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RNAi for insect resistant plants

Supervisor: **Candidate:**

Prof. F. Pennacchio **Alessandra Ruggiero**

Supervisory committee:

Lucio Filippini, Angharad Gatehouse, Gabriella Colucci

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INDEX

INTRODUCTION

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1. Strategies to control insect pest damage

Insect pests are a worrying problem in agriculture and, with a projected increase in the world population to ten billion over the next four decades, an immediate priority for agriculture is to achieve maximum production of food and other products in a environmentally sustainable and cost-effective manner. Losses due to insect herbivores, estimated at 10-20% for major crops, are significant factors in limiting food production (Ferry et al., 2004). Various types of strategies have been utilized to control insect pests; the most used is still the chemical treatment. Although the use of chemical pesticides is effective, the cost and toxicity to humans and the environment has motivated the search for alternative pest control strategies (Whangbo and Hunter, 2008). In fact, the production of transgenic plants as strategy to control insect pests has become widely used in America. The most successful strategy was the transformation of the plants with genes encoding *Bt* toxins. However, recent reports of resistance to *Bt* toxins being observed in field populations of insects exposed to transgenic plants (Tabashnik et al., 2008; Gahan et al., 2001) have been provided an additional impetus for the development of alternative crop-protection strategies.

Methods, based on the use of self-defensive plant proteins or of insecticidal proteins are applied, but have limited application to control pests in agriculture.

The most powerful alternative crop-protection technology remains RNA interference. It has a high specificity and is used for applications where Bt-based approaches have proved difficult, for example protection against flies (dipterans), or where no effective *Bt* toxins are known, for example protection against sap-sucking homopteran pests such as aphids, leafhoppers and whitefly (Borovsky, 2005; Gordon and Waterhouse, 2007; Price and Gatehouse, 2008).

1.1. *Bt* toxins

The source of the insecticidal toxins produced in commercial transgenic plants is the soil gram-positive spore-forming bacterium *Bacillus thuringiensis* (*Bt*). *Bt* strains show differing specificities of insecticidal activity toward pests, and constitute a large reservoir of genes encoding insecticidal proteins, which are accumulated in the crystalline inclusion bodies produced by the bacterium on sporulation (Cry proteins, Cyt proteins) or expressed during bacterial growth (Vip

proteins). The three-domain Cry proteins have been extensively studied, up to date the tertiary structures of six different three-domain Cry proteins, Cry1Aa, Cry2Aa, Cry3Aa, Cry3Bb, Cry4Aa and Cry4Ba have been determined by X-ray crystallography (Fig. 1, Bravo et al., 2007) (Li *et al.,* 1991; Grochulski *et al.,* 1995; Morse *et al.,* 2001; Galitsky *et al.,* 2001; Boomserm *et al.,* 2005; Boomserm *et al.,* 2006). The N-terminal domain (domain I) is a bundle of seven α-helices and this helical domain is responsible for membrane insertion and pore-formation. Domain II consists of three anti-parallel β-sheets with exposed loop regions, and domain III is a β-sandwich. Exposed regions in domain II and domain III are involved in receptor binding (Bravo *et al.,* 2005).

The mode of action of Cry toxins has been characterized principally in lepidopteran insects. It is widely accepted that the primary action of Cry toxins is to lyse midgut epithelial cells in the target insect by forming pores in the apical microvilli membrane of the cells (Aronson and Shai, 2001; de Maagd *et al.,* 2001). Nevertheless, it has been recently suggested that toxicity could be related to G-protein mediated apoptosis following receptor binding (Zhang *et al.,* 2006). Cry proteins pass from crystal inclusion protoxins into membrane-inserted oligomers that cause ion leakage and cell lysis. The crystal inclusions ingested by susceptible larvae dissolve in the alkaline environment of the gut, and the solubilized inactive protoxins are cleaved by midgut proteases yielding 60–70 kDa protease resistant proteins. Toxin activation involves the proteolytic removal of an N-terminal peptide and approximately half of the remaining protein from the C-terminus in the case of the long Cry protoxins. The activated toxin then binds to specific receptors on the brush border membrane of the midgut epithelium columnar cells before inserting into the membrane. Toxin insertion leads to the formation of lytic pores in microvilli of apical membranes. Subsequently cell lysis and disruption of the midgut epithelium releases the cell contents providing spores a germinating medium leading to a severe septicemia and insect death.

One of the major applications of *Bt* toxins is in the development of transgenic insect resistant plants. Transformation of the nuclear genome with genes encoding *Bt* toxins gives very low levels of expression unless extensive modifications, which include removal of AT-rich regions from the coding sequence and use of modified constitutive or tissue-specific promoters, are carried out.

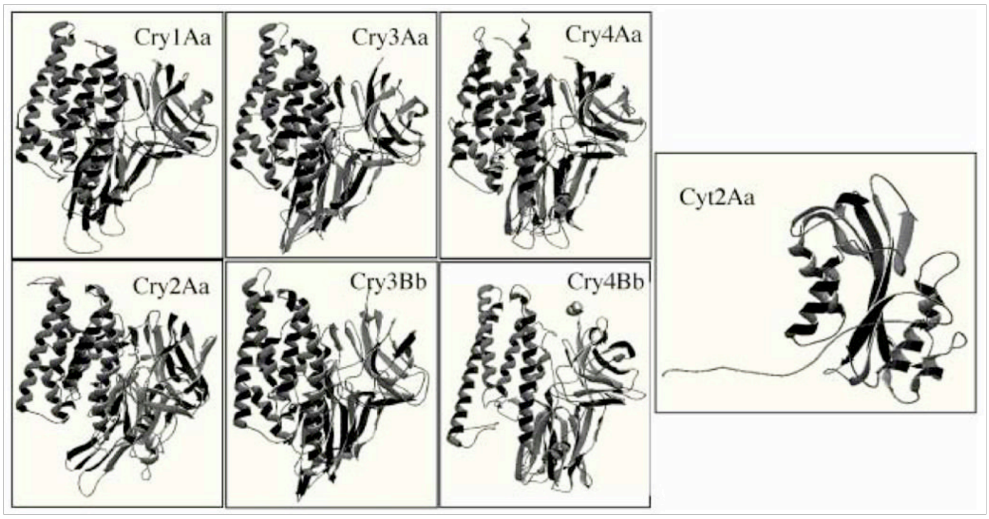

Figure 1.
Three dimensional structures of insecticidal toxins produced by *Bacillus thuringiensis* Cry1Aa, Cry2Aa, Cry3Aa, Cry3Bb, Cry4Aa, Cry4Bb and Cyt2A.

These methods were established within the first stage of the development of this technology and are now considered routine, although they do pose significant technical problems.

In contrast, introduction of unmodified *Bt* genes into the chloroplast genome results in high levels of toxin accumulation (3%-5% of total leaf protein; McBride et al., 1995), as the plastid genome is bacterial in origin. This method has not been widely adopted, due to significant technical problems in achieving stable transformation of the plastid genome and in transforming plastids in species other than tobacco (*Nicotiana tabacum*).

Although the specificity of *Bt* Cry toxins toward target pest species is a major advantage in agriculture, because effects on non-target insects and other organisms in the ecosystem are minimized, deployment of transgenic crops expressing a single specific *Bt* toxin can lead to problems in the field, where secondary pest species are not affected, and can cause significant damage to the crop (Gatehouse, 2008).

Introduction of additional *Bt* cry genes into the crop can afford protection against a wider range of pests and can also be beneficial in prevention of resistance to toxin activity in the target pest(s). Although the ‘‘approved’’ refuge strategy has been highly successful in containing pest resistance to *Bt* toxins expressed in transgenic plants (Tabashnik et al., 2005), targeting different receptors in the insect is (theoretically) more effective because multiple mutations are required to produce the loss of sensitivity to the toxins. Nevertheless results have shown that pests can even acquire resistance to multiple toxins; for example, a strain of the lepidopteran cotton pest *Heliothis virescens* has simultaneous resistance to Cry1Ac and Cry2Aa, with a different genetic basis of resistance to each toxin (Gahan et al., 2005).

The structural similarity of all members of the family of three-domain *Bt* toxins, and the separate roles of the domains in the processes of receptor binding and channel formation, suggested that combining domains from different proteins could generate active toxins with novel specificities. More remarkably, a hybrid Cry protein, containing domains I and III from Cry1Ba and domain II of Cry1Ia, conferred resistance to the lepidopteran pest potato tuber moth (*Phthorimaea operculella*) and to the coleopteran Colorado potato beetle (*Leptinotarsa decemlineata*) when expressed in transgenic potato (Naimov et al., 2003). The ‘‘parental’’ Cry proteins in this hybrid are lepidopteran specific, with no toxicity

toward coleopterans such as the potato beetle, demonstrating the creation of a novel specificity.

Finally modification of *Bt* toxins by site-directed mutagenesis to increase toxicity toward target pests has been employed as an alternative to the ‘‘domain swap’’ approach. However, recent reports of resistance to *Bt* toxins being observed in field populations of insects exposed to transgenic plants have provided an additional impetus for the development of alternative crop-protection strategies.

1.2. Self defense: exploiting plant defensive proteins

Since some plant pests, such as hemipteran, are not affected by known *Bt* toxins, alternative strategies as transgenic plants expressing particular lectins were exploited. Lectins are carbohydrate-binding proteins, involved in plant defense and are the only plant proteins that are capable of recognizing and binding glycoconjugates present on the surface of microorganisms (i.e. bacteria and fungi) or exposed along the intestinal tract of insect or mammalian herbivores (Peumans and Van Damme, 1995).

Expression of the Man-specific snowdrop lectin (GNA) in transgenic rice plants using constitutive or phloem-specific promoters gave plants that were partially resistant to rice brown planthopper (*Nilaparvata lugens*) and other hemipteran pests. Reductions of up to 50% in survival were observed, with reduced feeding, development, and fertility of survivors (Rao et al., 1998; Foissac et al., 2000). Concerns about possible consequences to higher animals of ingesting snowdrop lectin have limited further progress, although a recent study incorporating a 90-d feeding trial found no adverse effects resulting from consumption of transgenic rice expressing GNA by rats (Poulsen et al., 2007).

Another strategy, used to control insect pest damages, was producing plants expressing proteins that are end-products of the wounding response, such as proteinase inhibitors and polyphenol oxidase. Unfortunately, this technology failed, because it gave only partial protection against insect herbivores, due to pre-adaptation by the pests (Morton et al., 2000).

Engineering volatiles emitted by plants offers possibilities for new methods of crop protection as well (Wang et al., 2001; Aharoni et al., 2003): they can be used as repellents for insect colonization, and as attractants for natural

enemies of pests, but unfortunately these transgenic plants are not fully resistant (Schnee et al., 2006).

1.3. Novel approaches: insecticidal proteins

Nematodes of *Heterorhabditis* species that contain symbiotic enterobacteria are widely used for small-scale biological control of insect pests. When nematodes enter in insect host, bacterial cells from the nematode gut are released into the insect circulatory system. Toxins secreted by the bacteria cause cell death in the insect host, leading to a lethal septicemia. *P.luminescens*, the most well-investigated bacterial species of this type, contains a large number of potentially insecticidal components. One of the orally toxic components, toxin A, was selected for further study. The encoding gene *tcdA* was cloned and assembled into expression constructs, containing 5' and 3' untranslated region sequences from a tobacco osmotin gene to improve expression levels of mRNA and protein in transgenic plants. Expression of toxin A at levels >0.07% of total soluble protein in leaves of transgenic Arabidopsis (*Arabidopsis thaliana*) plants (Liu et al., 2003) gave almost complete protection against larvae of the lepidopteran tobacco hornworm (*Manduca sexta*). Leaf extracts from these plants were also toxic to corn rootworm, showing cross-species protection. Commercial development of this technique is likely.

Bacterial cholesterol oxidase has an insecticidal activity comparable to *Bt* toxins, dependent on its enzyme activity, which is thought to promote membrane destabilization. Expression constructs containing part or all of the coding sequence of the protein, or the coding sequence fused to a chloroplast-targeting peptide, resulted in production of active enzyme in transgenic tobacco (Corbin et al., 2001). However, phenotypic abnormalities were observed in transgenic plants unless the enzyme was localized in chloroplasts, possibly as a result of interference with steroidal signalling pathways. Leaf tissue from all transgenic plants was toxic to boll weevil larvae. The cholesterol oxidase gene appears to be an obvious candidate for introduction into the chloroplast genome rather than the plant nuclear genome, which would avoid potential problems caused by enzyme activity in the cytoplasm; however, no further development of this promising method has been reported.

1.4. RNAi

Plant expression of dsRNAs directed against genes in pathogens has become an established technique, and plants showing increased resistance to plant viruses (Niu et al., 2006; Waterhouse et al., 1998; Pooggin and Hohn, 2003) and bacteria (Escobar et al., 2001) through an RNAi effect have been described.

Several research groups have recently explored the possibility of conducting RNAi in insects through feeding: an overview is given in Table 1. It emphasizes interesting targets for RNAi in insect control: there is a wide pallet of target organisms from different insect orders (from pest insects of important crops in agriculture to vectors of human diseases), target genes and feeding methods, demonstrating the richness in application of dsRNA and the potentials of RNAi.

In 2007, Baum et al. published a break through paper on insect control through dsRNA feeding experiments. They provided evidence for the potential use of RNAi to control pest insects in crop protection and demonstrated the fact that it is possible to silence genes in insects when they consume plant material expressing hairpin dsRNA constructs against well chosen target genes. They utilised a screening approach where genes from Western corn rootworm (WCR; *Diabrotica virgifera virgifera* LeConte; Coleoptera) were identified in cDNA libraries, and genes encoding polypeptides predicted to provide an essential biological function were classified as 'targets'. The most effective dsRNA, directed against a gene encoding V-type ATPase A, demonstrated rapid knockdown of endogenous mRNA within 24 h of ingestion and triggered a specific RNAi response with low concentrations of dsRNA.

The specificity of RNAi-mediated insecticidal effects is an important consideration for the use of this technology in a practical application; effects on non-target insects should be minimised. dsRNAs directed against three target genes (β-tubulin, V-ATPase A and V-ATPase E) demonstrated an effective RNAi response in WCR that resulted in high larval mortality. These dsRNAs were also delivered to three other coleopteran plant pests: Southern corn root- worm (SCR; *Diabrotica undecimpunctata howardii*), Colorado potato beetle (CPB; *Leptinotarsa decemlineata*) and cotton boll weevil (*Anthonomus grandis* Boheman). The dsRNAs demonstrated significant larval mortality in SCR and CPB, although only at higher concentrations than those used for WCR.

Organism	Target gene	Location	Stage	Application Method	Amount dsRNA	Length (bp)	mRNA Silencing	Recovery	Gene expression evaluation method	Reference
Coleoptera										
Diabrotica virgifera virgifera	[a]	Gut	Neonates	Artificial diet	<5.4 ng/cm^2	–	–	–	LC50	Baum et al. (2007)
	Vacuolar ATPase subunit A	Gut	Neonates	Artificial diet	52 ng/cm^2	–	Drastic reduction	–	Northern blot and growth	
	Vacuolar ATPase subunit A	Gut	Neonates	Transgenic plant	–	246	–	–	Damage to the roots of the transgenic corn	
	α-Tubulin	Gut	Neonates	Artificial diet	52 ng/cm^2	–	Gradual decrease	–	Northern blot, growth	
	α-Tubulin	Gut	Neonates	Transgenic plant	–	–	–	–	Growth	
Diabrotica undecimpuctata howardii	Vacuolar ATPase subunit A and E, α-tubulin	Gut	Neonates	Artificial diet	780 ng/cm^2	–	–	–	Mortality	
Leptinotarsa decemlineata	Vacuolar ATPase subunit A and E	Gut	Neonates	Artificial diet	52 ng/cm^2	1842, 300, 134	–	–	Mortality	
Phyllotreta striolata	Arginine kinase	Gut	Adults	Leaves	0.05–3.2 ng/ml	332	Significant decrease	–	RT PCR, enzyme assay, mortality, development, fecundity and fertility	Zhao et al. (2008)
Diptera										
Glossina morsitans morsitans	Midgut protein TsetseEP	Midgut	Male adults	Blood meal	10 μg	315	30–55%	No	RT PCR, northern blot, protein assay and mortality	Walshe et al. (2009)
	Transferrin	Fat body	Male adults	Blood meal	10 μg	–	No effect	–	RT PCR, northern blot and protein assay	
Hemiptera										
Acyrthosiphon pisum	Water specific aquaporin	Gut	6 day aphids	Artificial diet	1 μg	–	50%	5 days	qPCR and change in osmotic pressure	Shakesby et al. (2009)
Rhodnius prolixus	Nitroporin 2	Saliva glands	2nd instars	Artificial diet	13 μg	502	42 ± 10%	7 days	RT PCR and plasma coagulation	Araujo et al. (2006)
			4th instars	Artificial diet	80 μg	502	No effect	–	RT PCR	
Hymenoptera										
Apis mellifera	Toll-related receptor 18W	Whole organism	Larvae	Soaking	1.26 μg	852, 330, 234	60%	–	qPCR, mortality and morphological abnormalities	Aronstein et al. (2006)
	Vitellogenin	Fat body carcass	2nd instar	Natural diet	0.5 or 3 μg	504	90%	–	qPCR, weight and development	Nunes and Simões (2009)
Isoptera										
Reticulitermes flavipes	Cellulose enzyme	Saliva glands	Workers	Artificial diet	13 μg	500	60%	8 days	qPCR, cellulase assay, mortality, fitness, feeding and weight	Zhou et al. (2008)
	Caste regulatory hexamerin storage protein	Fat body	Workers	Artificial diet	5.5 μg	500	50–70%	No	qPCR and protein assay, mortality, fitness, feeding and weight	
Lepidoptera										
Epiphyas postvittana	Larval gut carboxylasterase	Gut	3rd instars	Droplet	1 μg	370	<50%	No	qPCR	Turner et al. (2006)
	Pheromone binding protein	Antennae	3rd instars	Droplet	1.3 μg	–	60%	4 days	qPCR	

Table 1.
Overview on use of RNAi in insects with dsRNA being applied through feeding. The insect species and test stage, the target gene and its location are given. The application method with used amount and length of dsRNA is mentioned and if the data were present, the amount of silencing, the recovery of the gene expression and evaluation method are summarized.

