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Toward uncovering natural resistance to aphids in plants: study of proteins interacting with *Brevicoryne brassicae* and *Macrosiphum euphorbiae* effectors

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Abstract

Aphids are one of the major pests of temperate agricultural and horticultural crops, causing damage either directly by feeding or indirectly by transmitting plant viruses. The increasing interest and the immense progress made in the aphid salivary secretions research field, open the door to the use of aphid effectors to reveal the details of the intimate associations between the herbivore insects and their host plants as well as to discover candidate genes that convey tangible resistance against sap sucking pests. The goal of this work is to investigate in model plants a list of candidate genes that confer plant resistance to *B. brassicae* and *M. euphorbiae* in order to introgress the traits of interest through conventional breeding in economic important crops as *B. napus*, tomato, pepper. Two different approaches were assessed for the two aphid species taken in account. For *B. brassicae*, the effector C002, a protein secreted by aphid saliva with unknown function that showed to play an essential role in aphid-plant interaction in different species of aphids, was used as a bait protein for an in planta pull-down experiment. For the investigation of plant's targets interacting with *M. euphorbiae* effectors, it was performed a yeast two-hybrid assay between three potato aphid effectors, MeC002 and other two proteins that were showed to increase aphid's fecundity, and a library of pepper cDNA to identify possible aphid effectors-binding pepper proteins.

Sommario

Gli afidi rappresentano uno dei tipi di parassita più insidiosi in agricoltura e orticultura giacché causano sia danni diretti alle piante, succhiandone la linfa, sia indiretti agendo come vettori di virus fitopatogeni. I grandi progressi fatti nell'ambito della ricerca sulle secrezioni salivari degli afidi, aprono la strada all'uso degli effettori degli afidi negli studi finalizzati a rivelare i dettagli dell'associazione fra gli insetti fitofagi e i loro ospiti e a scoprire geni candidati che conferiscono resistenza contro i parassiti che si nutrono succhiando la linfa delle piante. Lo scopo di questo lavoro è quello di individuare in piante modello una serie di geni candidati che conferiscono resistenza a *B. brassicae* e a *M. euphorbiae* in modo da introdurre tali tratti di interesse tramite le convenzionali tecniche di breeding in colture importanti dal punto di vista economico come *B. napus*, pomodoro e peperone. Sono stati utilizzati due approcci differenti per le due specie di afidi prese in considerazione. Per *B. brassicae* è stato usato come *bait* per un *pull-down assay* in pianta, l'effettore C002, una proteina dalla funzione ignota, secreta tramite la saliva che ha dimostrato avere un ruolo essenziale nell'interazione afide-pianta in differenti specie di afidi. I possibili interattori vegetali che legano gli effettori di *M. euphorbiae*, sono stati studiati tramite il saggio del doppio ibrido in lievito: tre effettori dell'afide delle patate, MeC002 e altre due proteine che hanno dimostrato di aumentare la fecondità degli afidi, sono stati testati con una libreria di sequenze di cDNA di peperone

A Luna, Paola e Jacopo,

perché a qualunque regno essa appartenga

non c'è spettacolo più emozionante in natura

che la nascita di una nuova vita

Contents

Chapter 1

Introduction

1.1 Aphids

Aphids are a group of small insects consisting of about 4.400 species. They are members of the superfamily Aphidoidea in the homopterous division of the order Hemiptera. Aphids are small soft-bodied insects, ranging between 1.5 to 3.5 mm in length with piercing-sucking mouthparts used to feed from the phloem sap of plants. More than 250 species of Aphidoidea feed on agricultural or horticultural crop and are considered pest species [1].

Aphids are distributed worldwide, but are most common in the northern temperate regions of the world. They are diverse and have morphological structures that vary among the group [2] but there are some unique characteristics that distinguish aphid (Figure 1.1): paired siphunculi (or cornicles), prominent structures on the posterior dorsum of the abdomen through which they exude droplets of a wax secretion and pheromones; the antennae are usually five- or six-segmented with the last segment divided into a basal and distal part called terminal process; two tarsal segment; a cauda, a posterior tail-like protrusion on the tip of the abdomen; the wings with only strongly developed longitudinal vein [3].

Aphids display a diverse range of life cycles that include reproductive adaptations like having both parthenogenetic (or asexual) and sexual reproduction, production of eggs or living young nymphs and change of types of

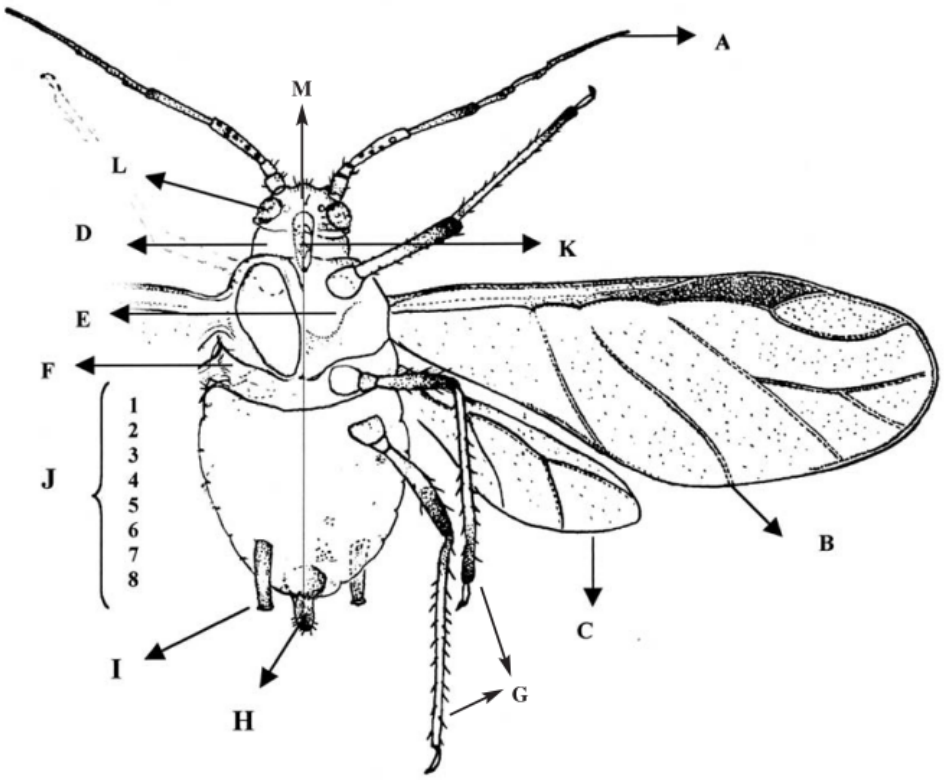


Figure 1.1: Morphological features of aphids. A antenna, B forewing, C hindwing, D prothorax, E, mesothorax, F metathorax, G legs, H cauda, I siphunculus, J abdominal segments I-VIII, K rostrum, L compound eye, M head (modified from Voegtlin et al. 2003)

host plant at different period of the year. The alternation of one bisexual generation with a succession of parthenogenesis associated with viviparity allows aphids a rapid turn-over of generations and they quickly develop in an immense population combined with genetic recombination [4]. Furthermore, each morph that characterizes a stage of the life cycle has a specific function as reproduction, dispersal and surviving in unfavorable nutritional or climatic conditions and not all morphs are able to infest crop plants. It is therefore important to know the life cycles of aphids to determine the impact an aphid can have on a crop and to establish the control measures [5].

1.1.1 Aphid feeding

Most aphid species are monophagous that means they are host specific and feed exclusively on species in a single host genus, or on species in closely related host genera. For example, *Acyrthosiphon pisum*, the pea aphid, feeds on leguminous host plants. Several aphid species are polyphagous and they have exceptionally broad host ranges, often feeding on hundreds of different species in many plant families. That is the case of many pest species such as *Myzus persicae* or *Macrosiphum euphorbiae* [1].

Aphids are phloem feeders: to reach the sieve elements they have specialized long flexible mouthparts, called stylets, composed of two outer mandibles and two inner maxillae, together forming a canal for saliva of about 0.3 μm and food canal of 0.7 μm. After landing on plants, aphids secrete on the plant surface gelling saliva before inserting the stylet within leaf tissues and assess the internal chemistry. These probes take less than one minute and are important to establish the plant rejection of aphid or, alternatively, the plant acceptance. In compatible interactions, the stylet enters the epidermis, between two cells and then follows an intercellular pathway between the primary and secondary cell wall layers [6]. During this apoplastic transit, gelling saliva is continuously excreted and envelopes the stylet as a protective sheath. On the path to the phloem, the mouthparts briefly puncture many cells, but then always withdrawn and continue along the intercellular way. These intracellular punctures help the aphid to locate the position of

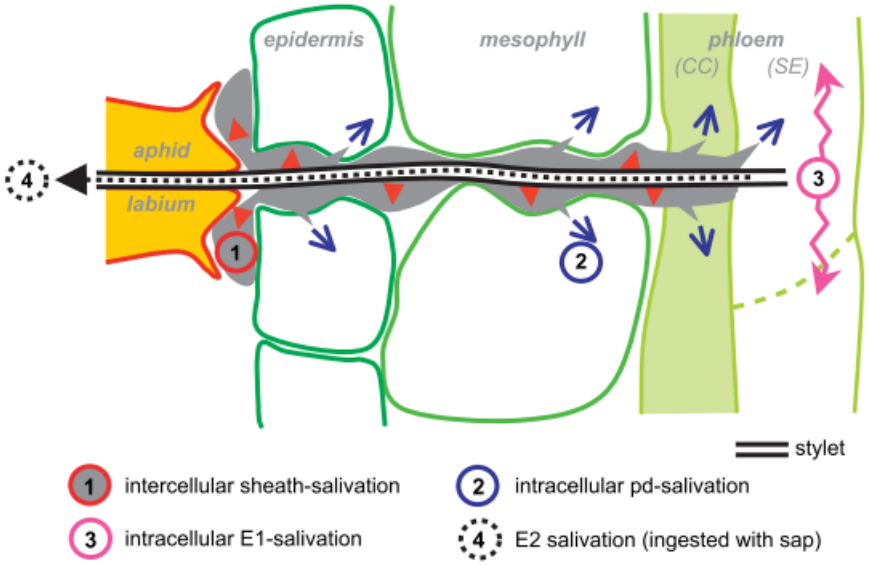


Figure 1.2: All salivation periods detected by the EPG (electrical penetration graph) technique that allows the electrical monitoring of plant penetration by aphids with piercing mouthparts and the recording of signal waveforms reflecting different insect activities. The mouthparts penetrate between two epidermic cells and mainly follow an apoplastic way; cells are punctured by the aphid's stylet that is protected by a sheath of gelling saliva (grey area)(1). Watery saliva is injected intracellular in the punctured cells (blue arrowheads)(2), in the sieve element (purple arrows)(3) and is secreted and directly ingested in a mixture with the phloem sap (dotted black arrow in the stylet)(4) (modified from Tjallingii et al. 2006).

the stylet in the leaf tissue: during the puncture a small amount of watery saliva is injected in cell's cytosol and after few seconds is ingested. In this way the aphid assesses the chemical composition and can find the sieve tubes. Once the sieve elements are reached, the watery saliva is injected in it and then a period of passive phloem sap ingestion will occur [7, 8, 9] (Figure 1.2).

Plant sap is an unbalanced diet for aphids: high amount of sugars, dominant compounds in the phloem sap, scarcity of essential amino acids, low levels of lipids, unbalance between K^+: Na^+ ions. To cope with this diet and overcome the high concentration of sugars and the associated osmotic pressure, aphids convert the simple sugars into long-chain oligosaccharides and then excrete excess honeydew [10]. Aphid guts have also developed special groups of cells containing symbiotic bacteria, which aid in synthesis of nutrients and provide essential amino acids [11].

1.1.2 Salivary glands and saliva composition

Aphid's salivary glands are paired and each half of the system is composed of the principal and the accessory gland. The salivary ducts of both glandular units on one side are joint together to form a common duct that discharges into the salivary canal [12, 13]. The principal gland is a symmetrical and bilobed organ and each lobe is composed of 6 cover cells and 15 main cells, consisting of 8 different cell types possibly secreting different components. This gland seems to be involved in excretion of the salivary sheath. The accessory gland is a group of 3-4 cells of uniform size that do not show much differentiation [8]. The function of the accessory gland is largely unknown; it plays an important role in virus transfer from the haemolymph to the salivary canal in the stylets and into plants, as seen in transmission studies of persistent/circulative plant viruses [14][15]. Since watery salivation into sieve tubes is responsible for inoculation of these viruses, it could be inferred that the watery saliva is produced by the accessory gland [16]. Anyway there is no evidence that the principal glands produce exclusively the sheath saliva and the accessory the watery saliva or that the saliva composition comes from both glands [8].

As already mentioned, during aphid feeding and stylet penetration in host plant tissues, four phases of salivary secretion can be identified [7, 8](Figure 1.2):

1. intercellular sheath-salivation;
2. intracellular watery salivation during internal cell probing by stylet;
3. salivation into the sieve elements;
4. phloem feeding salivation (ingested with sap).

Salivary secretions are the point of contact between aphid and plants and play critical roles in insect-host interactions. Proteins and enzymes in saliva allow continues feeding by aphids on the phloem, eliciting plant responses to wounds or detoxifying phytochemicals.

Aphid saliva is a mix of ions, amino acids, hemolymph and salivary enzymes secreted from principal and accessory salivary glands [17]. Gelling (sheath) saliva is primarily composed of phospholipids; conjugated carbohydrates; reducing agents; proteins especially oxidases (as phenoloxidases and peroxidases) that react with phenolic compounds released by the damaged plant tissues and convert them into less toxic substances, and pectinase and β-glucosidases that hydrolyze phenolic glycosides [18, 17]. The sheath saliva hardens on secretion to become an insoluble lining of the stylet path like a physical barrier that protects the feeding site from plant's immune response [4]. The gelling saliva seals also the puncture site in the sieve element cell wall before and during the stylet tip piercing and this presumably reduces loss of phloem sap through the wound and decreases a loss of turgor pressure as well [7, 19].

Watery saliva has a more complex composition that differs between aphid species and within the same species according to the diet [20, 21]. Numerous enzymes like pectinases, cellulases, amylases, oxidases, phenolic glycosides, glucose dehydrogenase, and enzymes that hydrolyze sucrose are present in soluble saliva [22, 23]. They have a role in establishing and maintaining feeding sites, facilitating stylet progress by degrading cell walls, inducing

changes in plant physiology to improve aphid nutrition. Recently, Will et al. (2007) identified in the vetch aphid saliva calcium-binding proteins that interact with the free calcium in the sieve elements and prevent phloem clogging [24]; Mutti et al. (2008) characterized and analyzed one of the most abundant *A. pisum* salivary protein, called C002, essential for aphid feeding on host plants [25]; De Vos et al. (2009) instead identified one or more proteins with a size of 3 and 10 kD in the saliva of green peach aphid *M. persicae* that show to induce defense response in Arabidopsis [26].

It seems obvious that the proteins of aphid saliva act as "effectors", term introduced to designate proteins and small molecules secreted by plant pathogens/pests for the purpose of altering host-cell structure and function and establishing "colonization" of the plant by the pathogen [27]. The alterations due to effectors may trigger defense responses of the plants or promote infection. The aphid effectors are expressed in the salivary glands and secreted into saliva; aphid saliva, being excreted both extra and intracellular into plant cells, the phloem and the apoplast, delivers the effectors into different host cell compartments that modulate host plant processes [28] (Figure 1.3).

Despite recent advances in peptide and cDNA sequencing which have resulted in the identification of numerous saliva proteins of unknown function, very little is known about salivary components and the role of aphid saliva in host plant interaction [29]. Further elucidation is essential to improve the use of host plant resistance against aphid pests.

1.1.3 Plant defense responses

Plants and herbivores have been interacting for millions of years. Over time, plants have evolved sophisticated defense systems to counteract attacks from insects [30]. According to the timing of the deployment, the plant defenses against insect herbivores can be categorized as "static" or constitutive defenses and "active" or induced defenses [31].

The constitutive defenses include physical barriers, such as cuticles, trichomes and thorns, lignification, resin production, allelochemicals such as

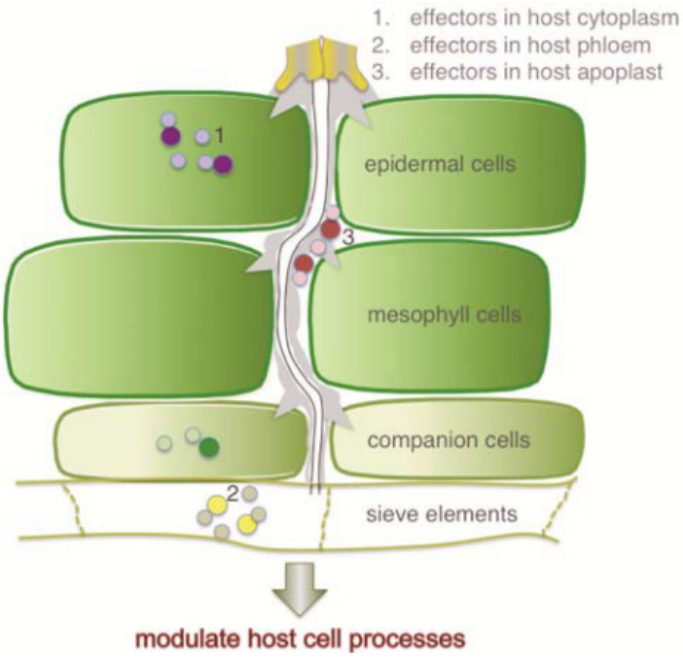


Figure 1.3: Representation of aphid effectors, proteins interacting with and/or modifying host proteins, delivery inside different plant cell types and compartments. This event results in suppression of host defenses and in a change of host physiology (modified from Rodriguez et al. 2012)

cyanogenic glucosides, glucosinolates, alkaloids, phenolics that reduce growth and development and have toxic, repellant or anti-digestive effects on herbivores [30].

On the other hand, an active mechanism results in the synthesis of proteins, acting as toxins, in the emission of volatile compounds or production of extrafloral nectar to attract predators of insect herbivores. Moreover, after being attacked by herbivores, plants quickly generate herbivory specific signals further converted to biochemical and physiological changes in the attacked leaves; certain signals move in different parts of the plant where they activate systemic defense [32].

The early plant responses to attacks by phytophagous insects or by pathogens involve cell membrane depolarization, mitogen-activated protein kinase (MAPK) activation, calcium influx and release of nitric oxide and reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) [9]. ROS and calcium signaling are both activators of three signal transduction pathways, based on different phytohormones. The main players in the regulation of signaling networks involved in induced defense to pathogens and insects are the plant hormones salicylic acid (SA), jasmonic acid (JA), and ethylene (ET). JA- and ET-dependent pathways regulate defense responses to necrotrophic pathogens, whereas SA-dependent pathway is mainly involved in response to biotrophic pathogens. The production of these three hormones varies greatly, depending on the type of pathogen or attacking insect [33]. Furthermore, the activation of other important regulators, often transcription factors, enables a cross-talk, mediates antagonistic or synergistic relationships between the pathways and defines gene expression profiles of induces resistance [34]. Phytohormones accumulation triggers both local and systemic plant responses, leading to production and accumulation of defense proteins and secondary metabolites with antixenotic or antibiotic properties in damaged and non-damaged parts of the plant [9].

1.1.4 Aphid's ability to reprogram defense responses

During compatible interactions, leading to successful feeding and reproduction, aphids cause a series of alterations in their host plants. Through saliva secretions, they inject into the plant effectors that play a crucial role in plant defenses suppression [35].

Firstly, in response to mechanical damage, plants immediately occlude injured sieve elements by callose deposition to prevent sap loss. This mechanism involves a sudden influx of Ca^{2+} ions from sieve element storage compartments by activation of voltage and mechano-sensitive Ca^{2+} channels [19, 36, 37]. However, aphid's stylet penetration does not affect sap flow: it means that saliva, secreted during the feeding into sieve tubes, prevents sieve tube occlusion and allows a continuous flow of phloem sap. Ca^{2+} - binding proteins were indeed found in watery saliva of different aphids species [19, 24, 36]: these proteins reduce Ca^{2+} ions availability in the phloem and inhibit sieve tubes clogging by proteins coagulation.

Aphids modulate the initial step of plant defense response, like signals related to oxidative stress and calcium [9]. The hypersensitive responses and cell death triggered by ROS are not observed in plants infested by aphid (or are limited around the feeding sites), suggesting that the saliva secretion could play a role in preventing ROS production [22]. Experimental studies on Arabidopsis transcriptome showed up-regulation of genes encoding for proteins involved in ROS detoxification and moderate induction or even down-regulation of genes producing proteins involved in ROS generation as a consequence of cabbage aphid infestation [38, 39]. In addition, *B. brassicae* seems to up-regulate several transcripts of calcium-binding proteins in Arabidopsis [38], suggesting a modulation of plant calcium-dependent signaling cascade by aphids.

In addition to these local reactions, aphids are able to modify the plant systemic responses, consisting of molecular, morphological or chemical events in non-damaged parts of the attacked plant. For example, aphids may alter the plant primary metabolism, especially nitrogen allocation and sugar metabolism, to better adapt phloem sap composition to their own nutritional

requirements [40, 41, 42, 43]. Aphid infections alter the expression pattern of genes encoding for enzymes involved in plant cell wall metabolism [41, 38, 44]: these events would facilitate stylet penetration of nymphs through cell walls or maybe are involved in adjustment of turgor pressure variations due to aphid feeding. Aphid infestations have been shown to reduce growth, especially stem height and dry-mass yield [45, 46]. When aphid feeding induces phytotoxicoses, plant damage is usally ascribed to toxic substances delivered by salivary secretions [47]. On the other hand, when the aphid species is "non toxic", it is assumed that the effect on growth is mainly due to removal of phloem sap from the host plant. Girousse et al. (2003) found a quantitative relationship between reduction in stem elongation rate and ^{14}C-assimilate withdrawal due to aphid feeding; this relationship is mainly a consequence of a strong reduction of the ^{14}C-assimilate allocation to the growing parts of the stems. Futhermore, they postulated that signals triggered by aphid punctures and feeding into plant tissues may affect one or several cellular activities, such as apoplastic and/or symplastic exchanges, gene expression and metabolism and may stimulated longitudinal more than radial expansion rates [47].

