Resistance mechanisms of *Solanum* species to *Myzus persicae*

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Para Lucas, Natalia y Mateo,
Para papá Roberto y mamá María Teresa

…

Universal delicia,
no esperabas
mi canto,
porque eres sorda
y ciega
y enterrada.
Apenas
si hablas en el infierno
del aceite
o cantas
en las freiduras
de los puertos,
cerca de las guitarras,
silenciosa,
harina de la noche
subterránea,
tesoro interminable
de los pueblos.

(…Universal delight, you did not wait for my song, because you are deaf and blind and buried. You hardly speak in the hell of boiling oil or sing in the kitchens of the harbors, near the guitars, flour of the underground night, interminable treasure of the peoples).

Oda a la papa, Pablo Neruda, 1904-1973

Pequeño valiente
creador de néctar,
pocos conocen
tu verde ausencia,
que cambia el destino
de los sembrados.

Small valiant
nectar creator,
only a few know
your green absence,
which changes the fate
of the crops.

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ABSTRACT

Alvarez, A.E. (2007), Resistance mechanisms of *Solanum* species to *Myzus persicae*, Ph.D. thesis, Wageningen University, Wageningen, the Netherlands.

The aphid *Myzus persicae* (Sulzer) constitute a threat to potato crops because of their efficiency to transmit viruses. Many wild *Solanum* species have been reported to have resistance to *M. persicae*. These species represent an important potential source of resistance, which can be used to enhance resistance in crops. The aim of this thesis was to find and study in tuber bearing *Solanum* species different mechanisms of resistance to *M. persicae* that also would reduced the spread of viruses. Resistance and susceptibility of wild *Solanum* genotypes and cultivated potatoes were characterized and linked to plant-aphid interactions with respect to aphid behaviour and performance and plant responses to aphid attack. A combination of aphid colony-development assay with the study of probing activities was used to localize resistance factors in tissues of *Solanum* genotypes. Genotypes with different degrees of resistance were found. *S. stoloniferum* showed pre-phloem resistance to *M. persicae*, and hence it was used as a model plant to unravel and characterise phenotypically and at molecular level the interactions of a single plant with different aphid species. Aphid performance, settling behaviour, and probing studies of *M. persicae* and *M. euphorbiae* on *S. stoloniferum* showed that while *S. stoloniferum* could be a host plant for *M. euphorbiae*, it is a poor host for *M. persicae*. The resistance found in *S. stoloniferum* against *M. persicae* seems to rely on constitutively expressed physical traits with some age effects.

The transcriptional response of *S. stoloniferum* to the attack of *M. euphorbiae* (compatible interactions) was stronger than in response to *M. persicae* (incompatible). This stronger response to *M. euphorbiae* involves up-regulation of genes related to pathogenesis (PR), regulatory, and protein metabolism and down-regulation of regulatory genes, general metabolism and photosynthesis related genes.

Infestation of *S. stoloniferum* leaves with *M. persicae* aphids leads to the development of pustules. Microscopic analysis of the pustules showed a burst of tissue on the surrounding of the vascular bundle. In contrast, the infestation with *M. euphorbiae* did not induce any visible cellular changes. The formation of pustules and the induction of some genes in *S. stoloniferum* suggest that a similar situation might exist between plants-pathogenic bacteria interactions, and plants-aphids interactions.

Response of *S. tuberosum* cv. Kardal to the attack of *M. persicae* was evaluated by studying gene expression. The plant responses depend on foliage maturity. Young leaves of cv. Kardal are resistant to *M. persicae* whereas mature to senescent leaves are susceptible. In old leaves *M. persicae* attack elicits higher number of differentially regulated genes than in young leaves.

The transcriptional results obtained with the two systems: (1) *S. stoloniferum* after the attack of *M. persicae* and *M. euphorbiae*, and (2) *S. tuberosum* cv. Kardal at different maturity leaf stages after the attack of *M. persicae* were compared. The gene-expression studies provide evidence that *S. tuberosum* and *S. stoloniferum* respond by activating the salicylic acid (SA) and ethylene (ET) pathways. Genes responsive to jasmonic acid were differentially regulated in low number in the *S. stoloniferum-M. euphorbiae* but not in the cv. Kardal- *M. persicae* interaction.

At the local level a compatible plant-aphid interaction resulted in a broader gene expression response than that a systemic level. Genes were identified related to changes in sink-source relationship at the feeding site which may indicate a plant manipulation by the aphids related to the process of changing the physiological status of the tissue towards a local metabolic sink; also genes related to signal-transduction pathways, regulation and signalling, protein metabolism, maintenance of cell homeostasis, transport, secondary metabolism, and structural features were found to be differentially regulated. On the contrary, in incompatible interactions the transcriptional response of the plant seems to be more restricted.

PLRV-infection in potato plants of cv. Kardal was found to affect aphid behaviour. *M. persicae* responses to volatiles emitted from PLRV-infected and non-infected plants depend on the age of the leaf. PLRV infection affects also the probing behaviour of *M. persicae*. On virus-infected plants stylet penetration into the plant tissue is enhanced. The transmission efficiency of PLRV is also expected to be affected in PLRV-infected plants because these plants attract more aphids than non-infected plants.

The results obtained on this research (e.g., aphid performance, probing behaviour, colony development, settling behaviour, and gene expression analysis of *Solanum* spp. attacked by aphids) contribute to the understanding of plant responses towards aphid attack as a basis for further unravelling the resistance mechanisms at the metabolic, molecular, and genetic levels.

CONTENTS

CHAPTER 1

General Introduction. *Solanum tuberosum*, *Myzus persicae*, and the Potato leafroll virus: a tripartite relationship

Abstract

The cultivated potato *Solanum tuberosum* L. is among the four most important crops worldwide, and it is generally susceptible to aphids. Aphids like *Myzus persicae* (Sulzer) constitute a threat to potato crops because of their efficiency to transmit viruses. The aim of this chapter is to review the present knowledge on potato-*M. persicae* interactions including a third player in this arena, the persistently-transmitted circulative Potato leafroll virus (PLRV).

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Introduction

Potato is the fourth most important food crop worldwide, after wheat, rice and maize. *Solanum tuberosum spp. tuberosum* L. is a vegetatively propagated and auto-tetraploid species of substantial importance on worldwide scale as carbohydrate source. Aphids are a main pest in potato and have therefore a significant economic importance. The main damage is caused by their role as virus-vectors (Radcliffe and Ragsdale, 2002). Hence, aphids together with host plant and environmental factors are crucial components in virus epidemiology (Robert et al., 2000). For most potato growers, and their pest management advisors, the vector is the least understood component of the "disease tetrahedron" (Radcliffe and Ragsdale, 2002).

To avoid virus spread in seed potato production, a common practice for growers is to monitor the arrival of aphids on the crop, after which corrective measures are taken. Aphids can be controlled by using insecticides. However, widespread use of these chemicals carries drawbacks not only for the environment but also for aphid control because important aphids like *Myzus persicae* have developed resistance to most major insecticide classes (Devonshire and Field, 1991; Robert et al., 2000). *M. persicae* is the prevailing species on potato crop (Kuroli and Lantos, 2006). Therefore, alternative approaches for its control are needed.

The potato degeneration

The potato crop was brought from South America to Europe in the early 16th century. Soon after its introduction, the potato started to suffer from a condition that was called 'potato degeneration' (van der Want, 1987). This 'degeneration' was first attributed to the continuous vegetative propagation of the tubers and to the unfavourable conditions of climate and soil causing the fatigue or deterioration of the crop. Much later, it was discovered that the real cause were plant viruses, mainly Potato leafroll virus (PLRV, genus *Polerovirus*, family Luteoviridae) (first described by Quanjer et al., 1916) and Potato virus Y (PVY, genus *Potyvirus*, family Potyviridae) (first described by Smith, 1931) (Beemster and Bokx, 1987; van der Want, 1987).

Many important potato diseases are caused by viruses. At least 54 viruses are known to infect potatoes (Brunt et al., 1996) and aphids are the most important, often the only, vectors of potato viruses. In seed potato production, the green peach aphid *M. persicae* (Insecta, Homoptera,

Aphididae) is the aphid vector of greatest concern, as it is very effective and distributed worldwide, from temperate to tropical climatic zones (Peters, 1987; Raman and Radcliffe, 1992).

PLRV and PVY are the most important viruses in potato crops, in terms of yield and quality reduction (Ragsdale et al., 1994). Infection causes yield reduction up to 90% for PLRV infection, and up to 80% for PVY infected potatoes (Jeffries, 1998). PLRV is found wherever potato crops are grown and it is the most damaging potato virus (Solomon-Blackburn and Barker, 2001).

Aphids must penetrate plants with their stylets (probe) to determine their suitability as a host (Pickett et al., 1992; Pollard, 1973). Viruses are acquired by aphids from infected plants during probing and subsequently after aphid migration other plants are inoculated.

Transmission of PLRV is very different from that of PVY. PVY is a non-persistently transmitted non-circulative plant virus. The aphid needs only a brief probe to acquire the virus, after which the virus remains attached to the aphid's stylets (stylet-borne virus) and can be transmitted also as fast as in a few seconds to other plants. The aphids will loose the virus particles very soon during probes in healthy plants. However, aphids can re-acquire the virus on their stylets numerous times. *M. persicae* is the most efficient and the principal vector for PVY (Beemster and Bokx, 1987).

PLRV is transmitted in a persistently circulative manner. It depends on aphids for dispersal and transmission to host plants. PLRV is restricted to the phloem; hence, aphids acquire PLRV during ingestion of phloem sap from infected plants and inoculate it during salivation into the phloem sieve elements of subsequent plants that they encounter. The virions ingested with phloem sap during aphid feeding, circulate from the digestive system, across the epithelial cells of the hindgut, diffuse through the haemolymph and finally pass through the accessory salivary gland membranes into the saliva (Gildow, 1987). Circulation is complete at transmission to another plant, which occurs via salivation into a phloem sieve tube of the plant. An aphid can acquire PLRV after a few minutes of phloem feeding, but generally it requires 24 to 48 hours before aphids are able to transmit the virus. Once infected with PLRV, an aphid will remain infected for its entire life. The haemolymph of an aphid acts as a reservoir in which acquired virus particles are retained in an infective form without replication for the life span of the aphid (**Figure 1**).

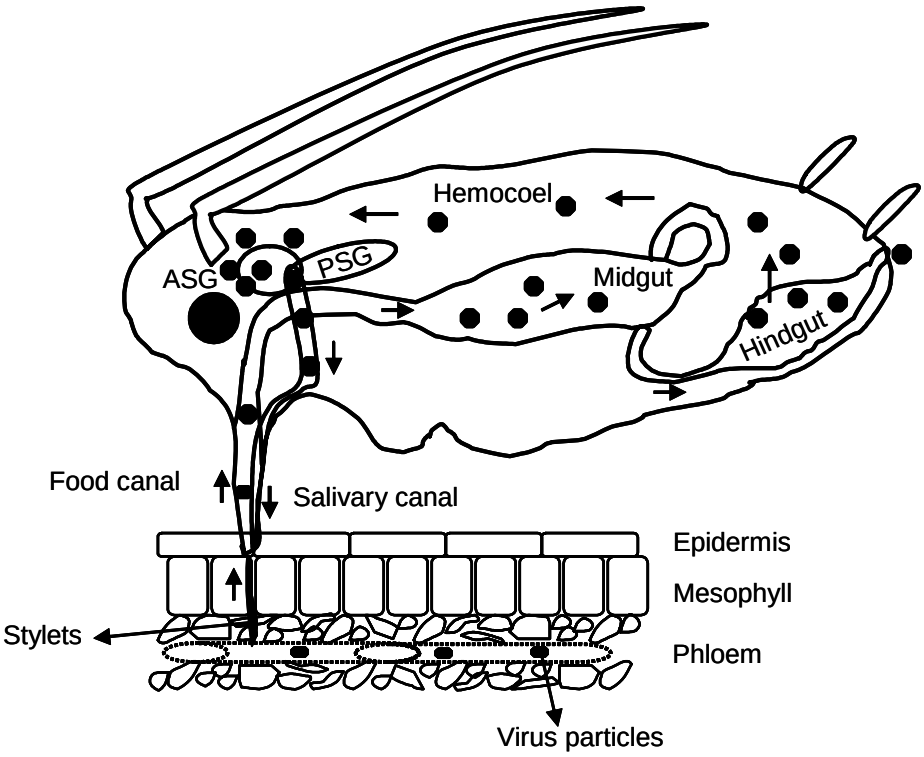

Figure 1. Schematic diagram of circulative route of Potato leafroll virus (PLRV) through aphids. Virus particles can be ingested from phloem into the aphid's food canal and arrive in the hindgut intact. Then some are transported through the hindgut epithelial cells into the hemocoel, and some are excreted. In the hemocoel particles migrate to the accessory salivary gland (ASG) and are released into the salivary canal. The principal salivary gland (PSG) is not involved. Modified from Gray and Banerjee (1999).

Aphids harbour endosymbiotic bacteria of the genus *Buchnera* in specialized cells, which are located in the abdomen and called bacteriocytes (Buchner, 1965). Aphids and bacteria have a long co-evolutionary history and are unable to survive separately (Douglas, 2003a). Virus circulation inside the aphid is made possible by the interaction with a chaperone, a protein homologous to GroEL from *Escherichia coli*, called symbionin from the aphid's endosymbiotic bacteria *Buchnera* sp. (Hogenhout et al., 2000). The role of symbionin in aphid metabolism is unknown. Chaperones are small, acidic and leucine-rich proteins that interact with effector's proteins. Their role is to stabilize and protect proteins from degradation. Symbionin plays a crucial role in determining the persistent nature of luteoviruses in the aphid's body fluid (Van den Heuvel et al., 1994; 1997; for a review see Gray and Banerjee, 1999).

In potato crops, the percentage of plants infected with PLRV strongly depends on the number of aphids that have previously visited PLRV-infected plants (Beekman, 1987). A low degree of PLRV infestation in seed potatoes normally poses no risk to commercial production when no green peach aphids are present (Flanders et al., 1999). Therefore, control of PLRV depends on effective control

of green peach aphids (Radcliffe and Ragsdale, 2002). Growers depend on insecticides to control *M. persicae* but selection pressure has resulted in several forms of insecticide resistance (Devonshire and Field, 1991). Moreover, the negative environmental impact of insecticides demands alternative control strategies, ideally based on host plant resistance to the vector and/or the virus.

Interactions at host plant level of *M. persicae* and Potato leafroll virus

PLRV is highly dependent upon *M. persicae* for its dispersal. In this tripartite relationship, direct interactions occur between virus, host and vector. Moreover, virus infections can change the hosts in such a way that interactions between host and vector are influenced. Vector activity and behaviour are important determinants for the rate and extent of epidemic virus development (Jeger et al., 1998; 2004). Any change in the virus-infected plant resulting in attraction or repellence of the aphid, or affecting its performance will influence the probability of virus dispersal.

In some plant-virus-aphid interactions the presence of virus negatively affects the performance of the vector. In wheat, the presence of Barley yellow dwarf luteovirus (BYDV) reduces the concentration of total amino acids in the phloem; furthermore, qualitative and quantitative analysis of honeydew indicated a lower efficiency of phloem sap utilisation by the aphid *Sitobion avenae* (F.) (Fiebig et al., 2004).

Benefits for the vector that favour virus transmissions have been described for different plant-pathogen-vector combinations. Belliure et al. (2005) showed that *Frankliniella occidentalis* (Pergande) benefits indirectly from Tomato spotted wilt virus, which it transmits, through effects of the virus on host-plant characteristics. They hypothesize that virus-infection has a negative effect on induced plant defences against the thrips vector. *Aphis gossypii* Glover transmits Zucchini yellow mosaic virus (ZYMV) to *Cucurbita pepo* L. and it lives longer and produces more offspring on ZYMV infected than on non-infected plants (Blua et al., 1994). BYDV benefits its vector *Sitobion avenae* (F.) by disrupting the development of a braconid parasitoid within the aphid vector (Christiansen-Weniger et al., 1998). BYDV-infection of wheat plants increases the attractiveness to the aphid *Rhopalosiphum padi* (L) as a result of producing more volatiles than non-infected plants (Jiménez-Martínez et al., 2004).

Myzus persicae performance is better on PLRV infected potatoes than on virus free potato plants, or PVY, or PVX (Potato virus X) infected plants (vector-independent mechanically-transmitted virus) (Castle and Berger, 1993). Furthermore, more *M. persicae* individuals settled on PLRV-infected leaves of *S. tuberosum* L. than on virus-free, PVY-infected, or PVX-infected leaves (Castle et al., 1998). Eigenbrode et al. (2002) found that *M. persicae* preferred PLRV-infected to non-infected, PVY- or PVX-infected potato plants in choice tests. In addition, they found an increased emission of several volatiles by PLRV-infected plants when compared to uninfected plants possibly acting as *M. persicae* attractants and arrestants. The role of plant volatiles in host recognition and settling behaviour by aphids has been reviewed by Pickett et al. (1992).

Solanum resistance to PLRV

Wild and cultivated *Solanum spp.* are known to contain resistance to PLRV but yet not one has been described as having absolute resistance (Jayasinghe and Salazar, 1998). The PLRV resistance found in potato cultivars is partial resistance that reduces the incidence of infection, restriction of virus multiplication or virus movements but can be broken down under high inoculum pressure (Solomon-Blackburn and Barker, 2001). This partial resistance is complex, apparently because of its multifactorial nature (Salazar, 1996) and unstable, since its effectiveness depends on factors such as temperature, virus strain, inoculum pressure, and infection by other pathogens (Jayasinghe, 1990). Until now, combining different types of host plant resistance in *Solanum tuberosum* has enhanced partial resistance to PLRV virus (Solomon-Blackburn and Barker, 1993).

Three components of resistance have been recognised: 1) resistance to infection by viruliferous aphids, 2) limitation of virus multiplication, 3) restriction of virus movement from foliage to tubers (Barker and Harrison, 1985; for a review see Solomon-Blackburn and Barker, 2001). In all cases, the plants can be infected but the virus does not reach high concentrations.

Solanum chacoense Bitter possesses a very strong resistance to PLRV multiplication, which seems to be controlled by a single dominant major gene (Brown and Thomas, 1994). A similar resistance was found in *Solanum etuberosum* (Chávez et al., 1988), *Solanum brevidens* (Jones, 1979) and in a clone of *Solanum phureja* (Franco-Lara and Barker, 1999). Resistance to virus multiplication found in *S. tuberosum* cultivars seems to be controlled by one locus, possibly involving two complementary genes both needed for the resistance or a group of closely linked genes (Barker and

Solomon, 1990). Hybrids from interspecific crossing of *S. tuberosum* L with the wild species *S. chacoense* Bitter and *Solanum yungasense* Hawk. carried resistance to PLRV multiplication. Quantitative trait locus (QTL) analysis of the hybrids revealed one major QTL (on chromosome XI, explaining more than 50% of the phenotypic variation) and two minor QTL in two different chromosomes (Marczewski et al., 2001).

Restriction of virus movements was found in *S. tuberosum* cultivar Bismark. This resistance was independent from resistance to infection and resistance to virus accumulation (Wilson and Jones, 1992). Recently a source of resistance to PLRV infection by viruliferous aphids combined with moderate resistance to virus accumulation was found on the native *S. tuberosum* ssp. *andigena* (Juz. Et Bukasov) at the International Potato Center (CIP). The resistance is located on chromosome V, and may represent a single gene or a cluster of tightly-linked resistance genes. Nevertheless, this resistance mechanism can be overcome either with grafting or with high inoculum pressure (Velásquez et al., 2007). Overall, no source of absolute resistance to PLRV has yet been found (Taliansky et al., 2003).

Resistant genotypes intended for agronomic purposes should be less affected by the pathogen (Swiezynski, 1994). But re-interpreted in terms of plant performance, this can be problematic because a tolerant plant may show no symptoms of infection but can be a source for infection in a crop (asymptomatic inoculum's reservoirs). Such plants are tolerant but not resistant; they do not show any symptoms or have reduced expression of symptoms (Beekman, 1987). Since the potato crop is vegetatively propagated, the virus can be easily transmitted through the tubers to subsequent generations.

Solanum resistance to *M. persicae*

Control strategies for plant viruses depend highly on understanding plant-vector interactions. *M. persicae* has a number of unique features that contribute to the success of the aphid as a virus vector. Their piercing-sucking mouthparts deliver the virions into plant cells with minute damage. Aphid vectors reproduce at a very high rate (asexually, viviparous) and can spread at short (apterae aphids) and long distances (alatae aphids) thus increasing virus disease epidemics (Ng and Perry, 2004).

Many wild *Solanum* species have shown effective resistance to aphids in general, and more than 60 were reported with resistance to *M. persicae* in particular (Flanders et al., 1999; Gibson and Pickett, 1983; Novy et al., 2002; Radcliffe and Lauer, 1968; Tingey and Sinden, 1982). These species represent an important source of resistance that can be exploited in cultivated potato breeding. Genes from at least 18 wild *Solanum* species have been incorporated into North American and European potato cultivars primarily for stress tolerance or disease resistance but so far, no commercial potatoes have been specifically developed for insect resistance (Flanders et al., 1999). Although wild *Solanum* resistance to *M. persicae* has been described and some plant features have been related to it, not much is known about their biochemical, physiological and molecular mechanisms. Aphids are phloem-feeding insects that must insert their mouthparts into the plant (probing) in order to find this tissue and to select a suitable host plant. Therefore, knowledge on probing behaviour of aphids is crucial for a better understanding of both aphid control and virus epidemiology.

The electrical penetration graph (EPG) technique, introduced by McLean and Kinsey (1964) and further improved by Tjallingii (1978; 1985; 1988) is a robust tool to study plant penetration by the aphid's stylets (**Figure 2**). EPG signals (**Figure 3**) have been correlated with aphid activities as well as with tissue locations of the stylet tips (Kimmins and Tjallingii, 1985; Tjallingii, 1978; 1988; Tjallingii and Hogen Esch, 1993). As such, EPG waveform variables can be used to identify the tissues containing the resistance factors (Tjallingii, 1995) and to infer the effects of the aphid-resistant plants on virus transmission (Martín et al., 1997; Prado and Tjallingii, 1994). In several studies, EPGs have been used to obtain more information on the location of the resistance factors in host plant tissues (Givovich and Niemeyer, 1991; Van Helden and Tjallingii, 1993; Cole, 1994; Gabrys et al., 1997; Lei et al., 1999; Garzo et al., 2002; Klingler et al., 1998; 2005).

Surface resistance is the first line of defence against attack and is especially important to avoid virus infection. Visual, mechanical, and olfactory stimuli at the leaf's surface, the cuticle-epidermal level, such as colour, the presence of trichomes, repellent volatiles, or the toughness of the cuticle and cell walls can have a defensive effect against aphids (Van Helden and Tjallingii, 1993). In wild potatoes, the role of glandular trichomes in the defence against insects is well-documented (Gibson, 1971; 1974; 1976; Gibson and Turner, 1977; Tingley and Laubengayer, 1981).

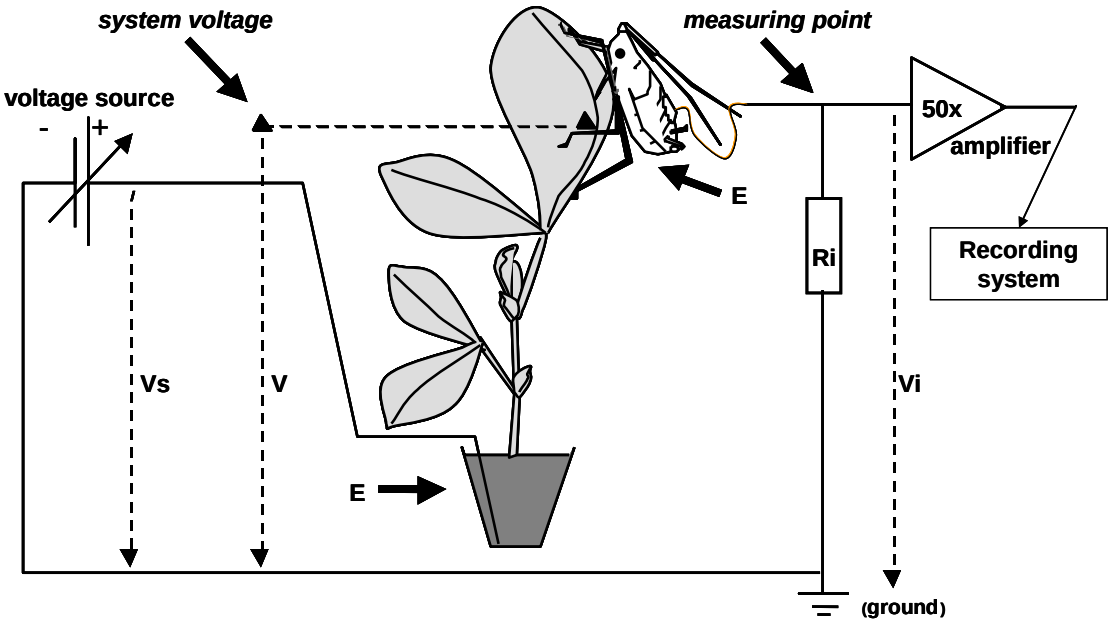

Figure 2. Electrical penetration graph (EPG) technique. Aphid and plant are made part of an electrical circuit by inserting a wire into the soil of a potted plant, and attaching a gold wire to the aphid dorsum. A low DC voltage source (Vs) and an input resistor (Ri) of 1 GΩ is applied. An amplifier connects at the measuring point between the insect and resistor. When the aphid starts probing (inserting the stylets in the plant) the circuit is closed and electrical signals (about 5 V) are received on a recording system. E, electrode potentials; V, circuit potential (Vs + E + E); Vi, signal potential (EPG signals) (Tjallingii, 2006).

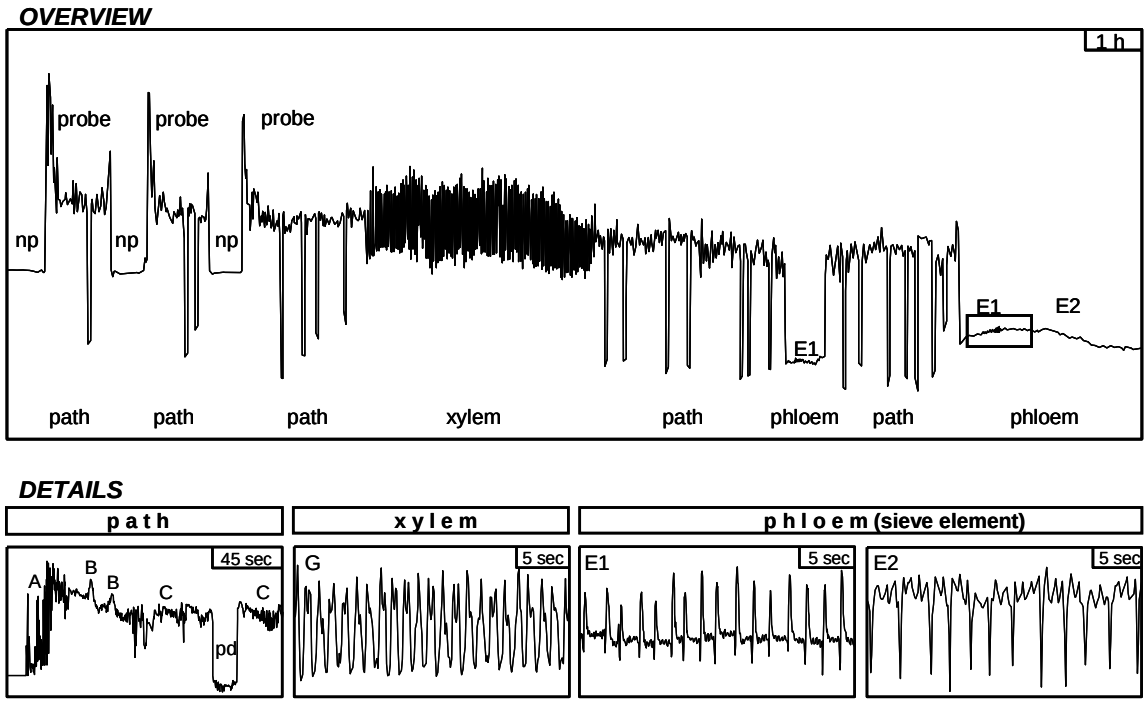

Figure 3. EPG signals (referred to as 'waveforms') are the result of voltage fluctuations due to different probing activities. np, no-probing; A, B, C, stylet extracellular pathway activities; pd, potential drops reflecting intercellular punctures; G, drinking from xylem E1, sieve element salivation; E2, phloem sap ingestion (Tjallingii, 1995).

The occurrence of glandular trichomes (type A and type B hairs) and their products in wild *Solanum* species contributes to aphid resistance (Tingey and Laubengayer, 1981; Tingey and Sinden, 1982; Lapointe and Tingey, 1986). These glandular trichomes produce several secondary metabolites constituting the basis for the trichome-based resistance. Type B hairs exude a volatile sesquiterpene, E-β-farnesene, a major component of aphid alarm pheromone that causes behavioural excitation and increased locomotion (Gibson and Pickett, 1983), and a viscous mixture of sucrose esters (King et al., 1987). Type A hairs contain polyphenol oxidases, and when their four-lobed gland at the tips are mechanically broken, the oxidases come into contact with phenolic substrates catalysing a phenolic oxidation reaction (Ryan et al., 1982). These glandular products may act alone or synergistically to cause physical and chemical deterrence, entrapment, reduced feeding, digestive disorders and/or reduced reproductive performance (Bonierbale et al., 1994).

Inheritance studies of trichome densities and trichome exudates have shown that the expression of resistance is the result of the interaction of several chemical and physical characteristics of the glandular trichomes (Mehlenbacher et al., 1983). QTL analysis of trichome-mediated insect resistance in potato has shown an incomplete correlation between the resistance and the trichome phenotypes. This may be explained by an incomplete understanding of the biochemical nature of the resistance mechanism, or the presence of multiple resistance mechanisms (Bonierbale et al., 1994). The potato species having a large amount of secretory pubescence are *Solanum berthaultii* Hawkes, *S. polyadenum* Grenm., and *S. tarijense* Hawkes.

In spite of the large number of wild potato species that have been described as resistant to aphids, no localized plant property have been identified so far as new source of resistance to *M. persicae*, other from glandular trichomes.

Solanum responses to aphid attack

Plants can respond to insect attack by inducing structural or chemical factors that could have a negative effect to the attacker. It has been shown that defence-related genes (associated to metabolic pathways) and direct defence genes, e.g., *pathogenesis-related proteins, acidic apoplastic β-1,3-glucanase, chitinases, defensin,* etc., are induced in plants in response to insect feeding and pathogen infection. These responses can act complementary to constitutive defences of the plant

(Brian and Bergelson, 2003; Traw and Bergelson, 2003; Kessler and Baldwin, 2002; Dicke et al., 2003).

Aphids salivate into the plant tissue and the saliva might contain chemical signals (inducer molecules), although they have not been identified yet. Some authors suggest that aphids (like compatible plant-pathogen interactions), by their particular mode of feeding, might control plant responses to their benefit, maybe by eliciting an anti-microbial defence pathway, activating ineffective genes and suppressing genes for critical defences (Zhu-Salzman et al., 2004; for review see Kaloshian and Walling, 2005). It is unclear whether induced or suppressed genes are related to the plant defence mechanism against aphids.

Aphid-plant interactions include complex local and systemically spread signals that are still largely unknown but gene expression studies in the last few years have shed some light on this (for review see Smith and Boyko, 2007). The studies on the interaction between *M. persicae* and *Arabidopsis thaliana* (Moran and Thompson, 2001; Moran et al., 2002) as well as between *Schizaphis graminum* and *Sorghum bicolor* (Zhu-Salzman et al., 2004), have shown that small and local damage of cells, related with stylet penetration, salivation, and phloem feeding by aphids, induces multiple plant defence pathways. Pathogenesis-related proteins (PR), salicylic acid (SA) responding genes and, to some degree, also jasmonic acid (JA) regulated genes are activated upon aphid attack (Moran and Thompson, 2001; Moran et al., 2002; Zhu-Salzman et al., 2004). In addition, the transcriptional changes in *A. thaliana* (wild type and mutants) in compatible plant-aphid (*M. persicae*) interaction showed some genes to be up-regulated that are also induced by pathogens and wounding. Transcription increases were reported for salicylic-acid regulated genes, like *PR1* and *BGL2* (*acidic apoplastic β-1,3-glucanase*), and for jasmonate/ethylene regulated genes, like *defensin* (*PDF1.2*), and also for genes related to resource allocation shifts (like sugar transport proteins) (Moran and Thompson, 2001). Moreover, *M. persicae* probing elicits the expression of genes involved in oxidative stress (superoxide dismutase, glutathione-S-transferase), and calcium-dependent signalling (calmodulin and touch-sensitive genes) (Moran et al., 2002). Resistant wheat infested with the Russian wheat aphid *Diuraphis noxia* (incompatible interaction) shows an increased activity of apoplastic PR enzymes, β-1,3 glucanase, peroxidase and chitinase as compared to susceptible wheat cultivars (van der Westhuizen et al., 1998a, 1998b).

