerging adults (Figure 20). In the test plants however, this fall was slower (Figure 17). Although lines 3 and 31 appear to stabilize the number of eggs over the first week, they all tended to converge along

with the remaining lines to show a steep fall by day 9 through day 13. In addition, several eggs appeared to be aborted. Although this abortion was observed in both transgenic and non-transgenic plants, it was not clear if it was higher in the former.

The emergence of crawlers followed similar pattern as deposition of eggs, with the number of crawlers being much lower (Figure 18). The difference between the control and test lines in terms of the population of nymphs becomes very clear when we consider the fact that day 1 is the peak day for their production. Whereas the control lines recorded between 107.41 and 124.91 crawlers, the highest number of crawlers generated on the transgenic lines on this day was 48 (Line 4) with the lowest being 13.92 (Line 6).

Both controls produced pupa earlier than and at higher rates than the test plants (Figure 19). On day 5, between 4.9 and 6.2 pupa emerged on control plants whereas there was no evidence of that emergence on any of the transgenic lines. The peak day for the emergence of pupa on both control and tests plants was day 13. However, whereas there were between 23.75 and 24.42 on control, the highest number of pupa from transgenic plants was 15.05 (Line 4) with the lowest (2) being common to Lines 6 and 19.

New adults started to emerge by the 18th day after infestation in all the control plants. However, this emergence was delayed by at least 2 days in most of the transgenic systems monitored (Figure 20). While the peak for the emergence of adults for all control plants was on day 17 (with 11.33 to 13.33 flies on the day), the peak for test plants was on day 13 with the highest number of adult being 1.94. From then on, adult emergence diminished in all transgenic lines. This puts emergence on transgenic line at a rate of 78% lower than in control. At any given day, there were more flies per leaf of each of the control plants than in the transgenic lines. While counting of adult flies was performed at interval of four days (Figure 21), sometimes-emerging adults died before they were counted. Carcasses of the flies were clearly visible at different stages over a period of 32 days (Figure 22).

Again, at the level of eggs, several aborted eggs were observable. It is not clear if the feeding triggered the systemic RNAi pathway and was responsible for this abortion or indeed the molecules acted at different stages of the development of the

whitefly, but what became apparent was that the death of emerging whiteflies often took place 3 days after it started to feed on the crop.
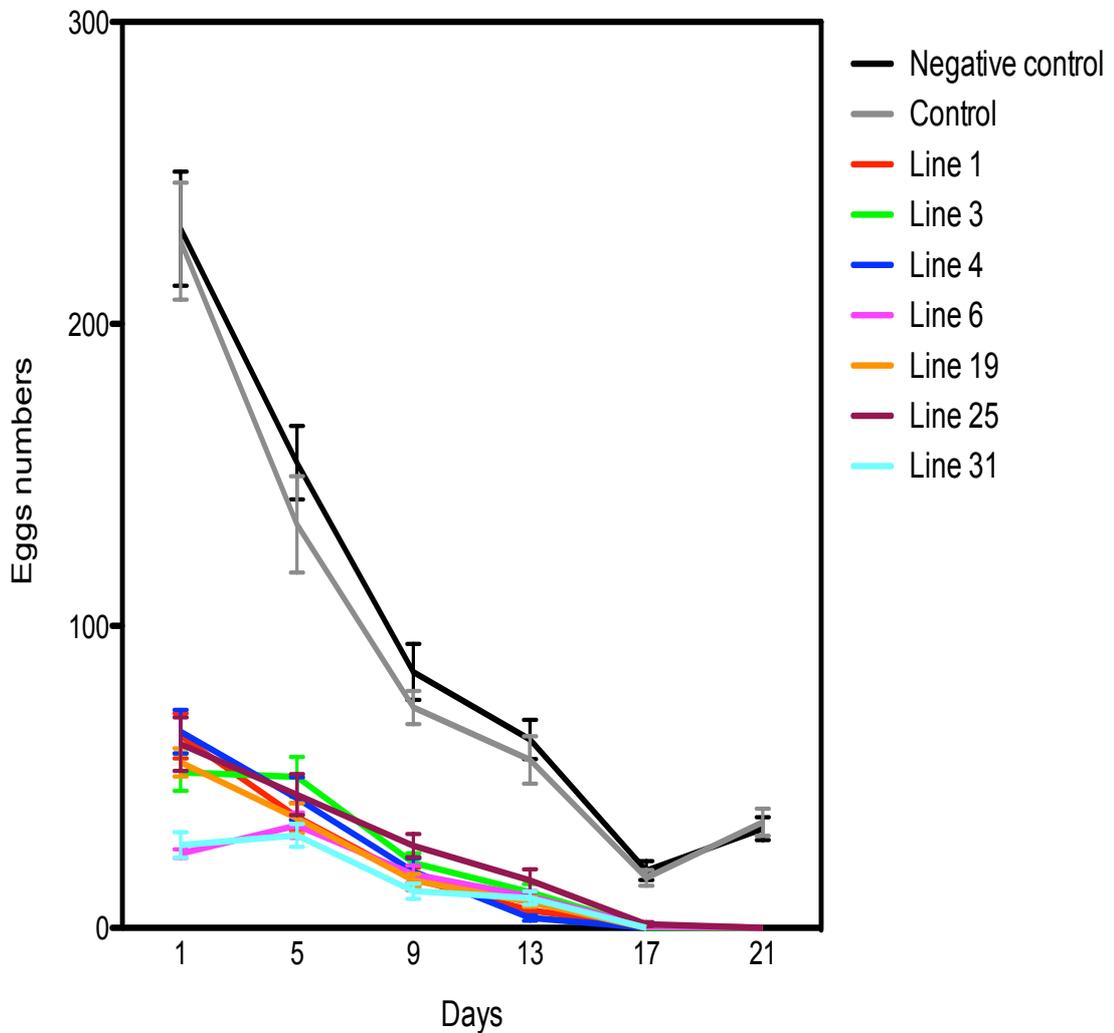
Figure 17. Oviposition by 20 whiteflies feeding on transgenic lettuce (*Lactuca sativa*) plants producing *v-ATPase* siRNAs (Lines 1, 3, 4, 6, 19, 25 and 31), control (expressing *bar*) and negative control plants. Counting of eggs started 12 days after inoculation (in this figure, considered day 1). All test plants produced lower number of eggs than both controls. Analysis was done using Tukey test with Prism software version 5.0 at $P < 0.05$ from mean values of 12 repetitions
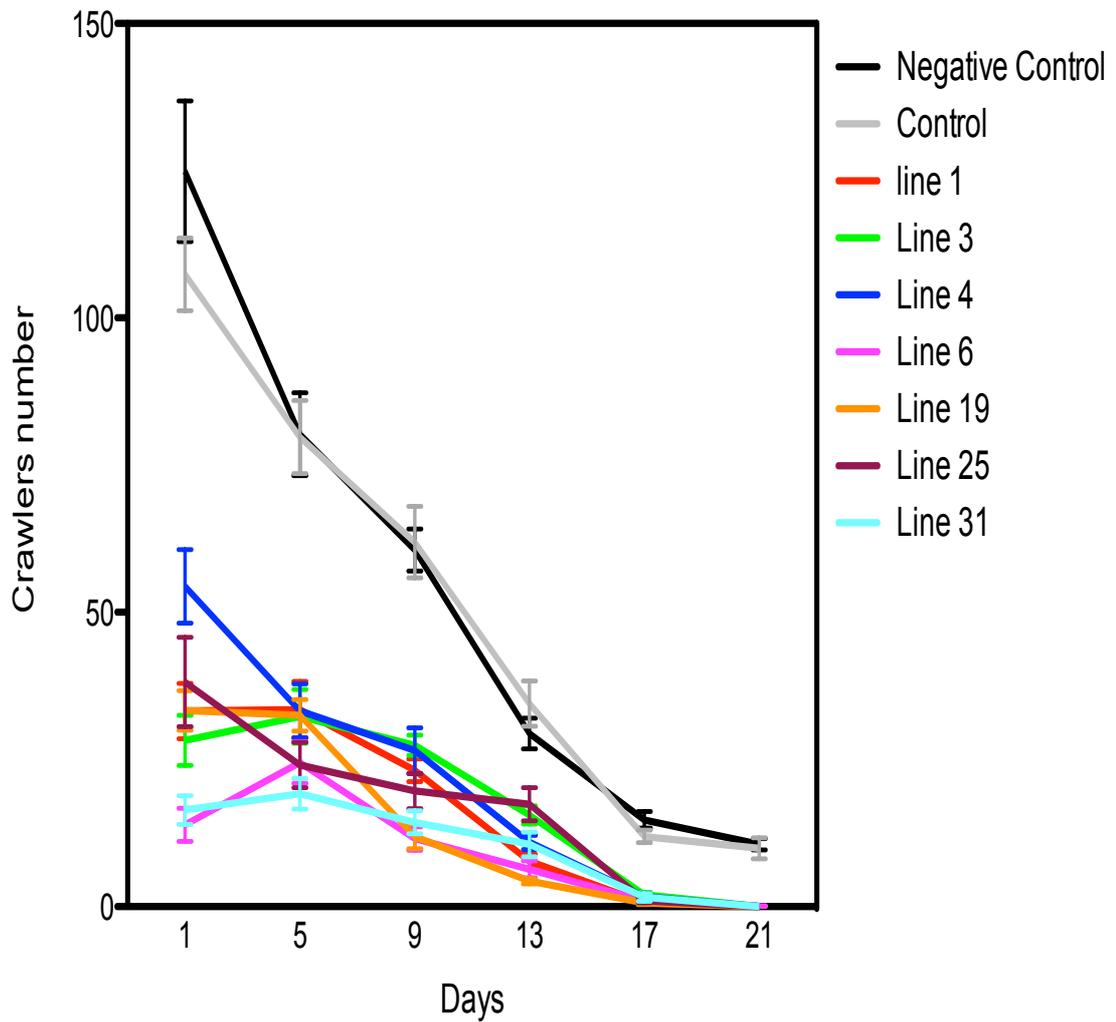
Figure 18. Emergence of crawlers (nymphs) from eggs of 20 whiteflies feeding on transgenic lettuce (*Lactuca sativa*) plants producing *v-ATPase* siRNAs (Lines 1, 3, 4, 6, 19, 25 and 31), control (expressing *bar*) and negative control plants. Counting of crawlers started 12 days after inoculation (in this figure, considered day 1). Both controls produced higher number of crawlers than test plants. Analysis was done using Tukey test with Prism software version 5.0 at P < 0.05 from mean values of 12 repetitions.
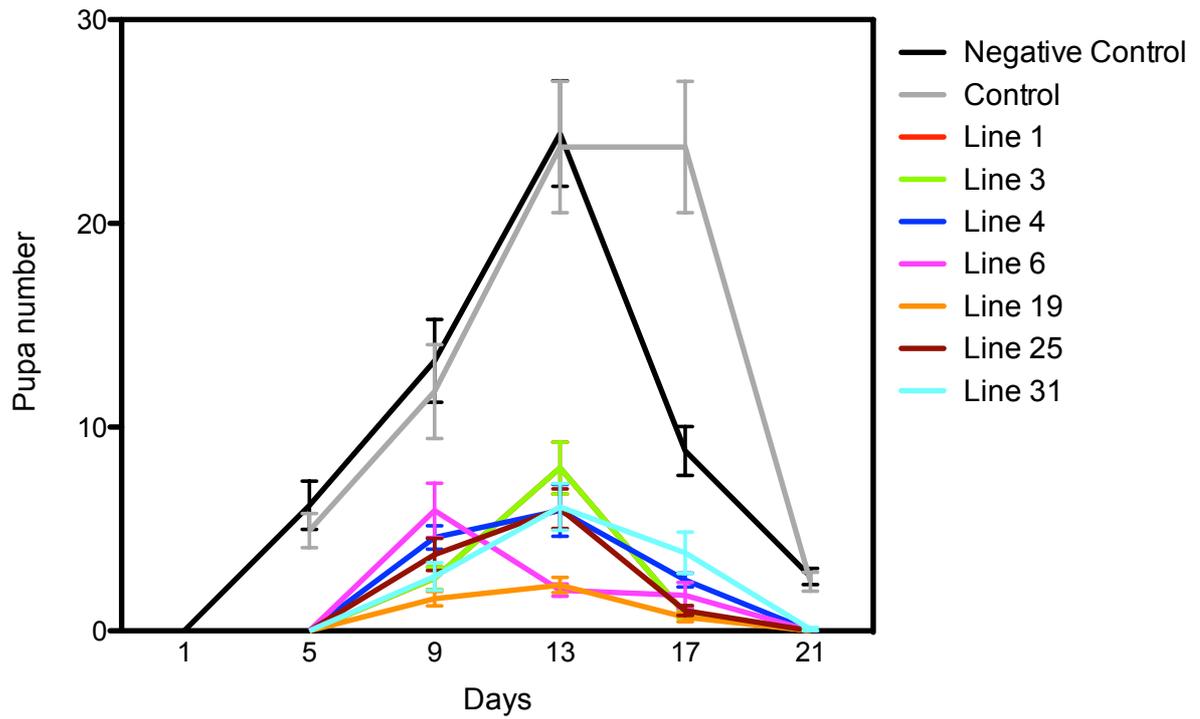
Fi**gure 19.** Emergence of pupa from eggs of 20 whiteflies feeding on transgenic lettuce (*Lactuca sativa*) plants producing v-ATPase siRNAs (Lines 1, 3, 4, 6, 19, 25 and 31), and on internal control (expressing *bar*) and negative control plants. Counting of pupa started 12 days after inoculation (in this figure, considered day 1). Both controls produced pupa earlier than test lines at higher rates than the test plants. Analysis was done using Tukey test with Prism software version 5.0 at $P < 0.05$ from mean values of 12 repetitions
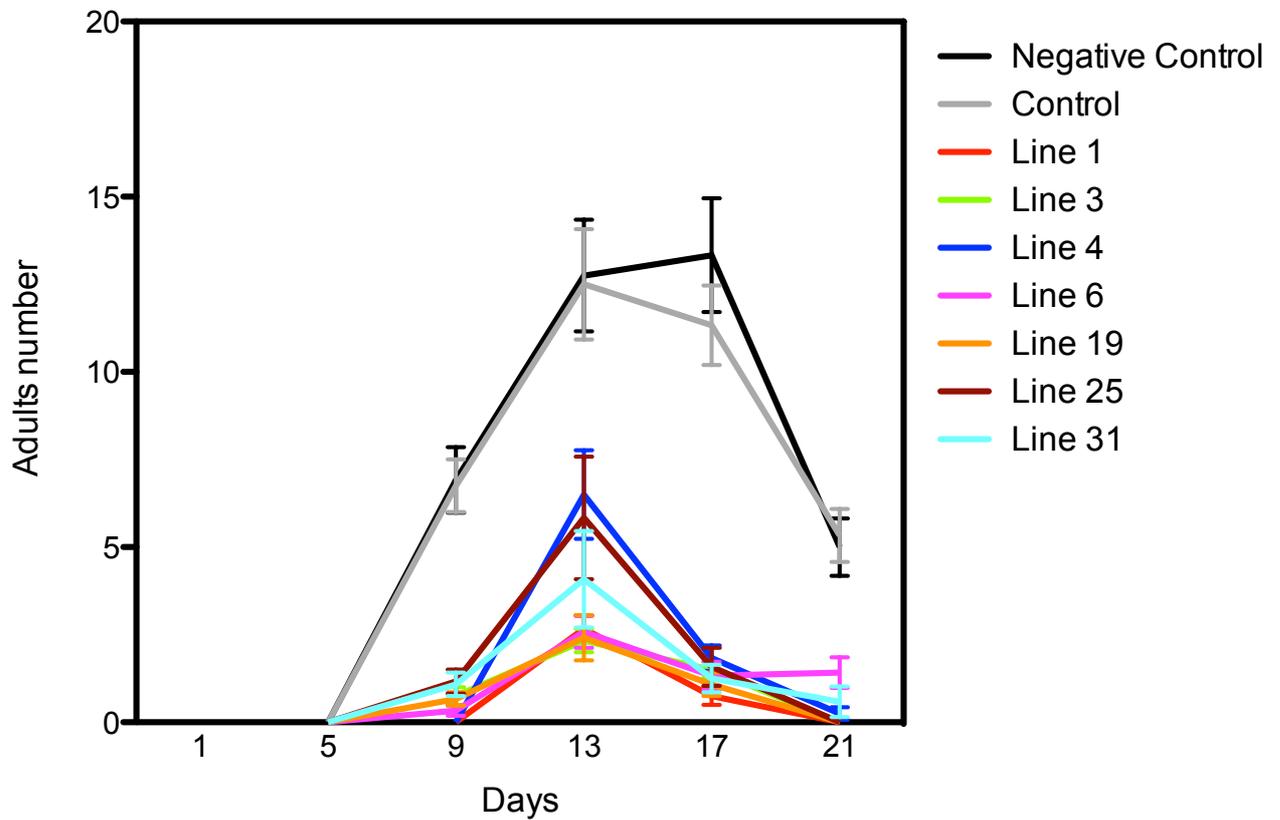
Figure 20. Emergence of adults from eggs of 20 whiteflies feeding on transgenic lettuce (*Lactuca sativa*) plants producing v-ATPase siRNAs (Lines 1, 3, 4, 6, 19, 25 and 31), and on internal control (expressing *bar*) and negative control plants. Adults started to emerge 12 days after inoculation (in this figure, considered day 5). Both controls produced higher number of flies than test lines at higher rates than the test plants. Analysis was done using Tukey test with Prism software version 5.0 at P < 0.05 from mean values of 12 repetitions.
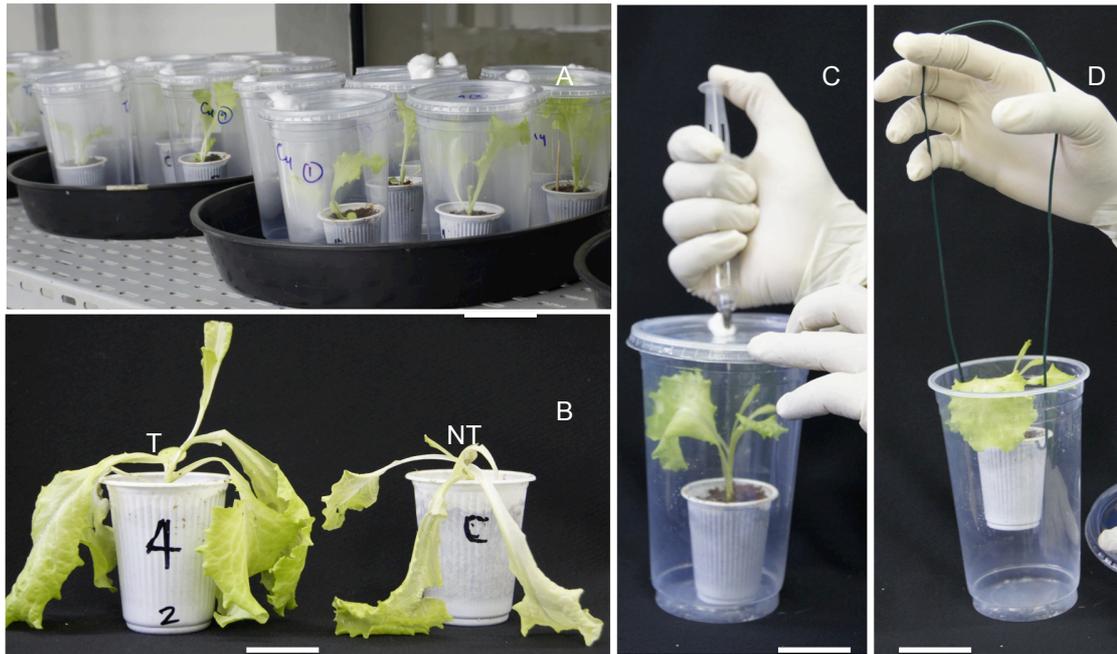
Figure 21 Whitefly toxicity assay using *L. sativa*. Infested plants were kept in a 16h photoperiod and 25±2°C (A). By the end of a complete whitefly cycle (B), control plants (NT) showed higher sap loss and drooping than the transgenic plants (T). Plants were watered by means of hypodermic syringe (C) and removed for counting of eggs, crawlers, pupa and adults after every 4 days starting 11 days after inoculation. (Bar = 1cm)
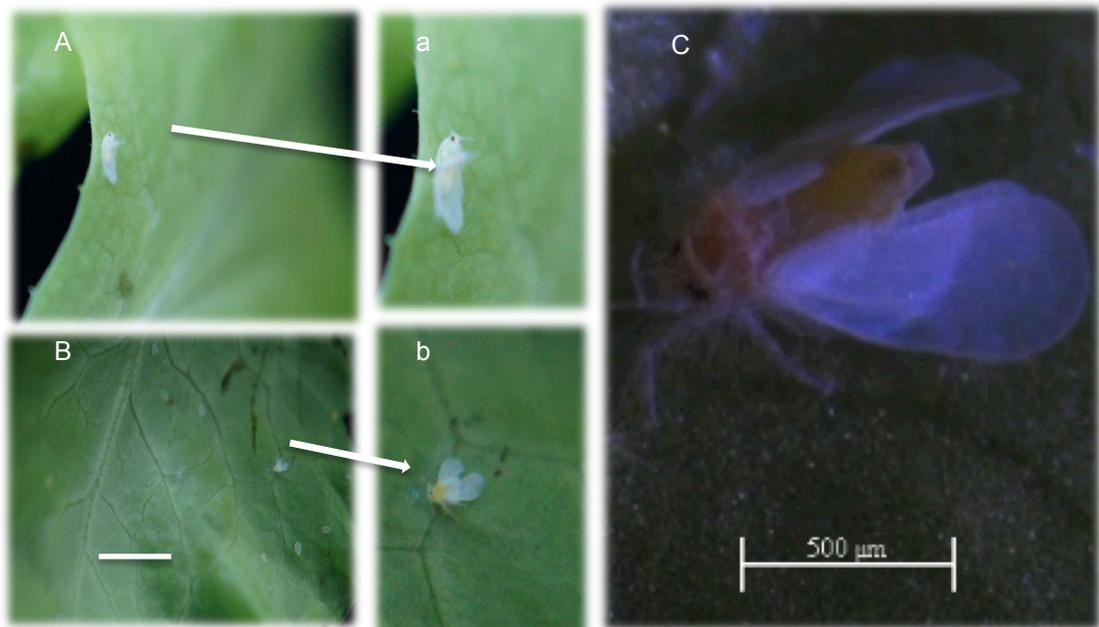
Figure 22 siRNA induced mortality on *Lactuca sativa*. Infestation of 20 whiteflies into a system containing transgenic plants leads to death of the flies within 3 to 5 days (A). As new adults emerge towards the end of a cycle of 32 days, death results within 3 days. Plates a and b are close up views of A and B respectively and plate C is an enlarged view of a dead fly from a different leaf. Bar = 1cm