Table 1. (continued)

Organism	Target gene	Location	Stage	Application Method	Amount dsRNA	Length (bp)	mRNA Silencing	Recovery	Gene expression evaluation method	Reference
Helicoverpa armigera	Cytochrome P450 (CYP6AE14)	Midgut	3rd instars	Transgenic plant	–	Diced	Decrease	–	Northern blot and gossypol tolerance	Mao et al. (2007)
	Gluthatione-S-transferase	Midgut	3rd instars	Transgenic plant	–	Diced	Decrease	–	Northern blot and growth	
	Acetylcholinesterase	Whole organism	Neonates	Artificial diet	0.7–1.4 μg	21	Drastic reduction	Yes	RT PCR, AChE activity, mortality, weight, growth and fecundity	Kumar et al. (2009)
		Whole organism	Neonates	Leaves	0.7–1.4 μg	21	–	–	Mortality	
Plutella xylostella	Cytochrome P450 (CYP6BG1)	Midgut + carcass	4th instars	Droplet	0.250 μg	345	98%	–	qPCR and permethrin resistance	Bautista et al. (2009)
Spodoptera litura	Aminopeptidase N	Gut	Neonates	Artificial diet + soaking	–	756	No effect	–	qPCR	Rajagopal et al. (2002)
Spodoptera frugiperda	Allatostatin C	Brain	5th instar	Droplet	3 μg	331	80 ± 10%	–	qPCR	Griebler et al. (2008)
	Allototropin 2	Brain	5th instar	Droplet	3 μg	195	97 ± 2%	–	qPCR	
	Cytochrome P450 (CYP6BF1v4)	Midgut + carcass	4th instars	Droplet	0.4 μg	381	85%	–	qPCR and permethrin resistance	
Orthoptera										
Gryllus bimaculatus	Sulfakinins	Brain	Adults	Droplet	10–60 μg	342	–	–	Food uptake	Meyering-Vos and Muller (2007)

–, not determined.

[a] Putative COPI coatomer, ESCRTIII, ribosomal protein S4V-ATPase subunits, mov34, ribosomal protein rps-14, actin, apple ATPase, ESCRTI-Vps28 and ribosomal protein L9 and L19.

Cotton boll weevil was not only completely insensitive to the three WCR-directed dsRNAs, but was also insensitive to dsRNAs directed against orthologous boll weevil genes, emphasising the differences between insect species in susceptibility to orally delivered RNAi strategies.

To demonstrate the practical application of this technology, transgenic corn was engineered to express dsRNA directed against WCR V-ATPase A. The plants were subjected to WCR infestation and demonstrated a significant level of protection compared to controls; that is, they showed reduced damage from WCR feeding.

A different approach was used by Mao et al. (2007). By studying the interaction between cotton bollworm (*Helicoverpa armigera*; Lepidoptera) and cotton, they identified a cytochrome P450 gene, *CYP6AE14*, which is highly expressed in the insect midgut and whose expression is correlated with larval growth when gossypol, a cotton secondary metabolite, is added to artificial diets. The authors concluded that expression of *CYP6AE14* is causally related to gossypol tolerance, presumably via detoxification of this compound, and that suppression of the expression of this gene could increase the sensitivity of the insect larvae to the plant endogenous defence. Tobacco and *Arabidopsis* plants were engineered to produce dsRNAs directed against the bollworm *CYP6AE14* gene. When plant material of both species was fed to larvae, effective repression of the endogenous *CYP6AE14* transcript was observed, and the insects showed increased sensitivity to gossypol when transferred to artificial diets.

When the striped flea beetle (*Phyllotreta striolata*) was fed specific dsRNA, the invertebrate specific phosphotransferase arginine kinase was successfully silenced in the gut, and due to disruption of cellular energy homeostasis, the development of the beetle was severely impaired (Zhao et al., 2008). Bautista et al. (2009) studied the influence of silencing the cytochrome P450 gene CYP6BG1 that is over-expressed in a permethrin-resistant diamondback moth (*Plutella xylostella*) strain. When the gene was silenced after consumption of a droplet of dsRNA solution, the moths became significantly more sensitive to the pyrethroid insecticide. Instead, in the Zhou et al. research (2008), the eastern subterranean termites (*Reticulitermes flavipes*) were fed with cellulose disks supplemented with dsRNA, silencing of the digestive cellulose enzyme and caste regulatory hexamerin storage protein led to reduced termite fitness and increased mortality

Based on the literature, it's possible to summarize five important factors largely influencing the silencing effect and therefore the efficiency of RNAi as insect control technique:

- **Concentration of dsRNA:** for every target gene and organism an optimal concentration has to be determined to induce optimal silencing. It is not true that exceeding that optimal concentration results in more silencing (Meyering-Vos and Muller, 2007; Shakesby et al., 2009).
- **Nucleotide sequence:** the sequence used will determine possible off-target effects in the target organism, but also in other insects. (Araujo et al., 2006).
- **Length of the dsRNA fragment:** this is a determinant of uptake and silencing efficiency in intact organisms and cell lines (Saleh et al., 2006). In feeding experiments most sequences range between 300 and 520 bp. However, there is a study using only one siRNA (Kumar et al., 2009).
- **Persistence of the silencing effect:** the silencing effect on aquaporin in *A. pisum* persists for 5 days and is then reduced (Shakesby et al., 2009). As reported by Turner et al. (2006) this transient effect of dsRNA against the pheromone binding protein in the light brown apple moth (*Epiphyas postvittana*) may be correlated with the turnover rate of the target protein.
- **Life stage of the target organism:** although older life stages are more efficient for handling, the younger stages often show larger silencing effects. For instance, no silencing effect was observed after treating 4th instars of *R. prolixus* with nitropin 2 dsRNA compared to 42% silencing when using 2nd instars (Araujo et al., 2006). Also in the case of the fall armyworm (*Spodoptera frugiperda*) a stronger silencing effect was observed in 5th instar larvae compared to adult moths (Griebler et al., 2008).

It is striking that there are no reports on feeding experiments with the red flour beetle, *T. castaneum*, probably due to the success of the injection experiments. Intriguing is the presence of five successful experiments with four different Lepidoptera species in the list since it is presumed that it is very difficult to establish RNAi in this insect order. Also, in some feeding experiments environmental RNAi worked, but systemic RNAi did not (Walshe et al., 2009), and some injection experiments succeeded to cause silencing of targets, but feeding experiments could not (Rajagopal et al., 2002). This discrepancy might be due to factors depending on the uptake mechanism of the different cell types, but not due to insensitivity of the

target. Besides, these experiments clearly illustrate that RNAi is not a knockout, but a knockdown method: there is no total silencing and often the effect is transient (Shakesby et al., 2009). When the technique is used to control pest insects, this implies that the dsRNA should be present until the pest insect is killed to minimize the risks of resistance development. However, it should not be an insurmountable drawback to use this technique, because in many cases partial silencing of certain genes is known to cause severe damage and irreversible detrimental/lethal effect on the insect.

Although there are several factors that largely influence the silencing effect and therefore the efficiency of RNAi as insect control technique, today the RNA interference (RNAi) still represents a breakthrough technology for conducting functional genomics research in non-model organisms and for the highly targeted control of insect pests.

2. RNA interference pathway

To understand the RNAi technology and its applications in agriculture, it is important to have clues on how it works and what are the important pathways leading to the generation of the silencing molecules.

A hallmark of RNAi is that short (~20-30 nucleotide) dsRNAs, known as small RNAs, are generated by the activity of RNaseIII enzymes (either Dicer alone or Drosha and Dicer). Two main categories of small RNAs have been defined on the basis of their precursors. The cleavage of exogenous long dsRNA precursors in response to viral infection or after artificial introduction generates short interfering RNAs (siRNAs), whereas the processing of genome-encoded stem-loop structures generates microRNAs (miRNAs) (Siomi and Siomi, 2009).

2.1. siRNA biogenesis

Dicer processes long RNA duplexes and generates siRNAs. These small RNAs are ~21-25-nucleotide duplexes with a phosphate group at both 5′ ends, and hydroxyl groups and two-nucleotide overhangs at both 3′ ends, all hallmarks of RNaseIII-mediated cleavage. The Dicer protein contains a PAZ domain, which binds to the 3′ end of a siRNA, and two RNaseIII domains, which have the catalytic activity. It functions as a monomer (Zhang et al., 2004) but the RNaseIII domains

associate with each other to form an 'internal dimer'. The distance between the PAZ domain and the two RNaseIII domains is the length spanned by 25 base pairs (bp) of RNA (MacRae et al., 2007). Thus, Dicer itself is a molecular ruler.

2.2. Loading and sorting by the RISC

In gene silencing pathways initiated by dsRNA precursors, Dicer-mediated cleavage yields small dsRNA intermediates (small RNA duplexes). These small RNA duplexes are converted from a duplex into a single-stranded form as are loaded into effector complexes, called RISCs. For each small RNA duplex, only one strand, the guide strand, is loaded onto a non-sequence-specific RNA binding protein (Argonaute) and assembled into the active RISC; the other strand, the passenger strand, is destroyed. Many eukaryotes express more than one Argonaute protein, and these proteins bind to small RNAs in a sequence-independent manner. RISC has two forms - precursor form (the pre-RISC), which contains the small RNA duplex and mature form (the holo- RISC), which contains the guide strand. The key steps in converting pre-RISC to holo-RISC are small RNA strand unwinding and preferential strand selection.

The prevalent view of RISC loading is that thermodynamic asymmetry along small RNA duplex determines which RNA strand is retained and which is discarded. More specifically, the strand that has its 5′ end at the thermodynamically less stable end of the small RNA duplex is preferentially loaded into the RISC as the guide strand, a phenomenon referred to as the asymmetry rule (Schwarz et al., 2003; Khvorova et al., 2003).

For siRNAs, the known interactions between Dicer and the Argonaute proteins (Hutvagner and Simard, 2008) indicate that the production of the small RNA and the assembly of the RISC might be physically coupled. For example, in *D. melanogaster*, DCR-2 (RNaseIII) does not simply transfer siRNAs to a distinct RISC but, instead, forms part of the RISC together with the siRNAs, indicating that the role of DCR-2 extends beyond the initiation phase. The loading of siRNA duplexes onto AGO2 is facilitated by the RISC-loading complex, which contains DCR-2 and its dsRBD-containing partner (double-stranded-RNA-binding domain), R2D2 (Liu et al., 2003; Liu et al., 2006). The particular strand of the siRNA duplex that is loaded onto AGO2 seems to be determined by the orientation of the DCR-2–R2D2

heterodimer on the siRNA duplex (Tomari et al., 2004). R2D2 is thought to sense the thermodynamic stability of the siRNA duplexes and bind to the more stable end of the siRNA, whereas DCR-2 is recruited to the less stable end. The heterodimer probably recruits AGO2 through an interaction between DCR-2 and AGO2.

It is recently demonstrated, in *D. melanogaster*, that DCR-2 also interacts with D-elp1, elongator subunit 1 of the Drosophila pol II core elongator complex. D-elp1 has RdRP activity, RNA-dependent RNA polymerase, and cytoplasmic location and its role in RNAi suggested it might be interacting with components of the RISC (Lipardi and Paterson, 2009).

The unwinding of the siRNA duplex and the loading of a single strand into the RISC are facilitated by the slicing of the unincorporated (passenger) strand by AGO2 (Matranga et al., 2005; Rand et al., 2005; Miyoshi et al., 2005), (Fig. 2). Cleavage in the middle of the passenger strand, as though the passenger strand were an mRNA target, would be expected to reduce the annealing temperature and the free energy of duplex formation, which in turn facilitates the separation of the siRNA strands. These data support a model in which siRNAs are initially loaded as duplexes onto an AGO2-containing pre-RISC (Fig. 3).

Once assembled, RISCs mediate a range of the effector steps in all RNA silencing mechanisms, from repressing translation to maintaining genome stability. The specialized functions of RISCs are likely to result from the particular proteins that associate with each Argonaute protein. In other words, the different RISC variants are distinguished by their constituent Argonaute protein. Thus, it is crucial that a specific set of small guide RNAs is directed to a specific Argonaute protein. Analyses of how different types of small RNA are channelled to different Argonaute proteins show that there are multiple mechanisms: the determinants for small RNA sorting vary from the structure of the small RNA duplex to the identity of the 5′ nucleotide and the presence and extent of modifications to this nucleotide.

In *D. melanogaster*, pre-miRNAs are processed by DCR-1, whereas exo-siRNA duplexes are produced by DCR-2 from long dsRNAs (Lee et al., 2004), (Fig. 3). Small RNAs then seem to be loaded onto either AGO1 or AGO2, depending on the structure of a small intermediate RNA duplex (Tomari et al., 2007). If the duplex has a bulge in the middle (frequently observed in miRNA precursors), the small RNA is routed to AGO1. If the duplex is perfectly matched, the small RNA is channelled to AGO2.

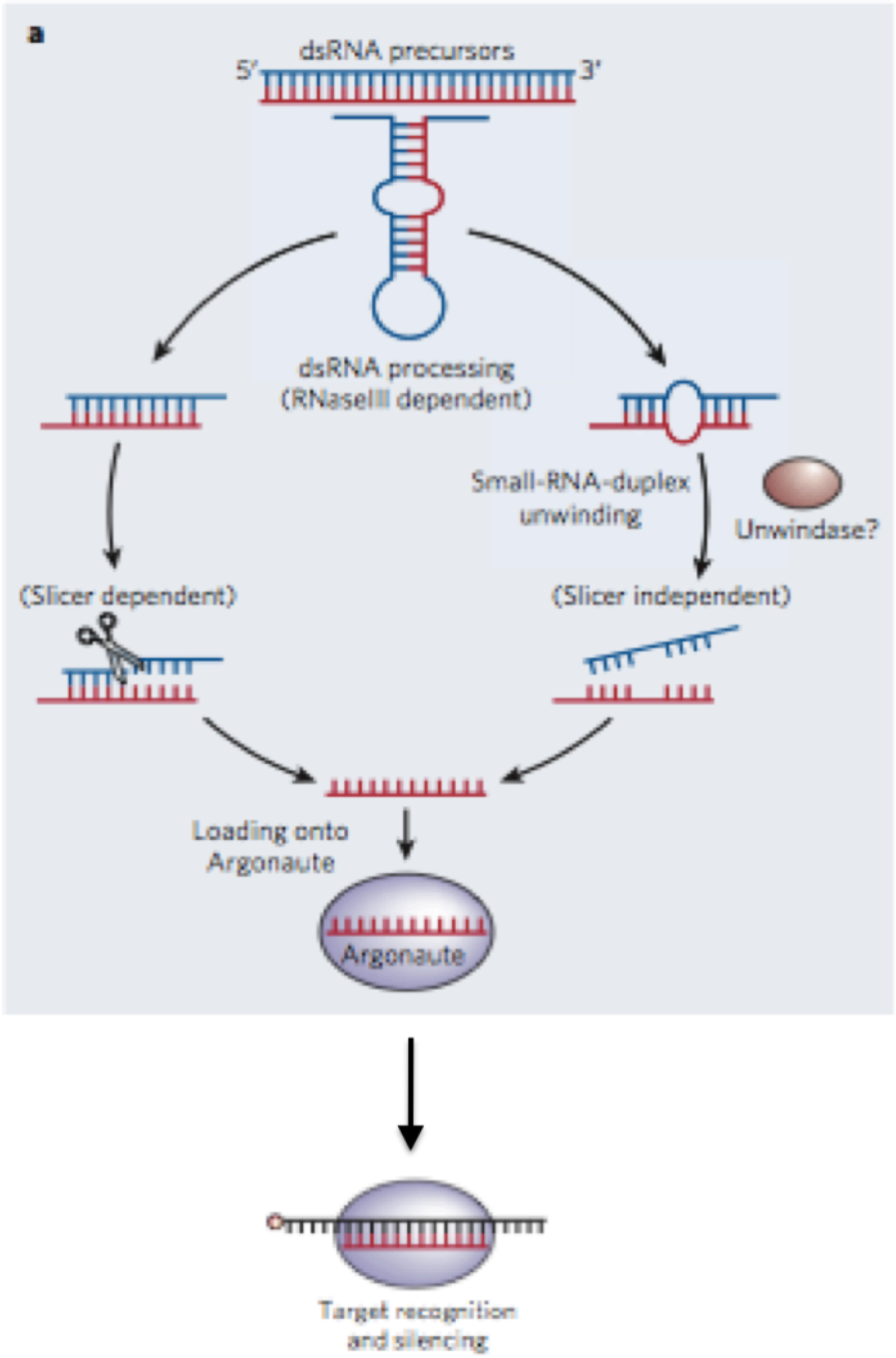

Figure 2.
Natural transcripts that form dsRNAs and hairpin-shaped structures can be sources of small RNAs. These precursors are processed by an RNaseIII enzyme (such as Drosha or Dicer), yielding small RNA duplexes. Duplexes with a perfect match (left pathway) are further processed by an enzyme with slicer activity (an Argonaute protein) into single-stranded small RNAs. By contrast, small RNA duplexes with a mismatch or bulge in the centre (right pathway) are not substrates for the slicer and thus become single- stranded in a cleavage-independent manner. The identity of the protein that carries out this unwinding is unknown. Single-stranded small RNAs are then loaded onto Argonaute proteins. The particular strand that is selected (sense or antisense) depends on thermodynamic stability. The loaded Argonaute proteins are guided to target mRNAs containing complementary sequence, and the expression of the corresponding genes is silenced.