Synthesis of PR proteins is a common plant response to pathogen infection (for review see van Loon et al., 2006). Aphid probing appeared to elicit similar gene expression patterns in tomato as

shown by *Phytophthora infestans* infection. Gene expression changes due to aphid feeding appear to differ from those by chewing herbivorous insects (Fidantsef et al., 1999). In a comparative transcriptional analysis of *Arabidopsis thaliana* in response to different plant attackers, aphids and pathogenic bacteria trigger expression changes for the largest number of genes. This study also included chewing insects (caterpillars), cell-content-feeding insects (thrips), and a necrotrophic leaf fungus. Although aphids caused the least symptoms, most of the transcriptional changes were unique to either bacteria or aphids. Moreover, a large proportion of genes affected by aphids are related to plant metabolism, suggesting aphid manipulation of the plant's physiology (De Vos, et al 2005; for review see Smith and Boyko, 2007). *Myzus nicotianae* on *Nicotiana attenuata* induces the accumulation of glutamate (Voelckel et al., 2004), which is one of the nitrogen transport molecules that could shift the nutritional quality of phloem sap (Karley et al., 2002).

Serial electron microscopy combined with EPG recording has shown that the stylet route followed by the aphid's stylets is intercellular, mainly between the secondary cell wall layers of cellulose fibres. Nevertheless, most cells encountered on the way to the sieve elements are briefly punctured. Stylets are withdrawn subsequently to follow their intercellular track. Only very few cells die because of these punctures. During phloem sap ingestion a sieve element is punctured continuously but generally remains alive (Tjallingii and Hogen Esch, 1993). Salivary secretions of different types occur in different tissue locations, inter- and intra-cellularly, allowing aphids to minimise plant damage and to maximise extended periods of phloem feeding for themselves and their progeny on the same plant (Tjallingii, 2006). Stylet penetration and feeding by aphids is a highly specialized mechanism and therefore, plant reactions are expected to differ from responses to other insect herbivores and mechanical wounding.

Resistance genes to one specific pathogen or pest can be tightly linked, forming a cluster of genes. These gene clusters evolve from common ancestors by local gene duplications followed by structural and functional diversification. Mapping experiments have shown that genes for resistance to distant pathogen species, a virus and a nematode, can occupy similar genetic positions (Van der Vossen et al., 2000). The potato genome has hotspots containing multiple genes for monogenic and polygenic resistance to different pathogens (fungi, bacteria and nematodes) on chromosomes V, XI and XII (Gebhardt and Valkonen, 2001). In tomato, an aphid-resistance locus is tightly linked to the *Mi* gene, a dominant locus that confers resistance to root knot nematodes (*Meloidogyne* sp) (Kaloshian et al., 1995; Rossi et al., 1998). The *Mi* gene is a member of a superfamily of plant

resistance genes that contains a common motif consisting of a putative nucleotide binding site (NBS) and a region of leucine-rich repeats (LRR) (Milligan et al., 1998). Proteins encoded by NBS-LRR genes seem to link the detection of a plant pathogen/attacker to the activation of an appropriate defence (Grant and Mansfield, 1999). *Mi*-mediated aphid resistance works for some genotypes of the aphid *Macrosiphum euphorbiae* (Thomas) but other genotypes are not affected or could overcome *Mi*-related resistance. The resistance did not extend to the green peach aphid *Myzus persicae* (Goggin et al., 2001). Moreover, it was found that the *Mi* gene is also responsible for the resistance to the whitefly *Bemisia tabaci* in *S. lycopersicum* (Nombela et al., 2003). Until now no pathogen resistance gene has been found to affect infestation by *M. persicae.*

Research aims and thesis outline

The main goal of this research project was to find and characterise different mechanisms of resistance to *M. persicae* in *Solanum* species aiming to reduce the spread of PLRV. For this, it is essential that we extend our knowledge on the plant-aphid interaction on susceptible and resistant host plants. To achieve our goal, we characterised phenotypic resistance and susceptibility of wild and cultivated potatoes and tried to link that to plant-aphid interactions with respect to aphid behaviour and performance and plant responses to aphid attack

In **Chapter 2**, aphid bionomical data are combined with probing data in order to identify resistance to *M. persicae* in wild tuber-bearing *Solanum* genotypes. Aphid probing has been studied in these genotypes to obtain information on tissue location of resistance types found and their possible mechanisms that may be important in limiting PLRV transmission.

Chapter 3 addresses the constitutive resistance of *Solanum stoloniferum* Schlechtd to *M. persicae* in comparison to susceptible responses in interactions with *Macrosiphum euphorbiae*. The differences in aphid performance and behaviour between these two generalist aphid species and their interactions with this plant species are reported.

In **Chapter 4**, the resistance or susceptibility of *S. stoloniferum* to *M. persicae* and *M. euphorbiae* is evaluated by studying gene expression, aphid settling behaviour, and morphological responses of the plant to attacks of the two aphids.

Chapter 5 focuses on the interaction between *M. persicae* and the cultivated *S. tuberosum* cv. Kardal by evaluating the plant gene-expression after aphid infestation. Kardal plants are resistance to the aphid in apical young leaves but susceptible in the mature-senescent leaves. The results are compared to those obtained in Chapter 4. Similarities in gene regulations in compatible and incompatible plant-aphid interactions are identified.

Chapter 6 addresses whether PLRV infection changes *S. tuberosum* in benefit of its vector *M. persicae.* Probing behaviour on young apical leaves of potato cv. Kardal that is partially resistant to the aphid has been investigated. Differences in vector attraction by plant volatiles on PLRV-infected and non-infected plants are documented and potential implications on PLRV transmission are discussed.

Chapter 7 is the general discussion, in which I discuss the findings of this thesis in an integrated, review-like way, adding into the discussion also the possible role of aphid endo-symbiotic bacteria *Buchnera* sp. From this, I hypothesise a new model for plant-aphids-endosymbiont interaction.

CHAPTER 2

Location of resistance factors in the leaves of potato and wild tuber-bearing *Solanum* species to the aphid *Myzus persicae*

Abstract

Analysis of electrically recorded feeding behaviour of aphids was combined with colony-development tests to search for sources of resistance to *Myzus persicae* (Sulzer) (Homoptera: Aphididae) in tuber-bearing *Solanum* species (Solanaceae), aiming at a reduction of Potato leaf roll virus (PLRV) transmission. Twenty genotypes, from 14 gene bank accessions, representing 13 wild *Solanum* spp., three *S. tuberosum* L. (potato) cultivars, and one *S. tuberosum* breeding line were selected. Colony-development tests were carried out in no-choice experiments by placing adult aphids on plants of each genotype and counting numbers of nymphs and adults on young plants after 8 and 15 days, and on flowering plants after 14 and 30 days. Large differences were observed among genotypes: some developed small colonies and others large ones. Also, in a few genotypes, resistance in mature plants was different for leaves of different ages; young leaves were resistant to aphids whereas old senescent leaves were susceptible. The electrical penetration graph (EPG) technique was used to study aphid feeding behaviour on each *Solanum* genotype for 6 h. EPG results also showed large differences among the genotypes, indicating resistance at three different levels of plant tissue (epidermis, mesophyll, and phloem). We concluded that different mechanisms of resistance to *M. persicae* exist among the genotypes analyzed. EPGs recorded from aphids on *S. berthaultii* Hawkes and *S. tarijense* Hawkes with and without glandular trichomes showed that strong surface resistance can bias EPG parameters associated with resistance located in deeper tissues. Experimental evidence is presented that the resistance to aphids in the genotypes with glandular trichomes strongly depends on these morphological structures.

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Introduction

With 235 recognised species, wild *Solanum* spp. (Solanaceae) represents a rich biological diversity, although only a few species gave rise to the cultivated potato (Hawkes, 1990). Until now, genes from at least 18 wild *Solanum* species have been incorporated into North American and European potato varieties. So far, no commercial potato varieties have been developed specifically for resistance to insects (Flanders et al., 1999). The green peach aphid, *Myzus persicae* (Sulzer) (Homoptera, Aphididae) has a worldwide distribution and causes damage principally indirectly by its ability to transmit plant viruses (Salazar, 1996). At least 54 viruses are known to infect potatoes (Brunt et al., 1996) and aphids are their most important vectors (Jeffries, 1998; Peters, 1987; Raman and Radcliffe, 1992). In potato crops, the percentage of plants infected with Potato leaf roll virus (PLRV) strongly depends on the number of aphids that have previously visited PLRV-infected plants (Beekman, 1987). A low degree of PLRV infestation in seed potatoes normally poses no risk to commercial production when no green peach aphids are present (Flanders et al., 1999). Therefore, control of PLRV depends on effective control of green peach aphids (Radcliffe and Ragsdale, 2002). Growers depend on insecticides for suppression of *M. persicae* but selection pressure has resulted in several forms of resistance to these insecticides. Moreover, the negative environmental impacts of insecticides indicate that alternative control strategies and host plant resistance are needed.

Some wild *Solanum* species have effective resistance mechanisms against aphids. *Solanum berthaultii*, *S. polyadenium*, and *S. tarijense* have been studied extensively because of their resistance to aphids due to the presence of glandular trichomes (Gibson, 1971; 1974; 1976; Gibson and Turner, 1977; Tingley and Laubengayer, 1981). However, the glandular trichome-based resistance is genetically complex and it is strongly associated with poor agronomical characteristics (Bonierbale et al., 1994; Kalazich and Plaisted, 1991). Therefore, it has not yet been used successfully in breeding for aphid resistance.

More than 60 wild *Solanum* species have been reported to possess genotypes with resistance to *M. persicae* (Flanders et al., 1999; Flanders et al., 1992; Gibson and Pickett, 1983; Novy et al., 2002; Radcliffe and Lauer, 1968; Tingey and Sinden, 1982). These species represent an important alternative source of aphid resistance, which can be exploited to enhance vector resistance in cultivated potato. Nothing is known about the underlying biochemical, physiological, and molecular

mechanisms in these species. Aphids, as phloem feeding insects, must insert their mouthparts into the plant (probing) in order to select a suitable host. Therefore, knowledge on probing behaviour of aphids is crucial for a better understanding of both aphid and virus control.

The electrical penetration graph (EPG) technique, introduced by McLean and Kinsey (1964) and further improved by Tjallingii (1978; 1985; 1988) is a robust tool to study plant penetration by the aphid's stylets. EPG signals have been correlated with aphid activities as well as with tissue locations of the stylet tips (Kimmins and Tjallingii, 1985; Tjallingii, 1978; 1988; Tjallingii and Hogen Esch, 1993). As such, EPG parameters can be used to identify the tissues containing the resistance factors (Tjallingii, 1995) and to infer the effects of the aphid-resistant plants on virus transmission (Martín et al., 1997; Prado and Tjallingii, 1994). In several studies EPGs have been used to obtain more information on the location of the resistance factors in host plant tissues (Cole, 1994; Gabrys et al., 1997; Garzo et al., 2002; Givovich and Niemeyer, 1991; van Helden and Tjallingii, 1993; Klingler et al., 2005; Klingler et al., 1998; Lei et al., 1999). The aim of the present study is to combine aphid bionomical data with EPG monitoring in order (1) to identify/confirm wild tuber-bearing *Solanum* genotypes resistant to *M. persicae*, and (2) to obtain information on tissue location and the possible mechanism of resistance active in different *Solanum* genotypes that may play a role in PLRV transmission.

Materials and Methods

Plants and aphids

Twenty *Solanum* genotypes were selected from the in vitro-culture collection maintained at the Laboratory of Plant Breeding, Wageningen University, Wageningen, The Netherlands. The original material was obtained from the Centre for Genetic Resources, Wageningen, The Netherlands (CGN). Most genotypes selected belong to species of which some accessions previously have been reported to show some level of resistance to *M. persicae* (Hanneman and Bamberg, 1986; Radcliffe and Lauer, 1968). However, at the start of the evaluation process we did not know whether the material selected possessed the reported resistance. The selected material included 13 wild tuber-bearing *Solanum* species, three *S. tuberosum* cultivars, and one breeding line of S. *tuberosum* (listed in **Table 1**). After some initial experiments, we selected the diploid breeding line *S. tuberosum* RH89-039-16 as the susceptible control. This genotype has frequently been used in our laboratory for interspecific crossings with wild *Solanum* spp. The propagation of plants was performed in

vitro on Murashige and Skoog medium including vitamins, sucrose 3%, pH 5.8. After 2 weeks the plantlets were transferred to soil in a glasshouse at 22 ± 2 ºC, about 70% r.h., and L16:D8 photoperiod.

In order to avoid a behavioural bias toward susceptible cultivated potato, we reared the aphids on radish (*Raphanus sativus* L.), hence the aphids could not adapt to any of the *Solanum* genotypes used. All aphids used in the experiments came from a newly established clone of *M. persicae* from a single virginoparous apterous individual from a colony maintained at the Laboratory of Entomology, Wageningen University. Colonies were reared in a climate chamber at 22 ± 2 ºC, 30-40% r.h., and L16:D8 photoperiod. A new colony was started weekly and newly moulted adult aphids were collected later for the EPG tests.

Aphid colony-development test

Young plants. For all genotypes (**Table 1**), resistance to *M. persicae* was tested in a no-choice experiment. Five recently-moulted (1-3 days old) adult apterae of *M. persicae* were transferred to the first fully-expanded leaf of one plant of each genotype (three plants per genotype, 1-2 weeks after transfer from agar to soil) enclosed in a Perspex cylinder, 130 mm high x 80 mm in diameter, sealed with nylon mesh at the cylinder's top and on two lateral holes of 25 mm of diameter. The assays were conducted in a climate chamber at 22 ± 2 ºC, 30-40% r.h., and L16:D8 photoperiod. The total numbers of aphids, adults, and nymphs were counted on day 8 and 15 of the experiment.

Flowering plants. Resistance to *M. persicae* was also tested in flowering plants, 5-8 weeks after transfer from agar to soil. Five adult apterae of *M. persicae* were transferred to the apical leaf of each plant. Four plants for each genotype were placed in cages (46 x 46 cm and 56 cm high) under glasshouse conditions (22 ± 2 ºC, about 70% r.h., at L16:D8 photoperiod). After 14 and 30 days, plants were inspected for the presence and location of aphids. Three plant regions based on the maturity stage of the leaves were taken into account: 1) young apical, not fully expanded leaves, 2) mature, fully expanded leaves, and 3) senescent, yellowing leaves. The scoring was performed in a qualitative way (**Table 1**).

EPG monitoring of aphid probing behaviour on 20 *Solanum* genotypes

The DC-EPG technique (Tjallingii, 1985; 1988) was used to monitor probing of young adult and apterous aphids during 6 h. Four plants of a genotype were placed in a Faraday cage; probing and

feeding behaviour of two aphids on each plant was recorded simultaneously during 6 h. Between 15 and 19 successful replicates were obtained per genotype. Each plant was used 20 days after being transferred to soil. Aphids were placed on the abaxial side of the third leaf from the apex, which was nearly fully expanded. Before exposure to the plant, the aphid was attached to the electrode – a 2-3 cm long gold wire (diameter 20 μm), conductively glued (water-based silver glue) to the dorsum – while immobilized by a vacuum suction device (van Helden and Tjallingii, 1993). The other end of the gold wire was attached to a 3-cm-long copper wire (diameter 0.2 mm) and connected to the input of the first stage amplifier with a 1 Giga-Ohm input resistance and 50 x gain (Tjallingii, 1985; 1988). The plant electrode, a 2-mm-thick, 10-cm-long copper rod, was inserted in the soil of the potted plant and connected to the plant voltage output of the EPG device (see diagram on Figure 2, Chapter 1) (Giga-4 or Giga-8, manufactured by Wageningen University). In addition to the plants, the aphids and the first stage amplifiers were set up in a Faraday cage. The recording was started immediately after aphid wiring, at about 20 °C and underconstant light in the laboratory, and about 1 h after collecting the aphids from the colony. Signals of eight plants, in two Faraday cages, were simultaneously acquired and recorded on a PC (one per setup) hard disk. Data acquisition and waveform analysis were mediated by Probe 3.0 software (Wageningen University, Laboratory of Entomology) at 100 samples per second.

EPGs on *Solanum berthaultii* and *Solanum tarijense* with and without intact glandular trichomes

To test whether *S. tarijense* 17861-8 and *S. berthaultii* 20650-3, in addition to the strong surface resistance, posses another type of resistance we recorded probing on *S. berthaultii 20650-3* and *S. tarijense* after washing the leaves, which eliminated glandular trichome effects. The abaxial side of the third leaf from the apex of 20-day-old *S. berthaultii* 20650-3 and *S. tarijense* 17861-8 plants was mechanically wiped off using a cellulose cleaning tissue under running tap water. After this procedure, the leaf surface was inspected under a stereomicroscope to check that all glandular parts and secretions of the trichomes were removed. When the leaf surface was dry, after 30 min, probing behaviour of aphids was studied using the EPG technique as described above. Four plants per treatment were used. Two intact plants and two treated plants of the same genotype were placed together in a Faraday cage and EPGs of two aphids on each plant were recorded simultaneously during 6 h. Fifteen successful replicates were obtained per genotype. Intact plants of each genotype were used as control.

EPG waveforms, waveform patterns, and parameters

The recorded EPGs were analysed by distinguishing the following waveforms or waveform patterns (see main waveforms on Figure 3, Chapter 1). Waveform C, stylet pathway phase, including four pooled pathway waveforms/activities, i.e., waveform A, epidermis first stylet contact; waveform B, intercellular sheath salivation; waveform C, stylet movements; and waveform pd (potential drop), an intracellular stylet puncture; waveform E, phloem phase, separated in waveform E1, sieve element salivation and waveform E2, phloem sap ingestion with concurrent salivation; waveform E1e, assumed watery salivation at extracellular voltage level; waveform F, derailed stylet mechanics (stylet penetration difficulties); and waveform G, active drinking of water from xylem elements (Tjallingii, 1990). Out of the 25 EPG parameters that we analysed, eight appeared to be most relevant for resistance to aphids in the analysed genotypes. These are as follows: 1) The time to the first probe, which represents the period between plant access (i.e., onset of the EPG recording) and the start of the first probe. A prolonged period before the first probe is thought to reflect the effects of repellent or deterrent surface factors; 2) The number of probes shorter than 3 min (test probes) that occur before the first phloem phase (E1) and 3) the minimum duration of waveform C within a probe before a phloem phase (a single E1 or an E12 period) likely reflect the role of epidermis/mesophyll and mesophyll/vascular parenchyma factors, respectively; 4) The time to first E1 and 5) the time to first sustained E2 [sE2 – any period of E2 longer than 10 min, previously called 'committed phloem ingestion' by Tjallingii (1995)] reflect the ease of phloem access and acceptance, respectively. Parameters 4) and 5) were calculated as time from the first probe in the recording; 6) The potential E2 index (van Helden and Tjallingii, 1993) was calculated as the percentage of time spent in E2 by an aphid with any sE2, after reaching the first sE2. The potential E2 index reflects how persistent phloem feeding is; 7) The average duration of E2 periods for aphids with E2 (total time spent in E2 divided by the number of E2 events per aphid); and 8) the number and percentage of aphids showing sE2 per accession, reflect phloem as well as general plant suitability.

Statistical analysis

The electrical penetration graph parameters were analysed individually for each aphid; the means and standard errors of the mean (SEM) of total number of aphids per *Solanum* genotype were calculated. To calculate parameters 5 and 6, only those aphids with E2 events were taken into account. For aphid colony development and probing and feeding behaviour the Mann-Whitney rank

sum test was used to test for significance of the difference with the susceptible control *S. tuberosum* RH89-039-16 (SPSS 12.0.1 for Windows). Fisher's exact test was applied to analyse the number of aphids that had shown sE2 (Software by Preacher and Briggs, 2001).

Results

Aphid colony-development test

Twenty *Solanum* genotypes were evaluated for susceptibility/resistance to *M. persicae*. Aphids were confined to one plant but were free to move around and choose any part of the plant for probing and feeding. Results of the aphid colony-development test on young plants were used to rank the plant species from susceptible to resistant (**Table 1**).

Young plants. When adult aphids are transferred from one host to a second one of a different species, it is expected that they will not be adjusted to that new host. In contrast, the nymphs produced by the adults will adjust. This is in agreement with our observations. On some plants of *S. tuberosum* RH89-039-16, *S. hondelmannii*, *S. jamesii* 18349-10, cultivar Kardal, *S. bulbocastanum*, and *S. spegazzini*, none of the initial five adult aphids survived until the first scoring day (day 8). In contrast to this, newborn nymphs appeared to survive and successfully developed into adults on day 15. Genotypes *S. capsicibaccatum* 18268-2, *S. capsicibaccatum* 18268-5, *S. multiinterruptum*, *S. cardiophyllum*, *S. berthaultii* 20650-3, *S. stoloniferum*, *S. polyadenium*, and *S. tarijense*, were highly resistant to *M. persicae*. On these genotypes fewer than five aphids, (nymphs or adults) were found after 8 and 15 days, which is significantly fewer than on the susceptible control *S. tuberosum* RH89-039-16 (Mann-Whitney: $P<0.05$). A less striking reduction in colony-development (moderate resistance) was observed for the genotypes *S. jamesii* 18349-1, cultivar Mondial and Kardal, and *S. spegazzinii*. On these genotypes only the number of nymphs on day 8 differed significantly from control *S. tuberosum* RH89-039-16 (Mann-Whitney: $P<0.05$) (**Table 1**).

Flowering plants. All plants showing susceptibility at the young stage of development were also susceptible to aphids at the mature stage. However, some of the plants that were resistant at the young developmental stage became susceptible at the mature flowering stage (**Table 1**). Some others remained resistant, even in the senescent stage.

Aphid probing behaviour on 20 *Solanum* genotypes

The electrical penetration graph data were processed into about 25 parameters. Most parameters related to stylet pathway showed no differences between accessions and the control line. The most relevant parameters with differences were divided into four groups to infer tissue location of resistance factors against aphids; these include parameters indicative of: (1) surface resistance, (2) epidermis/mesophyll resistance, (3) mesophyll/phloem resistance, and (4) phloem resistance (see **Table 2**).

Surface resistance. Eleven genotypes showed a significantly postponed first probe with respect to the susceptible control. This time to the first probe parameter can be considered as indicative of resistance factors located at the plant's surface level (**Table 2**). Eight out of 11 genotypes also showed some level of resistance in the aphid colony-development test. Three of them, *S. berthaultii* 20650-3, *S. polyadenium*, and *S. tarijense* carry glandular trichomes. The others, cv. Kardal, *S. spegazzinii*, *S. capsicibaccatum* 18268-2, *S. capsicibaccatum* 18268-5, and *S. stoloniferum* do not possess glandular trichomes; therefore these genotypes must have a different mechanism of surface resistance.

Epidermis/mesophyll resistance. Probes shorter than 3 min can be considered as test probes during which stylets do not penetrate deeper than a few cell layers (penetration rate is about 1 cell layer/min). The minimum duration of waveform C, which is preceding a first phloem phase (E1, sieve element salivation) during a probe, mainly reflects mesophyll interactions. Therefore, a large number of test probes and a long time until the first phloem phase activity are assumed to indicate resistance factors in peripheral layers of the plant tissue, i.e., epidermis and mesophyll. Only three genotypes (*S. okadae, S. berthaultii* 20650-3, and *S. stoloniferum*) have significantly larger numbers of short probes when compared to the susceptible control, and in almost all genotypes the pathway preceding the first phloem phase lasted longer (**Table 2**, epidermis/mesophyll column). It should be noted here, that *S. stoloniferum* was the only genotype with a significantly larger number and longer duration of the waveform F when compared to the susceptible control *S. tuberosum* RH89-039-16 (frequency of waveform F per aphid 2.6 ± 0.59 vs. 0.8 ± 0.31, respectively, and duration of waveform F 63.7 ± 13.58 vs. 13.3 ± 6.73 min, respectively; Mann-Whitney: $P<0.05$). Waveform F is associated with derailed stylet mechanics or stylet penetration difficulties (Tjallingii, 1990).

Mesophyll/phloem resistance. A long time before the first sieve element salivation (E1) and before the first sustained phloem sap ingestion (sE2) in the recording (i.e., time since the first probe) can be considered as indicative for mesophyll/phloem resistance factors. Genotypes *S. okadae*, *S. hondelmannii,* and *S. multiinterruptum* show a relative long time to the first E1 but not to the first sE2; and the other way around holds for *S. jamesii* 18349-10, *S. jamesii* 18349-1, and cultivar Kardal, which all show relatively long times to the first sE2 but not to the first E1 (**Table 2**; mesophyll/phloem column).

Phloem resistance. The potential E2 index (percentage of time spent in E2 after the first sustained E2) and the average duration of E2 periods both reflect how persistent phloem feeding is; low values for these parameters are related to phloem-located factors of resistance. Seven genotypes (*S. jamesii* 18349-10, *S. jamesii* 18349-1, cultivar Kardal, *S. capsicibaccatum* 18268-5, *S. multiinterruptum*, *S. polyadenium*, and *S. tarijense*) showed a reduced average duration of E2 periods compared to the susceptible control but *S. multiinterruptum* is the only one with a potential E2 index lower than the susceptible control *S. tuberosum* RH89-039-16 (19 ± 4 vs. 73 ± 11 min, respectively; Mann-Whitney: $P<0.05$; **Table 2**).

Resistance without behavioural effects. The electrical penetration graph parameters did not indicate any resistance factors in genotype *S. cardiophyllum* but nevertheless, in the colony-development test this genotype was a very poor host, especially as young plants. During the 6 h of EPG monitoring, 75% of the aphids reached sustained phloem ingestion (sE2) with an average of 51 ± 13 min. spent on E2 (this was 62.5% and 84 ± 33 min on the susceptible control) (**Table 2**).

Aphid probing behaviour on *Solanum tuberosum* cultivars

The probing behaviour of *M. persicae* on two of the cultivated potatoes in this study showed differences from the susceptible *S. tuberosum* RH89-039-16 control plants. Although probing and feeding on cv. Eersteling was very similar to the control, cultivars Mondial and Kardal appeared to have some degree of resistance to *M. persicae* (see aphid colony-development test, day 8; **Table 1**). However, the level of this resistance in cultivars Mondial and Kardal strongly depended on the development stage of the plants (**Table 1**).

Probing on *Solanum berthaultii* and *Solanum tarijense* with and without intact glandular trichomes

Removing the products of the glandular trichomes from leaves of *S. tarijense* and *S. berthaultii* 20650-3 made plants more accessible to *M. persicae.* On these 'cleaned plants', many aphids reached the sustained E2 phase (**Table 3**).

Discussion

Aphid performance on young vs. flowering plants

Host-acceptance by the aphids was strongly dependent on the developmental stage of the plants or leaves. On the genotypes *S. cardiophyllum, S. multiinterruptum,* and cv. Mondial host acceptance by the aphid increased dramatically from young plants to flowering stage (**Table 1**). In *S. polyadenium,* the resistance level changed from highly resistant in young to moderate resistant in mature plants. In *S. stoloniferum* and cultivar Kardal, young leaves always remained resistant and aphids were never found on apical leaves. In contrast, on susceptible potato cultivars the performance of *M. persicae* was superior on young plants when compared to mature plants, which was presumably caused by a better nutritionally amino acid composition in the phloem of young plants (Karley et al., 2002). Furthermore, aphids can compensate for a nutritionally deficient sap with the provision of supplementary amino acids from symbiotic bacteria (Douglas, 1998; Douglas et al., 2001). In wheat and barley, attack by the aphids *Schizaphis graminum* and *Diuraphis noxia* induces a change in the phloem amino acid composition that seems to be nutritionally better for the aphids' performance (Sandström et al., 2000). Voelckel et al. (2004) found that *Myzus nicotianae* can elicit differential transcriptional changes in source and sink leaves of *Nicotiana attenuata;* the expression of constitutive defence genes differed between source and sink leaves possibly correlated with feeding site preferences by aphids. All of this suggests that in response to aphid attack, a specific set of genes is induced in the host plant. In order to elucidate aphid-induced effects and their complex interplay with constitutive defences and nutritional factors, further studies at the biochemical, physical, and transcriptional levels will be necessary.

Aphid probing behaviour on twenty *Solanum* genotypes

Although in the colony-development tests, effects of aphid-plant interactions and induced phenotypical plant changes may have played a role, the EPG results most likely relate to constitutive properties of the exposed leaves. It is important to realize that wired aphids cannot

leave an unfavourable plant. Consequently, they may presumably probe longer and more frequently than free aphids would do on the same plant. Differences between susceptible and resistant plants may thus have a tendency to be underestimated by EPG analysis (Tjallingii, 1988). This would depend on the duration of EPG recording. The longer the EPG recording is continued after the time needed to decide to leave a plant by a free aphid, the more its resistance will be underestimated.

Surface resistance. Surface resistance is the first line of defence against attack and is especially important to avoid virus infection. A long time to the first probe mainly reflects the effects of mechanical or olfactory stimuli present at the leaf surface, i.e., at the cuticle-epidermis level, such as the presence of trichomes, repellent volatiles, colour, or toughness of the leaf surface (van Helden and Tjallingii, 1993). Clear surface resistance was observed in the glandular trichome containing *S. berthaultii* 20650-3, *S. polyadenium*, and *S. tarijense.* The role of glandular trichomes in the defense against insects is well-documented (Gibson, 1971; 1974; 1976; Gibson and Turner, 1977; Tingley and Laubengayer, 1981). It is interesting to notice that the *S. berthaultii* 20650-3 genotype has a high resistance to *M. persicae*, whereas *S. berthaultii* 20644-6 appeared to be as susceptible as the control *S. tuberosum* RH89-039-16 (Tables 1 and 2). Variation in resistance level from totally susceptible to highly resistant within the same plant species has been described for different *Solanum* genotypes (Radcliffe and Lauer, 1968). Traits may segregate within accessions; thus, different seedlings could have significantly different genetic properties (Bamberg et al., 1994). Aphids on *S. berthaultii* 20644-6 did not show any delayed first probe, suggesting that at least part of its susceptibility might be explained by the lack of surface components, which are so typical for *S. berthaultii* in general. The others species, e.g., *S. spegazzinii*, *S. capsicibaccatum* 18268-2, *S. capsicibaccatum* 18268-5, and *S. stoloniferum*, as well as the cultivar Kardal, do not possess glandular trichomes; therefore these genotypes must have a different mechanism of surface resistance. The basis of the observed surface resistance remains to be elucidated.

Epidermis/mesophyll resistance. Although only *S. berthaultii* 20650-3 and *S. stoloniferum* showed an increased number of test probes before the first phloem activity, almost all genotypes analyzed showed a prolonged pathway when compared to control plants (**Table 2**; epidermis/mesophyll column). In order to elucidate the cause of this prolonged pathway, further studies will be necessary. The number and total duration of the F waveforms were notably larger only in *S. stoloniferum.* Waveform F has been shown to occur in the mesophyll only (Tjallingii, 1987). Moreover, 73% of F waveform periods started during probes within 3 min, suggesting mechanical

causes of these derailed stylet mechanics located in the first tissue layers (stylets penetrate about one cell layer/min).