In conclusion, aphids induce profound alterations in their host plants though salivary secretions; further studies are necessary to better understand the means by which aphids manipulate their hosts.

1.1.5 Approaches to study salivary proteins

In recent years, great progress has been made toward identifying the components of aphid salivary secretions and understanding the functions of effectors injected in host plants.

The possibility to feed aphids on an artificial diet and collect saliva in combination with proteomics tools has allowed the identification of saliva proteins from several aphid species and the prediction of their enzymatic activities [20, 22, 21, 48]. However, the use of saliva collection in effector identification presents some limitations: the amount of produced proteins is too low for detection by mass spectrometry, proteins may be unstable during

the analysis time and genes encoding the effectors may have a different expression when aphids feed on artificial diets [28]. Therefore, new approaches that use transcriptome sequencing and proteomics of samples from dissected salivary glands have been developed. For examples, Carolan et al (2011) analyzed expressed sequence tag (EST) from two salivary gland cDNA libraries in order to find significantly over-expressed transcripts and they analyzed proteins isolated from salivary gland homogenates by mass spectrometry. Over 300 proteins were identified with predicted secretion peptide sequences, including proteins that had previously been identified directly from the secreted saliva [49]. Another multi-disciplinary approach to discover aphid candidate effectors, consists in combining bioinformatics (publicly salivary gland EST data) and functional assay to link sequence to phenotype [50].

Once a list of candidate effectors is generated, the next step is to understand their function and activity in the aphid-host plant interaction. One tool that is broadly used in gene function studies in aphids is RNA interference (RNAi). Because there are no approaches available for the genetic transformation of aphid, RNAi technique has been developed: it consists in delivering double-stranded (ds)RNA into aphids in order to silence a targeted gene. Different methods to deliver the RNA into aphid have been used: microinjection of RNAi directly into the aphid body [51], feeding of aphids on an artificial diet containing synthesized dsRNA, expressing dsRNA inside plant cells [52]. Other functional assays, like the leaf disc-based assay, exploit the overexpression of aphids proteins *in planta* and the consequent effect on aphid behavior to analyze their function [50].

Furthermore, in addition to identifying candidate aphid effector activities, it is essential to investigate the cellular biology underlying both compatible and incompatible plant–aphid interactions.

1.1.6 Economic importance of aphids

Aphids are among the most important insect pests of temperate agriculture and cause serious losses to cultivated plants. A pest aphid species may affect only a very specific crop, a group of related crop hosts (e.g. crucifers), or

may be polyphagous within and between plant families [1].

The dramatic negative impact that aphids can have on their host plants is partly due to their efficient colonization and settlement given to several biological characteristics. Their capacity to reproduce clonally and give birth to live young confers an explosive increase in aphid population under favorable environmental condition and a shortened pre-reproductive time [53]. Moreover, nymphs of certain aphid species can reach maturity in as little as five days. Secondly, winged adults disperse and colonize new plants while wingless adults morphs invest more resources in reproduction [6]. The wing dimorphism allows aphids to utilize different host plants in different seasons and to diffuse extensively because, under their winged form, they can be carried passively on long distance by wind [54]. In addition, long distance spread can occur through human activities and this can generate sudden disasters in specific crops. Thus, these reproductive and dispersal strategies contribute to aphids abundance in temperate zones [1].

The nature and extent of damages and symptoms caused by aphids vary widely among aphid and plant species [53]. One common symptom of aphid infestation is modification of plant growth and crop production: it can happen either directly through removal of plant nutrients, systemic manipulation of nutrient allocation, secretion of phytotoxic compounds, gall formation, leaf chlorosis and necrosis, wilting, leaf and/or fruit malformation, or involve indirect effects such as development of sooty molds on aphid's honeydew excretion that affect photosynthesis and promote other fungal disease, and virus transmission [1]. The latter is the most serious problem posed by aphids that often cause the major agricultural yields losses. Non-persistent viruses are present in the plant epidermis and are acquired and transmitted by aphids during stylet probing of the surface of plants. These viruses often are not aphid specific and are retained by the aphid for a short period of time [11]. Potato virus Y and alfalfa mosaic virus are examples of non-persistent viruses carried by aphids. In contrast, persistent viruses are located in the phloem and are acquired by aphids after feeding; once the insect is infected, the virus moves internally, migrates to gut and requires an incubation period before successful transmission. An infected aphid remains a vector during its whole

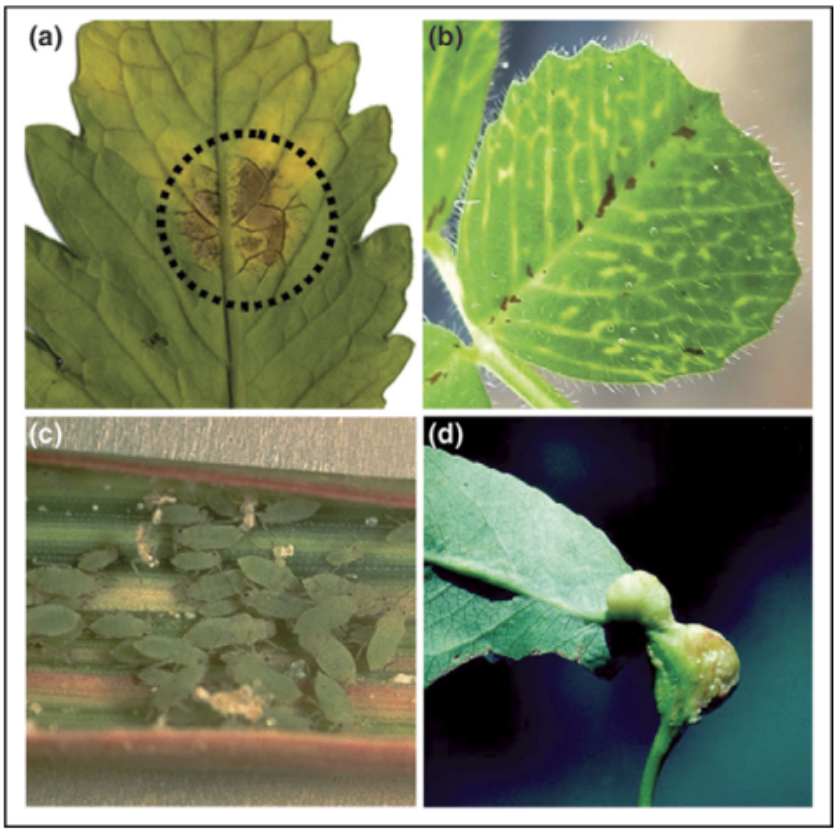


Figure 1.4: Different symptoms of aphid infestation. (a) Foliar chlorosis and necrosis caused by high density population of the potato aphid. (b) Systemic veinal chlorosis in new apical leaves due to alfalfa aphid feeding. (c) Leaf rolling and longitudinal streaks caused by Russian wheat aphid. (d) Foliar gall induced by sugarbeet root aphid (modified from Gogging et al 2008).

life. These type of viruses use only one or few aphid species as carriers [15].

1.1.7 Aphid control

Since a source for genetic host plant resistance is often not available for the majority of agricultural crop, control of aphids best uses an integrate pest management (IPM) strategy. The IPM, defined by FAO Panel of Experts as "a pest management system that, in the context of the associated environment and the population dynamics of the pest species, utilizes all suitable techniques and methods in a manner as compatible as possible and maintains the pest population at levels below those causing economic injury", relies both on agrochemical and biological control or a combination of few strategies [55]. IPM of aphids minimizes effects on non target species (i.e., biological control agents, vertebrates).

Historically, the most used method of aphids control is the application of high doses of agrochemicals, both contact and systemic insecticides. However, the contact pesticides are not very effective against aphids, because these insects often colonize the abaxial surface of leaves and feed directly from the phloem. Contrastingly, the systemic insecticides are absorbed by the plants and easily ingested by aphids through the phloem sap. The prevalent agrochemicals used against aphids include carbamates, organo-phospates, cyclodienes, pyrethroids [4]. The use of heavy doses of hazardous pesticides needs to be minimize because: it is cost intensive; it can be dangerous for the environment and the beneficial organisms like pollinators; there is the risk that toxic residuals enter in the human food chain; insecticides resistant aphid populations develop [56].

Alternative tactics in aphid control include cultural methods and biological control, that is the use of an organism to reduce the population density of another organism. Parasitoids and predators are natural enemies of insects and they can be released in the environment for biological control. Effective predators include predatory beetles, mites, lacewings, midges and bugs, all of which voraciously consume aphids [4]. The symbiotic bacteria present inside the aphids can confer resistance to parasitoid attack (causing death of

parasitoid eggs) associated to a decrease in aphids fecundity [57]. Secondary symbionts of aphids will be an interesting fields for studies about efficacy of biological control through parasitoids. Other biological control approaches include: use of spores of entomopathic fungi; small RNA virus biopesticides; phytopathogenic bacteria producing insecticidal toxins; entomophagous nematodes that contain symbiotic enterobacteria.

Finally, the adoption of aphid-resistant crops seems to be the most economic and eco-friendly strategy of pest management. Unfortunately, the introgression of aphid resistance into cultivars has not been possible mainly because of lack of resistance genes pool. There are few examples of aphid resistance genes in plants. The nematode resistance gene *Mi-1.2*, of the nucleotide-binding, leucine-rich repeat family, has been demonstrated to confer resistance against nematodes and potato aphids [58], causing aphid starvation and disseccation. The *Vat* (virus aphid transmission) gene from *Cucumis melo*, encoding for a cytoplasmic protein, determines a delay in growth of cotton aphid by decreasing fitness and fecundity [59].

The lack of knowledge about aphid resistance mechanisms is still a bottleneck for the development and the use of resistance crops in agriculture which may enable a minimization of agrochemicals spread. Further studies are thus necessary to develop strategies for aphid resistance.

1.2 *Brevicoryne brassicae*

Brevicoryne brassicae, commonly know as cabbage aphid, is one of the most destructive pests to members of family Brassicaceae. The aphid has a globally distribution, located in all but the coldest terrestrial habitats.

The apterae morphs are greyish green, with dark head, legs and tips of antennae (Figure 1.5a); their body is 1.9 - 2.7 mm long and is covered with greyish-white mealy wax. The siphuncoli are thick and very short and the cauda is triangular and broad. The alatae forms have a dark head, thorax and dorsal cross-bands (Figure 1.5b) and they stay with their group of offspring.

The cabbage aphid feeds on many plants of the genus *Brassica*, such as cabbage, cauliflower, broccoli, Brussel sprouts, radish, Arabidopsis, swede

and mustard, but does not occur on plants outside the Brassicaceae family. They do not alternate host but generally spend their entire life cycle in a sexual stage with eggs produced to overwinter. In climates where winters are mild, they retain a parthenogenic reproduction [1, 4].

B. brassicae is specialized towards crucifer feeding; it has evolved biochemical mechanisms to take advantages of the host defense systems: it sequesters glucosinolates, secondary metabolites of cruciferous plants that, during herbivore attack or mechanical wounding, are hydrolyzed by myrosinases into a range of toxic or deterrent products [60]. Moreover, the cabbage aphid produces an endogenous myrosinase, distinctly different from the plant one, and it apparently mimics the host plant defense mechanism [61, 62, 38]. This aphid glucosinolate-myrosinase system is probably involved in aphid colonies protection against natural enemies: when the aphid body is damaged, volatile compound from glucosinolates hydrolysis are released and can be directly toxic to natural enemies or act as repellant compounds [63] [64].

The cabbage aphid causes severe infestation on field crops and is one of the major pests of *Brassica napus*. It forms large colonies on leaves (Figure 1.5c), causing important bleaching and distortion of leaves and thus heavy losses of the crops. However, the greatest damage is done during the flowering and pod formation stages of plants, because the aphids attack stems and inflorescences and devitalize the crop by sucking the cell sap (Figure 1.5d). The presence of cast skins and honeydew at harvest can make the crop unmarketable. Furthermore, the cabbage aphid is implicated in the transmission of 17 plant viruses, including cabbage black ring spot, cabbage ring necrosis, cauliflower mosaic, radish mosaic and turnip mosaic virus [1]. Plants infested by *B. brassicae* show slow growth, reduce seed oil content and seed yield of 9-77% and do not develop marketable products.

B. brassicae control is based on repeated application of carbamate or organophosporus insecticides [65], but the reduction of pesticide inputs and the development of cheap and eco-friendly alternative control measures seem nowadays capital issues. Naturally occurring parasites and predators could be important factors in regulating population densities: biological control of cabbage aphid includes parasitic wasps, syrphid fly maggots, lady beetles and

lacewing larvae. Another possible alternative is to find and develop varieties of brassicas resistant to aphids. Different Brassica species demonstrated good levels of tolerance to *B. brassicae* [66, 67, 68], but the genetic source of this resistance is still unknown, so the introgression of the character into the crop cultivars has not been possible [4]. The resistance to cabbage aphid is a potentially complex trait and may be under the influence of several genes.

1.3 *Macrosiphum euphorbiae*

M. euphorbiae, commonly called potato aphid, is an highly variable and cosmopolitan aphid pest of field crop that is thought to have North American origin, but now it is found around the world [1].

It is a medium-size to large, spindle-shaped aphid, usually green, sometimes yellowish, pink or magenta (Figure 1.6 a, c). The most common morph, especially during the summer, is the adult apterous parthenogenic form: it measures about 3-4 mm, it is green or pink, has quite long and dark at the tips cornicles, long and pale-green legs and dark antennae. The other adult form is the alatae parthenogenetic morphs, also very abundant in summer when aphid population densities is high and the nutritional quality of host plant decreases. This form is smaller in size, about 2-3 mm long and is provided with transparent wings with dusky veins [5].

In north-eastern areas of USA, the potato aphid has a sexual phase in its life cycle on *Rosa*, using both wild and cultivated species as primary hosts, and overwinters in the egg stage. The sexual forms show sexual differences between oviparous female and male (Figure 1.5 b) and also differ in appearance in comparison with the parthenogenetic form. In Europe and in general in mild-winter regions, the *M. euphorbiae* life cycle does not include the sexual form overwintering (sexual morphs are produced occasionally) and the aphid reproduces parthenogenetically through four or five nymphal instars (Figure 1.5 d). The nymphs are small versions of the adults and undergo several moults in approximately ten days. In warmer climates, the viviparous females in early spring migrate from the winter hosts to young warm-season plants (like potato or weeds) and remain on their host until the plants dete-

(a)

(b)

(c)

(d)

Figure 1.5: *Brevicoryne brassicae*. (a) Apterae form, (b) Alata morph, (c) Big colony of cabbage aphids on a brassica crop. The greyish color is due to mealy wax secretions on aphids bodies, (d) Stems and inflorescences of *B. napus* severely infested by *B. brassicae*. (Figures (a), (b) and (c) modified from website http://aphid.aphidnet.org)

riorate because of overcrowding of aphids [69].

The potato aphid is highly polyphagous, feeding on more than 200 plant species of 20 different plant families. Its predominant hosts are potato, tomato, sometimes corn, pepper, eggplant, wild or cultivated rose, spinach and lettuce. Potato aphids may infest other crops such as clover, field corn, hops, peach, pawpaw, soybean, strawberry, sugar beet, sunflower, and tobacco or flowers as canna, geranium, gladiolus, hollyhock, iris, lily, poppy, rose, rudbeckia, and tulip. *M. euphorbiae* feeds indefinitely on the same host until the plat remain nutritionally suitable and then, when the quality of the plant deteriorates, moves to another host [5].

The potato aphids first attack young tissues, usually the growing tip of the plant, than multiply and colonize the entire plant causing removal of phloem sap. The plant leaves may take on a distorted appearance, and may be covered with honeydew and sooty mold. These symptoms are quite evident in potato plants and heavy infestation can also elicit the plant death. On other crops, such as tomato, pepper and eggplant the leaf deformities are less frequent, while the blossom drop or fruit deformities may occur more often. The potato aphid may be a contaminant, for example in lettuce, and may reduce the marketability of the product [69]. Furthermore, it can be a vector of more than 40 non-persistent and five persistent viruses like cucumber mosaic virus, potato leaf roll virus, beet yellow virus and potato virus Y [70].

Foliar insecticides, soaps, detergents, and oils are usually applied against aphids, but chemical suppression is not recommended unless half of the leaves are infested. Planting and cultural practices may influence aphid infestation and interfere with aphid host selection behavior. *M. euphorbiae* also has many natural enemies, as ladybirds, some lacewings, flower flies and the predatory midge and several species of fungi. Butterhead variates of lettuce are less susceptible to infestation but the basis for resistance are still uncertain [71]. Moreover, it was discovered than the *Mi-1.2* gene from *Solanum peruvianum* confers resistance to nematodes as well as some *M. euphorbiae* clones [72, 58]. However, the attempt to introduce this trait in eggplant failed: in fact eggplants that carried *Mi-1.2* displayed resistance to root-knot

nematodes but were completely susceptible to the potato aphid [73]. This potentially indicates that aphid resistance involves a number of additional genes that are not conserved between tomato and eggplant.

1.4 Examples of aphid proteins that modulate plant-insect interaction

In recent years, the biology of aphid salivary proteins and the role they have in plant-insect interaction have become an exciting area of research and great progress has been made in this research field. The availability of an aphid genome, transcriptome sequence data and the development of new bioinformatics and protein extraction and analysis tools allowed the identification of several effector proteins and the comprehension of their function in host cells modulation. However, more questions of interest still remain unanswered and more information needs to be collected as the nature of host target of aphid effectors, the mechanism the aphids effectors use to manipulated the host's metabolism and affect plant target functions, the role of aphid's secreted proteins in determining aphid host range. The identification of plant targets that interact with insect herbivore effectors seems to be of great importance because it can reveal the underlying molecular mechanism of the host manipulation as well as it can generate the possibility of developing novel pest resistance strategies [73, 35, 9].

Three newly identified aphid effectors, subject of this study, are described below; they showed to promote plant-insect interactions and they could be putative candidates for a program of sucking herbivores plant resistance.

1.4.1 *C002*, a salivary glands protein

Mutti and associates (2006) [51, 25] prepared a cDNA library from salivary glands of the pea aphid, *A. pisum*, and after an examination of about 4500 ESTs, selected on the basis of its abundance, one conting for detailed investigation. This highly abundant salivary transcript, arbitrary named C002, was predicted to encode for a protein of 219 amino acids residues and mass of 21.8

(a)

(b)

(c)

(d)

Figure 1.6: *Macrosiphum euphorbiae.* (a) Alatae, apterae, young nymphs and cast skins; (b) Male potato aphid on domestic rose; (c) Pink form; (d) Parthenogenic reproduction. (Figure (a) modified from http://commons.wikimedia.org; figures (b), (c) and (d) modified from http://www.flickr.com)

kDa. The N-terminal sequence is predicted to be a signal peptide for an extracellular protein and there are no O-glycosylation sites or N-glycosylation sites. The BLAST of *c002* did not reveal any similarity with protein of known function or homologs outside of the Aphididae family. Northern and Southern blot analysis suggested that the protein C002 is encoded by a single gene. Localization analysis showed that the transcript is present only in principal salivary glands and in only few of the secretory cells in each lobe. By Western blot experiments on protein extracted from plants that were exposed to aphids, the authors verified that C002 is a secreted protein that is transferred from aphid to the plant by saliva secretions during feeding.

Being an aphid salivary protein, it is postulated that it may have a function in aphid-plant interactions. RNAi experiments were done to prove the role of the protein in aphid life and feeding: small interfering RNA (siRNA) targeting C002 for cleavage were generated and injected into adult parthenogenetic *A. pisum* to lower the transcript level of the target gene. The injection of siC002-RNA had a great effect on reducing the life-span of aphids on fava bean leaves: half of them died 3 days after injection in comparison to the 11 days necessary for death of half of the aphids injected with the green fluorescent protein si-RNA and non-injected aphids (Figure 1.7). Furthermore, the siC002-RNA injected aphids had a comparable survival to the control when placed onto artificial diet; but when moved to fava bean leaves, 70 % of them died after 2 day compared to less than 25 % of the control. These results suggested that the C002 protein is not needed to feed on artificial diet but is essential for survival and feeding on a host plant (Figure 1.8). The behavior of C002-knockdown aphids during foraging and feeding was analyzed by EPG studies (electrical penetration graph): they resulted incapable to feed and likely this lacking of feeding is responsible for their premature death. The knockdown insects showed a probing phase, proving that they are searching the sieve elements and attempting to feed, but they did not identify a suitable location to penetrate in sieve elements or, if they did, they did not maintain the penetration (30 minutes compared to the 5-8 hours in the control). In conclusion, the C002 protein seemed to play a crucial role in the *A. pisum* ingestion of the phloem sap.

