Identificación de posibles factores de *Myzus persicae* implicados en la transmisión del virus del grabado del tabaco (TEV) y estrategias para interferir su expresión

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IDENTIFICACIÓN DE POSIBLES FACTORES DE MYZUS PERSICAE IMPLICADOS EN LA TRANSMISIÓN DEL VIRUS DEL GRABADO DEL TABACO (TEV) Y ESTRATEGIAS PARA INTERFERIR SU EXPRESIÓN

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2012

INTRODUCTION

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THE CHALLENGE OF INTERFERING WITH PLANT VIRUS TRANSMISSION

Since the onset of civilizations, Agriculture has been an essential activity for securing the supply of food and other useful products for human beings, like feed for farm animals and fibers. With plants on the basis of most sustainable agricultural processes, concerns around how to establish efficient strategies for protecting them against pests and pathogens have been a constant (Strange and Scott, 2005). Nowadays, the increased demand for agricultural products in our society, together with global changes, are creating new challenges to be faced, considering the economic impact of production losses due to known pests or diseases, and the emergence of new well adapted pathogens or pests.

Some studies have estimated that around 10% of the annual production of many crops on average can be lost (FAO, 2000), with a substantial part being consumed by herbivores, mainly insect pests. In some cases, this rate of damage can be well above the expected productivity of the crops, for instance in terms of plant biomass allocated to reproduction (fruits and seeds): this clearly indicates the importance of dealing with and controlling insect pests. Furthermore, and besides the direct damages caused, some insects are responsible of spreading plant diseases, in particular viruses, which can be also quite detrimental for crop production. In this thesis we are going to consider in particular this aspect of the problem, focusing on the transmission of viruses mediated by insect vectors, and how the ongoing research can provide innovative strategies to interfere with the process. Will these innovative strategies be succesful in avoiding virus spread? One of these strategies is pursued experimentally, and the results obtained in the laboratory will be presented and discussed.

Insects play an important role in the dissemination of most plant viruses (Hull, 2001). Being obligate parasites, the maintenance and survival of viruses in nature depends on the existence of mechanisms dedicated to allow them to reach new hosts. Consequently, the transmission process is one of the most important steps in the biological cycle of viruses, and the use of vector organisms is the most frequently adopted strategy to secure their dissemination. In addition to a few below ground

organisms like chytrids, plasmodiophorids and nematodes, the main vectors of plant viruses are arthropods, including mites and insects. Focusing on insects, plant viruses are spread from plant to plant by species belonging to several orders (Table I), being hemipterans, in particular species included in the suborder *Homoptera* like aphids, whiteflies and leafhoppers, the major vectors of plant viruses. Examples of representative virus taxons transmitted by the different categories of insects are also included in Table I.

Table I. **Main groups of insect vectors of plant viruses**, with examples of representative virus taxons transmitted by them

Order	Suborder	Superfamily	Family	Type of insect (common name)	Examples of plant virus taxons[1]
Hemiptera	*Homoptera*	*Aphidoidea*	*Aphididae*	Aphids	Potyvirus, Macluravirus, Cucumovirus, Caulimovirus, Luteovirus, Polerovirus, Alfamovirus, Fabavirus, Closterovirus, Nanovirus, Sequivirus, Umbravirus...
		Aleyrodoidea	*Aleyrodidae*	Whiteflies	Begomovirus, Ipomovirus, Crinivirus, Carlavirus, Torradovirus...
		Membracoidea	*Cicadellidae*	Leafhoppers	Curtovirus, Mastrevirus, Phytoreovirus, Waikavirus
			Membracidae	Treehopper	Topocuvirus
		Fulgoroidea	*Delphacidae*	Planthopper	Fijivirus, Oryzavirus, Tenuivirus
Thysanoptera		*Thripoidea*	*Thripidae*	Thrips	Tospovirus
Coleoptera				Beetles	Bromovirus, Comovirus, Sobemovirus, Tymovirus

[1] Virus group names according to the database of the International Committee for Taxonomy of Viruses, ICTV, accessible at http://ictvonline.org/index.asp

As shown in Table I, the transmission of members of a large number of virus groups is due to the action of insects in the *Aphididae* and *Aleyrodidae* families, aphids and whiteflies respectively, and also to other insects like leafhoppers, threehoppers and planthoppers. All these homopterans have in common a pierce-sucking type of mouthparts (Nault, 1997), with stylets that are able to pierce the cell walls of plant tissues without causing a major damage to the cell, in their way to target the phloem (Hewer *et al.*, 2011). Furthermore, the capacity for growth of the colonies, particularly in the case of aphids, and the easy dispersion of these insects, makes them collectively the most important groups of vectors of plant viruses.

When considering the types of insects listed in Table I, all of them are phytophagous, which means they depend on plants for obtaining their food. To do so, they have to surpass a diverse arsenal of plant defenses, evolved in a classical arms race to stop the damages caused by herbivore attacks. Insects adaptations to overcome plant defenses might consist for instance in anatomical changes like the pierce-sucking mouthparts that allow to reach rapidly and efficiently the vascular tissues of the host plant. The behaviour of vectors when feeding is also a feature that might affect their capacity for transmission of viruses, as acquisition and inoculations steps occurred during vector feeding.

1. MODES OF TRANSMISSION

Mechanistically, a successful transmission requires several steps to be fulfilled. In the case of insect vectors, the first step is the acquisition of the virions from an infected plant (Step 1). Next, the acquired virions need to be retained in the vector (Step 2), either at specific sites through binding of virions to receptor-like elements in the digestive tract, or circulating from different anatomical structures, mainly from the gut to the salivary glands. After this, delivery of virions from the retention sites is required (Step 3), in many cases following salivation. Finally, the virions have to be deposited in a susceptible cell of a different host plant (Step 4), where they can start again an infectious cycle (Figure 1).

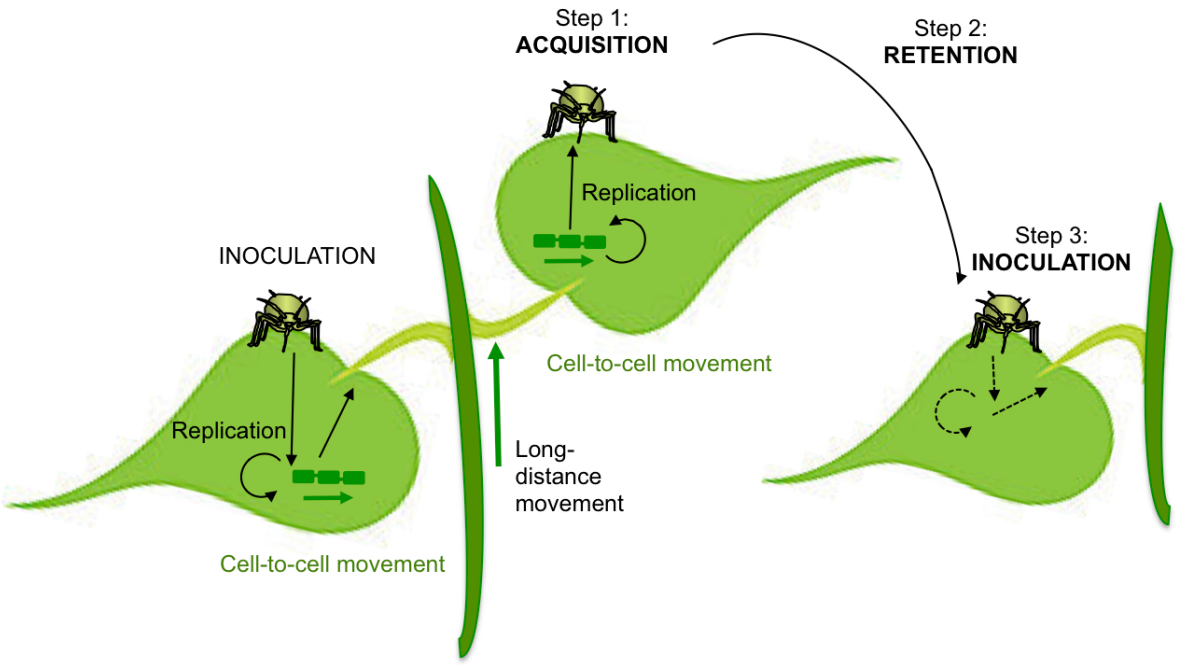

Figure 1. **Cycle of insect-disseminated plant viruses**. For a successful transmission are required several steps: (1) Acquisition of virions from an infected plant. (2) The stable retention of acquired virions in the vector. (3) Deliver of virions from the retention sites following salivation. And at last, the release of virions in a plant cell where they can start again the infection.