7.11 *In silico* off-target effect analysis

Although RNAi is an excellent functional genomic tool used in silencing genes in sequence-specific manner, the specificity of siRNA derived from the process is not absolute. There is an increasing number of evidence that siRNA sequences may sometimes recognize and pair with other genes for which minimal sequence homology required for their activities exists, leading to silencing of genes for which their design was not intended. This phenomenon, known as off-target effect, has been shown in a number of experiments (Birmingham et al., 2006; Jackson et al., 2006; Scacheri et al., 2004; Ui-Tei et al., 2008). It is caused by base pairing of the siRNA incorporated in RISC at position 2 to 8 of 5' region with their complimentary 3'UTR region of compatible mRNA. These sequences are referred to as seed-sequences. Off-target effect clearly highlights issues in biosafety because of its obvious potential to silence non-target genes. It also underscores the need to ascertain the specificity of any siRNA/dsRNA designed for use in such technologies as the one described in this thesis. In order to avoid off-target effects, it is important to design and select siRNAs whose seed sequences are not complimentary to any region on the 3' UTR of non-target genes. This is sometimes problematic because it has been demonstrated that in every 16,384 bp, there is a chance of having 7 nucleotides with the ability to pair with seed sequences of siRNAs (Naito and Ui-Tei, 2013; Naito et al., 2009). The availability of bioinformatics tools that allow for screening nucleotides in designing siRNAs allows for *in vivo* prediction of off-target prone sequences (Naito and Ui-Tei, 2013). At the center of off-target effect is the thermodynamic stability of the duplex formed when seed sequence of the siRNA pair with the guide sequence of corresponding mRNAs (Naito et al., 2009). The annealing temperature in the formation of this duplex determines the off-target effect directed by the seed sequence (Figure 23) In one of the analyses conducted using the siRNA forming sequence of this thesis, a T_m below 21.5 $^{\circ}$C served as a reliable parameter in ruling out the possibility of off-target effect. Based on this, the dsBtAPase RNA generated in this work may be considered highly specific with minimal off-target potential. *In silico* analysis of the BtATPase siRNA coding sequence using siDirect algorithm (Ui-Tei et al., 2008) returned only one sequence with a possible off-target potential in *Homo sapiens* and *Rattus norvegicus* genomes.

Considering that this sequence has seed-duplex stability with T_m of 19.2 ^{o}C and 19.1 ^{o}C for both guide and passenger siRNA, it poses no potential threat (Figure 22). In addition, analysis using DSIR (Vert et al., 2006) showed 8 siRNAs, none of which has off target effect in HumanRefSeq and MouseRefSeq (Figure 22). This was confirmed by using siRNA at Whitehead (Yuan et al., 2004), DEQOR (Henschel et al., 2004).

Figure 23 Search for possible off-target siRNA molecules generated by *L. sativa* transformed with pBtATPaseC3300, with the potential to silence genes in *Homo sapiens* genome. (A) Only one siRNA showed similarity with the sequence. Considering its T_m of below 21 ^{o}C, this siRNA presents no clear potential for off-target effect. (B) Using DSIR, all the 8 siRNA molecules generated present zero off-target effect in HumanRefSeq and MouseRefSeq.

7.12 Biosafety considerations

The establishment of biosafety regulations governing the use and consumption RNAi derived crops is an important component for their development (Petrick et al., 2013). The molecular mediators (dsRNA, siRNA and micro RNA) of engineering RNAi crops have been a source of concern to many stakeholders and experts. These concerns must not be dismissed given the potential for off-target effects of some of the siRNAs that may be generated by a given GM crop. Although it has been reported that rice miRNAs acquired orally from food was found in the serum and tissues of animals (L. Zhang et al., 2012), attempts to confirm these results (Y. Zhang et al., 2012) have failed miserably, because no measurable uptake of any rice miRNAs, including miR168a could be detected in mice (Dickinson et al., 2013; L. Zhang et al., 2012). This was further confirmed by other researchers showing that no evidence of any major plant-derived miRNA accumulation in animal samples could be detected (Chen et al., 2013). Attempts to develop drugs using siRNAs have also faced substantial challenges associated with biological barriers that limit oral absorption (Burnett and Rossi, 2012; Forbes and Peppas, 2012; O'Neill et al., 2011).

At any rate, double stranded RNAs have been shown to occur naturally in foods, indicating that humans have a history of consuming them. It has been demonstrated that endogenous dsRNAs in several plants species, such as lettuce, tomato, corn, soybean and rice, have sequence complementarity to human genes (Jensen et al., 2013). Many of these complementary long dsRNAs have perfect sequence complementarity of at least 21 nucleotides to human genes. From a total of more than 8 million long dsRNAs predicted in corn, soybean, rice, lettuce and tomato for example, 38,682 (0.5%) had complementarity with human gene transcripts. Some of the small RNAs would have enough complementarity to potentially trigger off-target effects in human cells. In addition, some of these genes encode for proteins, such as pantothenate kinase 1, TATA box binding protein, cytochrome P450 protein and insulin receptor substrate 2, which are critical for important biological functions. Current data, along with a history of safe consumption of such plant-derived foods, support the conclusion that consumption of dsRNAs present in food does not present any threat to human health. The high safety margin

for any small fraction of RNAs that might be absorbed following consumption of RNAi derived crops by mammals suggest that such crops are as safe for animal and human consumption as any other non-transgenic crop (Ibrahim and Aragão, 2015).

8. DISCUSSION

The emergence of strategies that employ RNAi in the control of insects, facilitated in part, by the deciphering of its molecular mechanism in *D. melanogaster* mutants, signal revolutionary agricultural boosts in productivity and crop management. Experiments on insect resistance in both model plants and crops using RNAi have demonstrated that the strategy is effective in both *in vitro* and *in vivo* studies (Baum et al., 2007; Mao et al., 2007; Thakur et al., 2014; Upadhyay et al., 2011). While it has been applied in several insects, there are only a few of such application in whitefly. This scarcity appears to be due to limited genomic information about whitefly (Leshkowitz et al., 2006). Although there are a number of initiatives for sequencing whitefly genome (Edwards and Papanicolaou, 2012; Wang et al., 2010), most of these initiatives stop short of making sequences of candidate genes available in the public domain for access and use in RNAi based research. Even with the 2011 launching of the i5k initiative, which sought to sequence the genomes of 5000 insect and related arthropod species ("i5K - ArthropodBase wiki," n.d.), including whitefly, this scarcity has persisted. Understandably, this may be due to patent deposition because of the obvious economic potential of the approach. Although patent disputes on RNAi have not emerged yet, they may eventually do so with the development of the first commercially available RNAi-insect based product (Schmidt, 2007). However, this has greatly limited the number of research works that directly employ the use of gene silencing approaches in the control of whitefly. To date, the number of peer-reviewed and publically available publications that have successfully reported silencing one gene or another in *B tabaci,* either through transgenic plants, in the form of diet or by siRNA injection, stands at 6 (Asokan et al., 2015; Chen et al., 2015; M Ghanim et al., 2007; Luan et al., 2013; Thakur et al., 2014; Upadhyay et al., 2011). This seems curious considering the great economic importance of the insect, given the fact that as early as 1990's, *B tabaci* had wrecked

havoc on crops with losses recorded in billions of dollars (Brown et al., 1995).

The impetus for the development of whitefly resistant plants derives from early reports showing that siRNAs can effectively serve as toxins, interfering with the physiology of insects leading to impaired growth of larvae, disruption of feeding habit and ultimately death (Baum et al., 2007; Mao et al., 2007). In the pioneering work of Baum et al., (2007), 14 genes were identified and knocked out using low concentrations of dsRNA leading to death of WCR larvae. With the demonstration that tissue-specific mRNAs of different genes of whitefly could be preferentially silenced by up to 70%, a critical point was reached in the history of whitefly research (Ghanim et al., 2007). However, any method that seeks to control whitefly via RNAi must take into cognizance, the medium of delivery of siRNAs and/or dsRNAs. Since it is impossible to inject every whitefly in the field, oral delivery and uptake of dsRNA in the gut became imperative (Upadhyay et al., 2011). Indeed, the use of this approach led to significant mortality of whiteflies based on artificial diet prepared from dsRNA corresponding to five different genes including *v-ATPase*. Although the above research reported similarity in pattern of toxicity of siRNA diets based on the 5 genes used, dsRNA from A subunit of *ATPase* was found to be most effective with a mortality of more than 80% whiteflies over 6 days. In this thesis, a 75% insect mortality within 5 days of feeding on transgenic lettuce expressing *v-ATPase* siRNA was observed (Figure 21). It may be argued that this result is an improvement on the all the reports so far on silencing of *v-ATPase* gene. Considering the fact that the method developed in this study does not only dispense with the preparation of artificial diets containing siRNA/dsRNA, it also eliminates the necessity of monitoring mortality based on the use of complex population dynamics in which so many parameters are out of control. The visibility of deaths caused by the transgenic plants is clear although it may have been due to natural causes in some cases (Figure 19). The effect of siRNA *v-ATPase* in a bioassay employing the use of leaf disc from transgenic tobacco showed mortality rate of between 15 and 38% on day 2 and between 34 and 85% on day 6 following infestation (Thakur et al., 2014). Feeding experiment using dsRNAs that target genes within the molting hormone-ecdysone synthesis and signaling pathway showed low survival and delayed development of whitefly during nymphal stages (Luan et al., 2013). An alternative strategy in which glutathione synthase transferase (GST), (an enzyme known to detoxify insecticides)

may be silenced by siRNAs designed to block its synthesis is quite attractive. Indeed, it has recently been demonstrated that dsRNAs synthesized based on *B. tabaci* GST and fed to the insect in dietary form, resulted in a mortality rate ranging from 40 to 77.43% over a period of 72 hours (Asokan et al., 2015).

Although the methods employed in evaluating the effect of siRNA on mortality of whiteflies by Upadhyay et al., (2011) and Thakur et al., (2014) are different from that used in this study, insect mortality in the three studies fall within the range of 75-85%. This mortality was recorded in the first ten days of insect release irrespective of number of whiteflies inoculated or the bioassay system used. The method developed in this thesis may be considered superior to the two studies above on two grounds: (1) For the first time, RNAi based strategy that employs transgenic crop was used to silence *v-ATPase*; (2) The bioassay system using simple, cheap and reproducible materials, without the need to neither synthesize siRNA/dsRNA for use in feeding experiment nor prepare diets for the same purpose, was developed (Figure 18).

A key factor in monitoring the mortality of whiteflies as developed in this method is the heterogeneity of the freshly emerged adults. While care was taken in introducing relatively synchronized and only emerging adult whiteflies of approximately the same age in to the bioassay system, some factors may have interfered with the overall toxicity of the siRNAs. For example, physiological barriers, behavioral factors and geographical orientation of individual whiteflies may have influenced the overall toxic effect of the siRNA consumed by the insects. In this study, in almost all of the bioassay units, the whiteflies tended to colonize the lower leaf of the host plant with the remaining leaves left uncolonized. With their deaths and subsequent emergence of crawlers and new adults, they migrated to the youngest available leaf, to repeat the cycle all over again. At any rate, this observation may be useful in future possible experiments in which individual leaves may be used as baits in a similar bioassay or diet-based analysis to allow for control that is more stringent and monitoring of the whitefly cycle. One scenario would be the introduction of cotton leaf side-by-side a dsRNA expressing lettuce plant or leaf.

The presence of several enzymes in insect gut, including v-ATPase, may have determined the toxicity of the siRNAs as well. For example, the siRNAs may be degraded in the gut before they could exert their toxic effect since there are

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detoxifying nucleases that may reduce their availability for gene silencing (Katoch and Thakur, 2012). While *in situ* studies are required to study the cellular location and physiological effect(s) of the siRNAs derived in this study, the pattern of death, which often took place by the third day of contact with lettuce plants, suggests that the silencing may be in *B. tabaci* gut and is most observable at the adult stage within the first three days of feeding. Clearly, the dramatic silencing effect leading to death of insects is intimately related to the transgenic plants' ability to express hairpin RNAs that target gut enzymes like v-ATPase at rates that are faster than they can be diced by the insect (Gordon and Waterhouse, 2007). This dosage and time dependent effect may explain why some of the whiteflies made it to the next generation unharmed. It will be interesting to establish an siRNA profile of the entire lines generated in this study in order to study the level of the molecules within a large population of individuals from the different lines. In addition, microscopic analysis of the insect gut within the first few days of feeding may churn out valuable physiological information on the effect of the siRNAs produced by the transgenic plants. Experiments targeting acetycholinesterase (AchE) gene in *H. armigera* using dietary siRNAs, reported reduction in larval growth, pupal weight loss and malformation and death (Kumar et al., 2009). Similarly, a HIGS-based approach was used to express TLR7 dsRNA of whitefly in recombinant *Isaria fumosorossea* on *Hibiscu rosasinensis*, knocking down the gene, leading to mortality of over 90% in nymphs (Chen et al., 2015). At the level of gene expression, decrease in target mRNA levels was reported in *Epiphyas postvittana* (Turner et al., 2006) and *Tribolium castaneum* (Tomoyasu et al., 2008). A 62% decrease in the expression level of ATPase RNA was reported following feeding with transgenic tobacco plants (Thakur *et al.,* 2014).

How soon RNAi technology may emerge as a an industrial-scale crop protection strategy against sap-sucking hemipteran pests such as aphids, leafhoppers and whiteflies, may ultimately depend on the speed with which approaches as described in this study are optimized (Price and Gatehouse 2008, Upadhyay *et al.*, 2011). Crucial optimization parameters include such vital components as siRNA delivery system as well as the enhancement of the tissue accumulation of miRNA, siRNA and dsRNA in sieve tubes by designing transformation vectors with phloem specific promoters (Buhtz et al., 2008; Kehr and

Buhtz, 2008; Pant et al., 2008; Zhang et al., 2013). Additionally, new candidate genes need to be discovered and their amenability to the approach tested.

9. CONCLUSION

In this works, 25 transgenic lines of lettuce expressing siRNAs derived from *v-ATPase* have been generated and screened. Northern analysis showed the presence of siRNA specific to *v-ATPase* hairpin forming sequence in 7 of the lines. When subjected to bioassay, these lines had protective effect against whitefly by interfering with its life cycle through induction of high mortality, reduced oviposition and delayed pupa to adult conversion. The method developed in this study may serve as a proof of concept for RNAi mediated silencing of genes for the control of whitefly and may form the basis for a field trial with the ultimate aim of breeding elite lines of the crop for protection against whitefly and other sap-sucking insects, contributing in the coming of new era in insect pest management.

10. PERSPECTIVES

The following lines of research may be pursued from this work:

1. Adoption of similar transformation and evaluation system in other economic crops like soybean, tomato and cotton.
2. *In situ* analysis of the toxic effect siRNA from lettuce in whitefly.
3. Population quantification of siRNAs from different lines generated for screening and use in field experiments.

11. Transgenic soybean lines were protected from whitefly by v-ATPase siRNA

Before the work on lettuce as reported in this thesis, soybean (*Glycine max*) was used as a host plant for the control of whitefly. Using the vector pBtATPase, soybean embryos were bombarded according to Rech *et al.* (2008). Following selection in baby-food jars containing 15 ml of the culture medium supplemented with 300nM of imazapry, emerging shoots were selected until fully grown. They were then acclimatized and maintained in a greenhouse. A total of nine (9) transgenic soybean lines designated 1, 2, 3, 4, 5, 6, 7, 8 and 11 were generated. All T_0 generation from each of the lines were confirmed to be positive by PCR. However, these lines did not adhere to the Mendelian law of inheritance. In all the lines, the insert was lost along the progenies, only to reappear in some of the individuals. Because line 4, 8 and 11 appeared to show higher detection level by PCR, individual plants that were confirmed to be positive from T_2 generation of these lines were selected to perform a preliminary bioassay with whiteflies. The inoculation of 20 whiteflies on these plants showed that they were resistant to the insect over a period of 40 days (Figure 24). This is demonstrated by lower number of adult whiteflies arising from nymphs, which had previously originated from the release of 20 whiteflies in caged transgenic plants as against the higher number of both nymphs and adult flies observed in non-transgenic plants. Indeed, transgenic line 11 appeared to show no sign of having been infested by whitefly even after 60 days (Figure 25). The results from this analysis however cannot stand statistical test because only one control was used. Although T_2 progenies from line 11 was used to repeat a similar bioassay at Embrapa Arroz e Feijão, and results from that analysis also showed that the plants were indeed protected from *B. tabaci*, the analysis could not be continued due to the inability to detect the v-ATPase transgene by PCR in most of the offspring of the lines. While no conclusion may be drawn from these results, it will be interesting to carry on with the study on the silencing of whitefly *v-ATPase* in soybean by analyzing the several seeds generated in this earlier attempt as well as by designing new and optimized vectors specific to other crops.
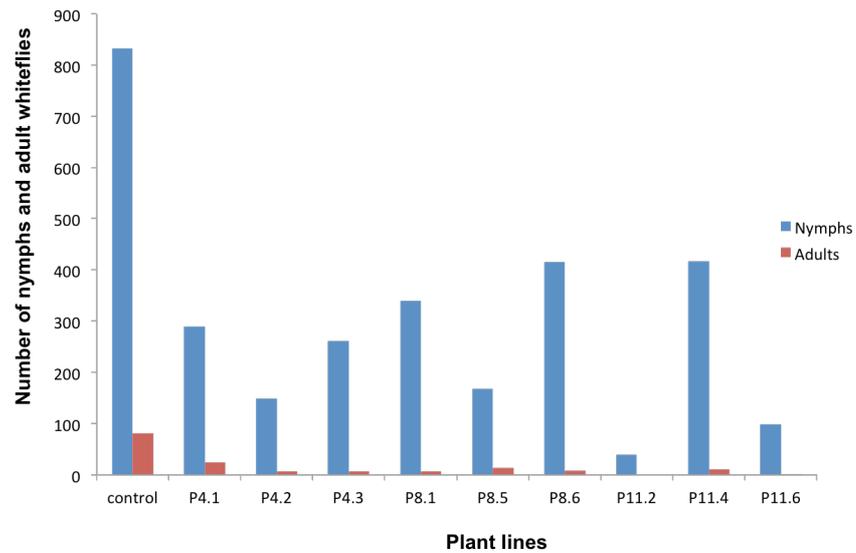
Figure 24 Whitefly toxicity assay using *Glycine max* transformed with pBtATPase. Twenty (20) whiteflies were released unto caged T_2 individual plants from lines 4 (4.1, 4.2 and 4.3), 8 (8.1, 8.5 and 8.6) and 11 (11.2, 11.4, 11.6). The number of nymphs per plant was counted after 20 days. Live adults on the leaves were counted after another 20 days. It is not clear if the transgenic status of the plants had anything to do with the differences in adults and nymphs between the control and the transgenic plants. No conclusion may be drawn from this observation since the control did not have sufficient repetitions. When line 11 was tested separately, similar observation was made.
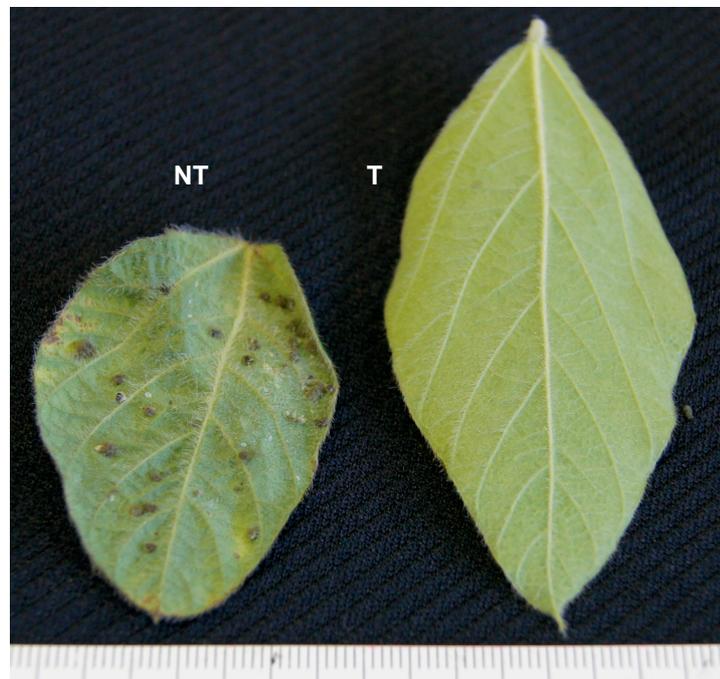
Figure 25. Morphological aspects of leaves from transgenic (T) soybean (*Glycine max*) plant, line 11, transformed with pBtATPase vector that targets the silencing of v-ATPase in whitefly and non transgenic (NT) plants. The figure is from leaves taken 60 days after the release 20 whiteflies onto caged plants for bioassay.