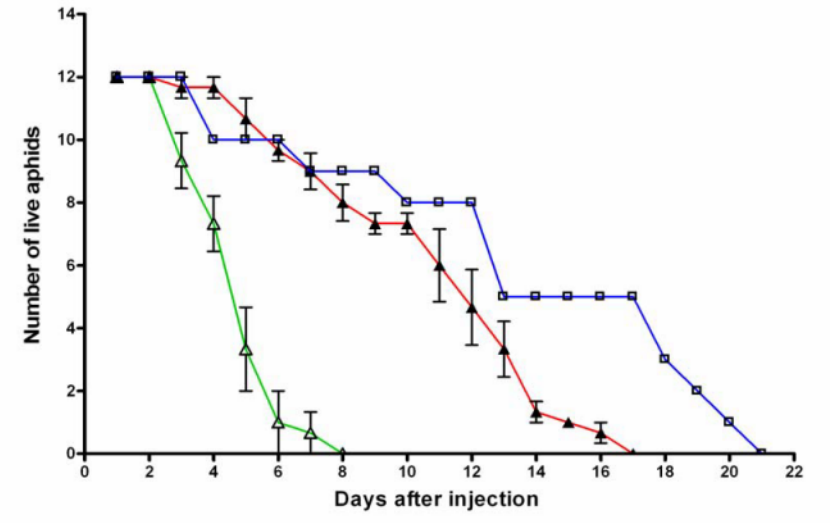


Figure 1.7: Survival rate of pea aphid after siRNA injection. Green line: injection with siC002-RNA; Red line: injection with siGFP-RNA (control); Blue line: uninjected aphids. (modified from Mutti 2006)

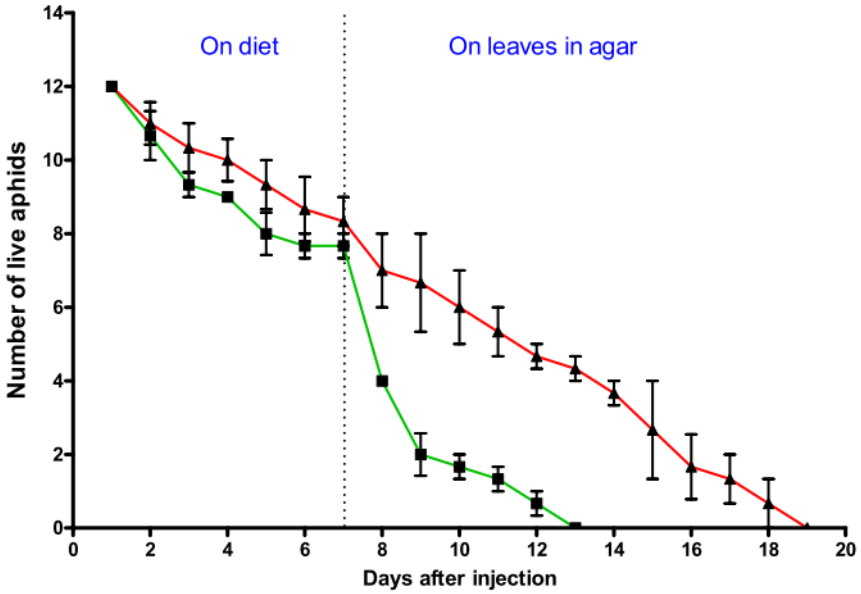


Figure 1.8: Survival of siC002-RNA injected aphids on artificial diet. They were kept for 7 days after injection on artifixial diet and then moved to fava bean leaves in plates. Green line: siC002-RNA injected aphids; Red line: siGFP- RNA injected aphids (control)(modified from Mutti 2006).

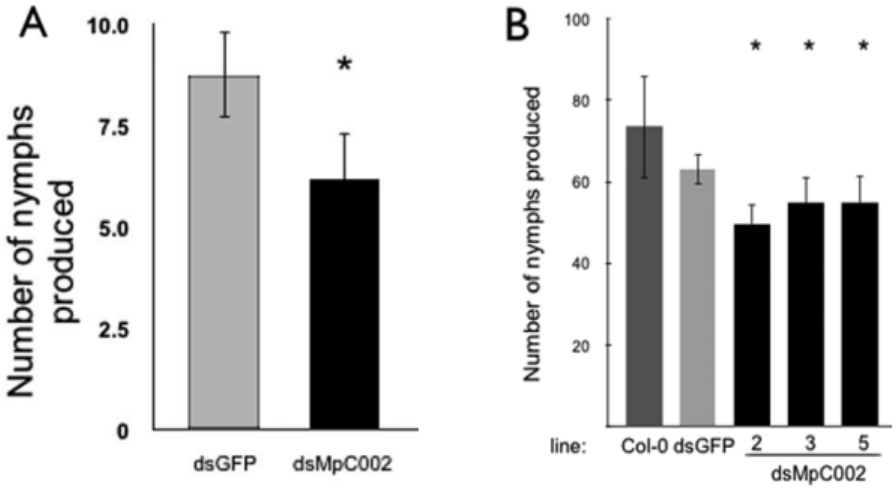


Figure 1.9: Silencing of *M. persicae MpC002* by plant feeding-mediated RNAi causes a reduction of aphids fecundity. The number of nymphs produced by the aphids analyzed for down-regulation of MpC002 is lower than the the nymphs produced from aphids fed on dsGFP transgenic leaves or on Col-0. (A) Feeding on transgenic *N. benthamiana* leaves. (B) Feeding on stable transgenic *Arabidopsis* line (modified from Pitino et al. 2011).

In a different study, Pitino et al. (2011) identified the homologue of C002 in *M. persicae* (*MpC002*) and silenced this gene in the aphids by feeding *M. persicae* on transgenic plants *Nicotiana benthamiana* and *Arabidopsis thaliana* expressing the dsRNA of *MpC002* [74]. Once assessed the silencing of the gene, they verified the fecundity and survival of aphids on the transgenic plants: whereas the survival rate was not affected by the knockdown of *MpC002*, the nymph production was significantly lower than the control (Figure 1.9).

The effects of C002 protein of *M. persicae* on aphid fecundity were also studied by Bos et al (2010). They performed the overexpression of this effector in *N. benthamiana* by agroinfiltration and then, assessed *M. persicae* fecundity on the leaves transiently transformed: *MpC002* enhanced aphid fecundity compared to the vector control (Figure 1.10) [50].

In a very recent study, Pitino and associates (2013) tried to identify whether effectors, among which C002, act in a plant-species-specific man-

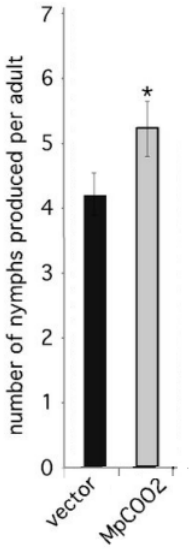


Figure 1.10: Overexpression of *MpC002* in leaves of transgenic *N. benthamiana* increases nymphs production (modified from Bos et al 2010).

ner. They discovered that *M. persicae* produces more progeny on transgenic *Arabidopsis* expressing *MpC002* but not on those that produce the *A. pisum* C002 ortholog or an *M. persicae* C002 mutant without a polymorphic amino acids repeat [74].

Taken together, these results provide evidence that C002 is an effector in *M. persicae* and *A. pisum* and has an essential role in aphid-plant interaction. However, the function of this protein still remains unknown, as well as the mechanism used to promote aphid infestation and alter plant response to infection. Further studies will be aimed to characterizing this candidate, to identify its plant targets and the molecular processes it perturbs.

1.4.2 *Me10* and *Me23*, *Macrosiphum euphorbiae* effectors

In a recent work, Atamian et al (2013) studied and identified a number of potato aphid effectors [75]. From the sequencing of an RNA-seq library produced from dissected salivary glands of adult aphids, they identified 159 predicted *M. euphorbiae* secreted proteins. In order to assess the roles of

these putative aphid candidate effectors *in planta*, they chose eight of them with putative orthologs in *M. persicae* and transiently expressed the *M. euphorbiae* selected proteins in *N. benthamiana*. The bioassay on infiltrated area with a population of *M. persicae* (aphid adapted to feeding on tobacco) was then performed: two candidate effector, named *Me10* and *Me23*, significantly increased the aphid's fecundity compared to the control. The same experiment was reproduced delivering the candidate effectors into tomato cells cytoplasm and assaying *M. euphorbiae* performance on the plants: only *Me10* significantly increased aphid's fecundity on tomato.

Me10 has orthologs among the *A. pisum* and *M. persicae* salivary proteins but it is uncharacterized and has no known function or functionally conserved domain, thus it's difficult to speculate how it manipulates plant responses. *Me23*, instead, encodes a glutathione peroxidase (GPX): it could be involved in reducing H_2O_2 and it could have a function as antioxidant to enhance aphid virulence and reduce the effect of the oxidative burst caused by aphid attack.

1.5 *Arabidopsis thaliana* as a plant model

Arabidopsis thaliana represent the best model system for plant physiology and genomic studies. It is a small herbaceous plant, few centimeters high, member of Brassicaceae family. Arabidopsis is diffused in whole Europe, Asia and North America and does not have any agronomic interest but, due to its favorable features, it is used as model plant by researchers all over the world. It shows a development and stress or disease response similar to most of crop plants; it has a very short life cycle and every single plant is able to produce up to 10.000 seeds. It is small, so it is suited to grown in limited spaces like laboratories and it is adapt for grand-scale genetic experiments. Its genome is quite small (about 157 million of bases pair) in comparison to most important crop plants genomes and it is organized in five chromosomes. Arabidopsis genomic regions were completely sequenced and the function on about 70% of its genes (2.700 in total) is well known. Finally, genetic and physic maps of both genes and molecular marker, insertional and chemical

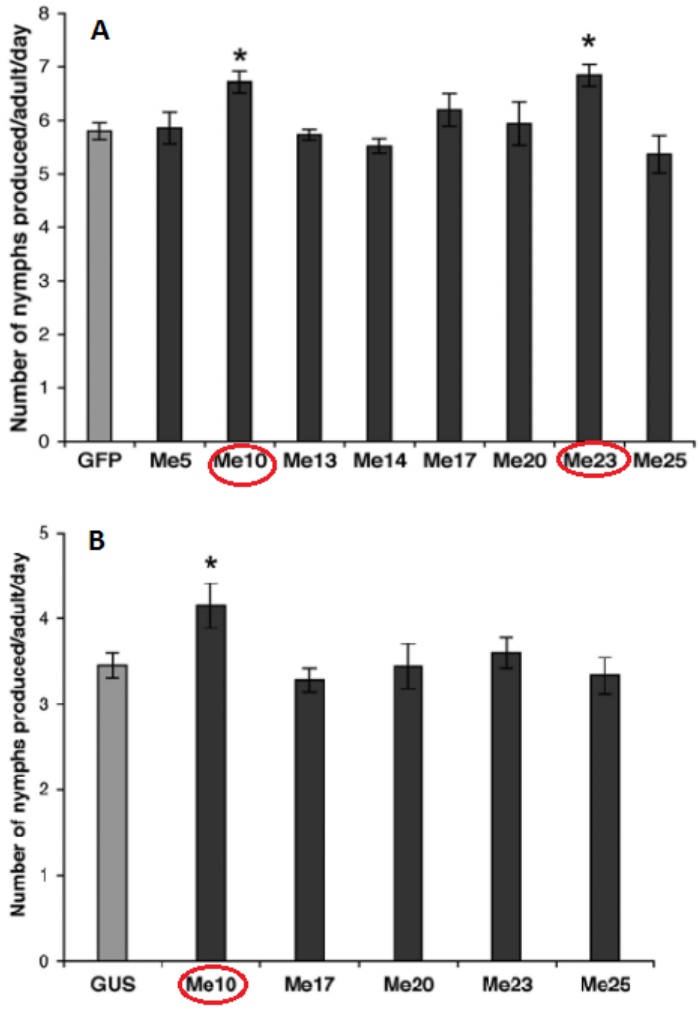


Figure 1.11: (A) *M. persicae* nymphs production on *Nicotiana benthamiana* plants expressing *M. euphorbiae* candidate effectors. Only *Me10* and *Me23* showed a significantly increasing of aphid fecundity. (B) *M. euphorbiae* nymphs production on tomato plants expressing *M. euphorbiae* candidate effectors. Only *Me10* showed a significantly increasing of aphid fecundity (modified from Atamian at al 2013).

mutagenesis protocol and transformation techniques are available.

All these characteristics ensure to Arabidopsis the role of international model in the research fields of plant physiology, functional genomics, proteomics, phylogenetic studies in plants. The information about relationships and interaction between genes and genetic products in Arabidopsis could be transferred in other plant species (especially Brassica crops) to play an important role in the agronomic sector.

Chapter 2

Objectives of this study

Aphids are one of the major pests of temperate agricultural and horticultural crops, causing damage either directly by feeding or indirectly by transmitting plant viruses. Some of them are host-specific feeding only on one species in a single host genus: this is the case of *B. brassicae*, the cabbage aphid, that is one of the most serious pests of brassicas crops throughout the world. The Cruciferous (Brassicacea) family includes both vegetable and oil seeds crops of great economic importance. The vegetable Brassicas consist of the cole group *Brassica oleracea* as cabbage, cauliflower, broccoli, kale, Brussels sprouts, savoy; the oil seed crops include *Brassica napus* and *Brassica rapa* producing the edible canola oil [76]. The most important producers in the world are Canada, USA, Europe, Australia, China and India. The members of Brassicaceae are attacked by a number of pests, but among these the cabbage aphid is the most destructive, quickly forming large colonies on leaves and during the flowering and pod formation stages. Aphid infested plants show slow growth resulting in seed yield loss and reduction in seed oil content up to 11% [77].

The control of this pest is primary based on treatments with insecticides, but several agrochemical applications are required to keep the crop free of aphids during a season (in 1991, 18.2 tonnes of demeton-S-methyl was applied to about 54,000 ha of UK brassica crops to control *B. brassicae* [78]). Growing concern over the dependence of farmers on insecticidal control of this insect

and the risk of contamination of the environment and non target organisms has increased the necessity to alternative control measures as the use of resistant cultivars. Several examples of resistance in brassicas to cabbage aphid have been reported but the genetic source remain unknown.

Other aphids, such as *M. euphorbiae*, have a broad range of host plants and are able to feed on a varieties of plants belonging to different genus and families. *M. euphorbiae* can be a major pest of potato, tomatoes, pepper, lettuce and it attacks both field crops and greenhouse-grown crops; its economic impact is mainly due to significant yield losses, transmission of plant viruses and reduction of the quality of vegetable and fruit crops. On the contrary to *B. brassicae*, an host-plant resistance against *M. euphorbiae* with a genetic basis has been recorded in tomato, but it was not possible to transfer the gene conferring resistance from tomato to other solanaceae species probably because of the complexity of the trait [69].

On the other hand, the increasing interest and the immense progress made in the aphid salivary secretions research field, open the door to the use of aphid effectors to reveal the details of the intimate associations between the herbivore insects and their host plants as well as to discover candidate genes that convey tangible resistance against sap sucking pests.

This experimental study was developed in the company Keygene N.V., based in Wageningen, The Netherlands and is part of an ongoing project focused on discovering genetic traits involved in aphid resistance in crops. The goal of this work is to investigate in model plants a list of candidate genes that confer plant resistance to *B. brassicae* and *M. euphorbiae* in order to introgress the traits of interest through conventional breeding in economic important crops as *B. napus*, tomato, pepper.

Two different approaches were assessed for the two aphids species taken in account, mainly due to the inability of *M. euphorbiae* to feed on Arabidopsis plants. Both the experiments, however, focus on the identification of targets in the plant that interact with some aphids effector proteins, in order to understand the mechanism that aphids use to manipulate plant response and generate information to develop novel pest resistant crops.

Study of *Brevicoryne brassicae* effectors The idea at the basis of this experiment is to exploit an *in planta* system to characterize putative plant proteins that bind the C002 effector (see section 1.4.1) of *B. brassicae*, test mutants of these C002 targets for increased aphid resistance and identify homologous sequences in *B. napus* and other brassica crops.

Arabidopsis plants expressing BbC002 protein under a phloem-specific promoter were generated. The recombinant C002 protein contained a C-terminus or N-terminus tag, a specific peptide sequence, for the purification of the protein from the phloem sap of the plant. Starting from the hypothesis that the C002 protein is actually present in the phloem sap and it interacts with some host target proteins to establish insect-plant interaction, an efficient protocol for Arabidopsis phloem sap extraction was established. Affinity tag purification of the recombinant protein was performed and, after digestion with the enzyme trypsin, the extract was analyzed through a mass spectrometry technique called MALDI-TOF (Matrix-assisted laser desorption/ionization-time of flight mass spectrometry) to check the presence of C002 protein in the extract and the possible presence of plant proteins that presumably had formed a complex with C002 and thus had establish an interaction with the aphid effector. From the MALDI-TOF analysis a list of 50 C002-binding proteins was created and Arabidopsis insertional mutant lines of these candidates proteins were ordered for aphid resistance bioassay. The goal that we would like to obtain was to identify some knock-out mutants that showed a loss of susceptibility or an increase in susceptibility to *B. brassicae* infestation in order to characterize the gene or genes responsible of the change in plant-insect interaction and look for homologous genes in other plant species, important from an economical point of view.

Together with this main study, the fecundity of *B. brassicae* on Arabidopsis plant expressing BbC002 was assessed to verify whether the overexpression of the aphid effector would affect nymph production as reported in previous works [50, 74].

Study of *Macrosiphum euphorbiae* effectors For the investigation on *M. euphorbiae* effectors, it was decided to perform a yeast two hybrid assay

between three potato aphid effectors, *Me10, Me23* and *MeC002* (see sections 1.4.1 and 1.4.2) and a library of pepper cDNA to identify possible aphid effectors-binding pepper proteins.

From *M. euphorbiae* aphids, RNA was extracted and from this the cDNA was synthesized. The entire sequences of the three examined effectors were amplified from the potato aphid cDNA and were cloned using Gateway® Cloning system first in the entry vector pDONR^{TM}221 and then into pDEST^{TM}32, the destination vector for the yeast two hybrid assay. Yeast cells were transformed with the baits and the different preys, representing the genes present in the pepper cDNA library, and the screening for positive interactions was performed.

The sequences of the three *M. euphorbiae* salivary proteins were cloned also in another binary destination vector, pK2GW7, in order to overexpress them in pepper plants and then assess potato aphid fecundity. *Agrobacterium tumefaciens* cells were transformed with these vectors and leaves of pepper plants were agroinfiltrated: the production of nymphs of aphids feeding on transformed plant material was checked to verify whether *Me10, Me23* and *MeC002* would affect potato aphid reproduction behavior as reported in a previous work [75].

Chapter 3

Materials and methods

3.1 Study of *B. brassicae* effectors

To identify putative plant proteins that interact with the C002 effector of *B. brassicae*, the pull-down technology was used: this strategy consists in biochemical purification of protein complexes, in which a bait protein is used to pull-down associated prey proteins, and identification of the proteins through mass spectrometry analysis [79]. This technology proves to be an useful tool for identification of protein interactions due to its ability to detect physiological complexes in natural settings [80].

In our experiment, the bait protein is BbC002 fused with a specific peptide sequence (tag) and the test is carried *in vivo* because the fused protein is expressed in Arabidopsis phloem: the aim is to evaluate the possible complexes BbC002:plant protein that form naturally in the sieve elements during aphid feeding and injection of the effector through saliva secretions.

3.1.1 Arabidopsis plants expressing BbC002 protein

Arabidopsis mutant lines expressing BbC002 protein have been produced previously in KeyGene's laboratories. The *C002* gene sequence of *B. brassicae* was identified by sequencing of cabbage aphid cDNA and mapping onto the reference genome of pea aphid *A. pisum*. Then, the obtained sequence without the signal peptide was cloned into the vector pK7m24GW,3 together

with:

1. two different tags for the pull-down, both bound in N-terminus and C-terminus of the protein. The used tags are: the Strep-II tag, a synthetic peptide consisting of eight amino acids (Trp-Ser-His-Pro-Gln-Phe-Glu-Lys) and the His-tag (also known as 6xHis-tag) an amino acids motif consisting of histidine (His) residues.

2. The promoter pSUC2 of a gene encoding a plasma membrane sucrose-H^+ symporter, for the specific expression of the protein in the phloem [81].

The Arabidopsis plants were transformed through floral dip technique, utilizing a solution of *Agrobacterium tumefaciens* containing the plasmid pK7m24GW,3 with the BbC002 sequence. The four obtained Arabidopsis lines are:

pSUC2:BbC002-StrepII-C
pSUC2:BbC002-StrepII-N
pSUC2:BbC002-His-C
pSUC2:BbC002-His-N

3.1.1.1 Genotyping Arabidopsis mutant lines

The progeny 3-weeks old of the transformed Arabidopsis plants were genotyped to select homozygous lines. 5 transgenic lines per each construct (N-His, C-His, N-StrepII and C-StrepII), 5 individuals per line (100 total) were analyzed by PCR to check the presence of C002 gene and the kanamycin resistance gene as control. The Phire Plant Direct PCR Kit (Thermo Scientific) was used to amplify the DNA directly from plant samples without any step of purification according to the manufacturer's instructions with the primers for C002 sequence (product length 348 bp) and the primers for kanamycin resistance gene (product length 500 bp).

Wild type Arabidopsis plants (Columbia-0) were took as negative control. 10 μl of PCR products, added with loading buffer, were load on a 1% agarose gel for electrophoresis analysis.

3.1.1.2 Arabidopsis RNA extraction and RT-PCR analysis

Total RNA was extracted from 3 plants per each type of C002 Arabidopsis mutant plants. Liquid nitrogen-frozen leaves were homogenized for 60 s to a fine powder using Tissuelyser (Qiagen) and 3 mm stainless steel beads. After homogenization, the samples were used for RNA extraction with RNeasy Mini Kit (Qiagen) and cDNA was synthesized from 1 μg of total RNA using Quantitect Reverse Transcription kit (Qiagen). The cDNA was diluted 10 times and used as template in RT-PCR to check the expression of *BbC002* gene. We used 5'-ATCGAAGATCTGGGACAACG-3' as forward primer and 5'-CTTCATCGGAGCCTAATTCC-3' as reverse primer. 2 μl of cDNA (10 ng) of each sample were used as template DNA in 50 μl of PCR reaction containing 5 μl of 10x PCR Buffer, 1.25 μl of each primer (10 μM), 0.5 μl of dNTPs (25 mM), 0.25μl of AmpliTaq DNA Polymerase (Invitrogen) (5 unites/μl). Cycle conditions were 94° C for 2 minutes, 94° C for 30 s, 55° C for 20 s, 72° C for 15 s and final extension at 72° C for 3 min. The RT-PCR was run for 20 and 30 cycles, to assess the level of *BbC002* expression. After PCR, 10 μl aliquots were analyzed by electrophoresis on agarose gel 1 % with the marker 1 Kb plus DNA ladder (Invitrogen).