The duration of these steps was used to propose the first classification of modes of virus transmission by vector organisms (Watson and Roberts, 1939) using two categories: nonpersistent and persistent. The length of the period during which the infectivity of the virus is retained in the vector serves to differentiate into these two modes of transmission. In the first case, nonpersitent transmission, the insect can transmit the virus to uninfected plants almost immediately after it has been acquired by feeding on an infected plant, in a timeframe ranging from seconds to few minutes. Most importantly, the capacity to transmit the virus is lost rapidly thereafter acquisition. On the second type, persistent transmission, the acquisition of virus requires longer periods of time (around hours), and afterwards a retention period (from hours to days) is also needed before the vector finally becomes viruliferous and is able to transmit the virus. In between these two, a third category named semipersistent transmission is reserved for viruses with intermediate requirements in terms of acquisition and retention periods.

A further refinement of this classification was proposed later, being based on the route followed by the virus inside the insect vector (Harris, 1977). In this case, noncirculative and circulative transmission can be differentiated. In noncirculative transmission, the virus is temporarily associated and reversibly retained in sites located in the anterior tract of the digestive system of the vector, like mouthparts or even the foregut. This type of transmission corresponds mainly with nonpersistent and semipersistent viruses. As the acquisition period gets longer, the virus transmission efficiency might increase, in the case of semipersistent viruses, or decrease rapidly, as it often happens in nonpersistent viruses. This important difference seems to be associated with the stability of virion retention in the vector, which allows in the first case to accumulate virions until the retention sites are saturated, increasing the chances of being transmitted later. This relationship has been observed in aphids and also in other vectors, notably in whiteflies and leafhoppers. On the other hand, nonpersistent viruses are only transmitted by aphids (Ng and Falk, 2006), and retention seems to happen through a weak interaction, meaning that the virions can be rapidly lost when the feeding period is extended over a certain limit. The virus is not retained for long, what means it can be immediately released and inoculated, and only during short periods of time. Both acquisition and inoculation are thought to occur during feeding probes. With such a narrow timeframe, it is indeed rather difficult to control the spread of these viruses using insecticide treatments targeted to kill vectors, since the window of opportunity passes quite rapidly.

Finally, circulative transmission requires the passage of virions through the insect. After feeding on the infected plant, the virus acquired must get across the gut wall to reach the haemolymph, and eventually could arrive to the salivary glands, getting access into the saliva for being inoculated. The complete process might takes days, and therefore a period of latency occurs, since immediately after acquisition the virus cannot be inoculated, because a rather long circulation inside the vector is required, which involves crossing of cellular barriers. Two additional categories can be established in this case: when the virus replication is restricted to the plant hosts, despite the movement of virions through the insect, the virus is considered nonpropagative. However, in certain cases the virus is able to replicate as well in the cells of the vector, and it is considered propagative. Interestingly, propagative viruses are parasites of both plants and insects, alternating between the two types of hosts to complete their cycles of dispersion.

2. MAIN GROUPS OF INSECT VECTORS OF PLANT VIRUSES.

Since the feeding behavior of pierce-sucking insects could define their capacities for virus transmission (Stafford *et al.*, 2012), in this section we will review some important aspects and peculiarities of the main groups of insect vectors, including aphids, whiteflies and leafhoppers.

2.1. - APHIDS

As shown in Table I, aphids can spread a large number of viruses infecting plants, including for instance potyviruses (a group of plant viruses comprising more than 150 definitive species), cucumoviruses, caulimoviruses, luteoviruses, poleroviruses and many others. Using their characteristic pierce-sucking mouthparts, aphids can feed on plant sap. Aphid mouthparts have stylet morphology, adapted to access and feed on the contents of even individual plant cells. This stylet is composed by a pair of mandibular elements surrounding a pair of tightly interlocked maxillary elements that comprise two internal canals, one for injecting saliva, and the other for sucking up plant fluids. Their searching behaviour for plants, the range of available host plants and their high reproduction rates contribute to the efficiency of aphids to act as virus carriers (Fereres and Moreno, 2009). They are capable to produce almost explosive population growths thanks to their parthenogenetic system of reproduction, with adults giving birth daily to many individual nymphs (Figure 2).

Figure 2. **Aphid (*Myzus persicae*)** feeding on a tobacco plant. An adult is shown. Photograph taken by M. Urizarna using a Olympus DP71

Aphids can transmit viruses in all the previously described modes of transmission. In particular, only aphids are capable of transmitting nonpersistent viruses. This might be related with their feeding behaviour, involving several short intracellular probes at the beginning of every feeding process, thought to be essential for acceptance of the plant as an adequate food source. These short probes are likely to involve sucking up cellular contents to be tasted in more internal chemorreceptors,

and acquisition of nonpersistent viruses is known to occur exactly during these probes. Furthermore, to facilitate penetration of the stylets, salivation is required, providing opportunities for inoculation of viruses previously acquired in other probes. The electrical penetration monitoring system (Tjallingii, 1990) has served to provide conclusive experimental evidence for these processes (Martin *et al.*, 1997). Regarding retention sites for nonpersistent viruses, recent works with caulimoviruses have shown that the specific auxiliar factors required for transmission are retained near the distal part of the stylet, where the food canal and the salivary canal merge into a common duct (Uzest *et al.*, 2007). In this site, a particular anatomical structure denominated acrostyle has been also recently described (Uzest *et al.*, 2010).

Other important elements for virus transmission are the barriers that circulative viruses are forced to cross inside the aphids (Gildow, 1993). These include the cells lining the gut, either midgut or hindgut, from where viruses are internalized. Once in the haemolymph, association with chaperon-like proteins of endosymbiont origin has been described (van den Heuvel *et al.*, 1994; Filichkin *et al.*, 1997), although it is not clear if this association is essential or not for the process (Bouvaine *et al.*, 2011). The entry into the salivary glands, normally occurs after the specific association of virions with the basal lamina, which constitutes another barrier for circulation (Gildow and Gray, 1993).

Another important aspect in the relationship of aphids with viruses and host plants is the possibility that the presence of the virus might increase the attractiveness of the plant for aphid vectors. Indeed, symptoms like yellowings, quite frequently caused by viruses, could make the infected plant easier to be spotted by aphids. Other changes associated with virus infections, like release of volatile compounds, might as well serve to attract vectors. Moreover, these changes could also affect aphid feeding behaviour to enhance the ability of acquisition and inoculation (Mauck *et al.*, 2010).

2.2. - WHITEFLIES

Whiteflies can be considered the second most important type of vector after aphids, due to their capacity to transmit many plant viruses, notably a large number of species in the genus *Begomovirus*, which currently comprises around 200 members. Other viruses transmitted by whiteflies include for instance ipomoviruses of the family *Potyviridae*, and criniviruses in the family *Closteroviridae* (Valverde *et al.*, 2004). Whiteflies are causing several important problems as a direct pest of many crops (Figure 3), although the major concern when they are present is usually associated

with their role as virus vectors, especially in the case of emerging virus diseases (Navas-Castillo *et al.*, 2011).

Whiteflies have piercing-sucking mouthparts similar to those of aphids, and also they feed by inserting their stylets in the plant to reach the phloem. However, some differences in the feeding behaviour exist, notably in the frequency and duration of short probes, which might serve to explain why most whitefly-transmitted viruses are classified as semipersistently transmitted.

Figure 3. **Whitefly (Bemisia tabaci)** feeding on a tobacco plant. Photograph taken by M. Urizarna using a Olympus DP71.

2.3. - LEAFHOPPERS

The first plant virus shown to be insect-transmitted was Rice dwarf virus, a phytoreovirus that was transmitted by a leafhopper (Fukushi, 1934). The phytoreovirus are circulative and propagative viruses, capable also of being transovarially transmitted to their descendants by prolonged periods of time without loosing their capacity to infect plants (Honda *et al.*, 2007). Leafhopper vectors, in general, show a considerable degree of specificity for transmission of particular viruses. Leafhoppers are the vectors that transmit the largest number of propagative viruses of any vector group, in addition to the capacity to transmit also semipersistent viruses.