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Publications

Chapter 5

RNAi-Mediated Resistance to Viruses in Genetically Engineered Plants

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Abstract

RNA interference (RNAi) has emerged as a leading technology in designing genetically modified crops engineered to resist viral infection. The last decades have seen the development of a large number of crops whose inherent posttranscriptional gene silencing mechanism has been exploited to target essential viral genes through the production of dsRNA that triggers an endogenous RNA-induced silencing complex (RISC), leading to gene silencing in susceptible viruses conferring them with resistance even before the onset of infection. Selection and breeding events have allowed for establishing this highly important agronomic trait in diverse crops. With improved techniques and the availability of new data on genetic diversity among several viruses, significant progress is being made in engineering plants using RNAi with the release of a number of commercially available crops. Biosafety concerns with respect to consumption of RNAi crops, while relevant, have been addressed, given the fact that experimental evidence using miRNAs associated with the crops shows that they do not pose any health risk to humans and animals.

Key words Biosafety, Gene silencing, Genetic engineering, RNA interference, Virus resistance

1 Introduction

In spite of the great advances in plant disease management, global food production and supply continue to be threatened by a large number of pathogens and pests. Among these, viruses cause the most devastating biotic stresses that hamper the production capacity of plants. Because of their ability to rapidly multiply and spread across the same or different plant species using their formidable arsenals, viruses are difficult to control [1]. Besides direct transmission, viruses may also be transmitted via insect vector. Techniques developed in the postgenomic era are increasingly being deployed using RNA interference (RNAi) and have greatly enhanced crop protection approaches that address the problem of plant viruses. Several strategies have been employed to genetically engineer resistance to viruses in plants, i.e., through the expression of coat protein (CP) genes, expression of truncated or defective viral genes, and antisense RNA.

Plants naturally process viral RNAs, leading to the generation of small sequences of a pathogen's genetic material, which can specifically be used against that pathogen through a RISC [2]. It has long been recognized that an RNA-silencing (posttranscriptional gene silencing or PTGS) mechanism is responsible for resistance against RNA viruses; a response that depends on the formation of double-stranded RNA (dsRNA) whose antisense strand is complementary to the transcript of a targeted gene [3, 4]. This has allowed for the introduction of constructs in transgenic plants to generate intracellular short interfering RNA (siRNA)-like molecules which target and silence viral genes, thus conferring resistance against the virus.

RNA silencing has been an important tool in the development of plants resistant to a large number of both DNA and RNA plant viruses [5–9]. Although the frequency of resistance obtained using RNA sense- or antisense-mediated strategies may vary, these approaches have often resulted in a maximum resistance frequency of 20 %. In some cases, lower frequencies were obtained [10–12]. In addition, not all viral genes used in transgenic constructs rendered plants resistant. However, the use of inverted repeat constructs resulting in dsRNA transcripts has allowed for the development of a highly efficient system in which a much greater frequency of transformed plant lines exhibit gene knockdown or virus resistance [10, 13]. This is perhaps due to the fact that, in this approach, dsRNAs are fed directly into the silencing pathway at the level of the RNaseIII-like enzyme Dicer without relying on the action of plant-encoded, RNA-dependent RNA polymerase proteins.

1.1 From Pathogen-Derived Resistance to RNAi

Long before the discovery and description of RNAi, scientists reported on the development of transgenic plants expressing viral coat proteins which conferred resistance to infection by homologous viruses. In what was earlier dubbed pathogen-derived resistance (PDR), researchers observed that several plants could be induced to develop resistance by a pathogen's genetic material [14]. Gene suppression as a cellular mechanism through which foreign nucleic acids are recognized and destroyed by host cells was thus well established even before the elucidation of the RNAi pathway [15]. Such reports were presented on different viruses, including Tobamovirus, Carlavirus, and Potyvirus, among others [16–18]. One of the pioneer experiments that employed a gene-silencing strategy was reported with the demonstration that tobacco plants could be genetically engineered to exhibit resistance against viral diseases [16]. In the work, a chimeric gene containing the CP of *Tobacco mosaic virus* (TMV) was introduced into cells of *Nicotiana tabacum* via *Agrobacterium tumefaciens*. The plants regenerated from the transformed cells expressed the CP gene and when inoculated with TMV showed delayed development of symptoms. It was further demonstrated that between 10 and 60 % of the plants showed no symptoms at all. In another experiment, transgenic plants transformed to express a complementary RNA

sequence (antisense RNA) of the coat protein gene of TMV were protected when challenged with the virus [19]. It was also shown that accumulation of antisense RNA was responsible for this protection. Although these early experiments implicated the presence of viral nucleic acids resulting from transgene expression being responsible for the resistance, the cellular mechanism involved was not fully understood at that time. Attempts to test the hypothesis that viral proteins may have triggered such resistance showed that even partial sequences expressing truncated proteins and their noncoding regions, like satellite RNA sequences, could induce disease resistance [20]. Based on these observations, scientists concluded that the main factor responsible for this gene silencing was the RNA molecule itself. These attempts paved the way for the development of RNAi-mediated silencing strategies to improve agronomic traits that combat viral infection in plants [21].

2 RNAi and Virus Resistance

When the term "RNA world" was first used in 1999 to describe the evolution of life on Earth, many were unaware that such a world may well still be here and thriving [22]. The observation that many organisms, when exposed to foreign genetic materials, elicit a highly specific counterattack to silence invading nucleic acid sequences before they integrate into their genomes led to the discovery of RNA silencing or RNA interference. The phenomenon, known as cosuppression in plants, quelling in fungi, and RNAi in animals, is indeed as old as the RNA world itself. Today, we know that in addition to its role in the immune response, RNAi guides endogenous gene regulation in specific biochemical and physiological pathways that control plant development.

2.1 The RNAi Pathway

Central to this sequence-directed immunity is double-stranded RNA (dsRNA) whose role in the mechanism was initially difficult to establish, given its seemingly nonspecific nature and thermodynamic instability [22]. However, with the deciphering of the RNAi pathway, dsRNA has today been established as the molecule at the heart of this important cellular response. By means of a mechanism designated virus-induced gene silencing (VIGS), plants evolved RNA silencing machinery that targets and processes dsRNA derived from pathogens or hosts to generate siRNA molecules which are recruited to host RISCs that ultimately inhibit gene expression and protein translation in viral genomes. The posttranscriptional ability of RNAi machinery to specifically target and degrade cytoplasmic RNA is the key to its antiviral function. Evidence that RNAi is clearly involved in the antiviral response emerged from the observation that *Arabidopsis thaliana* strains that were defective in transcriptional gene silencing pathways were more susceptible to infection by viruses [23].

2.2 Naturally Occurring RNAi-Derived Virus Resistance

Several plants are resistant to viruses by virtue of an inherent dsRNA and siRNA generating system whose targets are gene sequences essential for viral pathogenicity [1]. For example, siRNA sharing 100 % similarity with distinct genetic and intragenic regions of *Mungbean yellow mosaic India virus* (MYMIV), a Begomovirus which causes yellow mosaic disease, have been observed in mungbean. In the resistant line PK416, siRNAs found correspond to an intragenic region (IR) of MYMIV, while in the susceptible lines, most of the siRNAs correspond to the genetic regions and are present in low concentrations. It was also demonstrated that the viral genomes in resistant plants were methylated in the intergenic regions [24]. Earlier, siRNAs of different begomoviruses have been characterized in transgenic *A. thaliana*, *N. benthamiana,* and cassava, and shown to elicit inherent gene silencing in these viruses [25]. The role of PTGS has also been demonstrated in *N. benthamiana* infected with *Cymbidium ringspot tombusvirus* (CymRSV) using RNAi [26]. Transgenic tobacco plants were also used to demonstrate PTGS of *Tomato mosaic virus* (ToMV) genes, with suppression of symptoms following the silencing of a replicase gene [27].

3   Development of Virus-Resistant Crops by RNAi

3.1 Early Attempts in Regenerating Virus-Resistant Crops

Although RNAi-mediated resistance to viruses is a natural phenomenon in plants, it is not effectively present in many productive plant lines because siRNA molecules identical to viral sequences usually appear at later stages of infection in some of these lines, when it is not stoichiometrically favorable to control the infection [28, 29]. However, by mimicking the mechanism using recombinant DNA technology to generate siRNAs, endogenous RISCs may be induced to trigger and confer resistance to plants against viruses even before the onset of infection.

There are several reports in which RNAi strategies have been used to generate plants resistant to viruses based on either RNA or DNA viral genomes [6, 7, 9, 30–32]. This has been successfully reported in common bean [31], tomato [33, 34], and cotton [35]. The fact that many plant viruses possess RNA genomes makes their use as potent RNAi inducers quite easy. This is further facilitated by the fact that the viruses may replicate through the formation of dsRNA intermediates. By their very nature, several plants actually evolved an antiviral system that capitalizes on this pathway, and this has allowed scientists to develop transgenic plants using dsRNA derived from viruses. Transgenic plants have been engineered to silence a diverse group of viral proteins such as movement protein, viral suppressors of RNA silencing, replication-associated protein, and nuclear inclusion proteins [36].

Tobacco plants resistant to *Tobacco mosaic virus* (TMV) [37] and papaya resistant to *Papaya ringspot virus* (PRSV) have been in cultivation since 1998. To date, PRSV resistance is one of the most successfully established resistances in fruit crops [38]. Another equally successful induction of viral resistance has been reported in *Plum poxvirus* (PPV). The generation of cherry rootstocks with a high degree of resistance against *Prunus necrotic ring spot virus* (PNRSV) using RNAi technology has recently been reported [39]. Other commercially available crops in the U.S. include pumpkins resistant to *Watermelon mosaic virus* (WMV), *Zucchini mosaic virus* (ZYMV), and *Cucumber mosaic virus* (CMV), and virus-resistant potatoes [21]. A virus-resistant potato expressing both sense and antisense transcripts of viral helper component proteinase (HCPro) of *Potato virus Υ* (PVY) was developed and has been shown to present complete immunity [39]. This laid the background for the development of a resistant potato using dsRNA derived from the coat protein of PVY, which was further extended against *Potato virus X* [40]. In another experiment, tomato plants resistant to Potato spindle viroid were generated using RNA hairpin [41].

3.2 RNAi-Based Transgenic Approaches for Developing Virus Resistance

One of the first deliberate attempts at transformation to express a dsRNA (construct harboring intron-hairpin—hpRNA) was reported in 2000 using wheat in which gene sequences of a polymerase from *Barley yellow dwarf virus* (BYDV) were expressed. Plant lines arising from this were immune to the virus as confirmed by ELISA [42]. Using a transgene designed to produce hairpin-containing BYDV-PAV sequences, researchers generated up to nine lines of virus-resistant barley, two of which demonstrated Mendelian inheritance of the transgene whose presence consistently correlated with immunity against the virus. Shortly thereafter, transgenic tobacco plants expressing sense and antisense RNAs of DNA-A of *Cotton leaf curl virus* (CLCuV DNA A) and DNA-B of CLCuV were generated. The siRNAs of DNA-A inhibited viral replication while those of DNA-B conferred resistance against CLCuV to the plants [43]. In addition, researchers generated tomato plants with resistance against *Tomato yellow leaf curl Sardinia virus* (TYLCSV) using RNA hairpin constructs containing the truncated *rep* protein gene of TYLCSV [44].

3.3 Application in Model Plants

The development of transgenic *N. benthamiana* plants expressing the coat protein gene of *Sweet potato feathery mottle virus* (SPFMV) allowed for the elucidation of the role of RNAi mediated by RNA-dependent RNA polymerase (RdRp), which conferred resistance against the virus to the plant. Indeed, the RNAi signal was shown to be transmitted to a nontransgenic plant that had been grafted with a transgenic plant [45]. Similarly, RNAi was used to generate transgenic *N. benthamiana* that was resistant to *Chickpea chlorotic dwarf Pakistan virus* (CpCDPKV), a Mastrevirus that affects chickpea and other legumes [46].

3.4 Success Stories in Legumes

In leguminous plants, dsRNA construct was used to silence the promoter sequence of DNA-A of *Vigna mungo yellow mosaic virus* (VMYMV), leading to the expression of dsRNA of a conserved region of VMYMV in *Vigna* spp. and resulting in resistance against viral infection [47]. Similarly, RNAi has been applied using a viral *AC1* gene sequence encoding a multifunctional protein (Rep) of the *Bean golden mosaic virus* (BGMV) to generate a transgenic common bean (*Phaseolus vulgaris* L.) resistant to geminivirus [31]. The choice of this viral gene (*AC1* or *Rep*) for construction of the transformation vector was based on the fact that Rep protein plays an essential role in the viral infection cycle and that it is the only protein required for replication. The vector used was constructed from a DNA fragment of 411 bp of the *AC1* gene of BGMV. This resulted in the development of an event now known as Embrapa 5.1, the first transgenic line approved for commercial use following biosafety regulations set by the Brazilian technical biosafety commission (CTNBio) (Fig. 1) [21]. This strategy can also be applied to combat other devastating diseases such as geminivirus attacking maize and cassava in Africa, and tomato worldwide.

A comparative experiment was reported on RNAi-engineered soybean used to enhance resistance against the geminivirus *Mungbean yellow mosaic virus* (MYMV) [24]. In a more recent study, cowpea (*Vigna unguiculata*) plants were also engineered with an intron-hairpin construct to silence the proteinase cofactor gene from *Cowpea severe mosaic virus* (CPSMV) and the coat protein gene from *Cowpea aphid-borne mosaic virus* (CABMV). The resulting cowpea transgenic lines presented enhanced resistance to both viruses. However, northern blot analyses were carried out to detect the transgene-derived siRNA in leaves and revealed no correlation between siRNA levels and virus resistance. In addition, in the symptomless resistant lines, the resistance was homozygosis dependent. Only homozygous plants remained uninfected while hemizygous plants presented milder symptoms [48].

Cassava plants engineered using dsRNA derived from *African cassava mosaic virus* (ACMV) have also been generated, leading to the development of cassava lines expressing *AC1* siRNA that interrupts the Rep/AC1 function during viral replication, thus conferring resistance to the plants [49].

Researchers recently developed a plant virus vector-based *in planta* system by using recombinant TMV-containing sequences from *Bactericera cockerelli* to be used in a screening strategy for conferring resistance in tomato and tobacco [50]. What is remarkable in this experiment is the fact that a recombinant virus was used to silence genes in *Bactericera cockerelli* nymphs in a feeding experiment.
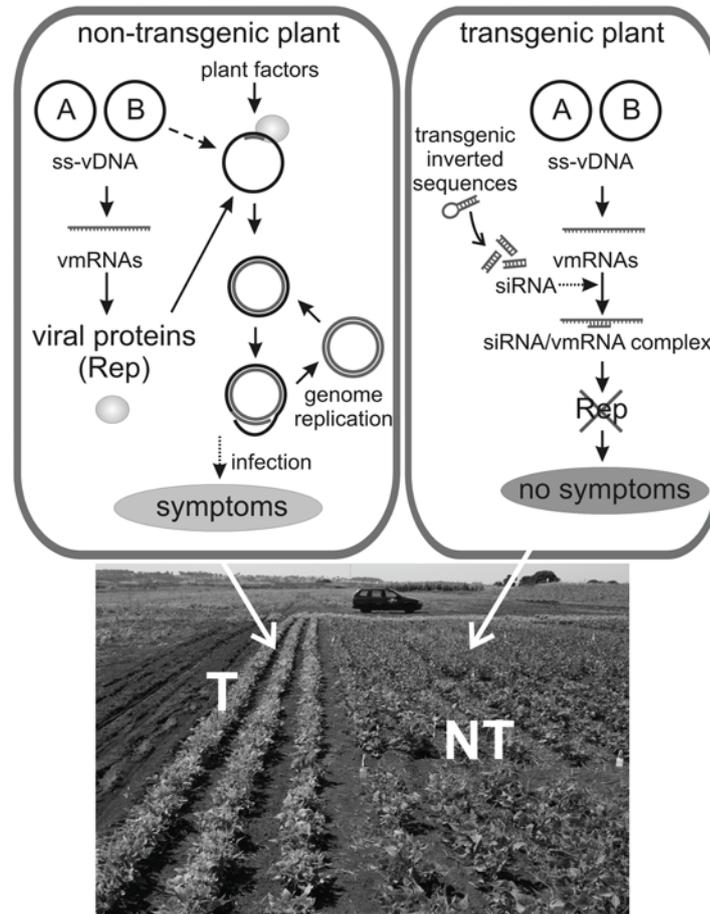
Fig. 1 Mechanism of the RNAi-engineered resistance to the *Bean golden mosaic virus* (BGMV) in transgenic *Phaseolus vulgaris*. Geminiviruses encode a Rep protein, the replication initiator protein, which is essential for viral genome replication. Rep is required, along with factors produced by the host plant, for initiation and termination of rolling-circle viral DNA replication. Transgenic common bean plants were generated to express a long dsRNA (hairpin), which is converted into shorter 21–25 base-length sequences (siRNA) to specifically silence the viral *rep* gene. Without the Rep protein, there is no virus DNA replication and no symptom appearance. These siRNAs are being expressed constitutively, meaning the virus infecting the first cells shuts down before it has a chance to get expression going. Under field conditions, transgenic plants (T) presented immunity against BGMV while nontransgenic plants (NT) showed typical virus symptoms, with yellow-green mosaic leaves and stunted growth

3.5 Designing Vectors for RNAi-Mediated Transformation

A drawback to using RNA-mediated virus resistance in transgenic crops is the high level of sequence specificity. Viruses containing 10 % nucleotide divergence are insensitive to this form of resistance. Indeed, viruses with this level of divergence are generally considered different species when designing a strategy for RNAi [8].

To overcome this problem, an alternative is to express transgenes of different viruses or different genes of a given virus. For example, the full-length coat protein gene of *Turnip mosaic virus* (TuMV) has been linked to 218-bp N gene segments from *Tomato spotted wilt virus* (TSPV) and transformed into *N. benthamiana*. A large proportion (4 of 18 %) of transgenic lines were found to be resistant to both viruses, and this resistance was transferred to the second generation [18]. A more impressive work was reported in which *N. benthamiana* was transformed with an RNA hairpin construct containing four 150-bp consecutive fragments of the N gene of four tospoviruses (*Tomato spotted wilt virus*, *Groundnut ringspot virus*, *Tomato chlorotic spot virus,* and *Watermelon silver mottle virus*) [11]. It was demonstrated that this construction was capable of rendering up to 82 % of the transformed plant lines heritably resistant against all four viruses [11]. In a remarkable experiment, researchers in China and the USA transformed soybean plants using a single transgene that expressed three separate hairpins specific to three different viruses, thus conferring robust RNAi-based resistance to mixed infection of the three viruses [51]. Using inverted repeats (IRs) containing highly specific sequences of 150 bp from AMV, BPMV, and SMV, a transgene was assembled in a 35S controlled vector to generate three lines of transgenic soybean that exhibited systemic resistance to the simultaneous infection of the three viruses.

4   Biosafety Considerations

A key component for the development of RNAi-derived crops, including those engineered to exhibit viral resistance, is the establishment of biosafety regulations governing their consumption [52]. Stakeholders have often raised questions about their safety assessment with particular emphasis on the molecular elements involved in generating these crops (dsRNA, siRNA, and micro RNA). Although it was shown that plant miRNAs acquired orally from food could be found in the serum and tissues of animals [53], progress in the development of siRNA-based drugs has been hampered by substantial challenges associated with biological barriers that limit oral absorption [54–56]. Indeed, attempts to confirm the results obtained in the oral experiment with miRNAi [53] have failed because no measurable uptake of any rice miRNAs, including miR168a, could be detected in mice [57]. It has additionally been reported that no evidence of any major plant-derived miRNA accumulation in animal samples could be detected [58].

Double-stranded RNAs have been shown to occur naturally in foods; thus, humans apparently have a history of consuming them. Reports have demonstrated that endogenous dsRNAs in several plant species, such as lettuce, tomato, corn, soybean, and rice, have

sequence complementarity to human genes [59]. Many of these complementary long dsRNAs have perfect sequence complementarity of at least 21 nucleotides to human genes. From a total of more than eight million long dsRNAs predicted in corn, soy, rice, lettuce, and tomato, 38,682 (0.5 %) had complementarity with human gene transcripts [59]. Some of the small RNAs would have enough complementarity to potentially trigger gene silencing in targeted human cells. In addition, some of these genes encode for proteins, such as pantothenate kinase 1, TATA box binding protein, cytochrome P450 protein, and insulin receptor substrate 2, which are critical for important biological functions. Current data, along with a history of safe consumption of such plant-derived foods, support a conclusion that consumption of dsRNAs present in food does not adversely affect human health. The high safety margin for any small fraction of RNAs that might be absorbed following consumption of RNAi-derived crops by mammals suggests that such crops are as safe for animal and human consumption as any other nontransgenic crop.

5   Conclusions

In recent years, old and emerging plant viruses have continued to pose a serious threat to food production while their effective control remains nearly impossible. In some regions, the only means of managing plant diseases caused by virus is the adoption of the so-called sanitary gap, in which an entire planting season is skipped in a given period when vectors known to transmit such viruses are most active, with its attendant economic losses. RNAi has therefore been increasingly adopted as a powerful new tool to target viruses due to its ability to control pathogens. Results from model plants and the development of a number of commercially available crops designed using the technique are a clear indication that RNAi can be highly effective in combating agricultural problems arising from viruses [21]. Advances in the genomic era, which permit for screening candidate sequences in different viruses, have allowed for the successful generation of the number of genetically modified plants derived using RNAi technology.