Mesophyll/phloem resistance. Sieve element salivation (waveform E1) always precedes phloem sap ingestion (E2) and sustained phloem sap ingestion (sE2). Therefore, the first E1 can be considered as the first established sieve element activity. However, a sieve element may have been 'reached' and punctured earlier already. Nearly every cell encountered along the intercellular pathway to the phloem is punctured and sampled (reflected as potential drops in EPGs; Prado and Tjallingii, 1994). The cells in the vascular bundle are punctured more frequently than those in the mesophyll (Tjallingii and Hogen Esch, 1993) and salivation by the aphid (E1 waveform) presumably indicates sieve element 'recognition' and not merely contact with the sieve element. The first sustained sieve element ingestion (sE2) has been considered as committed phloem ingestion or 'sieve element acceptance' (Tjallingii and Mayoral, 1992). Both events are central in the host plant selection process by the aphid. Genotypes showing a relatively long time to the first E1 but not to the first sE2 (i.e., *S. okadae, S. hondelmannii,* and *S. multiinterruptum*) most likely have resistance factors at the mesophyll level. However, the resistance factor(s) acting on the stylet's pathway to the phloem can be overcome by the aphid, as the result of the colony development test was similar to the susceptible controls (**Table 1**). In contrast, a relatively long time to first sE2 but not to first E1 most likely indicates resistance factors at the phloem level (i.e., *S. jamesii* 18349-10, *S. jamesii* 18349-1, and cv. Kardal). Nevertheless, some aphids are able to overcome these resistance barriers and can develop successfully on these plants (**Table 1**). The aphid colony-development tests show that aphids on *S. jamesii* 18349-1 and *S. jamesii* 18349-10 must be able to feed, but the 6-h-period of EPG recording appeared to be too brief for the aphids to achieve sustained phloem feeding (sE2). The potential E2 index (van Helden and Tjallingii, 1993) gives an indication of the aphid reaction after having the first successful phloem sap ingestion, irrespective of how long it takes for the aphid to reach sustained E2 for the first time (Tjallingii and Mayoral, 1992). In *S. multiinterruptum,* the reduced E2 index suggests the presence of a chemical factor in the phloem sap of young plants that stopped ingestion and reduced further phloem feeding.

Resistance without behavioural effects. Electrical penetration graph parameters do not indicate any resistance in genotype *S. cardiophyllum,* but nevertheless there was no colony development of aphids on young plants of this genotype. Therefore, in young plants the resistance factor appeared

to work in a delayed way, suggesting an antibiotic effect or nutritional deficiency of the phloem sap affecting the development and survival of aphids rather than a behavioural interference (**Table 2**).

Probing on *Solanum berthaultii* and *Solanum tarijense* with and without intact glandular trichomes

Electrical penetration graph results from *S. berthaultii* 20650-3, *S. polyadenium*, and *S. tarijense* plants with intact glandular trichomes (**Table 2**) suggest that resistance to *M. persicae* differs between these genotypes. *Solanum berthaultii* 20650-3 showed resistance at surface and first tissue layers while *S. polyadenium* and *S. tarijense* showed resistance factors at nearly all tissue levels: epidermal, mesophyll, and phloem (**Table 2**). Our results of plants with washed leaves indicate that the resistance in *S. berthaultii* 20650-3 and *S. tarijense* mainly depends on the trichomes (**Table 3**). Bonierbale et al. (1994) reported an incomplete correlation between trichome phenotype and aphid resistance in the progenies of crosses between *S. tuberosum* and *S. berthaultii* and one of their explanations was that different mechanisms of resistance - independent of trichomes - might be expressed in the hybrids. Previous work showed that removal of the trichomes from *S. berthaultii* leaflets resulted in increased adult feeding initiation, preference, and consumption of foliage by the Colorado potato beetle, *Leptinotarsa decemlineata* (Yencho and Tingey, 1994). Furthermore, the removal of type B droplets on trichomes of *S. berthaultii* resulted in a decreased time to the first probe by *M. persicae* as compared to intact plants; in contrast, when type B exudates of trichomes were transferred to *S. tuberosum* cv., first probes were delayed (Lapointe and Tingey, 1984).

Resistance based on glandular trichomes in wild tuber-bearing *Solanum* spp. has some drawbacks for breeding. The expression of resistance in *S. berthaulthii* appeared to be (a) due to the interaction of several chemical and physical characteristics of glandular trichomes, (b) a quantitative trait (Mehlenbacher et al., 1983), and (c) linked to undesirable agronomic characteristics such as late tuber development (Bonierbale et al., 1994; Kalazich and Plaisted, 1991). Additionally, it was found that resistance in field experiments is lower than under glasshouse conditions, probably because of the repeated influx of colonizing aphids in field trials that depletes the type-A trichomes, which are not renewed. In addition, precipitation and overhead irrigation may wash away trichome secretions in mature and senescent foliage (Tingey et al., 1982).

Table 1. Aphid colony development test. *Solanum* species are ordered according to the number of aphids (adults and nymphs) per plant on day 15 in the aphid colony-development test on young plants. Values are means ± SEM of number of aphids (adults and nymphs).

Solanum species	Identity number or cultivar name[1]	Young plants				Flowering plants[3]		
		Adults on day 8	Nymphs on day 8	Adults on day 15	Nymphs on day 15	y	m	s
S. okadae Hawkes et Hjerting	18108-3	29.7 ± 6.1	33.3 ± 4.1	29.3 ± 8.8	237 ± 81.2	++	++	++
S. tuberosum L.	cv. Eersteling	4.7 ± 0.7	14.7 ± 2.7	21.0 ± 3.6	95.3 ± 27.8	nd		
S. phureja Juz et Buck.	17667-1	1.0 ± 0.6	27.3 ± 3.7	9.7 ± 1.2	80.0 ± 10.8	nd		
S. tuberosum L.	RH89-039-16[2]	0.7 ± 0.7	14.0 ± 1.0	11.0 ± 2.6	65.3 ± 16.2	++	++	++
S. hondelmannii Hawkes et Hjert	18182-2	1.3 ± 0.7	25.7 ± 8.8	10.3 ± 2.3	49.3 ± 30.7	nd		
S. jamesii Torr.	18349-10	1.0 ± 0.6	13.7 ± 3.2	5.3 ± 2.3	31.0 ± 6.2	nd		
S. berthaultii Hawkes	20644-6	7.7 ± 1.2	12.7 ± 4.8	5.0 ± 1.7	31.0 ± 5.2	++	++	++
S. bulbocastanum Dun.	17693-2	0.7 ± 0.7	11.7 ± 4.4	4.0 ± 1.7	13.3 ± 6.0	nd		
S. jamesii Torr.	18349-1	1.0 ± 0.0	7.0 ± 2.1*	2.3 ± 0.9	7.3 ± 3.2	-	-	+
S. tuberosum L.	cv. Mondial	4.0 ± 1.2	3.0 ± 0.6*	2.0 ± 1.2	7.3 ± 4.1	++	++	++
S. tuberosum L.	cv. Kardal	0.7 ± 0.7	2.0 ± 1.2*	1.0 ± 0.6	8.0 ± 4.0	-	+	++
S. spegazzinii Bitt.	17839-2	0	3.3 ± 1.2*	2.0 ± 0.6	5.0 ± 0.6	+	+	+
S. capsicibaccatum Cárd.	18268-2	0	1.7 ± 0.3*	0.7 ± 0.3*	3.0 ± 1.0*	-	-	+
S. capsicibaccatum Cárd.	18268-5	0	0.7 ± 0.3*	0*	1.3 ± 0.9*	nd		
S. multiinterruptum Bitt.	17829-2	0	0.3 ± 0.3*	0*	1.0 ± 1.0*	++	++	++
S. cardiophyllum Lindl.	18326-1	0	3.0 ± 0.6*	0*	0.3 ± 0.3*	++	++	++
S. berthaultii Hawkes	20650-3	0	0*	0*	0*	-	-	-
S. stoloniferum Schlechtd.	17605-4	0	0*	0*	0*	-	+	++
S. polyadenium Greenm.	17749-1	0.3 ± 0.3	1.7 ± 1.2*	0*	0*	+	-	+
S. tarijense Hawkes	17861-8	0	0.3 ± 0.3*	0*	0*	-	-	-

Dotted lines group species according to relative level of resistance. [1]Identity numbers from CGN followed by the genotype in vitro collection number (e.g., -2) or cultivar name (cv.); [2]RH89-03916, breeding line chosen as susceptible control (diploid line used in interspecific crossings with wild *Solanum*); [3] y, young leaves; m, mature leaves; s, senescent leaves, - no aphids found, + few aphids, ++ leaves covered by aphids and nymphs; nd, not determined (experiment not performed); *smaller number of aphids than the susceptible control *Solanum tuberosum* RH89-03916 (Mann-Whitney; $P<0.05$).

Table 2. Electrical penetration graph (EPG) results and the tissue location of aphid-resistance factors as inferred from the parameters shown. Values are means ± SEM of EPG parameters described in Materials and methods.

Solanum species	Identity number or cultivar name[1]	n[2]	Surface	Epidermis/mesophyll		Mesophyll/phloem		Phloem		Aphids with sustained E2	
			Time to first probe (min)	Probes <3min before first E1	Minimum C prior to E1 (min)	Time to first E1 in experiment (min)	Time to first sE2 in experiment (min)	Potential E2 index %	Average period of E2 (min)	n	%
S. okadae	18108-3	15	1.7 ± 0.3	20 ± 5*	9.8 ± 1.0**	114 ± 24*	219 ± 34	60 ±10	56 ± 24	10	66.7
S. tuberosum	cv. Eersteling	19	2.9 ± 0.8	6 ± 1	7.3 ± 0.6*	45 ± 11	237 ± 31	55 ± 9	26 ± 7	10	52.6
S. phureja	17667-1	16	4.3 ± 1.0*	7 ± 1	9.4 ± 0.9**	60 ± 19	87 ± 18	61 ± 6	39 ± 6	16	100.0
S. tuberosum	RH89-039-16	16	1.8 ± 0.3	7 ± 1	5.5 ± 0.6	58 ± 13	219 ± 36	73 ± 11	84 ± 33	10	62.5
S. hondelmannii	18182-2	15	11.6 ± 2.7**	8 ± 1	8.1 ± 1.3	167 ± 26**	260 ± 27	87 ± 9	80 ± 23	9	60.0
S. jamesii	18349-10	18	6.5 ± 3.0	6 ± 1	5.6 ± 0.6	51 ± 9	359 ± 1**	100[c]	4 ± 2*	1[4]	5.6
S. berthaultii	20644-6	16	4.5 ± 1.2	11 ± 3	8.9 ± 1.0**	97 ± 24	181 ± 25	73 ± 6	48 ± 14	14	87.5
S. bulbocastanum	17693-2	16	8.3 ± 2.3*	4 ± 1	7.4 ± 0.6*	39 ± 9	287 ± 29	80 ± 9	56 ± 27	6	37.5
S. jamesii	18349-1	17	1.7 ± 0.4	9 ± 2	5.2 ± 0.3	62 ± 14	346 ± 8*	88 ± 3[3]	6 ± 2**	2[4]	11.8
S. tuberosum	cv. Mondial	19	8.7 ± 4.5	4 ± 1	9.7 ± 0.8**	49 ± 8	284 ± 20	75 ± 10	29 ± 9	11	57.9
S. tuberosum	cv. Kardal	16	7.2 ± 2. 8*	10 ± 2	12.5 ± 1.4**	81 ± 23	336 ± 19*	99 ± 1[3]	13 ± 10*	2[4]	12.5
S. spegazzinii	17839-2	15	6.5 ± 1.9*	17 ± 5	6.9 ± 0.5*	111 ± 29	285 ± 29	51 ± 15	31 ± 14	6	40.0
S. capsicibaccatum	18268-2	16	9.9 ± 3.0*	17 ± 4	13.4 ± 2.8**	194 ± 36**	322 ± 21*	27± 10	18 ± 5	3[4]	18.8
S. capsicibaccatum	18268-5	15	15.7 ± 3.9**	10 ± 3	12 ± 1.8**	146 ± 33*	349 ± 9*	54 ± 46[3]	6 ± 5*	2[4]	13.3
S. multiinterruptum	17829-2	19	5.0 ± 1.3	13 ± 3	7 ± 0.4**	96 ± 13*	246 ± 29	19 ± 4**	10 ± 1*	9	47.4
S. cardiophyllum	18326-1	16	2.1 ± 0.4	10 ± 3	7 ± 0.7	68 ± 23	177 ± 33	64 ± 8	51 ± 13	12	75.0
S. berthaultii	20650-3	16	13.5 ± 3.1*	14 ± 2*	8 ± 0.9*	95 ± 16	305 ± 19	53 ± 12	29 ± 11	7	43.8
S. stoloniferum	17605-4	18	8.6 ± 1.6**	14 ± 2*	10 ± 1.2**	179 ± 27**	311 ± 24*	65 ± 19	39 ± 25	4[4]	22.2
S. polyadenium	17749-1	15	20.4 ± 6.0**	12 ± 3	8 ± 1.3*	188 ± 40*	358 ± 2**	100[3]	6 ± 3*	1[4]	6.7
S. tarijense	17861-8	16	23.9 ± 14.9*	10 ± 2	12 ± 1.5**	185 ± 34**	360 ± 0**	0[3]	0*	0[4]	0.0

Dotted lines group species according to relative level of resistance with aphid colony development test (**Table 1**). [1] Identity numbers from CGN followed by the genotype in vitro collection number (e.g., -2) or cultivar name (cv.); RH89-03916, breeding line chosen as susceptible control (diploid line used in interspecific crossings with wild *Solanum*); [2]n, EPG replicates; * P<0.05, ** P<0.005, Mann Whitney; [3]The number of aphids (replicas) is lower than 2, thus statistical analysis is not possible (potential E2 index, is the percentage of time spent on E2 after subtraction of the time needed to reach the first sE2 and is calculated only for aphids showing sustained E2); [4]Fisher's test at the 6th hour of EPG recording: P<0.05.

Table 3. Electrical penetration graph (EPG) results of Myzus persicae adults feeding on *Solanum berthaultii* 20650-3 and *Solanum tarijense* 17861-8 with intact glandular trichomes and devoid of glandular trichomes content during a 6-h-recording period. Values are means ± SEM of EPG parameters described in Materials and methods.

Species and treatment	EPG n	EPG results and inference of the location of aphid-resistance factor in plant tissue								
		Surface	Epidermis/mesophyll		Mesophyll/phloem		Phloem		Aphids with sustained E2	
		Time to first probe (min)	n probes < 3 min before first E1	Shortest C prior to E1 (min)	Time to first E1 in experiment (min)	Time to first sE2 in experiment (min)	Potential E2 index %	Average duration of E2 (min)	n	%
S. tarijense intact trichomes	15	11.6 ± 4.0**	5 ± 1	14.1 ± 2.2	109 ± 30	360 ± 0.0**	0[2]	4 ± 1	0[1]	0.0
S. tarijense no glandular trichomes	15	1.6 ± 8	8 ± 3	10.6 ± 1.2	68 ± 15	311 ± 16	37 ± 11	26 ± 9	7	46.7
S. berthaultii intact trichomes	15	12.1 ± 4.0**	5 ± 2	12.9 ± 2.1	85 ± 22	360 ± 0.0**	0[2]	3 ± 1**	0[1]	0.0
S. berthaultii no glandular trichomes	15	4.0 ± 1.7	7 ± 2	12.4 ± 1.5	82 ± 24	217 ± 30	40 ± 11	68 ± 23	10	66.7

* P<0.05, ** P<0.005, Mann Whitney; [1] P<0.05 Fisher's test at the 6th hour of EPG recording; [2] The number of aphids showing sustained E2 (replicas) is 0, thus statistical analysis is not possible (potential E2 index, is the percentage of time spent on E2 after subtraction of the time needed to reach the first sE2 and is calculated only for aphids showing sustained E2).

Comparison of EPG parameters from *S. tarijense* plants with and without glandular trichomes indicates that a strong surface resistance may bias EPG parameters associated with resistance at deeper tissue levels. Thus the trichome effects completely prevented sustained phloem feeding, although some short periods of phloem ingestion may have occurred (**Table 2** and **Table 3**). It is likely that the glandular secretions and tarsal irritation interfered persistently with probing activities. The presence of a second line of defence depending on other mechanisms could not be demonstrated in *S. berthaultii* 20650-3 and *S. tarijense*.

Aphid colony development in relation to feeding behaviour

When interpreting EPG results, we should take into account that the differences observed between each test accession and the *S. tuberosum* control represents fine details of duration and occurrence of events or activities. An aphid can spend more or less time in activities on its stylet's path to the phloem, but the ultimate time of phloem feeding can be similar or longer. This final settling may occur after the 6 h we used in our EPG experiments. Therefore the colony-development tests cover a completely different time frame than the initial events recorded by EPGs.

In studying plant resistance to aphids, EPGs of initial events of probing and feeding behaviour and colony-development tests of the overall plant suitability should be considered as complementary techniques, rather than parallel approaches. When we combined colony development results with EPG data on probing and feeding some paradoxical results appeared: EPGs from aphids feeding on *S. okadae*, *S. phureja*, cv. Eersteling, S. *hondelmannii*, *S. jamesii* 18349-10, S. *berthaultii* 20644-6, and *S. bulbocastanum* showed that aphids encountered some constraints at different tissue levels when compared to the susceptible control, *S. tuberosum* RH89-03916. The aphid colony-development test showed that these accessions are similar in susceptibility to *S. tuberosum* RH89-03916. Thus, suitable host plants may contain resistant features at some tissue levels, but this resistance does not affect overall susceptibility.

Within the *S. tuberosum* varieties analyzed, Mondial and Kardal showed some resistance with respect to colony-development at the young stage (**Table 1**) and EPG results showed factors at the surface, mesophyll, and phloem levels. On our susceptible control *S. tuberosum* RH89-039-16, some individual aphids on young fully expanded leaves also showed an EPG pattern indicative of resistance, similar to Kardal, although on average, the effect was low and aphid colonies developed

well. Other studies have reported antibiotic resistance to *M. persicae* in some *S. tuberosum* varieties (Radcliffe and Lauer, 1968; Bintcliffe and Wratten, 1982). On the basis of our present knowledge however, we think that these 'antibiotic' effects might have been caused by avoidance of phloem feeding, i.e., 'antixenotic' effects at the phloem level. As pointed out by Karley et al. (2002) and in accordance with our results, many cultivated potato plants seem not to be optimal hosts for *M. persicae*. Most EPG data about resistance point in the direction of antixenotic effects, i.e., delays in showing phloem activities or no sustained phloem activity at all, which might be interpreted as phloem avoidance. The wired aphids could not leave the plants. Our aim was to study probing and feeding when aphids had access to the plants and how that would affect their plant penetration behaviour. Also free aphids have been observed to probe into non-host, resistant, or partially resistant plants frequently and even may spend considerable time on them. As long as they stay on such plants, feeding will be reduced, leading to reduced performance, which has been the classical criterion for antibiosis. As we learn more about the details of host plant resistance to aphids, Painter's terminology (Painter, 1951) is often not very appropriate. However, one exception should be made here for *S. cardiophyllum*, on which aphid colony development was very poor despite the absence of evidence in the EPGs that probing and feeding were affected. Consequently, resistance in this genotype appears to be due to antibiosis sensu strictu.

Virus transmission

The partial plant resistance present in cv. Kardal and *S. stoloniferum* can be important for limiting the transmission of phloem restricted viruses such as PLRV. Slow aphid population build-up on young plants will reduce secondary virus spread. Therefore, it would be interesting to elucidate the mechanism of the resistance in young leaves. Furthermore, the spread of viruses can be reduced by combining young plant resistance to aphids with 'mature plant resistance' (MPR) to viruses. Beemster (1987) showed that the older the plant at the time of inoculation, the fewer viruses are translocated to the tubers.

In general, vector efficiency of *M. persicae* will be reduced when the normal feeding behaviour is impaired. Genotypes with surface resistance factors combined with mesophyll- and phloem-localised resistance factor(s) can be expected to have a lower probability of inoculation by persistently transmitted viruses such as PLRV. Genotypes on which a small percentage of aphids reach sustained phloem ingestion and on which E2 has shorter duration (phloem factor resistance) are likely to lower the efficiency of aphids to acquire persistent virus.

Conclusions

The combination of an assay for free aphid colony development with the EPG measurement of initial probing and feeding has been shown to be an effective way to obtain information on the location of resistance factors in tuber-bearing *Solanum* genotypes. Mechanisms of resistance to *M. persicae* were detected at the surface and at three plant tissue levels.

The expression of resistance to *M. persicae* varies with the age of the plants and with plant parts. Some of the plants that are resistant at a young developmental stage became susceptible at the flowering mature stage, either by a change in the whole plant or by reduced resistance in senescent leaves. We did not include other aphid species in this study. Therefore we can only speak about resistance to *M. persicae*. Resistance to one aphid species often has no implication for resistance to other species. Van Helden and Tjallingii (1993) investigated a case of total resistance to *Nasonovia ribisnigri* in lettuce, which implied no resistance whatsoever to *M. persicae* or *Macrosiphum euphorbiae*.

The results of the present study were obtained under greenhouse and laboratory conditions, and can be used as a basis for further unravelling of resistance mechanisms at the metabolic, molecular, and genetic levels. Field experiments will be necessary to evaluate resistance under agricultural crop conditions.

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CHAPTER 3

Aphid-plant interactions: probing and performance differences between *Myzus persicae* and *Macrosiphum euphorbiae* on two *Solanum* species

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Abstract

Previously, we found that the wild potato species *Solanum stoloniferum* Schlechtd has a constitutively expressed resistance towards *Myzus persicae* (Sulzer) in the apical leaves. This resistance is located at the epidermal/mesophyll level, and declines as soon as the leaves become senescent. On the other hand, *Macrosiphum euphorbiae* (Thomas) is able to naturally colonise the apical leaves of *S. stoloniferum*. Hence, *S. stoloniferum* was chosen as model plant to unravel differences between compatible and incompatible aphid-plant interactions. In this chapter, we describe several tests to assess the interactions between *S. stoloniferum* and each of the two aphid species. We studied the performance, settling and feeding behaviour of *M. persicae* and *M. euphorbiae* on *S. stoloniferum* and *Solanum tuberosum*. We found that *S. tuberosum* and *S. stoloniferum*, can be host plants for *M. euphorbiae*, even when *M. euphorbiae* salivates more and for a longer period on *S. stoloniferum* than on *S. tuberosum*. On both plant species, it succeeds in performing sustained phloem-sap ingestion. In contrast to *M. euphorbiae*, *S. stoloniferum* is a poor host for *M. persicae*. The survival of both aphid species was affected when they developed on young leaves of *S. stoloniferum* but not on senescent leaves. To find out whether the resistance of *S. stoloniferum* to *M. persicae* was mainly due to repellence or to physical constraints we studied the aphid settling behaviour in 'free aphid' trials. We found no evidence for an induction of a repellence or deterrence in plants that had previously been infested with *M. persicae* compared to plants that had never been infested. The resistance found in *S. stoloniferum* against *M. persicae* most likely relies on constitutively expressed traits.

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Introduction

Potato crops can be severely affected by aphids, both directly by feeding or indirectly as pathogen vectors. To our knowledge, no commercial potato varieties have been developed specifically for resistance to insects. Aphids are phloem-sap feeding insects that have extremely-fine specialised mouthparts, the stylets, which they use to penetrate the plant tissue (Blackman, 1974). Hence, in order to feed, aphids have to establish a close interaction with their host plant. The plants protect themselves against attack by different aphids via specific defence mechanisms. Several studies on plant resistance to aphids have shown that mechanisms effective to one aphid species, do not provide protection to others. *Lactuca sativa* L., carrying the NR resistance gene from *Lactuca virosa* L., has absolute resistance to *Nasonovia ribisnigri* (Mosley), but is susceptible to other aphid species (*M. persicae*, *M. euphorbiae*, and *Uroleucon sonchi*) (Reinink and Dieleman, 1989; van Helden and Tjallingii, 1993). Furthermore, the resistance to aphids can be even aphid-isolate- or biotype-specific. For example, the *Mi* gene of tomato that confers resistance to the root knot nematode and to *M. euphorbiae* (Rossi et al., 1998), has been reported as not effective against *M. persicae* while it is also not effective to all the *M. euphorbiae* biotypes. (Goggin et al., 2001).

The generalist aphid *M. persicae* is the major problem in potato crops, mainly because it transmits viruses. Development of plants with a pre-phloem mechanism of resistance would be advantageous to avoid inoculation of persistently transmitted viruses. In previous works, we have localized several factors of resistance to *M. persicae* in leaf tissues of wild *Solanum* genotypes. This was inferred from performance and feeding behaviour differences on 13 wild potatoes species. In one of these, *Solanum stoloniferum* Schlechtd, we found pre-phloem resistance, which was not based on glandular trichomes. Moreover, we showed that young, fully developed leaves, are resistant to *M. persicae*, whereas senescent yellowing leaves of flowering *S. stoloniferum* plants are more or less susceptible (Alvarez et al., 2006). On the other hand, we observed that *M. euphorbiae* is capable of colonising young apical leaves of *S. stoloniferum.*

In general, *S. stoloniferum* is an acceptable host for *M. euphorbiae* but not for *M. persicae*. Here, we use *S. stoloniferum* as a model plant to unravel and characterise the differences in host plant attack between the two generalist aphid species, while using *S. tuberosum* as a control plant.

In order to compare the degree of resistance/susceptibility that *S. stoloniferum* has to each of these aphid species we studied (1) the performance of *M. persicae* and *M. euphorbiae* on *S. stoloniferum* and *S. tuberosum*; (2) the feeding behaviour of both aphids on the two plant species, and (3) the settling behaviour of *M. persicae* to investigate whether, besides the constitutive resistance present in *S. stoloniferum*, there is also an induced resistance that might be activated by aphid preinfestation.

Materials and Methods

Plants and aphids

The *S. stoloniferum* clone 17605-4 was selected for this study because of its high level of resistance to *M. persicae* (Chapter 2). The clone is derived from accession 17605 of the Centre for Genetic Resources, Wageningen, The Netherlands (CGN) and maintained *in vitro* at the Plant breeding department of Wageningen University. Propagation of plants was performed *in vitro* on Murashige and Skoog medium including vitamins, sucrose 3%, pH 5.8. *Solanum tuberosum* RH89-039-16, is a breeding line that was used as susceptible control plant for both aphid species. After two weeks on agar, the plantlets with developed roots were transferred to soil in 22 cm diameter pots in a glasshouse at 22 ± 2ºC, about 70% relative humidity and L16:D8 h photoperiod.

M. persicae was reared in a climate chamber in cages on radish, *Raphanus sativus* L., plants at 22 ± 2ºC, about 30–40 % relative humidity under L16:D8 photoperiod. An *M. euphorbiae* colony was reared in a glasshouse in cages on *S. tuberosum* cv Bintje at 22 ± 2ºC, about 70% relative humidity and L16:D8 h. All aphids used in the experiments came from a newly established clone of a single virginoparous apterous individual, the colony was maintained at the Laboratory of Entomology, Wageningen University. A new colony of this clone was started weekly. Newly moulted adult aphids were used in all experiments.

Aphid Performance

Performance of *M. persicae* and *M. euphorbiae* on *S. stoloniferum* was tested by measuring nymph mortality and pre-reproductive time. Three recently-moulted (1-3 days old) adult apterae were transferred to a leaf and enclosed in clip-on cages (20 mm diameter) on the abaxial side of two leaves of each test plant. After 24 h adults and nymphs were removed leaving 3 new-born nymphs per leaf. The condition of these 3 individuals, dead or alive, was recorded daily until production of

the first progeny. The mean number of pre-reproductive days was registered. *Solanum tuberosum* RH89-039-16 was used as susceptible control plant. Two stages of the plants were used for this assay, i.e. young plants and fully-grown flowering plants. On young plants (2 weeks after transfer to soil), aphids were placed on 2 young leaves (3^{rd}-4^{th} nearly fully expanded leaves from top); 5 plants, that is 10 leaves were used per aphid species, with a total of 30 aphids per species. On flowering plants (5 weeks old) aphids were placed on the yellowish basal (senescent) leaves; 8 plants, that is 16 leaves, were used per aphid species, with a total of 48 aphids per species. Experiments were done under glasshouse conditions (at 22 ± 2 ºC, about 70% RH, and L16:D8 photoperiod).

Myzus persicae settling behaviour on untreated plants

Five recently moulted (1-3 days old) adult apterous *Myzus persicae* were placed on the apical leaves of 4 plants per replicate, thus 20 aphids were used in each replicate. Numbers of aphids remaining on plants after 15, 30 min, 1 h, 2 h and 24 hours were counted (for 10 replicates aphids were counted from 15, 30 min, 1 h, 2 h, and on the last 6 replicates the counting was extended to 24 h). Plants were placed on a tray with water to avoid movements from one plant to the other. Experiments were done under glasshouse conditions (22 ± 2 ºC, about 70% r.h., at L16:D8 photoperiod).

Myzus persicae settling behaviour on preinfested plants

Preinfestation

To measure induced antixenosis to *M. persicae* at the local and systemic level after pre-infestation of the leaves by the two aphid species, we modified a test developed by Martin and Fereres (2003) referred to as ‘settling test’. We scored aphid settling on plants preinfested with *M. persicae*. Two leaves per plant of *S. stoloniferum* and *S. tuberosum*, were preinfested each with 40 aphids (80 per plant) during 96 hours. These leaves (leaves 5 and 6 from apex at start of preinfestation) were enveloped by non-woven ‘agrotextile’ bags and were regarded as ‘local leaves’. To test for systemically induced resistance, two nearly expanded leaves (leaves 3 and 4 from apex at start of preinfestation) of the same plants were enveloped by agrotextile bags without aphids and those were regarded as systemic leaves. Leaves on non-infested control plants were also covered with bags but without aphids. After 4 days of preinfestation, all aphids were counted and removed with a soft brush from the local leaves and plants were used for the aphid settling test. All the plants were then at flowering stage and 35 days old. Nine replicates were performed per treatment.

Settling tests

Three treatments were used to test *M. persicae* settling and within each treatment local and systemic leaves were used from: 1) *S. stoloniferum* preinfested with *M. persicae;* 2) *S. tuberosum* preinfested with *M. persicae*; 3) *S. stoloniferum* non-infested (control); 4) *S. tuberosum* non-infested (control).

In order to avoid aphids moving to non-tested leaves, all leaves except those tested were entirely enveloped with agrotextile bags. Also, to avoid aphid migration to other plants, all tested plants were placed on a tray with water. At the start of the experiment (time 0), 10 aphids were transferred to each of the two leaves per plant, and the number of aphids remaining on the leaf was counted every 5 min until 30 min, the total observation time. After 30 minutes, aphids remaining on test leaves were removed, and the missing aphids were tracked down and mostly found in the water or walking on the bags.

Aphid settling was tested first on local leaves of treated plants. Then, the local leaves were enveloped by agrotextile bags and the two systemic leaves of the plant were uncovered and used for tests, following the same procedure.

Electrical penetration graph (EPG) monitoring of probing behaviour

The DC-EPG technique (Tjallingii, 1985; 1988) was used to monitor probing of aphids during 6 h. Four plants of both species were placed in a Faraday cage and EPGs of two aphids on each plant were recorded simultaneously during 6 h, 11-15 successful replicates per plant species. Each plant was used after 20 days since transfer to soil. Aphids were placed on the abaxial side of the third leaf from the apex, which was nearly fully expanded. Before exposure to a plant, the aphid was immobilized by a vacuum suction device (van Helden and Tjallingii, 1993) to attach a gold wire electrode – 2-3 cm long, 20 μm in diameter – conductively connected by water-based silver glue to its dorsum. The other end of the gold wire was attached to a 3-cm long copper wire (diameter 0.2 mm) and connected to the input of the first stage amplifier with a 1 Giga-Ohm input resistance and 50 x gain (Tjallingii, 1985; 1988). The plant electrode, a 2 mm thick, 10 cm long copper rod, was inserted in the soil of the potted plant and connected to the plant voltage output of the EPG device (Giga-4 or Giga-8, manufactured by Wageningen University). Plants, aphids and the first stage amplifiers were set up in a Faraday cage. Recording was started immediately after plant access, about 1 h after aphids were collected from the colony and wired, at about 20 °C and constant light

in the laboratory. Signals of 16 aphids on 8 plants, 4 per Faraday cage, were simultaneously recorded on PC hard disks. Data acquisition and waveform analysis were mediated by Probe 3.0 software (Wageningen University, Laboratory of Entomology) at 100 samples per s.