Differently to other vectors, leafhoppers can feed actively in the xylem and in the mesophyll, as well as in the phloem (Wayadande, 1990). Actually their mouthparts, due to their larger size compared with those of other homopterans, could produce more damage on plants during feeding.

2.4. - OTHER INSECT VECTORS

As shown in Table I, other insects are vectors of certain plant viruses. It is worth mentioning here the peculiar relationship of thrips and tospoviruses, with virus acquisition occurring during larval stages, while inoculation is restricted to adults (Whitfield *et al.*, 2005). Modification of vector behaviour by virus presence has been recently described in this system (Stafford *et al.*, 2011). Also the transmission of viruses by species of phytophagous beetles shows peculiarities derived from their chewing mechanism of feeding (Walters, 1969; Mello *et al.*, 2010)

3. CONTROL OF INSECT VECTORS FOR CONTROLLING VIRUS DISEASES

Once the importance of vector organisms in the dispersal of plant viruses was fully recognized, a logical follow up was the idea of controlling plant viruses through actions against their vectors. However, the effectiveness of the use of insecticides was variable, and depends on the mode of transmission (Perring *et al.*, 1999). Most recently, a demand for safer and more societally acceptable systems of pest and pathogen control, mainly based on Integrated Pest Management (IPM) concepts (Birch *et al.*, 2011) is occurring, stimulated by serious environmental concerns raised by the massive use of pesticides in Agriculture, and the increasingly tightness in regulations.

An important component of any IPM programme is the use of host plant resistances, which can target the vector (Smith, 2005), the virus (Maule *et al.*, 2007) or both. When analysing resistance traits, it is critical to consider that the different terms used by plant breeders might have different meanings. For instance, a cultivar claimed to be resistant to a pest or a pathogen could possess only tolerance, meaning that the cultivar exhibits less damage than another one, despite the fact that the pest or the pathogen are present at similar levels in both cases. In the case of pests, the term resistance should be reserved for antixenosis or antibiosis. Antixenosis occurs when there is non-preference for the resistant plant compared with a susceptible one. When the life-history parameters (survival, development, fecundity) of the insect are affected, the term antibiosis can be applied. Obviously, both tolerances and true resistances are highly desirable in IPM programmes, in terms of ecology, economy and environmental protection, but for virus control a simple tolerance against the vector might be totally useless because the transmission of the virus might be unaffected.

Antibiosis and antixenosis reactions can derive from the enhancement in resistant plants of natural defensive traits against the damage caused by herbivores. For instance, there is a wide range of direct physical and/or chemical defenses that plants might set in place to force out the insect feeding on them. Direct defenses include thorns, trichomes or incorporation of silica, and also accumulation of components like toxins or inhibitors of digestive enzymes. Furthermore, plants can produce indirect defenses, like chemicals to attract other animals that can be predators/parasites of the herbivores.

Focusing on resistance genes specific for particular pests or pathogens, our current knowledge has improved recently thanks to the use of the powerful molecular biology tools (Kaloshian and Walling, 2005; Westwood and Stevens, 2010). In the case of vector transmission of viral diseases, two genes have been identified in plants, the *Mi* and *Vat* resistance genes, which correspond to the CC-NBS-LRR (coiled coil–nucleotide binding site–leucine rich repeat) subfamily of NBS-LRR resistance proteins. The *Mi1.2* gene of tomato is known to confer resistance against root-knot nematodes (Milligan *et al.*, 1998), to the potato aphid (Martinez de Ilarduya *et al.*, 2003), and to some biotypes of *Bemisia tabaci* (Nombela *et al.*, 2003). Resistance occurs through activation of a programmed cell death response, following the interaction between *Mi1.2* and elicitors from the pathogen side. Even more interesting is the *Vat* gene identified in melon, which confers resistance to the transmission process of viruses by *Aphis gossypii* (Silberstein *et al.*, 2003; Boissot *et al.*, 2010). It has been observed that after aphid landing on melon varieties carrying the *Vat* gene, there is an activation of typical plant responses such phenol synthesis, callose deposition in the cell wall, and an oxidative burst (Villada *et al.*, 2009). However, and despite these two most interesting examples, the availability of other natural genes putatively targeting vectors in other plants species is not clear, and the process to identify new sources of resistance traits could be a limiting factor in the use of this technology. As an alternative, genetically engineering methods can speed up the introgresion of resistance traits (Collinge *et al.*, 2010), although there are important issues that should be considered, like biosafety, societal opposition and regulations.

One important aspect to consider when managing both natural and genetically engineered resistances is the possibility that the targeted organism could evolve to break the resistance. There are many examples of this, like the case of Bt crops expressing insecticidal crystalline proteins derived from *Bacillus thuringensis*, with insects evolving resistance against the toxins (Tabashnik *et al.*, 2003). This is not a surprise, since adaptations in herbivores/pathogens and their host plants are potent driving forces in their respective co-evolution. Therefore, an intelligent use of this technology is required in order to assure its sustainability.

3.1. - INTERFERENCE WITH TRANSMISSION

Research in the fields of virus-plant, virus-vector and plant-vector interactions could eventually lead to design novel strategies aiming to interfere with virus transmission. Filling the gaps in our current knowledge might serve to gain a better understanding of the transmission mechanisms, and to identify key steps and/or specific elements essential for the process, which could turn out to be new targets for strategies aiming to block virus dissemination. In this section, we focus on recent work devoted to search specific virus receptors, and on available systems to interfere with gene expression in insects.

3.1.1. - Identification of virus receptors

A good example of research that might be used to interfere with the transmission process is the characterization of vector elements acting as receptors for viruses. These receptors are likely implicated in transmission through conferring specificity to the virus-vector interaction.

In the case of circulative viruses, it was soon recognized that vector specificity and tissue tropism might respond to the presence of some specific receptors or agents capable to interact with viral proteins. The glycoproteins present in the surface of *Tomato spotted wilt virus* (TSWV) particles (German *et al.*, 1992; Bandla *et al.*, 1998); were found to interact with a 50 KDa protein identified in the thrips vector *Frankliniella occidentalis*, considered to be a candidate receptor essential for TSWV entry (Medeiros *et al.*, 2000). Another example can be found in luteoviruses and poleroviruses, which can be specifically transmitted in a circulative manner by a limited number of aphid species. The involvement in transmission of a readthrough minor form of the capsid protein (CP) present in virus particles has been demonstrated (Brault *et al.*, 1995). Several approaches have been used to identify putative receptors implicated in luteovirus transmission using aphid extracts (Miles, 1972). Concretely, Barley yellow dwarf virus particles were used as a bait to identify aphid factors involved in virus recognition (Li *et al.*, 2001). Another protein proposed to be an important interactor for transmission of these viruses was GroEL, a chaperonin encoded by the *Buchnera* endosymbiotic bacteria of aphids (van den Heuvel *et al.*, 1994; Filichkin *et al.*, 1997). Mutants of *Beet western yellows virus* allowed mapping the virion-GroEL binding to the readthrough domain of the CP, and it was suggested that this interaction might serve to

protect virions during their circulation inside the aphid (van den Heuvel *et al.*, 1997). However, recent results question the availability of GroEL in the haemolymph, indicating that perhaps the *in vitro* observed interaction could not be essential for the transmission process (Bouvaine *et al.*, 2011).

Considering noncirculative plant viruses, an interesting case can be found in viruses of the genus *Potyvirus* (family *Potyviridae*). Because we will be using a virus belonging to this genus for the experimental part of this work, details of the transmission process will be provided in another section.

3.1.2.- RNA interference (RNAi) in insects

The recent discovery of the postrancriptional gene silencing phenomenon in all eukaryotic organisms, including insects, has lead to a large number of potential applications, ranging from its direct use in strategies for pest management (Huvenne and Smagghe, 2010) to functional studies in insects (Belles, 2010). The possibility of rapidly generating loss of function phenotypes using RNA interference (RNAi) in vectors could be a system to validate elements presumably essential for the process of virus transmission. In short, upon introduction in the insect of a given amount of the triggering element, like for instance a double stranded RNA (dsRNA) to be processed into small interfering RNAs (siRNAs), the endogenous silencing machinery will be activated, and the incorporation of siRNAs into the RNA-induced silencing complex (RISC) will serve to target specific sequences, based on complementarity with the triggering element. In this way, specific silencing of certain genes could be achieved.

