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**The effects of RNA interference on the expression level of
pea aphid (*Acyrtosiphon pisum*) sugar transporter gene
(*Ap_ST1*)**

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Abstract

The pea aphid (*Acyrtosiphon pisum*) is a serious pest which attacks a number of important crops worldwide, including grains, corn, potatoes and alfalfa, and it spreads viral diseases among the plants it feed on. It reproduces sexually and asexually and, since it occurs within a short period aphids can establish many colonies and spread easily. Interestingly, it has been found that pea aphids have a high number of sugar transporter facilitator encoding genes, and the most highly expressed of these is *Ap_ST1*, which is expressed in the aphid's gut, which functions as a transporter for the sugar molecules; mainly mannose and glucose.

The newly discovered tool RNA interference (RNAi) has become a powerful technique to improve plants resistance against insects due to its high specificity. This study demonstrates that using conventional dsRNA against the sugar transporter gene *Ap_ST1* reduces the gene's expression level. Using a range of dsRNA concentrations proves that RNAi trigger effect can be occur when applying dsRNA in a small quantity. The effect of RNAi was only observed on the gene expression level and there was no alteration noticed on the phenotype or the pest movement. This is due to the presence of the other sugar transporter encoding gene *Ap_ST3*, which facilitates the transportation of the sugar molecules into the gut. Thus, this study shows that RNAi could be a promising alternative biological tool in the control and management of pea aphids.

Abbreviations

Amino acids abbreviations

<u>Amino acid</u>	<u>Single letter</u>
<u>Glycine</u>	<u>G</u>
<u>Alanine</u>	<u>A</u>
<u>Valine</u>	<u>V</u>
<u>Leucine</u>	<u>L</u>
<u>Isoleucine</u>	<u>I</u>
<u>Methionine</u>	<u>M</u>
<u>Phenylalanine</u>	<u>F</u>
<u>Proline</u>	<u>P</u>
<u>Serine</u>	<u>S</u>
<u>Threonine</u>	<u>T</u>
<u>Cysteine</u>	<u>C</u>
<u>Tyrosine</u>	<u>Y</u>
<u>Asparagine</u>	<u>N</u>
<u>Glutamine</u>	<u>Q</u>
<u>Aspartic acid</u>	<u>D</u>
<u>Glutamic acid</u>	<u>E</u>
<u>Lysine</u>	<u>K</u>
<u>Arginine</u>	<u>R</u>
<u>Histidine</u>	<u>H</u>

DNA	Deoxyribo Nucleic Acid
cDNA	Complementary DNA
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
TMV	Tobacco mosaic virus
siRNA	Small interfering RNA
dsRNA	Double stranded RNA
mRNA	Messenger RNA
ssRNA	Small stranded RNA
RISC	RNA-induced silencing complex
ATP	Adenosine triphosphate
GSP	Gene specific primers
nt	Nucleotide

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Chapter 1 INTRODUCTION

1.1 The need to protect crops

Crop protection and world food security are two faces of the same coin because crop yields need to increase to allow more food to be grown on a land area that is shrinking. They have become real global concerns as a result of the continuous growth in the world's population, which is predicted to rise to more than 8 billion within the next fifteen years. According to McCalla (1994), this prediction, along with increases in individual incomes and urbanization, means that food supplies need to be more than doubled by 2025. Therefore, the factors that can limit agricultural production need to be studied in order to increase crop yields.

Pest infestations cause both direct and indirect damage to crops, and pose a grave threat to agriculture. Worldwide, it is estimated that there are 10,000 species of insects and mites, 30,000 weed species and 100,000 species of plant pathogens that attack crops, and more than 60% of crops pests are considered to have serious effects on world crop production (Hall, 1995; Dhaliwal, *et al.*, 2007; UNEP, 2010). These pests cause crop losses estimated at millions of dollars.

Direct losses are caused where the pest consumes or damages crops. For example, locusts, which spread in warm regions, destroy grasses and cereals. The average daily consumption of locust nymphs is estimated to be 100-450 mg of green plants per day, and the adults each consume 0.2g per day (Roberts, 2010). The moth *Helicoverpa zea*'s larva causes significant damage to a wide range of crops, including cotton, tomato and corn, and it is difficult to control (Capinera, 2000).

Pests can also cause indirect damage by acting as vectors for plant diseases or by damaging beneficial organisms. For example, the whitefly *Bermisia tabaci* transmits 140 different viruses that cause plant diseases and thus causes significant losses in crops such as melon, tomato and beans (Jones, 2003). A serious pest in honeybee hives, the *varroa mite*, causes annual losses in free pollination services from feral bees estimated at 30 million dollars a year (CSIRO, 2008). Lately, the same mite invaded beehives in New Zealand where it is

anticipated that it will cost between 267 and 602 million dollars to deal with its effects and cause beekeepers to change the way they manipulate their hives (GISP, 2008).

Pest and pathogen invasions have caused serious reductions in crop yields in the most food insecure and poorest regions in Africa, where the damage has been assessed to cost \$12.8 billion annually (Oerke *et al.*, 1994). Oerke (1994) reports that pests may reduce potential crop yields by 50% in developing countries. Environmental change, including climate change due to increasing atmospheric CO₂, could promote and alter insects' distribution and cause further invasion (Macdonald, 1994; Vilà *et al.* 2006; Wallingford, *et al.*, 2009).

For many years, insect pest control in agriculture has been based on the use of chemical pesticides. However, widespread use of these compounds has had harmful consequences, such as soil and water contamination, and harmful side-effects on human and animals. Hence, there is a need to find plant protection methods that are friendly to the environment, consumers and farmers.

Genetic modification (GM) techniques offer some alternative options to improve and protect crops against environmental conditions, including insect interactions, by manipulating the genetic materials of plants and introducing resistant genes from different species into them. For example, rice, cotton and maize have been manipulated to produce *Bacillus thuringiensis* δ -endotoxin protein (*Bt* toxin), a toxic protein which interacts in the insects' gut to produce lethal lesions (Mazier *et al.*, 1997). The introduction of the encoding gene sequence to plants has been shown to have a toxic effect in a number of insects, such as the European corn borer, potato beetle and cotton bollworm. Crops which produce *Bt* toxin proteins show a biological resistance to insects without the need to use chemical pesticides (Feitelson and Payne, 1992; Knowles and Dow, 1993). The specificity of *Bt* toxins to specific insect species is an advantage because using such a technique reduces the effects on humans, animals, other organisms and non-target species. Also, some modified plants can express two or more toxic proteins and this has raised their protection efficiency against a number of pests and has reduced the damage to crops (Gatehouse, 2008).

However, since *Bt* toxins are not effective against some important insect pests, such as phloem feeders, alternative methods for engineering plants for insect resistance have been studied. Effective bio-insecticide approaches, such as inserting genes encoding toxic insecticidal proteins into plants, have been described (Celia, *et al.*, 2002). For example, the expression of the biotin-binding protein avidin enhances the resistance of plants to insects. It

has been reported that avidin expressed by maize showed resistance to three different coleopteran larvae (Karmer, 2000). Producing secondary metabolite compounds in suitably engineered transgenic plants is another approach which can result in increased insect resistance (Gatehouse, 2008). Currently, research is being carried out on processes which can be used to activate specific genes to stimulate plant pest management (Thayer, 1999), or to knockdown important genes in plant enemies. Using the RNA interference technique to knockdown genes in pests has been shown to be a feasible method of controlling insects and other invertebrates (Baum, *et al.*, 2007).

In spite of all the controversies around the genetic engineering of crops, there is little doubt that GM applications will play an important role in the near future in increasing food supplies to the growing world population. Not only is GM beneficial and less harmful to the environment than agrochemical approaches, furthermore, it also has the potential to enhance nutritional values, increase crop yields and decrease inputs of chemicals and energy in crop production. Also, GM can enable farmers to selectively target and destroy weeds without damaging crops (Uzogara, 2000).

1.2 Aphids as a model agricultural pest

Aphids are small plant sucking insects which belong to the super family Aphidodidea. This group of insects are serious pests which infest almost all the major crops, including grains, corn, potatoes and alfalfa. They are usually found on plant stems or the undersides of leaves. They cause damage to their host plants by feeding on their phloem sap, which weakens the plants and causes curled and faded leaves, or by transmitting plant pathogens by acting as a vector to transfer viruses causing diseases. Although the damage caused to plant tissues by these insects is usually minimal, the holes created by their probing can help to cause fungal infection (Jones and Jones, 1974). Aphids generally do not cause phloem blockage, as is the case for some other phloem feeders, such as the rice brown planthopper. However, in addition to phloem feeding, aphids secrete honeydew, which is composed of unused sugary liquid from the phloem sap and some other waste products, and which is a medium for the growth of a black fungus called 'sooty mould'. The growth of this surface fungus negatively affects the quality and the quantity of the crop yield. But, the primary damage that aphids

cause to crops is their ability to transmit and spread plant viral diseases (Blackma and Eastop 2000; Nault, 2004). Jones and Jones (1974) report that they transmit more than thirty viral diseases. However, feeding can also result in plant death or deformation of crops. Thus, aphids cause considerable financial loss to farmers. Oerke (1994) and Morrison and Peairs (1998) estimate that, in the US alone, aphids cause an annual loss of hundreds of millions of dollars.

There are around 5,000 species of aphids (Remaudiere and Remaudiere 1997), divided into 10 families, and 250 aphid species are considered to be significant agricultural pests. Aphids are mostly found in the temperate climate regions but their worldwide distribution is due either to the climate factor or to humans transporting of plants carrying aphids to different regions.

1.2.1 Pea aphids (*Acrythosiphon pisum*) Morphology and life cycle

Pea aphids are small green insects with a soft, pear shaped body. Adults are usually less than 3 mm in length. They have long antennae folded back over the body, and a pair of cornicles in the fifth segment of their back, through which they excrete honeydew fluid that is believed to play a role in their defence mechanism (Figure 1). They penetrate plant surfaces with their proboscis and use the partially enclosed stylet to probe between cells to the vascular tissue, where they penetrate the sieve elements to suck phloem sap, ingesting it passively using the pressure of the vascular system. The phloem sap contains high levels of sucrose (up to 1M) (Ashford *et al.*, 2000) as a carbohydrate source, and free amino acids, which are used as a source of nitrogen for nutrition. Pea aphids are normally wingless but the adults produce offspring able to develop wings when colonies become overcrowded or when the host plants become senescent or moribund. These enable them to travel to other plants and form new colonies.



Figure 1: Pea aphid (*Acyrtosiphon pisum*). It is a common pest in some host plants such as alfalfa, red clover. It causes a serious damage to crops due to its ability to propagate fast and the feeding habit (Rice, 2006).

Aphids have an interesting and complex life cycle, which seasonally alters between sexual and asexual reproduction. Their reproduction process involves asexual (parthenogenetic) reproduction which is followed by sexual reproduction (holocyclic/Monocyclic) in the autumn, or it may involve only parthenogenetic generation (anholocyclic). This mainly occurs in warm climate regions and tropical zones or during the spring and summer. Interestingly, the anholocyclic aphids show a distinctive variation in the genotype among the population (Blackman, 1979) because each female produces genetically identical female offspring which continue to produce another such generation. Daughter embryos carry their embryos within the mothers' ovarioles; thus aphids can develop their daughters and granddaughters within the same generation. Aphid 'mothers' give live birth to first instar nymphs. Around 7-10 days later, after the nymphs have progressed through four nymphal instars, they become adult females which are able to produce offspring. Female aphids produce up to 200 live young during their lifespan, with an active reproduction phase of up to 20 days. In winter, aphids respond to the shorter day length and colder weather and produce sexual generation asexually (Lees, 1990). The males are produced through the random loss of one of the X chromosomes because of a lack of attachment to spindle fibres in the metaphase through the maturation division (Wilson *et al.*, 1997). After intercourse between male and female aphids, the female produces an egg containing a female embryo holding the chromosomes XX. This may be because the sperm which carry chromosome X are the only viable sperm. The eggs adapt to a very low temperature during the winter. In the spring,

aphids' eggs are hatched, resulting in new asexual female nymphs, which enter the same production cycle and found new colonies.

It is clear that the aphids' life cycle is controlled by genetic and environmental factors (Dixon, 1963; Lees, 1964; Via, 1991). Most aphids' generations are parthenogenic, carrying live nymphs. Their high fecundity and short generational production period allows them to grow their colonies very rapidly and become huge populations that are capable of destroying crops.

1.2.2 The specialization of host plants

Although aphids are subject to genetic adaptation and speciation, some pea aphids are generalist feeders while others prefer particular host plants, such as alfalfa, pea, leguminous weeds or clover (Sandstrom, 1994; Via, 1991). The preference refers to the number of aphids that accumulate on one plant species compared to another. When pea aphids are introduced to a host plant, they determine if it is a suitable diet source by using their stylets to probe it several times (Cailaud and Via, 2000). Field and laboratory studies indicate that an aphid's preference for a particular plant is established by inserting the stylet. The discrimination occurs through brief probes of the plant (Johnson, 1958; Wensler, 1962;). The actual host plant specialization is determined by the acceptability of the plant as a feeding resource. In turn, this is determined by the ingestion of the food and breeding on the host plant while the aphid probes the plant's phloem sap (Schoonhoven *et al.*, 1998; Tosh *et al.*, 2003)

1.3 Sugar transporter proteins

Sugar molecules (monosaccharide) are an important biological resource for energy and carbon, as well as providing monomeric components from which oligosaccharides and complex carbohydrates, found as structural and recognition molecules in cells, are built up. The transportation of sugar molecules into and out of cells is a vital process, which requires transporter proteins to pass the molecules through the membranes of cells or organelles.