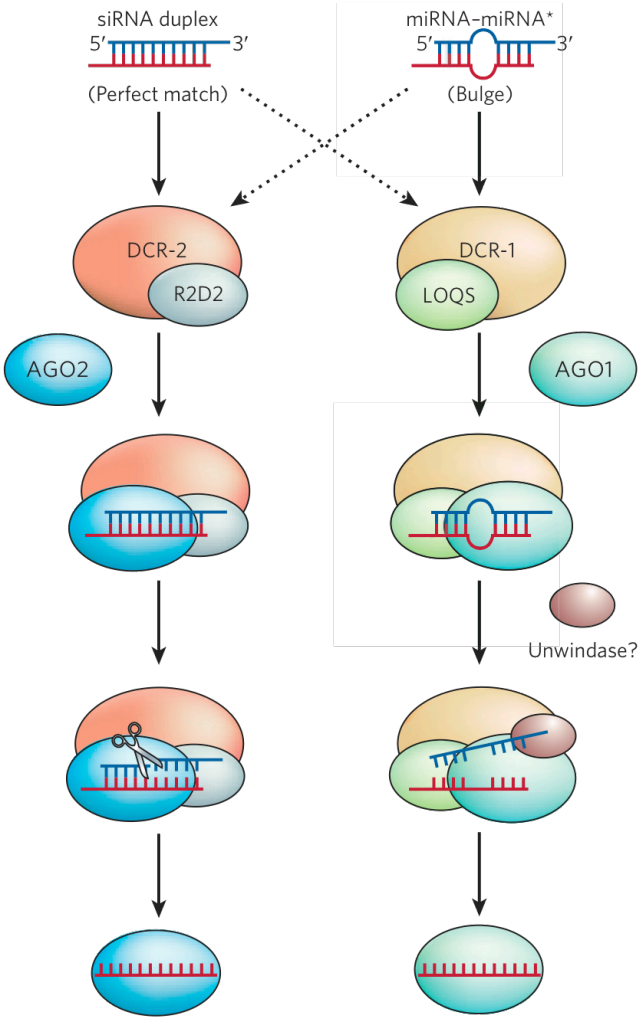

Figure 3.
Small RNAs are sorted onto specific Argonaute proteins, and this process occurs by several mechanisms. In *Drosophila melanogaster*, small RNAs originating from a duplex are loaded onto one of two Argonaute proteins (AGO1 or AGO2), on the basis of the structure of the small RNA duplex. If the duplex has a mismatch or a bulge in the centre (as miRNAs do), then the RNA is routed to AGO1. If the duplex is perfectly matched (as siRNAs are), then the small RNA is routed to AGO2. This selectivity occurs because the small RNAs are loaded onto Argonaute proteins from a Dicer-containing complex, and the two forms of Dicer, DCR-1 and DCR-2, associate with different RNA structures. DCR-2 pairs with R2D2, and this heterodimer binds to highly paired small RNA duplexes but recognizes small RNA duplexes with a central mismatch only poorly. AGO2 favours binding to DCR-2–R2D2 over binding to the other Dicer- containing complex, DCR-1–LOQS, which binds to small RNAs with bulges.

This is because the DCR-2-R2D2 heterodimer, which recruits AGO2 to form the pre-RISC, binds well to highly paired small RNA duplexes but poorly to duplexes with central mismatches. Thus, the DCR-2–R2D2 heterodimer not only determines the polarity of siRNA loading on the basis of thermodynamic stability rules but also functions as a gatekeeper for AGO2-containing RISC assembly, promoting the incorporation of siRNAs over miRNAs. These observations suggest that each siRNA duplex dissociates from the active site of the Dicer protein after it is produced and is subsequently recaptured by the DCR-2–R2D2 heterodimer.

The identity of the nucleotide at the 5′ end and the extent to which this nucleotide is phosphorylated also influence which Argonaute protein the small RNA associates with.

2.3. Safeguards in silencing pathways

To avoid 'off-target' silencing, gatekeepers, which ensure that Argonaute as non-sequence-specific RNA-binding protein can bind to small guide RNAs but not to degraded small RNAs, are required: such gatekeeper systems seem to depend mainly on structural features specific for small guide RNAs.

As described earlier, Dicer helps to load siRNAs into the RISC, preventing siRNAs from diffusing freely in the cytoplasm after their production. This function of Dicer probably also aids in the discrimination of genuine siRNAs from various RNA-degradation products in the cell. Processing by RNaseIII enzymes (such as Dicer) characteristically yields small RNAs with 5′ monophosphates and 3′ two-nucleotide overhangs. The PAZ domain of Argonaute proteins might, as a first step, distinguish degraded RNAs (derived from unrelated pathways) from these small RNAs by binding to the characteristic 3′ overhangs of the small RNAs (Chapman and Carrington, 2007). In addition, to become incorporated into the RISC and mediate cleavage of the target mRNA, the guide strand of a siRNA must have a phosphate group at the 5′ end (Pham and Sontheimer, 2005).

2.4. Target-sensing modes and effector modes of the RISC

Most of the binding energy that tethers a RISC to a target mRNA is from nucleotides in the seed region of the small RNA (Haley and Zamore, 2004). It seems that the accessibility of the target site can be sensed by the intrinsic, nonspecific

affinity of RISC for ssRNA, which follows the initial specific association between the RISC and the target (through the 5′ seed region of the small RNA) (Ameres et al., 2007). But the accessibility of the target site correlates directly with the efficiency of cleavage, indicating that the RISC cannot unfold structured RNA.

Target mRNAs are present in the cell in complex with ribonucleo-proteins (RNPs) (Dreyfuss et al., 2002), so target accessibility is also controlled by several RNA-binding proteins that either mask the target binding site or facilitate unfolding of the target. Therefore, the function of a RISC seems to be context-dependent, with its effector mode influenced not only by the structures of the small-RNA-binding sites on the target but also by the particular proteins associated with each Argonaute protein.

2.5. Transport of RNAi information between cells

An important aspect of the RNA interference pathway is the transport of RNAi information, because it is important to understand when a siRNA enters in a cell what is its diffusion. The transport mechanism is different from insects to plants.

In insects

RNAi can be divided in **cell-autonomous** and **non-cell-autonomous** RNAi (Fig. 4). As the name suggests, in the case of **cell-autonomous** RNAi, the silencing process is limited to the cell in which the dsRNA is introduced/expressed and encompasses the RNAi process within individual cells.

In case of **non-cell-autonomous** RNAi, the interfering effect takes place in tissues/cells different from the location of application or production of the dsRNA. There are two different kinds of non-cell-autonomous RNAi: **environmental** RNAi and **systemic** RNAi (Fig. 4).

Environmental RNAi describes all processes in which dsRNA is taken up by a cell from the environment. Therefore, this process can also be observed in unicellular organisms.

Systemic RNAi can only take place in multicellular organisms because it includes processes in which a silencing signal is transported from one cell to another or from one tissue type to another.

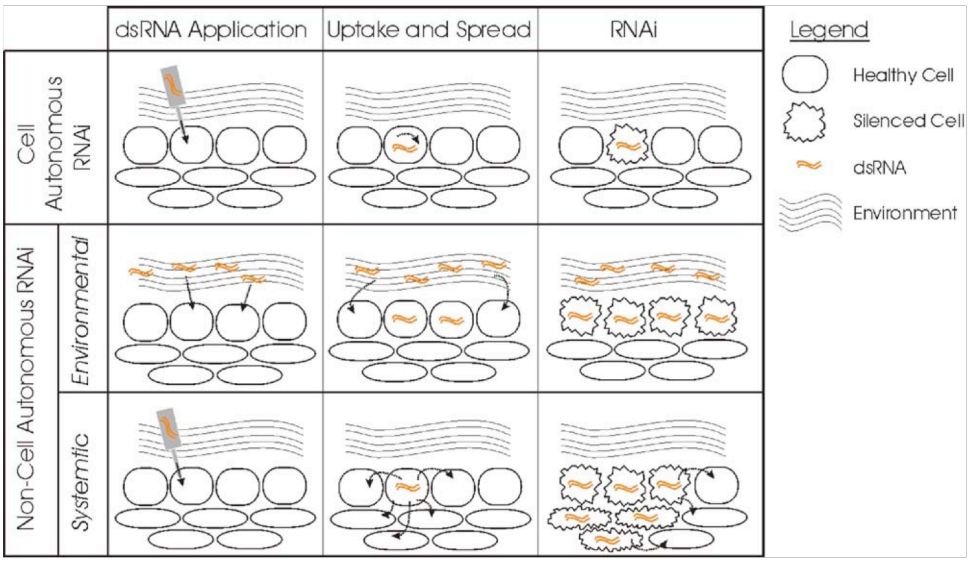

Figure 4.
A schematic overview of the different types of RNAi, explained through the silencing effect of an essential gene for cell viability in healthy cells. The first row represents the cell-autonomous RNAi. dsRNA of a gene essential for cell viability is applied to or expressed in a healthy cell. The silencing effect is limited to the cell. In case of environmental RNAi, the dsRNA is taken up from the environment of the cell, the silencing effect is observed in all cells which can take up the dsRNA. This can take place in unicellular and multicellular organisms. Systemic RNAi encloses all processes in which the silencing signal is transported from the cell in which the dsRNA is applied or expressed to other cells, also to other tissues, in which the silencing will then take place.

In multicellular organisms environmental RNAi can be followed by systemic RNAi and non-cell-autonomous RNAi will always be followed by cell-autonomous RNAi.

For the efficient application of RNAi in insect control, we have to focus on non-cell-autonomous RNAi. The insect will have to internalize the dsRNA of a target gene through feeding. In order to silence the target gene, this dsRNA must be taken up from the gut lumen into the gut cells, representing environmental RNAi.

The insect midgut consists of a single layer of columnar cells with microvilli, endocrine cells, and stem cells at the base, grouped in the so-called nidi. The midgut is designed to absorb nutrients from the gut lumen with its large absorption area created by the microvilli, with many channels and endocytosis apparati (Lehane and Billingsley, 1996; Hakim et al., 2010). These characteristics make the tissue very interesting as a potential dsRNA uptake location. If the target gene is expressed in a tissue outside of the gut, the silencing signal will also have to spread via cells and tissues, which is systemic RNAi.

The best studied dsRNA uptake mechanism is that of *C. elegans*. Research with systemic RNAi defective mutants (*sid*) resulted in the description of two proteins involved in non-cell-autonomous RNAi. SID-1 is a multispan transmembrane protein essential for systemic RNAi. It functions probably as a multimer, transporting dsRNA passively into the *C. elegans* cells. However, it is not essential for the export of dsRNA from the cell (Winston et al., 2002; Jose et al., 2009).

The other protein, SID-2, is mainly found in the intestine tissue of the worm and facilitates environmental RNAi (Winston et al., 2007). Three hypotheses are proposed on the relation/cooperation/coordination between the two proteins:

(i) SID-2 modifies the SID-1 molecule to activate the transport

(ii) SID-2 binds the dsRNA from the environment and delivers it to SID-1

(iii) SID-2 induces the endocytosis pathway of the dsRNA, in which case SID-1 delivers the dsRNA to the cytoplasm (Whangbo and Hunter, 2008).

A *sid-1* gene orthologue was found in the cotton aphid (*Aphis gossypii*). The online analysis of the topological structure showed large similarities with SID-1 of *C. elegans*, suggesting a possible role in the dsRNA uptake, however neither the expression nor its functionality were determined (Xu and Han, 2008). In the honey bee (*Apis mellifera*) and in *T. castaneum* the role of the orthologue genes in dsRNA

uptake were evaluated, and the results are contradictory. In an RNAi experiment with honey bee, the expression of the *sid-1* orthologue increased just before the target gene was knocked down, leading to the conclusion that *sid-1* was involved in the uptake of dsRNA (Aronstein et al., 2006). However, the direct correlation between increased *sid-1* expression and the silencing effect was not proven. When the three *sid-1* orthologues of *T. castaneum* were silenced individually or all together, RNAi was not influenced. However involvement was not excluded because of possible complementary of the genes versus weakening of the RNAi effect due to competition for the cell-autonomous core RNA machinery of the cells.

Interestingly, from the in silico analysis of the presence of *sid-1* orthologues in insects, it appears that the *sid-1* orthologues show more similarities with the *tag-I30* genes than with the *sid-1* genes of *C. elegans*. In the worm these genes are not involved in systemic RNAi. These data suggest that SID-1 is not essential for systemic RNAi/dsRNA uptake in certain insects. Moreover, it also suggests an alternative dsRNA uptake mechanism in insects since systemic RNAi is very robust in *T. castaneum* (Tomoyasu et al., 2008).

Intriguingly, in the best known model insect, *D. melanogaster*, which has no robust systemic RNAi, no *sid* gene orthologs were found. However, there is a cell-autonomous RNAi mechanism present and hemocytes are able to respond to environmental RNAi (Roignant et al., 2003; Gordon and Waterhouse, 2007; Miller et al., 2008).

In plants

Plant cells transport silencing information to adjacent cells through intercellular pores called plasmodesmata and to distant cells through the vascular tissue called phloem (Figure 5a).

Most plant cells are connected with each other through dynamic pores called plasmodesmata (Zambryski, 2004), thus forming a cytoplasmic continuum. Mature guard cells that regulate gaseous exchange in the leaf are not part of the cytoplasmic continuum and consequently, transport of the silencing signal can cause silencing in guard cells only if the signal is received by immature guard cells that have not yet lost their plasmodesmatal connections (Himber et al., 2003; Voinnet et al., 1998).

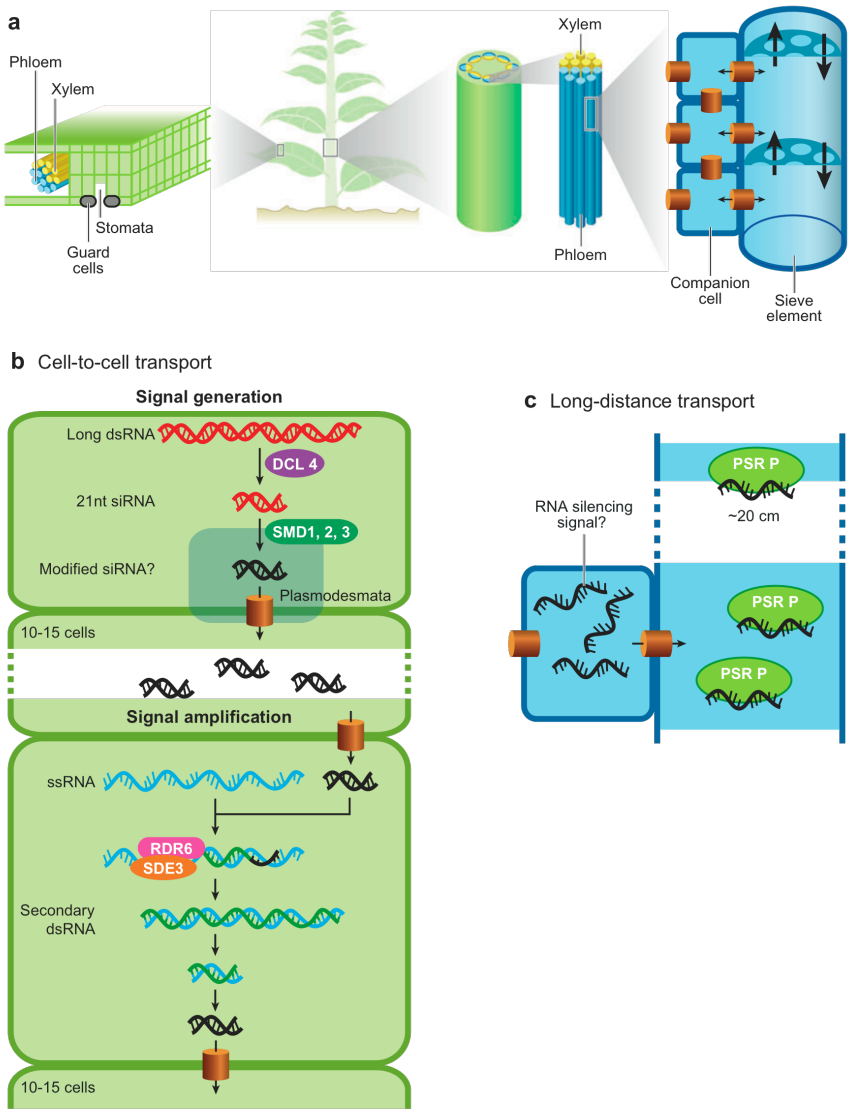

Figure 5.
Plants use distinct mechanisms for cell-to-cell and long-distance transport of silencing information. (a) Schematic showing the organization of plant vasculature. Long-distance transport of silencing information occurs through the phloem, comprised of companion cells that support associated sieve elements. Cell-to-cell transport in leaves occurs through plasmodesmal connections, which connect the cytoplasm of all cells except guard cells that surround sites of gaseous exchange (stomata). (b) Model for the generation and amplification of a cell-to-cell silencing signal. The RNAi trigger, long dsRNA, is converted to 21-nt siRNAs by DCL4, and modification of these 21-nt siRNAs for transport and/or their transport through plasmodesmata is controlled by the silencing movement defective genes (SMD1, 2, 3). This signal can travel for ~10-15 cells without any amplification. However, in the presence of target mRNA, a cellular RNA-dependent RNA polymerase (RDR6) and an RNA helicase (SDE3) can direct the synthesis of secondary dsRNA, which may then be processed just as the trigger long dsRNA. (c) Model for the long-distance transport of a silencing signal. The silencing signal (likely RNA) may be transported as single-stranded short RNAs through the phloem since the phloem small RNA-binding protein 1 (PSRP1) of pumpkin specifically binds short single-stranded RNAs.

Water and minerals are transported through xylem tubes, which are lined with dead cells, while photoassimilates, RNA, proteins, and silencing information are transported through phloem tubes, which are made of living enucleated sieve elements supported by companion cells. Transport through the phloem occurs from mature, photosynthetically autonomous organs (phloem sources) to new growth (phloem sink), and transport of silencing information over long distances also occurs from phloem source to phloem sink (Crete et al., 2001; Palauqui et al., 1997; Tournier et al., 2006).

The physiological status and surrounding environment of a tissue dictate the extent of transport of silencing information. When silencing is initiated in single cells or small groups of cells, the movement of silencing information is restricted to about 10-15 cells in diameter in phloem source tissues such as mature leaves (Palauqui et al., 1996). Conversely, in phloem sink tissues, such as new growth, silencing information progressively spreads from the phloem into the entire lamina of the leaves. Further, when mature leaves are converted into phloem sinks by shading, the transport of silencing information within the leaf becomes more extensive. Nutrient depletion may thus increase phloem flow bringing more silencing signals and nutrients to the leaf and/or may induce signal amplifiers such as RdRPs (RNA-dependent RNA polymerase) in the leaf.

Plant cells appear to use 21-nt siRNAs to transport silencing information through plasmodesmata. In the absence of amplification the spread of silencing is limited to 10-15 neighboring cells (Figure 5b), while more extensive spread of the silencing information can be achieved by a relay mechanism, whereby a silencing signal received by a cell in a leaf is amplified by the action of cellular RdRPs allowing additional cell-to-cell spreading within the leaf.

The results suggest a model (Figure 5b) whereby long dsRNA is processed into 21-nt siRNAs by *DCL4* (*Dicer-like-4*) during silencing. The movement of these 21-nt siRNAs through plasmodesmata is dependent on the *silencing movement defective* genes *SMD1, 2, 3*. This results in the spread of silencing to ~10-15 neighboring cells. *RDR6* (RNA-dependent RNA polymerase) and *SDE3* (RNA helicase) in these neighboring cells amplify the signal, producing secondary dsRNA beyond the original trigger. These cells then process the secondary dsRNA and relay the silencing information further by spreading the 21-nt siRNAs. The extent of spread depends upon the physiological state of the tissue and this

regulation may occur through modulation of RDR6 and SDE3 activity.