Despite the tremendous potential of this technology, it was soon recognized that not all species were equally susceptible, and these variations were specially clear in the case of insects, with some species being more sensitive to systemic RNAi than others. These differences are likely due to still unknown particularities during the amplification and spreading of the RNAi signals. In practical terms, empirical approaches have to be adopted for each species, finding for instance the best system to deliver the triggering molecules, the most effective doses for the expected response, and the timeframe of the effect. Among the delivery routes assayed we can mention microinjection of *in vitro* synthesized dsRNA into the insect haemocel, which can provide transient knockdown of a given target gene (Dzitoyeva *et al.*, 2001). In some cases it was also demonstrated a systemic effect throughout the insect body, showing knockdown of the targeted genes in different tissues. Interestingly, the microinjection system was very useful for the study of circulative virus, delivering viral particles into

the body cavity (Tamborindeguy *et al.*, 2008). However, not all insects can survive the wound caused during the injection, and it is difficult to quantify the volume injected.

Another system to deliver the triggering element to a given organism is feeding on an artificial diet supplemented with *in vitro* generated dsRNAs. This system was demonstrated to work with the nematode *Caenorhabditis elegans* (Timmons *et al.*, 2001). A system for feeding aphids and other piercing-sucking insects through artificial membranes (Pirone, 1964) can be used for these experiments. Providing dsRNA in artificial diet confers silencing on targeted genes in different insects. In some cases oral delivery has failed to produce the expected effect, for instance in *Spodoptera litura* (Rajagopal *et al.*, 2002), suggesting that this system could be not suitable for all insect species.

In the case of phytophagous insects, oral delivery can be easily combined with the use of transgenic plants expressing siRNAs. This system can be denominated vegetal diet, and it has the potential to target insect genes specifically without causing alterations in the plant. The method has been tested in lepidopteran, coleopteran and hemipteran species (Zha *et al.*, 2011). Interestingly, a recent work shows that aphid genes are susceptible to be targeted in this way (Pitino *et al.*, 2011). This system has the potential to be adopted in crop protection programmes, since it can be directed against specific pests, serving to knockdown transcripts in the insects feeding on the transgenic plants. In addition to plants stably transformed, transient expression systems can be used for the same purpose. A recent study shows for instance that a plant virus-based expression vector can be used to generate in the plant the dsRNAs triggering elements (Kumar *et al.*, 2012). This is indeed an attractive switch to the idea of using RNAi to interfere with plant virus transmission: a viral expression vector, based on a plant virus, can be used to avoid spreading of another unrelated plant pathogenic virus.

A diagram summarizing the available methods for RNAi in insects is shown in figure 4.

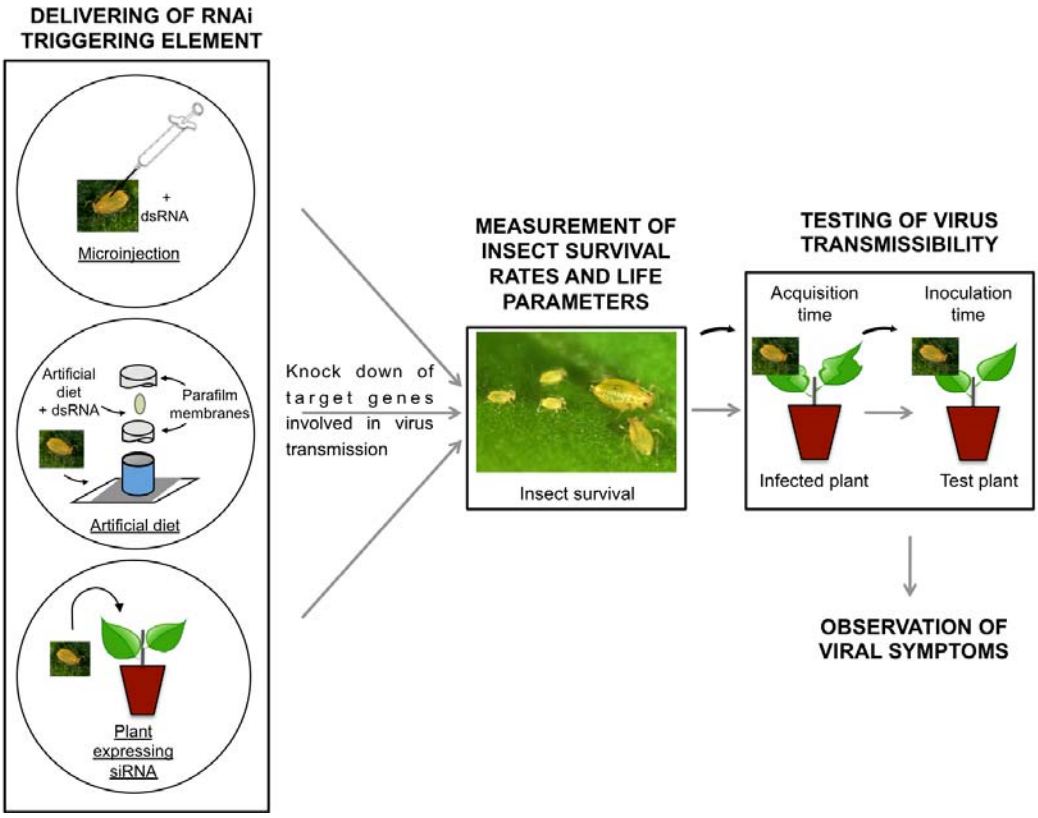

Figure 4. **Experimental procedures to characterize insect vector genes putatively involved in virus transmission.** Aphids are shown as examples of insect vectors. On the left side of the scheme, delivery systems to induce RNAi in insects are depicted: microinjection of dsRNA (above), an artificial feeding device with diet supplemented with dsRNA (center), or direct feeding on plant tissues expressing siRNAs (below). In this third case, the siRNAs can derive from stable transformation of plants, or from transient expression mediated by agroinfiltration, or from infection with a plant virus-based expression vector. In the central part of the scheme, the effect of these treatments on the life parameters of the insect needs to be considered. Finally, on the right side, a plant-to-plant transmission test is shown, which could be used to quantify the effect on virus transmissibility of the knocking-down treatment on specific target genes

4. PLANT POTYVIRUSES TRANSMISSION

4.1- THE POTYVIRIDAE FAMILY

The *Potyviridae* family is considered the largest group of plant viruses with RNA genome, including nearly 200 individual viral species, constituting about 30% of total plant viruses described. These viruses are collectively responsible of a large number of crop diseases, being the cause of significant worldwide economic damage, affecting cereals, legumes, vegetable crops, fruit and ornamental (López-Moya, 2009). In this section we will review the currently available knowledge about these viruses, with a concentrated interest in their transmission.

4.1.1. - General properties and taxonomic classification criteria into genera

Phylogenetically, this family is considered close to proposed supergroup "picorna-like", because it shares general aspects of genomic structure, including homologies in their gene products once processed (Le Gall *et al.*, 2008). They are viruses with single stranded positive sense RNA genomes, and their gene products are expressed through a polyprotein that is autoproteolitically processed.

Before the availability of genomic data, the criteria for determining genus and species within the family were established considering the composition and genomic structure of their genetic material (RNA), and other biological properties like pathogenicity and host range, transmission mechanisms and antigenic properties of the particles and other components. After obtaining many viral sequences, phylogeny studies are mainly based on the degree of their nucleotide and amino acid conservation (Adams, 2005).

The ICTV (ICTV, 2012) recognized in its previous report six genera within the family, although nowadays up to 8 genera are considered included in the *Potyviridae* family. On line updated taxonomical information can be obtained at http://ictvonline.org/index.asp. The current classification is based on the genomic structure and composition of potyvirids (1 or 2 RNA molecules, being 2 only in the *Bymovirus* case), the level of similarity of their sequences, and the vector involved in their transmission. In the Table II the criteria used for the classification into genera are summarized.