3.1.2 Phloem exudation from Arabidopsis inflorescences

BbC002 recombinant protein, expressed in Arabidopsis transformed plants, is under control of pSUC2 promoter, so its presence is predicted to be in the phloem of transgenic plants. To extract the recombinant protein and the possible complexes formed with plant proteins, the phloem sap of Arabidopsis mutants was extracted.

Several methods of phloem sap collection have been described in literature: direct collection of phloem after incisions in the plants works only for trees, cucurbits and legumes; aphid stylets method and collection of honeydew present inherent difficulties and small amount of sample is obtained; exudation through ethylenediaminetetraacetic acid (EDTA) chelation presents some advantages [82, 83, 84, 85]. King and Zeevaart (1974) for the first time

(a) (b)

Figure 3.1: Phloem exudation from Arabidopsis inflorescences experiment. (a) Plant at the suitable developmental stage for the phloem bleeding. (b) Setup of the phloem bleeding: different volumes of collect solution were tried (500 μl in the image) and at the end it was established as more effective to use a greater number of inflorescences, about 25, in a bigger volume of 4 ml of EDTA solution.

described an EDTA-promoted exudation of phloem sap from detached leaves: the exudation in most plants is stopped by the formation of a callus in the wounded part of the phloem, but the treatment of the cut plant surfaces with EDTA, a chelating agent that forms stable complexes with divalent ions as Ca^{2+}, inhibits callus formation and allows a continuous exudation from cut plant material [86]. This method is an easy technique that can be used on organs (leaves, fruits) previously detached from the plant or at the sites of the removed organs to gain access to the sieve elements [83].

In our experiment, we adapted the phloem exudation protocol described by King and Zeevaart to obtain an efficient phloem bleeding from Arabidopsis inflorescences. The extraction was repeated several times to establish the suitable volume of EDTA buffer and the suitable time and condition of incubation to avoid that the buffer was sucked up by inflorescences .

Procedure of phloem sap collection from Arabidopsis inflorescences:

1. The day before the start of the experiment water plants well.
2. Cut the inflorescence close to the rosette and place it with the wounded surface in $\sim$ 300 ml EDTA buffer in a big beaker. Collect the inflorescences as fast as possible.
3. Re-cut ($\sim$ 0.5 cm from the wounded surface) the inflorescences while submerged in EDTA buffer in an petri dish and wash them extensively in the buffer.
4. Transfer the inflorescences to a clean glass beaker with $\sim$ 50 ml EDTA buffer and let them 'bleed' for 30 minutes.
5. Re-cut ($\sim$ 0.5 cm from the wounded surface) the inflorescences while submerged in the EDTA buffer in an petri dish and wash them extensively in the buffer.
6. Transfer the inflorescences to a clean 15 ml tube (about 25 inflorescences per tube) with 4 ml EDTA buffer (now supplemented with 1 tablet of protease inhibitor).
7. Collect the phloem in the growth chamber in normal light conditions for 4 hours, with high (90-100%) relative humidity. To achieve this, cover the set up with a hood and place wet paper underneath it.

Solutions:

- 5mM Phosphate buffer pH 6: NaH_2PO_4 monohydrate 0.0607 g, Na_2HPO_4 heptahydrate 0.0161 g in 100 ml of MilliQ water.
- EDTA chelation bleeding buffer: 1 mM EDTA, 5 mM sodium phosphate buffer. Add 1 SigmaFAST™ Protease Inhibitor Cocktail Tablet (Sigma-Aldrich) to 100 ml buffer. Since we were interested in protein complexes in the phloem, to preserve their integrity from degradation by endogenous enzymes, such as proteases, we added to the EDTA buffer a broad spectrum of protease inhibitors.

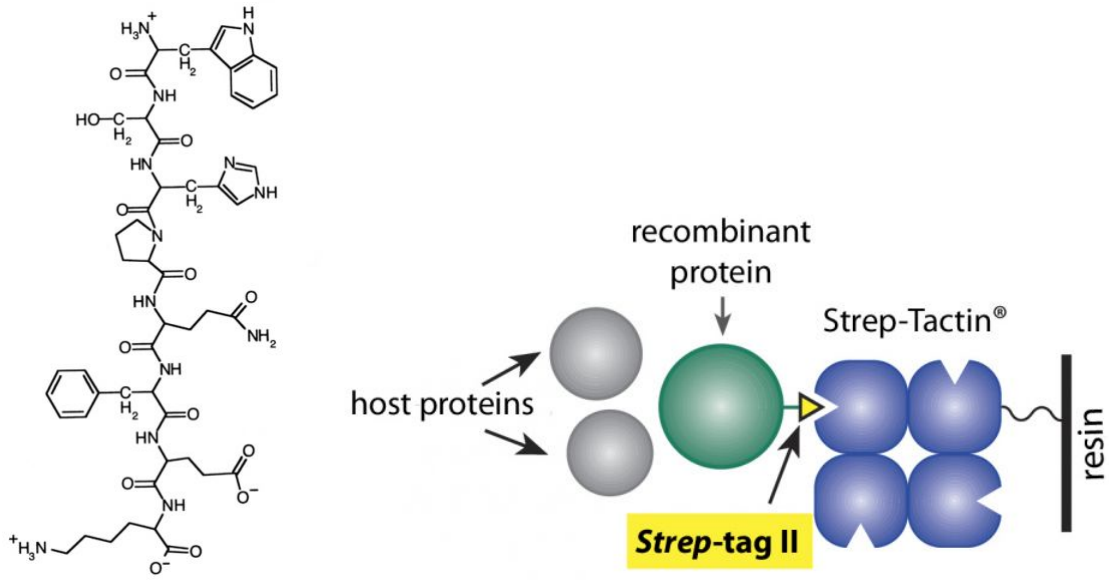
The collected phloem sap was then re-buffered into the buffers needed for the His-tag and Strep-tag purification (see section 3.1.3) by using Amicon® Ultra - 4 Centrifugal Filter Units (Millipore), filter devices consisting in a vertical membrane that provides fast sample ultrafiltration, high sample recovery (typically greater than 90% of diluted starting solution) and the capability for 80 fold concentration. The columns, containing the phloem sap, were centrifuged at 4,000 × g for approximately 10–40 minutes to decrease the volume; then 4 ml of wash buffer were added and centrifuged again: this step was repeated two times to make three washes in total and obtain very low EDTA contamination in a volume of 50 μl. The concentrate was collected from the filter device sample reservoir using a pipettor, while the ultrafiltrate was collected in the provided centrifuge tube.

3.1.3 Pull-down of Strep-II/His-tagged protein

Protein and peptide affinity tags have become highly used tools for purifying recombinant proteins and native protein complexes because they provide purification from crude extracts without prior steps of nucleic acid or other cellular material removal and use a simple and accurate protocols in contrast to conventional chromatography assay [87]. The most available affinity tags can be divided into three classes depending on their nature and on the nature of their target. The first class uses peptide or protein fusions that bind to small ligands linked to a solid support; in the second class, a peptide tag binds to a protein-binding partner immobilized on chromatography resin and in the third group the protein-binding partner is an antibody which recognizes a specific peptide sequence [88].

The principle and procedure of affinity tags purification of the two peptide tags used in this work are shown below.

Strep-II tagged protein purification The Strep-tag II is a short peptide (8 amino acids WSHPQFEK) (Figure 3.2a) that was developed as an affinity tool for the purification of corresponding fusion proteins on streptavidin columns. A streptavidin variant, called Strep-Tactin, has an higher affinity



(a) (b)

Figure 3.2: (a) Strep-tag II structure formula. (b) Strep-tag II principle: the recombinant protein, forming a complex with host proteins, binds through the Strep-tag II to Strep-Tactin immobilized molecules. The entire complex can be eluted by the addition of biotin and derivatives. (modified from the website http://www.iba-lifesciences.com/strep-tag.html)

(100 times higher) for the octapeptide Strep-tag II than the native form. The active Strep-tagged proteins bind to immobilized Strep-Tactin under physiological buffer conditions and can be purified in a single step (Figure 3.2b). After a short washing step, the recombinant proteins can be eluted gently by addition of low concentration of biotin or desthiobiotin. The shot peptide tag can be placed at the C- or N-terminus of the recombinant protein and it does not have any negligible effect on protein structure and function.

To purify the BbC002-Strep II complex from phloem sap, Strep-Tactin Magnetic Beads (Qiagen) were used. Procedure:

1. Resuspend Strep-Tactin Magnetic Beads by vortexing for 2 s and then immediately add 200 μl of Strep Beads to 50 μl of re-buffered phloem sap (see 3.1.3). 200 μl of Strep-Tactin Magnetic Beads suspension have a binding capacity of 40-60 μg protein.

2. Mix the suspension gently on an end-over-end shaker for 30 min at 4°C.

3. Place the tube on a magnetic separator for 1 min and remove supernatant with a pipet. Tubes may be briefly centrifuge to collect all droplets of suspension, before placing on the magnetic separator.

4. Remove the tube from the magnet, add 500 μl Buffer NP-T, gently vortex the suspension, place the tube on a magnetic separator for 1 min and remove buffer.

5. Repeat the previous step.

6. Add 50 μl Buffer NPB-T, gently vortex the suspension, incubate the tube for 5 min, place the tube on a magnetic separator for 1 min and collect the eluate in a clean tube.

7. Repeat the elution three times to give four eluate fractions.

Required buffers:

- Buffer NP-T (1 L): 50 mM NaH_2PO_4 (6.90 g of NaH_2PO_4 * H_2O); 300 mM NaCl (17,54 g); 0.01% Tween 20 (1 ml of a 10% Tween 20 stock solution). This buffer was supplemented with SigmaFAST™ Protease Inhibitor Cocktail Tablet (1 tablet to 100 ml buffer), to preserve the stability of protein complexes.

- Buffer NPB-T (1 L): 50 mM NaH_2PO_4 (6.90 g of NaH_2PO_4 * H_2O); 300 mM NaCl (17,54 g); 10 mM biotin (2,44 g); 0.01% Tween 20 (1 ml of a 10% Tween 20 stock solution).

His-tagged protein purification Purification of proteins, containing polyhistidine residues as affinity-tag, by immobilized metal-affinity chromatography is the most commonly used method. Immobilized metal-affinity chromatography (IMAC) is a technique based on the interaction between a transition metal ion, such as Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, immobilized on a matrix and a specific amino acid side chain: histidine is the amino acid that exhibits

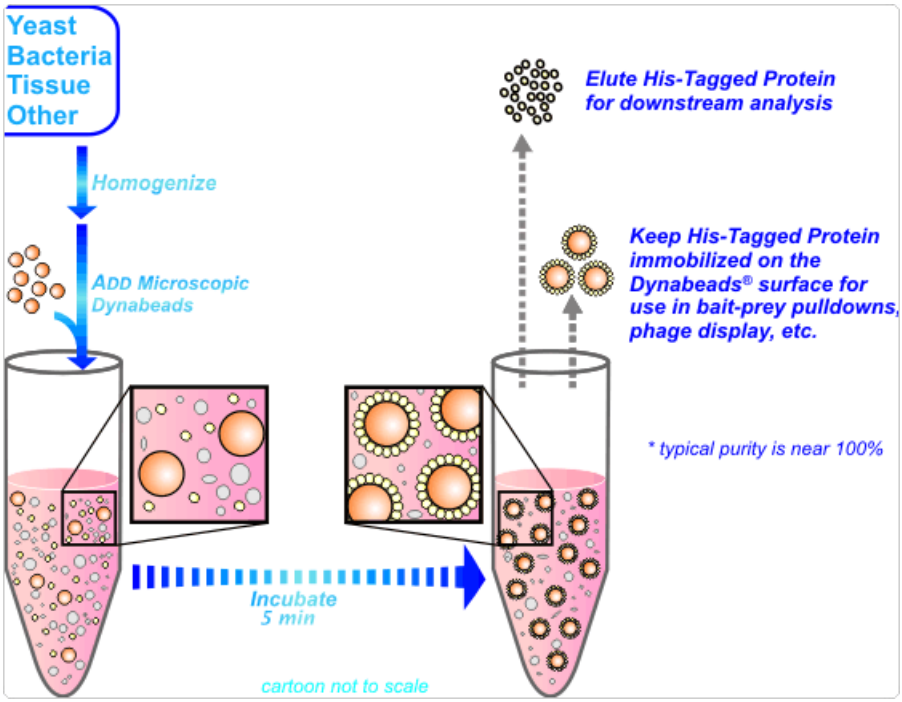


Figure 3.3: Schematic representation of isolation of His-tagged proteins through magnetic Dnabeads (modified from the website http://www.lifetechnologies.com)

the strongest interaction with immobilized metal ion matrices, because electron donor groups on the histidine imidazole ring readily form bonds with the immobilized transition metal. Thus, IMAC is suitable for an efficient purification of peptides containing a polyhistidine tag. After washing of the matrix material, peptides containing polyhistidine sequences can be easily eluted by adjusting the pH of the column buffer or by adding free imidazole.

To purified the BbC002-His complex from phloem sap, Dynabeads® His-Tag Isolation & Pulldown (Novex) were used. The magnetic beads are coated by cobalt ions and the His-tagged samples are eluted by addition of imidazole (Figure 3.3). Procedure:

1. Resuspend the Dynabeads in the vial by vortexing for 30 sec.

2. Transfer 50 μl of Dynabeads to a microcentrifuge tube and place it on a magnet for 2 min. Aspirate and discard the supernatant.

3. Add the phloem sap to beads and mix well.

4. Incubate on a roller for 5 min at 4^oC.

5. Place the tube on a magnet for 2 min, then discard supernatant.

6. Wash the beads 4 times with 300 μl Binding/Washing Buffer by place tube on a magnet for 2 min and discard the supernatant. Resuspend beads thoroughly between each washing step.

7. Add 100 μl His-Elution Buffer. Incubate the suspension on a roller for 5 min.

8. Apply on a magnet for 2 min and transfer the supernatant containing the eluted His-tagged proteins to a clean tube.

Required buffers:

- 1X Binding/Wash Buffer: 50mM NaH_2PO_4; 300mM NaCl; 0.01% Tween-20. One tablet of SigmaFAST™ Protease Inhibitor Cocktail was added to 100 ml of buffer, to preserve the protein complexes stability. Adjust pH to 8.0 with NaOH.

- His Elution Buffer: 300 mM imidazole; 50mM NaH_2PO_4; 300mM NaCl; 0.01% Tween-20

The purified proteins can be stored at -20^oC before further analysis.

3.1.4 Protein sequencing and analysis

3.1.4.1 Protein re-buffering and measurement of concentration

Prior to preparing the samples for mass spectrometry analysis, the eluted Strep-II/His-tagged proteins were re-buffered to concentrate them in a small volume of ammonium bicarbonate (50 mM NH_4HCO_3 pH 7.5-8.5). The re-buffering was performed by using Vivaspin 500 (Sartorius), centrifugal concentrators with a vertical ultrafiltration membrane that retains proteins

while salts and buffer can pass freely through. The desalting and concentration method constists in the following steps: spin at 15.000 rpm for about 20 minutes the device with the sample solution to decrease the volume to 5 μl, add 500 μl NH_4HCO_3 and spin again to 5 μl. Repeat this step once more (3 washes in total) to finally obtain re-buffered solution in a volume of 5 μl and recover the concentrated and de-salted protein samples from the bottom of the pocket with a pipette.

The obtained re-buffered samples were quantified by using the Qubit® Protein Assay Kit in combination with the Qubit® 2.0 Fluorometer (Invitrogen). The Qubit® 2.0 Fluorometer utilizes specifically designed fluorometric technology using fluorescent dyes that emit signals only when bound to specific target molecules, even at low concentrations. The Qubit® Protein Assay Kit is highly selective for proteins and allows the quantification of proteins ranging from 12.5 $\mu g/ml$ to 5 mg/ml, using a small starting volume of sample. The kit provides concentrated assay reagent to dilute in a dilution buffer and to be added to the samples and pre-diluted BSA standard to calibrate the Qubit® 2.0 Fluorometer.

3.1.4.2 MALDI-TOF analysis

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was invented in the late 1980s and it is a technique for analyzing peptides and proteins in relatively complex samples (peptide mass fingerprinting) [89].

The MALDI process is a two-step soft ionization technique: the so-called "desorption" and the ionization of the analyte. The sample is uniformly mixed with a matrix solution composed by crystallized molecules (such as acid sinapinic, DHB, alphacyano) in a mixture of highly purified water and an organic solvent (acetonitrile) that allows both hydrophobic and hydrophilic molecules to dissolve in the solution. The mixture matrix solution-analyte is deposited onto a MALDI plate and the solvents vaporize leaving only the recrystallized matrix with the analyte molecules embedded into crystals. The MALDI grid is then exposed to a nanosecond-duration laser pulses (gener-

ally UV light laser such as nitrogen laser light, wavelength 337 nm): the matrix absorbs the laser energy and some molecules become ionized through protonation. The matrix is then thought to transfer protons to the analyte molecules, generating charged ions of various sizes.

In time of flight (TOF) mass spectrometry, ionized molecules are accelerated in an electrostatic field in the mass analyzer within a vacuum. Ions with low mass/charge (m/z) ratio (lighter ions) are accelerated to higher velocities, so move faster through the drift space and reach the detector before ions with an high m/z. The time of ion flight is dependent only on the mass-to-charge ratio value of the ion and not on other factors because the separation occurs in vacuum. The standard detector for MALDI-TOF mass spectrometers is a microchannel plate that act as an electron multiplier for ions reaching the detector. Furthermore, the modern MALDI-TOF instruments are equipped with an electronic mirror, or reflectron, that reflects ions using an electric field, doubling the ion flight path and increasing resolution (Figure 3.4).

High-performance MALDI-TOF MS instruments are able to measure the masses of peptides with a relative molecular mass of 1000 – 3000 with an accuracy approaching 10 parts per million; as m/z increases, resolution and mass accuracy progressively decrease, although the instrument has no absolute upper analytical limit [90]. To produce peptides with molecular masses in the optimal range for MS analysis, the analytes are usually digested with specific protease that generates a mixture of peptides unique to that protein. The measurement of the molecular masses of these peptides gives a characteristic dataset (peptide mass fingerprint) that can be compared with a database containing peptide molecular masses of proteins theoretically digested by the same protease, to find the best match. In order to judge the validity of protein identification by this method, some means of scoring the quality of the match must be used [91].

The MALDI-TOF analysis of this project were carried out in the Department of Microbiology of Radboud University Nijmegen.

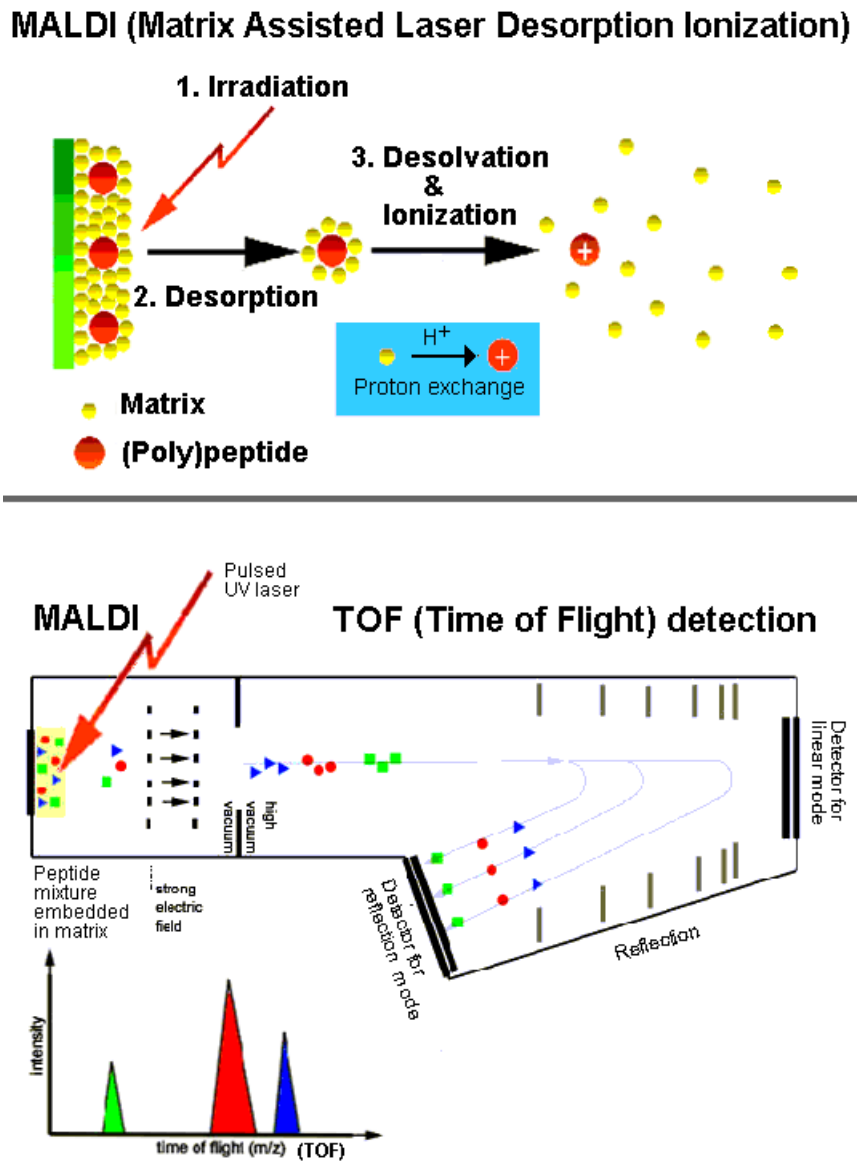


Figure 3.4: Schematic representation of MALDI TOF instrument working principles: the biomolecules are pre-coupled to a UV-light absorbing matrix. The matrix when irradiated with a nanosecond laser pulse, absorbs most of the energy and allows the transformation of the sample molecules into ionized gas. The nature of the ions is detected depending on their time of flight that is determined by the mass/charge ratio value (modified from the website http://www.ru.nl/science/gi/facilities/other-devices/maldi-tof/)

MW 66,000 Da	35 μl 50 mM NH_4HCO_3	13 μl trypsin
MW 24,000 Da	43 μl 50 mM NH_4HCO_3	5 μl trypsin
MW 12,000 Da	45 μl 50 mM NH_4HCO_3	3 μl trypsin

Table 3.1: Table to estimate NH_4HCO_3 and trypsin amount necessary for trypsin digestion

Preparation of the samples: trypsin digestion and peptide purification Trypsin digestion is the most frequently used step in mass spectrometry analysis for protein sample preparation due to the robust nature of this enzyme and the availability of extensive databases and software tools to analyze proteins digested by trypsin [92]. Trypsin is a serine protease that specifically cleaves at the carboxylic side of lysine and arginine residues and produces peptides of molecular weight that can be analyzed by mass spectrometry. The pattern of peptides is then used to identify the protein.