Sugars are hydrophilic molecules which cannot cross biological membranes by diffusion. There are many transporter proteins in the cellular membrane and they show diversity in respect to the substrate and the transporting mechanism. Although many different types of transporter exist, it is possible to use predicted amino acid sequences to extrapolate the transporting proteins' functions and structure, using information obtained from sequence comparisons, and inferred phylogenetic trees which depend on the proteins phylogenetic distances and their relationship to each other (Saier *et al.*, 1999a). This method is only appropriate if these proteins are related phylogenetically (Saier *et al.*, 1999a).

Sugar transporter proteins belong to the major facilitator superfamily (MFS). This superfamily consists of 29 families, in addition to possibly five other distant families (Saier *et al.*, 1999b). Eight families of MFS are classified as transporters for sugars and their derivatives; three of them exist in almost all the living organisms (Saier *et al.*, 1999b). Like other proteins belonging to the MFS superfamily, sugar transporter proteins have 12 transmembrane α -helices and comprise of two sub-groups, symporters and uniporters. Symporter proteins transport a specific substrate against its concentration gradient, using energy obtained by co-transporting a second substrate (usually an ion) down a concentration gradient. Uniporters move substrates down a concentration gradient, and thus transport is determined by the concentration of the substrate on each side of the membrane spanned by the transporter (Saier, 2000).

1.3.1 Sugar transporter encoding genes in pea aphids

In aphids, disaccharide sucrose is present at high concentrations in gut contents as a result of phloem ingestion, with phloem sap having a higher osmotic pressure when compared to the osmotic pressure of the aphid's haemolymph (Karley *et al.*, 2005). Sucrose is not transported, but is digested to the hexoses glucose and fructose by a sucrase enzyme in the gut. Glucose and fructose are transported through the cells of the gut epithelium to the aphid haemolymph. Due to the high level of sugar in the gut, the transportation of sugar molecules from gut to hemolymph could be facilitated by uniporter proteins, which mediate the transportation of solute molecules from the gut to the hemolymph, across the epithelial cells, and the movement could be controlled by concentration gradients. Supporting this hypothesis is the fact that no sodium-sugar symporter proteins that facilitate transportation against the

concentration gradient are present in a pea aphid's genome, according to the bioinformatics data (The international aphid genomics consortium, 2010).

Genomic studies (Price *et al.*, 2010) show that the pea aphid genome contains a large number of genes encoding transporter proteins, including about 200 genes encoding transporter proteins belonging to the MFS. Superfamilies can be annotated with predicted functions based on sequence similarity analysis. A pea aphid has 34 sugar/inositol transporter genes, which is higher than the number of transporter genes in other insects such as *Drosophila melanogaster*, which has 15 sugar transporter genes. The beetle *Tribolium castaneum* is an exceptional since it has the highest number of the sugar transporter genes, it is containing 54 sugar transporter genes. The most highly expressed sugar transporter gene in pea aphids is *Ap_ST3*, which encodes a uniporter protein with specificity towards both glucose and fructose (Price *et al.*, 2010).

1.3.2 The sugar transporter gene *Ap_ST1*

Ap_ST1 (LOC100160486) is a highly expressed gene in the digestive tract of *A. pisum*. It is found specifically in the midgut region of the tract, with 5 expressed sequence tags (ESTs) found in libraries from this tissue, which is higher than in other parts of the pea aphid's body (Price *et al.*). The gene *Ap_ST1* encodes a sugar transporter protein (XP_001942953) consisting of 489 amino acids residues (figure 3), giving 53.70 kDa as the predicted molecular weight. It has a glycosylation site at the extracellular loop 4 (amino acid 303), as shown in figure 2. The protein belongs to MFS superfamily and, like other members of this protein superfamily, it is predicted to contain 12 transmembrane α -helices. It shares other features with the major facilitator superfamily proteins. It has GRR/K motif in the intracellular loop 1 and in the intracellular loop 4, and it has highly conserved glutamine residues present in transmembrane helices 5 and 7, which are considered to be part of the exofacial ligand binding site (Hashimoto *et al.*, 1992; Mueckler *et al.*, 1994). Although the motif -QLS-, which presents in some glucose transporters and is absent from fructose/glucose transporters, does not exist in transmembrane helix 7 in the *Ap_ST1* encoded protein, a -QFS- motif sequence is present in this site. The *Ap_ST1* product functions as a carrier for mannose and glucose (Price *et al.*, unpublished).

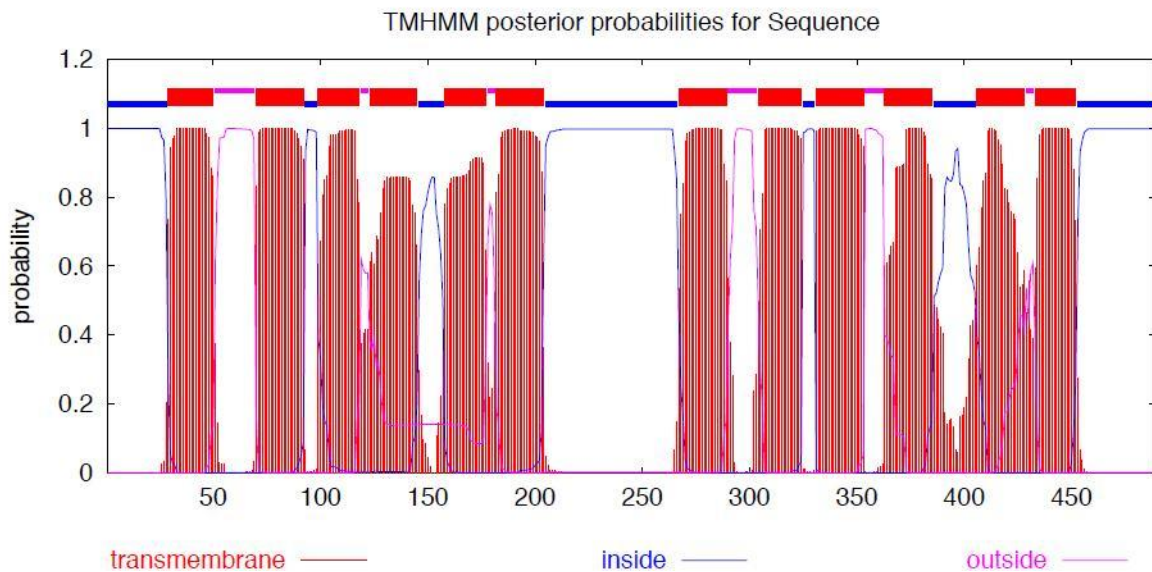


Figure 2: Predicted structure of the sugar transporter protein *Ap_ST1* and its 12 transmembrane helices. The structure was predicted by using the program TMHMM.



Figure 3: The sequence of the predicted protein sequence of the gene *Ap_ST1* contains 490 amino acids residues. The sugar transporter protein *Ap_ST1* is highly expressed in aphids' gut (Price *et al.*, unpublished). The produced protein transports mannose and glucose molecules. The gene sequence is obtained from the NCBI, National Centre of Biotechnology Information, and processed by 5 Geneious.

Multiple alignment of *A. pisum* sugar transporter genes, with a subsequent Clustal analysis to construct a phylogenetic tree, as previously described (Price *et al.*, 2009), shows that *Ap_STI* forms a sub-group with two other predicted sugar transporter proteins: *Ap_STI6* (XP_001943804), which has 83% similarity to *Ap_STI*, and *Ap_STI7* (XP_001950031), which has 84% similarity to *Ap_STI*. Similar predicted genes are present in other aphids, such as the aphid *Sitobion avenae*, which contains a homologous gene (*Sa_ST1*) with 96% identical coding sequence to *Ap_STI*, and sequences with a high similarity to *Ap_STI* are present in other aphid species, such as *Myzus persicae* and *Aphis gossyii*. Similar proteins are also present in other sap-sucking insects; the glucose transporter protein in the hemipteran species *Nilaparvata lugens* (rice brown planthopper) has 70% similarity to the *Ap_STI* protein (Price *et al.*, 2009).

1.4 RNA interference

1.4.1 RNA interference discovery

RNA interference (RNAi) is a newly discovered biological process which occurs in the cytoplasm of cells and affects the expression level of specific genes in a selective process by degrading the mRNA of target genes through a post transcriptional gene silencing process (PTGS). The process is mediated by RNA sequences which are introduced into the cell as exogenous RNA, usually as double stranded RNA (dsRNA), and it occurs after export of mRNA from the nucleus. Studies of the nematode *Caenorhabditis elegans* have shown that the injection of both sense (the strand which has an identical sequence to mRNA) and anti-sense RNA strands (the complementary sequence of mRNA) separately causes gene silencing (Guo & Kemphues, 1995). Later, Fire and Mello (1998) conducted an experiment by injecting a mixture of sense and anti-sense strands as dsRNAs into *C. elegans* and showed that the dsRNA was ten times more effective in decreasing expression of the homologous endogenous mRNA than using either the sense or the antisense strand alone. Since then, the silencing of a certain gene using double stranded RNA has been called RNA interference.

RNAi shares similar mechanisms with a previously observed phenomenon, namely, homology dependent gene silencing. Several years before Fire and Mello's (1998) discovery,

Richard Jorgensen and colleagues aimed to deepen the purple pigment of petunias by introducing the colour producing gene, controlled by a powerful promoter. Surprisingly, the experiment showed the unexpected result that many flowers were colourless or variegated. This phenomenon was called ‘co-suppression’, due to the suppression of both the endogenous and the homologous exogenous genes (Napoli *et al.*, 1990; Jorgensen, 1990). Other studies found that plants suppress the expression of viral RNA by a similar response (Dougherty *et al.*, 1994; Kumagai *et al.*, 1995). It is clear that dsRNA, when exogenously introduced or transcribed from engineered inserted DNA, is a potent gene silencing trigger in a plant’s system (Bernstein *et al.*, 2001). Co-suppression has been observed not only in plants, but also in some other unicellular organisms, such as the fungus *Neurospora crassa*, where it was called ‘quelling’ (Romano and Macino, 1992; Bernstien *et al.*, 2001). It was also observed in nematodes, protozoa, metazoans, and in mammals such as mice oocytes and *Drosophila* (Fire *et al.*, 1991; Pal-Bhadra *et al.*, 1997).

In fact, the initial idea of inhibiting genes by using an external molecular material or part of a pathogen's genetic material as a tool to generate a resistance was first put forward by Sandford and Johnston (1985) who proposed that, if a host cell genome expressed a modified genetic portion of the pathogen’s genetic material, it could act against the pathogen itself and inhibit it. In 1986, the theoretical proposition was shown to be valid in a practical application when a transgenic plant that contained and expressed the coat protein gene of the tobacco mosaic virus (TMV) was generated. When the transgenic plant was exposed to TMV, it exhibited resistance and delayed display of the symptoms of TMV infection (Abel *et al.*, 1986). Subsequent work linked this phenomenon to silencing of gene expression by RNA interference. RNAi has been characterised as a naturally occurring gene expression regulator (Ding *et al.*, 2004), and also as a function in the defence mechanism against viruses (Hunter and Poethig., 2003), and chromatin structure (Lippman and Martienssen, 2004).

1.4.2 RNA interference mechanisms

In the last decade, a large amount of research has been carried out in order to understand the mechanisms of RNAi and to characterize the components which contribute to the gene silencing process. According to recent studies, the RNAi process can be divided into three

basic steps (Meister and Tuschl, 2004; Tomari and Zamore, 2005). The first step is the processing of the long double-stranded RNA by an RNase III endonuclease termed Dicer (Carmell and Hannon, 2004) into small interfering double stranded RNA (siRNA) fragments consisting of 21-23 base pairs. The structure of the double stranded RNA is formed as a result of matching two separated RNAs strands (sense and antisense) or by forming a stem-loop structure within the strand itself and it could be exogenously introduced via a vector such as virus or directly introduced or expressed in cells. The yielded siRNAs have phosphate groups at their 5' ends and 2 nucleotides overhangs at their 3' ends (Hannon and Rossi, 2004; Meister and Tuschl, 2004). Some Dicers have ATP-binding sites which suggest that ATP is required for siRNA production (Ketting *et al.*, 2001; Nykanen *et al.*, 2001). siRNA has two strands, sense and antisense, corresponding to a specific mRNA sequence. The antisense strand can hybridise to this specific gene sequence, and therefore it acts as a guide to recognize the target mRNA, as shown in figure 4.