Plants can also transport silencing information over long distances (~20 cm) through the phloem (Figure 5c).

The molecular mechanism that controls phloem-dependent transport of silencing information is largely unknown. Although in one study phloem-dependent transport was found to be strictly correlated with the presence of ~24-nt siRNAs (Hamilton et al., 2002), conflicting results suggest that phloem-dependent transport does not correlate with any RNA species (Mallory et al., 2003). However, a phloem small RNA-binding protein, PSRP1, has been isolated biochemically from pumpkin phloem sap and found to bind small RNAs with high affinity (Yoo et al., 2000). Although PSRP1 preferentially binds to small RNAs, the precise nature and size of the RNA bound by PSRP1 during the transport of silencing information is unknown. Similar small RNA binding proteins were detected in the phloem saps of cucumber and lupine plants, suggesting that small RNAs involved in long-distance silencing may be transported through the phloem as parts of ribonucleoprotein complexes. Thus, the sequence-specific silencing signal that is transported through the phloem as well as the pathway responsible for its biogenesis and transport remain largely unclear.

3. G protein coupled receptors

An ideal target for RNAi would be a protein involved in many different insect vital functions, such as development, feed, reproduction and movement; interfering with all these functions at the same time can drastically affect the insect pest vitality.

G-protein-coupled receptors (GPCRs) represent nearly half of the current market for therapeutic agents in human, constitute annual revenues in excess of $40 billion, and remain a primary focus of many biomedical research and pharmaceutical drug discovery programs.

GPCRs comprise a diverse family of integral membrane proteins that are responsible for conveying extracellular signals to the inside of the cell *via* interactions intracellular heterotrimeric G proteins, which in turn affect enzymes, ion channels, and other intracellular messengers. Nearly a thousand GPCRs exist, mediating a host of molecular physiological functions by serving as receptors for hormones, neurotransmitters, cytokines, lipids, small molecules, and various sensory

signals (such as light and odors), to name a few. All GPCRs possess seven transmembrane helices, three extracellular loops, and three intracellular loops, with an extracellular N-terminal tail and an intracellular C-terminal tail (Figure 6; Bridges and Lindsley, 2008). The heptahelical transmembrane domain is largely hydrophobic, whereas the extracellular (e_1-e_3) and intracellular (i_1-i_3) segments, or loops, are generally hydrophilic. The seven transmembrane helices are each ~24 amino acids long, while the C- and N-terminal tails as well as the loops can vary widely in length with up to hundreds of amino acids. Based on sequence homology and functional similarity, all known GPCRs are divided into six major categories, including Rhodopsin-like receptors (Class A), Secretin receptors (Class B), Metabotropic glutamate/pheromone receptors (Class C), Fungal mating pheromone receptors (Class D), Cyclic AMP receptors (Class E), and Frizzled/Smoothened GPCRs (Class F) (Sadowski and Parish, 2003). However the most common division is into three main families (or classes): A (rhodopsin-like), B (secretin-like), and C (metabotropic receptor-like) (Figure 7). The families are readily distinguished by comparing their amino acid sequences, wherein Family B is characterized by a large extracellular loop and Family C has a large, bilobed extracellular Venus-flytrap-like domain. A second major difference between the families concerns the location of the orthosteric binding site and the nature of the orthosteric ligand. As shown in Figure 7, the orthosteric binding domain (OBD) of Family A GPCRs is located with the 7TM domain, whereas the OBD is located in the large extracellular loop within Family B and within the extracellular Venus- flytrap-like domain in Family C.

According to traditional two-state models of receptor theory, GPCRs can be conceptualized as operating in equilibrium between two functional conformations, an active (R*) and inactive (R) state (Limbird, 1986). In the R* state, the receptor has higher affinity for G-proteins, which normally exist apart from the receptor as a GDP-bound Gαβγ heterotrimer in their inactive form. Ligand binding to the receptor alters the equilibrium, with agonists shifting it toward the R* state, inverse agonists shifting it toward the R state, and antagonists preventing other ligands (such as endogenous agonists) from binding without altering the basal R*: R equilibrium.

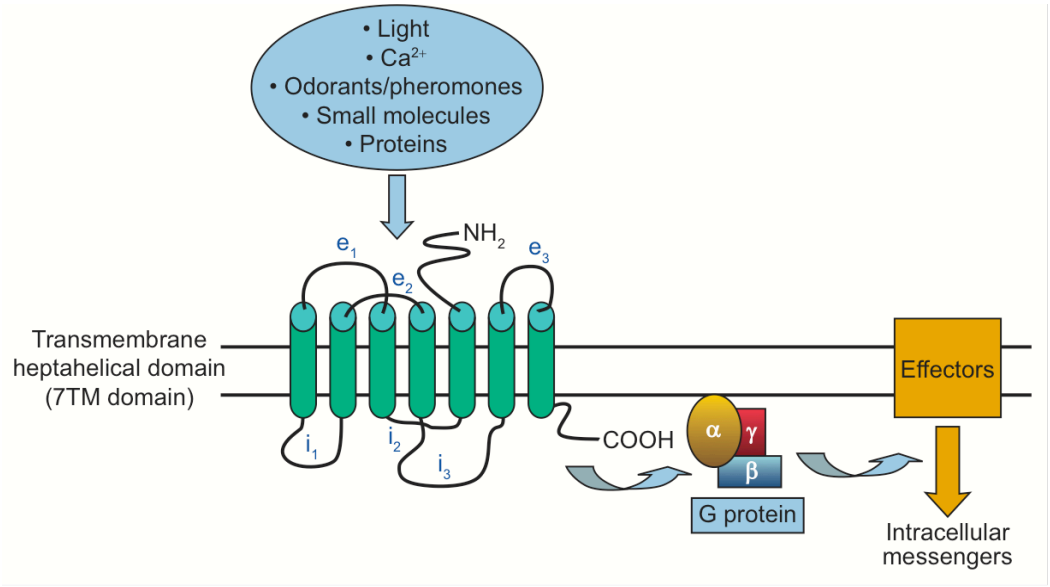

Figure 6.
Representative structure of a generic GPCR. GPCRs all have a common core composed of seven transmembrane helices (the 7TM domain composed of TM-I TM-VII) with an extracellular N-terminal domain and an intracellular C-terminal domain. The TMs are connected by three extracellular loops (e_1-e_3) and three intracellular loops (i_1-i_3). The GPCR receives an extracellular stimulus (light, calcium, odorants, pheromones, small molecules, proteins) that induces a conformational change in the receptor that either facilitates or inhibits the coupling of the receptor to a G-protein, composed of α-, β-, γ-subunits. The G-protein, in turn, interacts with a diverse group of effectors that control intracellular messengers.

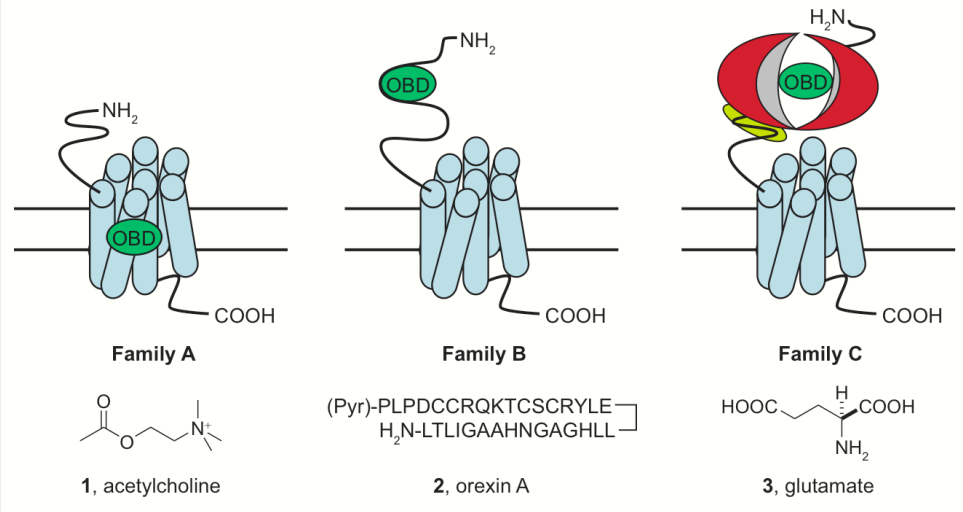

Figure 7.
Representative structures of the three families of GPCRs, Family A, Family B, and Family C. Note the location of the OBD varies for the families, as does the structure of the extracellular domain. The nature of the orthosteric ligand also varies across GPCR families. For Family A, a prototypical native agonist is acetylcholine (1), for Family B, a large 33-amino acid peptide such as orexin A (2), and for Family C, glutamate (3) is a representative native ligand.

Upon receptor activation, the GDP-bound G-protein interacts with the intracellular face and C-terminus of the receptor, inducing GDP to GTP exchange on the Gα subunit and concurrent dissociation of the Gα and Gβγ subunits. The now active GTP-Gα and Gβγ subunits then bind to their respective downstream effectors, which include kinases, phosphatases, small GTPases, integral membrane proteins, and a multitude of additional targets and signalling cascades. These downstream effectors exist in complex regulatory networks that control cellular functions such as movement, metabolism, membrane potential, neurotransmitter release, and gene expression.

The specific effectors influenced by a given GPCR depend on the type of G-protein that the receptor activates. There are many types of Gα, Gβ and Gγ subunits, allowing for diverse combinations, although the most commonly used simple categorization of GPCRs is by designation of coupling to either Gαq, Gαi, or Gαs (Oldham and Hamm, 2006). The mutual effector for both Gαi and Gαs is adenylyl cyclase (AC), which resides on the inner leaflet of the plasma membrane and generates cyclic-AMP in response to stimulation or inhibition by Gαs and Gαi, respectively. The primary effector for Gαq by contrast is phospholipase Cβ, a membrane-bound enzyme that converts phosphatidylinositol-4,5-bisphosphate into diacylglycerol and inositol-1,4,5-trisphosphate. Following effector binding, the GTP-Gα subunit hydrolyzes its γ-phosphate by augmentation of its intrinsic GTPase activity *via* binding of GTPase activating proteins (GAPs), resulting in conversion to GDP-Gα. This GDP-bound form possesses higher affinity for its Gβγ subunit partner, which causes reformation of the inactive heterotrimer, marking completion of the G-protein activation cycle. Figure 8 depicts the cycle in this simplified form. Many accessory proteins and lipids are also involved in regulating G-proteins, which play important roles in controlling the G-protein cycle.

Classical GPCR ligands modulate receptor signalling by directly stimulating a receptor response (agonism), blocking the binding of the native agonist (competitive antagonism), or blocking constitutive activity (inverse agonism) of the GPCR.

All GPCRs possess a distinctive binding site for their respective endogenous ligand(s) that is known as the **orthosteric** site. Ligands that bind to this site are considered classical or traditional orthosteric ligands.

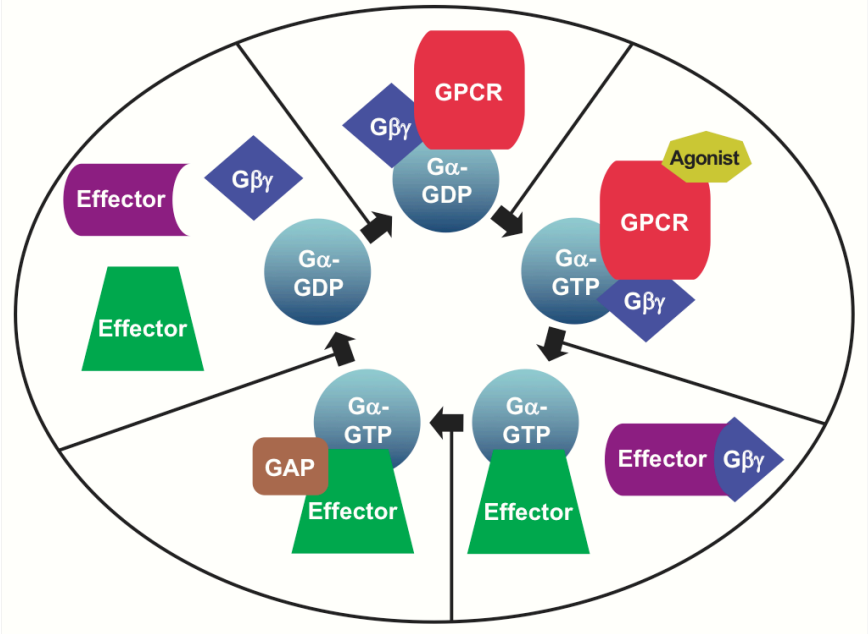

Figure 8.
Generalized diagram of the G-protein cycle. Upon agonist activaion of the receptor, GTP binds to the Gα subunit, displacing GDP, which causes dissociation of the protein complex from the receptor, allowing respective effector activation by Gα-GTP and Gβγ. GAPs then bind Gα and accelerate hydrolysis of GTP to GDP, which deactivates Gα and causes disengagement of the effector. Finally, Gα reassociates with Gβγ, marking cycle completion.

This group includes small-molecule agonists, partial agonists, antagonists, and inverse agonists; in general, the most physiologically common and relevant of these ligands are the endogenous agonists.

In addition to orthosteric sites, many GPCRs have been found to possess **allosteric** (Greek, "other site") binding sites that are spatially and often functionally distinct (Christopoulos, 2002; Christopoulos and Kenakin, 2002; May et al., 2007). The presence of allosteric sites allows for numerous additional ligand-receptor interactions beyond those associated with the orthosteric site. Allosteric agonists, antagonists, and inverse agonists for a given GPCR will bind to the allosteric site and induce a similar effect as their orthosteric relatives. Beyond such types of ligands, allosteric modulators bind to an allosteric site where they stabilize a receptor conformation and equilibrium shift that increases or decreases the affinity and/or efficacy of an orthosteric agonist at the receptor, without activating the receptor on its own.

4. G protein coupled receptors in insects

As well as in the other eukaryotic organisms, also in insects GPCRs have key roles in the regulation of vital functions, and thus may represent excellent targets for RNAi technology.

In addition to the above GPCRs classes (A, B, C, D, E and F), in insects there are two other classes: olfactory receptors and gustatory receptors. These receptors in insects were previously considered as Class A GPCRs. However, later studies indicated that they possess a distinct 7TM topology with the amino terminus located at the intracellular side, and function as heteromeric ligand-gated ion-channels (Sato et al., 2008; Smart et al., 2008; Wicher et al., 2008). Therefore, the involvement of G proteins in insect olfactory signal transduction is still under question (Pellegrino and Nakagawa, 2009).

The following section is not a faithful list of all insect GPCRs classes but only the classes of the GPCRs chosen for this work were reported.

4.1. Rhodopsin-like family (Class A)

This family encompasses receptors that bind a large variety of ligands, such as biogenic amine neurotransmitters, neuropeptides, peptide hormones, lights, nucleotides, prostaglandins, leukotrienes, chemotactic peptides, and chemokines. The rhodopsin-like family is the largest family of *B. mori* GPCRs, which can be divided further into 4 subfamilies: biogenic amine receptors (16 sequences), neuropeptide and protein hormone receptors (46 sequences), opsin receptors (6 seq) and purine receptors (1 sequence).

4.1.1. Biogenic amine receptors

In insects, as well as in mammals, amines play a prominent role in the physiology of the nervous system. They are involved in the transmission and integration of sensory information, in the control of muscular and glandular activities, and in complex processes such as learning, memory, and behaviour. But they are also involved in many other physiological processes, such as diuresis and immune responses (Blumenthal, 2003; Roeder, 2005; Birman, 2005). The known insect biogenic amines are dopamine, tyramine, octopamine, serotonin, acetylcholine and histamine.

Octopamine (OA) is a physiologically important invertebrate biogenic amine that has structural and functional similarities to the vertebrate biogenic amines adrenaline and noradrenaline (Roeder, 1999). OA is engaged in sensory inputs, rhythmic behaviors, endocrine regulation, mobilization of lipids and carbohydrates, sleep and aggression as well as more complex physiological events, such as learning and memory, as a neurotransmitter, neuromodulator or neurohormone (Farooqui, 2007; Crocker and Sehgal, 2008; Zhou et al., 2008).

The first insect OA receptor was isolated from the fruit fly *Drosophila melanogaster* (Han et al., 1998). Following the initial isolation in fruit flies, a variety of OA receptors were cloned from several other insect species. To classify the various OA receptors, Evans and Maqueira (2005) proposed a novel classification system in which OA receptors are designated as α-adrenergic-like OA receptors (OctαRs), β-adrenergic-like OA receptors (OctβRs) or OA/ TA (or tyraminergic) receptors. This classification is based on the similarities of these proteins to

vertebrate adrenergic receptors in terms of amino acid sequence and signaling pathway. Activation of α-adrenergic-like OA receptors expressed in cell lines primarily leads to an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), whereas activation of β-adrenergic-like OA receptors induces an increase in intracellular cAMP concentration ($[cAMP]_i$) but no increase in $[Ca^{2+}]_i$ (Balfanz et al., 2005; Maqueira et al., 2005).

The physiological role of TA is less understood than that of OA. While TA is a synthetic precursor of OA, several lines of evidence have indicated that TA is also involved in a variety of physiological processes, including carbohydrate metabolism, muscle contraction, locomotion, excretion, reproduction, oviposition, olfaction and behavioral sensitization in insects (Downer, 1979; Huddart & Oldfield, 1982; McClung & Hirsh, 1999; Kutsukake *et al*., 2000; Nagaya *et al*., 2002; Sasaki & Nagao, 2002; Blumenthal, 2003; Donini & Lange, 2004; Saraswati *et al*., 2004). Most of the cloned insect TA receptors, although categorized as OA/TA receptors in the newly proposed classification, are negatively coupled to adenylate cyclase via the Gi protein to reduce $[cAMP]_i$ (Arakawa *et al*., 1990; Saudou *et al*., 1990; Vanden Broeck *et al*., 1995; Blenau *et al*., 2000; Ohta *et al*., 2003).

The receptors of Octα class have mayor affinity to octopamine than tyramine like the octopamine receptor of *A. mellifera*, AmOA1, (Grohmann, 2003) or *Bombyx mori* receptor, BmOAR1, (Ohtani et al., 2006).

To date, only β-adrenergic-like OA receptors from *D. melanogaster* have been cloned, and they are DmOctβ1R, DmOctβ2R and DmOctβ3R (Maqueira et al., 2005). Recently, it is cloned a β-adrenergic-like OA receptors from *Bombyx mori*, BmOAR2, (Chen et al., 2010).