EPG waveforms, waveform patterns, and parameters

The recorded EPGs were analysed by distinguishing the following probing phases and waveforms. Stylet pathway phase: including four pooled pathway waveforms, i.e., A, B, C, and pd (potential drop); waveform A, the first electrical stylet contact with epidermis; waveform B, intercellular sheath salivation; waveform C, stylet movements; and waveform pd, intracellular stylet punctures. Phloem phase: including waveform E1, sieve element salivation; and waveform E2, phloem sap ingestion with concurrent salivation. Xylem phase, only waveform G, active drinking from xylem elements (Tjallingii, 1990). In addition waveform E1e was distinguished, representing watery salivation at extracellular voltage level as well as waveform F, derailed stylet mechanics [stylet penetration difficulties (Tjallingii, 1990)]. Waveform occurrence was retrieved and 34 EPG parameters were subsequently calculated, of which we selected 28 as relevant in this study (**Table 1**).

Statistical analysis

Nymph mortality was compared by Fisher's exact test; two contrasts were made for each aphid species, 1) at the same leaf stage (young or old), *S. tuberosum* vs. *S. stoloniferum*, 2) at the same host plant at different leaf stage, young vs old. Mann-Whitney rank sum test (SPSS 12.0.1 for Windows) was used for each aphid species to test for differences on pre-reproductive time on different host (*S. tuberosum* vs. *S. stoloniferum*).

To analyze aphid settling behaviour, the mean number of aphids remaining on the plant and the standard error of the mean (SEM) at every recording time were calculated for all treatments; then Mann-Whitney rank sum test was used to test for differences with non-infested controls.

The EPG parameters were analysed individually for each aphid and, after means and standard errors of the mean (SEM) were calculated for aphids per *Solanum* species, the Mann-Whitney rank sum test was used to test for differences with susceptible control data from *S. tuberosum* RH89-039-16. Fisher's exact test was applied for analysis of the number of aphids showing sE2. In order to

characterize probing and feeding of the two aphid species independent of the host plant that they were on, we analysed the data by Kruskal-Wallis followed by multiple comparisons with Bonferroni correction (Weisstein, 1999).

Table 1. Summary of EPG parameters considered and their relation to aphid feeding activity. Only the most relevant parameters are numbered. Capital letters refer to waveform patterns (Tjallingii, 1990). See the main waveform patterns on Figure 3, Chapter 1.

Nr	Related to	Parameter	Unit	Statistics
1	Probing	time to 1st probe (= 1st non-probing period)	s	Absolute
2		number of probes	#	Absolute
3		average probe duration	min	Average
4		number of brief probes < 3 min until 1st phloem contact	#	Absolute
		total probing time	s	Sum
5	Pathway	number of pathway periods	#	Absolute
6		average pathway duration, with pd without E1e, F and G	min	Average
7		total pathway time with pd without F, G and E1e	h	Sum
8	cell puncture	number of pd	#	Absolute
9		average duration of pd	s	Average
10		total time pd	min	Sum
	pathway (unknown)	number of E1 extracellular (E1e) periods	#	Absolute
11	derailed mechanics	number of F periods	#	Absolute
12		average F duration	min	Average
		total F	s	Sum
13	pathway + probing	time 1st probe - 1st E1 in the experiment	min	Absolute
14		time 1st probe - 1st E12 in the experiment	H	Absolute
15		time 1st probe - 1st sE2 > 10 min in the experiment	H	Absolute
	xylem contact	number of G periods	#	Absolute
		average G duration	s	Average
		total G	s	Sum
16	phloem contact	number of single E1 (without E2)	#	Absolute
17		maximum single E1 duration	s	Maximium
18		average single E1 duration	s	Average
19		total time of single E1	min	Sum
20		number of E1 periods (sgE1 and E1fr)	#	Absolute
21		maximum E1 duration (before or after E2)	min	Maximium
22		average E1 duration	min	Average
23		total time of E1 (sgE1 and E1)	min	Absolute
24		number of E12 phloem periods i.e. with both, E1 and E2	#	Absolute
25		average E12 duration	min	Average
26		average E2 duration	min	Average
27		total time of E2	min	Sum
28	host acceptance	Number of aphids with sE2	#	Absolute

Results

Aphid performance

Performance of *M. euphorbiae* and *M. persicae* on the wild potato *S. stoloniferum* in clip cages differed from the *S. tuberosum* control. The nymph mortality of *M. euphorbiae* on young leaves of *S. stoloniferum* was significantly higher than on young leaves of *S. tuberosum* control plants. *M. persicae* had also significantly higher mortality on *S. stoloniferum* than in *S. tuberosum* plants (**Table 2**). On the contrary, the nymph mortality of both aphids on *S. stoloniferum* and *S. tuberosum*, was similar on old leaves (see P-values Fisher's exact test in **Table 2**).

Table 2. Nymph mortality of *M. euphorbiae* and *M. persicae* on *S. tuberosum* (tub) and *S. stoloniferum* (sto). Values are percentages of aphids that died during the pre-reproductive period.

Leaf stage[1]	*M. euphorbiae*			*M. persicae*		
	tub	Sto	P-value[2]	tub	sto	P-value[2]
Young	0a[3]	40b	0.000	30d	79f	0.000
Old	8a	10c	0.500	13e	13g	0.620

[1] young, 3-4 nearly fully expanded leaves from top of 2 weeks old plants; senescent, basal leaf of 5 weeks old plants Each percentage is calculated for 30 and 48 individuals on young and old leaf stage respectively.
[2] Within each row, Fisher's exact test P-values are for contrast between each aphid species on different host.
[3]Within each column, numbers followed by different letters indicates mortality significantly different between young and old leaf stage on the same host plant at $P \leq 0.05$, Fisher's exact test.

Pre-reproductive time of *M. euphorbiae* on *S. stoloniferum* was delayed on young and old leaves, when compared to *S. tuberosum.* Surprisingly, the pre-reproductive time for *M. persicae* was similar on *S. stoloniferum* and *S. tuberosum*, irrespective of leaf age (**Table 3**).

Table 3. Pre-reproductive time of *M. euphorbiae* and *M. persicae* on *S. tuberosum* (tub) and *S. stoloniferum* (sto). Values are mean ± SEM of days from birth to reproduction.

Leaf stage[1]	*M. euphorbiae*			*M. persicae*		
	tub	sto	P-value[2]	Tub	sto	P-value[2]
Young	9.3 ± 0.1 n = 29	12.3 ± 0.4 n = 18	0.000	10.9 ± 0.2 n = 21	9.8 ± 0.8 n = 6	0.065
Old	9.0 ± 0.0 n = 44	10.3 ± 0.3 n = 18	0.000	8.3 ± 0.2 n = 42	8.4 ± 0.2 n = 42	0.922

[1] young, 3-4 nearly fully expanded leaves from top of 2 weeks old plants; senescent, basal leaf of 5 weeks old plants;
[2] P-values are from Mann-Whitney U test

Myzus persicae settling behaviour on untreated plants

Fewer aphids remained 'settled' on *S. stoloniferum* than on *S. tuberosum* plants. This was already clear after 15 minutes, and became even more pronounced towards the end of the experiment. After 24 h, only 20 % of aphids remained on *S. stoloniferum* and 70 % on *S. tuberosum* (**Table 4**).

Table 4. Settling behaviour of *M. persicae* on *S. tuberosum* and *S. stoloniferum*. Values are mean ± SEM of number of aphids on the plant at different times.

Time	n[1]	*S. tuberosum*	*S. stoloniferum*	P – value[2]
Time 0	10	20	20	
15 min	10	20 ± 0.0	19 ± 0.2	0.002
30 min	10	20 ± 0.2	18 ± 0.4	0.001
1 h	10	19 ± 0.4	15 ± 0.9	0.003
2 h	10	17 ± 1.0	11 ± 1.5	0.004
24 h	6	14 ± 1.5	4 ± 0.8	0.004

[1] n, number of replicates of 4 plants each; [2] P-values are from Mann-Whitney rank sum test

M. persicae settling behaviour on preinfested plants

Settling by *M. persicae* on preinfested and control plants was similar. This was the case for both *S. tuberosum* and *S. stoloniferum.* Also, no differences were observed for local and systemic leaves (**Table 5**).

Table 5. Settling behaviour of *M. persicae* on *S. tuberosum* and *S. stoloniferum*, on non-infested controls and preinfested (by *M. persicae)* plants. Values are mean ± SEM of number of aphids on the plant at different times.

	S. tuberosum			*S. stoloniferum*		
	Control n = 8	Preinfested n = 9	P value[1]	Control n = 8	Preinfested n = 9	P value
Local leaves						
time 0	20	20		20	20	
5 min	20.0 ± 0.0	19.7 ± 0.2	0.277	19.0 ± 0.4	18.7 ± 0.5	0.673
10 min	19.9 ± 0.1	19.6 ± 0.2	0.481	18.8 ± 0.5	18.1 ± 0.5	0.370
15 min	19.8 ± 0.2	19.6 ± 0.2	0.743	17.8 ± 0.6	17.6 ± 0.5	0.743
20 min	19.9 ± 0.1	18.8 ± 0.5	0.094	17.4 ± 0.8	17.1 ± 0.7	0.743
25 min	19.8 ± 0.2	18.8 ± 0.5	0.200	16.4 ± 0.8	16.2 ± 0.7	0.815
30 min	19.6 ± 0.3	18.3 ± 0.6	0.093	15.6 ± 0.8	15.6 ± 0.6	0.963
Systemic leaves						
time 0	20.0	20.0		20.0	20.0	
5 min	20.0 ± 0.0	19.8 ± 0.1	0.481	19.5 ± 0.3	18.6 ± 0.6	0.236
10 min	19.8 ± 0.2	19.7 ± 0.2	0.815	19.3 ± 0.3	18.0 ± 0.7	0.236
15 min	19.5 ± 0.2	19.6 ± 0.2	0.888	19.0 ± 0.3	17.2 ± 0.7	0.074
20 min	19.6 ± 0.2	19.4 ± 0.2	0.536	18.9 ± 0.3	16.8 ± 0.9	0.114
25 min	19.5 ± 0.2	19.4 ± 0.2	0.888	18.6 ± 0.5	16.4 ± 1.0	0.114
30 min	19.5 ± 0.2	19.4 ± 0.2	0.888	18.4 ± 0.5	15.7 ± 1.0	0.064

[1] P-values are from Mann-Whitney rank sum test

EPG monitoring of aphid probing and feeding behaviour

The main EPG parameters for which we found differences are summarized in **Table 6** (see the numbered 28 parameters of **Table 1**). For *M. euphorbiae*, probing behaviour during pathway periods (stylet route to the phloem) did not differ between *S. tuberosum* and *S. stoloniferum* (parameters number 1 to 15, **Table 6**). However, differences emerged in the phloem. *M. euphorbiae* salivated in sieve elements (E1) more often and longer in *S. stoloniferum* than in *S. tuberosum* (parameters 16 to 23, table 6) but ultimately, phloem sap ingestion and the number of aphids succeeding in sustained phloem feeding (sE2, any period of E2 longer than 10 min) did not differ between *S. stoloniferum* and *S. tuberosum* (parameters number 24 to 29, **Table 6**) thus showing phloem acceptance as well as the plant's general suitability.

On the contrary, results on probing and feeding behaviour of *M. persicae* on *S. stoloniferum* suggest the presence of pre-phloem factors of resistance. This wild potato showed stylet-pathway constraints (parameters 13 to 15, **Table 6**). The longer time to the first phloem salivation (E1) and to the first period of sustained phloem ingestion (sE2) as compared to susceptible *S. tuberosum*, reflect difficulties in phloem access and acceptance, respectively. Moreover, the number of brief probes (stylet withdrawals within 3 min) before the first phloem contact was significantly higher, reflecting resistance at the epidermis/mesophyll level. Also, the average duration of individual cell punctures with sampling (waveform pd, potential drop) was higher on *S. stoloniferum* than in *S. tuberosum* (parameter number 4 and 9 respectively, **Table 6**).

Myzus persicae also showed differences at the phloem level between *S. stoloniferum* and *S. tuberosum*. Although no parameters related to salivation (E1) were different between both plant species (parameters 16 to 23, table 6), *S. stoloniferum* negatively affected phloem sap intake (E2) parameters (24 to 28, table 6) and the number of aphids succeeding in feeding was substantially higher on *S. tuberosum* than on *S. stoloniferum* (parameter 28, **Table 6**).

Probing differences between *M. persicae* and *M. euphorbiae*

The fact that, irrespective of the plant species, *M. persicae* always showed higher numbers of probes, and higher numbers of pathway periods of shorter average duration than *M. euphorbiae* (**Figure 1**, parameters 2, 5 and 6, **Table 6**), seems indicative of intrinsic differences in probing and feeding behaviour between the two aphid species.

Table 6. Electrical penetration graph (EPG) results. Values are means ± SEM of EPG parameters during 6 h monitoring described in Materials and Methods.

	EPG parameter	Me-tub n[1] = 11	Me-sto n = 12	P[2]	Mp-tub n = 13	Mp-sto n = 15	P[2]
1	Time to first probe (sec)	2.2 ± 0.54	2.6 ± 0.76	0.667	1.9 ± 0.23	2.7 ± 0.75	0.712
2	Number of probes	11 ± 3.21	12 ± 2.64	0.666	57 ± 8.81	64 ± 5.49	0.475
3	Average probe duration (min)	15.4 ± 4.10	37.3 ± 12.99	0.109	7.6 ± 2.49	3.7 ± 0.48	0.102
4	Nr of probes < 3 min until first phloem contact (E1)	4 ± 1.24	3 ± 1.06	0.686	10 ± 2.30	20 ± 3.52	0.020
5	Number of pathway periods	17 ± 3.83	20 ± 2.97	0.423	71 ± 9.22	77 ± 5.65	0.580
6	Average pathway duration (min)	7.9 ± 1.30	7.9 ± 0.83	0.498	2.6 ± 0.30	2.1 ± 0.19	0.394
7	Total pathway time (h)	1.8 ± 0.35	2.3 ± 0.26	0.196	2.7 ± 0.26	2.6 ± 0.16	0.369
8	Number of potential drop (pd)	126 ± 24.61	161 ± 21.09	0.325	232 ± 20.4	211 ± 12.64	0.420
9	Average duration of pd (sec)	4.26 ± 0.07	4.44 ± 0.11	0.236	3.49 ± 0.08	3.97 ± 0.10	0.002
10	Total time pd (min)	8.8 ± 1.68	11.6 ± 1.45	0.268	13.5 ± 1.19	13.9 ± 0.81	0.765
11	Number of F periods	0.9 ± 0.39	0.9 ± 0.42	0.942	0.7 ± 0.33	1.4 ± 0.51	0.199
12	Average F duration (min)	12.4 ± 6.32	12.6 ± 6.64	0.942	2.0 ± 1.48	6.6 ± 2.13	0.084
13	Time 1st probe to first E1 in the experiment (min)	93.2 ± 22.98	57.3 ± 17.44	0.667	33.1 ± 7.82	87.6 ± 14.34	0.004
14	Time 1st probe to first E12 in the experiment (h)	1.6 ± 0.38	1.7 ± 0.44	0.854	1.3 ± 0.35	4.1 ± 0.52	0.000
15	Time 1st probe to first sE2 in the experiment (h)	1.7 ± 0.37	2.4 ± 0.62	0.580	2.8 ± 0.59	5.3 ± 0.31	0.002
16	Number of single E1 (without E2)	1 ± 0.48	4 ± 0.97	0.003	8 ± 1.13	9 ± 1.21	0.517
17	Maximum single E1 duration (sec)	23.7 ± 18.05	192.9 ± 47.21	0.001	323.6 ± 55.64	343.5 ± 73.28	0.730
18	Average single E1 duration (sec)	12.0 ± 7.38	90.2 ± 17.58	0.001	139.4 ± 21.20	104.1 ± 14.76	0.189
19	Total time single E1(min)	0.8 ± 0.59	7.3 ± 2.58	0.001	17.2 ± 2.58	17.0 ± 3.19	0.945
20	Number of E1 (single E1 and E1 fractions)	5 ± 1.14	8 ± 1.19	0.026	13 ± 1.06	11 ± 1.26	0.220
21	Maximum E1 duration (min)	2.8 ± 1.21	7.5 ± 2.35	0.007	5.6 ± 0.89	5.9 ± 1.18	0.730
22	Average E1 duration (min)	1.5 ± 0.48	2.8 ± 0.90	0.036	1.8 ± 0.25	1.8 ± 0.21	0.872
23	Total time E1(min)	5.7 ± 1.54	21.2 ± 4.99	0.005	22.9 ± 3.04	20.5 ± 3.34	0.420
24	Number of E12 periods (with both E1 and E2)	3 ± 0.70	2 ± 0.45	0.216	4 ± 0.82	1 ± 0.39	0.005
25	Average E12 duration (min)	87.9 ± 27.99	69.4 ± 19.01	0.667	24.2 ± 6.51	5.7 ± 3.19	0.005
26	Average E2 duration (min)	78.7 ± 27.08	43.5 ± 9.96	0.356	20.8 ± 5.36	4.2 ± 3.08	0.001
27	Total time E2 (min)	175.4 ± 28.27	118.6 ± 23.97	0.124	82.3 ± 21.89	16.4 ± 12.45	0.001
28	Number (%) of aphids with sE2	11 (100%)	10 (83%)	0.261	10 (77%)	3 (20)	0.007[a]

[1]n, EPG replicates; [2] Mann Whitney test, P-values; [a] Fisher exact test at the 6[th] h of EPG recording. Gray boxes P-values significantly different (P ≤0.05)

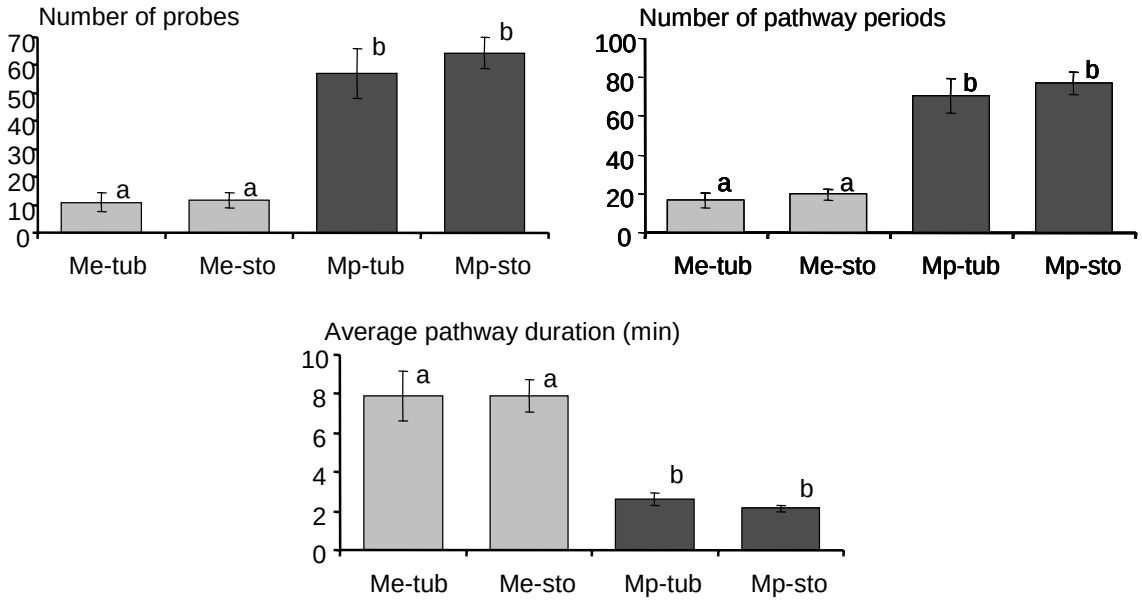

Figure 1. Electrical penetration graph parameters differing among *M. persicae* (Mp, dark grey bars) and *M. euphorbiae* (Me, light grey bars) independently if are on *S. tuberosum* (tub) or *S. stoloniferum* (sto). Bars within each graph with different letters are significantly different at $P < 0.008$ according to Kruskal-Wallis test followed by multiple comparisons with Bonferroni correction.

Discussion

The *S. tuberosum* breeding line we used as a susceptible control appeared to be a better host to *M. euphorbiae* than to *M. persicae*. In a previous study on *M. persicae* on wild and cultivated potatoes – in which we found highly susceptible to highly resistant plants – *S. tuberosum* cultivars appeared not to be the most susceptible species for *M. persicae* (Alvarez et al., 2006), which is in agreement with other studies suggesting that several cultivated potatoes are not optimal hosts for *M. persicae* (Karley et al., 2002).

Previously we found that *M. persicae* colony-development on *S. stoloniferum* was limited compared to that on *S. tuberosum* (Chapter 2). Our results indicate that *M. persicae* performance depends on the leaf physiological stage. In young leaves of *S. stoloniferum* the probing constraint found by *M. persicae* strongly affected its survival. On the contrary on *S. stoloniferum* old leaves *M. persicae* performance was similar to that on *S. tuberosum*. The performance of *M. euphorbiae* on *S. stoloniferum* is also relative to the leaf physiological stage. However on young leaves of *S.*

stoloniferum the nymph mortality of *M. euphorbiae* was significantly lower than that of *M. persicae* (40 % and 79 % respectively, Fisher exact test, P = 0.003).

S. stoloniferum showed antixenotic effect towards *M. persicae* compared to *S. tuberosum* control plants at any time point. However, in the free aphid settling tests – specially designed to study induced resistance at the surface level – *M. persicae* showed no increased repellence or deterrence on pre-infested plants as compared to non-infested plants. No extra induced surface resistance was detected in *S. stoloniferum* in addition to the constitutive resistance to *M. persicae* already described (Alvarez et al., 2006). In this respect, our results support Traw's optimal defence theory (Traw, 2002) predicting that plants with strong constitutive resistance will have weaker induced resistance responses.

***S. stoloniferum* vs. *S. tuberosum* as host plant**

The host plant range of an aphid is ultimately determined by successful phloem feeding, hence sustained phloem sap feeding as measured by EPG recording is a crucial feature. The 10 minute threshold used for sustained or committed phloem ingestion has been discussed earlier (Tjallingii, 1990). Both plant species, *S. stoloniferum* and *S. tuberosum*, can be regarded as host plants for *M. euphorbiae* with respect to this criterion. However, for *M. persicae*, in contrast, only *S. tuberosum* is an accepted host (see parameters related to E2, number 26 to 28 in **Table 6**).

The higher number of brief probes (shorter than 3 min) before first phloem contact (during which stylets do not penetrate deeper than a few cell layers; penetration rate is about one cell layer/min) by *M. persicae* on *S. stoloniferum*, as well as the longer time before the first E1, E12 and sE2 indicate that constraints to stylet penetration are located in tissues before the phloem. This resistance is constitutive and in agreement with previous results (Chapter 2).

M. euphorbiae showed more and longer periods of phloem salivation on *S. stoloniferum* than on *S. tuberosum* suggesting that the phloem sap of these two host plants is not equally acceptable. The aphid invests more time and energy therefore, to accept the plant, which perhaps explains the longer pre-reproductive time and the higher nymphal mortality of *M. euphorbiae* on *S. stoloniferum* than on *S. tuberosum*. Alternatively, the phloem sap of *S. stoloniferum* might have a lower nutritious quality, which cannot be measured by EPG.

S. tuberosum is an optimum host for *M. euphorbiae*. However, our results (especially EPG parameters) indicate that *M. euphorbiae* also accepts relatively well *S. stoloniferum* as a host.

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CHAPTER 4

Solanum stoloniferum responses in compatible and incompatible interactions with aphids

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Abstract

The wild potato *Solanum stoloniferum* Schlechtd has a constitutively expressed resistance factor at the epidermal/mesophyll level that is effective against attack of the aphid *Myzus persicae* (Sulzer) (resistant-incompatible-interaction). On the other hand *S. stoloniferum* is an accepted host plant for the aphid *Macrosiphum euphorbiae* (Thomas) (susceptible-compatible-interaction). We characterized the plant response to attack by either of these aphid species. We evaluated the resistance or susceptibility of the host plant by studying gene expression, aphid settling behaviour, and morphological responses of the plant. At the local level (infested sites of the plant), *M. euphorbiae* attack elicits a substantially higher number of differentially regulated genes than *M. persicae*. At the systemic level (non-infested sites of an infested plant) both aphids elicit a weak response in terms of gene expresion. While both aphid attacks elicited the expression of PR genes and the repression of mainly photosynthesis-related genes, only *M. euphorbiae* elicits genes related to regulated-proteolysis via the ubiquitin-proteasome system, to the plasmodesmata gateway system, and to extracellular transport. Also genes related to the ethylene signal-transduction pathway are differentially activated in the compatible interaction. The morphological reactions of the plant at the site of infestation, in response to *M. persicae* and *M. euphorbiae* attack were different. Infestation of *S. stoloniferum* leaves with a high number of *Myzus persicae* aphids leads to the development of obvious water-soaked pustules. Microscopy showed that these pustules contained burst cells by hypertrophy and hyperplasia of cells of the vascular parenchyma and the vacuolated bundle sheath cells surrounding vascular bundles. In contrast, the infestation with *M. euphorbiae* did not induce any visible cellular changes. We studied the induction of antixenosis resistance by assessing the aphid settling behaviour on previously infested leaves (local responses), and on non-infested leaves of infested plants (systemic responses). There was no evidence of induction of a repellence or deterrence in plants that had previously been attacked by *M. persicae* or *M. euphorbiae*. Factors related to host acceptance and effects of aphid attack on the physiology of the plant are discussed.

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Introduction

Phloem feeders, such as aphids have developed a specialized feeding mechanism by which they take up nutrients without killing their host plant. As such the strategy of these highly specialized herbivorous insects resembles that of biotrophic plant pathogens. In contrast to extensive studies on plant-pathogen interactions, little is known about local and systemic signalling in plant-aphid interactions. Nevertheless, in the last few years some gene expression studies have been performed (reviewed by Thompson and Goggin, 2006). Studies on the interaction between *Myzus persicae* (Sulzer) and *Arabidopsis thaliana* (Moran et al., 2002; Moran and Thompson, 2001) as well as *Schizaphis graminum* and *Sorghum bicolour* (Zhu-Salzman et al., 2004), have shown that small and localized damage to cells, coupled to the penetration, salivation and ingestion processes of phloem feeding by aphids induces multiple plant defence pathways. It appears that inter- and intra-cellular salivation activities in host tissue presumably are the most important triggering factors of plant response (Tjallingii, 2006). Pathogenesis-related (PR) proteins, salicylic acid (SA) responsive genes and, to a lesser extent also jasmonic acid (JA) regulated genes are activated upon aphid attack (Moran et al., 2002; Moran and Thompson, 2001; Zhu-Salzman et al., 2004).

Transcriptional analysis of a compatible plant-aphid interaction, i.e., *A. thaliana* (wild type and mutants) and *M. persicae*, showed that some induced genes are also induced by pathogens and wounding. There is an increased transcription of genes that are regulated by salicylic acid, like *PR1* and *BGL2* (*acidic apoplastic β-1,3-glucanase*), and also jasmonate/ethylene regulated genes, like *defensin* (*PDF1.2*). Other up- regulated genes are related to resource allocation shifts (like sugar transport protein) (Moran and Thompson, 2001). Moreover cDNA micro- and macro-array response profiles of *A. thaliana* to *M. persicae* feeding have shown increased expression of genes involved in oxidative stress (*superoxide dismutase*, *glutathione-S-transferase*), and calcium-dependent signaling (*calmodulin* and *touch-sensitive* genes) (Moran et al, 2002). There are suggestions that aphids, possibly through their particular mode of feeding, are able to manipulate the plant response to their own benefit, maybe by a misguided anti-microbial defence pathway activating ineffective genes and suppression of effective defence genes (Zhu-Salzman et al 2004). This seems supported by *Myzus nicotianae*-induced accumulation of glutamate synthase in *Nicotiana attenuata*, suggesting that the aphids induced synthesis of glutamate (Voelckel et al., 2004), one of the nitrogen transport molecules that could improve the nutritional quality of phloem sap (Karley et al., 2002.

A comparative transcriptional analysis of different plant attackers showed that aphids, like pathogenic bacteria, trigger expression changes in a large number of genes, compared to chewing insects (caterpillars), cell-content-feeding insects (thrips), and a leaf fungus, although aphids even caused the least intensive symptoms (De Vos et al., 2005). Nevertheless, most of the transcriptional changes found were unique to either bacteria or aphid attacker. Moreover, a large proportion of genes affected by aphids are related to plant metabolism, suggesting manipulation of the plant's physiology (De Vos et al., 2005; for review see Thompson and Goggin, 2006).

Potato crops can be infected by at least 54 different viruses (Brunt et al., 1996) and aphids are their most important vectors (Jeffries, 1998; Peters, 1987). The green peach aphid, *M. persicae* is a generalist aphid, worldwide distributed and a very efficient vector of virus diseases (Jeffries, 1998; Peters, 1987; Raman and Radcliffe, 1992). Therefore, potato cultivars with resistance to aphids can be important to control virus transmission. Previously we studied *M. persicae* resistance in different wild tuber bearing potato species by analyzing population development, probing and feeding behaviour (Chapter 2), aphid settling behaviour, and aphid performance (Chapter 3), we found that *Solanum stoloniferum* is highly resistant to the aphid; *S. stoloniferum* resistance appeared constitutively expressed and located at the epidermal/mesophyll level, and the resistance declined as soon as the leaves became senescent (Alvarez et al., 2006). On the other hand, *S. stoloniferum* has shown no or only low levels of resistance to *Macrosiphum euphorbiae* (Chapter 3). Therefore, we selected *S. stoloniferum* and the two aphid species *M. persicae* and *M. euphorbiae* as a model to study plant responses in compatible and incompatible interactions. To characterize both plant-aphid interactions we analyzed the transcriptional responses of *S. stoloniferum* towards aphid feeding at the local and systemic level. We also studied induced direct-resistance effects at these levels and the morphological changes at the site of infestation, after *M. persicae* and *M. euphorbiae* attack.

Materials and Methods

Plants and aphids

The *Solanum stoloniferum* Schlechtd clone 17605-4 was selected for this study because of its high level of resistance to *M. persicae* (Alvarez et al., 2006). The clone was derived from accession 17605 (Centre for Genetic Resources, Wageningen, The Netherlands, CGN) and maintained *in vitro*

at the Plant breeding department of Wageningen University. Propagation of plants was performed *in vitro* on Murashige and Skoog medium including vitamins, sucrose 3%, pH 5.8.

After two weeks in agar the plantlets with developed roots were transferred to soil in 22 cm diameter pots in a glasshouse at 22 ± 2 ºC, RH about 70%, and L16:D8 h photoperiod.

Aphids used in experiments came from newly established clones from a single virginoparous apterous individual taken from a colony maintained at the Laboratory of Entomology, Wageningen University. *M. persicae* was reared on radish *Raphanus sativus* L. in cages in a climate chamber at 22 ± 2 ºC, RH 30–40 %, and L16:D8 photoperiod. *M. euphorbiae* was reared in a glasshouse in cages on *Solanum tuberosum* cv *Bintje* at 22 ± 2 ºC, RH about 70%, and L16:D8 h photoperiod. New synchronous colonies were started weekly from which newly molted adults were used to perform infestations.

To study the presence of inducible resistance in *S. stoloniferum* to *M. persicae* we performed a settling test (see 'Aphid settling behaviour test' below). Then pre-infestation was carried out with either *M. persicae* or *M. euphorbiae* aphids. The test was designed to determine whether a pre-infestation with aphids has an effect on the next (secondary) infestation by *M. persicae*.

Aphid pre-infestation

Plants of thirty five days old were pre-infested by aphids for 96 h in a glasshouse at 22 ± 2 ºC, RH about 70%, and L16:D8 h photoperiod. Only one pair of fully expanded non-senescent leaves, numbers 5 and 6 from the apex, of *S. stoloniferum* was pre-infested per plant.

To analyze locally induced responses we used from each plant the pre-infested pair of leaves, and for systemically induced responses we used from each plant the first nearly fully expanded pair of leaves at time of pre-infestation (leaves 3 and 4 from top). Aphids were confined to the leaves by enveloping each leaf individually with non-woven bag ('agrotextile') and plants were placed on individual trays in the greenhouse. Leaves of control plants were also enveloped with non-woven bags, but without adding aphids (**Figure 1**).

Myzus persicae (Mp) pre-infestation was performed by placing 20 apterous adults aphids on the leaves specified. However, after the first infestation most aphids were found on the bag instead of

feeding on the leaf. In order to assure the attack effects, a second infestation with 20 aphids was performed on the same leaves 24 h after the 1st infestation. One *M. euphorbiae* (Me) pre-infestation by 20 apterous adults was sufficient (**Figure 1**).