Secondly, siRNAs assemble into a multiprotein RNA-induced silencing complex (RISC), which consists of Dicer, RNA binding proteins, the TAR-RNA-binding protein (TRBP), Argonaute (Ago), which forms the core component of RISC, and PACT (Lee *et al.*, 2006). Recently, the vasa intronic and dFMR product proteins were characterized as additional RISC proteins (Caudy *et al.*, 2002). There is a strict requirement that the siRNA is 5' phosphorylated to be attached into the protein complex, and the unphosphorylated siRNAs are immediately phosphorylated by endogenous kinase (Schwarz *et al.*, 2002). Short double stranded RNA has to be unwound to allow the antisense strand to guide the complex proteins to its homologous target mRNA during the incorporation, whereas the second strand is degraded (Hammond *et al.*, 2000; Schwaez *et al.*, 2003; Khvorova *et al.*, 2003). Dissociation of the antisense and sense strand in siRNA needs Dicer-2 and R2D2 protein heterodimer (Lee *et al.*, 2004; Liu *et al.*, 2003). Therefore, the antisense strand governs the remarkable specificity of gene silencing in RNAi (Elbashir *et al.*, 2001).

Thirdly, the guided strand (ssRNA) and RISC attach to the target mRNA and cleave it at a single site, 10-11 nt upstream from the 5' end of the siRNA. This complementary interaction causes an endonuclease specific degradation of mRNA around the central region of the mRNA strand (Elbashir *et al.*, 2001) and the cleavage of the complementary mRNA occurs by the activity of RNaseH in Argonaute (Hammond, 2005). Eventually, the RISC dissociates from the degraded mRNA and enters another catalysing cycle. This feature of RISC gives the

silencing effect. In some organisms, ssRNAs are not produced from a dsRNA precursor but they present naturally; thus RNase III is not involved in their production.

RNA-dependent RNA polymerase in some living organisms plays an important role in siRNA amplification, thereby increasing the silencing affectivity of siRNA (Cogonu and Macino 1999; Fire, 1999). In some organisms, such as mammals, long dsRNAs ≥ 30 nt are able to stimulate an interferon response (Elbashir *et al.*, 2001; Rutz and Scheffold, 2004), causing nonspecific knockdown to gene expression. The introduced long dsRNA induce an antiviral response, either by stimulating a series of activations starting with 2'-5' oligoadenylate polymerase which in turn activates RNase L, causing all RNA molecules to be degraded and non-specific gene silencing, which results in a change in cellular gene expression that leads to apoptosis (Minks *et al.*, 1979), or by activating the protein kinase (PKR). The activated PKR causes inactivation of the translation initiation factor, eIF2a, by phosphorylation, resulting in translation suppression (Manche *et al.*, 1992). However, not all cells have the same response to long dsRNA and this made long dsRNA a possible choice in specific gene silencing studies (Paddison *et al.*, 2002; Yang *et al.*, 2001).

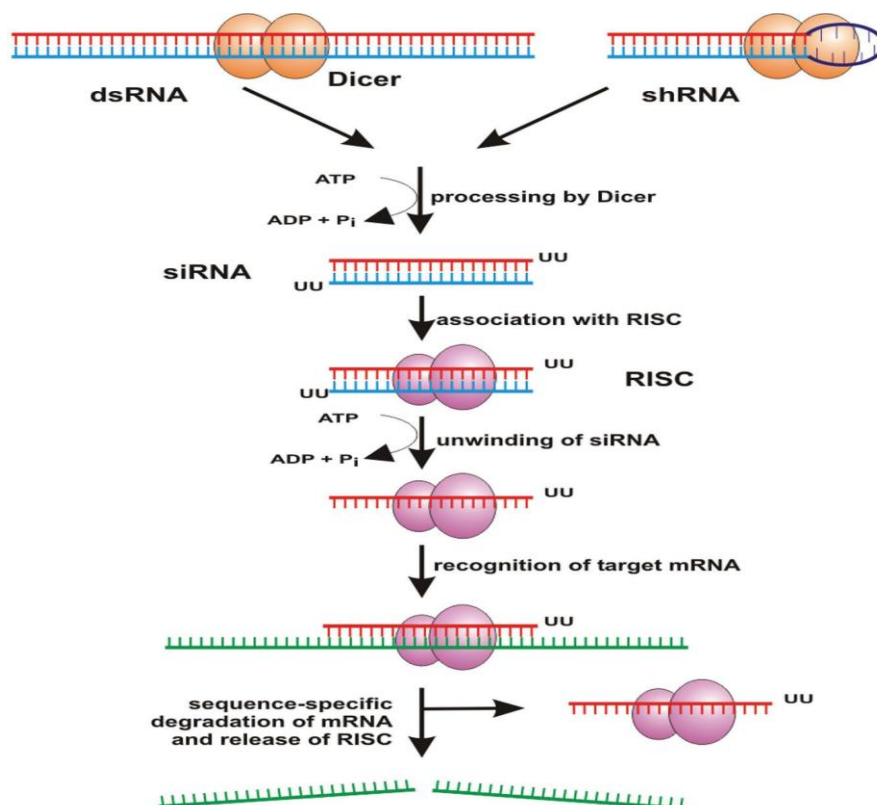


Figure 4: The pathway of RNA interference. Small hairpin (shRNA) or dsRNA are cleaved by Dicer depending on ATP to produce small interfering RNA (siRNA). The siRNA sequences bind to RISC

depending on ATP molecule to form a complex which targets mRNA based on the corresponding sequence to siRNA, then the complex siRNA RISC is released and enter another cycle (Hutvagner and Zamore, 2002).

Interestingly, two genes encoding important proteins identified in the effector step in post-transcriptional gene silencing have been found in *C. elegans*, *mut-7* and *rde-2*, which also affect the transposon activity. *C. elegans* with inactivating mutations in *mut-7* and *red-2* showed a defective RNAi response, but also the transposon activity was increased. Therefore, the suppression of transposons is controlled by a mechanism correlating to RNAi (Bastiaan *et al.*, 2005; Hannon, 2002).

1.4.3 RNA interference uptake

Several studies have described the uptake mechanisms of dsRNA by cells in different organisms. The research has identified two required transmitter agents for systematic RNAi by inducing mutations in the SID gene, using *C.elegans* as an animal model. The first characterized protein is SID-1, which is a transporter protein consisting of a single polypeptide and 11 transmembrane domains (Winston *et al.*, 2002). This protein passively transports dsRNA into the cells of *C.elegans*, but it does not export it to the neighbouring cells (Winston *et al.*, 2002; Jose *et al.*, 2009).

SID-1 orthologs are found in different organisms, including the cotton aphid (*Aphis gossypii*), the honey bee (*Apis mellifera*) and *Tribolium castaneum* (Xu and Han, 2008; Aronstein *et al.*, 2006; Tomoyasu *et al.*, 2008). The study of the SID-1 ortholog gene expression level using RNAi in the honey bee indicates a correlation between the expression level of the gene SID-1 and the knock down of the target gene (Aronstein *et al.*, 2006). The study of the three ortholog genes in *T.castaneum* shows no effect on systemic RNAi when silencing one or all SID-1 orthologs (Tomoyasu *et al.*, 2008). Intriguingly, further analysis of the SID-1 orthologues in insects showed that SID-1 orthologs are more similar to tag-130 genes than to SID-1 in *C.elegans*. Tag-130 genes are not involved in the systemic RNAi in *C.elegans*, which suggests that the SID-1 is not important to the uptake mechanisms in some insects (Tomoyasu *et al.*, 2008), thus clarifying the results obtained from the RNAi experiment in *T.castaneum*. SID-2 is another protein which participates in RNAi by facilitating the uptake of exogenous homologous RNA. This protein is mainly expressed in the worm intestine

tissue (Winston *et al.*, 2007). The relationship between SID-1 and SID-2 remains unclear. One hypothesis is that SID-2 attaches to the exogenous dsRNA molecule and passes it to SID-1, while another proposes that SID-2 activates the transport by inducing a modification to the SID-1 protein molecule, and a third proposes that SID-2 stimulates the endocytosis pathway in which SID-1 transport dsRNA to the cellular cytoplasm (Whangbo and Hunter, 2008).

However, some organisms, like *D. melanogaster*, lack SID gene orthologs but are still able to uptake dsRNA from the environment and trigger RNAi (Saleh *et al.*, 2006; Ulvila *et al.*, 2006;). This suggests an alternative mechanism present in these organisms to enable take up of dsRNA. In a study conducted to screen 50% of the *D.melanogaster* genes, 23 genes involved in dsRNA uptake mechanisms were identified. Some of these genes are known, and all are related directly or indirectly to the endocytic pathway (Saleh *et al.*, 2006). Uptake experiments also showed that scavenger receptors (SR-CI and Eater) in S2 cells in *D.melanogaster* (Ulvila *et al.*, 2006) and vascular H⁺ ATPase (Saleh *et al.*, 2006) participate in the dsRNA uptake pathway.

1.4.4 RNA interference applications

The rapid growth of genomic studies increases the need to find a feasible and efficient method to analyze the immense amount of generated data. RNAi technology has become a powerful experimental tool for biologists in the analysis of gene functions and genomic studies because of its simplicity and its easy delivery methods. RNAi is a direct method to knockdown or reduce the expression level of the target genes and generate mutation in the phenotype in order to determine the gene function.

The RNAi technique has been used in animals for different purposes. For example, using a library of homologous dsRNAs helped to screen chromosomes I and III in *C. elegans* in order to characterize the genes responsible for the division of cells and embryonic development (Fraser *et al.*, 2000; Gonczy *et al.*, 2000). Recently, using the RNAi approach, the functions of about 86% of predicted genes in *C. elegans* were analysed by identifying phenotypes (Kamath *et al.*, 2003).

The same process was applied in insects using *D. melanogaster* as a model. It is used to identify genes with important roles in embryonic development, studying the biochemical signalling cascades and the contributing proteins by blocking their expression using RNAi, and other fundamental cellular mechanisms. The insulin signalling pathway in *Drosophila*, which has a similar receptor to the mammalian, is an example of signalling pathways that have been studied by using RNAi. The insulin receptor triggers a series of intracellular effector activations, including mitogen activated protein kinase (MAPK), which is activated by insulin and, in turn, activates several effectors starting with DSOR1, which subsequently activates ERK-A. Using a homologous dsRNA to DSOR1 resulted in the absence of both phosphorylated and unphosphorylated DSOR1 and, subsequently, ERK-A did not activate. Applying dsRNA corresponding to ERK-A resulted in a knock-out to the encoded protein's gene, causing a total loss of this protein. The absence of this protein leads to activation of DSOR1 in the presence or the absence of insulin (Clemens *et al.*, 2000). Furthermore, the high specificity of RNAi has worked effectively to hamper virus replication in cultured cell lines (Bitko and Barik, 2001; Gtlin *et al.*, 2002; Jacque *et al.*, 2002; Takanori *et al.*, 2003). In addition, functional analysis of genes has also been used in plants where RNAi has worked efficiently when transgenes are in microRNA (hairpin) form.

The high specificity of RNAi allows this technique to be applicable as a method for silencing genes whose activity is essential for insect survival, resulting in an insecticidal effect. Recent study has proved that transgenic plants which produce dsRNAs with perfect homology to certain target mRNAs encoding subunits of V-type ATPase (V-ATPase A and V-ATPase E) in Western corn rootworm (WCR; *Diabrotica virgifera virgifera* LeConte; Coleoptera), have effectively suppressed gene expression in WCR, and caused an insecticidal effect. The same dsRNAs have also been delivered to other coleopteran plants pests, such as the Colorado potato beetle (CPB), which has 83% similarity in V-ATPase A and 79% in V-ATPase E orthologues to the ones in WCR. In this case, dsRNAs showed a significant response but it required higher concentrations than that used in WCR. On the other hand, synthesis of specific dsRNAs for the CPB genes V-ATPase A and V-ATPase E demonstrated an effective reduction of mRNA compared to the orthologues dsRNAs in WCR (Baum *et al.*, 2007). All these studies corroborate the specificity feature of RNAi, and that it is conserved amongst species.

RNAi is a feasible tool to use for crop improvement, whether it is for protection against pests or for improving nutritional values. Using the RNAi technique on barley has shown that the crop develops a resistance against the barley yellow dwarf virus (BYDV) (Wang *et al.*, 2000). Furthermore, RNAi has been shown to improve rice plants by reducing its glutenin level and producing low glutenin content-1(LGC). This product is helpful for people who are unable to digest glutenin. The change in phenotype was stable and passed through several generations (Abdolhamid *et al.*, 2010).

Thus, although the use of RNAi is a relatively new field, many studies have been conducted and have proved the role of RNAi in the functional analysis of cellular genes. They have also demonstrated the advantage of its applications in different organisms and for different purposes, especially in crop improvement and in plant resistance mechanisms.

1.5 Aims and the objectives of the study

The aim of this project is to investigate the effects of RNA interference on the sugar transporter encoding gene *Ap_STI* in *A.pisum* by considering the possible alterations it causes in both the morphology and the genotype, and estimating the knockdown level.

Chapter 2 MATERIALS AND METHODS

2.1 Chemicals and materials

All the chemical reagents used in these experiments were supplied by the Promega Corporation (Madison, USA), Thermo Scientific Fermentas (Canada), or the Sigma chemical company (St.Louis, USA), unless stated otherwise.