4.1.2. Neuropeptide and protein hormone receptors

The neuropeptide and protein hormone receptors are the largest subfamily in the rhodopsin-like family. This subfamily of GPCRs plays a central role in control of insect behaviour, reproduction, development, feeding and many other physiological processes. They exert their functions through a large variety of peptide transmitters and hormones, indeed, similar to other insects, *B. mori* rhodopsin-like neuropeptide and protein hormone receptors can be classified into eighteen groups based on their ligands, i.e. the Allostatin receptors, Allatotropin receptors,

FSH/TSH/LH-like receptors, Sulfakinin receptors, Myosuppressin receptors, Diapause hormone receptors, Adipokinetic hormone/corazonin/ACP receptors, Neuropeptide Y receptors, Neuropeptide F receptors, Tachykinin receptors, Leukokinin receptors, Capa receptors, CCAP receptors, PBAN receptors, ETH receptors, SIFamide receptors, FMRFamide receptors and several orphan GPCRs (Fan *et al.*, 2010).

4.1.2.1. Allatostatine receptors

The insect allatostatins are neuropeptides that obtained their names because of their ability to inhibit juvenile hormone biosynthesis in the corpora allata, two small organs (commonly fused) near the insect brain (Woodhead et al., 1989). Juvenile hormone is a terpene that plays crucial roles in insect development and reproduction. There exist three families of allatostatins that are structurally unrelated, the allatostatins- A, -B, and -C. It appears that all insects have all three types of allatostatins, but that, in each species, only one allatostatin type inhibits juvenile hormone biosynthesis, while the other allatostatins have different inhibitory functions (Lenz et al., 2000a; Williamson et al., 2001a,b; Nassel, 2002).

C-type allatostatins are 15 amino-acid residues long cyclic neuropeptides that have originally been isolated from the moth *Manduca sexta*, where they inhibit juvenile hormone biosynthesis (Kramer et al., 1991).

In *Drosophila*, two allatostatin-C receptor genes have been identified, Drostar1 (CG7285) and Drostar2 (CG13702) (Kreienkamp et al., 2002). The *honey bee* genome contains one close orthologue (Am 31) to these two *Drosophila* receptor genes. Recently, it is cloned allatostatin-C receptor gene in *Bombyx mori*, BNGR-A1 (*Bombyx* neuropeptide GPCR), that it is expressed at high level in the corpora allata and the corpora cardiaca (Yanamaka et al., 2008).

4.1.2.2. FSH/TSH/LH-like receptors

Drosophila produces a receptor that was structurally and evolutionarily related to the LH/FSH receptors from mammals. Mammals have at least four glycoprotein hormones (LH, FSH, choriogonadotropin (CG), and thyroid-stimulating hormone (TSH)) and at least three glycoprotein hormone receptors (the LH/CG, FSH, and TSH receptors) that are all closely related. A characteristic of

these glycoprotein hormone receptors is the presence of a very large, extracellular amino terminus that constitutes about half of the receptor protein and that contains 9 Leu-rich repeats, each measuring about 24 amino acid residues. These nine Leu-rich repeats probably form a horseshoe-like structure to which the glycoprotein hormone ligand binds (Jiang et al. 1995; Kajava et al. 1995). *Drosophila* has four Leu-rich repeats-containing G protein-coupled receptor (DLGR) that we named DLGR1–4 (Hauser et al., 1997; Eriksen et al., 2000). It was also identified the natural ligand for DLGR2, which is a heterodimeric cystine-knot protein with bursicon bioactivity (Mendive et al., 2005; Luo et al., 2005). Bursicon was described more than 40 years ago as a neurohormone that causes hardening and tanning of the soft cuticle from a newly hatched fly after adult ecdysis (Fraenkel and Hsiao, 1962; Fraenkel et al., 1966). Later studies showed that bursicon also induces apoptosis of the wing epithelial cells after completed wing expansion (Kimura et al., 2004). The *honey bee* genome contains a clear orthologue of the Drosophila bursicon receptor gene (CG8930/DLGR2).

4.1.2.3. Adipokinetic hormone receptors

Adipokinetic hormones (AKH) are insect neuropeptide produced by the corpora cardiaca, a neuroendocrine organ closely associated with the insect brain. They are a large family of small peptides, all being 8-10 amino acid residues long; they are involved in the mobilization of sugar (trehalose) and lipid from the insect fat body during energy-requiring activities such as flight or locomotion (Gäde et al.,1997). They are also reported to contribute to hemolymph sugar homeostasis, regulating energy homeostasis and orchestrating different processes within a physiological context (Staubli et al., 2002). Multiple adipokinetic hormone (AKH) like receptors (4 sequences) had been identified in *Bombyx*, as in the case of *Drosophila* (2 sequences), *Anopheles* (3 sequences) and *Apis* (2 sequences).

4.1.2.4. PBAN receptors

Pheromone-Biosynthesis-Activating Neuropeptide is a member of the PBAN/Pyrokinin neuropeptide family, characterized by a common amino acid sequence FXPRLamide motif in the C-terminus. PBAN is released into

the hemolymph of females during the scotophase and is drastically reduced after mating, contributing to the loss in female receptivity. Pheromone production is age-dependent and Juvenile Hormone is involved in its regulation. PBAN activates pheromone production through its binding to a PBAN-Receptor (PBAN-R) and subsequent up-regulation of key enzymes in the biosynthetic pathway. Differential expression studies reveal its localization in pheromone glands, neural tissues and the male aedeagus (Rafaeli, 2009).

The pyrokinins can be subdivided into two groups, depending on their peptide structures, and *Drosophila* has representatives from each class, pyrokinin-1 and -2 (Cazzamali et al., 2005b). Furthermore, *Drosophila* has three pyrokinin receptors, one specific for pyrokinin-1 (CG9918), and two for pyrokinin-2 (CG8784 and CG8795) (Park et al., 2002; Rosenkilde et al., 2003; Cazzamali et al., 2005b). The *honey bee* has two receptor genes (Am 25 and Am 26) that clearly are the orthologues to the three Drosophila pyrokinin receptor genes. The *Bombyx* receptor is related to *Drosophila* pyrokinin-2 receptors (CG8784, CG8795) and *Anopheles* GPRghp1.

4.2. Secretin-like receptors family (Class B)

The secretin-like family includes receptors for many hormones such as secretin, calcitonin, vasoactive intestinal peptide, and parathyroid hormone and related peptides; furthermore it comprises several members having key functions in the regulation of water balance and diuresis. The secretin-like receptors are characterized by long NH_2-terminal domains containing five conserved cysteine residues that may form disulfide bonds and by short third cytoplasmic domains.

4.2.1. Diuretic hormones receptors

Fluid secretion (diuresis) in insects occurs in the Malpighian tubules and reabsorption of water and other small molecules takes place in the hindgut. Several insect hormones control this important process of water and salt homeostasis.

There are two diuretic hormones (named DH) acting on the insect Malpighian tubules that are longer neuropeptides (Coast et al., 2001; Coast and Garside, 2005). One is structurally related to mammalian calcitonin and the *Drosophila* calcitonin-like peptide is called Drome-DH_{31}, (Coast et al., 2001),

whereas the other is structurally related to mammalian corticotropin-releasing-factor (CRF) and in *Drosophila* is called Drome-DH_{44}, (Cabrero et al., 2002). Drome-DH31 and Drome-DH44 are not structurally related. Two *Drosophila* DH receptors have recently been identified, CG32843 (for Drome-DH_{31}) and CG8422 (for Drome-DH_{44}) (Johnson et al., 2004, 2005). DH44-R1 has a receptor paralog, encoded by CG12370 (DH44-R2), (Hewes and Taghert, 2001), which, based on the high degree of sequence similarity, is predicted to be an additional target of DH44 activation. It has now found a *honey bee* gene, Am 55, that is a clear orthologue to CG32843, and it has also found another *honey bee* receptor gene, Am 53, that is clearly related to CG8422. BNGR-B1 and BNGR-B2, *Bombix* calcitonin-like receptors, are orthologous to *Drosophila* CG32843. Additionally, one *Bombyx* diuretic hormone-like receptor was identified in this family, which is homologous to the *Drosophila* diuretic hormone 44 (DH44-R1), (Hector et al., 2009) and the *Anopheles* receptors GPRdih1 and GPRdih2.

4.3. Odorant receptors

Insect chemosensory systems detect a wide range of volatile and soluble chemicals and are important for finding and assessing the quality of food sources, in addition to identifying mates and oviposition sites. Chemosensory neurons are present in specialized sensory hairs called sensilla. In many insects olfactory sensilla are present on two pairs of olfactory organs on the head, the antennae and the maxillary palps.

Odorant receptors are expressed in distinct subpopulations of olfactory neurons in either the antennae or the maxillary palps. Individual sensory neurons appear to be functionally distinct and perhaps express only a single odorant receptor gene (Vosshall et al., 2000). These neurons send axonal projections towards topographically invariant glomeruli in the antennal lobe. Therefore, different olfactory signals are probably "transmitted" to the brain via spatially distinct pathways of sensory information input (Gao et al., 2000; Vosshall et al., 2000).

Olfactory receptors are implicated in one step of the olfactory cascade of events, which consist of combinatorial systems from stereochemical recognition to the generation of an odor code in the brain. Insect chemical odorant messages are translated into neuronal electrical activities through specialized organs, principally

the antennae, and processed by brain centers to elicit behavioral–physiological responses.

Or83b is an unusual Or gene; it is the most conserved chemoreceptor gene in insects (Robertson et al., 2003; Hill et al., 2002; Melo et al., 2004; Jones et al., 2005; Nakagawa et al., 2005), and unlike other Or genes, it is expressed in most olfactory neurons, (Ng et al., 2002; Larsson et al., 2004). Epitope mapping of OR83b indicated that this protein (and likely other insect ORs) had a flipped topology in comparison with GPCRs, with an intracellular N-terminus and extracellular C-terminus (Benton et al. 2006).

Or83b orthologues have been found in other flies, *Anopheles*, AgOR7, (Pitts et al., 2004), *Bombix mori*, BmorR2, and *Heliothis virescens*, HvirR2 (Krieger et al., 2003).

OR83b, which heterodimerizes with other ORs (Neuhaus et al. 2005; Benton et al. 2006), is required for normal functioning of ORs and OR-expressing OSNs (Olfactory sensory neurons), (Larsson et al. 2004; Laissue and Vosshall 2008; Sato and Touhara 2008; Benton et al. 2006). The function of Or83b is also conserved across insect orders: Or83b orthologues from the medfly, the mosquito and the moth can all substitute for Or83b in *Drosophila*. In mosquitoes, the expression of the Or83b orthologue is also detected in a subset of neurons in the proboscis and legs, suggesting a role in the gustatory system (Pitts et al., 2004).

AIM
OF
THE WORK

As widely described previously, insects represent one of the major challenges for the plants in their natural environment. *Spodoptera littoralis* is certainly one of the most destructive lepidopterans in the tropical and sub-tropical areas. It is a polyphagous noctuid, that feeds on plants belonging to different families: *Malvaceae*, *Cruciferaceae*, *Graminae* and *Rosaceae*. Various types of strategies have been utilized so far to control this insect pest, in particular the chemical treatment with methyl-parathion, organophosphorus compounds, synthetic pyrethroids and others insecticides. However, the use of these compounds has generated the phenomena of acquired resistance. Some recent papers reported the potential use of RNA interference (RNAi) induced by hairpin RNAs as a new strategy to defend plants against coleopteran and lepidopteran pests.

On the basis of this information, I decided to use the RNA interference strategy to interfere with important molecular targets in the insect *Spodoptera littoralis*. An ideal target would be a protein involved in many different insect vital functions, such as development, feed, reproduction and movement; interfering with all these functions at the same time can drastically affect the insect pest vitality. I chose as molecular targets the receptors coupled to G protein (GPCRs), involved in the signaling transduction of variety extracellular signals through the G proteins activation.

Thus the aim of my work is the production and characterization of transgenic *Nicotiana tabacum* plants, expressing dsRNA specific to G protein coupled receptors of *Spodoptera littoralis* as new strategy to protect plants against insect pests. After an attentive selection of the most appropriate GPCRs, I will characterize the plant lines for the expression of the dsRNA and check the stability of this molecule over time. The positive plants will be employed in bioassays where *Spodoptera* larvae will be fed on the plants in order to find any interesting phenotypic effects on the insect vitality.

MATERIALS AND METHODS

1. Database search

The insect cDNA sequences corresponding to chosen receptors are available in database NCBI (www.ncbi.nlm.nih.gov). For the alignments the algorithms of *blast* (www.ncbi.nlm.nih.gov/BLAST) and the software of multiple alignment *multalin* (http://multalin.toulouse.inra.fr/multalin) have been utilized. The algoritms utilized for the prediction of the GPCRs transmembrane domains are: phobius.sbc.su.se; www.cbs.dtu.dk/services/TMHMM/;
bioweb2,Pasteur.fr/seqanal/interfaces/toppred.html.

2. Growth of *Spodoptera littoralis* larvae

The *Spodoptera littoralis* larvae were maintained at temperature of 23°C, 70% of relative humidity, under 16/8 hours light/dark period. The artificial diet utilized for the larvae growth is composed from wheat germ, brewer's yeast, cornmeal, ascorbic acid, benzoic acid and agar. The larvae were maintained in these conditions until VI instar. After this, the larvae were transferred in boxes with vermiculite to arrive to the pupa instar. After one week, the pupae became nymphs. The nymphs didn't feed for two weeks until the adult instar. The adults were left to couple to obtain the eggs.

3. Isolation and cloning of AlstC receptor from *Spodoptera littoralis*

RNA extraction and cDNA synthesis

Total RNA was isolated from *Spodoptera littoralis* using Promega kit. The RNA samples were treated with DNAses (Ambion) in order to eliminate potential genomic DNA contamination. 2 μl of RNA are loaded on 1% agarose gel in presence of denaturant loading dye and quantified using as standard a specific marker for RA (Fermentas). For the quantification it was used the software Gene tools (Perkin Elmer). The cDNA was synthesized using 0,3-1 μg of RNA with Reverse Transcriptase (Fermentas) using 500 ng of oligodT primer.

Enzymes and conditions for PCR reactions

For the cloning I used a high fidelity DNA polymerase: Phusion of Finnzymes.

The conditions for this enzyme are the following:

Denaturation: 98°C for 2 s

25-35 cycles: 98°C for 10 m

45°C-72°C (depends on the primers Tm) for 30 m

72°C for 30 m / 1 kb

Extension: 72°C for 10 s.

The degenerate PCR reactions were performed in 50 μl reaction volume with 2,5 μM of primers, 0,2 mM of dNTPs (Fermentas) and 1 unit of High-Fidelity Phusion DNA Polymerase (Finnzymes). The reactions with specific primers were performed in the same mix reaction but the primers concentration is 0,25 μM.

The fragments obtained have blunt ends then it is necessary to treat them 15 minutes with DNA polymerase (Euroclone) to insert them in the cloning vector pCR2-TOPO (Invitrogen).

4. Sub-cloning of selected receptors in the plant expression vector

The selected receptor genes were sub-cloned into the plant expression vector. pH7GWIWG2(I), using the gateway technology.

The receptor genes were first transferred into the gateway entryvector (pENTR 2B-Invitrogen) (Fig. 9), which is designed to clone DNA sequence using restriction endonuclease and ligase to create a gateway entry clone. This vector contains attL1 and attL2 sites necessary for the next cloning and the gene for the resistance to the kanamycin. The destination binary vector (pH7GWIWG2(I)-Plant Systems Biology) (Fig. 10) contains attR1 and attR2 sites. These sites are recognized by LR clonase, enzymes that does homologue recombination recognizing attL1 and attL2 sites gateway entry vector. Moreover, this plant expression vector contains the gene for the resistance to the spectinomycin, the 35S promoter, necessary for the constitutive expression of the downstream gene, and due to the presence on an intronic sequence between the two strands (sense and antisense) of the newly transcribed RNA, a dsRNA is finally produced in the plant. The recombinant plasmids have been characterized using restriction endonuclease and verified by sequencing.

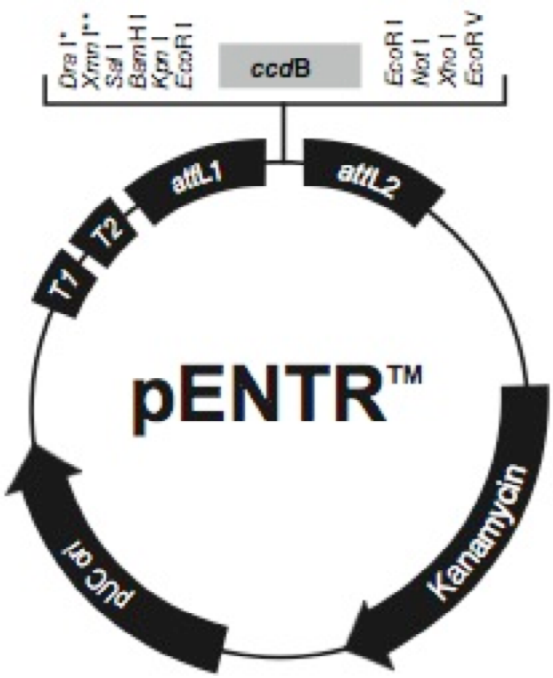

Figure 9.
Map of pENTR 2B-Invitrogen, gateway entry vector.