Plants were arranged in the greenhouse in a randomized complete design, and were assigned to 3 groups, A (pre-infested with *M. persicae*), B (pre-infested with *M. euphorbiae*), and C (control non-infected). After 96 hours aphids and nymphs were counted and removed carefully by brushing with a soft brush. Subsequently, complete leaves were taken from 4 plants per treatment for analysis of gene-expression at the local and systemic level. To study induced resistance, an aphid settling test was performed using 8 plants per treatment (see experimental set-up on **Figure 2**).

Figure 1. Pre-infestation of potato plants (left); leaves covered with non-woven bags on water trays. One pair of leaves (leaf 5 and 6 from top) was pre-infested and used to study local induced responses, another pair (3 and 4) was used to study systemic effects. Plants entirely enveloped with bags to perform aphid settling behaviour tests (right).

Aphid settling behaviour test

To measure induced antixenosis to *M. persicae* at the local and systemic level after pre-infestation of the leaves by the two aphid species, we modified a test developed by Martin and Fereres (2003) referred to as 'settling test'. To observe treatment effects, 20 adult aphids were placed per plant (at time 0) for the 3 treatments, 10 aphids on each of the two leaves. In order to avoid aphids moving to the untreated leaves, plants were entirely enveloped with non-woven bags, leaving only the test

leaves accessible to aphid infestation. Also, to avoid aphid migration from one plant to the other, the plants were individually placed on a tray with water as a barrier (**Figure 1**). The aphids moving away from the plant drowned in the water or kept on walking around.

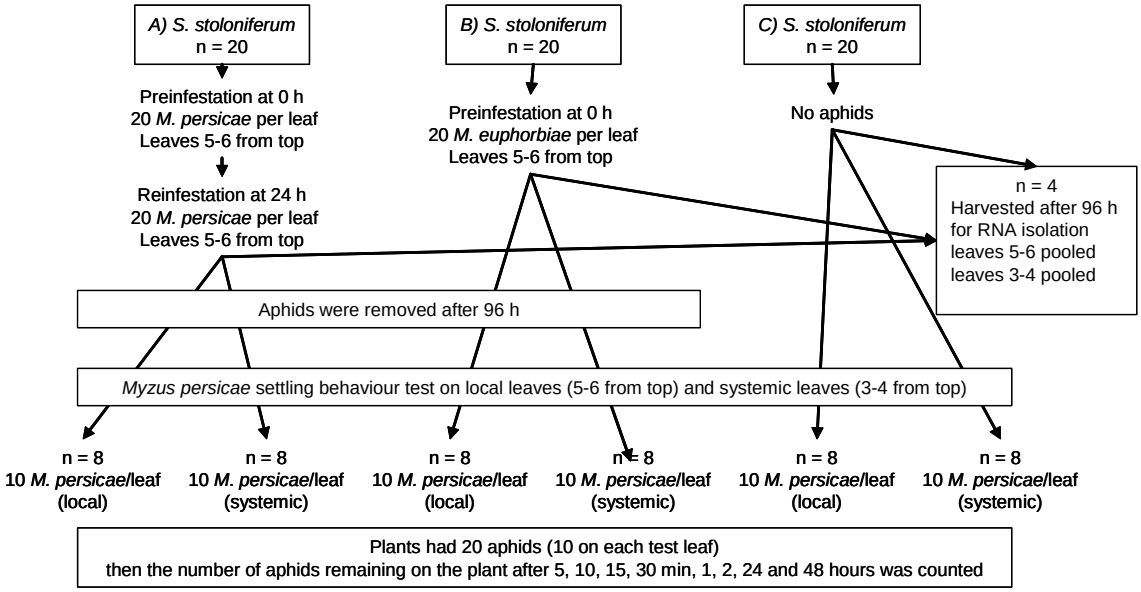

Figure 2. Experimental setup. A, B and C are the treatment groups with 20 plants each. On plants in groups A and B, two leaves (5-6 from top) were pre-infested with 20 *Myzus persicae* or *Macrosiphum euphorbiae* for 96 h, local leaves. On the same plants the leaves at position 3-4 (untouched by aphids) were the systemic leaves. Plants in group C (control) were kept without aphids. After the preinfestation of 96 h on each group 4 plants were used for cDNA microarray experiment: the 2 leaves at the local level were pooled, and the 2 leaves at the systemic level were pooled, but each plant was harvested individually. The settling behaviour test was performed with 8 plants per treatment (see Materials and Methods for details).

At time 0 all plants had 20 aphids, 10 on each test leaf. Subsequently, the number of aphids remaining on the plant after 5, 10, 15, 30 min, 1, 2, 24 and 48 hours was counted. Eight replicates were performed for each treatment. We tested: a) *M. persicae* pre-infested local (Mp L) and non-infested systemic (Mp S) leaves; b) *M. euphorbiae* pre-infested local (Me L) and non-infested systemic (Me S) leaves; c) control local (CL) and systemic (CS) leaves, i.e. leaves 5/6 for CL and 3/4 for CS, respectively.

Means and standard error of means (SEM) of the numbers of aphids per plant were calculated for all treatments. A Kruskall-Wallis test, and multiple comparisons with Bonferroni correction (Weisstein, 1999; SPSS 12.0.1 for Windows) were used to analyze the data on aphid settling behaviour.

Microscopy

Ten adult aphids of *M. persicae* were confined to small clip cages (2 cm diameter) on *S. stoloniferum* leaves. After 2 days most of the aphids were dead, hence 10 more aphids were added to the same clip cage. After 4 days, pustules visibly developed on the leaf middle veins and leaf tissue was collected for microscopy. Hand-cut cross-sections with a razor blade were made transversally to the main vein. Slides were observed under light microscope at 10X and 20X magnification.

Sample preparation for cDNA microarray analysis

The leaf samples for RNA isolation were harvested after 96 h of *M. persicae* and *M. euphorbiae* pre-infestation. After removing the aphids and nymphs from the leaves they were cut from the plant, weighed, immediately frozen in liquid nitrogen and stored at – 80 °C until use. We pooled the two pre-infested leaves of each plant for local responses, and also, the two non-infested leaves for systemic responses. Total RNA was extracted from frozen leaves with TRIzol (Invitrogen) and purified using the RNeasy mini elute kit (Qiagen). cDNA was obtained from total RNA by Super Script II reverse transcriptase (Invitrogen) with POLYdT primers and was purified with QIAquick PCR purification kit (Qiagen). cDNA was labeled with cyanine 3 (Cy3), and cyanine 5 (Cy5) fluorescent dyes (Amersham).

cDNA micro-array

The arrays used have a collection of 3564 *S. tuberosum* cDNA clones (provided by Edwin van der Vossen). The clones on the array corresponded to genes known to be involved in plant defence responses. They were selected on the basis of sequence homology with known genes from the TIGR database related to plant defence. Arrays were spotted with DMSO 50 % spotting buffer in ultragaps slides (Service XS, Leiden). Each slide has three blocks (technical replicates) on the array.

To analyze differential gene expression, infested plants were combined with non-infested control plants, generating 4 combinations: 1) *M. persicae* preinfested local leaves vs. the non-infested control leaves (at the same 5/6 position), 2) *M. euphorbiae* pre-infested local leaves vs. non-infested control leaves, 3) *M. persicae* pre-infested systemic leaves vs. non-infested control leaves (at the same 3/4 position), 4) *M. euphorbiae* pre-infested systemic leaves vs. non-infested control leaves.

Three slides were hybridized for each treatment using cDNA of different plants each time (3 biological replicates).

Microarray hybridizations

Spots on printed slides were immobilized by UV-cross linking at 150 mJ. Slides were pre-hybridized for 2 hours at 42 ºC in hybridization buffer (50% formamide, 5x Denhardt's reagent, 5x SSC, 0.2% SDS, 0.1 mg/ml fish-DNA denatured 3 min in boiling water), washed in de-ionized water , next washed in isopropanol and dried by centrifugation. The Cy5/Cy3 labelled cDNA was dissolved in hybridization buffer, denatured at 95 ºC for 1 min and then 70 μL mixed hybridization solution was loaded onto the hybridization slide. Slides were placed in a hybridization chamber (Genetix) with pre-loaded wet filter paper to avoid desiccation and incubated for 24 hours at 42 ºC. Hybridized arrays were washed by shaking for 5 min in 1x SSC, 0.1% SDS, followed by 5 min in 0.1x SSC, 0.1% SDS, and finally 1 min in 0.1x SSC, dried by centrifugation and scanned on a ScanArray^{TM} 3000 Express HT (Perkin-Elmer).

Microarray data analysis

Signal and background fluorescence intensities of the arrays were analyzed using the ScanArray Express program (Perkin-Elmer) version 2.22. Arrays were checked manually to exclude from the analysis anomalous spots with high background. Spots with fluorescence intensities lower than half the background were raised to half the background to avoid extreme expression ratios; and were excluded when both dyes had intensities lower than half the background. Data were converted by Express Converter ver 1.5, then log 2 ratios of Cy5/Cy3 were calculated and normalized to avoid spatial bias within each slide using Locfit (Lowess) normalization method by the TIGR-MIDAS-Microarrays Data Analysis System, Version 2.19. TIGR-MEV version 3.0.3 was used to perform t-test statistical analysis of the log 2 ratios; genes with expression ratio two folds higher (log 2 ratios ≥ 1) were consider up-regulated, and genes with expression ratio two fold lower (log 2 ratios ≤ -1) were considered down-regulated, when statistically different from 0 (P value ≤ 0.05). We also included some genes clearly up-regulated in all three biological replicates with an expression log 2 ratio ≥ 1 but with P value between 0.05 and 0.1 due to a high variability between replicates. The criterion to be included was that at least 2 technical replicates out of three on each of the 3 slides must be up regulated.

Results

M. persicae settling behaviour on *S. stoloniferum*

To study the presence of inducible resistance of *S. stoloniferum* to *M. persicae* we performed the settling test. Pre-infestation was carried out with either *M. persicae* or *M. euphorbiae* aphids. The test was designed to determine whether the pre-infestation with aphids has an effect on the next (secondary) infestation by *M. persicae*.

In local leaves the number of *M. persicae* aphid settled on test leaves of *S. stoloniferum* during a secondary infestation did not differ from control leaves at any time ($P > 0.05$, **Figure 3**). Constitutive resistance may explain why during the pre-infestation with *M. persicae* the aphids showed plant avoidance and mortality that urged us to replace aphids after 24 h (see materials and methods).

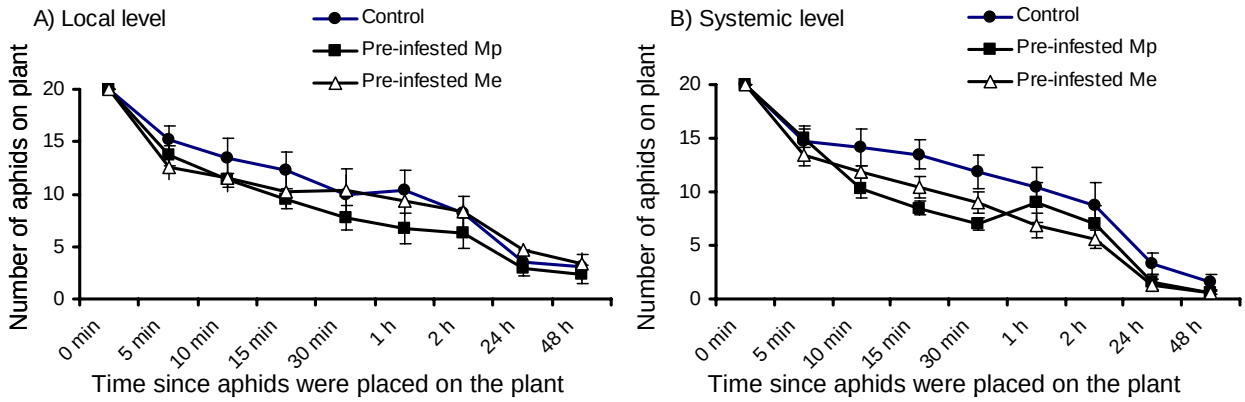

Figure 3. Aphid settling behaviour of *Myzus persicae* during a secondary infestation in plants previously infested with either *M. persicae* (Mp) or *Macrosiphum euphorbiae* (Me). A) settling tested on local (preinfested) or B) on systemic (uninfested) leaves of the pre-infested plant. Number of aphids present ('settled') on the potato leaves was scored at 5, 10, 15, 30 min, and 1, 2, 24 and 48 h. For each replicate 20 aphids per plant (10 on each leaf) had been used. Dots and error bars are means and SEM of 8 replicates. At systemic level there is a difference between preinfested Mp and uninfested control at 15 minutes (Kruskal Wallis P = 0.034; Bonferroni P = 0.014).

On the systemic test leaves we found a significantly reduced number of aphids only after 15 minutes from the start of the experiment on *M. persicae* pre-infested plants, as compared to control plants. Twenty-four hours after the start only 20 % of the aphids at the local level and 10 % at the systemic level were found on the control and test plants. After 48 hours only one or two of the aphids at the local and systemic levels were found on the control and test plants (**Figure 3**). Thus, the constitutive resistance of *S. stoloniferum* to *M. persicae* expressed during probing and feeding

by the aphids as described previously (Chapters 2 and 3) can be considered as the main cause for aphids leaving the plants that had been pre-infested and non-infested plants at similar rates.

Induction of pustules on plant leaves

The pre-infestation of *S. stoloniferum* leaves during 4 days with a high number of confined *M. persicae* caused visible water-soaked pustules developed on the midrib and/or secondary veins. Later on (from day 4 to 5) these pustules collapsed in the centre, the tissue became necrotic and finally holes were formed from the centre (**Figure 4**).

Figure 4. Pustules developed on the midrib and secondary veins on *S. stoloniferum* after infestation with *M. persicae* for 96 h (left). These water soaked lesions collapsed in the centre, the tissue became necrotic and finally holes are formed (right).

Microscopic analysis of the leaf pustules showed hypertrophy (cell enlargement) in the tissue at the abaxial side of the leaf together with hyperplasia (cell division) of the vascular parenchyma cells and the vacuolated bundle sheath cells surrounding the vascular bundle (**Figure 5**). Remarkably, a similar infestation with *M. euphorbiae* did not induce any visible cellular changes.

Gene expression in response to aphid feeding

We compared the transcriptional responses of *S. stoloniferum* to infestation by *M. euphorbiae* and *M. persicae*. In the local leaves 81 genes were up-regulated in response to *M. euphorbiae* and 33 in response *M. persicae*, of which 24 overlapped with those upregulated in response to *M. euphorbiae* feeding. Forty-nine genes were down-regulated by *M. euphorbiae* feeding and only 29 by *M. persicae* feeding, of which 14 overlapped with those down-regulated by *M. euphorbiae* feeding.

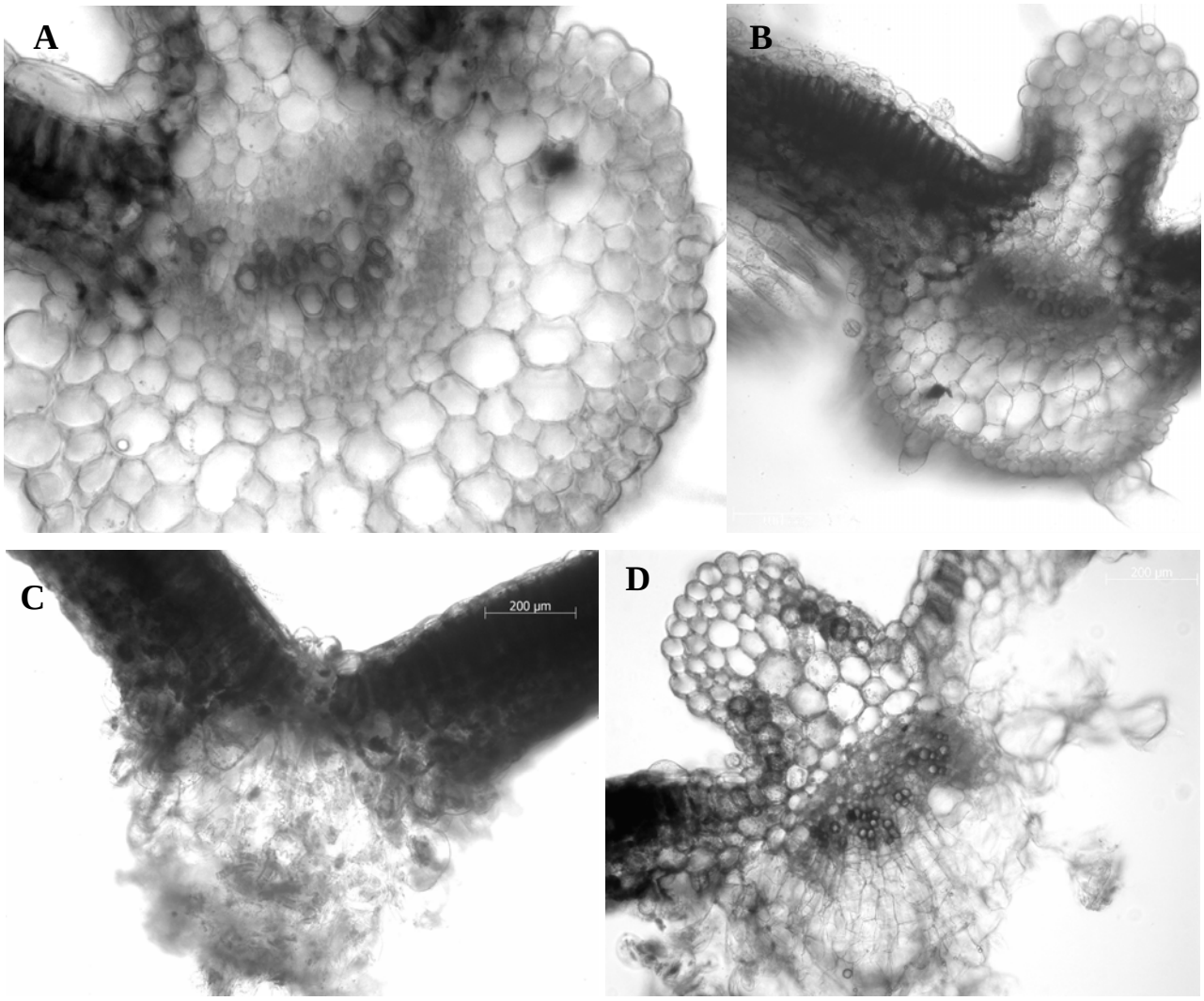

Figure 5. Sections of *S. stoloniferum* at the midrib leaf tissue. A-B, Non-infested control leaf tissue. C-D, leaf pustules after infestation with *M. persicae* for 96 h; C, burst of water soaked tissue to the abaxial side of the leaf causing epidermis rupture; D, the pustule is caused by hypertrophy (cell enlargement) in the tissue together with hyperplasia (cell division) of the vascular parenchyma cells and the vacuolated bundle sheath cells surrounding the vascular bundle at the abaxial side of the leaf. A, 20X enlargement; B, C, and D, 10X enlargement.

Thus, about twice as many genes were differentially regulated (i.e. up or down) in response to feeding by *M. euphorbiae* than to feeding by *M. persicae* (130 vs 62). Also the number of genes up and down regulated was differently distributed in *M. euphorbiae* or *M. persicae* infested leaves. The proportion of down/up regulated genes was 1.5 folds higher for *M. persicae* (29 /33 = 0.9) compared to *M. euphorbiae* (49/81 = 0.6).

The differentially expressed genes after aphid infestation are listed in **Table 1**. Genes have been assigned to functional groups based on their TIGR annotation. This grouping confers a potential function for each encoded protein based on similarity to known proteins, but the function of the genes was not confirmed.

Table 1. List of differentially regulated genes in *Solanum stoloniferum* after infestation for 96 h with either *Macrosiphum euphorbiae* or *Myzus persicae*.

Functional category	Clone name	Gene annotation (putative function), degree of homology with other spp (%)	Fold change[1]		Process category	Response to[2]
Local leaves. Up-regulated genes after the attack for both aphids spp			Me	Mp		
PR	PPCCF17	PR protein STH-21, *Solanum tuberosum* (100%)	3.67	3.05	Defence related	P/W
PR	PPCBF82	PR-1 protein isoform b1, *S. tuberosum*, *Solanum lycopersicum* (100%)	2.74	4.44	Defence related	P/SA
PR	PPCCP54	PR-2 protein P2 precursor, *S. lycopersicum* (100%)	3.15	4.57	Defence related	P
PR	PPCBP46	PR-2 protein 1,3-beta-glucanase precursor, *S. tuberosum* (37%)	3.48	4.29	Defence/antifungal	P/SA
PR	PPCAT19	Endo-1,3-beta-glucosidase; acidic isoform GI9, *Nicotiana tabacum* (85%)	3.87	2.55	Defence/antifungal	P/SA
PR	PPCAC06	Class II (acidic) chitinase, *S. tuberosum* (100%)	2.99	4.26	Defence/antifungal	P/SA
PR	PPCAT70	PR protein R major form (Thaumatin-likeprotein E22), *N. tabacum* (100%)	2.94	4.87	Defence/antifungal	P/ET
Regulatory	PPCBM08	WRKY-DNA-binding protein 4, *N. tabacum* (66%)	2.18	3.67	Regulation of transcription	P/SA
Regulatory	PPCAI63	LRR protein (CALRR1), *Capsicum annum* (100%)	4.96	3.84	Regulation	P/AS/W/ABA
Regulatory	PPCCM26	Auxin-induced/SAUR-like protein, *C. annum* (100%)	2.26	3.40	Signalling	P
Regulatory	PPCBS17	Enhanced disease susceptibility 1 protein (EDS1), *S. tuberosum* (100%)	3.96	2.42	Signalling/Lipid metabolism	P
Regulatory	PPCBO08	Calmodulin putative, *Arabidopsis thaliana* (19%)	2.54	1.98	Signalling	
Regulatory	BPLI1G3	Protein kinase-like protein, *A. thaliana* (38%)	2.41	2.38	Signalling	
Regulatory	PPCBG74	VQ motif putative, *Oryza sativa* (17%)	2.04	2.04	Regulation	
General metabolism	PPCAQ72	Short-chain type alcohol dehydrogenase, *S. tuberosum* (87%)	2.00	2.38	Metabolism	GA/ET/Ck
General metabolism	PPCAH56	Short-chain type alcohol dehydrogenase, *S. tuberosum* (46%)	2.54	1.96	Metabolism	
Protein metabolism	PPCBJ33	Isoleucyl-tRNA synthetase, *A. thaliana* (5%)	3.16	6.20	Protein translation	
Lipid metabolism	PPCAS26	Non-specific lipid transfer protein, *S. tuberosum* (83%)	2.64	2.58	Lipids transport	
Lipid metabolism	PPCAH78	Xylogen protein 1/non-specific lipid transfer protein, *A. thaliana* (55%)	2.92	3.43	Cell differentiation	
Lipid metabolism	PPCAN32	Enoyl CoA hydratase, *A. thaliana* (96%)	2.12	2.02	Lipid metabolism	
Unknown	PPCAM94	Unknown protein	4.34	1.98	Unknown	
Unknown	STMJC14	Unknown protein	2.72	2.01	Unknown	
Unknown	PPCAC18	Unknown protein, *A. thaliana* (96%)	2.47	2.91	Unknown	
Unknown	PPCBD84	Unknown protein	2.31	2.44	Unknown	

Table 1 (Continued)

Functional category	Clone name	Gene annotation (putative function), degree of homology with other spp (%)	Fold change[1]		Process category	Response to[2]
Local leaves. Down-regulated genes after the attack for both aphids spp			Me	Mp		
Regulatory	cSTB12H2	Mitogen-activated protein kinase (StMPK1), *S. tuberosum* (100%)	0.44	0.37	Signalling	
Regulatory	PPCBQ86	Zinc knuckle (CCHC-type); step II splicing factor, *A. thaliana* (33%)	0.48	0.34	mRNA processing	
Protein metabolism	BPLI8L14	Germin like protein, *N. tabacum* (100%)	0.37	0.40	Not determined	AS
Protein metabolism	cSTB3E17	Mitochondrial processing peptidase, *S. tuberosum* (100%)	0.39	0.37	Protein catabolism	
Protein metabolism	PPCBZ61	Zinc finger (C3HC4-type RING finger), *A. thaliana* (56%)	0.38	0.40	Protein ubiquitination	
General metabolism	PPCBW90	UDP-glucose glucosyltransferase, *Lactuca sativa* (31%)	0.38	0.48	Metabolism	
General metabolism	cSTB34D7	Plastidic aldolase, *Nicotiana paniculata* (100%)	0.27	0.15	Glycolysis	
General metabolism	BPLI11J20	Plastidic aldolase NPALDP1, *N. paniculata* (100%)	0.37	0.23	Glycolysis	AS
Cell wall metabolism	BPLI2E16	Extensin, *A. thaliana* (16%)	0.49	0.34	Cell wall biogenesis	
Photosynthesis related	BPLI10G12	Chloroplast ferredoxin-NADP+ oxidoreductase (FNR), *N. tabacum* (100%)	0.47	0.35	Photosynthesis-related	
Photosynthesis related	cSTB11B3	16 kDa subunit oxygen-evolving enhancer (photosystem II), *A. thaliana* (90%)	0.38	0.39	Photosynthesis-related	
Photosynthesis related	PPCBX59	Chloroplast RNA helicase VDL1 (variegated-distorted leaf), *N. tabacum* (77%)	0.52	0.42	Plastid differentiation	
Photorespiration	BPLI5K11	Serine hydroxymethyltransferase(SHMT mitochondrial), *S. tuberosum* (100%)	0.49	0.38	Glycine/serine metabolism	
Unknown	cSTB38L15	Leucine-rich repeat family protein, *A. thaliana* (51%)	0.48	0.41	Unknown	

Table 1 (Continued)

Functional category	Clone name	Gene annotation (putative function), degree of homology with other spp (%)	Fold change[1]		Process category	Response to[2]
Local leaves. Up-regulated genes after *M. euphorbiae* attack			Me	Mp		
PR	BPLI1G22	Disease resistance protein Hcr2-2A (LRR), *Solanum pimpinellifolium* (41%)	2.72	1.33	Defence related	P
PR	BPLI4I8	Disease resistance protein Hcr2-5D (LRR), *S. lycopersicum* (21%)	2.84	1.32	Signalling	P
PR	STMHZ79	Disease resistance-responsive protein, dirigent protein, *A. thaliana* (47%)	2.76	1.24	Defence related	P
PR	STMEY20	Endochitinase (Chitinase), *S. tuberosum*, *S. lycopersicum* (100%)	2.17	1.77	Defence/antifungal	P
PR	PPCCJ62	Disease resistance protein Cf-2.1, *S. pimpinellifolium* (7%)	3.81	1.25	Defence related	P
PR	cSTB33M4	Cell death protein hsr203J, *N. tabacum* (100%); NgCDM1, *N. glutinosa* (100%)	2.91	1.47	Defence/cell death	P/V
Regulatory	POAE058	AP2 domain-containing transcription factor TINY, *A. thaliana* (6%)	3.36	1.64	Regulation of transcription	AS
Regulatory	BPLI1H2	NPR1-interactor protein 1, *S. lycopersicum* (94%)	2.04	1.92	Regulation of transcription	SA
Regulatory	cSTS9E9	EIN3-binding F-box protein 1 (EBF1), *A. thaliana* (74%)	2.00	0.71	Regulation of transcription	ET
Regulatory	PPCCA18	Jasmonic acid 2, *S. lycopersicum*, *S. tuberosum* (83%)	2.25	1.37	Regulation of transcription	
Regulatory	POACM38	RSH2-like protein, *N. tabacum* (56%)	2.47	1.77	Signalling	P/AS/JA
Regulatory	PPCBM14	Strubbelig receptor family 1 - LRR protein kinase, *A. thaliana* (35%)	3.18	1.24	Signalling	
Regulatory	PPCBD19	Receptor-like serine-threonine protein kinase, *S. tuberosum* (31%)	2.32	1.35	Signalling	P
Regulatory	PPCBL01	Receptor-like protein kinase, *N. tabacum* (36%)	3.05	1.27	Signalling	V/W
Regulatory	PPCCF08	Protein kinase-like protein, *A. thaliana* (31%)	2.27	1.10	Signalling	
Regulatory	PPCCS67	Protein kinase-like protein, *A. thaliana* (4%)	4.57	1.37	Signalling	
Regulatory	cSTA9O14	Protein kinase-like protein, *A. thaliana* (33%)	3.77	2.57	Signalling	
Regulatory	STMJF47	S-receptor kinase (SRK), *A. thaliana* (31%)	2.87	1.35	Signalling	
Regulatory	PPCCI13	S-receptor kinase (SRK), *A. thaliana* (27%)	2.26	1.48	Signalling	
Regulatory	cSTD3E14	Calcium-dependent protein kinase, *S. lycopersicum* (100%)	2.08	1.10	Signalling	
Regulatory	STMHQ95	Calmodulin-binding family protein, *A. thaliana* (39%)	2.67	1.30	Signalling	
Regulatory	PPCBC42	Calmodulin-like, NaCl-inducible protein, *A. thaliana* (38%)	2.06	1.05	Signalling	AS
Regulatory	PPCCE54	Calmodulin NtCaM9, *Solanum demissum* (100%)	1.96	1.03	Signalling	
Regulatory	BPLI6P22	Lectin (probable mannose binding), *O. sativa* (2%)	2.54	1.05	Signalling	
Regulatory	STMJE78	Silencing group B protein, Zea mays(100%)	3.28	1.59	Regulation growth	
General metabolism	STMDF49	AAA-type ATPase family protein, *A. thaliana* (48%)	3.44	1.71	Variety of cellular process	
General metabolism	PPCBP38	Short-chain type alcohol dehydrogenase, *S. tuberosum* (87%)	2.15	1.58	Metabolism	
General metabolism	STMEX88	Dicyanin, *S. lycopersicum* (27%)	2.28	0.90	Electron transport	

Table 1 (Continued)

Functional category	Clone name	Gene annotation (putative function), degree of homology with other spp (%)	Fold change[1]		Process category	Response to[2]
Local leaves. Up-regulated genes after *M. euphorbiae* attack (continued)			Me	Mp		
Protein metabolism	cSTD21B14	Hsc70 protein, *S. lycopersicum* (100%)	2.15	0.87	Protein folding	AS/ET
Protein metabolism	cSTB34G8	Luminal binding protein precursor (BiP), *S. lycopersicum* (100%)	2.46	1.66	Protein folding	AS
Protein metabolism	cSTB11N24	Calreticulin 3, *A. thaliana* (90%)	2.42	1.68	Protein folding/Ca sequester	AS
Protein metabolism	PPCAR85	Branched-chain amino acid aminotransferase, *S. tuberosum* (38%)	2.25	1.54	Amino acid metabolism	
Protein metabolism	PPCAS49	5-enolpyruvylshikimate-3-phosphate (EPSP) synthase, *S. lycopersicum* (33%)	2.11	1.07	Aromatic amino acid biosyn.	
Protein metabolism	cSTS18G22	Chloroplast nucleoid DNA-binding protease 41 kD (CND41), *N. tabacum* (92%)	2.88	1.94	Rubisco catabolism	S
Protein metabolism	PPCAW92	Ubiquitin protease (UBP), *A. thaliana* (4%)	1.99	1.09	Ubiquitin-protein catabolism	
Protein metabolism	STMFB53	20S alpha 3 proteasome subunit, *N. tabacum* (72%)	2.01	0.67	Ubiquitin-protein catabolism	cryptogein
Protein metabolism	cSTS24F8	Lon protease homolog 1 (mitochondrial), *A. thaliana* (85%)	2.29	1.39	Regulatory proteolysis	AS
Protein metabolism	PPCAH09	PR subtilisin-serine-like protease (P69B), *S. lycopersicum* (46%)	4.67	1.95	Protein catabolism	P/ET/SA
Protein metabolism	cSTB48K21	Subtilisin-serine-like endoprotease (P69A), *S. lycopersicum* (50%)	3.89	1.59	Protein catabolism	
Lipid metabolism	cSTA21L23	Phospholipase D (PLDa1), *S. lycopersicum* (71%)	2.14	1.25	Phospholipids metabolism	
Lipid metabolism	PPCBG78	Family II lipase EXL3, *A. thaliana* (18%)	2.05	1.04	Lipid metabolism	
Secondary metabolism	POADO52	Cytochrome P450 81B1(Isoflavone 2'-hydroxylase) *Helianthus tuberosum* (28%)	3.11	2.05	Electron transport	
Secondary metabolism	BPLI8K6	Ferulate-5-hydroxylase/cytochrome P450, *A. thaliana* (4%)	5.10	1.87	Phenylpropanoid metabolism	
Secondary metabolism	cSTC2J7	4-coumarate--CoA ligase 2 (4CL 2), *S. tuberosum* (100%)	1.96	0.84	Phenylpropanoid metabolism	P/W/JA
Intracellular transport	BPLI4I23	Vacuolar sorting receptor-like protein, *A. thaliana* (37%)	2.89	1.05	Intracellular protein transport	
Transport	PPCAI68	Hexose transporter, *S. lycopersicum* (39%)	5.37	1.92	Carbohydrate transport	
Transport	POCBT09	Hexose transporter, *S. lycopersicum* (83%)	2.89	1.68	Carbohydrate transport	
Transport	PPCBL51	Amino acid transport protein (AAT1), *A. thaliana* (65%)	2.06	0.97	Amino acid transport	
ET biosynthesis	PPCAI70	1-aminocyclopropane-1-carboxylate oxidase (ACO), *S. tuberosum* (72%)	3.74	1.89	ET biosinthesis/Signalling	V/W/S/AS
Cell wall metabolism	cSTS1A12	Late embryogenesis (Lea)-like protein ER5, *S. lycopersicum* (100%)	4.78	1.27	Cell wall biogenesis	ET/AS/ABA/W
Cell wall metabolism	STMJE77	Extensin (Class I), *S. lycopersicum* (21%)	2.12	1.37	Cell wall biogenesis	W
Cell wall metabolism	POABV85	Proline-rich family protein, *A. thaliana* (94%)	1.96	1.29	Cell wall biogenesis	
Unknown	PPCBJ22	Heavy-metal-associated domain-containing protein, *A. thaliana* (44%)	2.33	1.73	Unknown	
Unknown	cSTD21L20	B2 protein, *Daucus carota* (74%)	2.77	1.41	Unknown	
Unknown	PPCBI30	Unknown protein, *N. tabacum* (60%)	2.63	1.65	Unknown	V/SA
Unknown	PPCAR90	Unknown protein	2.12	0.98	Unknown	
Unknown	POCBI24	Unknown protein, *A. thaliana* (62%)	1.96	1.17	Unknown	