2.2 Common reagents, solutions and chemical recipes

A-Bacterial culture:

- **Luria Bertani Broth (LB):** 1% (w/v) of tryptone, 1% (w/v) sodium chloride, and 0.5% (w/v) of yeast extract.
- **Low salt Luria Bertani Broth (LS-LB):** 1% (w/v) of tryptone, 0.5% (w/v) sodium chloride and 0.5% (w/v) of yeast extract.
- **Agar plates:** 1.5% (w/v) of Bacto agar was added to LS-LB.

B- Agarose gel electrophoresis:

- **1xTAE:**buffer was prepared as following: 20 mM glacial acetic acid, 0.2 mM EDTA, 40 mM Tris, pH 7.2.
- **Gels** were made up with 1% (w/v) agarose, 1xTAE buffer and ethidium bromide (10 µg/µl).
- **DNA molecular weight ladder:** Lambda DNA fragmented by Eco471 (AvaII).
- **6x orange G loading dye:** 10 mM Tris HCl (pH 7.6), 0.15% orange G, 60% glycerol, 60 mM EDTA.

C-Proteinase inhibitor: 100 µg of proteinase K was added to Tris HCl in concentration of 100 µg/ml and stored at 4 °C.

D-Sodium dodecyl sulphate (SDS): 10% w/v was added to deionised, distilled water.

E- Aphids liquid artificial diet:

Aphid liquid diets were prepared following the protocols of [KUNKEL \(1976\) AND DOUGLAS \(1992\)](#), using a sterile technique. The artificial diets were composed of sucrose and phosphate solutions mixed with 5 ml aliquot of completely thawed 150 mM amino acids, 0.1 ml minerals and 0.5 ml vitamins stock solutions which were prepared previously and kept at -20°C . The prepared diets were filtered using a 5 ml sterile plastic syringe and 0.2 μm Acrodisc syringe filters.

The preparation of stock amino acids (150 mM):

Alanine	50.8 mg
Asparagine	213.9 mg
Aspartic Acid	189.7 mg
Cystiene	42.5 mg
Glutamic Acid	123.6 mg
Glutamine	241.1 mg
Glycine	9.0 mg
Proline	56.6 mg
Serine	59.9 mg
Tyrosine	10.9 mg
Arginine	300.2 mg
Histidine	182.4 mg
Isoleucine	114.1 mg
Leucine	114.1 mg
Lysine	158.9 mg

Methionine	42.5 mg
Phenylalanine	47.1 mg
Threonine	103.6 mg
Tryptophan	58.2 mg
Valine	101.9 mg

All these compositions were dissolved in 50 ml distilled water then divided into 10 ml lots and kept at -20°C .

Mineral stock solution:

$\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$	11 mg
$\text{CuCl}_2 \cdot 4\text{H}_2\text{O}$	2 mg
$\text{MnCl}_2 \cdot 6\text{H}_2\text{O}$	4 mg
ZnSO_4	17 mg

The salts were dissolved in 10 ml of distilled water, sterile filtered, and divided into 0.1ml aliquots which were kept frozen at -20°C .

Vitamins stock

Biotin	0.1 mg
Pantothenate	5 mg
Folic acid	2 mg
Nicotinic acid	10 mg

Pyridoxine	2.5 mg
Thiamine	2.5 mg
Choline	50 mg
Myo-inositol	50 mg

The vitamins were dissolved in 5 ml of water, sterile filtered, and divided into 10x0.5 ml lots.

Sucrose mix

A sucrose standard diet composition of 500 mM was freshly prepared and mixed with the thawed minerals, amino acids and vitamin solutions. The composition of the sucrose solution was as follows:

Ascorbic acid	10 mg
Citric acid	1 mg
MgSO ₄ .7H ₂ O	20 mg
Sucrose (500mM)	1.7 mg

150 mg of phosphate (K₂GPO₄.3H₂O) was weighed out, dissolved in 1 ml of water and added to the amino acids, vitamins, minerals and sucrose mixture solutions. The pH was checked by using an indicator strip (the pH should be between 7 to 7.5), after which the solution was made up to 10 ml with distilled water. Finally, the solution was filtered into sterile plastic tubes by using an acrodisc syringe and stored at -20 °C until needed for 3 months.

2.3 Standard molecular biology techniques:

All the standard molecular biology techniques were based on methods described in "Molecular Cloning: A Laboratory Manual" (Sambrook and Russell, 2001), unless otherwise stated.

2.3.1 Preparation of electrocompetent cells for transformation

The standard *E.coli* strain used for cloning operations was named TOP10 in the biological laboratories at Durham University. Electrocompetent cells were prepared as described by Sambrook and Russell; the Top10 cells were stored in glycerol at -80 °C, to be thawed when needed.

2.3.2 Bacterial culture

To grow *E. coli* cells after transformation Luria Bertani (LB) broth for bacterial cultures was prepared as follows: 1% (w/v) of tryptone, 1% (w/v) sodium chloride and 0.5% (w/v) of yeast extract. All were dissolved in deionised water and the volume was made up to 1000ml. The solution was then autoclaved. After cooling, an antibiotic was added at a concentration 100µg/ml. Then, it was aliquoted in sterile bottles, 5 ml in each, covered with the lid and stored at room temperature. The low salt broth (LS-LB) was prepared by adding half the concentration of sodium chloride 0.5% (w/v) to the same intergradient of LB.

For agar plates, 1.5% (w/v) of agar was added, and the plates were poured while the medium was liquid. Then the plates were kept at room temperature until the agar set and then they were stored at 4 °C.

2.3.3 Plasmid DNA purification from transformed *E.Coli*

To extract plasmid DNA from cultures of putative transformed TOP10 competent cells, plasmid DNA was purified from small-scale (5-10ml) *E. coli* cultures, which were grown overnight, by using the DNA purification system (Wizard *Plus* SV miniprep) supplied by Promega and following the instruction manual supplied with the kit. The DNA was eluted in the appropriate amount of free nuclease water (10-30µl) and stored at -20 °C until it was required.

2.3.4 Restriction endonuclease digestion

In order to prepare desired fragments of the cloned gene, restriction enzymes were used. Enzymes were selected according to the restriction sites in the DNA sequence, or from a plasmid map. The digestion reactions were performed in a reaction volume of 30 μl , using the buffer recommended by the enzyme supplier, and 2-15 units of enzyme per μg of DNA. Digestion reactions were carried out at 37 $^{\circ}\text{C}$ for at least 2 hours, except where a different temperature for digestion was recommended by the enzyme manufacturer.

2.3.5 Precipitation of nucleic acids

To purify and concentrate the extracted DNA plasmid and take it for further manipulations, DNA was precipitated from solution by adding 1/10 sodium acetate and 2 volume 100% ethanol and kept overnight at -20°C . Solutions were then centrifuged at 12,000g at 4 $^{\circ}\text{C}$ for 15 minutes. The DNA pellet was washed by adding 70% ethanol twice after removing the supernatant and centrifuged, as above, for 10 minutes. Then, the pellet was resuspended in 10 μl or 30 μl of free nuclease water after removing the ethanol before being stored at -20°C .

For isolation and purification of the synthetic dsRNA we used the aphids feeding experiment in section 2.5 or to pursue to qPCR (section 2.3.18), RNA was precipitated by adding 30 μl of LiCl (7.5 M in 50 mM EDTA) supplied in the kit by the factory, to 30 μl of solutions containing RNA, followed by adding 30 μl of nuclease free water, mixing it thoroughly and keeping it at -20°C for at least 30 minutes. Samples were centrifuged at 4 $^{\circ}\text{C}$ for 15 minutes at 14,000 rpm. The precipitated pellet was washed with 70% ethanol and centrifuged at the same speed for 5 minutes. This step was then repeated. After that, the supernatant was discarded and the pellet left to dry. Finally, the pellet was re-suspended in free nuclease water using the appropriate amount, that is, 20 μl .

2.3.6 Agarose gel electrophoresis

To check the digested fragments either for screening or to extract the desired gene fragment, DNA fragments were separated by agarose gel electrophoresis, carried out essentially as

described in Sambrook and Russell (2001). A standard molecular weight marker (lambda phage DNA digested with Eco47 I, Fermentas) was used to calibrate the sizes of the separated fragments. Gels were made up with 1% (w/v) agarose, 1xTAE buffer (20 mM glacial acetic acid, 0.2 mM EDTA, 40 mM Tris, pH 7.2) and ethidium bromide (10 µg/µl). Before loading the samples, 1/5 sample volume of 6x loading dye was added and mixed. Gels were submerged in a 1xTAE buffer containing ethidium bromide (10 µg/µl). Gels were run at room temperature, at 70-100V. After completion of the electrophoresis, DNA fragments were visualised on a UV-trans-illuminator (300nm).

2.3.7 Recovery of DNA fragments from agarose gel

For purifying the selected DNA fragments after running the gel for cloning, agarose gel blocks containing bands of interest were removed from the gels by using a sterile razor blade, weighed and then purified by using a QIAquick gel extraction kit (Qiagen, www.qiagen.com), according to the manufacturer's instructions. DNA was eluted in 30-50 µl of free nuclease water before being stored at -20 °C and thawed for use when needed.

2.3.8 Quantitation of nucleic acids

The concentrations of nucleic acids in solution were estimated by using the NanoDrop ND-1000 Spectrophotometer (Bosch Institute nano drop). 1.5-2µl of DNA or RNA was quantified by measuring absorbance at 260nm for DNA and 280nm for RNA using a NanoDrop spectrophotometer. A blank containing nuclease-free water or buffer, corresponding to the solution used for re-suspension, was subtracted from the readings obtained.

2.3.9 DNA ligation

To clone the gene of interest into pLitmus 28i or pJet vectors ligation of DNA fragments was carried out in a 10 µl standard ligation reaction. The reaction contained DNA fragments with compatible ends. 100ng of plasmid were used for a typical ligation reaction, with a 1:1 molar

ratio insert: vector 0.5 μ l of T4 DNA ligase, and a 1 μ l of ligation mix buffer (Promega). Reactions were incubated at 4 °C overnight. Ligation reactions were used for *E.coli* transformation without further treatment.

2.3.10 *E. coli* transformation

In order to propagate the ligated DNA, the ligation mixture was transformed into competent *E. coli* cells, by mixing 0.5-0.75 μ l of the ligation reaction with 50 μ l of competent *E.coli* using Biorad Electroporator set as following: electrical pulse 25 μ F, capacitance 2.5 kV, 200 ohm resistance, and voltage strength 12.5 kV/cm, for time constant (3.8-5 τ) milliseconds. The transformed cells were recovered immediately after the electroporation by adding 750 μ l LS-LB media. Then, the transformation mix was left at room temperature for 30 minutes. After that, 100-200 μ l was taken and plated out on selective LS-LB agar plates that contained a suitable antibiotic (carbenicillin) at 100 μ g/ml. The plates were incubated overnight at 37 °C. The bacterial colony, that had grown during the incubation period and which was expected to contain potential recombinant plasmids, was picked off and transferred to the LS-LB liquid media containing 100 μ g/ml of carbinecillin antibiotic. The cultures were incubated overnight on the shaker at a shaking rate of 220 rpm at 37 °C.

2.3.11 RNA extraction

To investigate the effects of the RNAi treatment on expression of *Ap_STI*, total RNA was extracted from the whole insect by using TRI reagent from SIGMA. Five pea aphids were collected and weighed each time. Then they were homogenised manually using a sterile glass homogenizer in a TRI reagent solution and processed following the instructions provide by the manufacturer. The concentration of extracted RNA was checked by using nano-drop and the integrity was checked by running 3 μ l on agarose gel electrophoresis. The extracted RNA was re-suspended in nuclease-free water and snap frozen in liquid nitrogen, then kept at -80 °C and thawed when needed. All the extraction and thawing steps were performed on a cleaned and sterilized surface. All the equipment used in the RNA preparation was baked in an oven overnight at 200 °C to eliminate any enzymatic activities from RNAase

contamination, which causes RNA degradation. New and clean plasticware were used for each extraction.

2.3.12 Amplification of the plasmid carrying the gene *Ap_ST1*

The construct, which has the sugar transporter gene *Ap_ST1*, was provided by Dr Daniel Price. It was used for amplifying the inserted gene fragment using the conventional polymerase chain reaction method (PCR). A specific set of primers were designed and synthesised by SIGMA Genosys Service, as follows:

Ap_ST1 Fwd 5' TCTGACTATTATTTGTGCTATCATTCC 3'

Ap_ST1 Rev 5' CCTAATCTGTCAACGATCAGTGTA 3'

The PCR reaction (50µl), set up in a standard PCR reaction, contained 1 µl of a mixture of 0.2 mM dNTPs (dATPs, dGTPs, dTTPs and dCTPs), 1.25 units of *Taq* polymerase/ 50µl PCR reaction, DNA template (50-100ng), and 0.2 µM specific primers. A master mix containing all the components was used for a multiple PCR reaction to avoid pipette error. The PCR reaction was run under the following conditions: 40 seconds at 98 °C in the initiation step, followed by 30 cycles of amplification starting with 98 °C for 10 seconds, 56 °C for 10 seconds in the elongation step and then 72 °C for a minute in the termination step. Then, in the final step, which was run for 7 minutes at 72 °C, the PCR product was visualized on the gel, as described earlier.