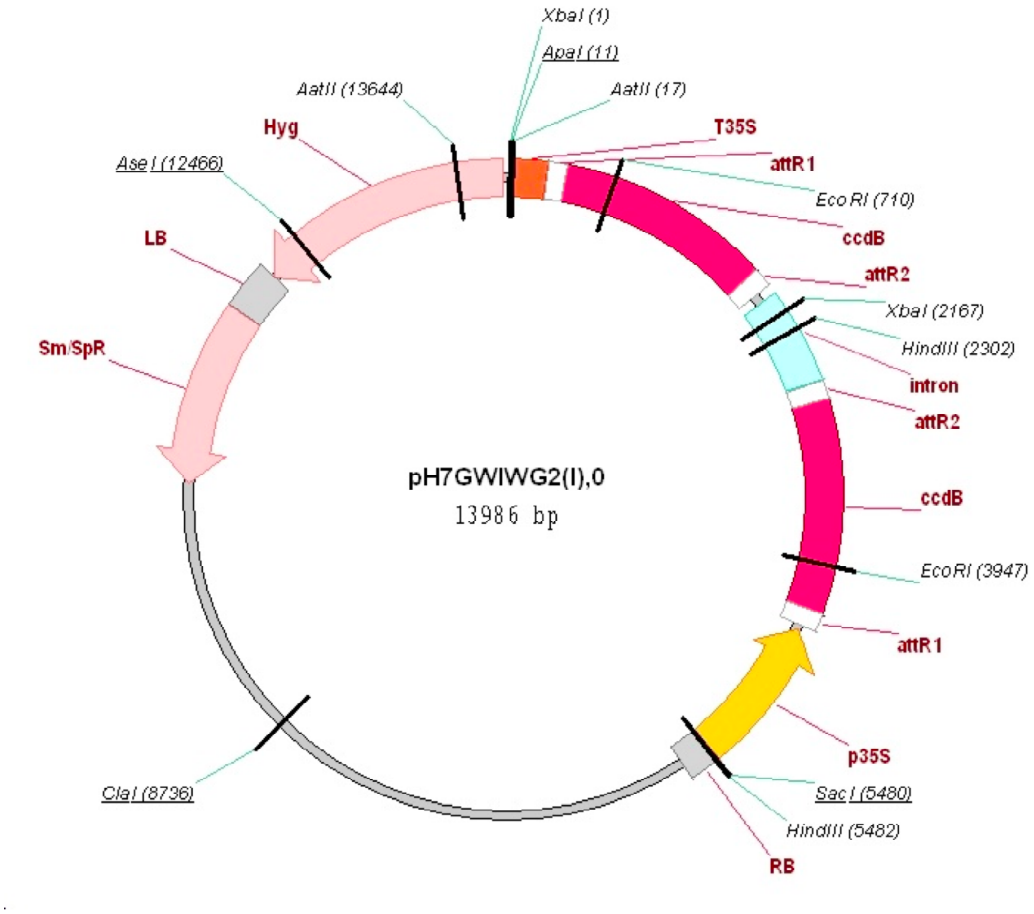

Figure 10.
Map of pH7GWIWG2(1)-Plant Systems Biology, destination binary vector.

5. Bacterial transformation

The competent cells DH5α (Invitrogen) were transformed with 100 ng of plasmidic DNA and put in ice for 30 minutes, then they were incubated at 42°C for 1 minute and subsequently in ice for 2 minutes. After the adding of 1 ml of LB medium the cells were incubated at 37°C for 1 hour, then they were centrifuged for 3 minutes at 5000 rpm. The sediment obtained, after the elimination of medium, was resuspended in the remaining volume (about 100 μl) and plating on LB agar containing 100 μg/ml kanamycin or 100 μg/ml spectinomycin.

6. Preparation of *Agrobacterium tumefaciens* (strain C58) competent cells and transformation

A bacterial single colony was grown for two days at 28°C in YEP medium with rifampicin 100 μg/ml. The saturated culture was diluted 1:100 and grow until O.D. 500 = 0,5. The cells were made competent with 20 mM of $CaCl_2$. 5 μg of plasmidic DNA were utilized for the transformation of the cells. These were incubated for 5 minutes in ice, 10 minutes in dry ice with ethanol and the thermic shock is performed at 37°C for 15 minutes. After that it was added 1 ml of YEP. The cells were grown for 2-4 hours at 28°C. the bacteria were plating on YEP agar with the appropriate antibiotics and grown.

7. *In vitro* growth of *Nicotiana tabacum* plants

The wild type and transgenic plants, variety N-N Samsung, were grown in growth chambers in these conditions: 16 hours of light, 8 hours of dark, 250 μEinsten $m^{-2}s^{-1}$, 23°C of temperature and 70% of humidity.

8. Plant transformation by agro infection

The tobacco leaves were cut into 0,5-1 cm^2 squares, avoid the mid-rib and the primary nerves, and don't damage the surface of the squares. Of course, all work was done in a laminar flow hood. 10-20 leaf squares were put to float on 10 ml of A10 medium (Table 2) in a Petri dish, upside up.

	A10	A11	A12	A13
B5	+	+	+	+
NH_4NO_3 (250mg/l)	+	+	+	+
MES (500 mg/l)	+	+	+	+
Glucose (2%)	+	+	+	+
Agar (0.75%)	-	+	+	+
Adenine (40 mg/l)	-	+	+	+

Added prior to use medium:

	A10	A11	A12	A13
BAP	0.1 μg/ml	1 μg/ml	1 μg/ml	1 μg/ml
NAA	0.1 μg/ml	0.1 μg/ml	0.1 μg/ml	-
Cefotaxime	-	500 μg/ml	200 μg/ml	200 μg/ml
Hygromycin	-	30 μg/ml	30 μg/ml	30 μg/ml

Table 2.
Medium used in the tobacco plants transformation by agro infection.

After that I infected the 10 ml with 100 μl of Agrobacteria overnight culture (in MGL medium) grown at 28°C and I added also acetosiringone 10 μM, substance that attracts the bacteria into the leaves. A Petri dish without Agrobacteria is used as positive and negative control. The plates were kept for 2-3 days under low light density, avoid the direct light, to promote the bacteria growth and then its access in the leaves. I transferred the leaves to a fresh dish with 10 ml of A10 and incubated for 15 minutes, swirling gently 2-3 times to allow bacteria to come off the plant cells. I repeated a second wash in the same conditions instead the final wash is carried out using A10 supplemented with 500 μg/ml cefotaxime, bacteriostaticum that stops the bacteria replication and doesn't cause them death. I transferred the leaves to solid A11 medium (Table 2), pressing them very gently to the surface to allow good contact. The leaves were transferred to a fresh plate after 7 days, and after 10 more days. The calli obtained (1-2 mm) (Fig.11) were transferred on A12 medium (Table 2), this allows good contact with the medium to ensure proper selection as well as nutrition. This incubation can be up to 2 weeks but no longer, because the cefotaxime instability. From this moment on, the orientation of the callus should not be changed. The calli of 5 mm were placed in small jars containing A13 medium (Table 2) and incubated for a further 2 weeks. If no shoots of good quality appear after 2 weeks, this step can be repeated. The shoots (Fig. 11) obtained were cut with a sharp scalpel and placed on MS30 medium in presence of selection. Each shoot with roots is directly analyzed using RT-PCR to verify the presence of transgene, and after that they were propagated *in vitro*.

9. RT-PCR in *Nicotiana tabacum* transgenic plants

To verify the presence of transgene in the different *Nicotiana tabacum* lines, the total RNA was extracted from 0,05 g of fresh tissue using Genelute mammalian total RNA kit (Sigma).

The cDNA was synthesized using 2 μg of total RNA with RevertAid^{TM} M-MuLV Reverse Transcriptase (Fermentas Life Sciences). All PCR reactions were carried out using as internal control the ribosomal RNA QuantumRNA^{TM} 18s Internal Standards (Ambion). I utilized different combinations of Internal Standards 18s for each analyzed gene. In all PCR reactions I utilized the enzyme Taq DNA polymerase (Euroclone) and the master cycler ep-gradients (Eppendorf).

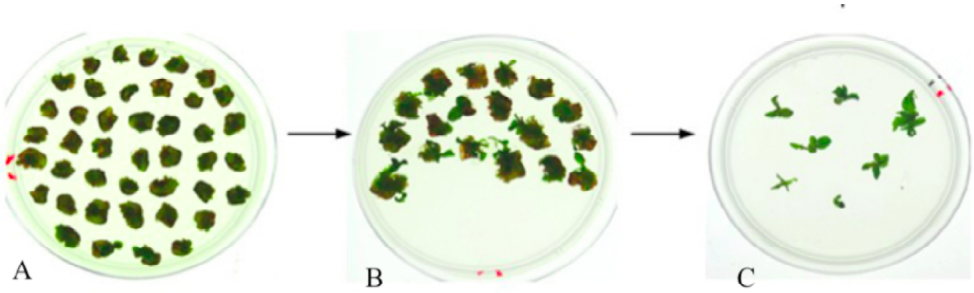

Figure 11.
Agrobacterium tumefaciens mediated transformation: (A) callus formation, (B) shoot differentiation and (C) root differentiation.

The typical scheme utilized for amplification is the following:

Initial denaturation: 94°C for 2 minutes; denaturation: 94°C for 30 second, annealing: 52-60°C for 1 minute (different temperatures for each primers couples), extension: 72°C for 30 seconds. The cycles were repeated for 30 times, after that there was a final extension of 10 minutes at 72°C, followed by the cooling of the samples at 4°C. The amplified fragments were controlled by electrophoresis on agarose gel with ethidium bromide 0.5 μg/ml, and displayed with the instrument Geliance 200 Imaging system (Perkin Elmer). The quantification of amplified bands it was performed using the software Gene tolls (Perkin Elmer).

10. Feeding bioassays of *Spodoptera littoralis* caterpillars on transgenic *Nicotiana tabacum* plants

To evaluate the toxicity of transgenic plants I performed feeding bioassays using the insect *Spodoptera littoralis*. The bioassays were carried out using the first instar larvae fed on artificial diet and on plant leaf disk until the second instar. The leaves were placed in a Petri dish containing 2% agar, in order to keep them turgid and thus more appetizing for the insects. At the third instar, 15-20 larvae were selected and were placed on cut leaves, in plastic glasses, until the pupa instar. All larvae were fed with a fixed quantity of leaf (1cmx1cm), to be sure that all larve have the same food quantity; obviously this quantity increased according to larva growth. Every day the leaf was changed and the mortality rate was measured.

11. Real-time PCR

500 ng of the extracted total RNA was reverse-transcribed primed by random hexamers in a final reaction mixture volume of 20 μl as described in the provide protocol of the high-capacity cDNA Reverse Transcription kit (Applied Biosystem). To minimize variations during the cDNA synthesis step, all RNA samples were reverse-transcribed simultaneously.

Real-time PCR reactions were performed in triplicate each in a 20 μl reaction mixture volume following the manufacturer's instructions for the PowerSYBR Green PCR MasterMix (Applied Biosystem) on an ABI Prism 7300 sequence detection system (Applied Biosystem) using the following thermal cycling profile starting with

2 m at 95°C, 41 repetitions of 15 s at 95°C, 1 m at 60°C. I used as endogenous control the β-actin. 10 to 20 ng of the reverse transcription reaction mixture was used as a cDNA template. The entire experiment was performed at least twice. For each transcript, values were analyzed by means of the ABI Prism 7300 SDS software and normalized relative to the endogenous β-actin control values. Calculations were done by relative quantification with the aid of the standard curve.

RESULTS
AND
DISCUSSION

1. Selection of *Spodoptera littoralis* GPCRs

The first step of my thesis involved the selection of some *Spodoptera littoralis* GPCRs to be used as targets of RNA interference (RNAi) experiments. Several receptors have been described in literature as potential targets in insects, since they are involved in vital functions during different developmental stages, such as water balance, nutrition, chemoreception, reproduction and regulation of metabolism.

The rhodopsin-like family (Class A), as previously described, encompasses receptors that bind a large variety of ligands, such as biogenic amine neurotransmitters, neuropeptides, peptide hormones, lights, nucleotides, prostaglandins, leukotrienes, chemotactic peptides, and chemokines.

Among the receptors belonging to the rhodopsin-like family, I chose some members of neuropeptide and protein hormone receptors since, according to literature, they represent the most important regulators of vital functions in Lepidoptera (Fan et al., 2010).

In particular, I selected:

- The allatostatin-C receptor (AstCR), which binds allatostatins, small neuropeptides acting on the corpora allata and blocking the release of juvenile hormone. Moreover this receptor is expressed in the insect gut where it regulates the smooth muscle contraction. Based on this information, I believed that the AstCR could be an ideal target to interfere with several mechanisms involved in insect development and feeding.
- The Leu-rich repeats-containing GPCR 1 (LGR1), which is a glycoprotein hormone receptor whose expression is developmentally regulated and is also involved in sexual reproduction. In this case, by interfering with its activity may lead to dramatic dysfunctions in insect development.
- The adipokinetic hormone receptor (AKHR), which is involved in the mobilization of sugar (trehalose) and lipids from the insect fat body during energy-requiring activities such as flight or locomotion. Thus, it may represent an ideal target to interfere with feeding and food searching.
- The Pheromone-Biosynthesis-Activating-Neuropeptide receptor (PBANR), which activates pheromone production, control reproduction and communication between the two sexes. The interference with its activity may easily lead to reproductive alterations.

- The octopamine/tyramine receptor (Oct/TyrR), which belongs to the tyraminergic receptors, and is capable of recognizing both the tyramine and the octopamine. This receptor, interacting with the major biogenic amines in insects, has a prominent role in the physiology of the nervous system and represents an excellent target for the RNAi experiments.

The Secretin-like receptor family (Class B), widely described in the introduction, includes also receptors for many hormones, such as secretin, calcitonin, vasoactive intestinal peptide, parathyroid hormone and related peptides. Furthermore it comprises several members having key functions in the regulation of water balance and diuresis. Within this class, I chose the diuretic hormone receptor (DHR), that represents an attractive target for the discovery of novel insecticides because it has a central role in the regulation of fluid and ion secretion, a very important function for the insect survival.

Finally, among the family of the odorant receptors (OR), I decided to choose a particular receptor: the OR83b. This receptor, unlike the conventional odour ligand-binding OR expressed in small subpopulations of Olfactory Sensory Neurons, is expressed in all neurons and is needed for the membrane localization of all the odorant receptors. Due to this specific role, this receptor represents a good target because in case the OR83b was inhibited, the whole insect olfactory system would be shut down causing severe behavioural disorders.

In conclusion, the selected *Spodoptera littoralis* receptors are the following: Adipokinetic Hormone receptor (AKHR), Allatostatin C Receptor (AstCR), Diuretic Hormone Receptor (DHR), Leucine-rich Repeat-containing GPCR 1 (LGR1), Octopamine/Tyramine receptor (Oct/TyrR), Odorant Receptor 83b (OR83b), and Pheromone-Biosynthesis-Activating-Neuropeptide receptor (PBANR).

2. Cloning of the selected GPCRs

Most of the receptors chosen had already been cloned at Arterra Bioscience and their sequences deposited in the gene bank with the following accession numbers: SlAKHR=HC321173; SlDHR=FJ374690; SlLGR1=FJ374692; SlOct/TyrR=FJ374691; SlOR83b=FJ374688 (Tito, 2007). The *Spodoptera littoralis* PBANR sequence was cloned by other authors (Arciello et al., 2006) and present in the gene bank with the accession number HC321177.

For the receptor AstCR, no sequence data were available thus I had to clone part of the sequence using as template the total RNA extracted from adult heads. RT-PCR was performed using degenerate primers based on homologous sequences of different insect species published on the gene bank. The protocols used for RT-PCR experiments are reported in the "Materials and Methods" section. After various attempts, I was able to clone a fragment of the AstCR sequence of 800 bp and, in order to extend the fragment to 1372 bp, I used the 5' and 3' Race technique. The obtained sequence is not complete as about 100 bp are still missing at the 5' end.

In order to express the dsRNA, corresponding to each receptor, in *Nicotiana tabacum* plants, all the receptor sequences were sub-cloned in an appropriate plant expression vector, pH7GWIWG2(I), using the Gateway technology.

Gateway Technology provides an innovative and highly efficient method for transferring DNA fragments across multiple systems and into multiple vectors, replacing tedious and time-consuming cloning and sub-cloning steps. Based on the well-characterized lambda phage site-specific recombination, any DNA fragment flanked by a recombination site can be transferred into any vector that has a corresponding site (Landy, 1989).

I decided to clone the whole coding sequence of each receptor gene, except for AstCR, where I used the 1372 bp fragment

The receptor genes were transferred into the gateway entry vector (pENTR 2B-Invitrogen) (Fig. 9 - Materials and Methods), which is designed to clone DNA sequence using restriction endonuclease and ligase to create a gateway entry clone. This vector contains attL1 and attL2 sites necessary for the subsequent cloning step. The destination binary vector (pH7GWIWG2(I)-Plant Systems Biology) (Fig.10 - Materials and Methods) contains attR1 and attR2 sites. These sites are recognized by LR clonase, enzyme that allows homologue recombination recognizing attL1 and attL2 sites of gateway entry vector. Moreover, this plant expression vector contains the 35S promoter, necessary for the constitutive expression of the downstream gene, and an intronic sequence between the two strands (sense and antisense) of the newly transcribed RNA, necessary to produce a dsRNA in the plant.

The recombinant plasmids have been characterized using restriction endonuclease and verified by sequencing. These plasmids have been transferred into *Agrobacterium tumefaciens* competent cells (strain C58) (de la Riva et al., 1998) for

the subsequent transformation of *Nicotiana tabacum* leaf discs by *Agrobacterium* infection.

3. Production, selection and characterization of transgenic *Nicotiana tabacum* plants expressing dsRNA

I produced transgenic *Nicotiana tabacum* plants expressing the double-strand RNA (dsRNA) of the each cloned *Spodoptera littoralis* GPCR, using *Agrobacterium tumefaciens* mediated transformation. The *Nicotiana tabacum* plant transformation procedure using *Agrobacterium* infection consists of 3 basic steps: transgenic callus formation, shoot differentiation and root differentiation. The protocol used to produce transgenic plants is reported in the "Materials and Methods" section. All the obtained transgenic plants were propagated and analyzed for dsRNA expression level using semi-quantitative RT-PCR. I analyzed 20 transgenic plants for each receptor, and selected 10 positive transgenic plants for the following receptors: AKHR, AstCR, DHR, Oct/TyrR and PBANR. For LGR1 and OR83b only 2 positive transgenic plants were obtained (Table 3). In figure 12, I reported only three examples of RT-PCR analysis, relative to AstCR, Oct/TyrR and PBAN-R: the results of the experiment show that there is a detectable expression level of the dsRNA in all the transgenic lines. This result was expected since the sequences are all under the control of the 35S promoter.

I also decided to produce *Nicotiana tabacum* transgenic plants expressing two dsRNA-GPCRs (double transformants), in order to produce a stronger interference in the insect. In particular, I produced transgenic plants expressing both the dsRNA of AstCR and of DHR, expecting to interfere with the insect development, feeding and regulation of the fluid at the same time. I also produced plants expressing both the dsRNA of DHR and of Oct/TyrR, which would interfere with the insect regulation of the fluid and physiology of the nervous system. I analyzed 40 transgenic plants for each double transformant and obtained 2 positive transgenic plants expressing both dsRNA of AstCR and DHR, and 5 positive transgenic plants expressing both dsRNA of DHR and Oct/TyrR (Table 3). In figure 13, I reported the RT-PCR analysis of the positive transgenic plants transformed with DHR and Oct/TyrR.