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11 RNA Interference

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Introduction

The second half of the 20th century brought with it great advances in biology, which allowed scientists to better understand biochemical pathways in living organisms and develop methods of manipulating these pathways with a high degree of precision in attempts to either unveil the mysteries behind such pathways or address biological problems for optimal utilization of natural resources in agriculture, health, and industry. In agriculture, as in other sectors, the development of recombinant DNA technology in the 1970s ushered in a new era and the emergence of genomic and proteomic tools, which could compliment already existing traditional methods of breeding, to counter both old leading to new biotic and abiotic stresses that hamper agricultural productivity. Among the several strategies that rely on genetic engineering, post-transcriptional gene silencing (PTGS), or RNA interference (RNAi), stands out as a method of choice for its practicality and specificity. The search for plants with resistance against viruses led to the accidental discovery of the phenomenon of PTGS, paving the way for the development of RNAi models (Angell and Baulcombe, 1997). The manipulation of naturally occurring gene silencing pathways in the laboratory has led to the generation of genetically modified plants capable of suppressing the expression of endogenous genes and invasive nucleic acids (for a review on this see Souza *et al.*, 2007; Aragão and Figueiredo, 2008).

In recent decades, few concepts in biotechnology have been th subjects of greater advances in terms of practical applications than RNAi. Indeed the experimental demonstration that has led to the comprehension of the mechanisms involved in gene silencing mediated by RNA represents an important milestone in understanding the biological function of genes. This has opened new avenues for understanding biological systems and

serves as a powerful tool for studying interaction amongst organisms, development of elite varieties for agriculture, and design and development of therapeutic agents for human health. Additionally, RNAi techniques are relevant in studies involving the search for improved nutritional values in plants, and in the development of plants that are better adapted to different ecosystems, as well as optimal utilization of raw materials derived from plants for industrial use.

Discovery of RNAi

The existence of naturally occurring gene silencing phenomena in organisms as diverse as viruses, fungi, plants, and animals is a clear indicator that it is an evolutionary stable strategy. Although gene silencing strategies have been shown to be much more efficient in viruses, RNAi technologies that rely on the phenomenon have been widely applied in pests and pathogens such as bacteria, fungi, nematodes, and insects.

One of the pioneer experiments involving the application gene silencing strategy was reported in 1986 when workers demonstrated that plants could be genetically engineered to exhibit resistance against viral diseases (Abel *et al.*, 1986). In this experiment, a chimeric gene containing the coat protein gene (CP) of *Tobacco mosaic virus* (TMV) was introduced into cells of *Nicotiana tabacum* via *Agrobacterium tumefaciens*. The plants regenerated from the transformed cells expressed the CP gene, and when inoculated with TMV showed delayed development of symptoms. Indeed 10–60% of the plants showed no symptoms at all. In another experiment, transgenic plants transformed to express a complementary RNA sequence (antisense RNA) of the coat protein gene of TMV were protected when challenged with the virus. It was further demonstrated that the accumulation of antisense RNA was responsible for this protection (Powell *et al.*, 1989). Although these pioneer experiments showed that the presence of viral RNAs resulting from transgene expression was responsible for the viral resistance observed in the plants, the mechanisms of resistance involved were not fully understood at the time.

Experiments conducted by Napoli *et al*. (1990) helped in elucidating the mechanism of endogenous gene silencing. By introducing the gene for chalcone synthase in petunia, Napoli *et al*. (1990) expected that the gene could be overexpressed thereby increasing pigmentation in flowers. In this way, plants may be generated with dark phenotype due to accumulation of anthocyanin. To their dismay, the introduced gene actually blocked the synthesis of anthocyanin and led to the generation of plants with white flowers. This phenomenon was referred to as co-suppression. Two years later, a similar phenomenon was observed in *Neurospora crassa* by Romano and Macino (1992). The fungus was transformed to super

express the *albino-1* gene (*al*-1), involved in carotenoid biosynthesis, which confers an orange color to the fungus. However, the introduction of an extra copy of the *al*-1 gene produced colonies with the albino phenotype. This phenomenon was referred as quelling.

It was not until 1998 that the phenomenon was fully understood, and the term RNA interference was thus coined by Fire *et al*. (1998) in their now famous experiments with *Caenorhabditis elegans*. By injecting double-stranded RNAs (dsRNA) into nematodes, Fire *et al*. (1998) demonstrated that specific genes could be silenced at the post-transcriptional level. They further demonstrated that this silencing can indeed be "propagated" over a wide section of the nematode following injection of dsRNA into its extracellular abdominal cavity. The same effect was also observed when *C. elegans* were fed with *Escherichia coli* that transcribed recombinant dsRNA or indeed when the nematode was immersed in preparations containing dsRNA.

Mechanism of RNA Interference

RNAi evolved as a natural cellular defense mechanism against viruses, genomic confinement of retrotransposons, and as a cellular strategy for post-transcriptional regulation of gene expression. Knowledge of this mechanism has transformed into a technology used to silence specific genes leading to the creation of knock-out phenotypes in both transgenic plants through the production of sequence specific RNA hairpin, and by infection with recombinant RNA viruses harboring sequences of the target gene. Today, we know that co-suppression and virus-induced gene silencing share mechanistic similarities, thanks to biochemical studies conducted over the years. The pathway leading to gene silencing mediated by RNAi involves several steps, key among which is the generation of small RNA molecules *in vivo* (Figure 11.1).

A central feature in the mechanism of RNA inference is the participation of small RNA molecules. There are two types of small RNA molecules: small interfering RNAs (siRNA) and microRNA (miRNA). The process is initiated by an endonuclease RNase III known as dicer, which processes double stranded RNA generating small RNA molecules that range in size from 20–30 nucleotides which ultimately mediate the degradation of their complimentary RNAs (Angaji *et al*., 2010; Czech and Hannon, 2011). The siRNA are processed by dicer-like enzymes (DCL2, DCL3, and DCL4) from a long double strand of RNA. On the other hand, DCL1 processes the precursors of miRNA exported from the nucleus (Xei *et al*., 2004) (Figure 11.1).

Following dsRNA processing, siRNAs are assembled unto a multicomponent nuclease known as RNA induced silencing complex (RISC) (Hammond *et al*., 2000; Figure 11.1). Originally identified by
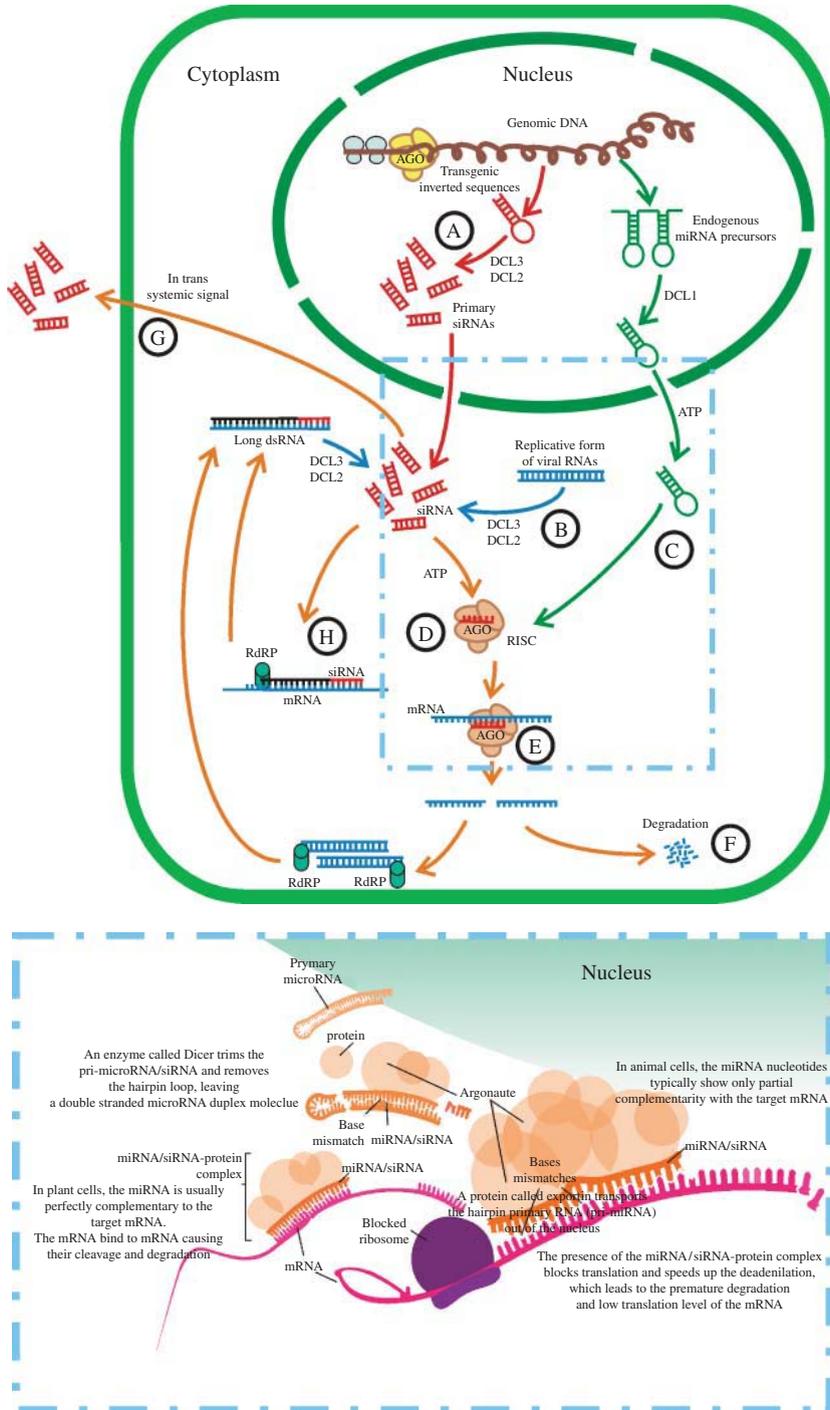
Figure 11.1 Gene silencing pathway. Dicer-like proteins processing transcripts containing inverted sequences (A), derived from viral RNA replication (B), and precursors of miRNA exported from the nucleus (C). Formation of siRNAs/RISC complex (D) directed to target RNA (E), which is subsequently, degraded (F); systemic silencing (G); and amplification by RdRP (H). (Source: Based on Souza *et al.*, 2007; Aragão and Figueiredo, 2008). (See color figure in color plate section).

fractionating an extract of specific nuclease from *Drosophila melanogaster* (Hammond *et al.*, 2001), RISC is a member of the Argonaut family. It is responsible for directing and cleaving of specific sequence of RNA in the cell (Martinez and Tuschul, 2004; Czech and Hannon 2011). This is achieved by cleaving the target mRNA at complimentary region of ten nucleotides upstream of a 5′ residue of the RNA. A helicase in the RISC complex unwinds the siRNA duplex, pairing it with the antisense strand of the target mRNA, which, on its part, has a high degree of complementarity with the siRNA sequence. The cleavage leads to gene silencing by preventing the protein synthesis machinery from reading the mRNA, resulting in its degradation (Tolia and Joshua-Tor, 2006).

siRNAs are classified into primary and secondary siRNAs. While the primary siRNAs are generated through the activity of dicer, secondary siRNAs arise from an alternative pathway, which involves the activity of RNA dependent RNA polymerase (Pak and Fire, 2007). It appears that secondary siRNAs regulate gene expression involving signal transduction where they initiate the process of RNAi in the absence of the original signal for RNAi (Figure 11.1).

MircoRNAs are endogenous RNA molecules and play an important regulatory role during mRNA cleavage and repression of translation. They constitute one of the most abundant classes of regulatory molecules in multicellular organisms (Aukerman and Sakai, 2003; Bartel, 2004). In plants, miRNAs have been implicated in the control of cell division, leaf and meristematic patterning, environmental responses, heterochromatin maintenance, embryogenesis and development of meristem, leaves, anthers ,and vascular system (Palatnik *et al.*, 2003; Vazques *et al.*, 2004; Jover-Gil *et al.*, 2005).

For the formation of the primary miRNA, a transcript of a primary micro-RNA (pre-miRNA) synthesized from the introns of the RNA polymerase II enzyme gene is processed in the nucleus by a protein complex containing a ribonuclease specific to the double-strand producing an intermediary hairpin with 70 nucleotides. This pre-miRNA is then transported to the cytoplasm where it is cleaved by dicer. Following separation of the duplex strands, single stranded miRNA is incorporated into RISC forming the complex that inhibits translation or induces the degradation of target mRNA (Angaji *et al.*, 2010) (Figure 11.1).

Applications in Plant Breeding: Naturally Occurring Gene Silencing and Modification by Genetic Engineering

Over the years, interest in the use of RNAi mechanisms in plant breeding has been on the increase particularly due to the specificity and efficiency of the technique. Several crops have been used as targets of

this technique to improve different characteristics of plants of agronomic importance. Efforts from various laboratories in research centers around the world have been rewarded with remarkable success. Such attempts have, in some cases, led to the development and commercialization of plants with improved agronomic traits. With advances in the post genomic era and the availability of high-throughput techniques, which have allowed for the generation of omics data for several species, the number of successful examples of genetically modified plants derived using RNAi technology has increased significantly (Sunilkumar *et al.*, 2006; Kusaba *et al.*, 2003; Bonfim *et al.*, 2007, Wang, *et al.*, 2011) (Table 11.1).

The application of RNAi techniques for improving plants evolved from studies of mutation in different plant species, which often led to the accidental discovery of RNA hairpin structures. The most common examples of such phenomenon have been observed in plants that display easily discernible phenotypic changes, such as seed and flower color. One such example is the change in color of the seed coat of soybean (*Glycin max*). In the plant, seed coat color is determined by the accumulation of anthocyanins. A key enzyme in the biosynthetic pathway of anthocyanins (besides other secondary metabolites such as isoflavones) is chalcone synthase (CHS) (Palmer *et al.*, 2004). At the chromosomal level, control of pigmentation is mediated by four alles (I, i^i, i^k, i) of locus I (inhibitor). Of these, I, i^i, and i^k are dominant alleles where I is responsible for the phenotypic features when seeds are colorless or bear yellow coloration, i^i gives rise to pigmented husk, and i^k gives rise to seeds with patches of pigment. In contrast, the i allele is recessive and produces seeds with brown or black pigment (Todd and Vodkin, 1993). Structural studies of the I locus (located on chromosome 8) revealed two inverted repeat clusters on the genes *CHS1*, *CHS3*, and *CHS4* (Todd and Vodkin, 1996; Tuteja and Vodkin, 2008). Six other CHS coding genes (*CHS*2, *CHS*5, *CHS*6, *CHS*7, *CHS*8, and *CHS*9) are also found in soybean and varieties with colorless seeds have reduced transcript level of *CHS* (Tuteja *et al.*, 2004). Subsequent studies further reported having found large quantities of siRNAs (predominantly 22nt), which corresponded to the regions of the *CHS* genes (Figure 11.2). These small RNA molecules are indeed specific to seed coat and arise from the transcription of *CHS*1, *CHS*3, and *CHS*4 arranged in inverted repeat regions, leading to the formation of dsRNA (Tuteja *et al.*, 2009). Similarly, when *C2-Idf* allele (*colorless2*; containing a mutated chalcone synthase gene) occurs in the homozygous state, different seed parts are colorless (pericarp, aleurone layer of the endosperm, and vegetative organs). Plants with functional heterozygous *C2* allele exhibit an intermediary phenotype, characterized by lesser pigmentation (Vedova *et al.*, 2005). Cloning and sequence analysis of *C2-Idf* allele showed that its structure is quite different from the normal *C2* allele as two of its

Table 11.1   Examples of crops engineered using RNAi technology.

Crop	Target gene	Strategy	Application	Reference
Apple	*Mal d 1*	Silencing of the gene coding for Mal d 1	Development of apple free of the allergen Mal d 1	Gilissen *et al.*, 2005
Banana	*rep* gene of BBTV	Transformation using an RNAi vector with hpRNA of *rep* gene of *Banana bunchy top virus* (BBTV)	Development of banana resistant to *Banana bunchy top virus*	Wang, Abbott, and Waterhouse, 2000
Barley	Sequence of BYDV-PAV	Transformation of plants with RNAi vector with hpRNA of BYDV-PAV	Development of plants resistant to BYDV	Ogita *et al.*, 2003
Coffee	*CaMXMT1*	Silencing of the gene coding for 7-N-methylxanthine methyltransferase	Development of plants with reduced caffeine content	
Cotton	δ-cadinene synthase	Silencing of the gene for δ-cadinene synthase in seeds	Reduction in gossypol	Sunilkumar *et al.*, 2006
Ground nut	FAD2	Use of RNAi to regulate oleate desaturase (FAD2)	Increase in oleic acid	Yin *et al.*, 2007
Maize	DHPS	Silencing of zein	Reduction in the catabolism of lysine (accumulation of lysine) and improved seed germination	Tang *et al.*, 2004
Plum	CP-PPV	Silencing of the gene for CP-PPV during germination	Development of plants resistant to *Plum pox virus* (PPV)	Hily *et al.*, 2005
Poppy	*COR*	Silencing of the gene coding for codeinone reductase (COR)	Elimination of narcotic alkaloids (morphine)	Allen *et al.*, 2004
Rice	Multigene family of *lgc*	Plants with low glutenin Content-1	Nutrition for patients with celiac disease	Kusaba *et al.*, 2003
Soybean	*GmMIPS*	Silencing of the gene coding for the enzyme myoinositol-1-phosphate synthase (GmMIPS)	Reduction in the level of phytates	Nunes *et al.*, 2006
Sweet potato	SBEII	Silencing of the gene for the protein SBE, which generates starch with branches	Increase in the level of amylase	Shimada *et al.*, 2006
Tomato	*DET1*	Suppression of the expression of *DET1*	Increase in the levels of carotenoids and flavonoids in fruits without affecting growth regulators	Davuluri *et al.*, 2005
Tomato	*Lyc e 1.01*	Silencing of the gene coding for profilin	Reduction in allergic reactions associated with profilin found in many fruits	Le *et al.*, 2006
Wheat	BYDV	Construction using *Barley yellow dwarf virus* (BYDV) sequences	Immune lines resistant to virus	Wang, Abbott, and Waterhouse, 2000
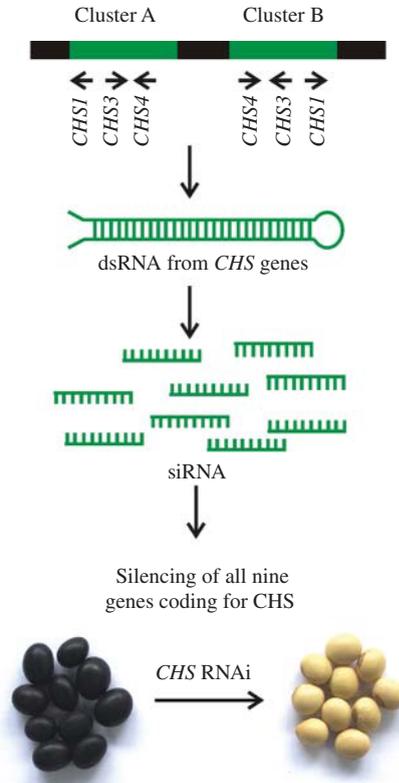
Figure 11.2 Silencing of *CHS* genes coding for chalcone synthase (a key enzyme in the biosynthesis of anthocyanins) in soybean. The presence of inverted repeat sequences of *CHS1*, *CHS3*, and *CHS4* leads to formation of RNA hairpin (dsRNA), which is processed to form siRNA, leading to silencing of all of the nine CHS genes. This manifests in the phenotypic characteristics of the seeds as colorless (yellow). (See color figure in color plate section).

three copies of the *CHS* gene lay side by side in an inverted orientation, leading to reduction in the level of its mRNA and consequently the enzyme (Dooner, 1983; Franken *et al.*, 1991). Indeed siRNAs have been found in plants containing C2-*Idf* allele and not in normal homozygous containing C2, indicating that the colorless phenotype is mediated by RNAi (Vedova *et al.*, 2005).

Another well characterized example is seen in rice with reduced levels of glutenin. The consumption of food substances with reduced levels of glutenin is important in patients with celiac disease whose diet must not contain this protein. The phenotype with a low level of glutenin is generated through an RNAi mechanism as a result of two inverted copies of genes near the glutenin coding gene on *Lgc1* locus (Kusaba *et al.*, 2003).

A number of techniques that rely on RNAi pathways have been widely developed and applied in order to knock out genes of interest in different plants with a view to unlocking the full agronomic potentials of the crops under physical and biological conditions that would otherwise make such feats impossible. These strategies often seek to improve productivity, confer resistance and/or tolerance to many pests and diseases (Wang *et al.*, 2000; Bonfim *et al.*, 2007).

Furthermore, the technique has been used to improve the nutritional value of plants, in addition to optimizing use of raw materials derived from plants for industrial use (Ossowski *et al.*, 2008; Sunilkumar *et al.*, 2006; Yin *et al.*, 2007; Shimada *et al.*, 2006).

A key component of the strategies involved in developing genetically modified plants in this respect is the use of RNAi vectors. Several of such vectors are now available and a representation of chief among them is given in Figure 11.3. In rice for example, the expression vector pNW55, derived from a natural miRNA (osa-MIR528 of rice) in which an artificially inserted miRNA sequence, was designed to silence *Pds*, *Spl11*, and *Eui1/CYP714D1* (Warthmann *et al.*, 2008). A similar approach had earlier been employed to silence the *P69* gene of *Turnip yellow mosaic virus* and the HC-pro gene of *Turnip mosaic virus* in *Arabidopsis thaliana* by using miR159 from the plant to construct a vector that expressed artificial miRNAs (amiRNA). Plants generated from this work were reported to be resistant to the two viruses even under low temperature (15 °C) (Niu *et al.*, 2006). This approach is immensely important in attempts to silence endogenous genes of plants especially where complete genomes of such plants are available. However, in cases where the target organisms are pathogens (virus, fungus, nematodes), specificity constitutes a problem in attempts to confer wide and reliable resistance in both *cis* and *trans* approaches.