Table 1 (Continued)

Functional category	Clone name	Gene annotation (putative function), degree of homology with other spp (%)	Fold change[1]		Process category	Response to[2]
Local leaves. Down-regulated genes after *M. euphorbiae* attack			Me	Mp		
PR	cSTB31B17	Hypersensitivity-related protein (hsr201), *N. tabacum* (94%)	0.45	0.74	Defence related	P
PR	PPCBD76	Dirigent-like protein, *A. thaliana* (7%)	0.30	0.39	Defence related	P
Regulatory	PPCBR06	WRKY transcription factor 72, WRKY DNA binding protein, *S. tuberosum* (55%)	0.45	0.84	Regulation of transcription	P
Regulatory	cSTB32G23	Transcription factor MYC7E, Z. mais (16%), *O. sativa* (16%)	0.40	0.58	Regulation of transcription	
Regulatory	STMGM47	F-box family protein, *A. thaliana* (10%)	0.47	0.52	Regulation of transcription	
Regulatory	BPLI4A23	Receptor protein kinase-like protein, *C. annum* (64%)	0.45	0.39	Signalling	
Regulatory	cSTB29K12	mRNA binding protein, *S. lycopersicum* (100%)	0.41	0.62	RNA processing/turnover	
Regulatory	cSTB38M11	Cryptochrome 1b, *S. lycopersicum* (58%)	0.42	0.58	Light-dependent responses	
Regulatory	PPCBN68	Protein phosphatase 2C (PP2C); GB-Ser/thr phosphatases, *A. thaliana* (69%)	0.47	0.85		
Regulatory	PPCBX66	bZIP (leucine zipper) protein, *A. thaliana* (22%)	0.43	0.56	Regulation of transcription	
Regulatory	PPCBV51	Small nuclear ribonucleoprotein, *A. thaliana* (100%);*O. sativa* (100%)	0.47	0.77	mRNA processing	
General metabolism	cSTB14C6	Xylulose kinase [Bacillus subtilis], *A. thaliana* (34%)	0.50	0.96	Carbohydrate metabolism	
General metabolism	cSTB35B12	Malate dehydrogenase (glyoxysomal precursor), *Cucumis sativus* (100%)	0.44	0.68	Tricarboxylic acid cycle	
General metabolism	PPCCA73	ALG3 (mannosyltransferase), *A. thaliana* (19%)	0.46	0.49	Glycan biosynthesis	
General metabolism	cSTB5G4	Glycosyl hydrolase, *A. thaliana* (33%)	0.49	0.63	Carbohydrate metabolism	
General metabolism	cSTE11P6	Glucose-1-phosphate adenylyltransferase small chain, *S. tuberosum* (100%)	0.45	0.53	Biosynthesis	
General metabolism	BPLI3F3	Aldo/keto reductase, *A. thaliana* (87%)	0.48	0.65	Metabolism	
General metabolism	PPCBF80	Acid phosphatase, *S. lycopersicum* (44%); *Hordeum vulgare* (65%)	0.29	0.51	Acid phosphatase	
Lipid metabolism	cSTB35H22	Proline-rich protein APG-like; GDSL-motif lipase/hydrolase, *A. thaliana* (62%)	0.43	0.69	Lipid degradation	

Table 1 (Continued)

Functional category	Clone name	Gene annotation (putative function), degree of homology with other spp (%)	Fold change[1]		Process category	Response to[2]
Local leaves. Down-regulated genes after *M. euphorbiae* attack (continue)			Me	Mp		
Cell wall metabolism	cSTB27J22	Polygalacturonase-like protein, *A. thaliana* (86%)	0.47	0.63	Carbohydrate metabolism	
Photosynthesis	BPLI2J15	Thylakoid membrane phosphoprotein 14 kDa, *A. thaliana* (60%)	0.43	0.54		
Photosynthesis	cSTB29H19	Carbonic anhydrase (chloroplast precursor), *N. tabacum* (100%)	0.48	0.67	Carbon utilization	
Photosynthesis	PPCBT17	Inorganic carbon transport protein, *A. thaliana* (45%)	0.49	0.73	Carbon transport	
Photosynthesis	cSTB27C7	Cytochrome B6-F complex iron-sulphur subunit 2, *N. tabacum* (100%)	0.44	0.52	Photosynthesis	
Photosynthesis	cSTB30L7	Photosystem II protein W-like protein, *A. thaliana* (92%)	0.45	0.87	Photosynthesis	
Photosynthesis	cSTB40A1	Photosystem II reaction centre W (PSII 6.1 kDa), *Spinacia oleracea* (66%)	0.41	0.76	Photosynthesis	
Lignin biosynthesis	cSTB18J19	Catechol O-methyltransferase, *N. tabacum* (100%)	0.43	0.52	Lignin biosynthesis	
Sterol biosynthesis	PPCAC27	Sterol-C5(6)-desaturase, *N. tabacum* (100%)	0.36	0.85	Sterol biosynthesis	
Dormancy-related	cSTA43M16	Dormancy/auxin-repressed protein, *Solanum virginianum* (100%)	0.42	0.51	Dormancy	
Unknown	STMJC89	Vacuolar ATP synthase/ V-ATPase-related, *A. thaliana* (19%)	0.46	0.66	Unknown	
Unknown	PPCCB13	Genomic DNA, chromosome 3, P1 clone: MVI11, *A. thaliana* (25%)	0.44	0.51	Unknown	
Unknown	cSTA5E12	Haloacid dehalogenase-like hydrolase, *A. thaliana* (79%)	0.37	1.04	Unknown	
Unknown	cSTB3G11	Unknown protein, *A. thaliana* (17%)	0.48	0.77	Unknown	
Unknown	STMJC38	Unknown protein, *A. thaliana* (8%)	0.48	0.89	Unknown	
Unknown	STMJG02	Unknown protein, *A. thaliana* (53%)	0.46	0.95	Unknown	

Table 1 (Continued)

Functional category	Clone name	Gene annotation (putative function), degree of homology with other spp (%)	Fold change[1]		Process category	Response to[2]
Local leaves. Up-regulated genes after *M. persicae* attack			Me	Mp		
PR	BPLI4D2	Class IV chitinase, *A. thaliana* (91%)	2.08	3.16	Defence/antifungal	P
PR	STMJD93	Class II (acidic) chitinase, *S. tuberosum* (60%)	1.68	3.70	Defence/antifungal	P/SA
Regulatory	PPCCL54	Nucleic acid binding protein, *A. thaliana* (47%)	2.17	5.56	Regulation of transcription	
Regulatory	POCCB81	AN1-like zinc finger, *A. thaliana* (53%)	1.77	2.50	Regulation of transcription	
Regulatory	PPCBB86	Wall-associated kinase 1 (WAK1), *A. thaliana* (27%)	1.04	2.22	Signalling	P
Regulatory	cSTS7C9	Calmodulin-like protein, *A. thaliana* (21%)	0.94	3.70	Signalling	
General metabolism	PPCCQ67	ATP synthase 8, *Embiotoca lateralis* (fish) (25%)	1.01	2.22	ATP synthesis	
Secondary metabolism	PPCBM31	Cytochrome P450, *A. thaliana* (5%)	1.06	2.09	Electron transport	P/I/AS
Intracellular transport	POCBT23	Coatomer beta' subunit (Beta'-coat protein), *A. thaliana* (28%)	1.07	3.82	Intracellular protein transport	
Local leaves. Down-regulated genes after *M. persicae* attack						
Regulatory	BPLI8E4	Protein kinase-like protein, *A. thaliana* (73%)	0.52	0.38	Signalling	
Regulatory	PPCBK19	Extra-large G-protein-like, *A. thaliana* (13%)	0.49	0.36	Signalling	
General metabolism	BPLI7J2	RNA binding protein, *A. thaliana* (100%)	0.86	0.40		
General metabolism	PPCBU51	Glycerophosphoryl diester phosphodiesterase, *Fusobacterium nucleatum* (4%)	1.04	0.45	Glycerol metabolism	
General metabolism	cSTB4D16	Allyl alcohol dehydrogenase, *N. tabacum* (100%)	0.64	0.44	Metabolism	
General metabolism	cSTD12G14	Acid phosphatase, H. vul (51%), *Glycine max* (42%)	0.39	0.44		
Cell wall metabolism	cSTA28P6	Beta-xylosidase, *A. thaliana* (52%); LEXYL1 *S. lycopersicum* (58%); L2 (53%)	0.63	0.49	Carbohydrate metabolism	
Cell wall metabolism	cSTB43H23	Extensin, *S. tuberosum* (95%); Cysteine-rich extensin 4, *N. tabacum* (80%)	0.51	0.47	Cell wall biogenesis	
Photosyntesis related	cSTB36B19	ATP synthase beta chain subunit II (chloroplast), S. ole (68%), *A. thaliana* (72%)	0.51	0.48	ATP synthesis	
Photosyntesis related	BPLI5J20	ATP synthase gamma chain (chloroplast), *N. tabacum* (100%)	0.53	0.43	ATP synthesis	
Photorespiration	cSTB14F19	Glycine decarboxylase (mitochondrial precursor), *S. tuberosum* (100%)	0.55	0.41	Glycine/serine metabolism	
Photorespiration	cSTB1C22	(S)-2-hydroxy-acid oxidase, peroxisomal/ glycolate oxidase, *A. thaliana* (100%)	0.58	0.40	Carbon pathway	
Unknown	BPLI10I16	Cleft lip and palate associated transmembrane protein-like, *A. thaliana* (91%)	0.56	0.38	Unknown	
Unknown	BPLI3B17	Epsin N-terminal domain-containing protein /clathrin assembly, *A. thaliana* (70%)	0.79	0.49	Unknown	
Unknown	PPCBF20	Unknown protein, *A. thaliana* (49%)	0.63	0.44	Unknown	

Table 1 (Continued)

Functional category	Clone name	Gene annotation (putative function), degree of homology with other spp (%)	Fold change[1]		Process category	Response to[2]
Systemic leaves. Up-regulated genes after *M. euphorbiae* attack			Me	Mp		
Unknown	PPCCS21	Unknown protein, *Drosophila melanogaster* (4%)	2.15	1.25	Unknown	
Systemic leaves. Down-regulated genes after *M. euphorbiae* attack						
Regulatory	BPLI10E21	Auxin-induced protein TGSAUR22, *A. thaliana* (56%)	0.46	0.96	Signalling	
Intracellular transport	cSTB41F4	Coatomer delta subunit (delta-COP), *A. thaliana* (27%)	0.47	0.74	Intracellular protein transport	
Unknown	STMDK87	KED protein, *N. tabacum* (48%)	0.22	0.41	Unknown	
Systemic leaves. Down-regulated genes after *M. persicae* attack						
	STMJC22	Protein prenyltransferase alpha subunit repeat, *O. sativa (21%)*	0.99	0.44	Amino acid prenylation	
Unknown	cSTS12A24	Unknown protein, *A. thaliana* (31%)		0.48	Unknown	
Unknown	cSTE10A20	Putative proline-rich protein, *S. lycopersicum* (97%)	0.77	0.35	Unknown	

[1] Values were calculated as relative transcript abundance (ratios of values for aphid infested plants/values for control plants). Underlined values indicate genes with expression ratio two folds higher (up-regulated), and genes with expression ratio two fold lower (down-regulated) than the control that are statistically different from 0 ($P \leq 0.05$). The grey boxes are genes clearly up-regulated in all 3 biological replicates with expression ratio two folds higher but with $0.05 < P < 0.1$ due to a high variability between replicates. The criterion to be included was that at least 2 out of 3 technical replicates in the slide showed differential regulation; Me, fold change after *Macrosiphum euphoebiae* attack; Mp, fold change after *Myzus persicae* attack; [2] P, pathogens; W, wounding; AS, abiotic stress; S, senescence; SA, salicylic acid; ET, ethylene; JA, jasmonic acid; ABA, absicic acid; GA, gibberellic acid; Ck, citokinins; I, insects; V, viruses.

Genes were assigned in 3 groups: 1) genes regulated in the same direction (up or down) after *M. euphorbiae* and *M. persicae* infestation, 2) genes regulated (up or down) only after *M. euphorbiae* infestation, 3) genes regulated (up or down) only after *M. persicae* infestation. The number of genes differently expressed after *M. euphorbiae* or *M. persicae* infestation across gene-functional categories is shown in **Figure 6**.

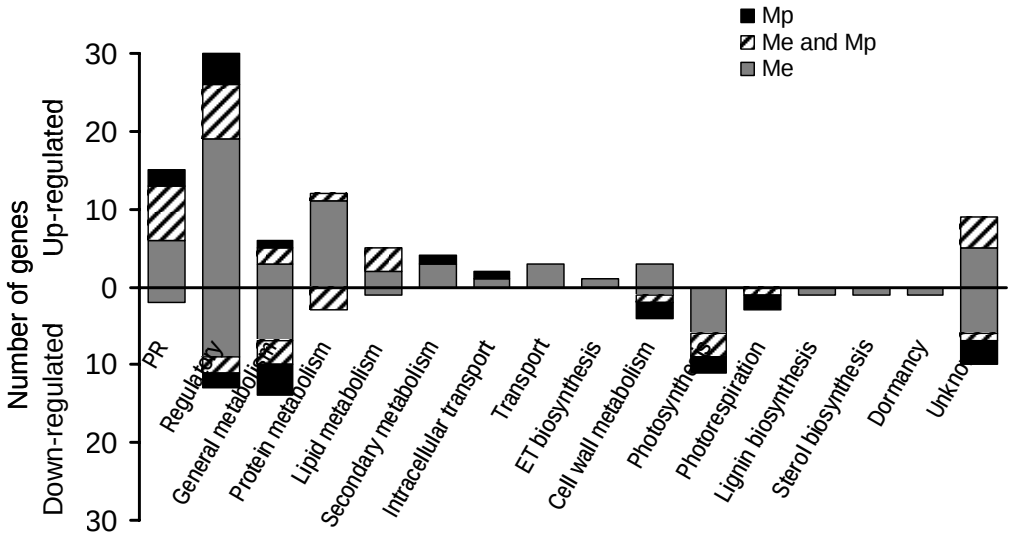

Figure 6. Differences in number of genes up- or down-regulated in *Solanum stoloniferum* after infestation by *Macrosiphum euphorbiae* or *Myzus persicae* across gene-functional categories. Me, means number of genes only differentially regulated after *M. euphorbiae* attack; Mp and Mp , means number of genes differentially regulated after both aphids attack; Mp, means number of genes only differentially regulated after *M. persicae* attack.

Up-regulated genes

Attack by *M. euphorbiae* and *M. persicae* elicit the expression of a common set of genes in the following functional categories: pathogenesis related (PR), regulatory, general metabolism, protein metabolism and lipid metabolism. It is interesting to note that in all these categories a higher number of genes were up-regulated after *M. euphorbiae* attack than after *M. persicae* attack (**Figure 6**). Both plant-aphid interactions elicit the accumulation of transcripts related to secondary metabolism and intracellular transport of molecules, but those genes were specific to either infestation by *M. euphorbiae* or *M. persicae*.

M. euphorbiae differentially induced the expression of genes involved in: transport and cell wall metabolism, and one gene involved in ethylene (ET) biosynthesis (**Figure 6**).

Down-regulated genes

Attack by *M. euphorbiae* and *M. persicae* elicit the repression of a common set of genes in the following functional categories: regulatory, general metabolism, protein metabolism, cell wall metabolism, and genes related to photosynthesis and photorespiration. *M. persicae* attack suppresses a larger number of genes related to photorespiration and cell wall metabolism, conversely *M. euphorbiae* suppresses a larger number of genes related to regulatory, general metabolism, photosynthesis. *M. euphorbiae* attack differentially suppresses genes related to pathogenesis, lipid metabolism, lignin and sterol biosynthesis, and dormancy (**Figure 6**).

Gene expression at the systemic leaf level

At the systemic level the plant response was weak for both aphid infestations (**Table 1**). In response to *M. euphorbiae* infestation only one gene was systemically up-regulated and 3 down-regulated. In response to *M. persicae* infestation no genes were systemically up-regulated and 3 genes were down-regulated. At the systemic level no overlapping transcription changes were found in response to both aphid attacks.

Discussion

Resistance of *S. stoloniferum* to *M. persicae*

The settling test provided no evidence for a local induction of repellence or deterrence in plants to *M. persicae* by pre-infestation of any of the two aphid species. Compared to control plants we did not detect any extra resistance other than the constitutive resistance to *M. persicae* that has been described previously (Chapter 2). Only in systemic leaves our results might suggest an early escape of the aphids (at 15 min), especially con-specifically induced.

The optimal defence theory predicts that resistance induction will be negatively correlated with constitutive resistance of a plant (Traw, 2002). *S. stoloniferum* has a strong constitutive resistance (Chapter 2) that seems not enhanced after aphid attack at the local level, which supports this theory. Dugravot et al. (2007) found that in *S. tuberosum* preinfestation by *M. euphorbiae* and *M. persicae* have beneficial effects on the probing behaviour of *M. persicae* (induced susceptibility) at the local leaves, whereas at the systemic leaves (on the non-infested leaves of infested plants) the opposite detrimental effects for *M. persicae* occurs (induced resistance). In *S. stoloniferum* with the aphid

settling test the induction of resistance at systemic level was not clear, therefore further long term studies taking into account aphid performance and probing behaviour are needed.

Gene expression upon *M. persicae* and *M. euphorbiae* attack

Although none of the two aphid species seemed to induce measurable resistance in *S. stoloniferum*, gene-expression was affected. We used a dedicated cDNA micro-array containing 3564 *S. tuberosum*, mostly pathogen-responsive clones, to measure this. At the systemic level we observed a weak response in our plant-aphid system, similar to what has been reported for an infestation by *M. nicotianae* on *N. attenuata* (Voelckel et al., 2004).

We found on the pathogen-responsive gene array, that 4 % of the genes respond to aphid infestation, suggesting that the mechanism of plant recognition to aphids in part overlaps but mainly diverges from the plant's response to pathogens.

In the compatible interaction with *M. euphorbiae* 130 genes were differentially regulated, whereas in the incompatible interaction with *M. persicae* 62 genes were differentially regulated, showing that the response was stronger in the compatible interaction. This stronger response in *M. euphorbiae* involves mainly up-regulation of genes in the functional categories pathogenesis related (PR), regulatory, and protein metabolism and down-regulation in the functional categories regulatory, general metabolism and photosynthesis related genes (**Figure 5**). Differential regulation of genes in these categories has been shown in other plant-aphid interactions as well Zhu-Salzman et al., 2004).

The results from our gene-expression studies provide evidence that *S. stoloniferum* responds to aphid infestation by activating the salicylic acid (SA), ethylene (ET) pathways, and to a lesser extent the jasmonic acid (JA) pathway.

Similar to pathogens, both aphid species induced a number of PR genes. PR genes have been associated with aphid feeding in diverse plant-aphid interactions (De Vos et al., 2005; Moran et al., 2002; Moran and Thompson, 2001; Park et al., 2006; Zhu-Salzman et al., 2004). Most of these genes are SA-responsive, indicating that the SA signaling pathway is activated upon the attack by either aphid species as was demonstrated for several other plant-aphid interactions (Zhu-Salzman et

al., 2004). The interaction with *M. euphorbiae* additionally induced six transcript homologues of PR-protein genes that were not induced by *M. persicae* (**Table 1**).

Both aphid species induced the expression of a number of ethylene-responsive genes. The infestation with *M. euphorbiae* directly activates genes responsible for ET biosynthesis and ET regulation; *1-aminocyclopropane-1-carboxylate oxidase* (*ACO*) is the key enzyme controlling and regulating ethylene production in plants (Nie et al., 2002; Yang and Hoffman, 1984). Also *M. persicae* feeding on *A. thaliana* (which is a compatible interaction) leads to *ACO1* accumulation (Moran et al., 2002). In addition, in the *M. euphorbiae*-infested plants we found the up-regulation of *EIN3-binding F-box protein 1* (*EBF1*) gene. *EBF1* is induced by ET, and *EBF1* is a negative regulator of the ethylene-signalling pathway, therefore there is a negative-feedback mechanism which may allow fine tuning of the expression of ET dependent genes (Guo and Ecker, 2004).

All together, our results suggest that according to the number of genes differentially regulated that are responsive to each of these phytohormones, the extent of the response depends on the type of interaction established (compatible or incompatible). The ET signal-transduction pathway plays a larger role in compatible plant-aphid interactions than in incompatible interactions. Regarding the JA-responsive genes, we observed the up-regulation of a transcript for *jasmonic acid 2*, and two more JA-responsive genes in the compatible interaction and no activation of JA-responsive genes in the incompatible interaction (**Table 1**).

It has been proposed that endogenous ET production triggered by aphid probing and feeding may suppress JA-induced defence genes (Zhu-Salzman et al., 2004). This regulation of plant defence mechanisms has also been observed in other plant-insect interactions, e.g. *Manduca sexta* on *Nicotiana attenuata*, where plant-ethylene induced by insects suppressed JA-dependent responses (Winz and Baldwin, 2001).

Do aphids manipulate the physiological state of the plant?

Since resistance at the local level was not improved in response to the induction of genes, the question is raised, why *S. stoloniferum* changes the expression of all these genes? For the compatible interaction it is conceivable that this is at least partly a general reaction to stress, which is also evidenced by the type and number of genes induced (**Table 1**). We hypothesize that

especially the high number of regulatory genes up-regulated in local leaves by the compatible plant-aphid interaction could be related to a change in the physiology of the plant as a consequence of aphid attack. Subsequently the shift on plant condition might contribute to the acceptance of *S stoloniferum* as a host for *M. euphorbiae.* In addition it seems likely that the local level is the affected area more than the remote systemic areas.

Activation of regulatory genes

Macrosiphum euphorbiae infestation elicits the expression of three *calmodulin-like* genes. Moran et al. (2002) also find similar results for *M. persicae* on *A. thaliana*. Hence, directly or indirectly aphids elicit the Ca^{2+} signaling pathway in the host plant. Calmodulins are major Ca^{2+} sensors that orchestrate regulatory events through their interaction with a diverse group of cellular proteins. Ca^{2+} concentration in resting plant cells is at low basal level but Ca^{2+} flux and calmodulin are produced in response to a variety of biotic and abiotic environmental stimuli (Braam and Davis, 1990; Mithöfer and Mazars, 2002). Moreover, Ca^{2+} plays an important role in a range of processes, for example, on growth, differentiation, mitosis, stomatal regulation, stress adaptation, and induction of plant defence responses (Klusener et al., 2002; Sanders et al., 1999).The calmodulin role on compatible interaction needs further study.

Signalling processes in plant cells are mediated by a number of receptor-protein kinases. After *M. euphorbiae* attack we found nine protein kinase-like transcripts that are differentially up-regulated (**Table 1**). Among them there is *RSH2*-like protein (*RelA/SpoT* homologues) that is similar to bacterial guanosine tetraphosphate (ppGpp) synthetases. The compound ppGpp is a secondary messenger of general stress and mediates global reprogramming of the transcriptional output of the cell (Toulokhonov et al., 2001). In tobacco RSH2 synthesizes ppGpp, which is induced by pathogenic bacteria and jasmonic acid. RSH2 is localized in chloroplasts and there is evidence that it regulates gene expression in chloroplasts in response to plant defence and environmental stress signals (Givens et al., 2004).

Activation of genes related to protein metabolism

Macrosiphum euphorbiae feeding induces the accumulation of a variety of genes related to protein catabolism (proteases) (**Table 1**). Two up-regulated genes are homologues to genes involved in the ATP/ubiquitin-dependent non-lysosomal proteolytic pathway. The first gene encodes for a protein that is homologous to the *20S α3 proteasome subunit* (part of the proteasome-multicatalytic

proteinase complex). The second transcript is homologue to *ubiquitin-specific protease 12* (*UBP12*), whose function is related to the proteasome (Wolf and Hilt 2004). The ubiquitin/proteasome pathway is involved in regulated proteolysis, which is needed for controlled cellular processes (Hochstrasser, 1996). Perhaps this proteasome related protein turnover may contribute to increase the phloem quality, which would be in benefits of the aphids.

Macrosiphum euphorbia attack induced also two up-regulated transcripts homologues to *subtilisin-like endoprotease P69*. Some *P69 RNAs* are activated in tomato plants upon *Pseudomonas syringae* infection and also by SA and ET (Jorda et al., 1999). Plants under *M. euphorbia* attack accumulate the *chloroplast nucleoid DNA-binding protease 41 kD* (*CND41*). This protease is involved in ribulose biphosphate carboxylase oxygenase (rubisco) degradation and the translocation of nitrogen during senescence in tobacco (Kato et al., 2004). Rubisco is the major protein in chloroplasts (composes >50% of the protein in leaves). *CND41 protease* is highly expressed in senescent leaves where chlorophyll loss is accompanied by the release and breakdown of rubisco (Wittenbach, 1979). Breakdown of the photosynthesis apparatus and subsequent mobilization of the breakdown products could result in nutritious enrichment of the phloem that is an advantage to the aphid, as was suggested also by Zhu-Salzman et al. (2004). Moreover, nine transcripts related to photosynthesis were down regulated by *M. euphorbiae* attack. The same effect has also been reported for *Manduca sexta* L. - *Nicotiana attenuata* Torr. Ex Wats. Interaction (Hermsmeier et al., 2001). The suppression in photosynthesis related genes might indicate a shift in use of plant resources toward defence and stress response.

Leaves infested with *M. euphorbiae*- accumulated three transcripts related to folding of proteins; one transcript is homologous to the *S. lycopersicum* gene for the molecular chaperone *Hsc70* protein. Cells increase chaperone concentrations as a response to diverse stresses. When proteins become unfolded chaperones recognize them and whenever possible promote re-folding. Alternatively, Hsc70 may promote the degradation of proteins by the ubiquitin/proteasome system (Bercovich et al., 1997; Esser et al., 2004).

Activation of genes related to the mobilization of molecules

Macrosiphum euphorbiae infestation induced the expression of a variety of transcripts related to mobilization of molecules. The *vacuolar sorting receptor-like protein* plays a role in trafficking between the endoplasmatic reticulum (ER) and the plasma membrane, and in mobilization of

storage proteins (Laval et al., 2003). *Phospholipase D* (*PLDα1*) is involved in lipid mobilization and catabolism associated with cell death (Laxalt et al., 2001) *Branched-chain amino acid aminotransferase* is related to amino acid production and N mobilization, and accumulates in leaf tissues undergoing rapid cell division (Campbell et al., 2001). Finally, *M. euphorbiae* attack accumulated three transcripts homologous to genes involved in extracelullar transport: two *hexose transporters*, and one *amino acid transport protein* (*AAT1*).

All together, these proteases, chaperones, and transport proteins are enzymes involved in mediating general protein turnover (by degrading misfolded and denatured proteins) and maintaining protein quality control in response to cell stress (for review see Esser et al., 2004; Maupin-Furlow et al., 2005; Wolf and Hilt, 2004). It is interesting to note that none of these genes are activated in the incompatible interaction with *M. persicae.* Our data suggest that, regarding manipulation of plant physiology by aphids, *M. persicae* probably faces a feeding constraint in *S. stoloniferum* because it is not capable of benefiting from the changes on the physiological status of the plant tissue that would enrich the phloem quality. Moreover, it is also not capable of re-allocating resources from the photosynthesis/photorespiration to improve phloem quality.

Genes related to changes on sink/source relations

Aphids feeding on phloem sap are hydraulically equivalent to plant sinks (Douglas, 2003b), such as meristems, growing roots and developing leaves. If aphids are able to manipulate sink/source relations in the feeding place through modifying the physiological status of the tissue this will become apparent in shifts in the expression of certain genes. For both *S. stoloniferum*-aphid interactions we found up-regulated candidate genes that could play a role in switching the tissue status from source to sink. The *Xylogen protein 1/nonspecific lipid transfer protein* (*nsLTP*) is a proteoglycan-like factor involved in plant tissue development and differentiation. It mediates inductive cell-cell interactions to direct continuous vascular development (Motose et al., 2004). In potato the expression of *nsLTP* increases just prior to active metabolic processes, e.g., tuberization, sprout development, dormancy breakage (Horvath et al., 2002). These cell differentiation and developmental processes occurs on sink tissues.

Two other up-regulated transcripts are homologous to *short-chain type alcohol dehydrogenase*. These genes are likely to be indirectly involved in the breakdown of gibberellic acid (GA) (Bachem et al., 2001). GA levels decrease in the stolons just before tuberization (Ewing and Struik, 1992).

Macrosiphum euphorbiae attack elicits a transcript homologue to *calreticulin 3*. Sink tissues, e.g., tissues in development and nectaries, have abundant expression of this calcium-binding chaperone (Nelson et al., 1997). Interesting, nectaries received a direct vascular supply from phloem to produce and secrete nectar which is composed of sugars and amino acids (Fahn, 2000). Furthermore calreticulin accumulates in zones which are active in transport (Oparka et al., 1994). In tobacco plants *calreticulin* transcripts increase upon treatment with elicitors related to pathogen attack, cell wall-degrading enzymes from pathogenic bacteria and salicylic acid (Denecke et al., 1995). Calreticulin may be involved in tissue-sink-strength through sink plasmodesmata, which are gateways for phloem un/uploading (Baluska et al., 2001).

The *dormancy/auxin-repressed protein* (*DRM*) was down-regulated after *M. euphorbiae* attack. The physiological function of the gene is unknown. It is highly expressed in non-growing buds of plants and down-regulated in growing ones. Growing tissues have a high sink strength (Herbers and Sonnewald, 1998), suggesting that the change on expression of these cell differentiation and development related genes in leaves infested by aphids could be related with a process of changing the physiological status of the tissue for generation of a local metabolic sink on the feeding place. Consequently the phloem sap would be enriched on the sink places.

Concerning the hypothesis of aphid manipulation related to sink-source strength an exhaustive study of the expression of sink-source related genes in different plant parts is necessary.