Spodoptera littoralis **receptors**	**Tested transgenic plants**	**Positive transgenic plants**
AKHR	20	7
AstCR	20	7
DHR	20	11
LGR1	20	3
Oct/TyrR	20	9
Or83b	20	2
PBANR	20	8
AstCR+DHR	40	2
DHR+Oct/TyrR	40	5

Table 3.
Transgenic plant lines transformed with *Spodoptera littoralis* receptors, number of tested transgenic plants and number of positive transgenic plants.

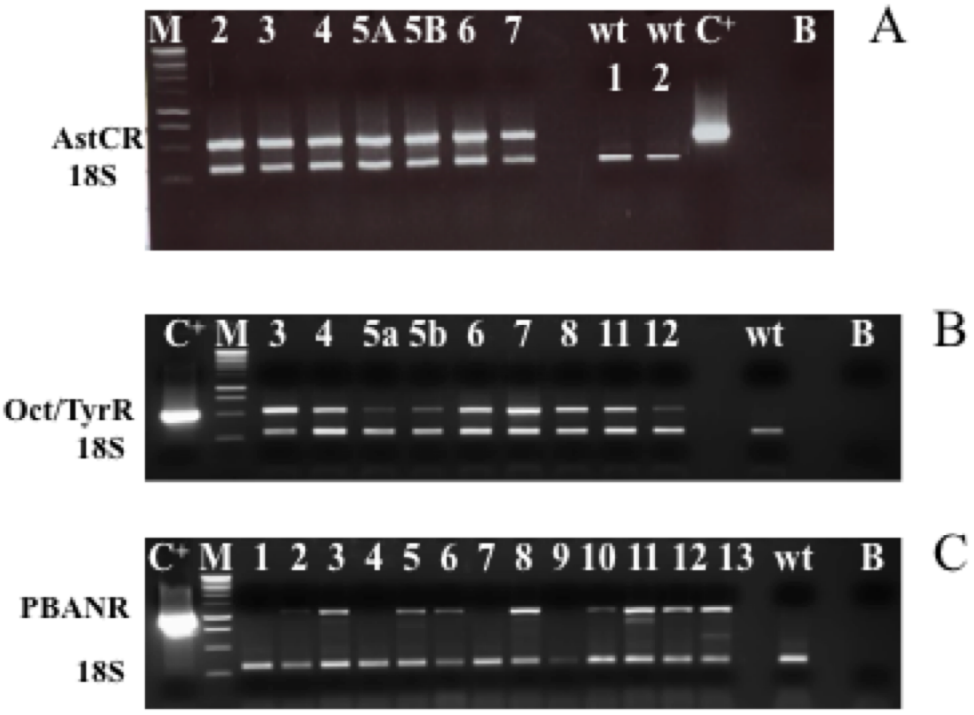

Figure 12.
RT-PCR of *Nicotiana tabacum* transgenic plants.
(A) M: 1Kb DNA ladder; Lanes 2-7: different transgenic plants expressing AstCR-dsRNA (520 bp band); wt1 and wt2: untransformed tobacco plants; C^+: pENTR-2B+AstCR (positive control of the PCR); B: negative PCR control.
(B) C^+: pENTR-2B+Oct/TyrR (positive control of the PCR); M: 1Kb DNA ladder; Lanes 3-12: different transgenic plants expressing Oct/TyrR-dsRNA (520 bp band); wt: untransformed tobacco plants; B: negative PCR control.
(C) C^+: pENTR-2B+PBANR (positive control of the PCR); M: 1Kb DNA ladder; Lanes 1-13: different transgenic plants expressing PBANR-dsRNA (1200 bp band); wt: untransformed tobacco plants; B: negative PCR control.

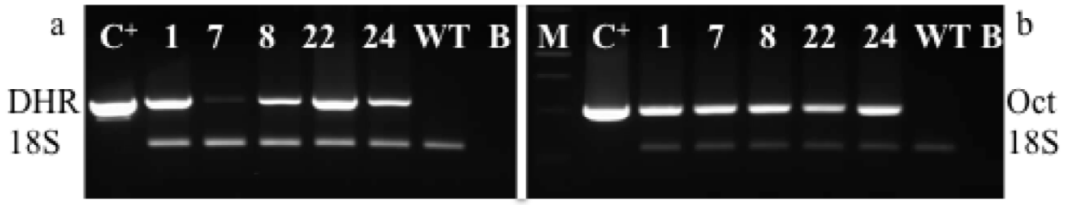

Figure 13.
RT-PCR of transgenic plants expressing both dsRNA of Oct/TyrR and DHR.
(a) C^+: pENTR-2B+DHR (positive control of the PCR); lanes 1, 7, 8, 22, 24: samples of the transformed plants; wt: untransformed tobacco plants; B: negative PCR control; (b) M: 1kb DNA ladder; C^+: pENTR-2B+Oct/TyrR (positive control of the PCR); lanes 1, 7, 8, 22, 24: samples of the transformed plants; wt: untransformed tobacco plants; B: negative PCR control.

Once obtained and characterized all the single and double transformants, before performing the feeding bioassays, I evaluated the stability of the dsRNA molecules in the *Nicotiana tabacum* leaves by performing a time course experiment.

4. Analysis of the dsRNA stability in the transgenic plants

Considering that many of the feeding bioassays had to be carry out on cut leaves, I wanted to verify the time stability of the dsRNA, to understand how long the dsRNA remains undegraded in the excised leaves of the plant.

To verify that, I conducted a time-course RT-PCR experiment by using excised leaves. Leaves were collected from transgenic *Nicotiana tabacum* plants expressing dsRNA for AlstCR, and total RNA was extracted after 3, 6 and 24 hours after the excision. The results of the semi-quantitative RT-PCR analysis (Fig. 14) showed that the transgene expression level was stable during the 24h time period, suggesting that no significant degradation of the dsRNA occurred in the leaves after the cut.

5. Feeding bioassays of *Spodoptera littoralis* caterpillars by using transgenic *Nicotiana tabacum* plants

All the feeding bioassays were performed by me in the laboratories of Arterra Bioscience, by the group of Dr. Chiara Sargiotto at Isagro Ricerca, and by the PhD student Ilaria Di Lelio in the laboratory of Prof. Francesco Pennacchio at the Department of Entomology, University of Naples "Federico II". ,

For these assays I decided to start with the plants transformed with AlstCR, DHR and OCT/TyrR, since, among all the cloned receptors, were those that could give the highest chances of success. According to literature data (Mao et al., 2007) and unpublished results obtained at Arterra, both AlstCR and DHR represent accessible targets for RNAi technology because mostly expressed in the gut or Malpighian tubules. Although the Oct/Tyr receptor appears a more difficult target to reach by RNAi, it acts in the central nervous system and is the most important regulator of the basal metabolism.

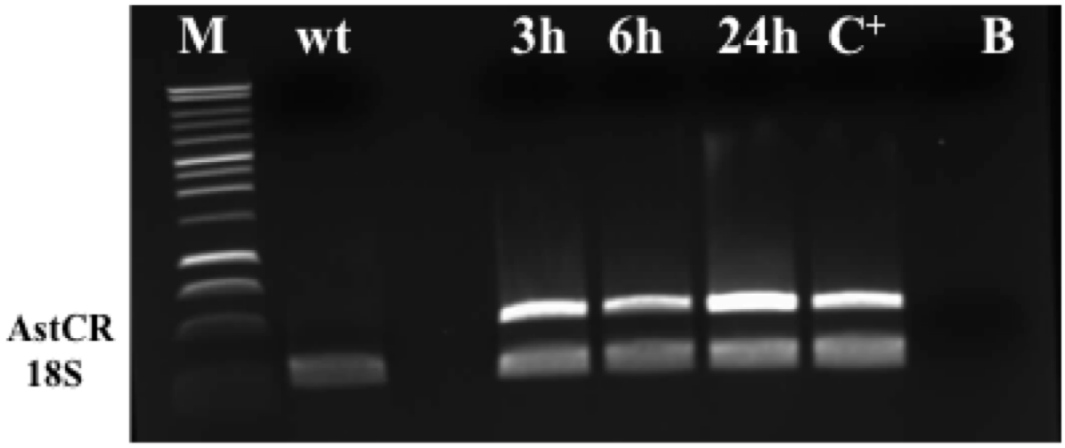

Figure 14.
Time course analysis of dsRNA stability in transgenic plants expressing the AstC receptor.

5.1. Feeding bioassays with AlstCR-dsRNA expressing plants

A preliminary feeding experiment on the whole plants was performed in the labs of Isagro Ricerca. The purpose of this experiment was to screen a large number of transgenic AlstCR plants and to choose those producing the most evident phenotypic effects in the larvae.

The bioassay was performed using 30 *Spodoptera littoralis* larvae which were fed on 7 different transgenic tobacco lines (AlstC 2, 3, 4, 5A, 5B, 6, 7). The first instar larvae were fed on artificial diet and on plant leaf disks until the second instar. Then, 15-20 larvae were selected and transferred onto the transgenic plants, and onto wild type (wt) plants, used as control. At the fourth-fifth instar, larvae were transferred again and placed on cut leaves, in plastic glasses, until the pupa instar. During the experiments the mortality rate was calculated and the larvae observed for eventual phenotypic defects. As shown in figure 15, although the mortality rate of the larvae fed on wt plants was unexpectedly high (50%), the plants of the transgenic line AlstC-6 produced an 80% mortality rate. Since in this preliminary screening only the transgenic line AlstC-6 gave positive results, I decided to use this line in all the other following feeding tests. In the laboratory of Prof. Francesco Pennacchio, Ilaria Di Lelio measured the mortality rate in larger populations of larvae (200 individuals), which were fed on plant leaf disks during the entire life cycle. The results of this experiment, showed in figure 16, revealed that after five days the mortality of the larvae fed on the transgenic plants was 25% higher than that of larvae fed on wt plants. After 10 days, all the larvae fed on transgenic plants died, while the mortality of larvae fed on wt plants reached 65%. To validate these results, the experiment was repeated 2 times and similar mortality percentage values were obtained. Although in all the experiments the mortality rate of the control larvae was unexpectedly high, the transgenic plants expressing dsRNA of AlstCR gave always a significant effect of mortality, most likely due to the interference with the GPCR expression in the insects.

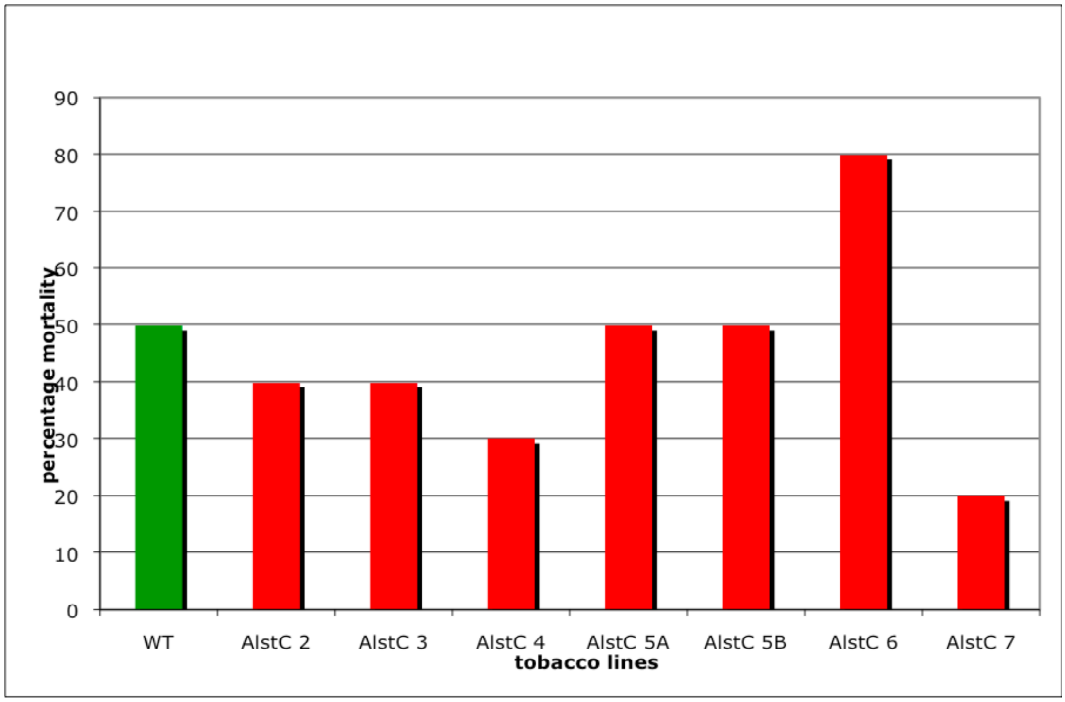

Figure 15.
Percentage of mortality of *Spodoptera littoralis* larvae fed on transgenic AlstCR-dsRNA plants (red bars) and wt plants (green bar).

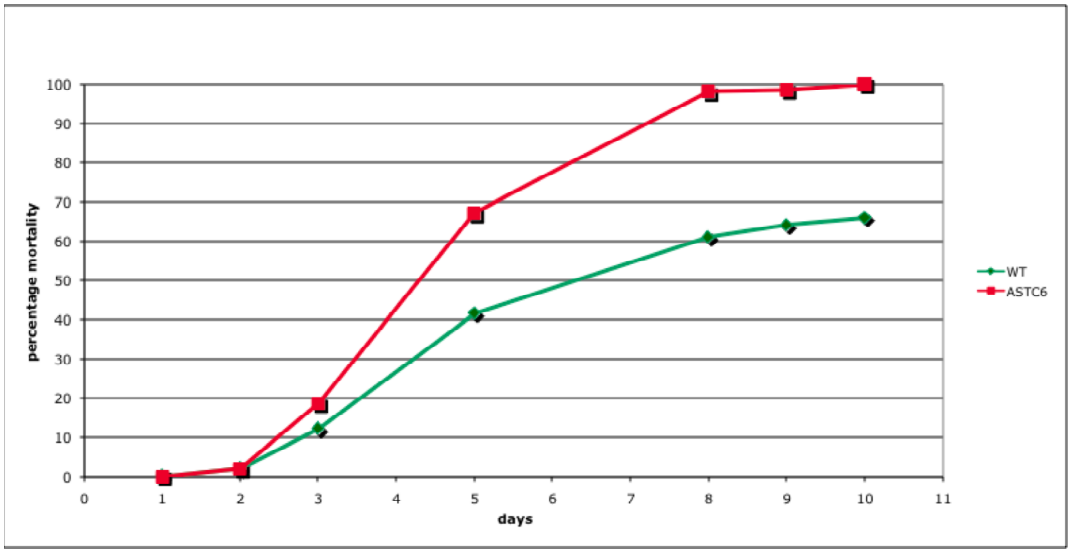

Figure 16.
Daily percentage of mortality of *Spodoptera littoralis* larvae fed on transgenic AlstCR-dsRNA plants (red line) and wt plants (green line).

5.2. Feeding bioassay with DHR-dsRNA expressing plants

Analogously to what previously done for the AstCR plants, the transgenic tobacco lines expressing the dsRNA of DHR were used in a preliminary screening test to find those producing significant phenotypic effects on the *Spodoptera* larvae. Among all the 11 transgenic lines analyzed (DHR 5, 7, 8, 9, 11, 13, 14, 15, 18, 19, 20), 4 lines showed a significantly higher mortality rate than those fed on wt plants (Fig. 17). Thus, I decided to use the plants belonging to lines DHR 7 and DHR 19 for further experiments of feeding. 200 larvae were fed on leaf disks excised from DHR-dsRNA and wt plants, and the mortality rate was measured over 12 days after the beginning of the experiment. The results, reported in figure 18, show the daily mortality percentage of the larvae fed on transgenic plants in comparison with that of larvae fed on wt plants: the mortality rate of the larvae fed either on the plant DHR7 or DHR19 reached 100% after 8 and 12 days, respectively, while the mortality rate of the control larvae was only 60% and 85%. The experiment was repeated one more time, and the results were consistent with what previously obtained. I can conclude that also the plants expressing DHR-dsRNA produced significant effects on insect vitality, suggesting the validity of the employed RNAi strategy.

5.3. Feeding bioassay with Oct/TyrR-dsRNA expressing plants

The feeding assays on the transgenic plants transformed with *Spodoptera littoralis* Oct/TyrR didn't follow the experimental protocol used for AlstCR and DHR. To save time, the bioassays were performed at the same time by the 3 different laboratories - Isagro Ricerca in Novara, the Department of Entomology (University of Naples "Federico II") and Arterra Bioscience. All the experiments followed the same protocol reported in "Material and methods" section. *Spodoptera littoralis* larvae were fed on plants belonging to 7 different lines of Oct/TyrR transgenic plants (3, 4, 6, 7, 8, 11 and 12), and the mortality rate was measured.

In the table 4 the results of all the bioassays are summarized. Delta (Δ) represents the difference of the mortality rate of larvae fed on transgenic plants and that of the larvae fed on WT plants (control), expressed in percentage. The lines Oct/TyrR 6, 7 and 11 gave the best results in these tests.

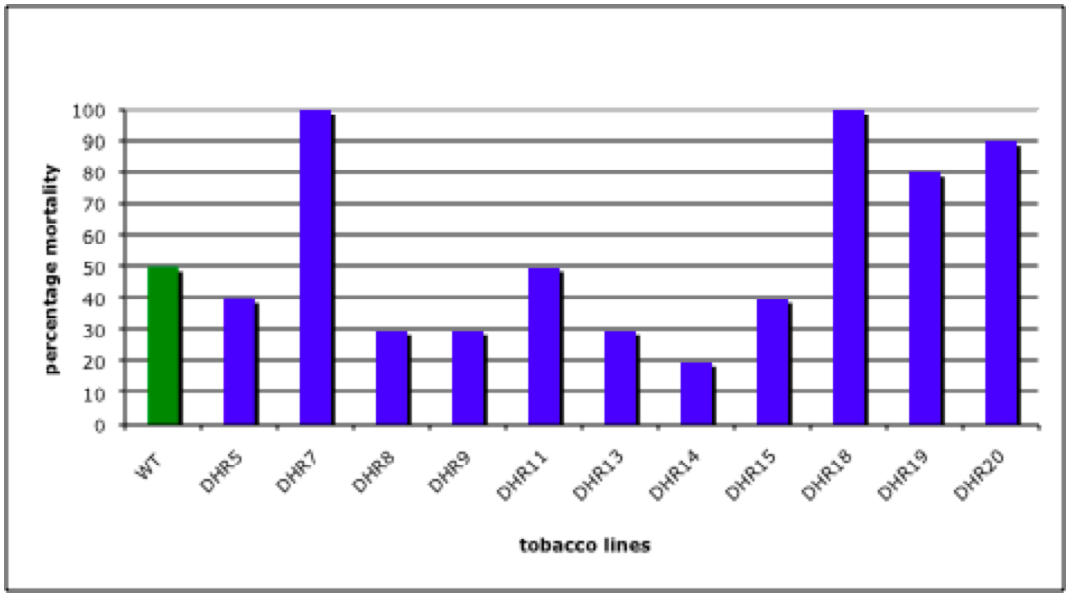

Figure 17.
Percentage of mortality of *Spodoptera littoralis* larvae fed on transgenic DHR-dsRNA plants (blue bars) and wt plants (green bar).