Our samples were digested with Trypsin Gold Mass Spectrometry Grade (Promega) as following: 10 μl aliquots of 100 ng/μl concentrated trypsin enzyme were prepared dissolving the solid powder in 50 mM acetic acid (storage at -20^{o}C). The protein solution was prepared diluting 1 nmol of proteins in 50 mM ammonium carbonate solution (see section 3.1.4.1) according to the table 3.1. 1 μl of 45 mM dichlorodiphenyltrichloroethane (DDT) freshly prepared was added to the proteins to reduce disulfide bonds. After 15 minutes of incubation at 50^{o}C, 1 μl of 100 mM iodacetamide (IAA) was added to allow alkylation of SH - groups. The samples were then digested by trypsin, adding the protease to obtain a final enzyme:protein ratio of 1:50 (see Table 3.1) and incubating overnight at 37^{o}C. The digestion was stopped by adding 5 μl of 10 % trifluoroacetic acid (TFA).

Before analyze the peptides by mass spectroscopy, it is important to concentrate and purify them by removing salts. Zip-Tip pipette tips C_{18} (Millipore) are special 10 μl tips that contain a small bed of C18 reverse phase resin to perform a reverse phase chromatography and purify peptides. In this procedure, peptides are mixed first with an ion pairing agent, such as trifluoracetic acid (TFA), that neutralizes their charge and then they are passed through the reverse phase resin, which is hydrophobic, and absorbed to the

column. The column is washed and peptides are eluted in a small volume of organic solvent.

Our samples were purified according to the following steps:

- Equilibration of Zip-Tip columns: using the maximum volume setting of 10 μl, aspirate with the pipettor the wetting solution 50:50 acetonitrile:H_2O and dispense to waste. Repeat 3 times. Aspirate the equilibration solution, 0.1 % TFA in H_2O, and dispense to waste. Repeat for a total of 3 times.

- Binding and washing of peptides: set the pipettor to 5 μl, aspirate and dispense the sample 7-10 times. Aspirate 10 μl of wash solution (0.1% TFA in H_2O) into the tip and dispense into waste. Repeat for a total of 3 times.

- Elution of peptides: dispense 3 μl of elution solution (50:50 acetonitrile:H_2O with 0.1 % TFA) into a clean tube. Carefully aspirate and dispense eluant through the ZipTip at least 3 times without introducing air into the sample.

1 μl of purified proteins were spotted on MALDI-TOF plate together with 1 μl of a matrix of alpha-cyano-4-hydroxycinnamic acid.

3.1.4.3 Analysis of MALDI-TOF spectra

The general approach to analyze the mass spectrometry data consists in comparing the experimental data with calculated mass values obtained by applying appropriate cleavage rules to the entries in a sequence database. Corresponding mass values are counted or scored in a way that allows the protein which best matches the data to be identified.

To identify the proteins from the peak list obtained from MALDI-TOF analysis, the software package Mascot from Matrix Science was used [93]. Mascot uses a "probability-based MOWSE" algorithm to estimate the significance of a match; once the MS data are submitted in form of peak list, Mascot calculates the probability that the observed match between the experimental data and mass values calculated from a candidate peptide or

protein sequence is a random event. The match with the lowest probability is reported as the best match. Due to the fact that some level of noise in the data and false or random matches caused by the software can occur, Mascot calculates a threshold score representing a 5 % confidence threshold and this score for statistical significance varies from experiment to experiment. In our analysis, a cut-off score of 50 was calculated for the 5 % confidence threshold. The peptide mass fingerprint report is shown as an histogram of the Mascot score distribution for the top 50 best matching proteins: scores are -10 x $Log_{10}(P)$, where P is the probability that the observed match is a random event. Scores under the fixed threshold represent random matches, while the scores superior to the threshold are considered statistically significant.

In our experiment, the peak list was compared to a database (Swiss-Prot) of Arabidopsis proteome *in silico* digested by trypsin. The Mascot model includes the possibility to set a number of allowed missed cleavage sites that can occur in the samples as a consequence of partial digestion. Increasing the number of missed cleavages increases the probability of identifying missed cleavages in the sample, but it is computationally very expensive for values greater than 2; 1 allowed missed cleavage site was chosen in our analysis. Moreover, Mascot considers different types of modifications that the protein samples can exhibit. Two types of deliberate modifications, introduced during sample work-up, were taken in account in our experiment: the carbamidomethyl modification caused by the alkylation agent iodoacetamide (IAA) and methionine-oxidation due to the DDT used during trypsin digestion. The putative identified proteins were then listed in order by protein score and beside the rank number the accession number representing the identified protein was shown.

4 biological replicates were used for identification of putative plant proteins interacting with BbC002 (N-StrepII, C-StrepII, N-His and C-His) and 4 technical replicates of protein extraction and analysis per each samples were performed. All the results obtained from the individual experiments were combined together to create a list of identified proteins with the respective number of hits, score, function, accession number: all the proteins with a hits number up to 4 were taken in account for further analysis.

3.1.5 Arabidopsis mutant lines

For all the identified proteins with a number of hits up to 4, the respective gene locus was annotated searching the atg number in The Arabidopsis Information Resource (TAIR) databases. Arabidopsis insertional mutant lines for all these genes were ordered from NASC, the European Arabidopsis Stock Center. With the help of T-DNA Express Arabidopsis Gene Mapping Tool and the Arabidopsis Information Resource (TAIR), homozygous SALK Arabidopsis lines containing insertion of *Agrobacterium* T-DNA in the genes of interest were identified. Only for few genes, homozygous mutant lines were not available: in that case, three different heterozygous lines were ordered per each gene.

SALK T-DNA primers were designed with the T-DNA Primer Design Tool: for each mutant line LP (left genomic primer) and RP (right genomic primer) primer pair was designed. It will be used in combination with the T-DNA border primer LB for the genotyping, to check whether the line is homozygous or heterozygous.

3.1.6 Aphid bioassay on transgenic Arabidopsis plants

B. brassicae fecundity assay was performed on Arabidopsis transgenic plants expressing C002 protein to verify whether the recombinant protein affects aphid reproduction behavior and validate for the cabbage aphid the same results obtained for *M. persicae* by Bos et associates (2010).

Arabidopsis seeds were sowed in small pots containing soil and vermiculite (3:1) and put in cold room at 4^oC for 2 days for vernalization. After 2 days the plants were moved in greenhouse and grown at 25^o C. 3-weeks old Arabidopsis plants were used for the aphid fecundity assay. One *B. brassicae* nymph was confined to single plant in sealed experimental cages containing the entire plant and the pots were incubated in growth chamber at 23^o C, long-day light conditions (16 hours of light and 8 h of dark) and 50 % of humidity. After 10 days the number of offspring was scored for each plant.

The experiment was carried out three times for pSUC2:BbC002-StrepII-N and pSUC2:BbC002-StrepII-C Arabidopsis transgenic lines on 20 plants

Figure 3.5: Setup of the aphid bioassay on Arabidopsis plants. One single aphid is entrapped in a cage covered with a mesh that contain the entire plant.

per lines the first and second time and on 30 plants the third time to create data from three independent biological replicates.

3.2 Study of *M. euphorbiae* effectors

To identify putative plant proteins that interact with *M. euphorbiae* effectors Me10, Me23 and C002, the yeast two-hybrid method was used: the assay is based on the expression of chimeric proteins that when interact, bind together re-combining two inactive parts of a transcription factor and activate a reporter gene in yeast cell nucleus. For this study, the yeast two-hybrid assay was chosen to try a different method to detect protein interaction; moreover, the potato aphid is not able to grow on Arabidopsis, there are no simple and fast protocols of stable transformation of pepper plants and mutant lines of pepper are not available.

3.2.1 RNA extraction and cDNA synthesis from *M. euphorbiae*

To isolate the sequences of the three effectors of interest, the RNA from one *M. euphorbiae* adult was extracted by using the RNeasy Mini Kit (Qiagen). The sample was homogenized by vigorously vortexing the tube containing the aphid and a 3 mm stainless steel bead. cDNA was synthesized from 1 μg of total RNA using Quantitect Reverse Transcription kit (Qiagen). The cDNA was diluted 10 times and used for further analysis.

3.2.2 Generation of *Me10, Me23* and *MeC002* sequences from potato aphid cDNA

The mRNA and protein sequences of Me10 (*M.euphorbiae* expressed sequence tag identification number Me_SG525; accession number GAAF01000080.1), Me23 (EST ID Me_SG130; accession number GAAF01000028.1) and MeC002 (EST ID Me_SG526; accession number GAAF01000085.1) were downloaded from GenBank database (National Center for Biotechnology Information). The presence and location of signal peptide cleavage sites in the proteins were predicted by using the SignalP 4.1 Server. Primer pairs to amplify the sequences of the three effectors from *M. euphorbiae* cDNA were designed: the forward primers were designed after the predicted signal peptide and the reverse primers were designed without the stop codon (Table 3.2).

Gene	Primer sequence
Me_SG130_Forward	5'-ATGGAGCCAATTGCTCCAAGGG-3'
Me_SG130_Reverse	5'-GCAACATTGGTCCTTTAACTGTTCTTG-3'
Me_SG525_Forward	5'-ATGCAATCAATACAACCATTAATAGACC-3'
Me_SG525_Reverse	5'-TGCTCCAACGACTGTTGGTTGGG-3'
Me_SG526_Forward	5'-ATGGGTGGGTCTTCTGACGATG-3'
Me_SG526_Reverse	5'-AAAACGTCGAAAGAAACTTCCAACC-3'

Table 3.2: Primers sequences

1 μl of cDNA was used as template in a 50 μl PCR reaction containing 10 μl 5x Herculase II reaction buffer, 1.25 of each primer (10 μM), 0.5 μl of

dNTP mix (25 mM each dNTP), 1 μl of DMSO, 0.5 μl of Herculase II fusion DNA polymerase (proof-reading). The PCR run 35 cycles at the following conditions: 95^o C for 2 minutes, 95^o C for 20 s, 55^o for 20 s, 72^o for 45 s and final extension at 72^o C for 3 minutes. After PCR, 5 μl aliquots were analyzed by electrophoresis on 1 % agarose gel.

3.2.3 Cloning of effector genes

Gateway® Technology (Invitrogen) was used as cloning method to insert the genes of interest into destination vectors. Primers containing *attB* sites were designed for each effector gene and *attB*-PCR products were generated by amplification of Me_SG130, Me_SG525 and Me_SG526 sequences isolated from aphid cDNA with the *attB*-primers. The *attB*-PCR products were introduced into pDONR^{TM}221 (Invitrogen) plasmid using Gateway® BP Clonase^{TM} II Enzyme Mix and transformed in One Shot® TOP10 Chemically Competent *E. coli* cells, according to the manufacturer's protocol. Colony PCR with specific primers for each gene was performed to check in the subsequent clones, grown on LB plates containing 100 $\mu g/ml$ kanamycin, the presence and the correct size of the inserts. The plasmids were extracted from *E. coli* cells cultures by QIAprep Spin Miniprep Kit (Qiagen) and used to introduce the inserts into destination vectors using Gateway® LR Clonase^{TM} II Enzyme Mix. The two used destination vectors were pK2GW7 for the introduction of the genes in *A. tumefaciens* cells and subsequent transformation of pepper leaves and pDEST^{TM}32 for the yeast two-hybrid assay.

3.2.4 *M. euphorbiae* bioassay on infiltrated pepper leaves

3.2.4.1 Transformation of *A. tumefaciens*

pK2GW7 plasmids containing the three potato aphid's effectors were introduced in *A. tumefaciens* strain GV3101 cells using the Cell-Porator® Electroporation System. 100 ng of DNA plasmids were added to 20 μl of electrocompetent cells and placed between the poles of a pre-chilled micro-

electroporation chamber. The electroporation mix was transformed at 360 Volts and then transferred to 250 μl SOC medium in a sterile tube and recovered at 28^o C for 2 hours with shaking.

After incubation, 50 μl of transformed cells were plated on LB agar plates containing 100 $\mu g/ml$ spectinomycin and incubated upside down at 28^o C for 2 days. The presence and the correct size of the inserts into *A. tumefaciens* colonies were verified by Colony PCR.

3.2.4.2 Pepper leaves infiltration

Single *A. tumefaciens* colonies harboring pK2GW7 transformed plasmids were inoculated into LB medium containing 100 $\mu g/ml$ spectinomycin and grown at 28^o C, 225 rpm for 2 days. The cultures were spun down and diluted in a volume of 10 mM $MgCl_2$ to reach the Optical Density (OD_{600}) of 0.8. Each construct was infiltrated into full-expanded leaves of 5-6 weeks old *Solanum annuum* cultivar Maor plants, using a sterile syringe without needle. The plants were grown in a growth chamber with daily temperature of 23^o C under a long day regime.

3.2.4.3 Aphid bioassay

Two days after infiltration, one single nymph of *M. euphorbiae* was placed on the lower surface of the infiltrated spot of the leaf and entrapped in a cage. For each construct two plants were used and 5 leaves per plant. As control the leaves of two plants were infiltrated only with 10 mM $MgCl_2$ solution. The plants were incubated in growth chamber with daily temperature of 23^o C under a long day regime. Aphid survival and fecundity were assessed after 10 days by counting the number of aphids in every single cage.

3.2.5 Yeast two-hybrid system

The yeast two-hybrid system is a method to identify protein-protein interactions: it exploits the feature of the transcription factor *GAL4* to be active only when the binding domain DB, that links to the promoter DNA region, and the activation domain AD, that activates the transcription, are physically

Figure 3.6: Setup of the aphid bioassay on agroinfiltrated pepper leaves. One single aphid is entrapped in a clip cage on the infiltrated spot on the leaf.

bound together. In general, in a yeast two-hybrid assay, the transcription factor is composed by a dimer of fused proteins: the so called "bait" contains the DB fused with the first protein of interest X, while the "prey" is made up of the AD linked to the second protein of interest Y. The two-hybrid proteins are inserted in two different plasmids containing independent selection markers, and yeast cells are transformed with these vectors. The yeast DNA contain some reporter genes, such as *lacZ* or auxotrophic marker like *HIS3* and *URA3,* and the regulator regions of these genes are modified to include the binding sited for DB-X (bait): if the protein X interacts with the protein Y, the activation domain will get close to the binding domain to form the functional transcription factor and activate the expression of the reporter genes (Figure 3.7). The interaction can be verified by selection on plates lacking auxotrophic marker as histidine and uracil (yest cells containing interacting bait and prey will grow and form colonies) or by an enzymatic activity assay such as the colorimetric assay of $\beta-$galattosidase activity.

In our experiments the kit ProQuest^{TM}Two-Hybrid System with Gateway® Technology (Invitrogen) was used. It contains the yeast strain MaV203,

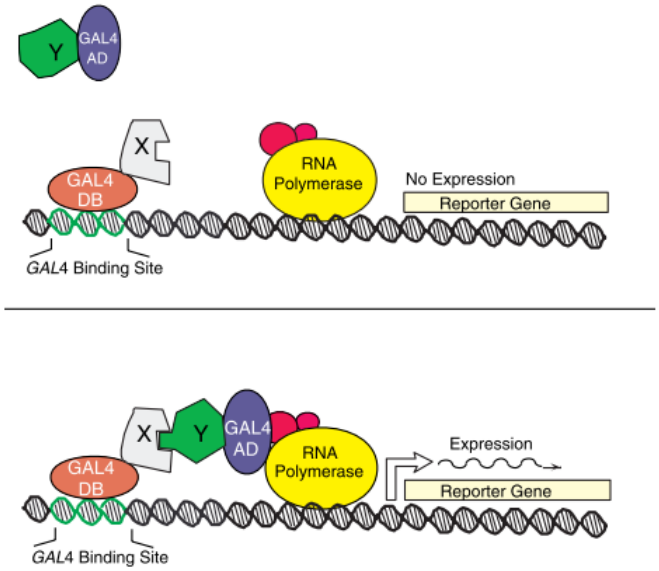


Figure 3.7: Basis of the two-hybrid system: Yeast cell expresses both the GAL4-AD-X fusion protein and the GAL4-AD-Y fusion protein. When X and Y do not interact, the GAL4-AD-Y fusion protein does not localize the promoter to activate transcription (above image). When the two proteins interact, the GAL4-AD-Y hybrid protein is able to localize the promoter and activate the transcription.

with the opportune DNA modifications (reporter genes and deletion of auxotrophic genes) and the plasmids for yeast transformation. As prey, a premade ProQuest™ library (Invitrogen) of pepper cDNA was used, consisting of a collection of expression plasmids in which the activation domain AD is fused to individual cDNAs. The major steps necessary to perform a two-hybrid library screen are: generation of bait plasmid; construction of two-hybrid library; testing the bait to determine 3-AT concentration for *HIS3* reporter gene's inhibition; transformation of yeast strain MaV203 with two-hybrid library and bait plasmid; screening of the reporter genes; confirmation of the positive interactions; isolation of prey plasmid DNA; biochemical or functional assay as sequencing of prey plasmids.

3.2.5.1 Cloning of effectors genes into pDEST^{TM}32

The three effector genes, inserted into the entry vector pDONR^{TM}221, were cloned into the destination vector pDEST^{TM}32 using the Gateway LR reaction to generate the hybrid protein DB-X (bait). pDEST^{TM}32 destination vector contains the following features:

- The sequences encoding for the binding domain *GAL4* for the fusion with the gene of interest. It is under control of the constitutive promoter and the terminator of the yeast gene *ADH1* (alcohol dehydrogenase).
- Two sites of recombination *att*R1 and *att*R2 for the Gateway LR reaction.
- *ARS4/CEN6* sequences for low-copy number maintenance in yeast.
- The *LEU2* gene for the yeast selection on media lacking leucine.
- A replication origin and the gene of resistance to gentamicin for the replication and retention in *E.coli*.

3.2.5.2 Pepper cDNA library in pDEST^{TM}22

Pre-made ProQuest™ Library of pepper cDNA, that we used to identify putative plant proteins interacting with *M. euphorbiae* effectors, was a normalized collection of pepper cDNA gene sequences expressed in different tissues of the plant. The library is inserted into Gateway vector pDEST^{TM}22 containing the following features:

- The sequences encoding for the activation domain *GAL4* fused with a signal of nuclear localization, to generate *GAL4* AD-cDNA fusion proteins.
- *ARS4/CEN6* sequences for low-copy number maintenance in yeast.
- The *TRP1* gene for the yeast selection on media lacking tryptophan.
- A replication origin and the gene of resistance to ampicillin for the replication and retention in *E.coli.*

The library was retained in *E.coli* host cells: before starting yeast transformation, the prey plasmids were extracted from *E.coli* cells by using the QIAGEN Plasmid Midi Kit. From 25 ml of cells culture, grown for 1 day at 28^{o}C with shaking, about 100 μg of purified prey plasmids were obtained.

3.2.5.3 MaV203 yeast strain

The yeast strain used as host for the two-hybrid screen, present in the ProQuest^{TM} System is MaV203 (Matα) [94, 95]. MaV203 DNA has the following features:

- A set of irreversible auxotrophic mutations: *leu2* and *trp1* that allow the selection for the fusion vectors bait and prey, and *his3* for the growth under control of the reported gene *GAL1::HIS3*;
- Deletion of genes *GAL4* and *GAL80* encoding respectively for GAL4 and its repressor GAL80. Lacking GAL80, galactose is not necessary for the activation of the GAL4-inducible promoters.

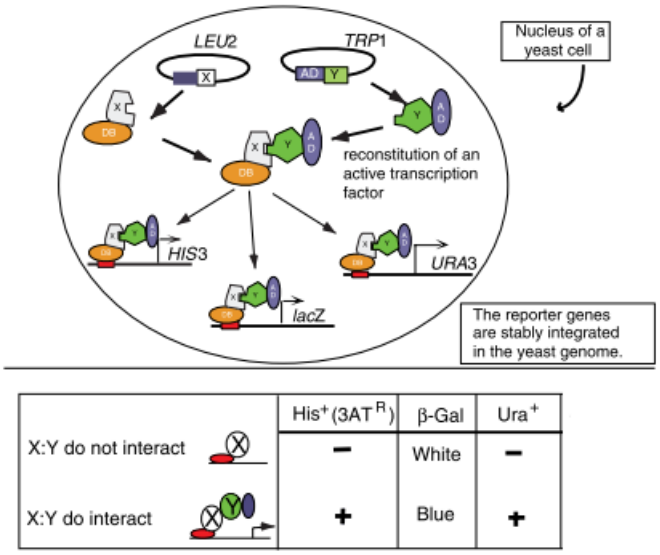


Figure 3.8: Two-hybrid screening with three reported genes. Mechanism of interaction between AD-Y and DB-X; expected growth and color depending on interaction or non-interaction of DB-X and AD-Y and induction or non-induction of reporter genes (modifies from ProQuest^{TM} Two-Hybrid System with Gateway Technology 2002)

- Single copies of each of the reporter genes, stably integrated in different loci of the yeast genome.