2.3.13 Sub-cloning of *Ap_ST1* fragment in pJet

DNA fragments were cloned into pJet using a PCR product sticky end cloning system, which was supplied by Thermo Scientific <http://www.fermentas.com/en/home>. This system was used to clone the amplified segments of both *Ap_ST1* and digested kanamycin resistance gene, which were extracted from the agarose gel and purified as described in section 2.3.7. The cloning system was used for simple cloning of PCR products. The reaction was set up separately according to the manufacturer's instructions. The constructs were transformed to the TOP10 *E.coli*, as mentioned section 2.3.10.

2.3.14 Screen of isolated plasmid DNA for correct insert

To investigate the success of DNA fragments insertion in the selected vectors isolated plasmid DNA was subjected to restriction enzyme digestion, as described in 2.3.4. Restriction enzymes were chosen to allow excision of the insert as a single fragment. The digested fragments were analysed by agarose gel electrophoresis, and fragment size was estimated by comparing band mobility to the marker.

2.3.15 DNA sequences analysis

In order to confirm the identity of DNA sequences the sequences were analysed by using the automated DNA sequencing service which is available at the School of Biological and Biomedical Sciences at Durham University. The newly constructed plasmids were sequenced in both strands of the DNA. The produced sequences were analysed on Sequencher™ software, version 4.5, on a Macintosh computer. The nucleotide sequences were blast on www.ncbi.nlm.nih.gov/BLAST/ to investigate the ID of the inserted genes. The alignment of the similar genes, *Ap_ST1*, *Ap_ST16* and *Ap_ST17* with accession numbers ACYI001780, ACYPI006113 and ACYPI006604 respectively, was analysed and compared using the online tool ClustalW2 server (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>). The prediction of the *Ap_ST1* protein structure was performed using the server THMHH (<http://www.cbs.dtu.dk/services/TMHMM/>). The translation of the gene sequence to amino acids was performed using Geneious 5 software.

2.3.16 Synthesis of double stranded RNA (dsRNA)

The dsRNA was prepared by transcription of a DNA template cloned into pLitmus 28i plasmid provided by BioLabs, New England <http://www.neb.com/>. The plasmid has an ampicillin encoding gene and T7 promoters either side of the plasmid into which the DNA to be transcribed can be introduced. Recombinant plasmids used as templates were prepared as described above. The sense and antisense RNA strands were synthesised in two separated

reactions using the MEGAscript Kit from Ambion Applied Biosystem. The yields transcribed were mixed and kept in 55 °C for five minutes to anneal and then left over the bench to cool.

To remove template DNA, the dsRNA product (1.6-1.2 µg/µl) was treated with 2U of DNaseI followed by incubation at 36 °C for an hour. Then the reaction was treated with lithium chloride, as described in section 2.3.5, to remove the enzyme and precipitate the dsRNA. The integrity of the treated dsRNA was analyzed by running 1-3µl of the solution on agarose gel electrophoresis.

2.3.17 Reverse transcription PCR (RT-PCR)

For the relative quantification of mRNA corresponding to *Ap_ST1*, single stranded complementary DNA (cDNA) was generated from the total extracted RNA in a standard reaction by using 1 µg of total extracted RNA, reverse transcriptase and an oligo (dT)₁₈ primer system provided with a Roche kit. The reaction was set up according to the manufacturer's instructions <http://www.roche-applied-science.com/pack-insert/4379012a.pdf>, in a 15 µl volume. The secondary structure of RNA was dissociated by heating to 70 °C for 5 minutes and then the tubes were immediately placed in ice to prevent the formation of the secondary structure again. The reaction components were added while the tube was in the ice as follows: 5 µl of 5 x reaction buffer (250 mM Tris/HCl, 150 mM KCl, 40 mM MgCl₂, pH approx. 8.5), 1 mM dNTPs, 10 units of the enzyme reverse transcriptase, 20 units of Protector RNase inhibitor, and free nuclease water up to a volume 20 µl. The components were mixed carefully, and then centrifuged briefly. Then, the reactions tubes were incubated at 55 °C for 30 min for production of the first cDNA strand. The reaction was stopped by incubating at 85 °C for 5 min to inactivate the reverse transcriptase enzyme, and then placed in ice. The reaction was snap shot in liquid nitrogen and then frozen at -80 °C. Later, 5 µl of produced 5 µl of cDNA was used for PCR.

2.3.18 Quantitative polymerase chain reaction (qPCR)

The cDNA was amplified and quantified by the qPCR reaction using Rotor Gene machine and analysis software 3000. The reaction was performed in 20 μ l of total volume, using the following specific primers set:

Ap_ST1 Fwr 5' CCC GGC ACA AAT CAA AGG T 3'
 Ap_ST1 Rev 5' CAA TGA AAG CGA AGA ACC AGT TG 3'

set up as follows: 2 μ g/ μ l of cDNA, 1 μ l of homemade Taq polymerase, 0.5 of each forward and reverse primers, 4 μ l of 10x buffer, 1.6 μ l of 25mM MgCl₂, 1 μ l of 4x SYBR Green, and 1.6 μ l of a mixture of (dATPs,dCTPs, dTTPs and dGTPs) 10mM each. The reaction conditions were set up as follows: 94 °C for 2 minutes for the denaturing step followed by 35 cycles of denaturing step at 95 °C for 30 sec, the annealing step at 50 °C for 30sec, extension at 72 °C for 40sec; followed by final extension at 72 °C for 10 min. The qPCR was normalised to a housekeeping gene; therefore a set of glyceraldehydes-3-phosphate dehydrogenase (GAPDH) primers were used.

GAPDH Fwd 5' TTGCCATCAATGACCCCTTCA 3'
 GAPDH Rev 5' CGCCCCACTTGATTTTGA 3'

To calculate the expression level of the target gene $\Delta\Delta$ Ct, as described by Livak and Schmittgen (2001), the data of both the sample and the calibrator were compared to the reference gene (GAPDH) to normalize for variation in the sample; that is called the Δ Ct values. Then the Δ Ct values for the treated sample with RNAi and the calibrator, which is the untreated sample, were used to calculate $\Delta\Delta$ Ct value as follows:

$$\Delta\text{Ct}_{\text{treated}} = \text{Ct}_{\text{target treated}} - \text{Ct}_{\text{reference treated}}$$

$$\Delta\text{Ct}_{\text{calibrator}} = \text{Ct}_{\text{target calibrator}} - \text{Ct}_{\text{reference calibrator}}$$

$$\Delta\Delta\text{Ct}_{\text{treated sample}} = \Delta\text{Ct}_{\text{treated sample}} - \Delta\text{Ct}_{\text{calibrator sample}}$$

2.4 Maintenance of pea aphids culture

Pea aphids, which are available in the School of Biological and Biomedical Sciences of Durham University, are maintained on fava bean plants at 18 °C in a daily cycle of 16 hours of light and 8 hours of darkness in a growth room. Aphids for these feeding experiments in this study were removed from the bean plants gently by using a fine paintbrush and collected in a Petri dish. They were transferred to feeding diet chambers, which were covered with two parafilm sheets and left to produce nymphs while they were supplied with 100 µl of artificial diet stuffed between the parafilm layers.

2.5 Feeding bioassay

To examine the effect of the transcribed dsRNA the following bioassays were conducted. Feeding was selected as a simple method to deliver the synthetic dsRNA, which was more feasible with small insects such as aphids than injection or other methods; the experiment was run as described below.

2.5.1 Feeding bioassay (1)

To determine the effects of dsRNAs on the expression level of the encoding gene of the sugar transporter gene *Ap_ST1*, aphid nymphs were collected from the culture and distributed in six chambers, ten aphids in each. The chambers were covered with two layers of parafilm, as shown in figure 6, and supplied with 100µl of one of the following: water only, diet only, diet mixed with 10ng/µl dsRNA corresponding to kanamycin-resistance gene, or dsRNA corresponding to *Ap_ST1* in a range of concentrations (10ng/µl, 50ng/µl and 100ng/µl). The nymphs were left to feed on the supplements for a week; meantime their movements, growth and survival were observed carefully. The supplements were replaced every 24 hours. After seven days, the aphids were collected to measure their growth by using ImageJ software. Then, as described in section 2.3.11, their total RNA was extracted and used for cDNA synthesis.

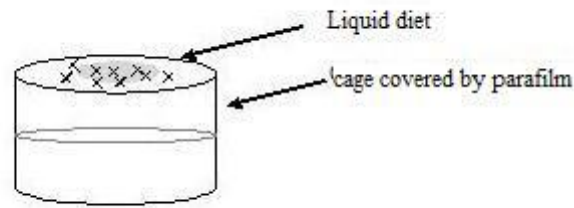


Figure 5 : Aphids feeding cage.

2.5.2 Measuring aphids bodies length

Following the treatment period, the aphids' growth was measured by using the program ImageJ to determine the length of their bodies, and examining any alteration in aphids' growth compared with control aphids. Initially, a single aphid was picked up and placed between two slices, then visualised alongside a ruler under a light microscope. Their images were then captured using Openlab software 'Modular Imaging Software'.

2.5.3 Feeding bioassay (2)

To study the effect of dsRNA on the different aphids' growth stages, the bioassay was repeated using the dose that the previous study had determined to be the most effective, based on the alteration produced to both phenotype and target gene expression. Seventy nymphs were collected and transferred to testing chambers. Ten were designated as the control group, which was fed on diet only, and the rest were distributed between three chambers. Each day, five aphids were collected from each chamber to perform RNA extraction, as mentioned in section 2.3.11. The collection time points started after 24 hours after these aphids started feeding and ended on day seven.

Chapter 3.0 RESULTS

To study whether the oral delivery of the conventional dsRNA to pea aphids has a possible effect on the expression of the sugar transporter gene *Ap_ST1*, dsRNA corresponding to *Ap_ST1* was synthesized and applied to pea aphids' diet in different concentrations. The aphids' growth was monitored to observe any morphological alterations during the experiment and after the experiment aphids were collected to take their body length measurements, followed by the detection of the transcription level of the target gene, which was determined after each test by running the qRT-PCR.

This chapter reports on the results of the experiments described in chapter 2 before discussing these results and conclusions that can be drawn from this study.

3.1.1 Clone and sub-clone of *Ap_ST1* in litmus 28i

The construct which carries the gene of interest *Ap_ST1* was provided by Dr. Price as stated in section 2.3.12. This construct was used as template for amplification of a selected region of the sugar transporter gene *Ap_ST1*, which is 433 nucleotides in length and correspond to the fragment between 541nt and 961nt in *Ap_ST1*, by using the designed set of primers in the PCR reaction. The PCR product, shown in figure 7, was cloned in plasmid p.JET2.1 because of the ease of cloning blunt-ended fragment and its availability in the lab. Kanamycin-resistance gene has chosen as a control to examine the effect of taking up dsRNAs. It has provided in pFA6 plasmid. The plasmid was treated with the enzymes Styl and HindIII to extract the desired fragment. Then it was ligated in p.Jet2.1. Both constructs were transformed into *E.coli*.

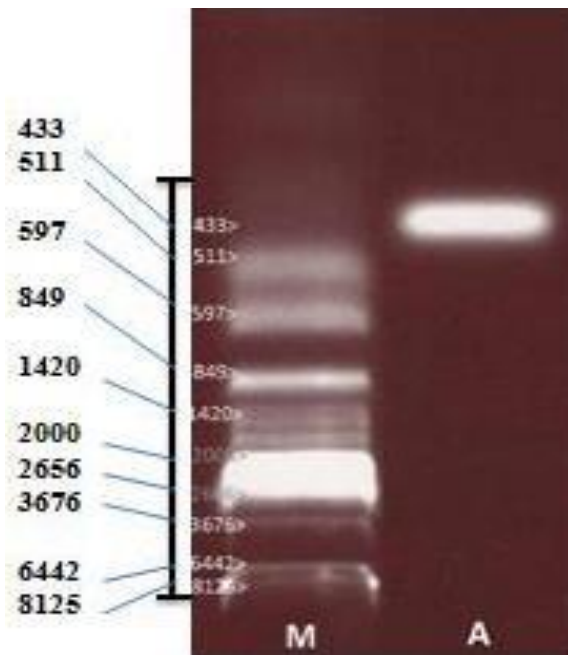


Figure 6: The PCR product of the amplified sugar transporter encoding gene *Ap_ST1* from the recombinant plasmid. The fragment of *Ap_ST1* was amplified by using a specific set of primers to produce 433 bp length segments of *Ap_ST1*. Lane M: DNA ladder / (Aval) marker. Lane A: the band *Ap_ST1* PCR product, 433 nt.