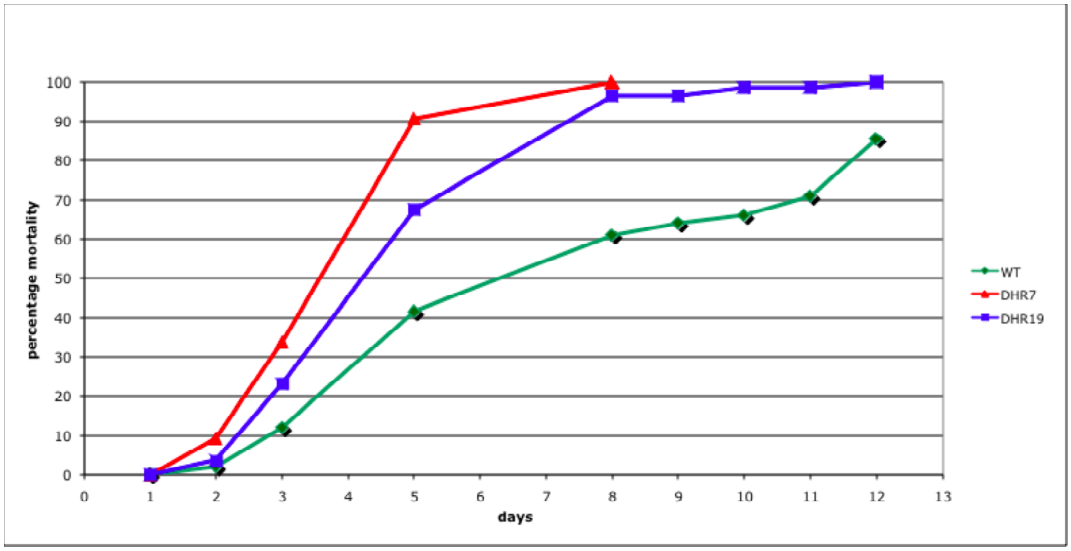

Figure 18.
Daily percentage of mortality of *Spodoptera littoralis* larvae fed on transgenic DHR-dsRNA plants belonging to line DHR7 (red line), line DHR19 (blue bar) and of larvae fed on wt plants (green line).

	I EXP.	II EXP.	III EXP.	IV EXP.	V EXP.
OCT 3	Arterra	Isagro			
	–Δ	Δ=6%			
OCT 4	University	University	Isagro	University	
	Δ=25%	–Δ	Δ=0%	Δ=9%	
OCT 6	Arterra	Arterra	Isagro	Isagro	University
	Δ=30%	Δ=50%	Δ=0%	Δ=57%	Δ=6%
OCT 7	University	University	Isagro	University	
	Δ=40%	–Δ	Δ=46%	Δ=9%	
OCT 8	University	Isagro			
	–Δ	Δ=7%			
OCT 11	Arterra	Isagro			
	–Δ	Δ=37%			
OCT 12	University	University	Isagro	Isagro	University
	Δ=5%	Δ=15%	–Δ	Δ=20%	–Δ

Table 4.
Feeding bioassays on different lines of *Nicotiana tabacum* transgenic plants (3, 4, 6, 7, 8, 11 and 12) expressing the dsRNA of Oct/TyrR.

Line 6 produced the highest percentage of mortality in three out of five experiments; line Oct/TyrR 7 gave the highest percentage of mortality in two out of four experiments, while line 11 only in one out of two experiments. On table 4, it is possible to note that the results of the tests, even though performed on plants of the same line, are often variable: for example, analyzing the results obtained on transgenic line 6, only three bioassays gave a Δ of about 30-50% (those performed at Arterra and at Isagro), the other one gave a Δ of about 0-6% (those performed at the University and at Isagro). Unfortunately, the results of the tests performed by the different labs don't always agree, which can be explained by the different protocols used, and maybe by the slight different experimental conditions.

6. Discussion of the results obtained from the feeding bioassays

Most of the results suggest that the transgenic plants expressing dsRNA corresponding to the insect receptor cause a significant mortality rate in the larvae that fed on them. On the other hand, the results are not always consistent, which indicate the presence of experimental problems giving:

i) an unusually high mortality rate of the control larvae

ii) lack of repeatability of the tests.

I can only hypothesize the following:

i) The unusual high mortality rate of the larvae fed on wt plants can be explained by the fact that there is always a basal mortality in insect populations, even in those grown under controlled experimental conditions. This basal mortality can be higher when the larvae are domesticated and thus become more susceptible to slight environment changes. A high mortality in the insect populations can be also due to the toxicity of nicotine, the alkaloid normally present in the leaves of *Nicotiana tabacum* plants. It was shown that insect fed on tobacco plants have problems in the neurotransmission because the nicotine acts as agonists at the nicotinic acetylcholine receptor (nAChR) that is widely and predominantly distributed in the neuropil regions of the central nervous system (CNS), (Tomizawa and Casida, 2003).

ii) Concerning the repeatability of the assays, the problems could be related to either the insect system or the plants. In the insect, there could be redundancy in the

receptor genes, which implies a bigger resistance to loose a specific gene functionality. In particular, the inconsistent results obtained for the Oct/TyrR suggest that a reduction of the activity of this gene may be very hard to achieve due to the presence of analogous genes which are expresses differentially in the various stages of the insect development. Indeed, in Lepidoptera there are three classes of Octopamine (OA) receptors (Evans and Maqueira, 2005): α-adrenergic-like OA receptors (OctαRs), β-adrenergic-like OA receptors (OctβRs) and OA/TA (or tyraminergic) receptors. Moreover, the Oct/Tyr receptor, which I studied, is expressed in the insect central nervous system and thus it may be a more difficult target to reach by the dsRNA molecules.

Finally, to exclude that the inconsistency of the results of the bioassays with Oct/TyrR plants was due to genetic recombination phenomena occurring in the plants, I checked the presence of genetic chimaeras. In botany, "chimaeras" are plants consisting of two or more genetically distinct kinds of cells that can arise by a mutation in a certain region of the plant after cells divide. If this phenomenon had happened in my transformed plants, some clones would have expressed the transgene and other ones not. To verify that, I performed the transgene expression analysis in all the clones derived from the same line (Fig. 19). The results obtained showed that all the clones had a comparable expression level of the transgene and thus they were not genetic chimaeras.

7. Validation of the strategy: expression study in the interfered larvae fed on dsRNA transgenic plants

To verify whether the effect of insect mortality I observed in the bioassays was effectively due to the interference of the dsRNA with the specific insect receptor, I performed an expression analysis in the interfered larvae fed on dsRNA transgenic plants. I conducted this analysis on the larvae fed on transgenic *Nicotiana tabacum* plants expressing DHR-dsRNA since I obtained the most reproducible results by using these plant lines. Moreover, preliminary results obtained at Arterra on RNAi in insects by microinjection indicated that dsRNA can actively reduce the level of the GPCR expression (unpublished results).

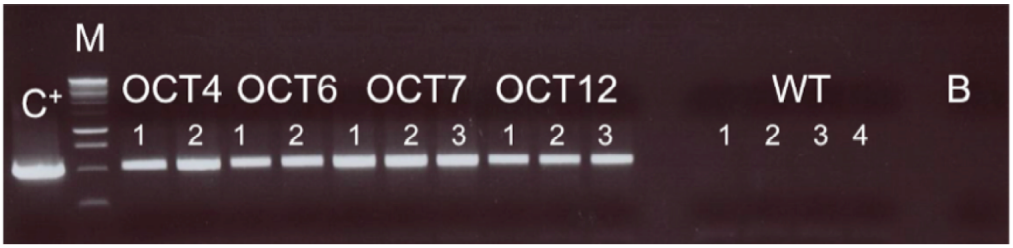

Figure 19.
RT-PCR of transgenic plants expressing dsRNA of Oct/TyrR.
C^{+}: pENTR-2B+Oct/TyrR (positive control of the PCR); M: 1kb DNA ladder; lane OCT4 (clones 1-2), OCT6 (clones 1-2), OCT7 (clones 1-3), OCT12 (clones 1-3): samples of the transformed plants; wt (1, 2, 3, 4): untransformed tobacco plants; B: negative PCR control.

To perform the expression studies on DHR gene in *Spodoptera*, I fed the larvae on *Nicotiana tabacum* transgenic plants belonging to the line DHR 7, and on *Nicotiana tabacum* wild type plants, as control. For each sample, 5 larvae were picked-up at days 3, 5 and 7 after the beginning of the experiment and total RNA was extracted. I chose these days for the pick-up on the basis of the results obtained from the feeding bioassays. In fact, recalling to figure 18, at the day 3 it is already possible to observe a slight difference between the percentage mortality of the larvae fed on transgenic plants and that of the larvae fed on WT plants (Δ=10%). Then, after the day 5 the Δ value overcomes 50%, to reach a maximum at day 7. The expression level of DHR was measured by using semi-quantitative RT-PCR on the larvae fed on the transgenic at days 3, 5 and 7. The values reported in figure 21, which refer to the amplification bands shown in figure 20, are averages of two independent experiments. On day 3, the expression of the DHR gene is higher in the interfered larvae than that in the control larvae, while on day 5 and 7 it goes down significantly only in the interfered larvae. At the day 7, the decrease of DHR gene expression in the interfered larvae is around 35% lower than that in the control larvae. These results clearly indicate that the interference caused by the transgenic plants had the specific effect of reducing the expression of the DHR gene in *Spodoptera littoralis* larvae.

To confirm the results obtained from RT-PCR analysis I quantified the expression level of the DHR gene also by using Real Time PCR technique. The results of the Real Time PCR analysis (Fig. 22), as expected, were more accurate and showed a trend very similar to the one obtained from the semi-quantitative RT-PCR: at the day 5, the expression of DHR gene started decreasing until reaching around 65% of the initial value at day 7. The result obtained at the day 3 was quite unexpected, but hardly due to an experimental artifact since confirmed even by the Real Time analysis. This could be explained by the fact that after the feeding the larvae accumulate siRNA molecules, as result of the RNAi mechanism, and also amplify these molecules due to activity of RNA-dependent RNA polymerase (RdRP) (Lipardi and Paterson, 2009).

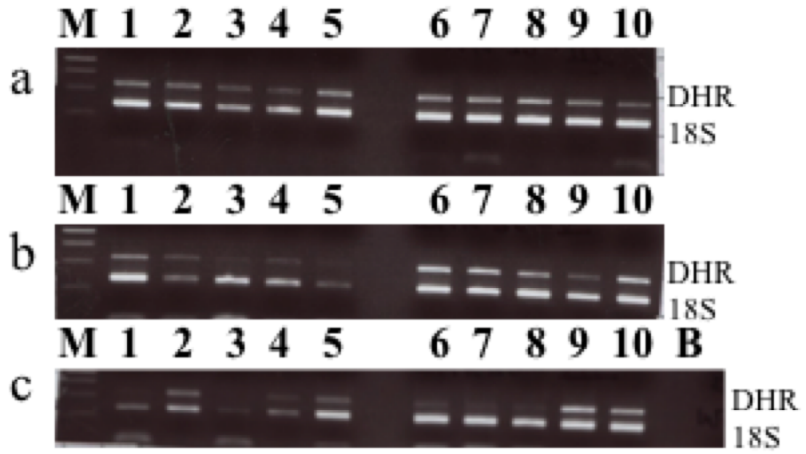

Figure 20.
RT-PCR of the interfered larvae.
a: day 3; **b**: day 5; **c**: day 7. From lane 1 to 5: larvae fed on WT plants; from lane 6 to 10: larvae fed on DHR-dsRNA transgenic plants; B: negative PCR control; M: 1kb DNA ladder.

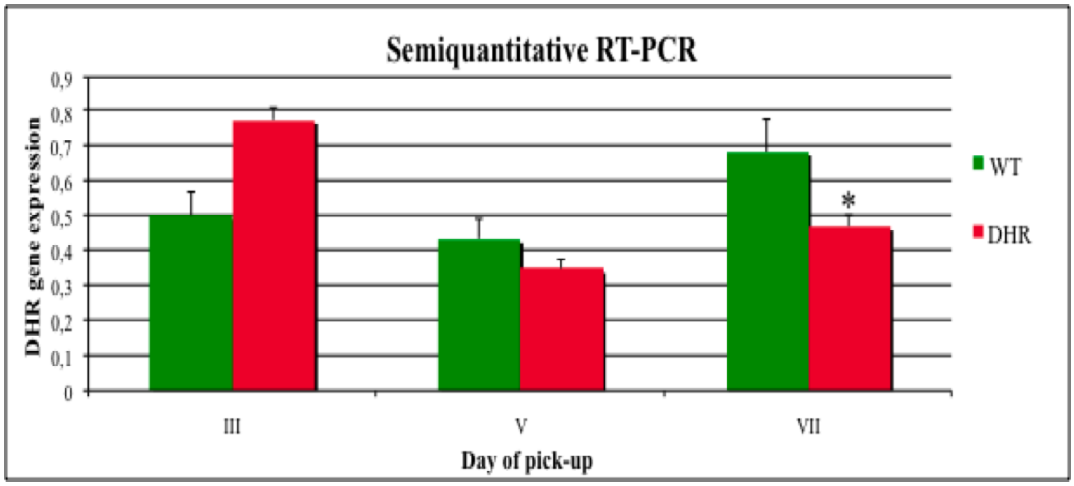

Figure 21.
DHR gene expression level, using semiquantitative RT-PCR analysis, in *Spodoptera littoralis* larvae fed on WT plants (green bar) and transgenic plants (red bar), at the days 3, 5 and 7; (* $p < 0,05$).

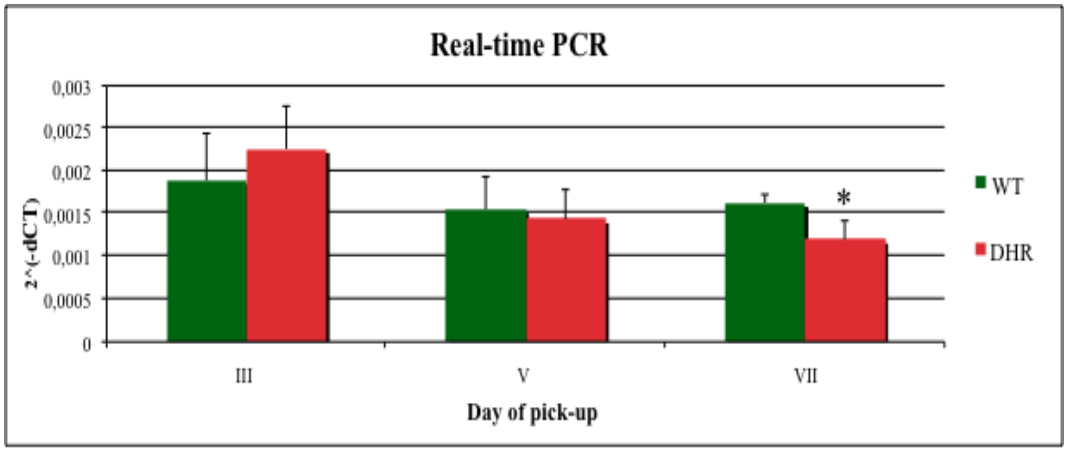

Figure 22.
DHR gene expression level, using real-time PCR analysis, in *Spodoptera littoralis* larvae fed on WT plants (green bar) and transgenic plants (red bar), at the days 3, 5 and 7; (* $p < 0,05$).

In conclusion, the results of the expression analysis obtained for DHR suggested that the effect of mortality observed in the populations of larvae fed on the transgenic plants was most likely due to a reduction of the amount of specific mRNA in *Spodoptera*, causing severe phenotypic effect on the metabolism and leading to the insect death.

CONCLUSIONS

The aim of my PhD project thesis was the production and characterization of transgenic *Nicotiana tabacum* plants, expressing dsRNA specific to G protein coupled receptors of *Spodoptera littoralis* as new strategy to protect plants against insect pest. For this purpose, seven *Spodoptera littoralis* GPCRs (AKHR, AstCR, DHR, LGR1, Oct/TyrR, OR83b and PBANR) were chosen and cloned in an appropriate plant expression vector. Using *Agrobacterium tumefaciens* mediated transformation transgenic *Nicotiana tabacum* plants were produced for each cloned GPCR. All the plants were characterized for the presence of the transgene using RT-PCR.

Nicotiana tabacum transgenic plants expressing dsRNA for AstCR, DHR and Oct/Tyr-R were also chosen for further feeding bioassays with the target insect *Spodoptera littoralis*. This because these 3 receptors represented the most attractive targets due to their central roles in the regulation of insect development and vitality. In particular, DHR has a key role in the regulation of fluid and ion secretion, and Oct/TyrR works as neurotransmitter in different functions, from insect metabolism regulation to modulation of respiration and muscles contraction. Moreover, AlstCR may represent an interesting target also for its localization in the larval body: literature data have highlighted that the RNA interference by feeding works better on genes expressed in the gut (Mao et al., 2007).

In all the performed bioassays the percentage of mortality was measured every day. Some of the lines produced significant mortality rates compared to wt plants. To verify that the observed mortality was due to the interference with the specific gene and to validate this new strategy, an expression analysis of DHR gene in interfered larvae was performed using semi-quantitative RT-PCR and Real-time PCR techniques. Some authors reported that is very difficult to interfere with genes expressed in Lepidoptera, and that the interference preferentially works with genes expressed in the gut (Huvenne and Smagghe, 2010).

The results obtained with both, semi-quantitative RT-PCR and Real-time PCR, show that the DHR expression level was significantly reduced in *Spodoptera littoralis* interfered larvae, proving that the interference technology can be used also in Lepidoptera. Moreover the DHR is not expressed in the gut but only in the Malpighian tubules. The reduction of the expression level of DHR that we observed in the larvae fed on DHR-dsRNA transgenic plants demonstrated that the interference technology can be applied to a wide range of insect genes and not only

in those expressed in the gut. In conclusion, all these results could be an input for further field trials and the development of a non-conventional crop protection strategy based on the use of the transgenic plants as bio-insecticides.

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