When the proteins X and Y interact and recombine the active transcription factor, it binds the DNA GAL4 binding sequences that are in the promoter region of the reporter genes and activates the transcription. Each reporter gene produce different genotypes (Figure 3.8). The *GAL1::HIS3* gene encodes for the enzyme imidazole glycerol-phosphate dehydratase involved in the biosynthesis of histidine; when the gene is expressed (because the two proteins of interest interact) the yeast cells can grow on media lacking histidine. The gene *SPAL10::URA3* encodes for an enzyme involved in the biosynthesis of uracil, so the positive selection of this gene reporter activation is carried out on media lacking uracil. Finally, the gene *GAL1::lacZ* allows the colorimetric analysis of $\beta-$galattosidase activity following the X-gal assay.

MaV203 colonies grow on YPAD medium plates or broth at 30^o C.

3.2.5.4 Yeast media

Clontech Yeast Media pouches were used to prepare all the media necessary for the two-hybrid assay. Each ready-to-go pouch provides a precise amount of premixed media and supplements to dissolve in 0.5 L of deionized water and autoclave at 121^o C for 15 minutes. The media used in the yeast two hybrid assay are:

- YPAD broth/agar for routine culturing of untransformed yeast;
- Minimal medium single dropout SD -Trp broth/agar for the selection of yeast transformed only with the prey plasmid;
- Minimal medium single dropout SD -Leu broth/agar for the selection of yeast transformed only with the bait plasmid;
- Minimal medium double dropout SD -Leu/-Trp broth/agar for the selection of yeast transformed with both the bait and the prey plasmids;
- Minimal medium triple dropout SD -His/-Leu/-Trp agar to test *HIS3* induction. 50 mM 3-Amino-1,2,4-triazole (3AT) was added on plates containing this medium for the inhibition of the basal level expression of *HIS3* (see section 3.2.5.6).
- Minimal medium triple dropout SD -Leu/-Trp/-Ura agar to test *URA3* induction.

3.2.5.5 Transformation of yeast with bait plasmids

The yeast transformation was performed according to the protocol that uses polyethylene glycol (PEG) and lithium ions (Li^+) [96]. The PEG helps the DNA plasmid to bind yeast cell surface, while lithium ions modify cell wall porosity: the plasmids can easily pass through the holes in the cell wall due to Li^+ and a major number of DNA molecules can enter into the cells once bound to cell membrane thanks to PEG.

The bait plasmids were inserted into yeast competent cells following the one step transformation procedure: 3 ml cultures of single MaV203 colonies

were grown at 30^0 C in YPAD broth; 500 μl of culture were transferred into an eppendorf tube and centrifuged briefly to collect the cells. The supernatant was discard and 1 μg of plasmid DNA was added and mixed with a sterile pipet tip. 5 μl of single stranded carrier DNA, earlier melted for 5 min at 98^o C, and 100 μl of One Step Buffer (1 ml of 1 M LiAc; 4 ml of 50 % PEG 3350 pH 5.0; 0.5 ml of 1 M DTT) were added to the eppendorf tube. The yeast suspension was incubated 30 minutes in a 45^0 C waterbath and than plated on -Leu medium. The plates were incubated upside down at 30^0C for 3-4 days.

3.2.5.6 Self-activation test

Before starting the analyses of putative interactions, it is necessary to test the bait for self-activation and determine the level of basal expression of the reporter gene *HIS3.* MaV203 strain expresses a basal level of *HIS3* and bait proteins often contain a certain level of transcriptional activity. This *HIS3* basal activity can be eliminated by determining a threshold of resistance to 3- Amino-1,2,4-Triazole (3AT, a dose-dependent inhibitor of an enzyme involved in histidine synthesis) above which the colonies growth in absence of histidine will be inhibited. This concentration of 3AT has be included in selective plates lacking histidine used for the interaction screen.

The test for self-activation was performed transforming the yeast cells containing the three bait plasmids with the empty pDEST^{TM}22 (without any insert), using the same procedure describe in section 3.2.5.5, and plating them on minimal medium -Leu/-Trp. The transformation of MaV203 competent cells only with the empty prey plasmid (without any bait) was also performed as control and plated on -Trp medium.

When single colonies appeared, using a disposable sterile inoculation loop, a single yeast colony was taken and resuspend in one well of a 96-wells plates containing 50 μl -Leu/-Trp liquid medium. From each transformation, 6 individual colonies were taken. A 96 pins stamp was sterilized with 70 % EtOH and flames; when cooled, the sterile stamp was put in the 96 well plate containing resuspended yeast colonies, then stamped on plates containing the

appropriate media. For each plate, the stamp was dipped again in the plate and then stamped. The yeast colonies were stamped on -Leu/-Trp medium as positive control and on -Leu/-Trp/-His + 0 mM, 10 mM, 25 mM, 50 mM, 75 mM and 100 mM 3AT media to determine the threshold of resistance to 3AT. The plates were incubated upside down at 30 degrees until colonies were visible on at least the -Leu/-Trp plate (5–7 days).

Comparing the growth of colonies on different plates, the 3AT concentration of 50 mM was chosen for the interactions screen of bait vectors GAL4-DB-Me_SG525 and GAL4-DB-Me_SG526, because at this concentration colonies did not grow anymore. Unfortunately, yeast colonies containing the bait GAL4-DB-Me_SG130 grew perfectly on 100 mM 3AT medium: this means that the bait causes self-activation of *HIS3* reporter gene. Me_SG130 effector will not be used in yeast two-hybrid library screen.

3.2.5.7 Yeast Two-Hybrid Library Screen

MaV203 yeast cells containing the bait vectors were transformed with plasmid DNA from the cDNA library to identify putative plant interactors of *M. euphorbiae* effectors. The procedure to perform the library transformation consists of the following steps:

- Inoculate a single colony containing the bait vector in 10 ml YPAD and let it grow overnight at 30^0 C with shaking.
- Inoculate the preculture in 100 ml of pre-warmed YPAD to give an OD_{600} of 0.25. Shake the culture for 4-6 hours at 30^0 C until the OD_{600} reach 1 ($1.5x10^7$cells/ml).
- Harvest the cells centrifuging for 5 minutes at 3000 rpm and wash the pellet with 50 ml of sterile water.
- Add 1 ml of 100 mM LiAc solution to the cell pellet, resuspend and transfer to a eppendorf tube.
- Pellet the cells by spin and add 100 mM LiAc to a total volume of 750 μl.

- Pellet the cells, remove the supernatant and transfer the pellet in a 5 ml tube.
- Add 3600 μl of 50 % PEG3350 and vortex. Add 540 μl of 1 M LiAc, 375 μl of single stranded Salmon Sperm DNA (2 mg/ml) and 750 μl of plasmid dilution (35 μg of plasmid DNA). Vortex until the suspension is homogeneous.
- Incubate at 30^o C for 30 minutes and at 42^o C for 20 minutes.
- Pellet the cells, remove partially the supernatant and resuspend the cells.
- Plate 10 μl of each transformation on -Leu/-Trp plates (as positive control and to calculate transformation efficiency) and 150 μl on -Leu/-Trp/-His + 50 mM 3AT plates for the interaction screen. Incubate the plates at 30^o C until colonies appear.

Each transformation was plated on 1 -Leu/-Trp plate and on 18 square plates containing -Leu/-Trp/-His + 50 mM 3AT media.

3.2.5.8 Testing *URA3* reporter gene

MaV203 cells that contain bait and prey proteins that strong interact grow on plates without histidine and induce all three reporter genes present in the system. To verify that grown colonies represent true interactors and not false positives, the expression of *URA3* reported gene was tested.

After 5 day of incubation when single colonies appeared on -Leu/-Trp/-His + 50 mM 3AT plates, using a disposable sterile inoculation loop, a single yeast colony was taken and resuspend in one well of a 96-well plate containing 50 μl -Leu/-Trp liquid medium. From each transformation, 192 individual colonies were taken (two 96-well plates). With a sterilized 96 pins stamp the resuspended colonies were stamped on -Leu/-Trp/-Ura medium (2 replicates per each plate). The plates were incubated upside down at 30 degrees until colonies were visible (5–7 days).

Chapter 4

Results and discussion

4.1 Analysis of Arabidopsis plants expressing BbC002 protein

The study of putative plant targets of C002 protein started with the analysis of Arabidopsis mutant plants expressing *C002* gene. Mutti et al. (2008) revealed through in situ hybridization the presence of transcript *c002* only in the principal salivary glands of aphids and detected the presence of C002 protein in fava bean extract after aphid feeding: this effector is a specialized salivary gland protein and is transferred from aphid to plant during feeding probably through saliva secretion. Since aphids feed on plant phloem and inject saliva mainly into sieve elements, it can be hypothesized that C002 protein is delivered into phloem where triggers a molecular mechanism still unknown but essential for the foraging and feeding of aphid. To recreate a condition as natural as possible, the *C002* gene inserted in Arabidopsis transgenic plants, was put under control of the promoter pSUC2 to direct the protein expression into plant phloem and allow the formation of BbC002: plant protein complexes that naturally occur during aphid feeding.

Arabidopsis plants expressing BbC002 protein were analyzed for the presence and expression of the recombinant protein. All the plants used for the pull-down assay were first genotyped to assess that stable transformation occurred properly: a small amount of plant material was used as template in

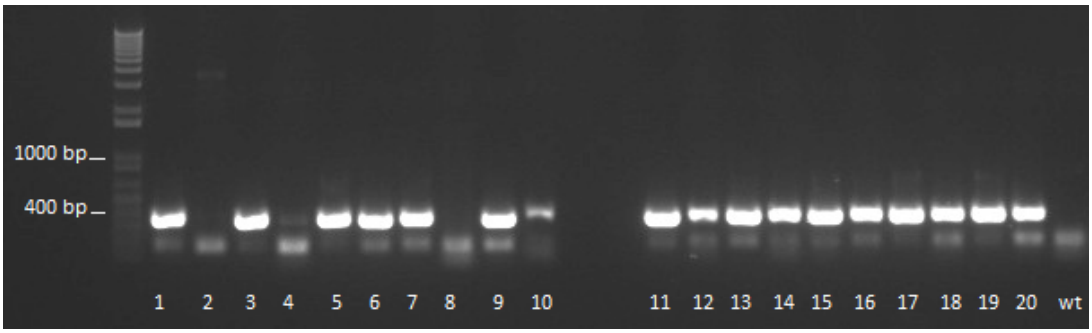


Figure 4.1: Example of Arabidopsis mutant lines genotyping: the 348 bp band corresponding to *C002* sequence is present in almost every sample while the wild type does not show any band (picture taken by ImageQuant 300 Imager).

a direct PCR procedure to amplify the *C002* sequence (it shows no matches to any gene outside of the family Aphididae, so it is not present in plants) and the gene of kanamycin resistance, a selection marker used to selected on medium containing kanamycin Arabidopsis transformed seeds. Samples taken from Arabidopsis wild types were included in PCR plates as negative control. As example, in Figure 4.1 the detection of C002 sequence in 20 plants of pSUC2:BbC002-His-N mutant line is reported: all the analyzed plants show the 348 bp band corresponding to C002 sequence except for samples number 2, 4 and 8; the wild type sample is negative so it means that the PCR worked fine. The plants both kanamycin and *C002* gene positive were selected and used in further analysis.

Three plants per each lines, that had showed positive results in genotyping analysis, were used to verify the expression and the level of expression of *c002* transcript. Total RNA was extracted from one leaf of each sample and the concentration was measured by nanodrop: on average more than 300 ng/μl of RNA were obtained from each extraction. 1 μg of cDNA was synthesized from the RNA and then used as template for RT-PCR. In Figure 4.2 the electrophoresis pattern of RT-PCR is reported: *c002* transcript is not produced in high concentration because after 20 cycles of PCR the band is not visible in none of the samples; moreover pSUC2:BbC002-His-N plants seem to show a weaker expression of the transgene.

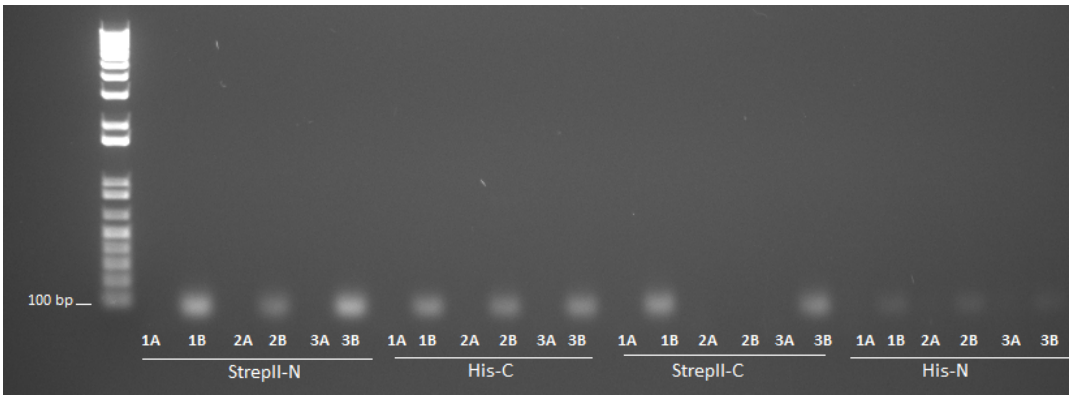


Figure 4.2: RT-PCR result: all the samples show the 98 bp expected band (except for sample number 2 of StrepII-C construct) after 30 cycles of PCR (B), whereas no amplification is visible after 20 cycles of PCR (A). The three His-N samples show less intense bands, probably because *C002* gene is low expressed.

4.2 Phloem sap collection

In order to pull-down BbC002:plant proteins complexes formed in the sieve tubes, phloem exudates from Arabidopsis transgenic plants were harvested and the method of facilitated exudation in EDTA solution was adopted. A major technical problem associated with collection of phloem sap is the sensitivity of sieve tubes to wounding: as result of cutting the vascular bundles, the release of wound calcium induces production of protein plugs and callose constrictions that cause occlusion of the sieve tubes. These problems can be avoided adding calcium-binding compounds to the collection medium: EDTA stimulates phloem exudation by chelating Ca^{2+} and prevents callose deposition [86]. Facilitated exudation technique, first described by King and Zeevart in 1974, is a quick and easy method widely used for collection of phloem sap from Arabidopsis plants [97, 98, 99, 100, 101] but it is not excluded that the wound and EDTA may be responsible for an artefactual composition of phloem exudates [85].

The EDTA facilitated exudation was performed from Arabidopsis inflorescences because they are easy to collect, cut and set for the experiment and because they contain a greater amount of phloem sap than leaves. The

protocol resulted quite quick and efficient but attention had to be payed on some critical steps. First of all, it is very important that the plants are well watered before starting the experiment and that the inflorescences are kept in high humidity condition during the bleeding to avoid that they collapse or suck up the collection medium. It is also crucial to establish the proper number of inflorescences and volume of EDTA solution to use per each tube in order to obtain the greater amount of phloem in as less volume as possible. The phloem sap collection was repeated four times on 50 plants per each transgenic line and at the end, an efficient protocol was established. For example, it was decided to collect the phloem of about 25 inflorescences in 4 ml of EDTA buffer in a growth chamber with 90 % of humidity for 4 hours, checking every 30 minutes the volume of buffer and eventually adding a volume of EDTA solution to maintain the cut part of the inflorescences submerged in the solution. Moreover, a cocktail of protease inhibitors was added to the bleeding buffer to prevent the degradation of native proteins by proteases.

The collected phloem sap was immediately concentrated in the volume needed for the pull-down protocols and re-buffered to obtain a very low concentration of EDTA that could interfere with the magnetic beads used for the tagged protein extraction.

4.3 Affinity tag purification and mass spectrometry analysis

To identify *in planta* molecular targets of the effector C002, recombinant BbC002 protein was expressed in Arabidopsis plants as fusion with an affinity-tag and the purified protein complexes were detected through MS technique. Affinity purification of complexes coupled to MS detection offer some advantages, but also some drawbacks. The entire approach is more physiological in comparison to other methods as yeast two-hybrid, because actual molecular assemblies made up by all combinations of direct and cooperative interaction are analysed *in vivo*, rather than re-constituted interaction *ex vivo* or

in vitro. The approach is not restricted to one cell type or organism and in this strategy only one component of the complex is expressed as a fusion protein, minimizing possible steric interference. Nevertheless, this method is not economical, is difficult to automatize for large scale approaches and is not very suited for the detection of transient or unstable interactions [80].

Several different affinity-tag systems exist but they share some common features: minimal effect on tertiary structure and biological activity, one-step adsorption purification, easy and specific tag removal to produce the native protein, applicability to a number of different proteins [87].

The ample choice of affinity tags for protein purification can make it difficult to decide the best fusion system for a specific protein of interest. This depends on the target protein itself, for example stability and hydrophobicity, the expression system and the application of the purified protein. To choose an effective selection, the advantages and disadvantages of various tags must be considered with respect to their ability to increase the yield, enhance the solubility, and facilitate the purification of their fusion partners. In our experiment, the protein of interest is fused with two different tag. The hexahistidine tag (His) combines the advantages of small size and charge which ensure that protein activity is rarely affected with the added benefit of interacting with a chromatography matrix that is relatively inexpensive and exhibits a high binding activity. Moreover, elution conditions are mild and flexible and His-tag works under both native and denaturing conditions. However, specificity of immobilized metal affinity chromatography is not high as other affinity methods and proteins with a His-tag may vary slightly compared to the native protein. StrepII-tags exhibit a high degree of specificity for their binding partner and a low metabolic burden, but the resins that they interact with tend to be expensive and the tag does not enhance recombinant protein solubility [88]. Because optimal placement of the tag is protein-specific in our experiment the two tags were placed on either N- and C-terminus of recombinant BbC002 protein.

Altogether, both the pull-down of StrepII-tagged complexes through Strep-Tactin magnetic beads and purification of His-tagged protein through magnetic beads covered of cobalt ions from phloem sap resulted easy and quick

to perform and allow the elution of on average 150 $\mu g/ml$ proteins per each construct.

The proteins obtained from the pull-down assay were digested by trypsin and the molecular masses of proteolytic peptides were detected by MALDI-TOF mass spectrometry. This is a sensitive high-throughput MS technique that requires only small amounts of proteins, provides a relatively fast identification and can easily be automated.

Ions are generated by Matrix-Assisted Laser Desorption/ionization, accelerated by a high electric potential and separated by the time taken to reach a detector (Time-of-Flight). The time-of-flight is directly proportional to the mass-to-charge ratio of an ion, and hence a mass spectrum is obtained. In Figure 4.3 it is shown an example of mass spectrum obtained from MALDI-TOF MS analysis: the x axis represents the ratio m/z whereas the y axis represents absolute intensity, that is the number of ions of each species that reach the detector. However, abundance in the gas-phase is not usually representative of abundance in solution. In the interpretation of MALDI Mass Spectra some features have to be taken in account. Ions are nearly always singly charged $[M^+H^+]$ or less often doubly or triply charged so to obtain an exact molecular weight it is necessary to subtract the mass of a single (or double and triple) hydrogen from the mass shown on a spectrum. When shown not at full scale, groups of peaks, called isotope distributions, are clearly noticeable; this is caused by naturally occurring of Carbon-13 (it has an abundance of about 1 % of 12C) that increases the mass of a peak of 1Da. As a peptide contains many carbon atoms, then the contribution from 13C can be significant. The peaks containing only 12C are called monoisotopic peak and are the only ones to be considered for peptide mass annotation. Finally, peaks resulting from autolysis of trypsin, from keratin and other contaminants have to be removed from the mass list.

The data from MALDI-TOF peptide mass fingerprinting in the form of a simple list of masses (peaklist) were used for database searches using the tool Mascot, Matrix Science. Mascot takes the mass spectrometry data and searches it against molecular sequence databases; then computes the probability that the observed match between the experimental data and mass

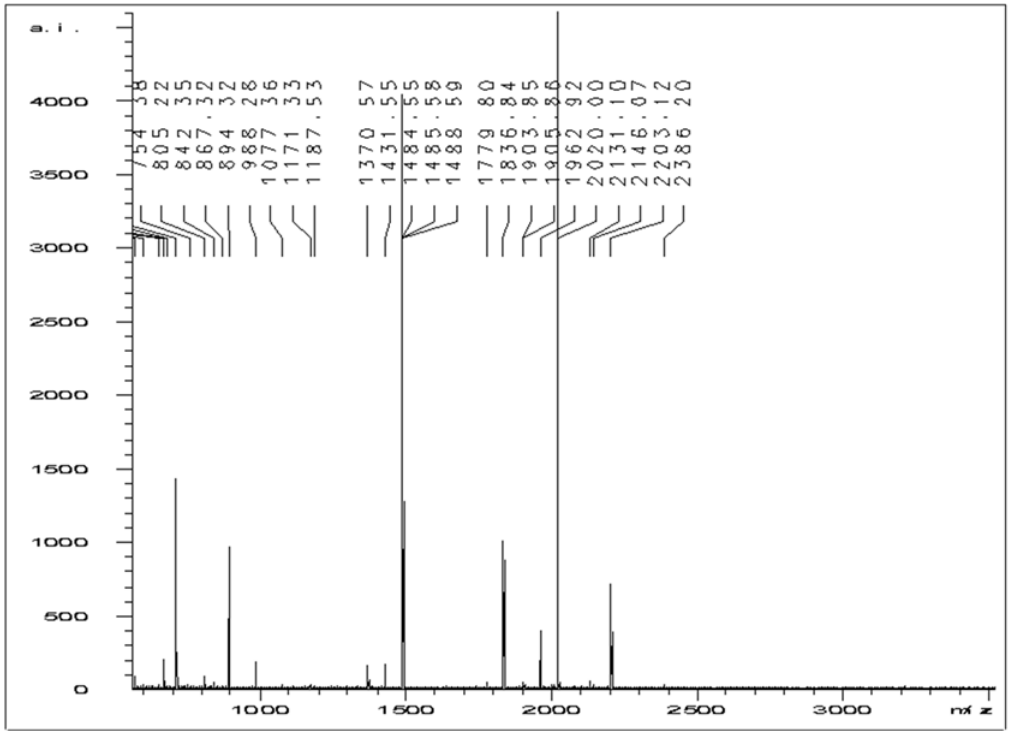


Figure 4.3: Example of MALDI-TOF Mass spectrum of a tryptic digest of proteins isolated from Arabidopsis phloem sap: on the x axis the mass-to-charge ratio is reported; the y axis represents the abundance intensity. The molecular mass values of the main peaks are reported.