Resistance to Viruses

Several plants are resistant to viruses by virtue of an inherent dsRNA and siRNA generating system whose targets are gene sequences essential for viral pathogenicity. For example, siRNAs sharing 100% similarity with distinct genetic and intragenic regions of *Mungbean yellow mosaic India virus* (MYMIV), a begomovirus which causes yellow mosaic disease, have been observed in mungbean. In the resistant line PK416, siRNAs were found to correspond to an intragenic region (IR) of MYMIV, while in the susceptible lines, most of the siRNAs correspond to the genetic regions and are present in low concentrations. It was also demonstrated that the viral genomes in resistant plants were methylated in the intragenic regions (Yadav and Chattopadhyay, 2011).
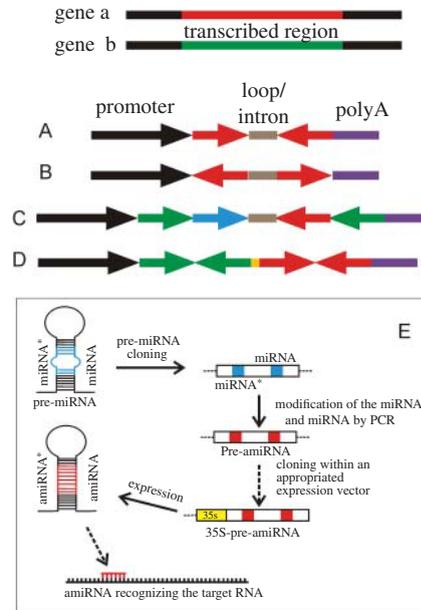
Figure 11.3 Vectors used in the stable transformation of plants are generally designed to produce hairpin structures (after transcription of RNA or dsRNA). Here, a transcribed sequence of a gene is amplified and placed under the control of a promoter in forward (sense) and reverse (antisense) directions spaced by an intron or a spacer region (loop) (A, B). Sequences of two or more genes can be used in the same expression cassette (C). It is also possible to join two expression cassettes harbouring gene fragments cloned in forward and reverse directions separated by a spacer (D). In (E), a vector designed to generate a modified miRNA by introducing a target gene sequence into a natural miRNA region (such as miR159 of *A. thaliana* and miR528 of *Oriza sativa*) is presented. The resulting vector expresses artificial miRNA (amiRNA) whose target may be the endogenous gene or that of an intracellular pathogen. (See color figure in color plate section).

Although RNAi mediated resistance to viruses is a natural phenomenon in plants, it is not effectively present in many productive lines because siRNA molecules identical to viral sequences usually appear at later stages of infection in some of these lines, when it is not stoichiometrically favorable to control the infection (Rodriguez-Negrete *et al.*, 2009; Aregger *et al.*, 2012). However, this can be circumvented by mimicking the mechanism using recombinant DNA technology to generate siRNAs that can confer resistance or immunity to plants against viruses even before the onset of infection. Currently, there are several reports of protocols in which RNAi strategies have been used to generate plants resistant to viruses based on either RNA or DNA genomes (Prins *et al.*, 2008; Runo, 2011; Prins, 2003; Vanderschuren *et al.*, 2007; Bonfim *et al.*, 2007; Aragão and Faria, 2009; Lucioli *et al.*, 2003; Fuentes *et al.*, 2006; Vanderschuren *et al.*, 2009; Hashmi *et al.*, 2011; Vanderschuren *et al.*, 2012).

However, the first report on gene silencing appeared in 1986 when tobacco plants were transformed with the coat protein gene of *Tobacco mosaic virus* (TMV) (Beachy *et al.*, 1987). Following this, more than 100 publications have appeared reporting on the development of genetically modified plants resistant to viruses of different groups.

At the commercial level, the first crop varieties resulting from this technology were tobacco resistant to *Tobacco mosaic virus* (TMV) in China, and papaya resistant to *Papaya ringspot virus* (PRSV), which has been in cultivation in the United States since 1998. All of these crops are now available to farmers. Other commercially available crops in the United States include pumpkins resistant to WMV, ZYMV, and CMV, and virus resistant potatoes.

However, the first deliberate transformation to express a dsRNA construct harbouring intron–hairpin RNA (hpRNA) was reported in 2000 using wheat in which gene sequences of a polymerase from *Barley yellow dwarf virus* (BYDV) were expressed. Plant lines arising from this were immune to the virus following tests using ELISA (Wang *et al.*, 2000). Shortly thereafter, transgenic tobacco plants expressing sense and antisense RNAs of DNA-A of *Cotton leaf curl virus* (CLCuV DNA-A) and DNA-B of CLCuV were generated. The siRNAs of DNA-A inhibited viral replication while those of DNA-B conferred resistance against CLCuV to the plants (Asad *et al.*, 2003). In addition, researchers generated tomato plants with resistance against *Tomato yellow leaf curl Sardinia virus* (TYLCSV) using RNA hairpin constructs containing truncated Rep protein gene of TYLCSV (Yang *et al.*, 2004).

In an attempt to extend this technique to leguminous plants, Poogin *et al.* (2003) used a dsRNA construct to silence the promoter sequence of DNA-A of *Vigna mungo yellow mosaic virus* (VMYMV), leading to the expression of dsRNA of a conserved region of VMYMV in *Vigna* spp., resulting in resistance against viral infection. Similarly, Bonfim *et al.* (2007) applied RNAi technology using a viral *AC1* gene sequence encoding a multifunctional protein (Rep) of the *Bean golden mosaic virus* (BGMV) to generate transgenic common bean (*Phaseolus vulgaris* L.) resistant to geminivirus. The choice of this viral gene (*AC1* or *Rep*) for the construction of the transformation vector was based on the fact that Rep protein plays an essential role in the viral infection cycle and as it is the only protein required for replication. The vector used was constructed from a DNA fragment of 411 bp of *AC1* gene of BGMV. This resulted in the development of an event now known as Embrapa 5.1: the first transgenic line approved for commercial use through the application of Brazilian technology and following Brazilian biosafety regulations set by the Brazilian Technical Biosafety Commission (CTNBio) (Aragão and Faria, 2009). This strategy can also be applied to combat other devastating diseases such as geminivirus attacking maize and cassava in Africa, and tomato worldwide.

Host-induced Gene Silencing

The discovery that genetically modified plants can be used to control pathogenic organisms when engineered to release siRNA specific to a vital gene in susceptible pathogens is another indication of the great potentials that RNAi techniques can unlock. Such a feat was reported by Tinoco *et al*. (2010) who demonstrated *in vivo* interference using the pathogenic fungus *Fusarium verticillioides*. In their experiments, inoculation of mycelium in transgenic tobacco plants, engineered to express siRNA from a dsRNA corresponding to a transgene, specifically silenced genes in the fungus. This proved a powerful tool for understanding the molecular interaction between plants and pathogens and symbiotic interactions. From the viewpoint of biotechnology, silencing fungal genes by siRNAs generated by host plant represents an important strategy for developing fungal resistance in plants and other organisms Koch *et al*., 2013). This movement of silencing signals in the form of siRNAs derived from one organism, exerting their effects on another, has also been observed in nematodes (Waterhouse, Graham, and Wang, 1998). These workers reported that gene silencing was triggered when nematodes were fed on a diet made from transgenic plants engineered to express dsRNA. The same phenomenon was observed in herbivorous insects fed with transgenic plants expressing dsRNAs of genes that are vital to insects (Baum *et al*., 2007; Mao *et al*., 2007).

In 2007 Roney, Khatibi, and Weswood reported on the systemic movement of mRNA through phloem between tomato and the parasitic plant *Cuscuta pentagona* Engelm. Experiments described by Tomilov *et al*. (2008) also showed that host plants transformed with constructs that generate interference hairpins can silence the expression of target gene in parasitic plants. Roots of transgenic *Triphysaria versicolor* expressing the reporter gene *gus* became parasitic to transgenic lettuce expressing RNA hairpin containing a fragment of the *gus* gene (hpGUS). Additionally, Aly *et al*. (2009) showed that a construct containing the binary vector pBIN-IR-M6PR inserted in the tomato genome can silence the expression of the *M6PR* gene in tubers of *Orobanche* that parasitize the roots of transgenic plants. The observation that molecules produced by host plants are responsible for silencing specific genes in parasitic plants suggests a new strategy for engineering plants resistant to parasites.

Insect and Disease Control

Although commercial biotechnology has made available protocols for the control of diseases transmitted by both Coleopteran and Lepidopteran insects through the expression of insecticidal protein from *Bacillus thuringiensis* (Bt toxin), the emergence of resistance to Bt toxin

in some insect biotypes underscores the need to develop new control strategies that require a different mode of action (Baum *et al.*, 2007). Silencing of essential insect genes mediated by dsRNA can interrupt feeding or lead to death of susceptible insects. In this respect, it has been demonstrated that ingestion of RNAs provided in an artificial diet induces RNA interference in Coleopterans such as *Diabrotica* sp. (Gordon and Waterhouse, 2007; Baum *et al.*, 2007; Gatehouse, 2008; Upadhyay *et al.*, 2011). The development of transgenic corn engineered to express dsRNAs against the V-ATPase of corn rootworm, which showed suppression of mRNA in the insect and reduction in feeding damage, is a powerful indicator that the RNAi pathway can be exploited to control insect pests in plants by expression of a dsRNA *in vitro* (Baum *et al.*, 2007). Similarly, transgenic cotton and *Arabidopsis* plants engineered to express dsRNA directed against Cyt P450, a detoxification enzyme (coded for by CYP6AE14) for gossypol in cotton bollworm, induced feeding damage in insects (Mao *et al.*, 2007).

Indeed the fact that the RNAi machinery is present in all living insects further highlights the potentials for the use of this approach for insect control by interrupting the expression of their essential genes. This is possible even for insect species that lack a systemic RNAi response because genes expressed in insect midgut are susceptible to silencing by dsRNA when ingested in a diet (Huvenne and Smagghe, 2010).

Improving Nutritional Values

Although many plants may be regarded as sources of proteins, a good number of them are deficient in certain essential amino acids or, when present, these important nutrients are accumulated in cellular compartments that make their utilization difficult or indeed toxic for human and animal consumption. Accordingly, various breeding programs seek to increase levels of amino acids in order to add value to crops and make such nutrients bioavailable (Tu, Godfrey, and Sun, 1998; Marcellin *et al.*, 1996). Among these amino acids are lysine and sulfur containing amino acids. For example, while a high level of lysine in seeds is beneficial, an increase in the level of this amino acid in vegetative tissues is undesirable because it may lead to abnormal growth or hamper flower development. The pathway for the biosynthesis of lysine is under tight regulation by a feedback inhibition mechanism in which the amino acid inhibits the activity of dihydrodipicolinate synthase (DHPS), the first enzyme in the committed step of lysine biosynthesis. It has been demonstrated that mutations in tobacco *DHPS* gene may cause its encoded DHPS lysine to become insensitive, leading to overproduction of lysine in all plant organs (Frankard *et al.*, 1992; Negrutiu *et al.*, 1984). The RNAi technique has thus been used to improve the germination of seeds of *Arabidopsis* by

silencing DHPS (Zhu and Galili, 2003; Zhu and Galili, 2004; Tang, Galili, and Zhuang, 2007). The same approach was used in maize to increase the level of lysine in seeds by manipulating the gene for zein, a protein normally associated with low nutritional quality. Using RNAi constructs derived from a fragment of the 22 kDa zein gene, researchers generated a dominant opaque maize phenotype with a low level of zein, which corresponded to an increase in the level of lysine improving the plant's nutritional value and promoting seed germination (Segal *et al.*, 2003).

The RNAi technique has also been used in soybean to silence the gene of myoinositol-1-phosphate synthase (*GmMIPS*), a key enzyme in the biosynthesis of phytic acid in seeds. Phytates are anti-nutritional factors that chelate divalent minerals such as zinc, calcium, iron, and others, present in food, reducing nutritional value. Phytates are also eliminated in the feces and thus may pose environmental contamination. In order to generate soybean plants with silenced *GmMIPS1*, a vector was constructed (pMIPSGm) in which *GmMIPS1* fragments were cloned in the reversed direction, generating sense and antisense arms. The resulting soybean plants showed partial silencing of this gene and led to the development of soybean lines with up to 94.5% reduction in phytates (Nunes *et al.*, 2006).

At the industrial level, potato (*Solanum tuberosum*) with high-amylose starch was developed using RNA interference to inhibit two genes coding for starch branching enzymes (*Sbe1* and *Sbe2*) resulting in transgenic lines with high-amylose, a quality desirable in the market (Shimada *et al.*, 2006).

Secondary Metabolites

Besides agronomic traits, industrial and pharmaceutical substances derived from plants can be enhanced using RNAi technology. This has been used to interfere with pathways of secondary metabolites in order to generate useful substances for pharmaceutical use and allelopathy. For example, gene silencing of codeinone reductase (COR) in opium (*Papaver somniferum*) led to accumulation of non-narcotic alkaloids (Allen *et al.*, 2004). In cotton, the technology was used to reduce the level of gossypol, a toxic compound that accumulates in seeds and restricts the use of cotton as a possible source of protein for humans. This was achieved by intervening in the gene expression of δ-cadinene synthase during seed development (Sunilkumar *et al.*, 2006).

Perspectives

Discovered less than 20 years ago, the RNAi mechanism has today become a powerful tool for understanding how genes function in

various biological processes, thus constituting an important tool in metabolomics. Its application in the development of plants with farmer preferred agronomic traits has opened new opportunities hitherto unthinkable even with the best methods of classical breeding. Already, several technologies have been approved for commercialization in the United States and Brazil. With the explosion of knowledge on the biological and biochemical mechanisms underlying the RNAi pathway, our ability to fully harness and unlock the potentials of this mechanism, as both an experimental tool and a problem solving strategy, will undoubtedly increase. Despite such limitations as dependency on other techniques, which are sometimes not reproducible, RNAi technology will continue to be used alongside conventional breeding approaches for the development of new cultivars in the coming years. With advances in the development of tools for genetic manipulation of the plant genome, the coming years seem promising when it will be possible to effectively use strategies involving zinc-finger nucleases (ZFNs) (Isalan, 2012), TALENS (Sanjana *et al.*, 2012), and other endonucleases to generate new transformation events with remarkable success. This is even more so because with these strategies, it is possible to selectively mutagenize multiple gene copies resulting in precise silencing that could yield desirable phenotypes with a high degree of accuracy and requiring less time and resources for selection, molecular analyses, and biosafety tests.

Issues related to biosafety of the use of RNAi in genetically modified plants have been discussed extensively in some reviews (Hollingworth *et al.*, 2003; Petrick *et al.*, 2013; Parrott *et al.*, 2010). However, based on the evidence that humans, and indeed all animals, have been consuming foods with naturally occurring RNA molecules (including miRNA, siRNA, long dsRNA, and mRNA), it is reasonable to posit, in principle, that there is no reason to expect that consumption of genetically modified foods derived from RNAi could pose any health risks. Plants have an average of 1 mg of total RNA per mg of tissue (Ivashuta *et al.*, 2009; Lassek and Montag, 1990). Of this total, the non-coding RNA (tRNA, rRNA, antisense-ssRNA, dsRNA from external sources, such as viruses, miRNAs, and siRNAs) constitute the larger percentage. It is worth remembering that man has been feeding on animals (with a history of safe consumption) and these animals contain miRNA and siRNA with a high similarity to human genes (Carthew and Sontheimer, 2009; Petrick *et al.*, 2013; Jensen *et al.*, 2013). Despite this, however, it is extremely important that for each product generated using RNAi technology, rigorous biosafety analyses are conducted. Already, a number of genetically modified plants expressing siRNA have been commercially released following such regulations. These plants include: Flavr Savr™ tomato modified to silence the gene for polygalacturonase in fruits; pumpkin resistant to *Watermelon mosaic virus 2* and *Zucchini yellow mosaic virus*: and papaya resistant to *Papaya risgspot virus* (http://www.agbios.com).

Scientists will continue to rely on RNAi technology to discover and validate gene function, but more importantly, to generate desirable products in plants. The great advantage of the technique, symbolized by its specificity in sequence, tissue, and time of expression, allows for its relative ease of gene targeting with high silencing efficiency and potency, as against other methods that have higher tendencies for missing targets. Indeed, RNAi technology, by its very nature, has the ability to predict the effect of off-target silencing. As new generations of RNAi based transgenic crops emerge, further research is needed to meet the growing human need.

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Capítulo 4

RNA interferente

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Introdução

Em meados do século 20, os cientistas passaram não apenas a entender as vias bioquímicas em vários organismos vivos, mas também passaram a manipulá-las de forma precisa para atingir objetivos específicos. Nesse contexto, a tecnologia do DNA recombinante, criada nos anos 1970, representou uma ferramenta importante, como aliada no melhoramento tradicional de plantas, pois contorna antigos e novos desafios da agricultura, como estresses bióticos e abióticos.

Visando resolver esses problemas, várias estratégias utilizando engenharia genética têm sido utilizadas, dentre elas o silenciamento gênico pós-transcricional (PTGS) ou RNA interferente (RNAi). Foi a busca por plantas resistentes a vírus que levou pesquisadores a acidentalmente descobrirem o fenômeno de PTGS e a estabelecer modelos de RNAi (Angell & Baulcombe, 1997). Atualmente, a capacidade de manipular técnicas de silenciamento gênico em laboratório tem produzido plantas transgênicas capazes de suprimir a expressão de genes endógenos e ácidos nucleicos invasores

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(para uma revisão ver Souza et al., 2007; Aragão & Figueiredo, 2008). Também se têm identificados exemplos de ocorrência natural de silenciamento, gerando características que foram mantidas devido às forças evolutivas e à seleção humana. Apesar de essas estratégias terem se mostrado muito mais eficientes para vírus, a tecnologia do RNAi tem sido amplamente utilizada para outros patógenos e pragas, como bactérias, fungos, nematoides e insetos. Adicionalmente, as técnicas de RNAi são relevantes na busca para melhorar valores nutricionais em plantas, desenvolvimento de plantas mais adaptadas aos distintos ecossistemas, bem como o aproveitamento otimizado de matérias-primas derivadas de plantas para uso industrial.

Um dos primeiros experimentos aplicando uma estratégia de silenciamento foi relatado em 1986, quando se demonstrou que plantas podem ser geneticamente transformadas visando à resistência a doenças virais (Abel et al., 1986). Nesse experimento, um gene quimérico contendo o gene da capa proteica (CP) do *Tobacco mosaic virus* (TMV) foi introduzido em células de *Nicotina tabacum* via *Agrobacterium tumefaciens*. As plantas regeneradas das células transformadas expressaram o gene CP do TMV e, quando inoculadas com TMV, apresentaram atraso no desenvolvimento dos sintomas e 10% a 60% das plantas não apresentaram sintomas. Em outro experimento, plantas de fumo transgênicas transformadas para expressar uma sequência complementar de RNA (RNA antisense) do gene da capa proteica do TMV foram protegidas quando inoculadas com o vírus. Mostrou-se que o acúmulo de RNA antisense foi o responsável por essa proteção (Powell et al.,1989). Embora esses experimentos pioneiros tenham mostrado que a presença de RNAs virais resultantes da expressão de transgenes fora eficiente para obtenção de plantas resistentes a vírus, os mecanismos envolvidos com a resistência ainda não eram bem compreendidos.

Outro experimento envolvendo o silenciamento de genes endógenos que ajudou a elucidar o mecanismo de silenciamento gênico foi conduzido por Napoli et al. (1990), ao introduzirem o gene da chalcona sintase em petúnia, esperando que ele fosse superexpresso e aumentasse a pigmentação nas flores. A intenção era gerar plantas com flores escuras com o acúmulo de antocianinas. Inesperadamente, o gene introduzido bloqueou a síntese de antocianina, produzindo flores totalmente brancas. Na época, esse fenômeno foi denominado "co-supressão". Dois anos mais tarde, um fenômeno similar foi observado em *Neurospora crassa* por Romano & Macino (1992). Eles

esperavam super expressar o gene *albino-1* (*al*-1), envolvido na biossíntese dos carotenóides que conferem a cor alaranjada ao fungo. Mas, ao contrário do esperado, com a introdução de uma cópia extra do gene *al*-1, foram observadas colônias exibindo um fenótipo albino; esse fenômeno foi denominado *quelling*.

Somente em 1998 este fenômeno foi entendido, assim, o termo RNA interferente foi cunhado por Fire et al. (1998) em experimentos com *Caenorhabditis elegans*. Nesses experimentos, RNAs dupla fita (dsRNA) foram injetados nos nematoides e induziram silenciamento gênico pós-transcricional específico. Além disso, foi observada propagação do silenciamento em ampla região do animal após a injeção de dsRNA na cavidade abdominal extracelular. O mesmo efeito também ocorreu quando *C. elegans* foi alimentado com a bactéria *Escherichia coli* que transcreve o dsRNA recombinante ou até mesmo quando esse nematoide foi embebido em preparações contendo dsRNA.

Nas últimas décadas, poucas biotecnologias tiveram grandes avanços em termos de aplicações práticas como a de RNAi. O entendimento dos mecanismos envolvidos no silenciamento gênico mediado por RNA tem sido importante para a compreensão da função biológica de genes, da interação entre organismos, bem como no desenvolvimento de variedades para a agricultura, como agente terapêutico na saúde humana, entre outros. Neste capítulo, concentrar-se-á nas aplicações e avanços na área de melhoramento de plantas empregando a estratégia de RNAi e será apresentado uma breve descrição do mecanismo de RNAi, bem como os papéis das principais enzimas da via e as pequenas moléculas de RNAs.