Table II**: Genera within the *Potyviridae* family**, with indication of the presence of one or two genomic segments, vector transmission organism, number of recognized species and an example.

Genera	Genome	Vector	Members[1]	Virus type
Bymovirus	Bipartite	Plasmodiophorids	6	Barley yellow mosaic virus
Ipomovirus	Monopartite	Whitefly	5	Sweet potato mild mottle virus
Macluravirus	Monopartite /Particles< 700 nm	Aphid	6	Maclura mosaic virus
Potyvirus	Monopartite/ Particles > 700 nm		146	Potato virus Y
Rymovirus	Monopartite	Mite	3	Ryegrass mosaic virus
Poacevirus	Monopartite		2	Triticum mosaic virus
Tritimovirus	Monopartite		4	Wheat streak mosaic virus
Brambyvirus	Monopartite	Unknown	1	Blackberry virus Y

[1.] Number of virus species officially recognized by the ICTV (2011), not including tentative ones. There are also three viral species with no genera assigned at this time.

Except for the 6 viruses in the genus *Bymovirus*, all members of this family have a genome consisting on one positive single stranded RNA molecule of 9-10 Kb of length. This genomic RNA is surrounded by approximately two thousand copies of the coat protein (CP) to form the virus particles (Shukla *et al.*, 1986). The genomic RNA molecule presents a covalently linked 5' VPg protein (Murphy *et al.*, 1990) and a poliadenine tail at the 3' end (Hari et al., 1979). The genomic expression of the RNA molecule involves the synthesis of a polyprotein that it is autoprocessed to give rise to the different mature viral products (Riechmann *et al.*, 1992).

All members of the Potyviridae family form virions characterized by a morphology with flexible and filamentous particles (Figure 5A) of about 650-950 nm length and 12-15 nm of diameter (excluding the members of the genus Bymovirus, which particles showing two sizes: 250-300 nm and 500 - 600 nm).

A very characteristic and representative trait of potyvirids is their capacity to induce the appearance of cylindrical cytoplasmic inclusions in the host cells (Figure 5B) which in cross section resembles a windmill (denominated "pinwheels") (Rubio-Huertos and Lopez-Abella, 1966; Hollings and Brunt, 1981). Some studies have also described other cytoplasmic amorphous inclusions, or crystalline inclusions in the nucleus (Rubio-Huertos, 1978; Christie and Edwardson, 1992).

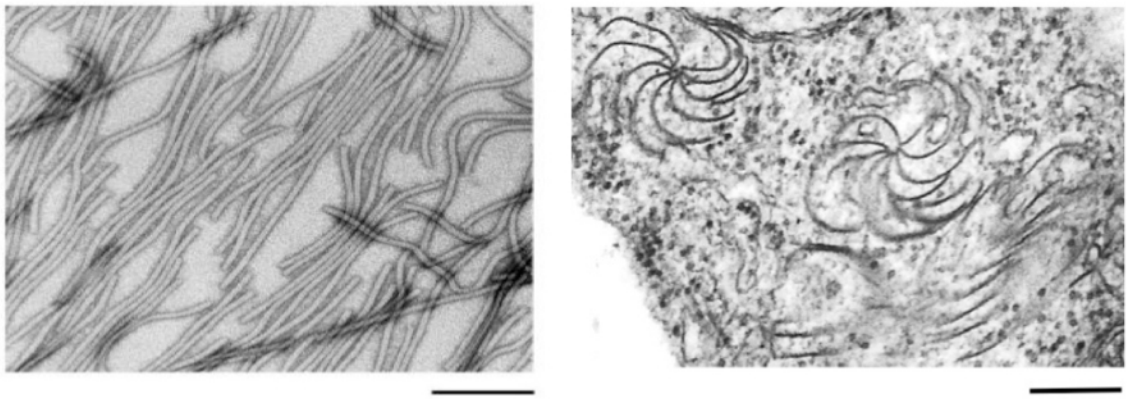

Figure 5. **Electron microscopy pictures of potyvirus and associated structures.** (A): Viral particles of *Tobacco etch virus* (TEV) by negative staining. (B) Section of a plant (Nicotiana benthamiana) tissue infected by the causal agent of the sharka disease, the potyvirus plum pox virus (PPV), showing the typical cytoplasmic “pinwheels” inclusions resulting from aggregation of the viral non-structural protein CI. Images from Dr. López Abella (CIB, CSIC, Madrid) (Bar: 200 nm).

4.2. - POTYVIRUS GENUS

As indicated above, the *Potyvirus* genus (family *Potyviridae*) is the largest group of plant RNA viruses (143 species in the ICTV Virus Taxonomy 2009) with members causing significant losses in many crops (Ward and Shukla, 1991; Hull, 2001).

4.2.1.- Genomic organization

The genetic information of potyviruses is comprised in a unique molecule of single stranded RNA, organized in one open reading frame (ORF) flanked by 5' and 3' noncoding regions.. This RNA molecule is around 10 Kb in size, and it encodes a polyprotein of approximately 350 kDa, that is cleaved by 3 different proteases (all encoded by the virus itself) into different mature proteins (Riechmann *et al.*, 1992)(Figure 6).

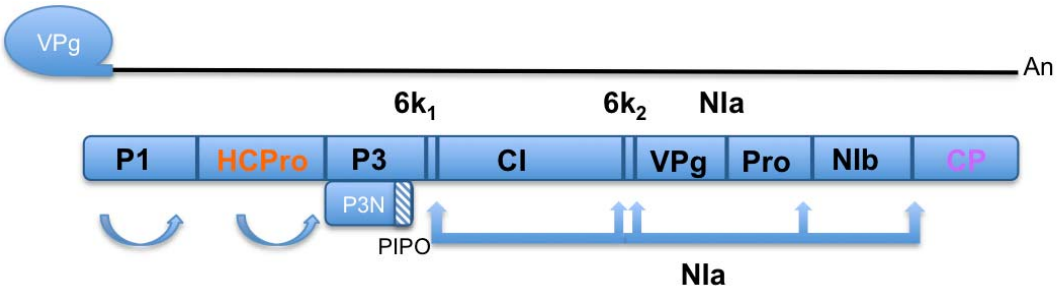

Figure 6. **Genome organization of viruses belonging to the genera Potyvirus in the family Potyviridae.** A single molecule of single stranded RNA positive sense is translated to generate by autoprocessing, mature viral proteins. Three gene products possess protease activity: P1 and HCPro, which are autoprocessed by cleavage at their C-terminus, and NIa, which generates all other viral products by cis and trans cleavages. Proteins known to be involved in vector transmission are highlighted (HCPro and CP).

The untranslated (UTR) 5' region could play an important role in viral processes as encapsidation, translation or genomic replication (Carrington *et al.*, 1990; Wu and Shaw, 1998). Although apparently there is no conserved secondary structure, this region presents a domain of conserved sequence among different potyviruses (Lain *et al.*, 1989a; Turpen, 1989; Simon-Buela *et al.*, 2000; Lain *et al.*, 1989b). In addition, the Tobacco etch virus (TEV) 5 'UTR has been proposed to contain a putative element similar to an Internal Ribosome Entry Site (IRES), with an RNA structural configuration type "Pseudoknot" that might regulate the process of translation independent of CAP (Zeenko and Gallie, 2005).

The 3 'UTR region presents heterogeneity among potyvirus in terms of size, sequence and secondary structure (Lain *et al.*, 1989b; Lain *et al.*, 1989a; Turpen, 1989; Quemada *et al.*, 1990a; Quemada *et al.*, 1990b). As functions it thas been proposed to interact with the viral replicase during the initiation of negative-sense RNA chain synthesis, and to provide protection against exonucleases degradation (Bryan *et al.*, 1992; Dolja *et al.*, 1992). Mutations in the 3 'UTR have shown also the involvement of this region in symptoms expression in plants infected with *Tobbaco vein mottling virus* (TVMV) (Rodriguez-Cerezo *et al.*, 1991), and in TEV genomic replication (Haldeman-Cahill *et al.*, 1998).