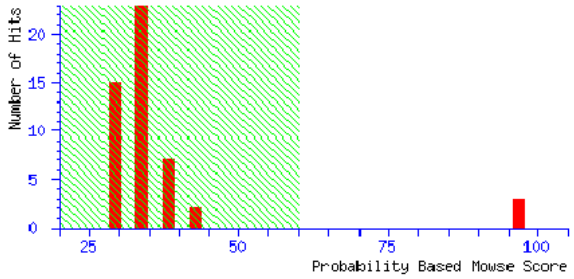
values calculated from a candidate peptide or protein sequence is a random event. The correct match, which is not a random event, has a very low probability. Figure 4.4 illustrates typical result report. The histogram of the score distribution for the 50 best-matching proteins is displayed: in this case a cut-off score of 60 was set for the confidence threshold; the number of protein matches at each scoring position is indicated by the height of the red bars whereas the non-significant area is shaded in green. The next section of the result report is a tabular summary of the matching proteins: for each protein the accession number, the molecular mass, the overall score and the FASTA title line are reported. The protein view report includes details on how individual MS spectra were matches to peptide sequences: a list of peptides expected from the digest; the observed mass/charge of the ion; the calculated uncharged mass; the theoretical mass of the closest-matching peptide and the theoretical-observed difference; the number of missed trypsin cleavage sites to identify peptides originated from incomplete proteolytic digestion of the original protein; the start and the end of peptides in protein sequence; the amino acid sequence of peptide eventually with fixed modifications (as oxidation). At the end, the reports displays sequence coverage map of identified protein: red amino acids correspond to those that were matched to experimental data.

4.4 Putative BbC002 interactors

The lists of best-matching proteins obtained from 4 different experiments on 4 biological replicates (N-StrepII, C-StrepII, N-His and C-His) were combined together and a big table containing respective number of hits (how many times in total the protein was detected in the different experiments), score, function, accession number was generated. All the proteins detected at least 4 times in the different experiments (number of hits of 4) were taken in account for further analysis.

The Arabidopsis-cabbage aphid system has been already successfully utilized to identify a number of plant genes and mechanisms that contribute to plant defense against aphids, but to date resistance gene against aphids in

MATRIX SCIENCE Mascot Search Results



Accession	Mass	Score	Description
gi|9294400	33517	33	fertilization-independent endosperm protein-like [Arabidopsis thaliana]
gi|9757873	32803	43	adenylylsulfate kinase-like protein [Arabidopsis thaliana]
gi|9758448	68914	37	unnamed protein product [Arabidopsis thaliana]

Observed	Mr(expt)	Mr(calc)	Delta	Start	End	Miss	Peptide
679.47	678.46	678.41	0.06	517 -	522	1	AYVKAK
867.32	866.31	866.46	-0.14	205 -	211	0	LALEYMK
894.32	893.31	893.47	-0.15	129 -	135	0	NEFLLMK
1077.36	1076.35	1076.59	-0.24	495 -	503	1	LFEEVKNAK
1488.59	1487.58	1487.80	-0.22	449 -	461	1	EALKNIEMGLNLK 1 Oxidation (M)
1962.92	1961.91	1962.04	-0.13	531 -	548	1	RMVLGGARPDAESYSLLK
2131.10	2130.09	2130.18	-0.09	136 -	151	1	ILLNLMEWIIRERPYR 1 Oxidation (M)
2386.20	2385.19	2385.19	0.00	285 -	306	1	KAGVEPNEVSYCILAMAHAVAR

No match to: 671.19, 711.31, 805.22, 842.35, 988.28, 1171.33, 1187.53, 1370.57, 1431.55, 1484.55, 1485.58, 1779.80, 1836.84, 1903.85, 1905.86, 2020.00, 2146.07, 2203.12

```
   1 MTDTTDDIAE EISFQSFEDD CKLLGSLFHD VLQREVGNPF MEKVERIRIL
  51 AQSALNLRMA GIEDTANLLE KQLTSEISKM PLEEALTLAR TFTHSLNLMG
 101 IADTHHSQLL QSGISPDELY KTVCKQEVEI VLTAHPTQIN RRTLQYKHIR
 151 IAHLLEYNTR SDLSVEDRET LIEDLVREIT SLWQTDELRR QKPTPVDEAR
 201 AGLNIVEQSL WKAVPQYLRR VSNSLKKFTG KPLPLTCTPM KFGSWMGGDR
 251 DGNPNVTAKV SLLIFYDLNS KPTGHERISL LSRWMAIDLY IREVDSLRFE
 301 LSTDRCSDRF SRLADKILEK DYMPPNLQKQ NEQDFSESDW EKIDNGSRSG
 351 LTSRGSFSST SQLLLQRKLF EESQVGKTSF QKLLEPPPLK RAGSAPYRIV
 401 LGEVKEKLVK TRRLLELLIE GLPCEYDPKN SYETSDQLLE PLLLCYESLQ
 451 SSGARVLADG RLADLIRRVS TFGMVLVKLD LRQEAARHSE ALDAITTYLD
 501 MGTYSEWDEE KKLEFLTREL KGKRPLVPQC IKVGPDVKEV LDTFRVAAEL
 551 GSESLGAYVI SMASNASDVL AVELLQKDAR LALTSEHGKP CPGGTLRVVP
 601 LFETVNDLRA AGPSIRKLLS IDWYREHIQK NHNGHQEVMV GYSDSGKDAG
 651 RFTAAWELYK AQENVVAACN EFGIKITLFH GRGGSIGRGG GPTYLAIQSQ
 701 PPGSVMGSLR STEQGEMVQA KFGIPQTAVR QLEVYTTAVL LATLKPPQPP
 751 REEKWRNLME EISGISCQHY RSTVYENPEF LSYFHEATPQ AELGFLNIGS
 801 RPTRRKSSSG IGHLRAIPWV FAWTQTRFVL PAWLGVGAGL KGVSEKGHAD
 851 DLKEMYKEWP FFQSTLELIE MVLAKADIPM TKHYDEQLVS EKRRGLGTEL
 901 RKELMTTEKY VLVISGHEKL LQDNKSLKKL IDSRLPYLNA MNMLQVEILK
 951 RLRRDEDNNK LRDALLITIN GIAAGMRNTG
```

Figure 4.4: Example of Mascot Search Result and Protein View report.

Arabidopsis has not been reported [102].

Almost one-third of the identified proteins have unknown function and are involved in biological processes that remain still undiscovered. These proteins constitute the most interesting and exiting aspect of further studies about plant-insect relationship: they likely interact with a salivary protein injected by aphid's stylet during penetration into plant cells and can be involved in the mechanism triggered by saliva secretion of reprogramming plant defense responses. To test these proteins not only will allow the identification of putative traits involved in plant resistance/susceptibility to aphid attack but also will allow the discovering of new pathways leading plant-aphid interaction.

Some of the proteins present in our list belong to the cytochrome P450 superfamily composed by proteins involved in glucosinolate metabolism. Glucosinolates are plant secondary metabolites, sources of thioacyanates and other breakdown products that are toxic to some aphids. They mediate numerous biological interactions between cruciferous plants and their natural enemies, such as herbivorous insects, pathogens, and other pests. There are several studies that show the negative impact that glucosinolates have on fecundity and performance of different types of generalist aphids and that report the induction of plant genes related to glucosinolates biosynthetic pathway during aphid salivation, but the effect of these metabolites breakdown products against the specialist cabbage aphid have not been reported yet [102].

One candidate protein is encoded by a disease susceptibility gene involved in defense response to molecule of fungal origin; another has oxidoreductase activity and has a function in toxin catabolic process and defense response to fungus; another one belong to the heavy metal transport/detoxification superfamily protein. Most of the identified proteins are located in mitochondria and in cytoplasm as some heat-shock proteins and there are also molecules that occur in nucleus, like transcription factors, proteins involved in plastid movement and proteins located on the plasma membrane, as kinases that can have a role in signal cascade triggered by aphid effectors.

Arabidopsis insertional mutant lines were ordered from the European

Arabidopsis Stock Center: these plants contain a knock-out in the genes encoding for the proteins listed after mass spectrometry analysis and they will be used in aphid bioassay to verify whether they exhibit altered resistance to cabbage aphid.

4.5 Aphid fecundity assay

In different studies [25, 50, 52, 74], it was demonstrated that C002 effector plays an important role in aphid feeding and reproduction and enhances fecundity in both *A. pisum* and *M. persicae* (see section 1.4.1). We attempted to confirm the same results in cabbage aphid using Arabidopsis plants over-expressing *BbC002*. The two mutant line BbC002-N-StrepII and BbC002-C-StrepII were used for an aphid bioassay: the hypothesis is that aphids feeding on transgenic Arabidopsis plants will show an increase in fecundity rate compared to aphids feeding on wild type plants. The number of nymphs produced 10 days after inoculation of a single small nymph on plants was assessed and mean values, standard errors and Student's t test one-tailed were calculated. As shown in Figure 4.5, the number of nymphs produced resulted greater in BbC002 transgenic lines than in the control Col-0 and for the construct C-StrepII the difference is statistically significant: in fact *B. brassicae* produced approximately 40% more progeny on BbC002-C-StrepII line that on Col-0.

Our study is thus in agreement with precedent published findings, because we demonstrated that *B. brassicae* shows an increased fecundity on transgenic plant producing BbC002 protein: this provides evidence of the importance of C002 salivary component in plant-aphid interaction and his role in facilitate aphid infestation.

4.6 *M. euphorbiae* effectors

The study of putative plant targets of *M. euphorbiae* effectors started from the analysis of the sequences of *Me10* (Me_SG525), *Me23* (Me_SG130) and

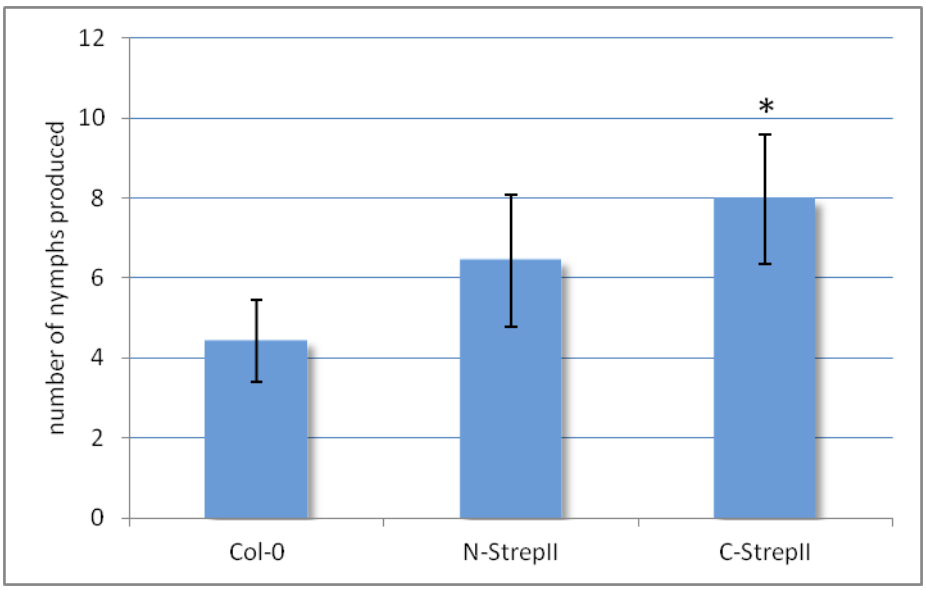


Figure 4.5: *B. brassicae* produces more progeny on Arabidopsis BbC002 transgenic lines in comparison to Col-0. Columns show the average nymph production plus standard error bars of the third biological replicate consisting of the progeny produced by 1 aphid per plant, 30 plants per genotype. Asterisk (*) indicates Student's t test one-tailed, $P < 0.05$ compared with aphid fed on wild type plants.

MeC002 (Me_SG526) effectors. The mRNA sequences were downloaded from GenBank database and from the reverse complement sequences the predicted signal peptide and primer pairs to amplify the genes were identified (Figure 4.6). The three genes were isolated from cDNA synthesized from 1 μg of RNA extracted from a single adult potato aphid and the right length of the amplicons was verified through electrophoresis on 1 % agarose gel (Figure 4.7). The three genes, added with *attb* sites, were cloned using Gateway® Technology into pDONR^{TM}221 vector and *E.coli* chemically competent cells were transformed with the obtained plasmids. To verify the transformation of bacterial cells with the donor vectors and the correct lenght of the insertions, the bacteria cells were plated on selective medium and Colony PCR with specific primers per each construct was performed on 12 colonies per each transformation. In Figure 4.8 the results on 1 % agarose gel of Colony PCR are shown: all tested colonies present the inserts of the right length. Once obtained the entry vectors, LR reactions were performed to transfer the inserts in two different destination vectors and also in this case, the correct insertion of the three effector sequences into plasmids were verified through Colony PCR. The generated constructs are the following: pK2GW7:Me_SG525, pK2GW7:Me_SG526, pK2GW7:Me_SG130 for the expression of the genes in pepper plants and pDEST^{TM} 32:Me_SG525, pDEST^{TM} 32:Me_SG526, pDEST^{TM} 32:Me_SG130 for the yeast two-hybrid analysis.

4.7 Aphid bioassay on pepper plants

As for the aphid bioassay on transgenic Arabidopsis expressing BbC002 gene, the attempt of this experiment was to confirm the results obtained by Atamian and associates (2002) that *Me10* and *Me23* effectors enhance aphid fecundity (see section 1.4.2). They demonstrated that these two candidate effectors increase the performance of *M. persicae* on tobacco plants expressing the two genes and that *Me10* expression in tomato plants increase *M. euphorbiae* production of nymphs. Our aim is to verify whether these two effectors and MeC002 expressed in pepper leaves affect *M. euphorbiae* re-

>gi|407017537|gb|GAAF01000080.1| TSA: Macrosiphum euphorbiae Me_SG525 mRNA sequence (Reverse Complement)

GAAGCCGACGGTTCCACCAGGGTGGTCACCATCATAAGTTATAGTGTACTAGAAGGCAATGCAATGCAATCAATACAACCATTAATAGACCAAGATTACA
TGAAAGTAAAAGCCGCATTGTATGACTTGGGAGAAGTTGGAATGAACTTGATGGACGATTCGCAGACATTGAACGATATGCAAAGAGAATATTTTGCTGG
AAAAGTCGATTATTCTGCTGTGGAAAGAGCAAGGAATGAACTTAACAAGACCAAAAATAAGTTGTTCCTAAAACTTATAAAATATATTTGGGCAACAAAT
GAATTTGAGCCTACAATCAATTATCAAACCGCAGATCCCCACAAACTTTACAAAACCATGGACGACTTGGAAAATTACAAAGATAGCATAGATGATATGC
ATGCCGATCTTCTTAACGCAATGTCCCAACCAACAGTCGTTGGAGCATAA

>gi|407017589|gb|GAAF01000028.1| TSA: Macrosiphum euphorbiae Me_SG130 mRNA sequence (Reverse Complement)

ATGAAGGTTCAAGAATATATTCTTCTAATTTTTGCCGCTATTTCATTTTGGGCGATGGAGCCAATTGCTCCAAGGGTTTCCCAAGGATTTCCCTACGAGT
CTCTCGACTGGCCGACAATGCCCGACATTTCTGGATCGTCGCCCACAGGATACGACAAATCGTCGTCCTCCGGATACGACAAATCGTCGGATGAGGATAA
CTGCAATGAGGACTCGTGCGGCAACATTTACAAATTCACCGCAAGAAAACCCAACGGACAAAACGTTTGTTTGCAACAATACGTTGGAAAAGTGTTGATC
ATTGTGAATTACGCTTCAGCATGTGGTTTCACATACGACAACGTTTGCACTCTATCGGACTTTGCTCAAAGGTACAGGAAATGTGGCTTGGAGATACTGG
TATTTCCCAGCAACGACTTCTTACAGAACTTCGGTGGGGATATCGCAGCCGAAGAGTTCGCAAATAATCATCCTGAATTTGAAGTATTTTCGGAAATTTG
TGTAAATGGCAGATCACAACATCCTTTGTACAGATTTCTCAAGAATAAGTTACCGGGTGCTTGTAACGCAAAACCTATTAAATGGAATTTTACCAAATTC
GTAGTAGACAGGAACGGATGTCCCGTTCAACGATATGCGGCCACAGACAGTTTTAAGGACATAGAAGATTTAGTACAAGAACTGTTAAAGGACCAATGTT
GCTAA

>gi|407017532|gb|GAAF01000085.1| TSA: Macrosiphum euphorbiae Me_SG526 (=MeC002) mRNA sequence (Reverse Complement)

ATGGGAAGTTACAAATTATACGTAGCCGTCATGGCAATAGCCATAGCTGTAGTACAGGAAGCTAGATGTATGGGTGGGTCTTCTGACGATGAACCTTTCG
ATGAGCAGGAAGAAGCGTCTGTTGAAATACAGATGGAGCACCGCCAGTGCGATGAATACAAATCGAAGATCGGGGACAAAGCATTTAGCAACCCAGCGGC
TATGCAGTTGATGGAACTACCGTTTAATACAGGTAAGGAATTAGGCTCCGACGAAGTGTGCTCGGACACAACGCGGGCCATTTTTAACTTCGTCAGTGTG
ATGGCCACCAGCCAGAACGCCCATTACTCGCTGGGTATGATGAACAAGATGCTGGCGTTCATCCTGAGGGAGGTGGACACGACGTCCGACAAATTCAAAG
AGACGAAGGCGGTTGTCGAACGCATCGCGAAAACTCCAGAGATCCGAGACTCTATCAGGCACACGACCGCCCGGACCGTCGACTTGCTCAAGGAGCCCGT
GATTAGAGGCCGACTGTTCAAAGTGGTGAAAGCCTTCGAGGGTCTGATAAAACCGTCCGAAAACGAGGAAATGATCAAGCAGAGGATTAAGGGGATAACC
AATGCTCCCGCCAAGATGGCTAAGGGAGCCATGAATAAGGTTGGAAGTTTCTTTCGACGTTTTTAA

Figure 4.6: mRNA Sequences of Me_SG525, Me_SG130 and Me_SG526 with the respective accession number. The underlined sequences encode for a predicted signal peptide (prediction performed by SignalP 4.1 Server) and the highlighted regions are the primer sequences designed to isolate the genes from aphid cDNA.

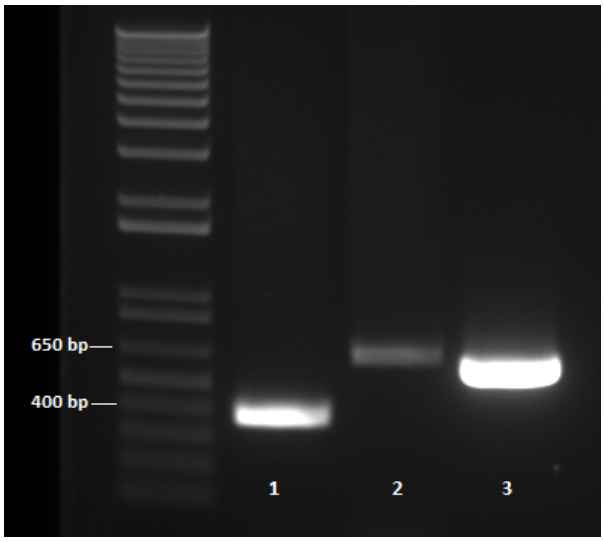


Figure 4.7: Amplification of *M. euphorbiae* effectors from cDNA. (1) Me_SG525 sequence, 385 bp. (2) Me_SG526 sequence, 594 bp. (3) Me_SG130, 648 bp.

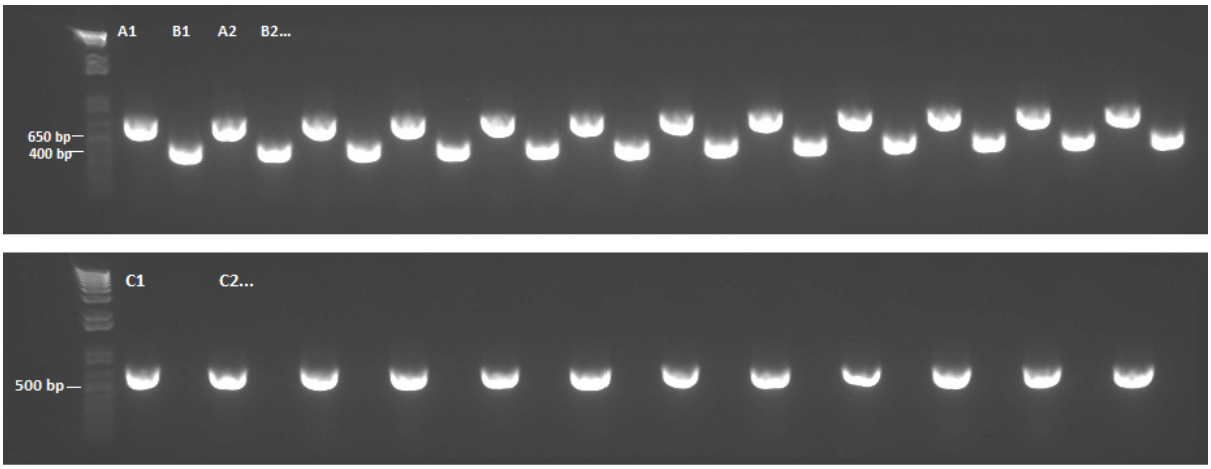


Figure 4.8: Colony PCR results: all 12 colonies transformed with donor vector containing Me_SG130 (A), Me_SG525 (B) and Me_SG526 (C) presented the correct bands of respectively 648 bp, 385 bp and 594 bp.

production rate. The three candidate genes were transiently overexpressed in pepper plants using *A. tumefaciens*; two days after infiltration each leaf sample was caged with one *M. euphorbiae* nymph. As negative control leaves were infiltrated with $MgCl_2$. As shown in Figure 4.9, after 10 day of incubation, as expected, the leaves infiltrated with *A. tumefaciens* showed the symptoms of chlorosis and browning on the infiltrated spots in comparison with the control leaves that maintained a normal green color and did not show any damage. The number of nymphs present in every cage was assessed: unfortunately the aphids showed a very low rate of reproduction in every sample, with an average of about 2 nymphs per spot. Only in few samples, more then 5 nymphs were produced and some aphids did not reproduce because they were parasitized by fungi. The small differences of reproduction rate between the three constructs and the control were not statistically significant.