Mecanismo de RNA interferente

O mecanismo de RNAi está envolvido na defesa celular natural contra vírus, confinamento genômico de retrotransposon e regulação pós-transcricional da expressão gênica. Atualmente, esse conhecimento foi transformado em uma tecnologia usada para silenciar genes específicos e individuais, criando fenótipos nocauteados, tanto em transgênicos que produzem o grampo de RNA necessário quanto por infecção com RNA recombinante de vírus que carregam sequências do gene alvo. Este mecanismo é um processo de muitas etapas que gera pequenas moléculas de RNAs *in vivo* (Figura 4.1).
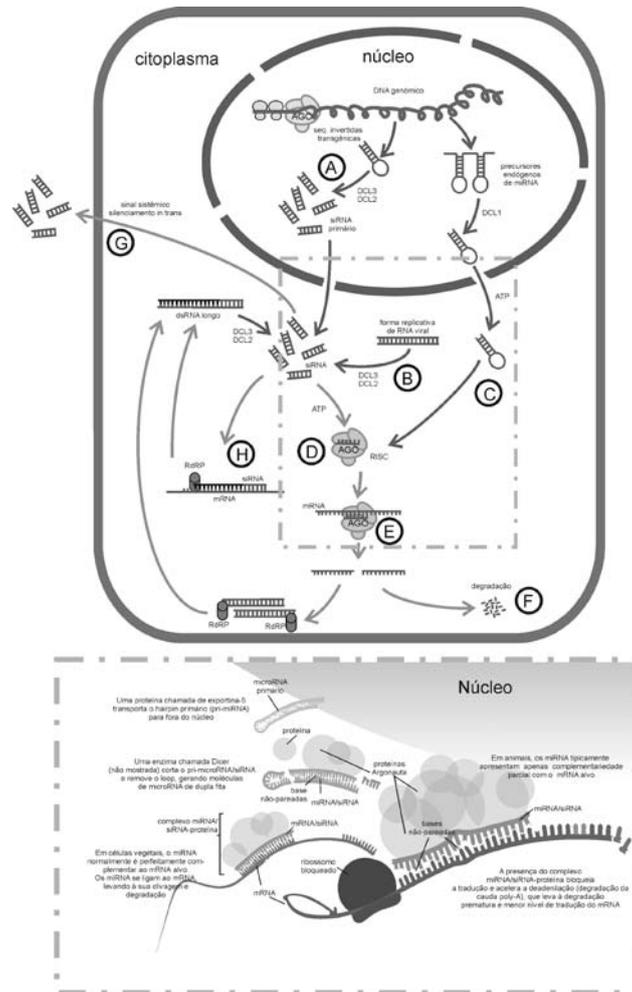
Figura 4.1. Vias de silenciamento gênico. Proteínas-tipo-Dicer processando transcritos contendo sequencias invertidas (A), derivados da replicação de RNA viral (B) e precursores de miRNA exportado do núcleo (C). Forma-se complexo siRNAs/RISC (D) que é direcionado para o RNA alvo (E) que é então degradado (F). G: Silenciamento sistêmico; H: Amplificação por RdRP. (Baseado em Souza et al., 2007; Aragão & Figueiredo, 2008).

Uma característica central nos mecanismos de RNA interferente é a presença de pequenos RNAs. Existem dois tipos de pequenos RNAs, os pequenos RNAs interferentes (siRNA) e os microRNA (miRNA). O mecanismo de RNAi envolve a ação de uma endonuclease RNase III chamada Dicer a partir da presença de dsRNA no citoplasma que pode variar tanto em tamanho quanto em relação à origem. Os siRNAs resultantes, de 20 a 30 nucleotídeos, mediam a degradação do seu RNA complementar (Angaji et al., 2010; Czech & Hannon, 2011). As enzimas DICER têm um papel importante na formação dessas moléculas no processo de RNAi e clivam longos dsRNA em siRNA e miRNA de um modo que é dependente de ATP (Angoji et al., 2010). Os siRNA são processados por enzimas tipo DICER (DCL2, DCL3 e DCL4) a partir de um longo RNA dupla fita. Por outro lado, DCL1 processa precursores de miRNA exportados do núcleo (Xei et al., 2004) (Figura 4.1).

Após o processamento de dsRNA, os siRNAs são rearranjados no Complexo de Silenciamento Induzido por RNA (RISC) (Hammond et al., 2000; Figura 4.1). Esse complexo foi originalmente identificado pelo fracionamento de uma nuclease específica de extrato de *Drosophila melanogaster* (Hammond et al., 2001). Quanto à composição de RISC, sabe-se que este inclui membros da família Argonauta e uma fita guia de um pequeno RNA. O complexo RISC é responsável pelo direcionamento e pela clivagem da sequência específica de RNA dentro da célula (Martinez & Tuschul, 2004; Czech & Hannon 2011) e age clivando o mRNA alvo no meio da região complementar, 10 nucleotídeos *upstream* do resíduo 5' do siRNA/mRNA alvo dupla fita. Uma helicase do complexo RISC desenrola o siRNA duplex, que se pareia com a fita antisense do RNA mensageiro; este, por sua vez, tem alto grau de complementaridade de sequência com o siRNA. Por fim, uma proteína da família Argonauta do complexo RISC age como uma endonuclease, clivando o mRNA alvo. Essa clivagem leva ao silenciamento do mRNA alvo pelo bloqueio da leitura da mensagem pela maquinaria de tradução, resultando na degradação do mRNA (Tolia & Joshua-Tor, 2006).

Os siRNAs são classificados em primários e secundários. Enquanto os primários são gerados da atividade da enzima DICER, os secundários são gerados por uma via diferente, com o envolvimento de uma RNA polimerase dependente de RNA (Pak & Fire, 2007). Os siRNAs secundários aparentemente regulam a expressão gênica nos casos em que a amplificação do sinal de silenciamento é importante e o

iniciador original para o desencadeamento do RNAi está ausente (Figura 4.1).

Os miRNAs são RNAs endógenos que têm um papel regulatório importante tanto por clivagem do mRNA quanto por repressão da tradução. Essas moléculas compreendem uma das classes mais abundantes de moléculas regulatórias em organismos multicelulares (Aukerman & Sakai, 2003; Bartel, 2004). Em plantas, miRNAs estão envolvidos no controle da divisão celular, polaridade foliar e meristemática, resposta ao meio ambiente, manutenção da heterocromatina, embriogênese, desenvolvimento de meristemas, folhas, flores, anteras e sistema vascular (Palatnik et al., 2003; Vazques et al., 2004; Jover-Gil et al., 2005).

Para a formação do microRNA primário, um transcrito de um micro-RNA primário (pré-miRNA) sintetizado a partir de íntrons de RNA codificante da enzima polimerase II é processado por um complexo proteico contendo uma ribonuclease específica para dupla fita no núcleo a fim de produzir um grampo intermediário de 70 nucleotídeos. Esse pré-miRNA, então, é transportado para o citoplasma, onde é clivado por outra ribonuclease dsRNA específica, a DICER, em miRNA duplex. Após a separação das fitas do duplex, o miRNA fita simples é incorporado ao RISC. Esse complexo inibe a tradução ou induz a degradação de mRNA alvo (Angoji et al., 2010). (Figura 4.1).

Aplicações no melhoramento de plantas: ocorrência natural e modificação por engenharia genética

Devido à sua especificidade e eficiência, há grande interesse em utilizar o mecanismo de RNAi no melhoramento de plantas. Desde a sua descoberta, várias culturas diferentes foram usadas como alvos dessa técnica visando melhorar diversas características de importância agronômica como resultado do desenvolvimento e comercialização de plantas oriundas de laboratórios nos centros de pesquisas públicos e privados. Com avanços nas técnicas complementares, como o de técnicas *high-throughput*, que disponibilizam dados 'ômicos' de diversas espécies, o número de exemplos de sucesso de plantas geneticamente modificadas empregando a tecnologia do RNAi tem aumentado expressivamente (Sunnilkumar et al., 2006; Kusoba et al., 2003; Bonfim et al., 2007; Wang, et al., 2011; Shekhawat et al., 2012) (Tabela 4.1).

Tabela 4.1. Exemplos de plantas modificadas com a estratégia de RNAi.

Cultura	Gene alvo	Estratégia	Aplicação	Referência
Algodão	•-cadineno sintase	Silenciamento do gene de enzima •-cadinene sintase na semente	Redução de gossipol	Sunilkumar et al., 2006
Amendoim	FAD2	Uso de RNAi para a regulação da dessaturase oleato (FAD2)	Aumento de ácido oleico	Yin et al., 2007
Ameixa	CP-PPV	Silenciamento de gene CP-PPV durante a germinação	Desenvolvimento de plantas resistentes a *Plum pox virus* (PPV)	Hily et al., 2005
Arroz	Família multigene de *lgc*	Plantas com nível reduzido de glutelina (Low Glutenin Content-1)	Alimentação para pacientes com problema de rim	Kusoba et al., 2003
Banana	Gene *rep* do BBTV	Transformação com o vetor de RNAi com hpRNA de gene *rep* do vírus *Banana bunchy top virus* (BBTV)	Desenvolvimento de banana resistente ao *Banana bunchy top virus*	Shekhawat et al., 2012
Batata-doce	SBEII	Silenciamento de do gene da proteína SBE, que gera do amido ramificado	Aumento no nível de amilase	Shimada et al., 2005
Café	*CaMXMT1*	Silenciamento do gene que codifica para a 7-N-metixantina metiltransferase	Desenvolvimento de plantas com nível reduzido de cafeína	Ogita et al. 2003
Cevada	Sequência do BYDV-PAV	Transformação de plantas com RNAi engenheirado com o vetor hpRNA do BYDV-PAV	Produção de plantas de cevada com resistência ao BYDV	Wang et al., 2000

Tabela 4.1. Cont.

Cultura	Gene alvo	Estratégia	Aplicação	Referência
Milho	DHPS	Silenciamento do gene da proteína zeina por RNAi	Redução do catabolismo da lisina e aumento na germinação de sementes, gerando uma variante dominante com alto nível de lisina	Tang et al., 2004
Maçã	*Mal d 1*	Sileciamento de gene codificante para Mal d 1	Produção de maçã sem a proteína alergênica Mal d 1	Gilissen et al., 2005
Papoula	*COR*	Silenciamento da codeinona redutase (COR)	Eliminação de alcaloides narcóticos (morfina)	Allen et al., 2004
Soja	*GmMIPS*	Silenciamento do gene que codifica para a enzima mioinositol-1-fosfato sintase de soja (GmMIPS).	Redução de nível de fitato nas sementes de soja	Nunes et al., 2006
Tomate	*DET1*	Supressão da expressão de *DET1*	Aumento dos níveis de carotenoides e flavonoides na fruta de tomate sem afetar os reguladores de crescimento	Davuluri et al., 2005
Tomate	*Lyc e 1.01*	Silenciamento dos genes da priflina	Redução de reações alérgicas associadas à proteína profilina encontrada em muitas frutas	Le et al., 2006
Trigo	Vírus BYDV	Construção com sequências do *Barley yellow dwart virus* (BYDV)	Linhagens resultantes foram consideradas imunes, pois não houve detecção do vírus	Wang et al., 2000

A ocorrência de mutações que levaram à geração "ao acaso" de estruturas de RNA na forma de grampo tem sido estudada em diversas espécies. A maioria dos exemplos mais estudados até o momento se refere a alterações fenotípicas facilmente identificadas, como a alteração de coloração. Um desses exemplos diz respeito à alteração na coloração do tegumento da semente em soja (Figura 4.2). A cor do tegumento é dada pelo acúmulo de antocianinas. Na via de síntese desses compostos, existe uma enzima chave, a chalcona sintase (CHS), essencial para a síntese de vários compostos secundários, como antocianinas, isoflavonas, etc. (Palmer et al., 2004). Quatro alelos (I, i^i, i^k, i) do lócus I (inibidor) governam a pigmentação da semente. Os alelos I, i^i, e i^k são dominantes e levam, respectivamente, à despigmentação das sementes ou sementes amareladas, hilo pigmentado e sementes com regiões pigmentadas. Em contraste, o alelo i é recessivo e gera sementes marrons ou negras (Todd & Vodkin, 1993). A estrutura do lócus I (que está no cromossomo 8) foi estudada e se identificaram dois *clusters* perfeitamente repetidos e invertidos nos genes *CHS1*, *CHS3* e *CHS4* (Todd & Vodkin, 1996; Tuteja & Vodkin, 2008). Seis outros genes que codificam para CHS (CHS2*, CHS*5*, CHS*6*, CHS*7*, CHS*8 e CHS*9) estão presentes no genoma da soja. Tuteja et al. (2004) demonstraram que havia uma redução de transcritos do gene CHS em variedades com sementes não pigmentadas. Mais tarde, esse mesmo grupo mostrou que havia grande quantidade de siRNA (predominantemente de 22 nt) correspondendo a regiões dos genes *CHS*. Esses pequenos RNA são específicos do tegumento das sementes e são resultantes (Tuteja et al. 2009) da transcrição dos genes *CHS1*, *CHS3* e *CHS4* arranjados em regiões repetidas e invertidas, resultando na geração de dsRNA (Tuteja et al., 2009). De forma similar, em milho observou-se que, quando o alelo *C2-Idf* (*colorless2*; contendo o gene mutado da chalcona sintase) está em homozigose, as plantas são despigmentadas (pericarpo, camada de aleurona do endosperma, órgãos vegetativos). Em plantas heterozigotas, com o alelo *C2* funcional, há um fenótipo intermediário, com plantas menos pigmentadas (Vedova et al., 2005). O alelo *C2-Idf* foi clonado e sequenciado e isso mostrou que sua estrutura é muito diferente do alelo normal *C2*. No alelo *C2-Idf*, duas das três cópias do gene da chalcona sintase estão localizados próximo e com orientações invertidas, levando a uma redução na quantidade de mRNA e enzima (Dooner, 1983; Franken et al., 1991). Foram encontrados siRNA em plantas contendo o alelo *C2-Idf*, mas não em plantas homozigotas

normais contendo o alelo *C2,* indicando que o fenótipo de despigmentação é mediado por RNAi (Vedova et al., 2005).
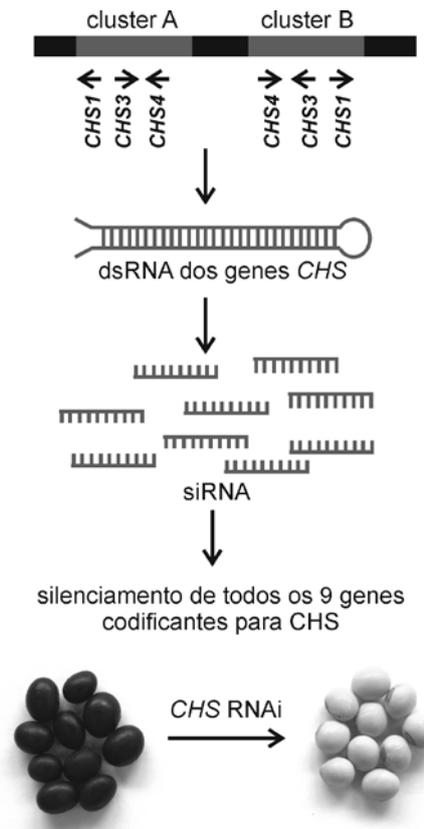
Figura 4.2. Silenciamento dos genes *CHS* que codificam para a enzima chalcona sintase (uma enzima chave da via de síntese de antocianinas) em soja. Devido à presença de sequencias repetidas e invertidas nos genes *CHS1*, *CHS3* e *CHS4*, há a formação de um RNA na forma de grampo (dsRNA) que é processado para a geração de siRNA, o que leva ao silenciamento de todos os 9 genes CHS. Com isso, as sementes apresentam o fenótipo despigmentado (amareladas).

Outro exemplo bem caracterizado é o do arroz com baixo teor de glutelina. O consumo de produtos com menores quantidades de glutelina é importante para pacientes celíacos, que têm restrição ao consumo dessa proteína. O fenótipo de baixo teor de glutelina é gerado pelo mecanismo de RNAi, causado por duas cópias invertidas e próxima do gene que codifica para glutelina no lócus *Lgc1* (Kusaba et al., 2003).

Uma série de técnicas que dependem de vias de RNAi têm sido desenvolvidas e aplicadas extensivamente para nocautear genes de interesse em vários culturas com a finalidade de melhorar produtividade, conferir resistência e/ou tolerância a diversas pragas e doenças (Wang et al., 2000; Shekhawat et al ,2012; Bonfim et al., 2007). Além disso, a técnica de RNAi tem sido usada para melhorar o valor nutricional em plantas, assim como o aproveitamento otimizado de matérias-primas derivadas de plantas para uso industrial (Ossowski et al., 2008; Sunilkumar et al., 2006; Yin et al., 2007; Shimada et al., 2005). Essas estratégias de engenharia genética levaram ao desenvolvimento de diversos tipos de vetores. Os principais estão mostrados na Figura 4.3. Por exemplo, em arroz, usou-se o vetor pNW55, que contém o precursor de um microRNA natural (osa-MIR528 de earroz) no qual se colocou a sequência de um microRNA artificial (desenhado para silenciar os genes *Pds*, *Spl11* e *Eui1/CYP714D1*) (Warthmann et al., 2008). Anteriormente, uma estratégia semelhante havia sido utilizada em *A. thaliana* para silenciar o gene *P69* do *Turnip yellow mosaic virus* e o gene HC-pro do *Turnip mosaic virus*. Foi construído um vetor para expressar miRNA artificiais (amiRNA) usando o miR159 de *A. thaliana*. As plantas geradas foram resistentes aos dois vírus, mesmo em temperaturas baixas (15°C) (Niu et al., 2006). Estratégias como essa podem ser muito úteis para silenciar genes endógenos de plantas cujo genoma esteja bem conhecido. No entanto, para o silenciamento de genes de patógenos (vírus, fungos, nematóides), quer *in cis* ou *in trans*, a especificidade gera o inconveniente de dificultar a obtenção de resistência ampla e duradoura.
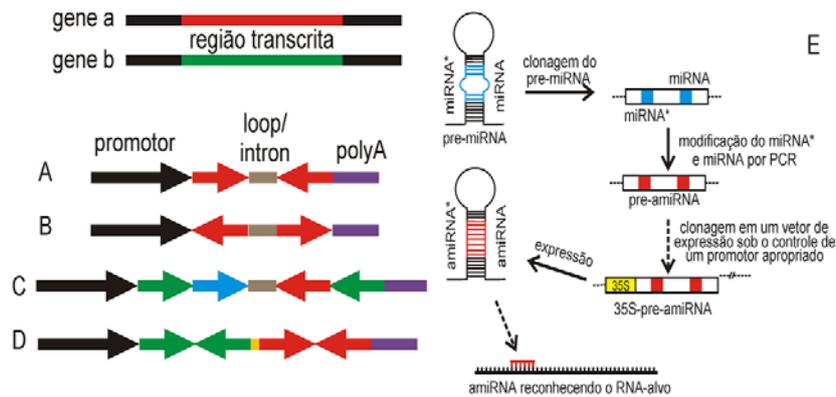
Figura 4.3. Vetores para silenciamento gênico em plantas transformadas estavelmente são, em geral, construídos para que se forme uma estrutura de grampo (após a transcrição (RNA de dupla fita ou dsRNA). Nesta figura, uma sequência da região transcrita de um gene é amplificada é posicionada sob o controle de um promotor, em orientação direta (sense) e reversa (antisense) intercaladas por um intron ou uma região espaçadora (loop) (A, B). Sequencias de 2 ou mais genes podem ser usadas em um mesmo cassete de expressão (C). Também é possível se utilizar da estratégia de ter dois cassetes de expressão com os fragmentos gênicos clonados em orientação direta e reversa intercalados por um espaçador (D). Em (E) tem-se o vetor para gerar um miRNA modificado pela introdução de sequencias do gene alvo em um miRNA natural, como o miR159 de *A. thaliana* e miR528 de *Oriza sativa*). Com esse vetor serão expressos miRNA artificiais (amiRNA) cujo alvo podem ser tanto genes endógenos quanto de patógenos intracelulares.

Resistência a vírus

Muitas plantas são resistentes a vírus devido à ocorrência natural de dsRNA e siRNA, cujos alvos são regiões gênicas virais importantes para o ciclo do patógeno. Em soja, por exemplo, identificou-se a ocorrência de siRNA com 100% de similaridade com distintas regiões gênicas e intergênicas do *Mungbean yellow mosaic India virus* (MYMIV), um begomovírus causador da doença conhecida como mosaico amarelo. Na linhagem resistente PK416, os siRNA encontrados correspondiam à região intergênica (IR) do MYMIV, enquanto que, na variedade suscetível, a maioria dos siRNA encontrados correspondiam a regiões gênicas e estavam em menor concentração. Também se demonstrou que o genoma viral em plantas resistentes estava metilado na região intergênica (Yadav & Chattopadhyay, 2011).

Embora a resistência a vírus mediada por RNAi seja um fenômeno natural, não está presente de forma efetiva em muitas linhagens produtivas. Em outros casos, o aparecimento de siRNA com identidade às sequencias virais ocorre apenas na infecção tardia, quando já não são estequiometricamente favoráveis para controlar a infecção (Rodríguez-Negrete et al., 2009; Aregger et al., 2012). No entanto, esse mecanismo pode ser mimetizado e introduzido com o uso da tecnologia do DNA recombinante, no sentido de gerar plantas resistentes ou imunes às viroses. Atualmente, há dezenas de exemplos em que estratégias de RNAi têm sido usadas para obter plantas resistentes a vírus tanto com genoma de RNA quanto de DNA (Prins et al., 2008; Runo, 2011; Prins, 2003; Vanderschuren et al., 2007; Bonfim et al., 2007; Aragão & Faria, 2009; Lucioli et al., 2003; Fuentes et al., 2006; Vanderschuren et al., 2009; Hashmi et al., 2011; Vanderschuren et al., 2012).