4.2.2- Genomic expression and processing of the viral polyprotein

The RNA genome of potyviruses contains one long open reading frame (ORF) expressed as a 350kDa polyprotein precursor (Riechmann *et al.*, 1992; Riechmann *et al.*, 1995), with a minor ORF embedded translatable through a +1 frameshift (Chung *et al.*, 2008). The long precursor is proteolytically processed by viral proteases (Langenberg and Zhang, 1997) into at least ten proteins named as P1 proteinase, helper component proteinase (HCPro), P3, cylindrical inclusion (CI), nuclear inclusion a (NIa), nuclear inclusion b (NIb), capsid protein (CP), as well as two small putative proteins known as 6K1 and 6K2, located flanking the CI region (Riechmann *et al.*, 1992). Furthermore, the NIa protein can be processed into the VPg and the proteinase domain. Finally, a frameshift at the P3 region produces a short polypeptide (PIPO) out of the frame of the polyprotein, generating the product denominated P3N+PIPO (Chung *et al.*, 2008).

The following table (table III) summarizes the main features and functions of the mature protein products from viral polyprotein processing. Among these proteins, it should be noted HCPro and CP are known to be factors involved in the transmission process.

Table III. **Table listing the size and main features and functions of the products derived from the polyprotein processing of the potyvirus.**

Viral protein	Weight (KDa)	Inclusions	Functions associated
P1	25-84		- Serine protease S30 (Verchot and Carrington, 1995b) - Pathogenicity and viral multiplication (Verchot and Carrington, 1995a) - Enhancer of PTGS suppression (Kasschau and Carrington, 1998; Carrington *et al.*, 1989a) - Host range determination? (Carrington *et al.*, 1989a; Valli *et al.*, 2007), - PTGS suppressor?
HCPro	56	Amorphous	- C6 cysteine protease (Carrington *et al.*, 1989b) - Transmission (Govier and Kassanis, 1974a) - Movement (Cronin *et al.*, 1995; Rojas *et al.*, 1997) - PTGS suppressor (Anandalakshmi *et al.*, 1998; Brigneti *et al.*, 1998; Kasschau and Carrington, 1998) - Pathogenicity and synergism (Pruss *et al.*, 1997) - Host determinant (Saenz *et al.*, 2002)
P3	40		- Pathogenicity? (Riechmann *et al.*, 1995; Saenz *et al.*, 2000; Salvador *et al.*, 2008)
P3N+ PIPO	25		- Pathogenicity? Movement (Wei *et al.*, 2010)
6k1	6		- Pathogenicity?(Merits *et al.*, 2002; Waltermann and Maiss, 2006)
CI	70	Pinwheels	- Helicase (Lain *et al.*, 1990; Lain *et al.*, 1991) - ATPase function and replication (Eagles *et al.*, 1994) - Movement (Carrington *et al.*, 1998; Gomez de Cedron *et al.*, 2006)
6k2	6		- Membranes cleavage? (Schaad *et al.*, 1997a) - Viral amplification (Merits *et al.*, 2002)
VPg	21	Nuclear	- Covalent binding to viral RNA (Oruetxebarria and Valkonen, 2001) - Primer in RNA synthesis during replication (Schaad *et al.*, 2000) - Host determination (Schaad *et al.*, 1997b) - Often appears fused to NIa because of incomplete processing of both products (Dougherty and Parks, 1991) - Pathogenicity (Charron *et al.*, 2008) - Nuclear inclusions with NIb
NIa-Pro	28	Nuclear	- C4 cysteine protease (Dougherty and Parks, 1991; Hellmann *et al.*, 1988) - DNAse activity?(Anindya and Savithri, 2004)
NIb	58	Nuclear	- RNA-dependent RNA polymerase (Hong and Hunt, 1996) - Nuclear inclusions
CP	30-40	Particles	- Viral particles assembly (Voloudakis *et al.*, 2004) - Transmission and infectivity (Blanc, 1997; Lopez-Moya *et al.*, 1999) - Movement (Dolja *et al.*, 1992; Rojas *et al.*, 1997) - Viral amplification (Mahajan *et al.*, 1996) - NTPase activity (Rakitina *et al.*, 2005)

4.2.3.- Viral factors involved in viral transmission by aphids

As mentioned above there are two proteins known to be involved in this process: CP, coat protein and the transmission factor, helper component proteinase HCPro. The members of this genus are aphid-transmitted in a non-persistent manner with the assistance of this viral encoded transmission factor (Govier and Kassanis, 1974a).. The HCPro has been proposed to act during transmission as a reversible connection (or a bridge) between the CP and hypothetical specific receptors in the aphid mouthparts (Raccah, 2001).

4.2.3.1.- CP, the coat protein.

The coat protein of potyviruses is involved in many functions throughout the viral cycle (see table III), including the transmission process conducted by insect vectors. The size of this protein ranges approximately between 30 and 37 KDa. The central region of the protein is highly conserved among different potyviruses, being more variable the amino and carboxyl terminal regions. These ends are exposed on the protein surface, being susceptible to protease degradation (Allison *et al.*, 1985; Dougherty *et al.*, 1985). They are supposed not to be involved in virus encapsidation since they are dispensable for the process (Shukla *et al.*, 1986; Voloudakis *et al.*, 2004)

The CP protein presents on its amino terminal a domain highly conserved among aphid transmissible isolates (Harrison and Robinson, 1988). It consists on three residues alanine-glycine-aspartic acid (DAG). Mutagenesis analysis has shown that alteration or elimination of the aminoacids in this domain might determine total or partial loss of transmissibility of the virus (Atreya *et al.*, 1990; Atreya *et al.*, 1991; Atreya *et al.*, 1995; Gal-On *et al.*, 1992).

Despite its conservation, the DAG domain is not present in all naturally occurring isolates of potyviruses, and other similar domains appear to be functionals for different potyviruses such as a DAS domain in the *Pea seed-borne mosaic virus* (PSbMV) (Johansen *et al.*, 1996) or a DAA domain in *Pepper mottle virus* (PeMV) (Flasinski and Cassidy, 1998). Surprisingly, mutations in the DAG domain of TVMV generating DAA or DAS mutants, produced non transmissible variants (Lopez-Moya *et al.*, 1999). This study also shows that mutations in residues close to DAG domain of TEV have an effect on the viral transmission as already observed in some positions next to DAG domain in TVMV (Atreya *et al.*, 1991; Atreya *et al.*, 1995). These results suggested that the molecular context of the DAG domain, and possibly the topology of

the region, might be essential for the transmission process. However there are no three-dimensional structure data supporting this hypothesis.

The CP interacts with the HCPro in the transmission process. In this interaction the amino terminal region of the CP and the PTK domain of HCPro (see next section) are involved (Peng *et al.*, 1998; Wang *et al.*, 1996). The loss of this interaction in mutated variants of the CP (with alterations in the DAG domain) correlates with the loss of transmission (Blanc, 1997).

4.2.3.2.- HCPro, the helper component proteinase.

The other viral protein involved in the transmission process is the HCPro proteinase. This protein is needed during transmission, and it provides specificity between virus and aphids (Dombrovsky *et al.*, 2005; Wang and Pirone, 1996). The involvement of a helper protein in transmission as a auxiliary factor different from virus particles was demonstrated for the first time in 1974 by Kassanis and Govier (Govier and Kassanis, 1974a) in experiments with *potato potyvirus C* (now considered an isolate of PVY) and *Potato aucuba mosaic virus* (PAMV), a potexvirus which requires the presence of a potyvirus for its transmission. These two viruses were transmitted only from mixed infections with the *potyvirus potato Y* (PVY). With these experiments, the authors demonstrated that a factor from plants infected with PVY was required for transmission (Govier and Kassanis, 1974b). This was the origin of the concept of "helper component" or HC (today called HCPro, since it was later shown to be a proteinase). The HC factor was purified later and shown to be a protein of viral origin (Thornbury and Pirone, 1983).

The monomeric form of HCPro has an approximate size between 53 to 58 KDa. However, in the transmission process, the active form was found at higher molecular weight, which serves as basis for the hypothesis that the transmission is mediated by at least a dimeric form of the factor (Thornbury *et al.*, 1985; Wang and Pirone, 1999). Later, this hypothesis was confirmed using yeast two-hybrid system to verify the ability of HCPro to form dimers (Guo *et al.*, 1999; Guo *et al.*, 2001; Kang *et al.*, 2004; Urcuqui-Inchima *et al.*, 1999a; Urcuqui-Inchima *et al.*, 1999b). Furthermore, structural proteomic studies confirmed this hypothesis (Plisson *et al.*, 2003; Ruiz-Ferrer *et al.*, 2005), although remains unknown whether the active form in transmission are dimers or higher order-two oligomers like tetramers or hexamers.