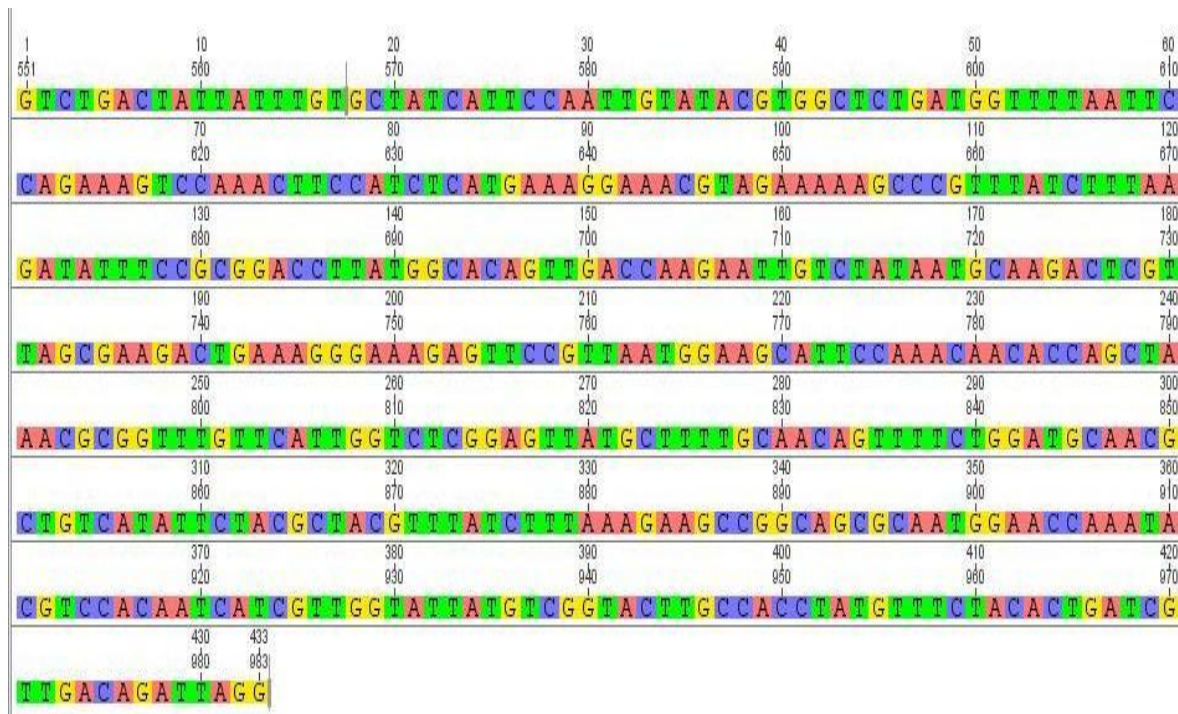


Figure 7: the selected sequence of *Ap_ST1* for dsRNA synthesis. A set of primers were designed to amplify the fragment that correspond to the sequence between 551 nt and 983 nt in *Ap_ST1*, located in the middle of its sequence, to synthesis the dsRNA, which was used in the expression knockdown.

The plasmids were prepared from the clone and sent for sequencing to ensure that the desired sequences had been cloned. The results were analyzed by using SEQUENCHER, which confirmed the sequences of the inserted fragments. By using the enzyme BglII, the inserts were released from the constructs and sub-cloned in the plasmid litmus28i, because it has the promoter T7 in both orientations which facilitates the synthesis of the conventional dsRNA.

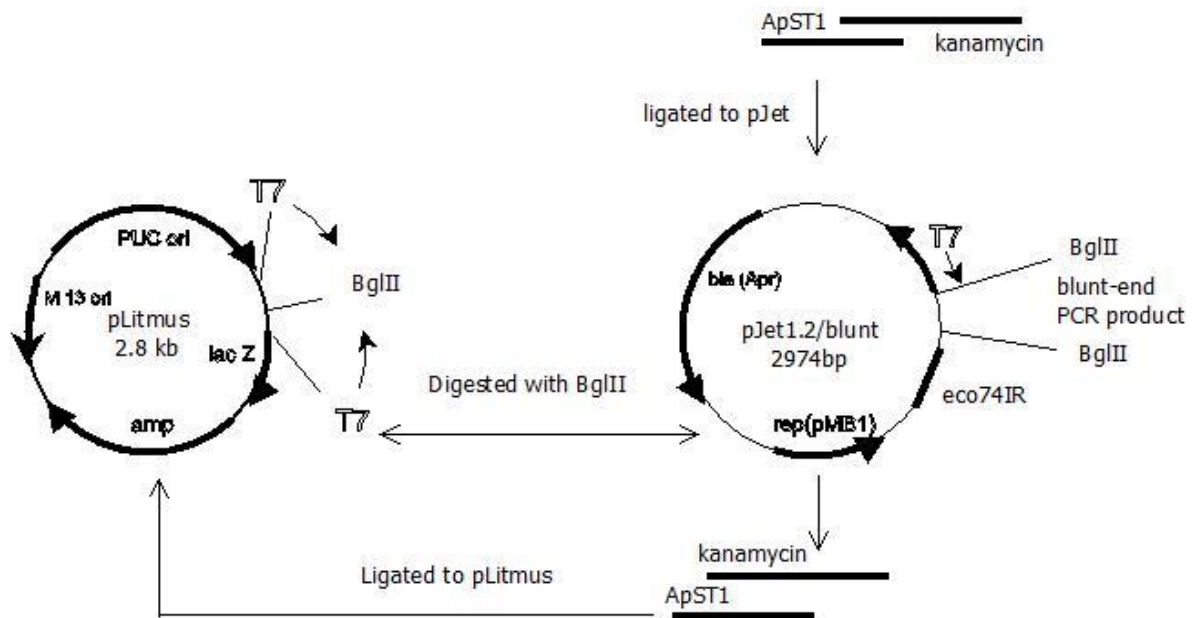


Figure 8: Schematic diagram for construction of Litmus 28i *Ap_ST1*, and Litmus 28i Kanamycin-resistance gene. The diagram illustrates the sub-cloning process of the selected fragments *Ap_ST1* and Kanamycin-resistance gene into the BglII site of the vector litmus28i, after extracting them from the pJet vector.

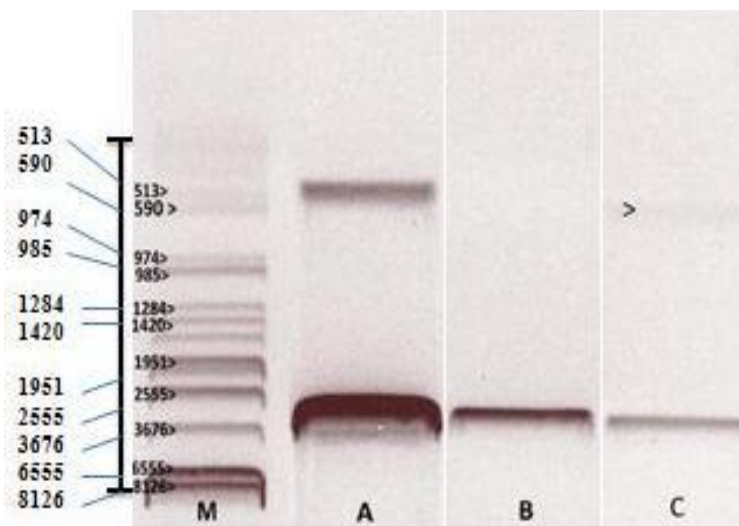


Figure 9: The digestion of the segments of cloned genes *Ap_ST1* and Kanamycin resistance gene in p.JET. The images show the fragments of interest which were extracted from the constructs by using the digestive enzyme BglII. Lane M: the DNA marker. Lane A: the small band (433 pb) shows the extracted *Ap_ST1* from p.Jet. Lane B: digested plasmid litmus 28i, with BglII. Lane C: kanamycin 545nt band.

3.1.2 Synthesis and preparation of dsRNAs

In order to synthesis a conventional dsRNA that targets the corresponding mRNA of the sugar transporter protein *Ap_STI*, the recombinant plasmids were linearized and prepared for transcription. The *in vitro* transcription was used for both the examined homologue *Ap_STI* and the control (kanamycin- resistance gene), as described in the section 2.3.16. The sense and anti-sense strands were produced in single reaction tubes. Then they were treated post transcriptionally to anneal the strands, as described in the method (section 2.3.16).

The generated dsRNAs were visualized and analysed on 1% agarose gel electrophoresis, as shown in figure 10. The result confirmed the success of producing an annealing complementary dsRNAs; the amounts produced were 1.6 μ g/ μ l and 1.3 μ g/ μ l in 20 μ l volume of dsRNAs corresponding to *Ap_STI* and kanamycin resistance gene respectively. It is important to point out that, in the analyzed bands representing the generated dsRNAs, the immigration of dsRNA was slower than the DNA fragment which had a similar length. This is due to the net charge density of dsRNAs, which is lower than DNA (Gast and Sanger, 1994). However, the reaction had incubated overnight; longer incubation could result in degradation in the produced RNA strands.

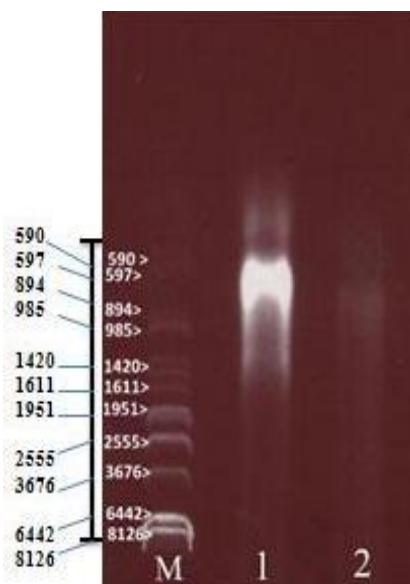


Figure 10: Visualization of the *in vitro* synthetic annealed dsRNAs. The bands represent the generated double stranded RNAs which were obtained after performing the *in vitro* transcription reaction using Ambion kit. Lane M shows 3 μ l of the DNA ladder. Lane 1: 1 μ l of the *Ap_STI*'s dsRNA fragment. Lane 2: 1 μ l of the kanamycin- resistance gene dsRNA. It is obvious that dsRNAs immigrate slower than the DNA fragments which have the corresponding size.

3.1.3 The stability of dsRNA

It was important to make sure that the exposure of the dsRNA to aphids' saliva during the experiment had no effect on its stability. Therefore, the stability of the dsRNA was examined. The dsRNA was extracted from the diet which the aphids were feeding on and some of it was kept at room temperature on the bench for a longer time. The obtained results, illustrated in figure 11, show two examined samples. The first lane, showing the extracted dsRNA 24 hours after the aphids had started feeding, clearly demonstrates that the dsRNA was intact during the test. Lane 2 shows the extracted dsRNA which had been left on the bench, and it shows the stability of dsRNA, despite the unexplained increased in the molecular weight compared to the dsRNA in lane 1. Thus, dsRNA is a very stable molecule in general and it was not been affected by the test conditions.

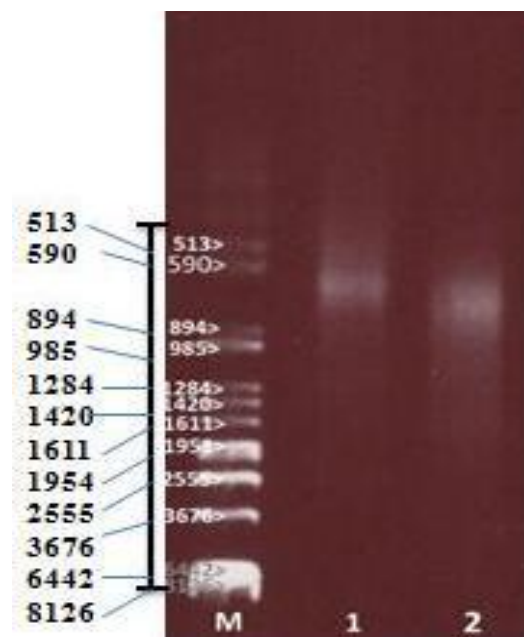


Figure 11: The stability of dsRNA during the test. The image shows the dsRNAs which were run and visualised on 1% agarose gel stained with ethidium bromide. Lane M: the marker, lane 1: the extracted *Ap_STI* dsRNAs after 24hr of aphids feeding. Lane 2: the extracted *Ap_STI* dsRNAs kept for a week over the bench at room temperature.

3.2 Bioassay

The bioassay was designed in the first trial to examine the effect of a range of different concentrations of the dsRNA, (10ng/μl, 50ng/μl and 100ng/μl), which corresponded to *Ap_STI* mRNA and, thus, determine the concentration that was effective on the pea aphids' growth and the gene expression level. The experiment was conducted over seven days, which is the period in which pea aphids become adults.

Following the bioassay, total RNA was extracted from whole aphid body and prepared for synthesis cDNA in a reverse transcriptase PCR reaction followed by a quantitative real time PCR (qRT- PCR) to determine the expression level of *Ap_STI* in both the control and the treated samples. Two sets of primers were used, a gene specific primer (GSP), which was designed to detect *Ap_STI* (section 2.3.18), and a set of primers for control, namely, the endogenous house keeping gene, GAPDH.

3. 2.1 Effects of dsRNA on the expression level of the target gene (experiment 1)

Experiment 1 aimed to study the effect of RNAi and select the optimum concentration to decrease the gene expression. The results, illustrated in figure 12, show that there was a 98% decline in the expression level of the target gene *Ap_STI* compared to the expression level for the same gene in the control group when using the lowest concentration, whereas there was no decrease in the expression was recorded when using the other concentrations. This proves that the RNAi affected the amount of the expressed mRNA of the target gene in the treated sample.