Pustule development on *S. stoloniferum* upon *M. persicae* attack

Solanum stoloniferum leaves that were insistently attacked by *M. persicae* in a clip cage developed pustules (**Figure 4**). The plant tissue reactions appeared very similar to the reactions observed in some plant species after the attack of some pathogens, e.g., *Xanthomonas campestris* pv. *vesicatoria* on susceptible pepper and tomato plants (Marois et al., 2002) and *Xanthomonas citri* on citrus plants (Duan et al., 1999; Swarup et al, 1991). In such reactions type III effector-proteins, like AvrBs3 from *X. campestris* pv. *vesicatoria* (Marois, 2002) or PthA protein produced by *X. citri* (Duan et al., 1999; Swarup et al, 1991) play an important role. These proteins are injected into the plant-cells, thereby triggering cell enlargement and division.

Moreover, the attack of both aphids elicits the expression of a gene homologue to *auxin-induced SAUR-like protein* (**Table 1**). Induction of the *SAUR-like protein* is also found after inoculation of pepper and tomato plants by the virulent bacterium *X. campestris* pv. *vesicatoria.* Most likely *SAURs* are involved in the disease response of the susceptible tissue (like pustules development) and the bacteria possess effectors that manipulate host gene expression (Marois et al., 2002).

Myzus persicae has feeding difficulties on *S. stoloniferum.* Aphids need 3 hours or more to penetrate through the mesophyll and vascular tissue (pathway phase) before the first phloem activity is apparent, and at least 5 h to start sustained phloem feeding (activity related with host plant acceptance) Alternatively, *M. persicae* probing on a susceptible *S. tuberosum* plant needs shorter time (1 h) before the first phloem activity is apparent, and an average of 3 to 4 h to start sustained phloem feeding (Alvarez et al., 2006). During the pathway phase there is salivation of intercellular sheath material and briefly intracellular punctures are performed regularly. During the punctures, some watery saliva is injected into de cell and there is ingestion of minute cytoplasmic content. The punctures are performed in all tissues and the cells show little damage and very few cells died (Tjallingii and Hogen Esch, 1993). These cells will be primary targets to salivary signals eliciting plant responses (Tjallingii, 2006).

On Chapter 3 we found that the penetration behaviour of *M. persicae* on the resistant *S. stoloniferum* is different from that on *S. tuberosum*. Although the total number and time of cell punctures is similar, the average duration of the individual cell punctures is longer on *S. stoloniferum,* which indicates that individual cells hold the stylet longer and perhaps more saliva is injected. Moreover whereas most *M. persicae* aphids on *S. tuberosum* will consume the phloem sap after 3 h of probing, on *S. stoloniferum* they will continue puncturing the cells and overloading them with saliva, and consequently will probably puncture more cells and the same cell multiple times (see Chapter 3) thereby injecting effectors present in the saliva.

Pustule development seems not likely to occur upon a natural *M. persicae* attack as a high number of aphids are needed to elicit it. High numbers of plant penetrations during 4 days on a non-host/resistant plant concentrated on a small area is rather unnatural as *M. persicae* will leave the *S. stoloniferum* plant when given a choice. Altogether we hypothesize that the aphid watery-saliva may contain effector-proteins similar to AvrBs3 or PthA. The aphid's endosymbionts may be responsible for the production of the effector protein that elicits the pustule formation. Zhu-Salzman

et al. (2001) and Moran et al. (2002) suggest that endosymbionts and/or aphid-vectored viruses could be responsible for the pathogen-responsive gene expression pattern observed in response to aphid feeding in Arabidopsis.

Conclusions

Our study shows that two different aphid species on the same plant genotype elicit the expression of a shared set of genes, but there are also transcriptional differences.

On the compatible plant-aphid interaction the high number of regulatory and metabolism related genes with differential expression that were triggered by aphid infestation suggest that like pathogens, aphids might benefit from the changes that they promote on plant metabolism. On compatible plant-aphid interactions the capability of the aphids to manipulate the plant apparently seems to occur mainly in local leaves, i.e. at probing/feeding sites but not on the remote systemic tissues. The ability of reprogramming the biochemical-physiological status of the cells and tissue at these sites might partly contribute to determine the host plant range for each aphid species.

Despite the differences that we find at gene expression level for the plants reaction in a compatible and resistant plant-aphid interaction, further research with other plants and aphids combinations would be necessary in order to have a complete picture of gene regulation patters after aphid attack.

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CHAPTER 5

Responses of *Solanum tuberosum* cv. Kardal to *Myzus persicae* infestation depend on foliage maturity

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Abstract

The green peach aphid *Myzus persicae* (Sulzer) is not able to colonise young leaves of the potato *Solanum tuberosum* L. cv. Kardal. Resistance factors apparently prevent normal probing behaviour, i.e. plant penetration and feeding of the aphid. However, *M. persicae* can survive and reproduce on mature to senescent leaves of the same plant without problems. We investigated the plant responses to the attack by *M. persicae* at different maturity stages of the leaves, young and old leaves, by studying gene expression and aphid settling behaviour. We compared the transcriptomics results with those obtained previously from *S. stoloniferum* after the attack of *M. persicae* and *Macrosiphum euphorbiae* (Thomas) (Chapter 4). In old leaves, *M. persicae* attack elicits a substantially higher number of differentially regulated genes than in young leaves. The response in young leaves of Kardal after *M. persicae* attack was weak, as far as the number of down-regulated genes is concerned. In both leaf stages, *M. persicae* attack mainly elicited the expression of pathogenesis related, regulatory, and protein-metabolism related genes. We also studied aphid settling behaviour on previously infested young and old leaves to assess possible induction of antixenotic resistance. No evidence was found that preinfestation of plants by *M. persicae* induced repellence or deterrence. Thus, aphid attack did not cause an increased resistance in addition to the constitutive resistance found earlier.

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Introduction

Leaves of different maturity stages offer different environments to aphids. In previous studies, we observed that *Myzus persicae* settled underneath older leaves on the point of turning yellow on the potato (*Solanum tuberosum* L.) cultivar Kardal. The aphids reproduced and accumulated against the midrib and secondary veins and moved upward progressively to the next leaf to become yellow soon. The Kardal apical young leaves remained free of aphids (**Figure 1**).

Figure 1. *Solanum tuberosum* cultivar Kardal infested by *Myzus persicae*. Aphids (black spots) were only found on senescent leaves

Moreover, by studying *M. persicae* probing behaviour and colony development on cv. Kardal, we found that Kardal has a certain degree of resistance to the aphids on young leaves (Chapter 2) (Alvarez et al., 2006). Potato resistance to some pathogens, like the oomycete *Phytophtora infestans*

(Mont.), seems to follow a similar pattern. In late blight resistance, leaf position is the most significant factor. Apical leaves are far more resistant than basal leaves (Visker et al., 2003). Aphid preference for mature and senescent potato leaves reflect differences in chemical composition of the leaves that affect the aphid distribution and behaviour on the plant.

Several studies have shown that differences in gene-expression occur during senescence of leaves and that those changes indicate changes in the physiology of the leaf. Senescence is an active developmental and highly regulated process that includes the modulated expression of many genes related to different functional categories (Gepstein et al., 2003; Buchanan-Wollaston, 2003; Buchanan-Wollaston et al., 2005). The degradation of chloroplasts is one of the key factors in leaf senescence for recycling and mobilizing the nutrients from senescing leaves to developing sink parts (Buchanan-Wollaston, 1997). Chlorophyll loss is usually accompanied by release and breakdown by ribulose biphosphate carboxylase oxygenase (rubisco), which comprises more than 50% of the protein in green leaves (Wittenbach, 1979). The resulting mobilisation of amino acids enrich the phloem sap and may stimulate phloem sap ingestion by aphids. Phloem feeding by aphids is hydraulically equivalent to natural plant sinks, such as fruits or roots. However, in susceptible plant-aphid relationships there are more complex interaction than between natural source-sink tissues in plants (Douglas, 2003).

On grasses *Schizaphis graminum* Rondani, the greenbug, induces chlorotic halos around a necrotic spot on mature leaves (Al-Mousawi et al., 1983). *Diuraphis noxia* Mordvilko, the Russian wheat aphid, induces long, narrow, chlorotic streaks in developing leaves (Fouché et al., 1984). These two aphids induce an increased amino acid concentration and a much higher proportion of essential amino acids. The induced changes in phloem appear to be systemic, affecting at least the whole leaf the aphids are feeding on. However, *Rhopalosiphum padi* L., the bird cherry-oat aphid, does not induce any visible macroscopic changes in its host plant and seems to have little effect on the amino acid content of phloem host (Sandstrom et al., 2000).

The effects on plants is very rapid in the case of *S. graminum* feeding on susceptible *Triticum aestivum* L. (wheat) cultivars; ultrastructural studies have shown severe degenerations in vascular cells of wheat plants after 1 h of infestation (severe organelle degeneration occurred rapidly in phloem parenchyma cells) (Al-Mousawi et al., 1983; Morgham et al., 1994). Two days after infestation, chloroplasts in mesophyll cells are also affected, in which an increased size and number

of plastoglobuli occurred (attributed to damage and loss of thylakoid membranes) (Jutte and Durbin, 1979). After 3-4 days of infestation, macroscopic colour changes appear (Sandstrom et al., 2000). In addition, after 1-4 days after infestation, an increased amount of rough endoplasmic reticulum indicated active protein synthesis. This suggests that damaged cells produce new metabolites used in cellular autolysis during the later stage of cell destruction (Morgham et al., 1994). Also the level of cytoplasmic polyribosomes and the rate of protein synthesis increases in senescent leaves (EiLamy et al., 1971; Brady and Tung, 1975).

Dorschner et al. (1987) found a clone of *S. graminum* that was unable to induce chlorotic lesions on a wheat cultivar. Therefore, aphids performed better on plants previously infested by another lesions inducing *S. graminum* clone. For greenbugs, the modification of the host plant's metabolism through the induction of senescence-like symptoms may improve the quality of the susceptible plant as a food source (Dorschner et al., 1987). The inability of greenbugs to modify the metabolism of resistant plants may explain why greenbugs performed poorly on them (Sumner et al., 1986).

A common hypothesis is that, on susceptible plants, aphids induce senescence-like changes to increase translocation and breakdown of leaf proteins (Dorschner et al., 1987). The drastic increase of glutamine in phloem exudates supports this hypothesis (Sandstrom et al., 2000). Glutamine is considered the major form of nitrogen translocation from senescent leaves to sink organs in rice and other plants (Kamachi et al., 1992; Watanabe et al., 1997).

All together, the foregoing indicates that, in some plant-aphid combinations, induced senescence is related to plant acceptance by the aphid although little is known about the genetic basis of susceptibility differences to aphids between mature and senescent leaves.

In this chapter, we specifically address the differences in gene expression between young and mature leaves of Kardal in response to *M. persicae* probing. In addition, we investigated whether aphid probing induced any direct resistance by studying aphid settling behaviour.

Finally, the differences observed in gene expression are compared to differences found in *S. stoloniferum* after the attack by *M. persicae* and *Macrosiphum euphorbiae* (Thomas) (Chapter 4), to elucidate common principles.

Materials and Methods

Plants and aphids

Solanum tuberosum L. cultivar Kardal was selected for this study because of its high level of resistance to *M. persicae* in apical leaves. This resistance decreases in mature and senescent leaves (Chapter 2; Alvarez et al., 2006).

The plants were maintained and propagated *in vitro* on Murashige and Skoog medium including vitamins, sucrose 3%, pH 5.8. After two weeks in agar the plantlets with developed roots were transferred to soil in 22 cm diameter pots in a glasshouse at 22 ± 2 ºC, RH about 70%, and a photoperiod of L16:D8.

Aphids used in the experiments came from newly established clones from a single virginoparous apterous individual taken from a *M. persicae* colony maintained at the Laboratory of Entomology, Wageningen University. Aphids were reared on radish, *Raphanus sativus* L., in cages in a climate chamber at 22 ± 2ºC, RH 30–40%, and L16:D8 photoperiod. New synchronous colonies were started weekly from which newly moulted adults were used to perform infestations.

Aphid preinfestation

To study the presence of induced resistance in Kardal to *M. persicae*, we preinfested 45 to 55 days old plants with aphids and then performed a settling test (see 'aphid settling behaviour test' below) and a transcriptomic analysis. Preinfestion went on for 96 h in a glasshouse at 22 ± 2ºC, RH about 70%, and L16:D8 photoperiod. Preinfestation was performed either on young or on old leaves. The young leaves used were 2nd and 3rd leaves from top while mature-old leaves were 7th to 9th leaves from top. Two young or two old leaves per plant were preinfested with 40 young adult apterous aphids per leaf. Aphids were confined to the leaves by enveloping each leaf individually with non-woven 'agrotextile' bags and plants were placed on individual trays. Leaves of control plants were also enveloped with cloth bags without adding aphids. Plants were assigned to 4 treatments (**Figure 2**): A (young leaves infested), B (control young leaves non-infested), C (old leaves infested), and D (control old leaves non-infested). For each treatment, plants were arranged in the greenhouse in a randomized complete design. After 96 hours, aphids and nymphs were counted and removed carefully by brushing with a soft brush. Subsequently, 8 plants per treatment were used for the aphid settling test. For each treatment, complete leaves from 4 plants were taken for analysis of

gene-expression in young and old leaves. RNA was isolated in leaves from 3 plants (biological replicates) while the leaves from the fourth plant were kept as a back up.

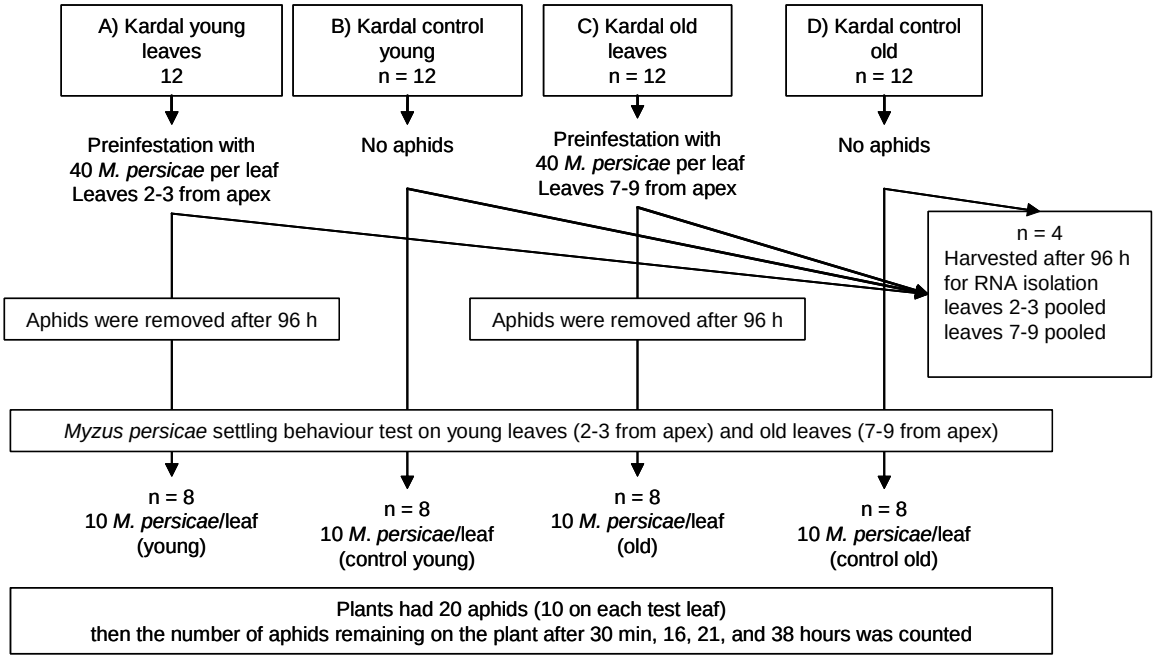

Figure 2. Experimental setup. A, B, C and D are the treatment groups (n, number of plants). In group A two young leaves, and group C two old leaves were pre-infested with 40 *M. persicae* for 96 h. The groups B and D (controls) were kept without aphids. After the pre-infestation of 96 h on each group 4 plants from each group were used for cDNA microarray analysis. The aphid settling behaviour test was performed with 8 plants per treatment (see Materials and methods for details).

Aphid settling behaviour test

To measure induced antixenosis to *M. persicae* in old and young leaves after preinfestation, we modified a test developed by Martin and Fereres (2003) referred to as 'settling test'. To observe treatment effects, 20 aphids were put on plants (time 0), 10 on each of the two test leaves. In order to avoid aphids moving to untreated leaves of the plants, the latter were entirely enveloped with cloth bags, while only the test leaves remained accessible to the aphids. To avoid aphid migration from one plant to another, the potted plants were individually placed on a tray with water. Aphids moving away from their plant either drowned in the water or kept on walking around. Plants had 20 aphids (10 per leaf) at time 0. Subsequently, the numbers of aphids remaining on the plants were counted after 30 minutes, 16, 21, and 38 hours. Seven or 8 replicates were performed for each treatment.

Means and standard error of means (SEM) of the numbers of aphids per plant were calculated for all treatments. A Kruskall-Wallis test, and multiple comparisons with Bonferroni correction (Weisstein, 1999) were used (SPSS 12.0.1 for Windows) to analyze the data on aphid settling behaviour.

cDNA micro-array

The sample preparation for cDNA microarray analysis and the microarray hybridizations procedure were described in Chapter 4. The arrays used contained a collection of 3564 *S. tuberosum* cDNA clones (provided by Edwin van der Vossen). The clones on the array corresponded to genes known to be involved in plant defence responses, selected from the TIGR database using the criterion of sequence homology. Arrays were spotted with DMSO 50% spotting buffer in ultragap slides (Service XS, Leiden). Each slide has three blocks (technical replicates) on the array. To analyze differential gene expression infested plants were combined with non-infested control plants, generating 2 combinations: (1) *M. persicae* pre-infested young leaves vs. the non-infested control leaves (at the same 2-3 leaf position), and (2) *M. persicae* pre-infested old leaves vs. non-infested control leaves (at the same 7-9 leaf position). Three slides were hybridized for each treatment using cDNA of different plants each time (3 biological replicates).

Micro-array data analysis

Signal and background fluorescence intensities of the arrays were analyzed using the ScanArray Express program (Perkin-Elmer) version 2.22. Arrays were checked manually to exclude from the analysis anomalous spots with high background. Spots with fluorescence intensities lower than half the background were raised to half the background to avoid extreme expression ratios; when both dyes had intensities lower than half the background, they were excluded. Data were converted by Express Converter ver 1.5, then log 2 ratios of Cy5/Cy3 were calculated and normalized to avoid spatial bias within each slide using Locfit (Lowess) normalization method by the TIGR-MIDAS-Microarrays Data Analysis System, Version 2.19. TIGR-MEV version 3.0.3 was used to perform t-test statistical analysis of the log 2 ratios; genes with expression ratio two folds higher (log 2 ratios ≥ 1) were consider up-regulated, and genes with expression ratio two fold lower (log 2 ratios ≤ -1) were considered down-regulated, when statistically different from 0 (P value ≤ 0.05). We also included some genes clearly up-regulated in all three biological replicates with an expression log 2 ratio ≥ 1 but with P value between 0.05 and 0.1 due to a high variability between replicates. The criterion to be included was that at least 2 out of 3 technical replicates on each slide showed

differential regulation. The absolute number of differentially expressed genes on young and old Kardal leaves was compared by chi-square test.

Results

M. persicae settling behaviour on *S. tuberosum* cv. Kardal

Pre-infestation by aphids had no effect on the number of settling aphids on the test leaves of Kardal. Control leaves, not previously infested, showed similar numbers at any time after time 0 on both young and old leaves (**Table 1**). However, young leaves as such, differed from old leaves. After 21 hours, there were significantly fewer aphids remaining on young leaves, control and preinfested than on old leaves, control and preinfested. Finally, after 38 h, only 10 and 23% of the initial aphids were found on the young control and test leaves respectively, compared to 52 and 56% on the old control and test old leaves, respectively (**Table 1**).

Table 1. Settling behaviour of *M. persicae* on *S. tuberosum* cv. Kardal on pre-infested and non pre-infested young and old leaves. Values are mean ± SEM of number of aphids remaining on the plant at different times

Time	Young leaves[1]		Old leaves	
	Control n = 8	Pre-infested n = 8	Control n = 7	Pre-infested n = 8
0	20	20	20	20
30 min	11.1 ± 1.5a	10.3 ± 1.7a	12.0 ± 0.8a	13.7 ± 0.9a
16 h	10.3 ± 2.0a	8.3 ± 1.3a	13.0 ± 1.1a	16.0 ± 1.8a
21 h	6.4 ± 0.6a	7.0 ± 1.0a	12.0 ± 0.7b	13.1 ± 1.3b
38 h	2.0 ± 0.5a	4.5 ± 1.3ab	10.4 ± 1.7bc	11.1 ± 1.1c

[1] young leaves, 2 to 3 leaves from top; old leaves, 7 to 9 leaves from top; numbers in a row followed by different letters are significantly different at $P < 0.008$ according to Kruskal-Wallis test followed by multiple comparisons with Bonferroni correction

Gene expression in response to aphid feeding

We compared the transcriptional responses of Kardal plants to *M. persicae* infestation at two developmental stages of the leaf, i.e., young and old leaves. In old leaves, *M. persicae* infestation elicited a stronger response than in young leaves, 99 genes (62 up- and 37 down-regulated) vs. 56 genes (48 up- and 5 down-regulated) were differentially expressed in old and young leaves respectively (χ^2 value = 11.6, d.f. = 1, P = 0.0007) (**Figure 3**). The proportion of commonly regulated genes between old and young leaves was 67% for up-regulated and 16% down-regulated genes. The number of genes up- or down-regulated was also differently distributed between old and

young leaves. The proportion of down/up regulated genes was 5.6 folds higher in old leaves (37/62 = 0.60) than in young leaves (7/49 = 0.14).

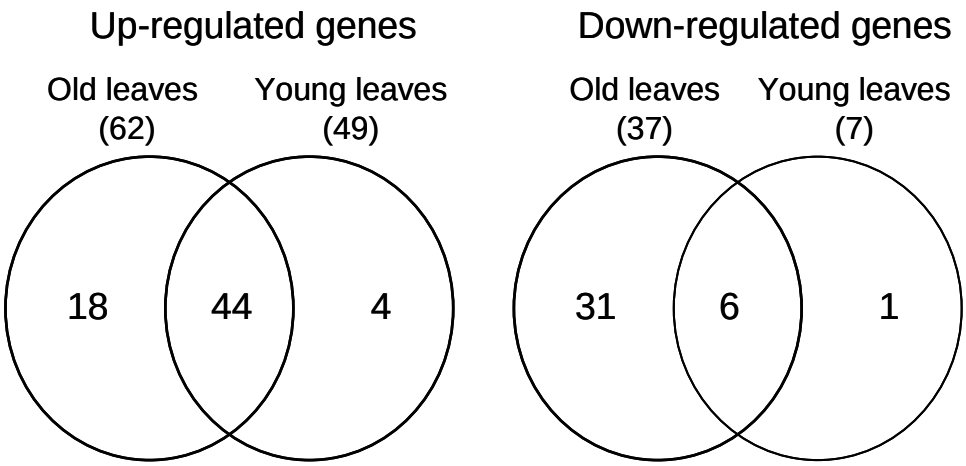

Figure 3. Number of genes differentially expressed in young and old leaves of *S. tuberosum* cv. Kardal plants infested with *M. persicae*

The differentially regulated genes are listed in **Table 2**. Genes have been assigned to functional groups based on their TIGR annotation. This grouping confers a potential function to each encoded protein based on similarity to known proteins, but does not indicate a confirmed function for most of the genes (**Figure 4**).

Young apical leaves of cultivar Kardal are resistant to *M. persicae* (incompatible interaction) and the mature senescent leaves are susceptible (compatible interaction). In Chapter 4 we performed transcriptome analysis of *S. stoloniferum* after the attack of *M. persicae* (incompatible interaction) and *M. euphorbiae* (compatible interaction). In order to identify genes that are commonly regulated in both systems, we analysed all gene expression data together and classified the differentially expressed genes in groups (**Figure 5**).

In cultivar Kardal, like in *S. stoloniferum*, we find a common response in genes that were up-regulated, which were primarily in the functional categories: pathogenesis related (PR) and regulatory, followed by protein metabolism, and transport (**Figure 4**, and see Figure 6 in Chapter 4). The response in genes down-regulated was more specific to the host plant (see column sto in **Table 2**). In Kardal, most of the down-regulated genes were found in old leaves. These were evenly

distributed across all functional categories (**Figure 4**). The number of down regulated genes in young leaves was scarce (**Figure 5**).

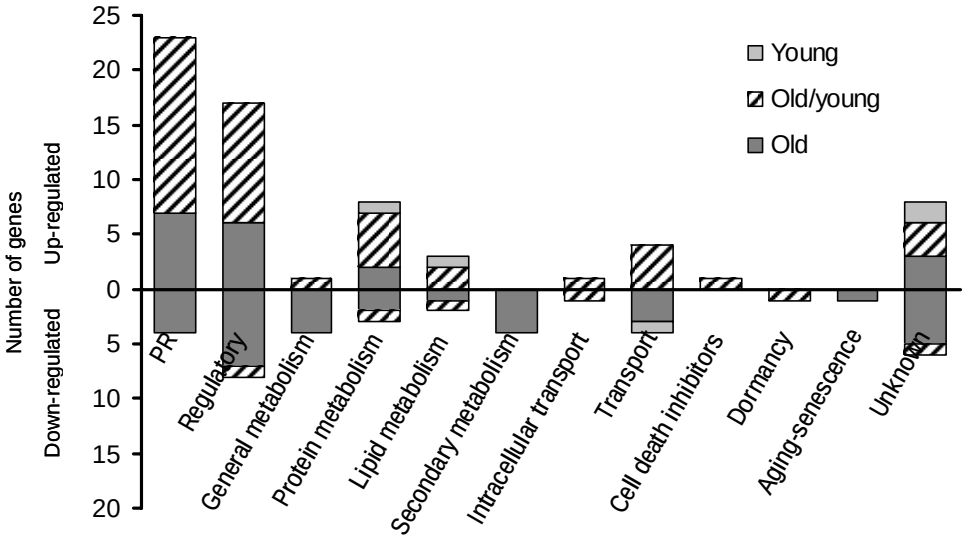

Figure 4. Number of genes expressed in *Solanum tuberosum* cv. Kardal after *Myzus persicae* attack across functional categories. Old, means genes only differentially regulated in old Kardal leaves; Old/young, means genes differentially regulated in old and young Kardal leaves; Young means, genes only differentially regulated in Kardal young leaves. Genes have been assigned to functional groups based on their TIGR annotation

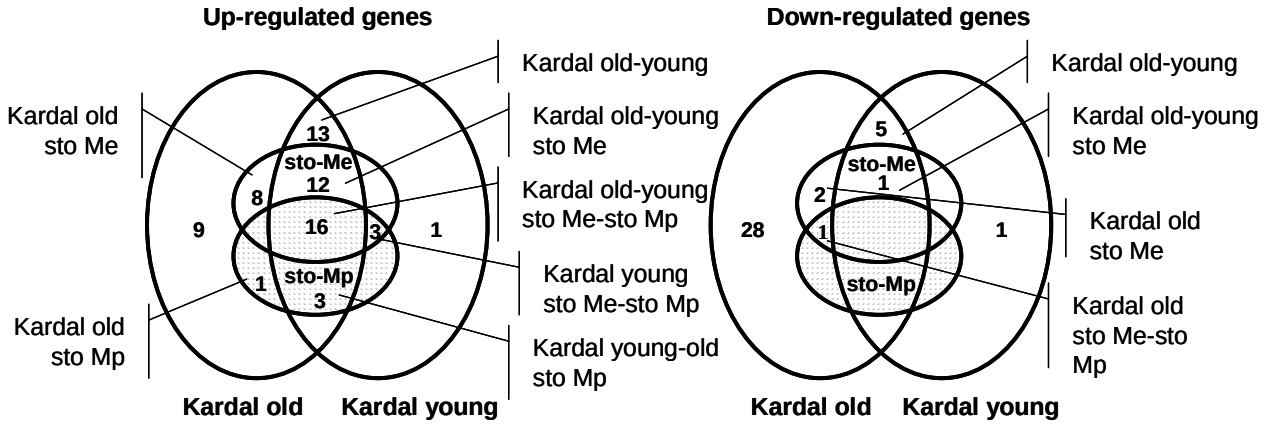

Figure 5. Number of genes differentially regulated in old leaves of *S. tuberosum* cv. Kardal (Kardal old) and young leaves (Kardal young) after *M. persicae* attack that were also differentially regulated in *S stoloniferum* after *M. persicae* (sto Mp) or *M. euphorbiae* (sto Me) attack. Genes that were exclusively regulated in *S. stoloniferum* are not shown (see Chapter 4).

Table 2. List of differentially expressed genes in young and old leaves of *S. tuberosum* cv. Kardal after infestation for 96 h with *Myzus persicae*.