HCPro is a multifunctional protein being involved in different processes, in addition to the viral transmission. Among these processes, it is important to mention its

role as a suppressor of post transcriptional gene silencing PTGS. HCPro acts suppressing the silencing phenomenon triggered by the plant as a defense mechanism. This is an important pathway acting as a defense mechanism against various agents like transposons and viruses, detected as foreign nucleic acids by the plant. Shortly, the silencing response is triggered by a double stranded RNA (dsRNA) element which is not detected as endogenous, and it is processed by the first enzyme acting in the mechanism, known as Dicer, into small double stranded RNA denominated small interfering siRNAs. Then, one of the chains of the ds siRNAs will be degraded while the complementary will be incorporated to the RISC complex (which contains an Argonaute AGO protein as the active element), thus guiding the complementary messenger RNA degradation in a specific manner. There are suppressors capable of inhibiting this route at different steps of the silencing pathway such HCPro, which acts sequestering siRNAs (Merai *et al.*, 2006; Lakatos *et al.*, 2006). The HCPro of potyviruses was the first RNA silencing suppressor of viral origin to be described (Anandalakshmi *et al.*, 1998; Brigneti *et al.*, 1998; Kasschau and Carrington, 1998).

Although the crystal structure of HCPro is still unknown, numerous studies have determined the domains and residues involved in various proposed functions (Gonzalez-Jara *et al.*, 2005; Kasschau *et al.*, 1997; Oh and Carrington, 1989). Since this work is focused on the function of this protein in the transmission process, we will be described in detail only the domains involved in this function.

The genomic data available of natural transmissible and non- transmissible isolates provide the first insights on putative functional domains and conserved residues presumably involved in the transmission process (Lecoq *et al.*, 1991; Legavre *et al.*, 1996; Thornbury *et al.*, 1990; Canto *et al.*, 1995; Llave *et al.*, 1999). Later, the availability of complete infectious virus clones allowed the mutational analysis which further served to determine domains and conserved residues involved in the process.

HCPro has a highly conserved domain in the N- terminal region, known as KITC, (Lys-Ile-Thr-Cys). Genomic studies, later confirmed using mutagenesis analysis, showed that this domain is involved in the transmission process. Changes in the first residue (lysine), a positively charged amino acid, caused loss of transmission (Atreya *et al.*, 1992; Atreya and Pirone, 1993; Blanc *et al.*, 1998; Huet *et al.*, 1994). Furthermore, there is a cysteine-rich region just before the KITC domain, where mutations in the conserved cysteines also caused loss of TEV transmissibility (Llave *et al.*, 2002).

Another conserved domain implicated in potyvirus transmission is located at the end of the central portion of the HCPro, and it is constituted by the PTK tripeptide (Pro-Thr-Lys). Alignments between transmissible and non-transmissible isolates, confirmed later by mutagenesis analysis, have verified that this domain plays a part in the transmission process. The change of threonine to alanine found in non-transmissible isolates of Zucchini yellow mosaic virus (ZYMV) (Granier *et al.*, 1993), when introduced into transmissible isolates, results in a drastic reduction of the transmission rate (Huet et al., 1994). Other changes in the first position of PTK domain, also affects ZYMV transmission (Peng *et al.*, 1998).

4.3. - BRIDGE HYPOTHESIS FOR NON PERSISTENT TRANSMISSION

Several hypotheses have been proposed to explain the process whereby potyviruses are transmitted by aphids (reviewed in (Raccah, 2001)) being the hypothesis known as "bridge hypothesis" the most widely accepted given the experimental evidence provided accumulatively in different experimental models. This hypothesis was postulated in 1974 by Govier and Kassanis (Govier and Kassanis, 1974a), and suggested a specific dual interaction of the HCPro with aphid mouthparts on one side, and with the viral particle on the other side, thus acting as a bridge between the two structures (Figure 7). This interaction, being reversible, allows subsequent release of the viral particle during the inoculation process. It has been proposed that the amino terminal region of the HCPro (KITC domain) could recognize a possible receptor present in the stylet (Blanc *et al.*, 1998; Llave *et al.*, 2002). Moreover the PTK domain appears to interact with the DAG motif of the CP.

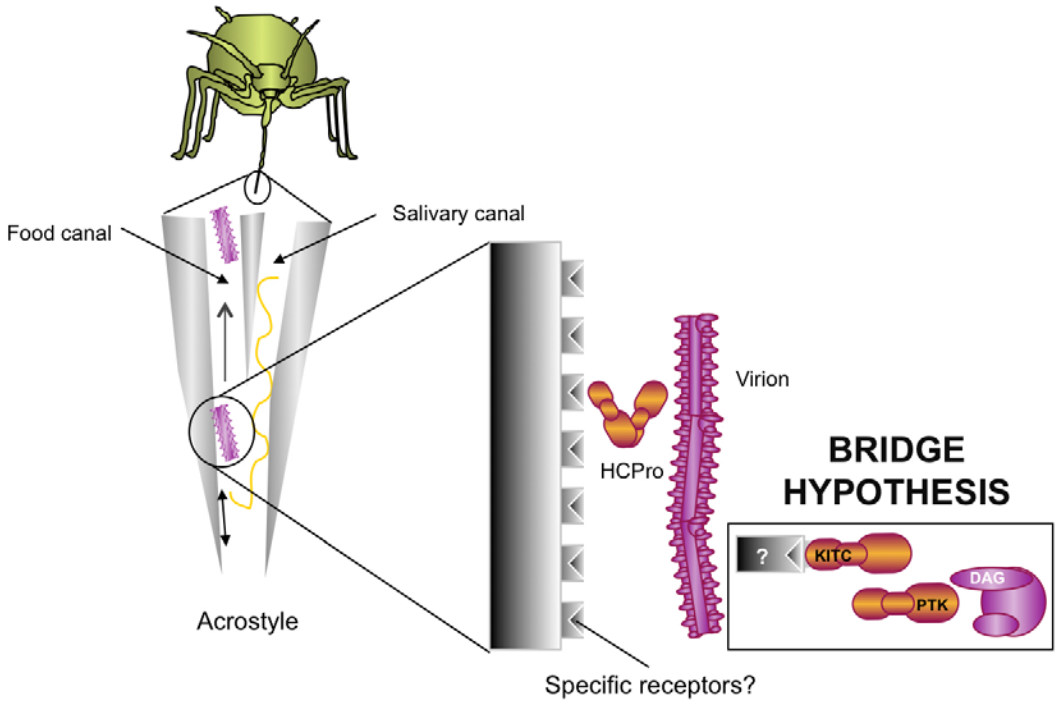

Figure 7. **Bridge hypothesis** suggested for non-persistent transmission. This hypothesis proposes that the HCPro might form a "bridge" between the virus particles and the aphid mouthparts, where the virions are likely to be retained and subsequently inoculated to plants. Two conserved motifs in which modifications were associated with loss of transmission activity might be essential: the KITC motif, located in the N-terminal region of the protein and presumed to be critical for virus retention in the stylets of the aphid vector; and the second motif, PTK, located in the C-terminal half of HCPro, probably implicated in binding to the coat protein of virions. In addition, a HC-Pro binding domain was identified at the N-terminal region of the viral coat protein, overlapping with a highly conserved DAG motif that is also essential for the transmission process

The first direct evidence that supported this hypothesis was the accumulation of radiolabeled viral particles in the distal tip of aphid stylet only in the presence of HCPro protein (Berger and Pirone, 1986). Similarly, the colocalization of HCPro with viral particles in the aphid food canal (Ammar *et al.*, 1994) reinforced the hypothesis. The lack of interaction between the viral particle and the HCPro correlated with loss of transmissibility (Blanc, 1997; Blanc *et al.*, 1998; Lopez-Moya *et al.*, 1999; Peng *et al.*, 1998), suggesting that these interactions were indeed essential for the transmission process.