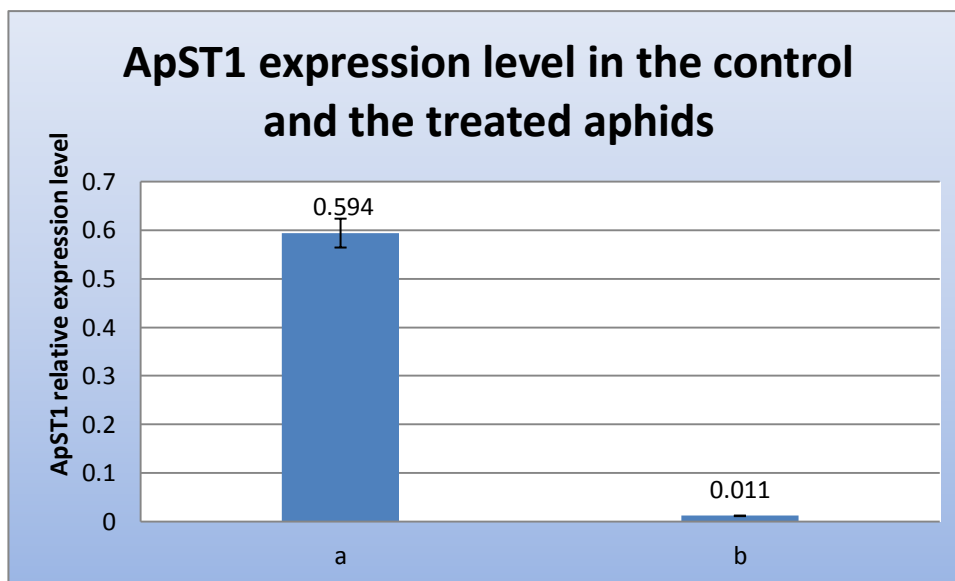


Figure 12: qRT-PCR quantitation of RNAi mediated gene silencing of *Ap_STI* expression in pea aphids. Column a represents the expression level of *Ap_STI* in the control group. Column b represents the expression level of *Ap_STI* in the specimen treated with (10ng/μl).

The experiment was repeated in order to confirm the result obtained from the first experiment and to support the effect of the examined concentration of *Ap_STI*'s dsRNA.

3.2.2 Effects of dsRNA on *Ap_STI* expression level (experiment 2)

Because there was evidence that the expression level of *Ap_STI* was decreased by applying RNAi at a 10ng/μl concentration, the experiment was repeated with this concentration of dsRNA over the same period of time. Ten pea aphids were placed in each cage and the groups were tested. Each day, for seven days, pea aphids were collected from each group to extract the total RNA, which was kept at -70 °C. Finally, the expression level of the target *Ap_STI* was analyzed by qRT-PCR (figure 13). There was a decrease in the detected *Ap_STI* mRNA in each of the examined groups, though the results varied day by day. On the first day, the expression level of *Ap_STI* mRNA was a 3.84%; on the second day, it was 6.66%; on day three, 5.44%; on day five, 6.25%; on day six, 5.44%; and on day seven it was 35.35%. These results are illustrated in figure 15.

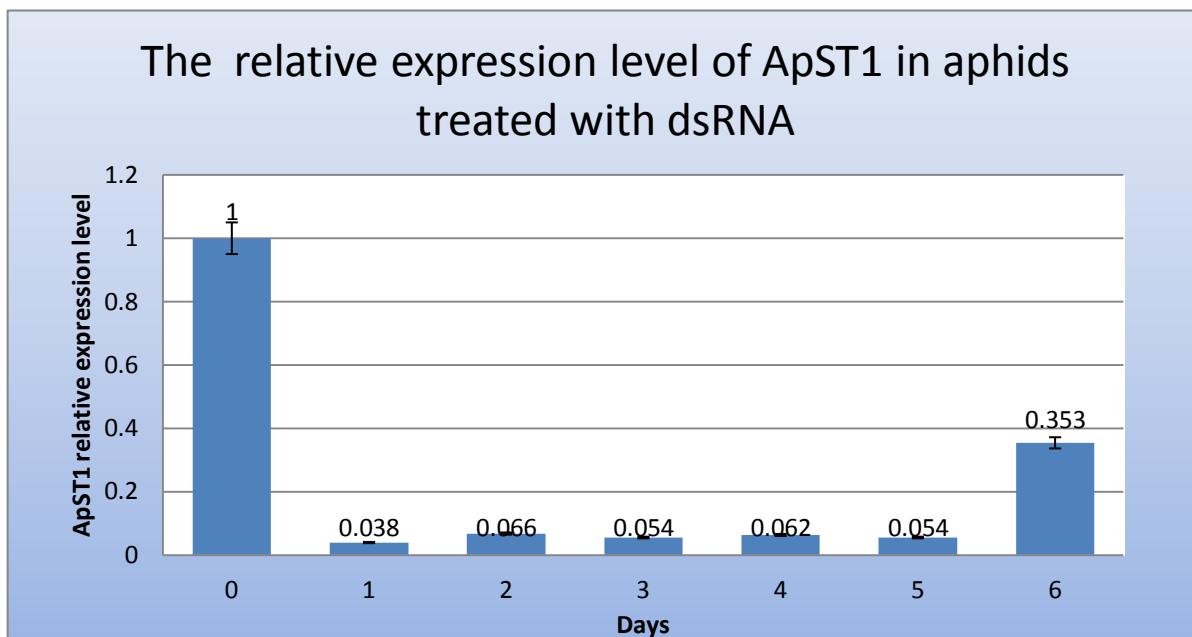


Figure 13: RNAi mediated effects on the expression level of *Ap_ST1*. The effect of RNAi was monitored during seven days by determining the level of mRNA using qRT-PCR. Clearly, there is a decrease in the expression level of the target encoding sugar transporter gene (*Ap_ST1*). Columns 0-6 show the expression level in the aphids treated with dsRNA from the first day to the seventh day. Column 0 shows the expression level in the untreated aphids, which represents the control.