O primeiro exemplo de desenvolvimento de estratégias contra doenças virais ocorreu em 1986, quando uma planta de fumo foi transformada com o gene da capa proteica do *Tobacco mosaic virus* (TMV) (Beachy et al., 1987). Depois disso, mais de 100 publicações têm relatado a obtenção de plantas geneticamente modificadas resistentes a vírus dos mais variados grupos. As primeiras variedades disponibilizadas para o produtor foram: o fumo resistente ao vírus do mosaico (TMV), na China, e o mamoeiro resistente ao vírus da

mancha anelar (PRSV), que vêm sendo cultivados nos Estados Unidos desde 1998. Outras plantas têm sido autorizadas para comercialização nos Estado Unidos, como abóboras resistentes aos vírus WMV, ZYMV e CMV, e batata resistente a viroses. O primeiro exemplo em que uma planta foi deliberadamente transformada com uma construção para expressar um dsRNA (construção do tipo *intron hairpin* - hpRNA) ocorreu em 2000. Foram transformadas plantas de trigo com sequências do gene da polimerase do *Barley yellow dwart virus* (BYDV). As linhagens desenvolvidas a partir dessa estratégia foram consideradas imunes, pois não houve detecção do vírus por ELISA (Wang et al., 2000).

Pouco tempo depois, foi gerado um tabaco transgênico expressando RNAs sense e antisense de *Cotton leaf curl virus* DNA A (CLCuV DNA A) que inibiu a replicação tanto do DNA-A quanto do DNA-B virais e conferiu resistência ao *Cotton leaf curl virus* nos fumos transgênicos, mantendo as plantas livres dos sintomas de infecção (Asad et al., 2003). Adicionalmente, pesquisadores obtiveram resistência contra *Tomato yellow leaf curl Sardinia virus* (TYLCSV) usando construções de RNA do tipo *hairpin* contendo o gene da proteína Rep truncado (Yang et al., 2004). Numa tentativa de aplicação dessa técnica em leguminosas, Poogin et al. (2003) usaram uma construção de dsRNA para silenciar a sequência de promotor de DNA A de VMYMV, levando à expressão de RNA dupla fita da região conservada de *Vigna mungo yellow mosaic virus* (VMYMV) em *Vigna* spp., o que resultou na resistência à infecção viral.

Bonfim et al. (2007) exploraram a tecnologia de RNA interferente usando uma sequência do gene viral AC1 que codifica um complexo multifuncional de Rep do *Bean golden mosaic virus* (BGMV) para gerar plantas transgênicas de feijão comum (*Phaseolus vulgaris* L.) altamente resistentes a geminivírus. O gene viral (*AC1* ou *rep*) foi escolhido para a construção do vetor de transformação porque a proteína Rep exerce uma função essencial no ciclo de infecção viral, sendo a única proteína requerida para a replicação do genoma viral. O vetor utilizado foi construído a partir de um fragmento de DNA de 411 pb do gene *AC1* do BGMV. Isso levou ao desenvolvimento do evento Embrapa 5.1, gerando a primeira linhagem transgênica aprovada para uso comercial aplicando tecnologia brasileira e seguindo as normas de biossegurança estabelecidas pela Comissão

Técnica Nacional de Biossegurança (CTNBio). Essa estratégia também poderá ser aplicada a outras doenças devastadoras, como geminivírus que atacam milho e mandioca na África, além do tomate por todo o mundo (Aragão & Faria, 2009).

Silenciamento *in trans*

O grande potencial de manipulação da técnica de RNAi foi demonstrado com a descoberta de que uma planta geneticamente modificada pode ser usada para controlar organismos patogênicos via siRNA. Tinoco et al. (2010) demonstraram o fenômeno de interferência *in vivo* no fungo patogênico *Fusarium verticillioides*, no qual a expressão de um transgene foi especificamente silenciada pela inoculação de células do micélio em plantas transgênicas de fumo transformadas geneticamente para expressar pequenos siRNA a partir de um dsRNA correspondente ao transgene. Isso se mostrou uma poderosa ferramenta para estudos sobre a interação molecular planta-patógenos e interações simbióticas. Do ponto de vista biotecnológico, o silenciamento de genes de fungos a partir de siRNAs no hospedeiro permite o desenvolvimento de estratégias de resistência a fungos nas plantas e outros organismos.

O movimento do sinal de silenciamento também foi observado por Waterhouse et al. (1998) quando, em nematoides, o silenciamento de genes foi desencadeado por uma dieta composta de plantas transgênicas modificadas para expressar dsRNA. O mesmo fenômeno foi observado em insetos herbívoros alimentados com uma planta geneticamente modificada para expressar dsRNAs de um gene vital dos insetos (Baum et al., 2007; Mao et al., 2007).

Ainda em 2007, Roney et al. constataram o movimento sistêmico de mRNA transitando através do floema entre tomate e a planta parasita cuscuta (*Cuscuta pentagona* Engelm.). Experimentos descritos por Tomilov et al. (2008) também mostraram que plantas hospedeiras transformadas com construções para gerar grampos de interferência podem silenciar a expressão do gene alvo na planta parasita. Raízes transgênicas da planta parasita *Triphysaria versicolor* expressando o gene repórter *gus* parasitaram raízes transgênicas de alface expressando o grampo de RNA contendo um fragmento do

gene *gus* (hpGUS). Adicionalmente, Aly et al. (2009) mostraram que uma construção contendo o vetor binário pBIN-IR-M6PR inserido em plantas de tomate pode silenciar a expressão do gene *M6PR* em tubérculos de *Orobanche* que parasitam as raízes transgênicas. A observação de que moléculas responsáveis pelo silenciamento produzidas no hospedeiro são funcionais no parasita sugere uma nova estratégia para a engenharia de plantas resistentes a parasitas.

Controle de insetos-praga

Embora existam protocolos para o controle de pragas transmitidas pelos insetos lepidópteros e coleópteros empregando a expressão da proteína inseticida de *Bacillus thuringiensis* (toxina Bt), com o surgimento da resistência à toxina Bt por alguns biótipos de insetos, tornou-se necessário o desenvolvimento de novas estratégias de controle envolvendo um diferente modo de ação (Baum et al., 2007). O silenciamento de genes essenciais para os insetos mediado por dsRNA por meio da técnica de RNAi pode provocar a interrupção da alimentação e morte. Foi demonstrado que a ingestão de RNAs fornecidos em uma dieta artificial provoca interferência de RNA em várias espécies de coleópteros; como *Diabrotica* sp. (Gordon e Waterhouse, 2007; Baum et al., 2007; Gatehouse, 2008; Upadhyay et al., 2011). Adicionalmente, plantas transgênicas de milho foram desenvolvidas para expressar dsRNAs contra genes de *Diabrotica virgifera*, sugerindo que a via de RNAi pode ser explorada para controlar insetos-praga nas plantas por meio de expressão de um dsRNA (Baum et al., 2007).

O fato de a maquinaria de RNAi estar presente em todos os insetos faz com que o potencial para a utilização de estratégias RNAi para o seu controle seja bastante promissor, o que permite a interrupção da expressão de genes essenciais. Isso é possível mesmo para espécies de insetos que não possuem uma resposta sistêmica de RNAi, pois genes expressos no intestino médio são suscetíveis ao silenciamento por dsRNA quando ingeridos (Huvenne e Smagghe, 2010).

Melhoramento quanto ao valor nutricional

Embora muitas plantas sejam consideradas fontes de proteínas, algumas são deficientes em aminoácidos essenciais ou esses são acumulados em compartimentos, o que torna a proteína e/ou aminoácido inacessível ao homem. Nesse sentido, vários programas de melhoramento genético buscam aumentar os níveis de aminoácidos de modo que eles sejam benéficos e biodisponíveis (Tu et al., 1998; Marcelino et al., 1996). Dentre esses aminoácidos estão a lisina e os aminoácidos contendo enxofre. Entretanto, enquanto se busca aumentar o teor de lisina em sementes, altas concentrações desse aminoácido nos tecidos vegetativos podem ter efeito negativo, acarretando desenvolvimento anormal da flor. A via da biossíntese deste aminoácido está sob regulação pelo mecanismo de *feedback* na qual a lisina inibe a atividade de dihidrodipicolinate sintase (DHPS), a primeira enzima da sua biossíntese. A técnica de RNAi foi usada para melhorar a germinação em sementes de *Arabidopsis*, silenciando a DHPS (Zhu e Galili., 2003; Zhu e Galili, 2004; Tang et al., 2007).

A mesma abordagem foi usada em milho, para aumentar o nível de lisina nas sementes, manipulando-se o gene da proteína zeína, uma proteína usualmente associada com uma baixa qualidade nutricional do milho. Usando construções de RNAi derivadas de um fragmento de 22-kd do gene da zeína, pesquisadores geraram um fenótipo dominante de milho opaco, com nível reduzido de zeína. A diminuição do nível de zeína levou ao aumento do nível de lisina nas plantas de milho transgênicas transformadas com as construções de RNAi, aumentando o seu valor nutricional, além de favorecer a germinação das sementes (Segal et al., 2003).

A técnica de RNAi foi utilizada para silenciar o gene de enzima mioinositol-1-fosfato sintase (GmMIPS) em soja. Essa enzima está em um importante passo para a síntese de fitatos em sementes. Os fitatos são fatores antinutricionais que quelatam minerais bivalentes (como zinco, cálcio, ferro e outros) presentes no alimento, reduzindo seu valor nutricional. Além disso, os fitatos são eliminados nas fezes e se tornam um importante fator de contaminação ambiental. Para produzir plantas de soja com o gene *GmMIPS1* silenciado, foi construído um vetor (pMIPSGm) no qual fragmentos do gene *GmMIPS1* foram clonados em sentido invertido, gerando braços sense

e antisense. As plantas de soja transformadas apresentaram silenciamento parcial desse gene e possibilitaram a geração de linhagens com baixo teor de fitatos, com uma redução de até 94,5% (Nunes et al., 2006).

Metabólitos secundários

Além de características agronômicas, matérias-primas industriais derivadas de plantas podem ser manipuladas com o uso de RNAi. Essa estratégia tem sido utilizada para a interferência em vias de metabólitos secundários com a finalidade de gerar compostos químicos para uso farmacêutico e alelopático. Cientistas mostraram que o silenciamento do gene da codeinona redutase (*COR*) em ópio (*Papaver somniferum*) levou ao acúmulo de alcaloides não narcóticos (Allen et al., 2004). Em algodão, a tecnologia foi usada objetivando um nível reduzido de gossipol, um composto tóxico acumulado nas sementes que inviabiliza o algodão como fonte de proteína para humanos. Esse objetivo foi alcançado intervindo na expressão do gene da •-cadineno sintase durante o desenvolvimento da semente (Sunilkumar et al., 2006).

Perspectivas

Apesar de ser descoberto menos de 20 anos atrás, o mecanismo de RNAi tornou-se uma poderosa ferramenta para o entendimento de como os genes funcionam, seu papel nos mais diversos processos biológicos. Além disso, sua importância para o desenvolvimento de plantas com características úteis para a agricultura vem sendo reconhecida. Já há diversas tecnologias aprovadas para comercialização nos Estados Unidos e Brasil. Com aumento de conhecimento sobre os mecanismos biológicos e bioquímicos subjacentes à via de RNAi, haverá aumento na nossa capacidade de utilizar esse mecanismo como uma ferramenta experimental. Apesar dos obstáculos como a dependência de outras técnicas que, em alguns casos, não são reproduzíveis, a RNAi continuará a ser usada lado a lado com abordagens convencionais de melhoramento para o desenvolvimento de novos cultivares nos

próximos anos. Por outro lado, com o avanço das ferramentas para manipulação genética do genoma de plantas, é possível que seja mais eficiente o emprego de estratégias envolvendo o uso de *zinc-finger nucleases* (ZFNs) (Isalan, 2012), TALENs (Sanjana et al., 2012) e outras endonucleases (ver capítulo 8). Com essas estratégias, é possível mutagenizar de forma precisa e direcionada várias cópias de um gene, levando ao silenciamento, com produção de fenótipos desejáveis para a agricultura.

Os aspectos de biossegurança de RNAi em plantas geneticamente modificadas têm sido discutidos em revisões recentes (Hollingworth et al., 2003; Petrick et al., 2013; Parrott et al., 2010). Com base na evidência de que as diversas moléculas de RNA estão presentes na alimentação humana e animal (incluindo miRNA, siRNA, mRNA e dsRNA longos), é razoável supor que a princípio não haja razões para se esperar que seu consumo a partir de plantas geneticamente modificadas poderia trazer riscos para a saúde. Plantas têm em média 1 mg de RNA total por miligrama de tecido (Ivashuta et al., 2009; Lassek e Montag, 1990). Desse total, os RNA não codificantes (tRNAs, rRNAs, ssRNA-antisense, dsRNA de fontes externas, como vírus, miRNAs e siRNAs) são abundantes. Também há se considerar que o homem se alimenta de animais (cujo conjunto tem um histórico de consumo seguro) que contêm miRNA e siRNA com alta similaridade aos genes humanos (Carthew e Sontheimer, 2009; Petrick et al., 2013). Mesmo assim, cada tecnologia gerada baseada em RNAi deverá ser analisada para que se verifique sua segurança. Já existem algumas plantas geneticamente modificadas para expressar siRNA que foram colocadas no mercado, como o tomate Flavr Savr™ modificado para silenciar o gene da poligalacturonase nos frutos, abóbora resistente ao *Watermelon mosaic virus 2* e *Zucchini yellow mosaic virus* e o mamoeiro resistente ao *Papaya risgspot virus* (http://www.agbios.com).

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Appendix

Appendix

Resistência a mosca branca (*Bemisia tabaci*) em plantas transgênicas expressando siRNA do gene de uma *v-ATPase*

Table 1: Monitoring 20 adult whiteflies on non transgenic lettuce plants over 11 days

Control -	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11
1	20	19	18	18	16	13	9	6	4	2	2	1
2	20	20	19	19	18	12	10	7	3	1	1	1
3	20	20	18	17	16	11	11	8	4	3	2	1
4	20	20	19	19	14	10	7	4	3	2	1	0
5	20	20	19	17	15	14	8	6	4	2	2	1
6	20	19	19	17	15	13	6	4	4	4	3	2
7	20	18	17	14	12	9	6	3	2	2	1	1
8	20	19	19	18	14	12	8	5	3	2	1	1
9	20	19	19	18	13	10	7	7	4	3	2	0
10	20	20	19	16	11	10	6	2	2	2	1	1
11	20	19	19	18	15	12	9	4	3	2	1	0
12	20	19	15	13	10	9	7	4	3	3	2	1
Mean	20	19.3333	18.33333	17	14.08333	11.25	7.833333	5	3.25	2.333333	1.583333	0.833333
SD	0	0.623	1.1785	1.77	2.17785	1.58	1.5723	1.732	0.7216878	0.745355992	0.640095479	0.552770798

Table 2: Monitoring 20 adult whiteflies on transgenic control lettuce plants over 11 days

Control	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11
1	20	20	16	16	15	13	6	5	3	2	1	1
2	20	17	16	13	11	9	7	5	3	1	1	0
3	20	20	19	16	16	11	10	4	3	2	1	0
4	20	18	18	17	13	11	8	4	3	2	1	1
5	20	20	19	18	17	12	9	5	4	3	2	1
6	20	20	17	16	16	13	7	5	3	2	1	0
7	20	19	19	17	17	10	6	2	2	2	2	1
8	20	20	19	17	17	13	10	7	5	3	2	1
9	20	19	19	14	14	10	6	5	4	3	2	1
10	20	20	19	15	15	13	9	4	3	2	1	1
11	20	19	18	13	13	12	7	3	1	0	0	0
12	20	19	19	11	11	9	7	3	2	2	1	1
Mean	20	19.25	18.1666	15.25	14.58333	11.333333	7.6666666	4.3333333	3	2	1.25	0.6666666
SD	0	0.92	1.1426	2.00	2.09993	1.490711	1.43372	1.24721912	1	0.816496581	0.595119036	0.471404521

Table 3: Monitoring 20 adult whiteflies on transgenic test lettuce plants over 11 days

Line 1	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11
1	20	12	10	9	7	2	1	1	0	0	0	0
2	20	13	12	10	4	3	3	1	0	0	0	0
3	20	11	11	10	6	2	1	1	0	0	0	0
4	20	14	13	11	3	2	1	0	0	0	0	0
5	20	12	10	8	7	3	2	0	0	0	0	0
6	20	14	13	10	7	2	1	1	0	0	0	0
7	20	10	9	9	3	3	2	0	0	0	0	0
8	20	12	10	10	4	2	0	0	0	0	0	0
9	20	10	11	10	5	2	1	0	0	0	0	0
10	20	10	9	9	7	2	1	0	0	0	0	0
11	20	11	10	8	6	2	1	0	0	0	0	0
12	20	11	11	9	5	1	0	0	0	0	0	0
Mean	20	11.666666	10.75	9.4166666	5.3333333	2.1666666	1.1666666	0.3333333	0	0	0	0
SD	0	1.3743685	1.2990381	0.8620067	1.4907119	0.5527707	0.7993052	0.4714045	0	0	0	0

Line 3	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11
1	20	13	9	5	5	2	1	0	0	0	0	0
2	20	11	10	4	3	1	0	0	0	0	0	0
3	20	12	11	7	4	2	0	0	0	0	0	0
4	20	11	10	6	4	2	0	0	0	0	0	0
5	20	12	9	6	3	1	0	0	0	0	0	0
6	20	11	9	6	4	2	0	0	0	0	0	0
7	20	11	10	8	7	3	0	0	0	0	0	0

	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11
8	20	12	10	7	5	3	1	0	0	0	0	0
9	20	11	9	6	6	2	1	0	0	0	0	0
10	20	17	10	7	5	2	1	0	0	0	0	0
11	20	11	9	5	3	2	0	0	0	0	0	0
12	20	10	9	5	4	3	0	0	0	0	0	0
Mean	20	11.833333	9.5833333	6	4.4166666	2.0833333	0.3333333	0	0	0	0	0
SD	0	1.7240134	0.6400954	1.0801234	1.1873172	0.6400954	0.4714045	0	0	0	0	0
Line 4	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11
1	20	13	11	10	7	4	4	3	1	1	0	0
2	20	13	11	8	6	5	4	2	1	0	0	0
3	20	15	13	6	3	3	3	1	1	1	0	0
4	20	13	12	7	6	6	6	5	1	0	0	0
5	20	11	8	7	4	3	3	1	0	0	0	0
6	20	13	13	5	4	2	2	0	0	0	0	0
7	20	11	8	5	4	3	3	1	0	0	0	0
8	20	15	10	6	4	2	2	0	0	0	0	0
9	20	11	11	9	6	2	2	2	1	0	0	0
10	20	14	8	4	3	2	2	1	1	0	0	0
11	20	13	10	8	5	3	2	0	0	0	0	0
12	20	14	12	11	6	4	4	1	1	1	0	0
Mean	20	13	10.583333	7.1666666	4.8333333	3.25	3.0833333	1.4166666	0.58333	0.25	0	0
SD	0	1.3540064	1.7539637	2.0344259	1.2801909	1.2332207	1.1873172	1.3819269	0.4930	0.43	0	0
Line 6	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11

	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11
1	20	10	9	9	7	2	1	0	0	0	0	0
2	20	11	10	9	5	2	1	0	0	0	0	0
3	20	12	11	10	4	2	2	0	0	0	0	0
4	20	18	10	7	3	3	1	0	0	0	0	0
5	20	11	10	8	4	3	2	1	0	0	0	0
6	20	15	13	9	5	6	3	1	0	0	0	0
7	20	11	10	9	7	3	2	0	0	0	0	0
8	20	15	11	7	6	2	1	0	0	0	0	0
9	20	11	10	7	5	3	0	0	0	0	0	0
10	20	13	11	10	7	4	2	0	0	0	0	0
11	20	13	10	7	4	3	2	0	0	0	0	0
12	20	12	12	11	5	4	1	0	0	0	0	0
Mean	20	12.666666	10.583333	8.5833333	5.1666666	3.0833333	1.5	0.1666666	0	0	0	0
SD	0	2.2110831	1.0374916	1.3202482	1.2801909	1.1149240	0.7637626	0.3726779	0	0	0	0
Line 19	**Day 0**	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11
1	20	16	12	10	4	3	2	1	0	0	0	0
2	20	17	13	10	3	3	2	1	0	0	0	0
3	20	13	11	8	4	2	1	1	0	0	0	0
4	20	15	12	7	6	3	1	1	0	0	0	0
5	20	13	10	7	5	3	1	0	0	0	0	0
6	20	15	13	9	7	3	1	1	0	0	0	0
7	20	14	11	9	5	3	2	1	0	0	0	0
8	20	16	11	7	4	3	2	1	0	0	0	0
9	20	13	10	9	6	4	0	0	0	0	0	0
10	20	17	14	10	5	2	1	0	0	0	0	0

	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11
11	20	15	10	8	6	2	1	0	0	0	0	0
12	20	14	11	7	6	3	2	1	0	0	0	0
Mean	20	14.8333333	11.5	8.41666666	5.08333333	2.83333333	1.33333333	0.66666666	0	0	0	0
SD	0	1.40435829	1.25830573	1.18731723	1.11492401	0.55277079	0.62360956	0.47140452	0	0	0	0
Line 25	**Day 0**	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11
1	20	17	10	9	3	1	1	0	0	0	0	0
2	20	16	11	9	3	2	1	1	0	0	0	0
3	20	14	10	9	6	2	2	2	0	0	0	0
4	20	17	11	8	4	3	2	0	0	0	0	0
5	20	11	10	8	6	2	1	0	0	0	0	0
6	20	10	10	8	4	1	0	0	0	0	0	0
7	20	17	12	10	5	2	2	2	0	0	0	0
8	20	13	12	12	3	1	1	1	0	0	0	0
9	20	11	10	7	5	2	0	0	0	0	0	0
10	20	15	12	10	4	1	1	0	0	0	0	0
11	20	16	13	9	5	3	2	2	0	0	0	0
12	20	14	10	6	4	3	3	2	0	0	0	0
Mean	20	14.25	10.9166666	8.75	4.33333333	1.91666666	1.33333333	0.83333333	0	0	0	0
SD	0	2.4195385	1.03749163	1.47901994	1.02740233	0.75920279	0.84983658	0.89752746	0	0	0	0
Line 31	**Day 0**	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11
1	20	13	10	6	6	1	1	0	0	0	0	0
2	20	13	10	6	5	3	2	1	0	0	0	0
3	20	16	12	10	7	3	1	0	0	0	0	0