Gene expression analysis was performed to verify the presence and expression of the three gene effectors in transformed leaves. Per each construct, plant material was collected from an infiltrated (local) and non-infiltrated spot (systemic) and reverse transcription PCR analysis was performed using cDNA prepared from collected plant material. All the samples were amplified with primer pair for the Ubi-3 gene as positive control to check the quality of cDNA, and every sample expressing one of the three transgenes was amplified with the primer pair for the respective gene. The Figure 4.10 shows the results of RT-PCR analysis: all the samples present the band of Ubi-3 gene demonstrating that the cDNA synthesis was well performed and the cDNA was of good quality; the systematic samples (non-infiltrated) and the control do not show the band of the transgenes as expected, whereas Me_SG525 and Me_SG526 local samples present the expected band of respectively 385 bp and 594 bp. Only Me_SG130 local gave a negative result, because the expected band of 648 bp was not visible: probably the gene was not expressed in the analyzed infiltrated leaf or something went wrong during *A. tumefaciens* transformation.

We did not succeed in verify the enhancement of fecundity of aphids feeding on leaves overexpressing the three effectors under consideration, because

(a)

(b)

(c)

Figure 4.9: Agroinfiltrated leaves used in aphid bioassay. The *A. tumefaciens* infiltration induces symptoms of chlorosis and browning both on upper (a) and lower (b) surfaces of leaves. In Figure (c) the nymphs produced on leaf expressing Me_SG526 are shown: it is possible to distinguish an adult, six nymphs and one white cast skin.

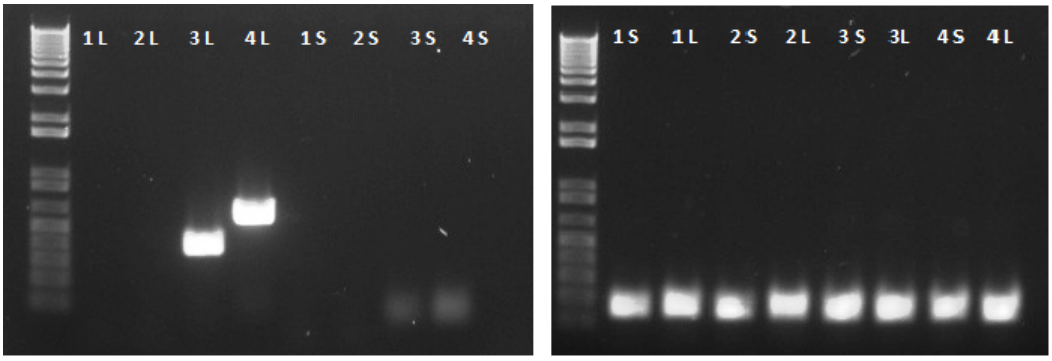


Figure 4.10: RT-PCR analysis on infiltrated pepper leaves: (left immage) Me_SG525 (1) and Me_SG526 bands are visible in the local samples (L) whereas are not present in the systemic samples (S). Me_SG130 (2) gene seems to be not expressed in the local spot. All the samples, included the control (1) showed the UBI-3 gene used as positive control for RT-PCR (right image).

the experiment needs some improvements: first of all, it is necessary to have more replicates per each sample (we only used 2 plants and 5 leaves per plants per each construct) to obtain enough data for statistical analysis. A strategy can be to utilize a leaf disk assay as reported in several studies [50, 52]: from the infiltrated leaves, disks are cut and placed in single wells of a 24-well plate on top of a plug of 1 % solidified agar; then aphids are inoculated onto leaf disks and wells are sealed with mesh lids. In this way it is possible to have a greater number of replicates in small space and to use less starting plant material; however this setup is an artificial system and does not reflect exactly the natural situation of aphid feeding. Finally, it can be considered to infiltrate leaves with *A. tumefaciens* harboring an empty plasmid or a plasmid containing a reporter gene (as GFP) as control: in this way control leaves will be exposed to the same stress of the treated samples (infection of *Agrobacterium*) and the differences in aphid fecundity will be attributed only to the different overexpressed genes.

4.8 Yeast two-hybrid assay

Interactions between the three effectors secreted by *M. euphorbiae* through saliva and plant proteins were analyzed by yeast two-hybrid assay. The yeast two-hybrid is a powerful and efficient method to assess binary physical interactions and to identify new interactors of proteins of interest. This system is an *ex vivo* assay that uses eukaryotic cells as "bio-reactor" (yeast cells); it does not require protein purification steps and heavy manipulation of insert sequences during the cloning; it is up to characterize either weak and transient interactions; it is economical, scalable and perfectly suited for automated high-throughput approaches. There are several ways of exploiting the yeast two-hybrid system for high-throughput approaches: the most elaborate one to screen entire genome is the library approach. In the library approach, each bait is screened against an undefined prey library containing random cDNA fragments or open reading frames (ORFs). Positives clones are selected based on their ability to grow on specific substrates and interacting proteins are determined by DNA sequencing. However the yeast two-hybrid method presents some limits: first, the interaction is forced to occur in the nucleus, which poses problems for certain protein classes; second transcription factors and other proteins can self-activate transcription of the reporter; third it is possible that expressed protein does not undergo post-translationally modification in the proper way in yeast cells. Finally, some proteins can be barely expressed, degraded by yeast proteases or can be toxic and inhibit growth of yeast cells.

In our study the ProQuest^{TM} system was used to screen the three *M. euphorbiae* effectors generated as baits against a pepper cDNA library. Before starting the interaction screening, the three baits were tested to determine the level of basal expression of the reported gene *HIS3.*

4.8.1 MaV203 transformation

The MaV203 yeast strain was used because it presents some specific features necessary for the experiment: deletion of genes *GAL4* and *GAL80* and presence of reporter genes inducible by the transcription factor GAL4 to identify

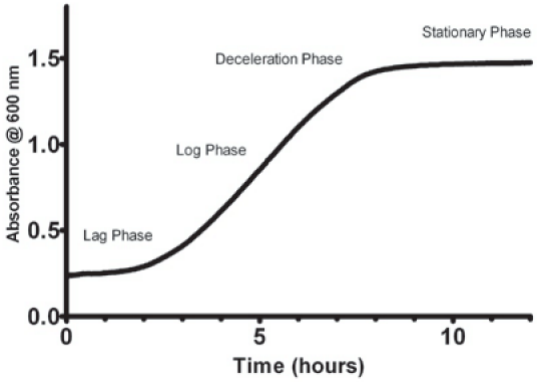


Figure 4.11: Growth curve of yeast cells.

protein interactions; the yeast strain is also auxotrophic for leucine and tryptophan to allow the selection of cells co-transformed with bait and prey vectors. MaV203 strain shows a growth curve typical of yeast cells (Figure4.11): after a lag phase of scarce and slow growth, an exponential growth follows (log phase) that then decelerates and becomes stationary when the nutritive substances are limiting and the produced secondary metabolites start to interfere with cellular development. The one step yeast transformation protocol used to introduce the three bait plasmids into MaV203 [96] requires yeast cells in the stationary phase when they show a density up to 2.5x10^8cells/ml. The procedure to perform the library transformation, instead, uses yeast culture grown until the middle of the log phase, when they have an optical density of 0.6-1 that means about 1,5x10^7 cells per milliliter.

In our experiment, the yeast cells were first transformed with only the three bait vectors and selected on -Leu medium. Then, one positive colony per each construct was transformed with the empty vector, pDEST^{TM}22, to perform the self-activation test and determining a threshold of resistance to 3AT above which the colonies growth in absence of histidine will be inhibited. In both the procedures, the one step transformation protocol described by Chen et al. (1992) that uses of a mix of transformation (one step buffer) composed of lithium acetate, PEG and DNA carrier single-stranded. was chosen: it showed a quite good transformation efficiency of about 10^4 transformant/μg of plasmid DNA. After 4-5 days of incubation, up to 50 big

colonies grew on selective plates, demonstrating the good efficiency of the used method.

4.8.2 Self-activation test

Before analyzing the interactions between the baits and the cDNA-preys, the self-activation test was performed. The three baits can activate a certain level of expression of the reported gene *HIS3* and allow the growth of yeast cells in media lacking histidine either when a real interaction between a protein bound to the activation domain does not occur. To eliminate this basal production of histidine a dose-dependent inhibitor (3AT) of histidine production is added into the selective plates used to test proteins interactions. The self-activation test is needed to determine the 3AT concentration to add in the selective media.

Six different 3AT concentrations, 0 mM, 10 mM, 25 mM, 50 mM, 75 mM and 100 mM, were added into -Leu/Trp/His medium and for each of them 2 replicated were tested. Moreover, the same colonies transformed with the three baits + pDEST^{TM}22 empty were stamped into -Leu/-Trp plate as positive growth control. In Figure 4.12 the results of the self-activation test are shown: in absence of 3AT the colonies grow normally as the control but as the 3AT concentration increases, the growth of colonies containing Me-SG525 and Me_SG526 baits decreases and it is totally blocked when 3AT is 50 mM (the pale visible trace represents the colony stamped on the medium). For these two baits the 50 mM 3AT concentration was chosen for the interaction screening. Me_SG130 protein, instead, could not be used for the yeast two-hybrid test because resulted a strong activator of the reporter gene *HIS3*: colonies transformed with this bait showed at 100 mM 3AT a growth comparable to the growth on control medium.

The occurrence of DB-X fusions that can activate transcription independently of an interaction with an AD-Y protein is one of the major limitation inherent to the two-hybrid system. because self activators can not be used in interaction screening. Self activators include proteins that act as transcriptional activators in their respective organisms and maintain this ability

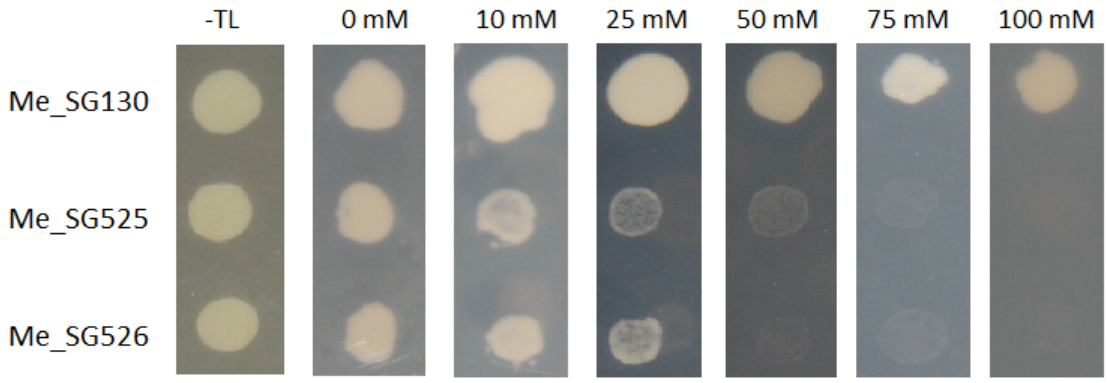


Figure 4.12: Self-activation test: the same colony per each construct is shown on plates containing different concentration of 3AT. The colonies transformed with Me_SG525 and Me_SG526 baits stop to grow at 50 mM 3AT, whereas the colony containing Me_SG130 bait plasmid continues to grow well also at 100 mM 3AT.

in yeast and also proteins that normally act in other processes but exhibit transcriptional activity when tethered to a promoter in yeast cells [103]. One possible solution is to generate bait plasmids that lack parts of the coding sequence of the gene of interest and test whether these self-activate the reporter. In our case, the aphid protein Me_SG130 is a glutathione peroxidase and it was supposed not to have transcriptional activation ability: however when expresses in yeast cells, seems to act as a strong activator of the reporter gene. This protein is thus not suitable for the yeast two-hybrid assay.

4.8.3 Test of interactions

Once determined the 3AT concentration for the selective media, the plates -Leu/-Trp/-His + 3AT 50 mM were prepared to test the interaction between the two bait proteins Me_SG525 and Me_SG526 and the cDNA preys through activation on *HIS3* reported gene.

35 μg of plasmid DNA representing the pepper cDNA library were introduced into yeast cells containing the two baits. The protocol used for transformation is similar to the procedure used for the introduction of bait plasmids in host cells because the transformation is carried out by a buffer

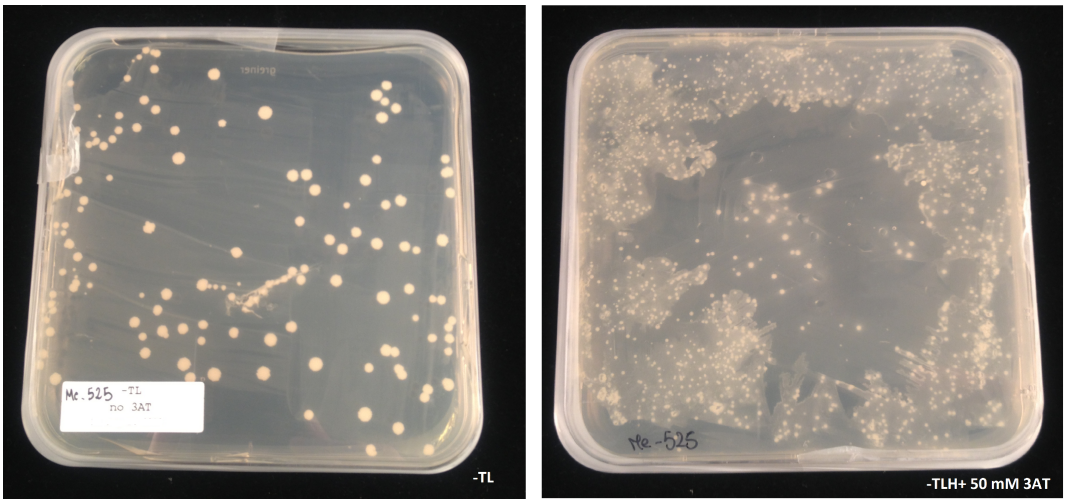


Figure 4.13: Comparison between library screen grown 4 days at 30^0 C plated onto -Leu/Trp and -Leu/-Trp/-His + 3AT 50 mM plates. The colonies grown on the control medium appear big and well isolated: about 100 colonies are present indicating a transformation efficiency of about $3x10^5$ colonies per reaction. The colonies on the interaction-selective medium are small, close each other and often impossible to isolate from the near colonies. In theory, they contain interacting bait-prey but, as demonstrated with the test of *URA3* reporter gene, they represent growth background and false positives.

mix consisting in lithium acetate, PEG and single stranded Salmon Sperm DNA; the difference is that the transformation of cDNA library is performed in a bigger volume of yeast culture and utilize a bigger concentration of plasmid DNA. The transformed cell suspensions were indeed plated onto 18 selective plates and one -Leu/Trp plate as positive control to calculate transformation efficiency. The transformation for the library screen was successful because showed a transformation efficiency (calculated as number of colonies per transformation reaction = colonies on a plate x dilution factor × total volume / plated volume) of about $3x10^5$ colonies per reaction. -Leu/-Trp/-His + 3AT 50 mM plates presented the growth of a great number of small colonies: theoretically every colony grown on plates without histidine harbors a couple of bait and prey that interacting activate *HIS3* reporter gene. However some of these may be false positives. To identify the true interactors, 192 single colonies per each transformation were resuspended in

liquid culture and stamped on -Leu/Trp/Ura plates: yeast cells that contain bait and pray proteins that strongly interact will induce the *URA3* reporter gene, too. Unfortunately, none of the tested colonies grew on medium lacking uracil: the interactions observed during the first screen were not confirmed by the second reporter gene screen.

Probably the colonies grown on plates lacking histidine represented only transformation background; there were a lot of dense and small colonies that could help each other in growing on minimal medium and could "hide" the colonies containing true interactors. The experiment has to be repeated with some improvements in the procedure: a smaller amount of cells suspension has to be plated into the selective media to obtain single isolated colonies; it can be considered to test cDNA preys for self-activation and to use a lower incubation temperature for yeast growth (some proteins can undergo modifications at 30^{o}C).

4.9 Comparison between the two systems used to analyze protein interactions

In this study two different methods were used to identify putative plant proteins interacting with various effectors secreted by aphid saliva during feeding: the pull-down technology coupled to mass spectrometry and the yeast two-hybrid system. Both the experimental methods are *in vivo* techniques that enable screening of a large number of protein using a protein as bait to "fish" putative interactors. The method used to study *B. brassicae* effector resulted to be a longer and more expensive procedure than the yeast two-hybrid and presented several critical step not simple to implement. In fact, once obtained the cDNA sequence of BbC002 effector and chosen the suitable tags to use, the gene of interest was bound to the tags in two different conformation and cloned into vectors for Arabidopsis transformation. *A. tumefaciens* cells were transformed with the generated plasmids and used for floral dip: the transformed plant were then selected, reproduced and characterized for insertion and expression of *BbC002*. An efficient protocol

of phloem sap collection was established and affinity purification of complexes containing tagged protein was performed. These were then purified, digested by trypsin and identified by mass spectrometry: the data obtained were analyzed through a software and a list of putative BbC002-interactors was obtained. Despite the numerous steps required, the method resulted powerful and efficient: the approach essentially recreates the natural situation of effector injection into plant cells and its mechanism of action; it primarily identifies higher-order complexes and their connectivity and the mass spectrometry then allows the detection of peptides in the lower femtomolar range with high accuracy. However, it can not be excluded that modifications occur to protein complexes formed into sieve tubes during the phloem collection due to EDTA and that proteins are degraded by proteases during the extraction or washed away by affinity purification step.

The yeast two-hybrid system offers clear advantages in comparison to the pull-down technology because is economical, easier to perform, no step of protein purification are needed and the method yields information about actual bimolecular physical interaction which are not necessary mapped by complex purification approaches. The only steps required were generation of bait plasmids, isolation of cDNA library from *E. coli* cells, transformation of yeast cells, test the baits for self-activation and finally screen of interactions. Despite the ease of implementation, the two-hybrid assay showed the disadvantage of high number of candidate colonies identified as harboring couple of proteins interacting but which do not truly interact. The growth properties of yeast cells on the screen plates can be influenced by several parameters: cells approaching stationary phase exhibit different expression levels of the hybrid proteins from cells growing in exponential phase; as the number of cells increases, the phenotypic differences between positive and negative controls decrease and the amount of growth of yeast patches on a particular selection plate will vary basing on incubation conditions. Furthermore, the method suffers of false positives that can result from different reason as proteins containing regions with surfaces having low affinities for many different proteins, proteins that normally interact with a large number of proteins or proteins containing regions functioning as activation domains.

Finally, as demonstrated for the effector Me_SG130, the yeast two-hybrid assay is not suitable to investigate all types of proteins, because of spurious activation of reporter genes.

Chapter 5

Conclusions and future works

This work is only a small part of a big project developed in the company KeyGene to discover genetic bases of aphid resistance in plant and then generate crops important from an economical point of view resistant to these phloem sucking insects. The aim of this study was to identify candidate plant proteins interacting with proteins secreted by aphid saliva during feeding: there are indeed several evidences that salivary secretions play an important role in establish colonization of plant by aphid through effector molecules that once injected in plant cells manipulate plant defense responses. The effectors of the two aphid species taken in consideration, *B. brassicae*, the cabbage aphid and *M. euphorbiae* the potato aphid were demonstrated to have an important role in aphid behavior during plant infection: especially it is reported that C002 protein is essential for different species of aphids for survival and feeding on plant host. The importance of C002 in aphid colonization promotion was verified in our experiment: *B. brassicae* feeding on plants overexpressing this effectors produces more progeny then the control. Furthermore, a list of candidate Arabidopsis proteins that interact with BbC002 were generated through affinity purification methods combined with mass spectrometry analysis. Next step is to test mutant Arabidopsis lines containing a knock-out in the genes encoding for these proteins in aphid bioassay to identify more resistant or more susceptible genotypes to aphid attack. Once validated the evidence that a certain mutant line exhibits al-

tered resistance to cabbage aphid, the idea is to identify homologous genes in other plant species, especially crops like *B. napus*, mutagenize populations of these crops and phenotype altered alleles for loss-of-susceptibility to *B. brassicae.*

We tried also to identify plant proteins interacting with C002 and other two effector proteins of *M. euphorbiae* by using yeast two-hybrid screen against a cDNA pepper library. Unfortunately this system did not give the expected results. The first problem was that Me_SG130 resulted a strong activator of *HIS3* reporter gene and thus was not suited to be tested in the two-hybrid assay: from analysis of the Me_SG130 cloned sequence, a point mutation causing a change in amino acids sequence was detected. Resulted of primary importance, to re-clone this gene effector to obtain the correct amino acid sequence and test it again for self-activation. If it will still self activate the reported gene, different strategies as using a truncated version of the gene or changing the position of GAL4 binding domain (N- or C-terminal) can be considered. The other problem we encountered was that positive colonies obtained in the first screen were not confirmed as true interactors in the screen of the second reporter gene. Modification of some steps of yeast two-hybrid protocol and repetition of the experiment seem to be crucial to obtain a number of colonies harboring bait and prey truly interacting. A positive control has to be included in the screening to minimize the presence of false positives. Once obtained from the pepper cDNA library a series of interactors of *M. euphorbiae* effectors the next steps can be to silence transiently the respective genes or induce a point mutation in pepper plants and test whether they affect aphid behavior during feeding and reproduction and thus identify putative resistance genes.

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