3.2.3 Bioinformatic Analysis

The nucleotide sequence of the expressed gene *Ap_ST1* and the most similar genes, *Ap_ST16* and *Ap_ST17*, in pea aphids were aligned with each other. Comparison between these sequences revealed that there are 307 nucleotides similar in the selected region for dsRNA synthesis; the similarity between these sequences in the selected region was estimated to be 79.9%. This part of the nucleotides sequences alignment was considered because it was the part which was used for the degradation by dsRNA. By blasting the primers set that designed for the qRT-PCR to detect the expression level of the gene *Ap_ST1*, using NCBI database shows the specificity of the primers set to the target gene.


```

Ap_ST1          TCAATGTATTGGGTCTGACTATTATTTGTGCTATCATTCCAATTGTATACGTGGCTCTGA 598
Ap_ST16        TAGATGTATTGGTCTATCTATTGCTTGTCATAGTTCAGCGGTCTATTTTGTGGTGA 927
Ap_ST17        TTAATGTATTGGTCTGTCTGTTGCATGTGCAATAGTTCAGTGATTTATATATGCTTGA 864
*   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *

Ap_ST1          TGGTTTTTAATTCCAGAAAAGTCCAAACTTCCATCTCATGAAAGGAAACGTAGAAAAAGCCC 658
Ap_ST16        TGTTTTTTAGTACCAGAAAAGTCCAATTTTCTACCTAACAAAAGGAAATATTATAAAGCTC 987
Ap_ST17        TGTTTTTGATACCAGAAAAGTCCAATTTTCTATCTTATGAAAAAAAATGTGAAAAAGCTC 924
**  **  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *

Ap_ST1          GTTTATCTTTAAGATATTTCCGCGGACCTTATGGCACAGTTGACCAAGAATTGTCTATAA 718
Ap_ST16        GATGGTCGTTAAAATATTTCCGTAGACCTTTTGGTCAGGTGGATCAAGAATTAATTACAA 1047
Ap_ST17        AGTTATCACTGAAATATTTCCGAAAACCTGTTGTTTCATGTAAATCAAGAATTAATACCA 984
    *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *

Ap_ST1          TGCAAGACTCGTTAGCGAAGACTGAAAGGAAAAGAGTCCGTTAATGGAAGCATTCCAAA 778
Ap_ST16        TGCAAGATTCTTTGGCTAAAACCTGAAAGAGAAAAAGTCCAATCATGAAAGCATTCCAAA 1107
Ap_ST17        TGCAGAGTGCATTAGCTAAAACCTGAAAGGAAAAGAGTCCAATCATGGAAGCATTCCAAA 1044
****          *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *

Ap_ST1          CAACACCAGCTAAACGCGGTTTGTTCATGGTCTCGGAGTTATGCTTTTGCAACAGTTTT 838
Ap_ST16        CTACACCAGCCAAGCGCGGACTATTCTTAGGCCTTGGGGTTATGGTTTTTATGCAGTTTA 1167
Ap_ST17        CTACACCAGCCAACGAGGATTATGCTTAGGTCCTGGAGTTATGGTTTTTCAACAGTTTA 1104
*  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *

Ap_ST1          CTGGATGCAACGCTGTCTATATTCTACGCCTACGTTTATCTTTAAAGAAGCCGGCAGCGCAA 898
Ap_ST16        CAGGATGTAACACTGTTATTTCTACACCACAACCTATCTCAATGCATCTGGTAGCACAA 1227
Ap_ST17        CTGGGTGTAATGCCGTTATTTTCTATGCAACAACCTATCTTCAACGCAACCGGGAGTTCAA 1164
*  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *

Ap_ST1          TGAACCAAATACGTCCACAATCATCGTTGGTATTATGTCGGTACTTGCCACCTATGTTT 958
Ap_ST16        TAAGCTCAAATGTATCCACAGTTATTGTTAGGTATTATGGCAGTTTGTCTACGTATGTGT 1287
Ap_ST17        TTGGATCAAATACATCCACAATTATTATGGCATCATGGCTGTAGTGTCCACATATGTAT 1224
*           *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *

Ap_ST1          CTACACTGATCGTTGACAGATTAGGAAGAAAAATTCTCTTTTGGAGCTCAATCATTGTCA 1018
Ap_ST16        CAACACTTGTGTTGATAAACTAGGACGAAAAGATTCTGCTTTTGTATTCTGTTATTGCAA 1347
Ap_ST17        CAACGCTTGTGTTGATAAATAGGACGAAAATTCTGTTGTTGATTCTGTTGTTGCAA 1284
*  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *

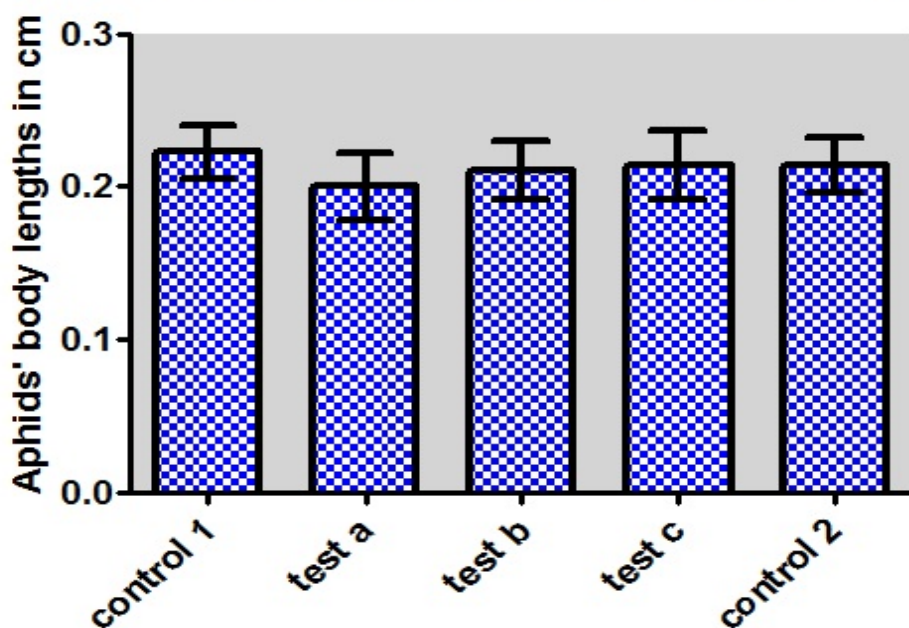
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Figure 14: Multiple sequences alignment of sugar transporter *Ap_ST1* and other predicted genes, *Ap_ST16* and *Ap_ST17*. The shaded sequence is the selected part of the gene to transcribe the dsRNA.

3.3 Measuring the examined aphids growth

The grown nymphs, which were feeding on a diet containing dsRNA, were observed during the experiment, and no incidence of death or abnormal movements were observed. At the end of this period, their body lengths were measured, as described in section 2.5.2. The mean length of the aphids in the control 1 group was 0.22 mm, whereas the mean length of the specimens treated with dsRNA of kanamycin- resistance gene, and dsRNA of *Ap_ST1* in concentrations of 50ng/ μ l and 100ng/ μ l was 0.219 mm in both groups. However, the specimens treated with the lower concentration demonstrated an estimated 10% smaller length than those in the control group. These results are illustrated diagrammatically in figure 15, and by photographs in figure 16.

The body lengths of the examined pea aphids



The measurement of specimens body lengths

Figure 15: The effect of RNAi on the pea aphids. The bars illustrate the effects of RNAi on the aphids' length in cm after seven days of treatment. The first column shows the length of the control 1 group of aphids, which were fed on a normal diet without additives; their growth was regular. The last column shows the length of the aphids in the control 2 group, which were fed on diet mixed with kanamycin to examine the effect of dsRNA uptake. The columns labelled test a, test b and test c show the lengths of the aphids treated with 10ng/ μ l, 100ng/ μ l and 50ng/ μ l, respectively.

The results show that the RNAi that targeted the expression of the encoding sugar transporter *Ap_ST1* gene in the pea aphids' gut has no effect on the growth of pea aphids even when it showed an effect on the expression level.

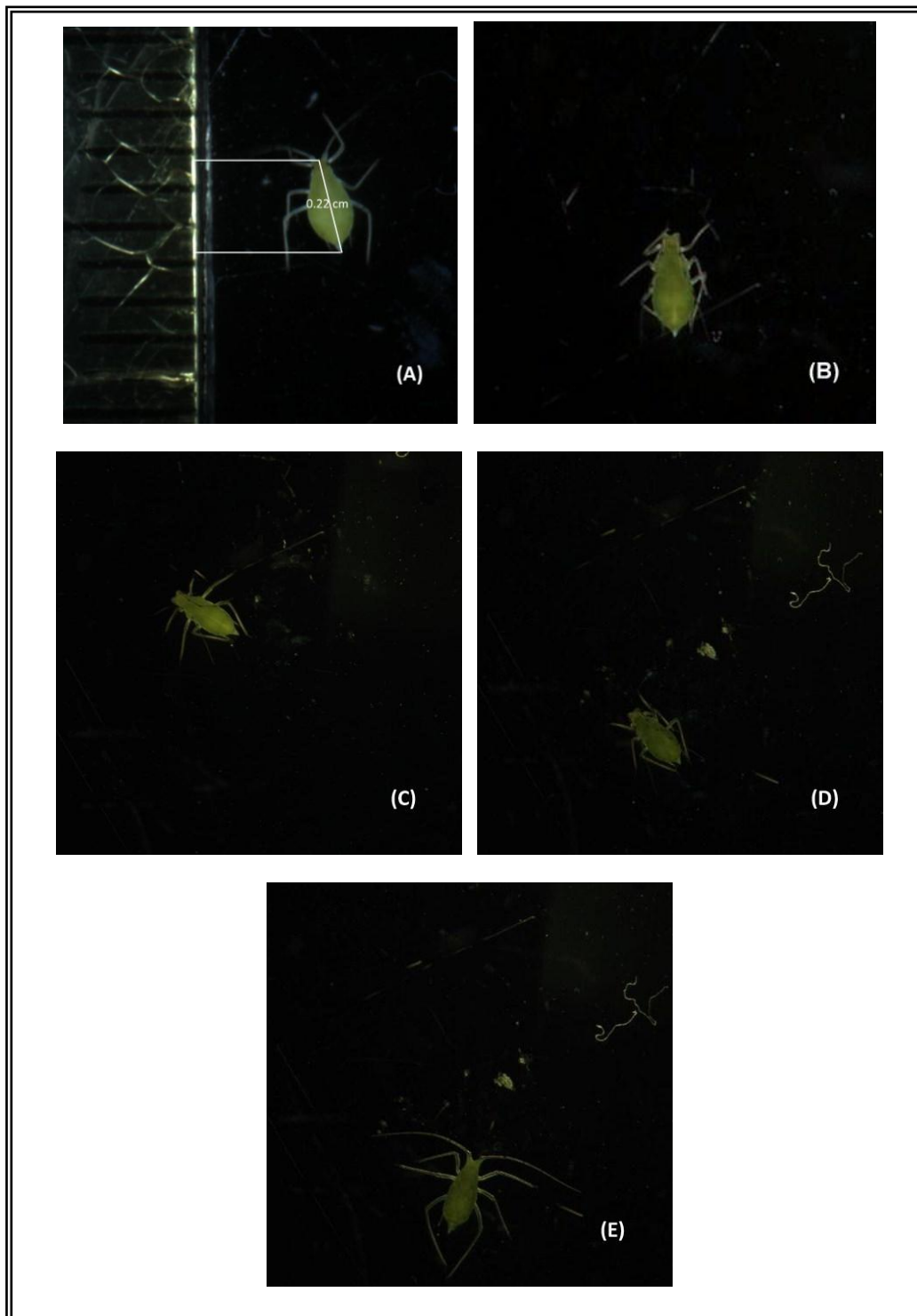


Figure 16 shows surviving pea aphids from different examined groups. 13A is a pea aphid from the control group which fed on a normal diet only. 13B is a pea aphid which fed on a normal diet mixed with dsRNA kanamycin. 13C, 13D and 13E are aphids which fed on a normal diet mixed with 10ng/ μ l, 50ng/ μ l and 100ng/ μ l dsRNA of *Ap_ST1*, respectively.

4.0 Discussion

Recognition of the importance of RNA interference came from research that produced a range of effective applications in different fields and for various purposes, as discussed in section 1.4.4. This technique has been examined in a number of agricultural studies to control pests and it is a promising tool in the development of environmentally friendly pesticides (Gatehouse, 2008). Thus, this research study has investigated the effects of RNAi, using pea aphids as a model organism because, as described in chapter 1, they are a serious pest problem. They feed on plants' phloem sap, which has a rich sugar content and cause serious damage to plants. Therefore, studying the role in aphids' nutrition of their sugar transporter proteins and their encoding genes could help to develop an effective and environmentally harmless solution to control the pest. The objective of this study was to apply the RNAi technique to pea aphids by using a corresponding dsRNA to *Ap_ST1* and examine any possible effects on the gene expression and the aphids' growth. This particular gene was selected because of its important nutritional role in transporting the sugar molecules, mainly glucose, from the aphid's gut to its hemolymph. A single segment of conventional dsRNA was examined to induce an RNAi response; this segment corresponds to the gene sequence that is located between 541nt and 961nt. It was expected that the expression level of the target gene would reduce due to the degradation effect of RNAi.

The first experiment used a range of concentrations of the selected long dsRNA in the artificial diet of groups of aphids, and the effects were observed. Studying the phenotype, by measuring the aphids' body length after they were exposed to the dsRNA for seven days, showed that there was no effect on the growth of the pea aphids groups that were supplied with the lowest concentration of the dsRNA. In further investigation, the expression level of *Ap_ST1* was estimated for each aphids group by running qRT-PCR. The results showed that the examined dsRNA was effective in low concentration, causing a reduction in the expression level of the target gene *Ap_ST1* of 98%. The deficiency in the *Ap_ST1* sugar transporter protein affected the uptake of the sugar molecules fructose and galactose and, as a result of reducing the expression level of the encoding sugar transporter, the aphids' growth was slightly reduced. This almost unnoticeable alteration in the aphids' growth is explained by the presence in the insect's gut of *Ap_ST3*, another expressed sugar transporter protein, which transports hexose sugar molecules, mainly fructose and glucose (Price *et al.*, 2010),

thus enabling it to continue to uptake sugar molecules. However, this result supports other studies which suggest that long dsRNA is effective in inducing specific gene silencing (Possamai *et al.*, 2007), and it emphasizes the correlation between the RNAi phenomenon and the administrated concentration. Surprisingly, higher concentrations did not display any further reduction of the expression of the target gene (results not shown). Similar results were observed by Willims *et al.* (1979) and Cheng *et al.* (2005) when a high concentration of dsRNA was applied in order to inhibit the protein synthesis in rabbit reticulocyte lysates.

Replicating the experiment using the concentration shown to be effective in the previous test demonstrated a reduction in the gene expression. Although the experiment was conducted under the same conditions for each group, different results (shown in figure 13 above) were obtained from qRT-PCR for the effects of RNAi on the target gene *Ap_STI*, which were estimated to be in the range of 65% - 96.2%. This might be due to the differences in the homogenised individual aphids and the different ages of the groups. A similar variation in RNAi efficiency was noticed by Jaubert-Possamai, *et al.* (2007) in testing the efficiency of RNAi on pea aphids using the micro-injection of dsRNA that corresponds to both encoding the *Ap-crt* and *Ap-cath-L* genes, which encode a calreticulin and the gut specific acathepsin-L, respectively. However, the results confirmed the effect of the RNA interference in inducing specific gene silencing.

This study depended on the oral delivery of dsRNA to mediate the gene silencing for two reasons: first, because this method is feasible and appropriate for crop protection against pests (Gatehouse, 2008), and secondly, because the relatively small size of pea aphids makes other delivery approaches, such as injection, difficult to conduct. Oral delivery of dsRNA has been used in previous studies. Two of the problems that have been addressed in the oral delivery of dsRNAs or siRNAs to insects are the ability of the organisms' cells to uptake RNAi inducing molecules and the persistence of its effect. This is due to the absence of both the sid-1 homologue gene, which is found in *c.elegans* and functions as a facilitator to uptake the dsRNA, and the RNA dependent RNA-polymerase (RdRP) homologue gene (Niu *et al.*, 2010), which amplifies the siRNA and maintains the RNAi effects. Although aphids lack these two genes, the results obtained from this experiment support the suggestion that there are alternative pathways to uptake dsRNA and the proposal that feeding dsRNA mediates a specific gene silencing.

To verify that the dsRNA was not affected by the aphids' saliva during the test, its stability was examined after being exposed to the aphids. The dsRNA was extracted from the diet that the aphids were feeding on, and running it on the gel showed that it was in the same band as the fresh dsRNA. This indicates that dsRNA is a quite stable molecule. It would also have been possible to examine the stability either by shearing some amount of the extracted dsRNA and run two lanes on the agaros gel to compare them to each other, or by reversely transcribing dsRNA to cDNA and then comparing the obtained sequence to the examined fragment of *Ap_STI*. However, it was sufficient to compare the migrant bands on a gel.

The possibility of interference between the target gene *Ap_STI* and the most similar genes, *Ap_STI7* and *Ap_STI6*, was considered but it was decided that the possibility was very low due to the very low expression of the latter genes in the organism, and also using primers which are specific only to *Ap_STI* eliminate any errors or misinterpreting the obtained results. However, further work is required to increase the affectivity of the RNAi technique as a biological pesticide to protect crops against pea aphids. In the light of this study, it is suggested that knocking down multiple targets of sugar transporter encoding genes in an aphid's gut might be a highly effective method to control this pest (Gatehouse, 2008), and this technique was examined previously in *Drosophila* (Schmid, 2002). It is also possible to use different strategies to fight the aphids, such as engineering crop plants that are able to produce a mixture of dsRNAs that target the sugar encoding genes in pea aphids to weaken them and express a toxic protein which might be lethal to aphids.

Rapid growth and proliferation are important features of aphids. Affecting proliferation by designing dsRNAs that target the conserved genes in the production system would be a feasible method of limiting the spread of aphids and accordingly reducing their effects on the plants. In general, in order to achieve an effective knockout method, it is important to choose the right targets which have a fatal phenotype.

4.1 Conclusions

The aim of this study was to examine the effect of RNAi on the encoding sugar transporter gene *Ap_ST1* by using a conventional dsRNA. dsRNA was effective in causing RNAi suppression to *Ap_ST1* transcript. However, this had no effect on the aphids' length, which is explained by the presence of another sugar transporter gene. The analysis of the results obtained from this research showed that dsRNA is a quite stable molecule. The corresponding dsRNA to *Ap_ST1* showed its effect and this genotypic level. Interestingly, the effect of RNAi was dose dependent, with the lowest dose of dsRNA used in the experiment proving to be the most effective.

4.2 Future Research

This study has examined the possibility of using RNAi to control pea aphids. Since dsRNA corresponding to *Ap_ST1* has down-regulated expression of the target gene, but had no effect on aphid growth, this suggests that *Ap_ST1* is functionally redundant, and that other sugar transporters are able to compensate for decreased activity of the encoded protein. Targeting the other sugar transporter genes which are highly expressed in the pea aphid gut (e.g. *Ap_ST3*) using dsRNA or siRNA would increase inhibition of sugar transport, and possibly demonstrate the effectiveness of RNAi as a pest control method. However, a reconsideration of possible gene targets in the aphid for RNAi, based on genes in other insects which are known to give lethality when down-regulated, might be a better approach.

Whatever the target, an examination of dose-response effects of fed dsRNA would be necessary. RNAi is known to be dose dependent, and feeding assays at various concentrations of dsRNA targeting the candidate gene is an essential step. It would also be interesting to use plant-mediated RNAi approach for aphid control. Such as developing fava plant to deliver siRNA corresponding to *Ap_ST1* and *Ap_ST3* to the pest and assess the silencing level of each gene and consequences of the exam. Further investigation to identify essential sugar transporter genes and assessment of other genes, which are necessary for aphids survival, would allow us to increase the potential success of RNAi in pest control.

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