4	20	14	11	7	6	2	2	0	0	0	0	0
5	20	12	10	8	6	2	1	0	0	0	0	0
6	20	16	10	6	5	2	2	0	0	0	0	0
7	20	13	13	10	7	2	1	0	0	0	0	0
8	20	15	11	7	5	3	2	0	0	0	0	0
9	20	14	10	6	4	4	0	0	0	0	0	0
10	20	14	11	6	5	3	1	0	0	0	0	0
11	20	17	11	6	5	2	0	0	0	0	0	0
12	20	11	9	7	4	2	1	0	0	0	0	0
Mean	20	14	10.666666	7.0833333	5.4166666	2.416666	1.166666667	0.083333333	0	0	0	0
SD	0	1.68325082	1.02740233	1.44096803	0.95379359	0.75920279	0.68718427	0.27638539	0	0	0	0

Table 4: Monitoring the emergence of eggs on lettuce plants

Eggs	Day					
Control -	1	5	9	13	17	21
1	280	190	59	36	19	51
2	302	181	72	55	17	29
3	260	204	95	61	26	40
4	216	96	99	100	21	36
5	310	148	165	51	20	18
6	163	112	92	70	4	2
7	191	198	72	72	20	44
8	173	204	68	44	2	29
9	283	174	108	62	21	43
10	269	116	42	50	16	39
11	237	122	90	109	45	33
12	95	104	55	39	17	31
Mean	231.5833333	154.0833333	84.75	62.41666667	19	32.91666667
SD	63.93809279	41.17081772	31.41621016	22.18286975	10.49223315	12.72080346
Eggs	Day					
Control	1	5	9	13	17	21
1	175	116	62	46	16	28
2	272	201	96	71	21	19
3	286	140	49	36	16	42
4	204	109	91	13	9	36
5	159	118	70	34	17	31
6	300	174	71	78	21	74
7	90	162	97	79	8	46

8	226	189	56	73	19	37
9	308	143	102	100	18	42
10	233	179	65	15	5	20
11	186	111	69	53	10	26
12	290	62	48	70	39	19
Mean	227.4166667	142	73	55.66666667	16.58333333	35
SD	65.74450988	39.7710841	18.58938454	26.6713764	8.642245414	15.11693551
Eggs	**Day**					
Line 1	**1**	**5**	**9**	**13**	**17**	**21**
1	58	33	15	10	2	0
2	91	34	5	4	0	0
3	54	24	5	0	0	0
4	53	31	8	11	1	0
5	95	53	37	3	1	0
6	17	19	10	9	1	0
7	87	61	32	13	4	0
8	90	66	41	5	0	0
9	63	44	10	6	0	0
10	81	25	16	2	0	0
11	45	32	7	4	0	0
12	29	20	10	3	0	0
Mean	63.58333333	36.83333333	16.33333333	5.833333333	0.75	0
SD	25.08232821	15.4178515	12.56865206	3.930556622	1.188715053	0
Eggs	**Day**					
Line 3	**1**	**5**	**9**	**13**	**17**	**21**
1	28	33	22	10	0	0

2	19	23	16	9	2	0
3	78	44	41	30	0	0
4	64	88	21	11	0	0
5	63	88	31	21	0	0
6	29	19	16	6	0	0
7	80	69	43	28	0	0
8	47	33	15	4	0	0
9	55	46	10	9	0	0
10	70	55	10	4	0	0
11	27	41	14	6	0	0
12	59	61	19	5	0	0
Mean	51.58333333	50	21.5	11.91666667	0.166666667	0
SD	20.74465183	22.44994432	10.88676899	9.0068412	0.564659703	0
Eggs	**Day**					
Line 4*	**1**	**5**	**9**	**13**	**17**	**21**
1	42	18	6	0	0	0
2	43	36	10	0	0	0
3	80	69	49	5	0	0
4	58	54	22	4	0	0
5	86	43	16	1	0	0
6	88	83	31	2	0	0
7	80	64	43	5	0	0
8	74	66	21	6	0	0
9	24	12	10	10	0	0
10	78	19	3	5	0	0
11	98	38	11	2	0	0

12	29	13	2	0	0	0
13	75	49	10	5	0	0
14	90	76	15	0	0	0
15	78	34	3	2	0	0
16	29	24	11	1	0	0
17	47	28	17	2	0	0
18	89	70	50	3	0	0
Mean	66	44.22222222	18.33333333	2.944444444	0	0
Eggs	**Day**					
Line 6	**1**	**5**	**9**	**13**	**17**	**21**
1	28	12	9	7	0	0
2	31	42	16	8	0	0
3	27	12	19	11	0	0
4	24	42	26	17	0	0
5	29	56	45	21	5	0
6	23	37	22	19	0	0
7	30	31	17	9	0	0
8	14	18	11	0	0	0
9	25	53	12	14	2	0
10	19	30	11	4	0	0
11	24	44	14	10	0	0
12	21	31	12	5	0	0
Mean	24.58333333	34	17.83333333	10.41666667	0.583333333	0
SD	4.817690829	14.26458064	9.698348881	6.191977044	1.471960144	0
Eggs	**Day**					
Line 19	**1**	**5**	**9**	**13**	**17**	**21**

1	55	31	12	3	0	0
2	63	75	20	4	0	0
3	41	44	20	13	0	0
4	64	25	5	2	0	0
5	71	36	18	5	0	0
6	21	12	7	11	0	0
7	80	31	25	6	0	0
8	36	27	21	9	0	0
9	63	21	6	21	0	0
10	54	61	10	5	0	0
11	49	33	27	13	0	0
12	61	40	18	11	0	0
Mean	54.83333333	36.33333333	15.75	8.583333333	0	0
SD	15.8132214	16.90553464	7.344030406	5.380453082	0	0
Eggs	Day					
Line 25	1	5	9	13	17	21
1	75	43	34	9	0	0
2	98	75	30	15	4	0
3	78	38	29	51	7	0
4	53	49	11	10	0	0
5	10	15	22	9	2	0
6	23	25	49	21	2	0
7	95	94	11	4	0	0
8	75	55	31	13	0	0
9	12	10	10	9	0	0
10	56	48	23	11	0	0

11	72	40	50	29	0	0
12	83	38	27	8	0	0
Mean	60.83333333	44.16666667	27.25	15.75	1.25	0
SD	29.99371915	22.97383515	12.96231997	12.66371056	2.171955641	0

Eggs	Day					
Line 31	1	5	9	13	17	21
1	15	45	4	4	0	0
2	65	40	12	30	0	0
3	21	18	10	9	0	0
4	17	10	11	6	0	0
5	32	39	6	4	0	0
6	19	10	9	9	0	0
7	22	37	5	8	0	0
8	31	48	19	20	0	0
9	28	37	19	11	0	0
10	44	36	35	7	0	0
11	13	29	10	7	0	0
12	23	19	6	4	0	0
	27.5	30.66666667	12.16666667	9.916666667	0	0
	14.3041496	12.93685892	8.493817018	7.511826425	0	0

Table 5: Monitoring the emergence of nymphs on lettuce plants

Nymphs	Day					
Control -	1	5	9	13	17	21
1	109	100	89	40	21	9
2	196	72	59	23	14	11
3	130	135	62	30	21	13
4	182	46	51	17	18	10
5	118	73	52	25	17	13
6	70	98	70	30	10	10
7	81	68	49	41	11	16
8	120	63	63	26	9	10
9	110	79	54	43	7	12
10	160	88	70	19	14	3
11	153	91	65	38	21	13
12	70	50	43	21	14	7
Mean	124.9166667	80.25	60.58333333	29.41666667	14.75	10.58333333
SD	40.50219175	23.84050262	12.03948817	8.900008142	4.793564526	3.269312741
Nymphs	**Day**					
Control	1	5	9	13	17	21
1	116	96	80	52	17	2
2	133	80	102	36	18	13
3	113	61	60	29	13	10
4	116	70	68	18	15	13
5	89	100	91	21	13	17

6	120	80	50	52	10	20
7	49	82	37	41	9	13
8	117	98	49	38	11	11
9	121	96	54	51	13	9
10	108	67	53	16	7	0
11	105	100	68	39	11	9
12	102	27	31	21	6	2
Mean	107.4166667	79.75	61.91666667	34.5	11.91666667	9.916666667
SD	20.96563786	21.01397051	20.61007914	13.09165018	3.598510159	5.948450048
Nymphs	**Day**					
Line 1	**1**	**5**	**9**	**13**	**17**	**21**
1	31	20	21	10	2	0
2	51	44	14	12	0	0
3	20	50	31	11	0	0
4	31	40	15	9	0	0
5	40	54	32	2	1	0
6	2	11	19	4	0	0
7	60	30	16	6	1	0
8	42	52	31	9	0	0
9	22	50	27	4	0	0
10	50	13	21	11	2	0
11	30	15	22	13	0	0
12	20	23	29	2	2	0
Mean	33.25	33.5	23.16666667	7.75	0.666666667	0
SD	15.90255653	16.24004712	6.458339177	3.870175819	0.868114732	0
Nymphs	**Day**					

Line 3	1	5	9	13	17	21
1	17	21	36	21	0	0
2	3	10	21	19	2	0
3	43	31	28	22	3	0
4	30	51	18	9	4	0
5	21	49	38	16	2	0
6	10	22	24	9	2	0
7	51	61	28	15	4	0
8	33	23	22	22	3	0
9	40	22	31	11	0	0
10	36	40	26	9	1	0
11	15	17	26	13	0	0
12	40	41	31	21	3	0
Mean	28.25	32.33333333	27.41666667	15.58333333	2	0
SD	14.51311251	15.45446795	5.800424772	5.199637669	1.444630237	0
Nymphs	**Day**					
Line 4*	1	5	9	13	17	21
1	24	7	3	6	0	0
2	56	15	29	11	0	0
3	58	56	55	13	2	0
4	60	41	36	10	0	0
5	102	35	14	17	5	0
6	46	49	40	11	0	0
7	84	47	28	12	2	0
8	52	53	24	15	2	0
9	45	21	27	15	4	0

10	39	25	23	10	0	0
11	58	21	26	9	5	0
12	29	29	13	2	0	0
13	22	20	27	13	3	0
14	65	86	28	16	3	0
15	98	41	43	4	0	0
16	36	23	12	2	0	0
17	32	38	29	13	3	0
18	43	85	21	16	3	0
Mean	52.72222222	38.44444444	26.55555556	10.83333333	1.777777778	0
Nymphs	**Day**					
Line 6	**1**	**5**	**9**	**13**	**17**	**21**
1	17	5	15	5	0	0
2	12	21	25	7	2	0
3	9	29	3	9	0	0
4	20	24	13	10	0	0
5	15	43	23	6	0	0
6	14	40	10	19	2	1
7	6	44	12	7	2	0
8	0	11	9	0	0	0
9	10	20	3	4	0	0
10	40	18	6	1	1	0
11	12	19	9	3	2	0
12	12	20	11	5	2	0
Mean	13.91666667	24.5	11.58333333	6.333333333	0.916666667	0.083333333

Nymphs	Day					
Line 19	**1**	**5**	**9**	**13**	**17**	**21**
1	43	42	7	2	2	0
2	55	48	16	3	0	0
3	16	30	11	7	0	0
4	28	19	4	3	0	0
5	40	24	8	5	0	0
6	14	25	11	6	2	0
7	39	38	31	3	2	0
8	30	40	17	4	1	0
9	40	30	8	4	0	0
10	30	42	9	3	0	0
11	26	31	6	6	0	0
12	39	21	15	6	1	0
Mean	33.33333333	32.5	11.91666667	4.333333333	0.666666667	0
Nymphs	Day					
Line 25	**1**	**5**	**9**	**13**	**17**	**21**
1	40	26	14	4	0	0
2	82	35	19	28	3	0
3	60	20	29	30	3	0
4	49	17	14	11	0	0
5	0	9	24	17	2	0
6	0	15	20	31	2	0
7	45	44	13	7	0	0
8	63	47	41	21	0	0

9	0	7	3	6	0	0
10	42	35	13	10	0	0
11	32	20	32	25	2	0
12	45	47	14	19	0	0
	38.16666667	26.83333333	19.66666667	17.41666667	1	0
Nymphs	**Day**					
Line 31	**1**	**5**	**9**	**13**	**17**	**21**
1	10	17	7	7	0	0
2	31	30	17	23	7	0
3	17	29	11	7	0	0
4	19	8	17	4	3	0
5	20	33	31	26	1	0
6	10	10	11	10	0	0
7	10	18	19	11	3	0
8	12	22	16	12	0	0
9	15	20	13	12	0	0
10	34	19	13	8	1	0
11	9	21	13	4	4	0
12	10	3	4	3	0	0
Mean	16.41666667	19.16666667	14.33333333	10.58333333	1.583333333	0

Table 6: Monitoring the emergence of pupa on lettuce plants

Pupa	Day				
Control -	5	9	13	17	21
1	4	11	21	9	3
2	6	9	25	11	3
3	17	3	12	15	3
4	9	14	29	4	4
5	5	25	23	10	1
6	4	4	21	6	2
7	4	13	37	16	0
8	4	15	29	5	5
9	10	16	34	6	2
10	4	9	10	5	4
11	5	14	36	13	2
12	2	26	16	6	3
Mean	6.166666667	13.25	24.41666667	8.833333333	2.666666667
Pupa	Day				
Control	5	9	13	17	21
1	4	4	10	5	5
2	3	3	27	6	2
3	4	25	29	13	3
4	5	23	14	9	0
5	6	4	26	7	3
6	7	17	38	10	1
7	4	5	15	6	5

8	13	17	44	14	2
9	3	6	10	11	3
10	5	17	24	11	3
11	3	6	33	9	0
12	2	14	15	8	2
Mean	4.916666667	11.75	23.75	9.083333333	2.416666667
Pupa	**Day**				
Line 1	**5**	**9**	**13**	**17**	**21**
1		0	6	1	0
2		2	4	0	0
3		3	12	1	0
4		6	7	2	0
5		3	3	1	0
6		6	11	1	0
7		2	14	1	0
8		1	11	0	0
9		3	5	0	0
10		2	11	1	0
11		0	12	0	0
12		3	0	2	0
Mean		2.583333333	8	0.833333333	0
Pupa	**Day**				
Line 3	**5**	**9**	**13**	**17**	**21**
1		3	1	0	0
2		2	2	2	0
3		7	11	3	0

	Day 5	Day 9	Day 13	Day 17	Day 21
4		5	4	4	0
5		6	2	1	0
6		3	6	3	0
7		7	5	3	0
8		7	13	3	0
9		4	2	4	0
10		3	10	2	0
11		2	3	3	0
12		6	12	2	0
Mean		4.583333333	5.916666667	2.5	0
Pupa	**Day**				
Line 4*	5	9	13	17	21
1		0	4	0	0
2		4	15	4	0
3		9	23	4	0
4		8	13	2	0
5		5	20	9	1
6		7	32	4	0
7		7	23	6	0
8		8	34	4	3
9		4	9	4	0
10		9	11	2	0
11		5	10	2	0
12		2	0	0	0
13		3	13	7	0
14		7	11	5	0

15		9	7	4	0
16		3	27	6	0
17		7	13	1	0
18		6	6	3	0
Mean		5.722222222	15.05555556	3.722222222	0.222222222

Pupa	Day				
Line 6	5	9	13	17	21
1		3	2	0	0
2		4	3	2	0
3		2	1	0	0
4		2	3	1	0
5		8	3	0	0
6		4	3	2	0
7		10	2	6	0
8		8	3	0	0
9		15	0	2	0
10		12	1	2	0
11		0	1	0	0
12		3	2	6	0
Mean		5.916666667	2	1.75	0
Pupa	**Day**				
Line 19	5	9	13	17	21
1		1	5	2	0
2		2	1	0	0
3		0	2	0	0

4		0	1	0	0
5		2	3	1	0
6		4	2	2	0
7		3	4	1	0
8		2	3	1	0
9		2	1	0	0
10		1	2	1	0
11		0	2	0	0
12		2	1	0	0
Mean		1.583333333	2.25	0.666666667	0
Pupa			**Day**		
Line 25	5	9	13	17	21
1		6	1	1	0
2		6	10	2	0
3		9	6	3	0
4		0	2	0	0
5		5	3	1	0
6		2	10	0	0
7		3	7	1	0
8		6	11	0	0
9		0	4	1	0
10		2	9	1	0
11		4	5	1	0
12		2	4	1	0
Mean		3.75	6	1	0

Pupa	Day				
Line 31	5	9	13	17	21
1		1	9	10	1
2		2	7	7	0
3		1	2	2	0
4		2	11	3	0
5		2	2	2	0
6		0	11	1	0
7		4	2	2	0
8		3	1	1	0
9		9	12	2	0
10		4	4	4	0
11		3	7	1	0
12		1	5	11	0
Mean		2.666666667	6.083333333	3.833333333	0.083333333

Table 7: Monitoring the emergence of adult whiteflies on lettuce plants

Adult	Day			
Control -	9	13	17	21
1	3	14	15	6
2	5	6	21	7
3	13	12	19	1
4	3	9	7	3
5	9	14	11	7
6	4	20	9	11
7	7	16	7	3
8	10	5	16	7
9	10	21	19	2
10	5	7	6	4
11	9	10	20	6
12	5	19	10	3
Mean	6.916666667	12.75	13.33333333	5
Adult	Day			
Control	9	13	17	21
1	3	4	13	3
2	3	16	18	3
3	5	7	11	6
4	5	16	9	4
5	6	10	8	5
6	6	12	16	7
7	7	15	7	11

8	7	20	6	6
9	9	9	17	9
10	9	5	11	4
11	10	19	10	3
12	11	17	10	3
Mean	6.75	12.5	11.33333333	5.333333333
Adult	**Day**			
Line 1	**9**	**13**	**17**	**21**
1	0	2	0	0
2	0	4	0	0
3	0	3	2	0
4	0	3	1	0
5	0	1	2	0
6	0	0	1	0
7	0	4	1	0
8	0	2	0	0
9	0	2	0	0
10	0	4	0	0
11	0	3	0	0
12	0	4	2	0
Mean	0	2.666666667	0.75	0
Adult	**Day**			
Line 3	**9**	**13**	**17**	**21**
	1	1	3	0
1	0	3	1	0

2	2	4	2	0
3	1	3	0	0
4	2	2	1	0
5	0	1	2	0
6	2	3	2	0
7	0	2	2	0
8	1	4	1	0
9	0	1	2	0
10	0	1	1	0
11	0	3	1	0
12	0.75	2.333333333	1.5	0
Mean				
Adult	**Day**			
Line 4*	**9**	**13**	**17**	**21**
1	0	2	2	0
2	0	9	1	0
3	0	4	2	0
4	0	4	1	0
5	0	7	5	1
6	0	16	3	0
7	0	9	2	0
8	0	10	2	2
9	0	5	1	0
10	0	9	1	0
11	0	3	2	0
12	0	0	0	0

13	0	4	2	0
14	0	10	3	1
15	0	3	2	1
16	0	3	2	1
17	0	2	2	0
18	0	1	2	0
Mean	0	5.611111111	1.944444444	0.333333333
Adult	**Day**			
Line 6	**9**	**13**	**17**	**21**
1	0	1	0	0
2	0	2	1	2
3	0	3	0	0
4	1	2	3	0
5	1	1	2	0
6	1	5	3	1
7	0	2	1	2
8	0	1	0	3
9	0	3	0	5
10	0	3	1	1
11	0	2	1	2
12	1	6	4	1
Mean	0.333333333	2.583333333	1.333333333	1.416666667
Adult	**Day**			
Line 19	**9**	**13**	**17**	**21**

1	1	5	1	0
2	1	1	0	0
3	0	1	0	0
4	0	1	0	0
5	0	7	1	0
6	1	1	2	0
7	2	6	1	0
8	1	1	3	0
9	0	2	0	0
10	1	1	3	0
11	0	2	2	0
12	1	1	0	0
Mean	0.666666667	2.416666667	1.083333333	0
Adult	**Day**			
Line 25	**9**	**13**	**17**	**21**
1	1	1	3	0
2	0	7	3	0
3	3	23	6	0
4	0	5	0	0
5	3	3	0	0
6	1	9	0	0
7	3	3	2	0
8	1	9	0	0
9	0	3	1	0
10	1	2	1	0

11	1	4	3	0
12	0	1	0	0
Mean	1.166666667	5.833333333	1.583333333	0

Adult	Day			
Line 31	9	13	17	21
1	1	2	1	5
2	1	16	3	0
3	1	1	0	0
4	1	1	2	0
5	1	10	2	0
6	0	0	0	2
7	0	6	2	0
8	2	3	0	0
9	4	6	0	0
10	0	0	1	0
11	0	2	4	0
12	2	2	0	0
Mean	1.083333333	4.083333333	1.25	0.583333333

Control- are Non-transgenic plants, Control are transgenic plants expressing *bar* gene
Transgenic test lines: 1, 3, 4, 6, 19, 25 and 31
Upper and lower values excluded before Tukey analysis to maintain n= 12 throughout