4.4.- TOBACCO ETCH VIRUS, A POTYVIRUS MODEL FOR TRANSMISSION STUDIES

Tobacco etch virus (TEV) is an important pathogen of plants belonging to the *Solanaceae* family. It is well known that this virus infects many agriculturally important crops that include several species of pepper (i.e. *Capsicum annuum*, *C. frutescens*), tomato (*Solanum lycopersicum*) and tobacco (*Nicotiana tabacum*). The classical symptom of TEV infection is a severe vein clearing on expanding leaves. This can be followed by necrosis of the tissues along the veins, resulting in an etched pattern. Subsequently, young leaves develop a classical mosaic design. Plant growth is retarded, resulting in stunting, especially when young plants are infected. TEV appears to be a virus that evolved in the New World, although it is present nowadays in many other regions. It has been reported from Canada, the USA (including Hawaii), Mexico and Puerto Rico in North America and from Venezuela in South America. In Europe and Asia it is also present in many countries, including Spain.

A complete infectious clone of TEV has been available for over 20 years, since a first version under the control of the bacteriophage SP6 promoter region was produced in the laboratory of W. Dougherty (Dolja *et al.*, 1992). Different versions of infectious TEV clones have been produced since then, and in particular the TEV-HCH10 has resulted very useful for studying aphid transmission. This clone has an histidine tail before HCPro, offering the possibility of purify easily an active form of HCPro from infected plants (Blanc *et al.*, 1999). This modification makes this virus an ideal model for the study of potyvirus transmission (Llave *et al.*, 2002; Ruiz-Ferrer *et al.*, 2005; Ruiz-Ferrer *et al.*, 2004). Tools developped to take advantage of the potential of TEV as expression vector have been also described (Dolja *et al.*, 1993), including a recent report of visually-traceable variant of the virus (Bedoya *et al.*, 2012)

4.5.- THE GREEN PEACH APHID, *Myzus persicae*, AS VECTOR OF TEV.

As it has mentioned above, aphids are considered the main insect vectors of viral diseases. Specifically, in this work we have been studying the transmission of TEV mediated by the aphid *Myzus persicae,* commonly named the green peach aphid.

Since it first scientific description in Europe in 1776, the broad geographical distribution and the wide host range of *M. persicae* has driven considerable attention to this insect, considered an important pest all over the world. This aphid has a very

complex life cycle having been found on more than 800 plant species as potential hosts. The green peach aphid is particularly important as a pest on legumes, cucurbits, crucifers, and solanaceous crops like potato, tomato, and tobacco, but also attacks a broad range of other crops such as peach, plum, apricot, cherry, citrus species, strawberry, and a large variety of ornamentals. Among vegetables, the green peach aphid is also frequently found on peppers and cole crops.

Green peach aphids are small, usually less than 2 mm long. The body color varies from pink to green, and the head supports long antennae and red eyes. As in other aphid species, adults may be winged or wingless (apterous). Winged forms are usually triggered by environmental changes (e.g., decreasing photoperiod or temperature, deterioration of the host plant, or overcrowding). On the back of the fifth abdominal segment, a pair of tube-like structures called "cornicles" are present in M. persicae as on most aphid species. These structures serve to phisiological purposes, and also can secrete a defensive fluid.

Most aphids reproduce sexually and develop through gradual metamorphosis (overwintering diapause egg, nymphs and winged or wingless adults) but also through a process called 'parthenogenesis' in which the production of offspring occurs without mating. In southern temperate regions, the green peach aphids might develop complete annual cycles through parthenogenesis only. Parthenogenic adult females can produce three to six fully formed young per day for several weeks, and the number of generations per year can be around 30. In colder areas, the winter season is normally passed as black shiny sexually-produced eggs, usually placed on the bark of peach, plum, apricot, and cherry (overwintering hosts). Once hatched, new generations might alternate hosts to complete complex cycles.

The green peach aphid has piercing sucking mouthparts forming stylets, and feeds by inserting these mouthparts into plant tissue and sucking out the sap, as described in general in section 2.1 . The feeding process might injure plants in three ways:

- First, as direct dammage, the feeding itself interferes with the proper nutrient transfer in the plant. Plants dammaged by aphid feeding will have leaves that appear curled, distorted and discolored.
- Secretion of honey dew (caused by the high sugar content of the diet, that requires elimination from the aphid bady) might stick to the leaves, and often becomes a substrate for fungus and other pathogens.
- - The green peach aphid can transmit many plant diseases, particularly viruses. It has been implicated in the transmission of over 180 plant viruses. Potyviruses, and in particular TEV, are efficiently transmitted by *M. persicae*.

In our laboratory, we keep a colony of *M. persicae*, originally found in a field nearby Madrid by A. Fereres (ICA, CSIC, Madrid). This colony is maintained on tobacco plants, and their individuals have been extensively used for transmission of potyviruses under experimental conditions since 1995 (Lopez-Moya *et al.*, 1995) including TEV among the viruses studied (see section 4.4).

4.6.- HYPOTHETICAL VIRUS RECEPTORS IN APHID VECTORS.

Following the bridge hypothesis and the recent identification of a particular anatomical structure at the tip of the aphid mouthparts (Uzest *et al.*, 2010), research in this field is currently trying to identify the receptor(s) presumably located in or near this site in vector stylets. Previous work had shown the retention of *Tobacco etch virus* (TEV) virions on maxillary stylets in the presence of functionally active HCPro (Wang *et al.*, 1996). Because the major components of vector stylet are cuticular proteins, characterized for the presence of conserved R&R consensus domains (Dombrovsky *et al.*, 2007b), using the HCPro of *Zucchini yellow mosaic virus* (ZYMV) as a bait, a set of interactor cuticular proteins was described (Dombrovsky *et al.*, 2007a). Later, the search was expanded to other proteins, finding a ribosomal protein S2 (RPS2) with a presumed dual function in the insect cuticle. The specific binding between RPS2 and the HCPro of TEV was confirmed after cloning and heterologous expression of the corresponding aphid gene (Fernandez-Calvino *et al.*, 2010), including as control a mutant version of HCPro impaired for aphid transmission through alteration of a conserved motif likely involved in binding to aphid mouthparts (Blanc *et al.*, 1998). In these experiments, the mutated version of HCPro failed to interact specifically with RPS2, while retaining its capacity to bind the viral CP. However, the presence of RPS2 at or near the acrostyle has not yet been demonstrated, and probably other candidates must be explored before the receptor of potyvirus transmission is fully characterized. These are scientific objectives that we have addressed among others in the present work.

5. INTERFERENCE WITH GENE EXPRESSION IN VECTOR ORGANISMS AND VIRUS TRANSMISSION

Once described the available knowledge about vector transmission of plant viruses, specially considering the case of aphid-transmitted potyviruses and TEV, we might envisage strategies to prevent virus dissemination acting directly on the vector. An attractive alternative will be the interference with the expression of essential elements (specific receptors, for instance).

5.1- ANTECEDENTS OF INTERFERENCE WITH GENE EXPRESSION IN APHIDS, AND POTENTIAL APPLICATIONS

Besides the general picture provided in section 3.1.2 about RNAi in insects, recently several cases have been reported in the literature that deal specifically with RNAi procedures in aphids like *A. pisum* (Jaubert Possamai et al., 2007) and *M. persicae* (Pitino et al., 2011; Bhatia et al., 2012). These antecedents open the door to the possibility of interfering with the expression of vector factors essential for transmission of plant viruses. Considering the case of aphid-transmitted potyviruses and particularly TEV as a model, among other objectives that we have addressed in the present work, we have explored the feasibility of altering the expression of particular genes in *M. persicae*. These experiments were aiming to demonstrate that this manipulation was indeed possible in the laboratory, using for instance artificial diets for delivering the dsRNAs required to trigger the silencing response, and also in conditions closer to the natural or field ones, like feeding aphids on plant tissues infected with a VIGS vector. A similar VIGS-based strategy has been recently described in experiments with *Manduca sexta*, (Kumar et al., 2012) but, to our best knowledge, this report is the first one applying the VIGS technology to target the expression of aphid genes. Compared with feeding on transgenic plants (Bhatia et al., 2012), our system might serve to speed up procedures. The potential uses of the different experimental methodologies tested will be explored, ranging from the validation of vector factors presumibly involved in transmission, to the design of practical systems to reduce/impede virus transmission based on the specific silencing of those factors in the vector organism.