Functional category	Clone name	Gene annotation (putative function), degree of homology with other spp (%)	Fold change[1]		Sto[2]	Process category	Response to[3]
Up-regulated genes in old and young leaves			old	young			
PR	PPCCF17	PR protein STH-21, *Solanum tuberosum* (100%)	3.2	3.6	Me-Mp	Defence related	P/W
PR	PPCBF82	PR-1 protein isoform b1 precursor, *S. tuberosum*, *S. lycopersicum* (100%)	3.8	3.8	Me-Mp	Defence related	P/SA
PR	PPCCP54	PR-2 protein P2 precursor, *S. lycopersicum* (100%)	3.9	4.8	Me-Mp	Defence related	P
PR	PPCBP46	PR-2 protein 1,3-beta-glucanase precursor, *S. tuberosum* (37%)	4.2	3.0	Me-Mp	Defence/antifungal	P/SA
PR	PPCAT19	Endo-1,3-beta-glucosidase, *Nicotiana tabacum* (85%)	2.7	2.5	Me-Mp	Defence/antifungal	P/SA
PR	PPCAC06	Class II (acidic) chitinase, *S. tuberosum* (100%)	2.7	2.3	Me-Mp	Defence/antifungal	P/SA
PR	PPCAT70	PR protein R major form (Thaumatin-like protein E22), *N. tabacum* (100%)	4.6	5.0	Me-Mp	Defence/antifungal	P/ET
PR	cSTB33M4	hsr203J (cell death related protein), *N. tabacum* (100%)	2.7	2.2	Me	Defence/cell death	P/V
PR	BPLI4D2	Class IV chitinase, *A. thaliana* (91%)	2.5	2.3	Mp	Defence/antifungal	P
PR	PPCBN57	PR-1 protein (Prb-1b), *N. tabacum* (94%); Basic PR-1, *C. annum* (91%)	3.4	2.6		Defence related	P/AS/ET
PR	PPCAI46	Endochitinase (Chitinase), *S. tuberosum*, *S. lycopersicum* (100%)	2.4	2.0		Defence/antifungal	P
PR	STMEY20	Endochitinase (Chitinase), *S. tuberosum*, *S. lycopersicum* (100%)	3.1	2.0		Defence/antifungal	P
PR	POAEE44	PR-5 protein, *S. lycopersicum* (92%); Osmotin-like protein, *N. tabacum* (95%)	3.2	3.1		Defence/antifungal	P/AS
PR	PPCAC11	Osmotin-like protein OSML13 (PA13), *S. commersonii*, *C. annum* (100%)	2.7	3.4		Defence/antifungal	P/AS/W/ABA/SA
PR	POABE75	Osmotin-like protein OSML15 (PA15), *S. commersonii*, *C. annum* (100%)	2.9	3.2		Defence/antifungal	P/AS/W/ABA/SA
PR	POADE25	Osmotin-like protein OSML81 (PA81), *S. commersonii*, *C. annum* (100%)	2.8	3.4		Defence/antifungal	P/AS/W/ABA/SA
Regulatory	PPCBM08	WRKY-DNA-binding protein 4, *N. tabacum* (66%)	2.2	3.1	Me-Mp	Regulation of transcription	P/SA
Regulatory	PPCAI63	LRR protein (CALRR1), *Capsicum annum* (100%)	3.1	2.6	Me-Mp	Regulation	P/AS/W/ABA
Regulatory	PPCCM26	Auxin-induced/SAUR-like protein, *C. annum* (100%), *A. thaliana* (76%)	4.6	2.6	Me-Mp	Signalling	P
Regulatory	BPLI1G3	Protein kinase protein, *Arabidopsis thaliana* (38%)	2.6	2.9	Me-Mp	Signalling	
Regulatory	PPCBG74	VQ motif putative, *Oryza sativa* (17%)	2.1	2.6	Me-Mp	Regulatory	
Regulatory	BPLI1H2	NPR1-interactor protein 1, *S. lycopersicum* (94%)	2.1	2.1	Me	Regulation of transcription	SA
Regulatory	POAE058	AP2 domain-containing transcription factor TINY, *A. thaliana* (6%)	2.8	2.2	Me	Regulation of transcription	AS
Regulatory	STMJF47	S-receptor kinase (SRK) precursor, *A. thaliana* (31%)	3.4	3.4	Me	Signalling	
Regulatory	PPCCL54	Nucleic acid binding protein, *A. thaliana* (47%)	3.2	4.2	Mp	Regulation of transcription	
Regulatory	PPCBH33	Zinc knuckle (CCHC-type) family protein, *A. thaliana* (9%)	2.6	2.2		Regulation of transcription	
Regulatory	PPCCI93	Lectin (probable mannose binding) protein kinase, *A. thaliana* (27%)	2.8	2.3		Signalling	
General metabolism	STMDF49	AAA-type ATPase family protein, *A. thaliana* (48%)	3.6	2.5	Me	Variety of cellular process	

Table 2 (Continued)

Functional category	Clone name	Gene annotation (putative function), degree of homology with other spp (%)	Fold change[1]		Sto[2]	Process category	Response to[3]
Up-regulated genes in old and young leaves (continued)			old	young			
Protein metabolism	PPCBJ33	isoleucyl-tRNA synthetase, *A. thaliana* (5%)	3.8	4.3	Me-Mp	Protein translation	
Protein metabolism	cSTS18G22	Chloroplast nucleoid DNA binding protein 41 kD (CND41), *N. tabacum* (92%)	3.4	2.5	Me	Rubisco catabolism	senescence
Protein metabolism	PPCAH09	PR subtilisin-serine-like protease (P69B), *S. lycopersicum* (46%)	5.1	3.0	Me	Protein catabolism	P/ET/SA
Protein metabolism	BPLI3K16	Multifunctional aminoacyl-tRNA ligase-like protein, *A. thaliana* (92%)	2.0	2.0		Protein translation	
Protein metabolism	POABC66	Ring finger protein, *Cicer arietinum* (65%)	2.0	2.0		Ubiquitin-protein catabolism	
Lipid metabolism	PPCAS26	Non-specific lipid transfer protein, *C. annum* (90%)	2.5	3.1	Me-Mp	Lipids transport	
Lipid metabolism	PPCAH78	Xylogen protein 1/non-specific lipid transfer protein, *A. thaliana* (55%)	3.8	4.0	Me-Mp	Cell differentiation	
Intracellular transport	POCBT23	Coatomer beta' subunit (Beta'-coat protein), *A. thaliana* (28%)	2.4	2.4	Mp	Intracellular protein transport	
Transport	PPCBL51	Amino acid transport protein AAT1, *A. thaliana* (65%)	2.3	2.2	Me	Amino acid transport	
Transport	PPCAI68	Hexose transporter, *S. lycopersicum* (39%)	4.7	4.2	Me	Carbohydrate transport	
Transport	POCBT09	Hexose transporter, *S. lycopersicum* (83%)	2.7	2.3	Me	Carbohydrate transport	
Transport	PPCCF11	NtEIG-A1 protein, *N. tabacum* (76%)	4.3	3.7		Electron transport	P
Cell death inhibitors	PPCBL95	MLO-like protein 12 (AtMlo12) (AtMlo18), *A. thaliana* (22%)	2.3	2.3		Cell death inhibitors	
Unknown	STMJC14	Unknown protein	3.1	3.8	Me-Mp	Unknown	
Unknown	PPCBI30	Unknown protein, *N. tabacum* (60%)	2.6	2.2	Me	Unknown	V/SA
Unknown	PPCAR90	Unknown protein	2.2	2.0	Me	Unknown	

Table 2 (Continued)

Functional category	Clone name	Gene annotation (putative function), degree of homology with other spp (%)	Fold change[1]		Sto[2]	Process category	Response to[3]
Up-regulated genes in old leaves							
PR	STMHZ79	Disease resistance-responsive protein, dirigent protein, *A. thaliana* (47%)	2.6	1.7	Me	Defence related	P
PR	BPLI4I8	Disease resistance protein Hcr2-5D (LRR), *S. lycopersicum* (21%)	3.7	2.2	Me	Signalling	P
PR	PPCAQ66	Putative pathogenesis related protein, *Oryza sativa* (43%)	3.8	2.7		Defence related	P
PR	STMJD51	Disease resistance protein Hcr9-9E, *S. lycopersicum* (12%)	2.6	1.3		Defence related	P
PR	BPLI7M2	Basic PR-1 protein precursor, *C.annum* (100%)	3.7	1.0		Defence related	P
PR	PPCAU07	Rx protein (LZ-NBS-LRR protein), *S.tuberosum* (96%)	2.3	1.9		Defence related	P/V
PR	PPCBB93	Endo-1,3-beta-glucosidase, basic isoform 2 precursor, *S. tuberosum* (100%)	2.7	1.7		Defence/antifungal	P/W/ET
Regulatory	PPCBD19	Putative receptor-like serine-threonine protein kinase, *S. tuberosum* (31%)	2.7	1.7	Me	Signalling	P
Regulatory	PPCBM14	Strubbelig receptor family 1 – LRR protein kinase, *A. thaliana* (35%)	2.7	1.3	Me	Signalling	
Regulatory	STMHQ95	Calmodulin-binding family protein, *A. thaliana* (39%)	2.2	1.5	Me	Signalling	
Regulatory	STMJE78	Silencing group B protein, *Zea mays* (100%)	2.9	1.9	Me	Regulation cell growth	
Regulatory	PPCBB86	Wall-associated kinase 1 (WAK1), *A. thaliana* (27%)	1.9	1.4	Mp	Signalling	P
Regulatory	PPCAQ85	Eukaryotic protein kinase domain, *A. thaliana* (30%)	2.8	1.5		Signalling	
Protein metabolism	PPCAR85	Branched-chain amino acid aminotransferase, *S. tuberosum* (38%)	2.1	1.4	Me	Amino acid metabolism	
Protein metabolism	PPCAW92	Ubiquitin-specific protease 12 (UBP12), *A. thaliana* (11%)	2.0	1.3	Me	Ubiquitin-protein catabolism	
Unknown	STMDK87	KED, rich in lysine (K), glutamic (E) and aspartic acid (D), *N. tabacum* (38%)	7.3	2.3		Unknown	W
Unknown	PPCBH37	Unknown protein	2.1	1.5		Unknown	
Unknown	STMJE25	Unknown protein	2.0	1.5		Unknown	

Table 2 (Continued)

Functional category	Clone name	Gene annotation (putative function), degree of homology with other spp (%)	Fold change[1] Old	young	Sto[2]	Process category	Response to[3]
Up-regulated genes in young leaves							
Protein metabolism	PPCCK15	Prot-methionine-S-oxidereductase (Fruit-ripening E4),*S. lycopersicum* (100%)	1.3	3.1		Protein catabolism	
Lipid metabolism	PPCAN32	Enoyl CoA hydratase, *A. thaliana* (93%)	1.8	2.3	Me-Mp	Fatty acid metabolism	
Unknown	PPCAC18	Unknown, *A. thaliana* (96%)	1.9	2.5	Me-Mp	Unknown	
Unknown	PPCBD84	Unknown	1.5	2.0	Me-Mp	Unknown	
Down-regulated genes in old and young leaves							
Regulatory	cSTE21I23	Ethylene-responsive element binding factor, *N. tabacum* (85%)	0.2	0.4		Regulation of transcription	AS/ET
Protein metabolism	cSTB43B10	SKP1-like protein- E3 ubiquitin ligase SFC complex, *N. benthamiana* (100%)	0.4	0.5		Protein catabolism	
Lipid metabolism	cSTD2E22	Patatin T5 precursor (Potato tuber protein), *S. tuberosum* (100%)	0.1	0.3		Nutrient reservoir	
Intracellular transport	cSTE17A9	Hypothetical protein, *Cicer arietinum* (72%), *Oryza sativa* (47%)	0.4	0.4		Intracellular protein transport	
Dormancy-related	cSTA43M16	Dormancy/auxin-repressed protein, *S. virginianum* (100%)	0.4	0.4	Me	Dormancy	
Unknown	PPCBB22	Unknown protein, *A. thaliana* (37%)	0.5	0.5		Unknown	
Down-regulated genes in old leaves							
PR	STMJG06	PR protein, *A. thaliana* (61%)	0.4	0.7		Defence related	P
PR	POCCL34	PR Thaumatin-like protein, *A. thaliana* (68%)	0.4	0.6		Defence/antifungal	P
PR	cSTE16K18	Major latex like protein homolog, *Beta vulgaris* (70%), *A. thaliana* (46%)	0.5	0.9		Unknown	P
PR	PPCAE52	Wound stimulated protein Sn-1 protein, *C. annum* (52%)	0.3	0.7		Defence related	P/W
Regulatory	PPCBN68	Protein phosphatase 2C putative (PP2C), *A. thaliana* (69%)	0.5	0.7	Me		
Regulatory	POACZ85	Speckle-type BTB/POZ, *A. thaliana* (62%)	0.4	1.0		Unknown	
Regulatory	cSTB43O7	MYB transcription factor, *A. thaliana* (49%)	0.4	0.6		Regulation of transcription	
Regulatory	POAB559	DaGAI-A (Giberrellic Acid-insensitive mutant protein), *S. lycopersicum* (97%)	0.5	0.7		Regulation of transcription	
Regulatory	cSTB36A4	CDC5 protein, *Z. mays* (57%)	0.3	0.8		Regulation of transcription	
Regulatory	cSTD18K14	Zinc finger protein 4, O. sat (29%); *A. thaliana* (21%)	0.5	1.0		Regulation of transcription	
Regulatory	cSTD17G10	Zinc-finger protein -Stress-associated protein-3 (SAP-3), *Oryza sativa* (73%)	0.4	0.6		Regulation of transcription	
General metabolism	BPLI3F3	Aldo/keto reductase, *A. thaliana* (87%)	0.4	0.6	Me	Metabolism	
General metabolism	cSTB24E4	Glycosyl transferase family 8 protein, *A. thaliana* (93%)	0.3	0.8		Carbohydrate biosynthesis	
General metabolism	cSTD1B23	Anthocyanidin 3-O-glucosyltransferase, *Petunia hybrida* (96%)	0.3	0.9		Metabolism	
Protein metabolism	PPCBZ61	Zinc finger (C3HC4-type RING finger), *A. thaliana* (56%)	0.4	0.5	Me-Mp	Protein ubiquitination	

Table 2 (Continued)

Functional category	Clone name	Gene annotation (putative function), degree of homology with other spp (%)	Fold change[1] Old	young	Sto[2]	Process category	Response to[3]
Down-regulated genes in old leaves (continued)							
Protein metabolism	cSTD3N22	Type I small heat shock protein 17.6 kD isoform, *S. lycopersicum* (100%)	0.4	0.9		Protein folding	
Lipid metabolism	cSTB47G10	Non-specific lipid-transfer protein 1 (LTP 1), *S. tuberosum* (94%)	0.5	0.7		Lipids transport	
Secondary metabolism	cSTD7D10	Chalcone-flavonone isomerase B, *Petunia hybrida* (95%)	0.2	0.6		Flavonoid biosynthesis	
Secondary metabolism	cSTB40I24	Naringenin-chalcone synthase 2, *S. tuberosum* (100%)	0.3	0.5		Flavonoid biosynthesis	
Secondary metabolism	cSTB25F9	Naringenin-chalcone synthase 1A, *S. tuberosum* (100%)	0.4	1.0		Flavonoid biosynthesis	
Secondary metabolism	cSTB45H11	Proteinase inhibitor type II TR8 precursor, *S. lycopersicum* (44%)	0.2	1.0		Secondary metabolism	
Transport	PPCAW72	Aquaporin 1, *N. tabacum* (100%); Tonoplast intrinsic protein, *Z. mays* (98%)	0.3	1.0		Water transport	
Transport	BPLI3P23	Proton-dependent oligopeptide transport (POT) protein, *A. thaliana* (11%)	0.4	0.6		Oligopeptides transport	
Transport	cSTB49A23	Copper chaperone -farnesylated protein ATFP6, *A. thaliana* (88%)	0.4	0.8		Metal-ion transport	
Aging/senescence	cSTB48I2	Ntdin-Senescence-associated protein DIN1, *N. tabacum* (83%)	0.3	0.6		Aging/senescence	
	cSTB11M8	CDH1-D gene for 18S rRNA, *S.tub* (100%), *N. tabacum* (100%)	0.3	0.7		No determined	
Unknown	PPCAD09	Unknown protein, *A. thaliana* (69%)	0.4	0.7		Unknown	
Unknown	cSTB6I18	Unknown protein, *Oryza sativa* (43%)	0.4	0.7		Unknown	
Unknown	cSTB1M4	Unknown protein, *Oryza sativa* (32%)	0.5	1.0		Unknown	
Unknown	PPCAY05	Unknown protein	0.4	0.5		Unknown	
Unknown	cSTB29G22	Unknown protein	0.3	0.6		Unknown	
Down-regulated genes in young leaves							
Transport	PPCBB35	Sodium sulphate or dicarboxylate transporter, *A. thaliana* (83%)	0.6	0.5		Transport/Cell maintenance	

[1] Values were calculated as relative transcript abundance (ratios of values for *M. persicae* infested plants/values for control plants). Underlined values indicate genes with expression ratio two folds higher (up-regulated), and genes with expression ratio two fold lower (down-regulated) than the control that are statistically different from 0 ($P \leq 0.05$). The grey boxes are genes clearly up-regulated in all 3 biological replicates with expression ratio two folds higher but with $0.05 < P < 0.1$ due to a high variability between replicates. The criterion to be included was that at least 2 out of 3 technical replicates in the slide showed differential regulation; old, Kardal old leaves; young, Kardal young leaves; [2] Sto, *S. stoloniferum* experiment (Chapter 4): Me-Mp, differentially expressed in Sto after *M. euphorbiae* (Me) and/or *M. persicae* (Mp) attack; [3] P, pathogens; W, wounding; AS, abiotic stress; SA, salicylic acid; ET, ethylene; JA, jasmonic acid; ABA, absicic acid; V, viruses.

Discussion

Aphid settling behaviour test

Aphid settling on preinfested and noninfested leaves of cv. Kardal was equal, we could not detect any extra resistance (repellence or deterrence) in addition to the constitutive resistance to *M. persicae* that has been described before (Chapter 2). Studies in probing behaviour report that, in *S. tuberosum*, preinfestation by aphids has beneficial effects on the probing behaviour of *M. persicae* (induced susceptibility) on local leaves, whereas at the systemic leaves (on the non-infested leaves of infested plants) the opposite detrimental effects for *M. persicae* occurs (induced resistance) (Dugravot et al., 2007). Prado and Tjallingii (1997) also reported induced plant susceptibility at local level for *Aphis fabae* on *Vicia faba* L. However, we did not test probing behaviour here and, therefore, it is difficult to compare these results. Probing behaviour and performance studies will be necessary to assess induced susceptibility in the system *M. persicae*-Kardal.

Gene expression in young and old leaves of cv. Kardal

We found 104 genes differentially regulated upon *M. persicae* attack, using the dedicated cDNA micro-array containing 3564 *S. tuberosum* mostly pathogen-responsive clones. Thus, only 3% of the genes in cv. Kardal responded to aphid infestation, which is similar to the percentage of genes responding in *S. stoloniferum* to *M. persicae* or *M. euphorbiae* attack (4%) (Chapter 4).

M. persicae attack to old Kardal leaves resulted in a larger number of differentially regulated genes than the attack to young leaves, probably due to a stress response triggered by the increased extraction of phloem sap from old leaves. Alternatively, this differential regulation of genes may facilitate the aphids feeding activities. In young leaves, plant stress might be limited because the lack of down-regulated genes probably prevents feeding. On the other hand, even minor changes in turgor pressure and electrical potential in plant tissue can stimulate defence signalling in plants (Yahraus et al., 1995).

The response in old and young leaves involves up-regulation of genes primarily in the functional categories: pathogenesis related (PR) and regulatory, followed by protein metabolism, and transport. Most of the down-regulated genes were from old leaves, and were evenly distributed across all functional categories. The number of down-regulated genes in young leaves was scarce (**Figure 4**).

Myzus persicae induced the expression of a number of PR genes. PR genes have been associated with aphid feeding in diverse plant-aphid interactions (De Vos et al., 2005; Moran and Thompson, 2001; Moran et al., 2002; Park et al., 2006; Zhu-Salzman et al., 2004) and most of these genes are SA responsive, validating that the SA signalling pathway is activated upon aphid attack.

In old leaves of cv. Kardal attacked by *M. persicae* we find 31 uniquely down-regulated genes (**Figure 3**, for a list of genes see **Table 2**). Among them there are homologues of genes related to secondary defence-compound biosynthesis, e.g. two *chalcone synthase*, one *chalcone isomerase* and one *proteinase inhibitor* (**Table 2**). Arabidopsis infested by *M. persicae* (compatible interaction) also represses *chalcone synthase* genes (Moran et al., 2002)

Interestingly, another transcript down-regulated in old leaves of cv. Kardal is homologous to an *aquaporin* gene. In drought-stressed *Nicotiana glauca* plants, the accumulation of transcripts homologous to *aquaporin* also diminished dramatically. This down-regulation of *aquaporin* gene expression may result in reduced membrane permeability and may encourage cellular water conservation during periods of dehydration stress (Smart et al., 2001), which is also a consequence of aphid feeding.

Comparing responses of *S. tuberosum* cv. Kardal and *S. stoloniferum*

The results obtained for cv. Kardal have similarities to the results obtained with *S. stoloniferum* upon *M. persicae* or *M. euphorbiae* attack (Chapter 4). The gene-expression studies provided indirect evidences that Kardal plants respond to aphid infestation by activating genes responsive to salicylic acid (SA) and ethylene (ET) transduction pathways. However, the attack of *M. persicae* in cv. Kardal does not lead to the up-regulation of any jasmonic acid (JA) responsive genes. The SA signalling transduction pathway seems to prevail in this case.

Up-regulated genes

There is a group of 16 genes that were up-regulated in both systems analyzed, Kardal after *M. persicae* and *S. stoloniferum* after either *M. persicae* or *M. euphorbiae* attack (**Figure 5**, see **Table 2** for list of genes). It is interesting to note that half of those genes are pathogenesis-related.

Like in both *S. stoloniferum*-aphid interactions (Chapter 4), we also found here up-regulated genes that could play a role in switching the tissue status from source to sink. One is the *xylogen protein 1/nonspecific lipid transfer protein* (*nsLTP*) gene, which encodes a proteoglycan-like factor involved in plant tissue development and differentiation; it mediates inductive cell-cell interactions to direct continuous vascular development (Motose et al., 2004). In potato, the expression of *nsLTP* increases just prior to active metabolic processes, e.g. tuberization, sprout development, dormancy breaking (Horvath et al., 2002), which are all processes involving source-sink relationships. Other transcript is a homologue of *auxin-induced SAUR-like protein*. Induction of the *SAUR-like protein* is also found after inoculation of pepper or tomato plants by the virulent bacterium *Xanthomonas campestris* pv. *vesicatoria*. It is most likely involved in the disease response (changes on plant metabolism) of susceptible tissue (Marois et al., 2002).

Myzus persicae attack of Kardal induced the expression of 12 genes that are also induced in *S. stoloniferum* after *M. euphorbiae* attack (both are compatible interactions), but those genes are not induced in *S. stoloniferum* after the attack of *M. persicae* (incompatible interaction) (**Figure 5**). Three transcripts are homologous to genes involved in molecule mobilization, i.e., two *hexose transporters*, and one *amino acid transport protein* (*AAT1*). Two other transcripts are homologous to genes involved in protein catabolism, one is *PR subtilisin-serine-like protease P69*, which is also activated in tomato plants upon *Pseudomonas syringae* infection, or treatment with SA or ET, (Jorda et al., 1999). The second transcript is homologous to the *chloroplast nucleoid DNA-binding protease 41 kD* (*CND41*). This protease is involved in ribulose biphosphate carboxylase oxygenase (rubisco) degradation and the translocation of nitrogen during senescence in tobacco (Kato et al., 2004). The *CND41 protease* is highly expressed in senescent leaves where chlorophyll loss is accompanied by the release and breakdown of rubisco (Wittenbach, 1979). Breakdown of the photosynthesis apparatus and subsequent mobilisation of the breakdown products could result in nutritious enrichment of the phloem sap, which is advantageous to the aphid, as suggested by Zhu-Salzman et al. (2004). In addition, in old leaves attacked by *M. persicae*, a transcript homologous to the *branched-chain amino acid aminotransferase* gene is up-regulated, which is related to amino acid production and N mobilization. This transcript is also up-regulated in leaf tissues undergoing rapid cell division (Campbell et al., 2001). Similarly, a *ubiquitin-specific protease 12* (*UBP12*), whose function is in protein catabolism related to the proteasome (Wolf and Hilt, 2004) is upregulated. The ubiquitin/proteasome pathway is involved in the degradation of regulated protein turnover (Hochstrasser, 1996). All the above genes could play a role on the acceptance of the plant

as a host, since they are all genes related to turnover and mobilisation of nutrients that can have beneficial effects on the aphid's diet.

Down-regulated genes

We did not find down-regulated genes in common between Kardal plants after infestation with *M. persicae* and in *S. stoloniferum* after infestation with either *M. persicae* or *M. euphorbiae.* Hence, down-regulated genes may be more specific to the plant-aphid combination than up-regulated genes.

Kardal young and old leaves infested with *Myzus persicae* down-regulate a transcript homologous to the *dormancy/auxin repressed protein*. We found the same in *S. stoloniferum* after *M. euphorbiae* attack (Chapter 4). Although the physiological function of the gene is unknown, it is highly expressed in non-growing buds of plants and down-regulated in growing ones. Growing tissues have a high sink strength (Herbers and Sonnewald, 1998), and the repression of this genes in leaves attacked by aphids could be related to the process of changing the physiological status of the tissue. We speculate that, in aphid-infested plants, this gene could be involved in changing the metabolic state of the plant tissue, namely from source to sink in the region of the leaf targeted by the aphids.

In *S. stoloniferum*, aphid attack resulted in down-regulation of a number of photosynthesis and photorespiration related genes (Chapter 4). In contrast, on Kardal, aphids did not down-regulate any of these genes, in relation to non-infested Kardal plants, at any leaf tissue level. It is likely that non-infested old leaves had already reduced the expression of these senescence-related genes as a result of the developmental stage the leaves were in. Hence, the gene expression ratio for infested vs. non-infested old leaves, which indicates relative transcript abundance, will be unchanged after aphid attack. We think that, in our Kardal system, the senescence on leaves contribute to the acceptance of the plant by the aphid. We do not known yet if *M. persicae* is able to induce senescence in mature leaves in Kardal, but we noticed that leaves should be entering to senescence stage to be successfully infested by *M. persicae.*

To find out the way induced genes affect aphid feeding, further studies on aphid probing behaviour and performance will be necessary. A transcriptomic analysis of non-infested old leaves relative to non-infested young leaves should be done next in order to evaluate whether *M. persicae* colonise only mature-senescent leaves in Kardal because it is unable to induce senescence in younger leaves.

This research contributes to the understanding of plant responses towards aphid attack by analyzing the differences in gene expression between susceptible and resistant plant tissues.

It is important to take into account that our results on the plant-aphid interaction were limited to one period, after 96 hours of preinfestation, and that the expression analysis included a limited set of genes. Therefore, our results provide only a partial representation of the complex plant-aphid interaction.

CHAPTER 6

Infection of potato plants with Potato leafroll virus changes attraction and feeding behaviour of *Myzus persicae*

Adriana Alvarez, Elisa Garzo, Martin Verbeek, Marcel Dicke, Ben Vosman, and Freddy Tjallingii

Abstract

Potato leafroll virus (PLRV; genus *Polerovirus*, family Luteoviridae) is a persistently transmitted circulative virus that depends on aphids for spreading. The primary vector of PLRV is the aphid *Myzus persicae* (Sulzer). *Solanum tuberosum* L. potato cultivar Kardal has a certain degree of resistance to *M. persicae*: young leaves appear resistant, whereas senescent leaves are susceptible. In this study we investigated whether PLRV-infection of potato plants affected aphid behaviour. We found that *M. persicae* responses to headspace volatiles emitted from PLRV-infected and non-infected potato plants appeared to depend on the age of the leaf. For young apical leaves no difference in aphid attraction was found between PLRV-infected and non-infected leaves; hardly any aphids were attracted. In contrast, for mature leaves headspace volatiles from virus-infected leaves attracted more aphids than non-infected leaves. We also studied the effects of PLRV infection on probing and feeding behaviour (plant penetration) of *M. persicae* using the electrical penetration graph (EPG) technique (DC system). Several differences were observed between plant penetration in PLRV-infected and non-infected plants, but only after infected plants showed visual symptoms of PLRV infection. The effects of PLRV-infection in plants on the behaviour of its vector are discussed related to the implications on virus transmission.

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Introduction

Potato leafroll virus (PLRV) (genus *Polerovirus*, family Luteoviridae) is a persistently transmitted circulative virus that depends on aphids for dispersal and transmission to host plants. PLRV is restricted to the phloem; hence aphids acquire PLRV during ingestion of phloem sap from infected plants and inoculate it during salivation into the phloem sieve elements of subsequent plants that the aphid feeds on. The green peach aphid *Myzus persicae* (Sulzer) is the most efficient vector of PLRV. In this triangular relationship, direct interactions occur between virus, host and vector. Moreover, virus infections can change hosts in such a way that interactions between host and vector are influenced. Vector activity and behaviour are important determinants of the rate and extent of epidemic virus development (Jeger et al., 1998, 2004).

Any change in the virus-infected plant attracting or benefiting the aphid vector will influence the probability of virus dispersal. In some plant-virus-aphid interactions the presence of virus negatively affects the performance of the vector. On wheat the presence of Barley yellow dwarf virus (BYDV) reduces the concentration of total amino acids in the phloem; moreover BYDV infection leads to a lower efficiency of phloem sap utilisation by the aphid *Sitobion avenae* (F.) (Fiebig et al., 2004). Benefits for the vector that favour virus transmissions have been described for different plant-pathogen-vector combinations. Belliure et al., (2005) showed that *Frankliniella occidentalis* (Pergande) benefits indirectly from Tomato spotted wilt virus, which it transmits, through effects of the virus on host-plant characteristics. They hypothesized that virus-infection has a negative effect on induced defences against the thrips vector. *Aphis gossypii* Glover transmits Zucchini yellow mosaic virus (ZYMV) to *Cucurbita pepo* L. and it lives longer and produces more offspring than on non-infected plants (Blua et al., 1994). BYDV, another persistent, circulatively-transmitted virus, benefits its vector *Sitobion avenae* (F.) by disrupting the development of a braconid parasitoid within the aphid vector (Christiansen-Weniger et al., 1998). BYDV-infection of wheat plants increases the attractiveness to the aphid *Rhopalosiphum padi* (L) as a result of producing more volatiles than non-infected plants (Jiménez-Martínez et al., 2004). Castle and Berger (1993) found that *M. persicae* performance increases in terms of growth rate, reproduction, and longevity on cultivated *Solanum tuberosum* L. plants when infected by PLRV (vector-borne virus) as compared to virus-free potato plants, or plants infected by Potato virus Y (PVY, stylet-borne virus, that can be briefly associated to aphids, but also mechanically transmitted) or Potato virus X (PVX, vector-independent mechanically-transmitted virus). Furthermore, more *M. persicae*

individuals settled on PLRV-infected leaves of *S. tuberosum* L. than on leaves of virus-free, PVY-infected, or PVX-infected leaves (Castle et al., 1998). Eigenbrode et al., (2002) reported that *Myzus persicae* preferred PLRV-infected to non-infected potato plants, PVY-infected or PVX-infected plants. In addition, they found an increased emission of several volatiles by PLRV-infected plants when compared to non-infected plants. These volatiles may act as attractants and arrestants of *M. persicae*. Furthermore, Srinivasan et al., (2006) found that the preference of *M. persicae* for PLRV-infected *Solanum* spp plants over non-infected plants relies primary on olfactory cues over visual cues. The role of plant volatiles in host recognition and settling behaviour by aphids has been reviewed by Pickett et al., (1992). Vargas et al., (2005) have recently shown that alate virginoparae of the tobacco-adapted subspecies *M. persicae nicotianae* recognised and chose their host plant more efficiently than the generalist *M. persicae s.s.*, on the basis of olfactory and visual cues and factors present at cuticular and sub-cuticular levels.

Apart from olfactory cues, aphids must insert their mouthparts into plant tissues (probing) in order to select a suitable host and find the phloem. Therefore, knowledge of mechanical or biochemical cues in host plants during probing by aphids is crucial. The electrical penetration graph (EPG) technique (Tjallingii, 1978, 1985, 1988) is a robust tool to study plant penetration by aphid stylets. EPG waveforms have been correlated with aphid activities as well as with tissue locations of the stylet tips (Tjallingii, 1978;1988, Kimmins and Tjallingii, 1985; Tjallingii and Hogen Esch, 1993). Strategies for plant virus control depend highly on understanding virus-plant-vector interactions. The partial plant resistance present in potato cultivar Kardal slows down aphid population growth on young plants and consequently, limits acquisition of PLRV and its spread. The resistance to aphids on Kardal is present in young apical leaves but declines in mature and senescent leaves (Chapter 2, Alvarez et al., 2006).

Here, we address whether the effects of PLRV changes the host plant in benefit of the aphid through improving probing and feeding behaviour in young apical leaves of cv Kardal that is partially resistant to the aphid. Also we were especially interested in changes regarding vector attraction.

Our specific aim was to study 1) the effects of PLRV infection of potato plants on vector attraction by using an olfactometer assay for apical and mature leaves, and 2) the impact of PLRV-infection on the resistance, as expressed in feeding behaviour by using the electrical penetration graph (EPG) technique. We hypothesized that the better performance of *M. persicae* previously found on PLRV-

infected potatoes plants is due to structural or chemical changes in the plant's tissues that enhance the probing and feeding behaviour.

Materials and Methods

Plants and aphids

Virus-free potato plants (*Solanum tuberosum* L. cultivar (cv.) Kardal) were propagated *in vitro* (Alvarez et al., 2006). After two weeks, the rooted plantlets were transferred to soil in a glasshouse at 22 ± 2 ºC, about 70% r.h, and L16:D8 h photoperiod. A new colony of *Myzus persicae* was established on radish, *Raphanus sativus* L, starting with a single virginoparous apterous aphid from a colony maintained at the Laboratory of Entomology, Wageningen University. The colony was reared in a climate chamber at 22 ± 2 ºC, 30-40% RH, and an L16:D8 photoperiod.

Virus infection

One week after transfer to soil, nine potato plants were exposed for 96 h to 15 viruliferous *M. persicae* nymphs (treatment) and nine potato plants were exposed for the same time to 15 virus-free nymphs (control). After 96 h aphids and nymphs were gently removed with a brush. Also, three *Physalis floridana* Rybd. plants – very susceptible to *M. persicae* and PLRV – were exposed for 96 h to 15 viruliferous nymphs as a positive control. Viruliferous aphids had been obtained by placing apterous adult aphids on PLRV-infected (isolate PLRV-Wageningen) *P. floridana* plants for 24 h. After adults were removed, newborn nymphs were allowed to feed for 24 h on the source plants before being used in the inoculation of the test plants.

Twenty-seven days after the inoculation with PLRV, infection was determined by enzyme-linked immunosorbent assay (ELISA) (van den Heuvel and Peters, 1989) using antisera from Prime Diagnostics, Wageningen. ELISA results were considered positive for PLRV infection when absorbance at 405 nm exceeded the mean absorbance of the non-infected control plants by 4 times the standard deviation or more.

PLRV infection symptoms were not visible 27 days after exposure of the plants to viruliferous nymphs. Seven out of nine potato plants were successfully inoculated and only these plants, positive by ELISA, were used for the EPGs recordings 27 days after virus-inoculation.

Static air two-chamber olfactometer test

The response of non-viruliferous *Myzus persicae* to headspace volatiles from PLRV-infected versus non-infected potato plants was tested at 65 days after infection when symptoms of PLRV infection were evident. The response was observed in a two-chamber olfactometer without airflow (**Figure 1**).

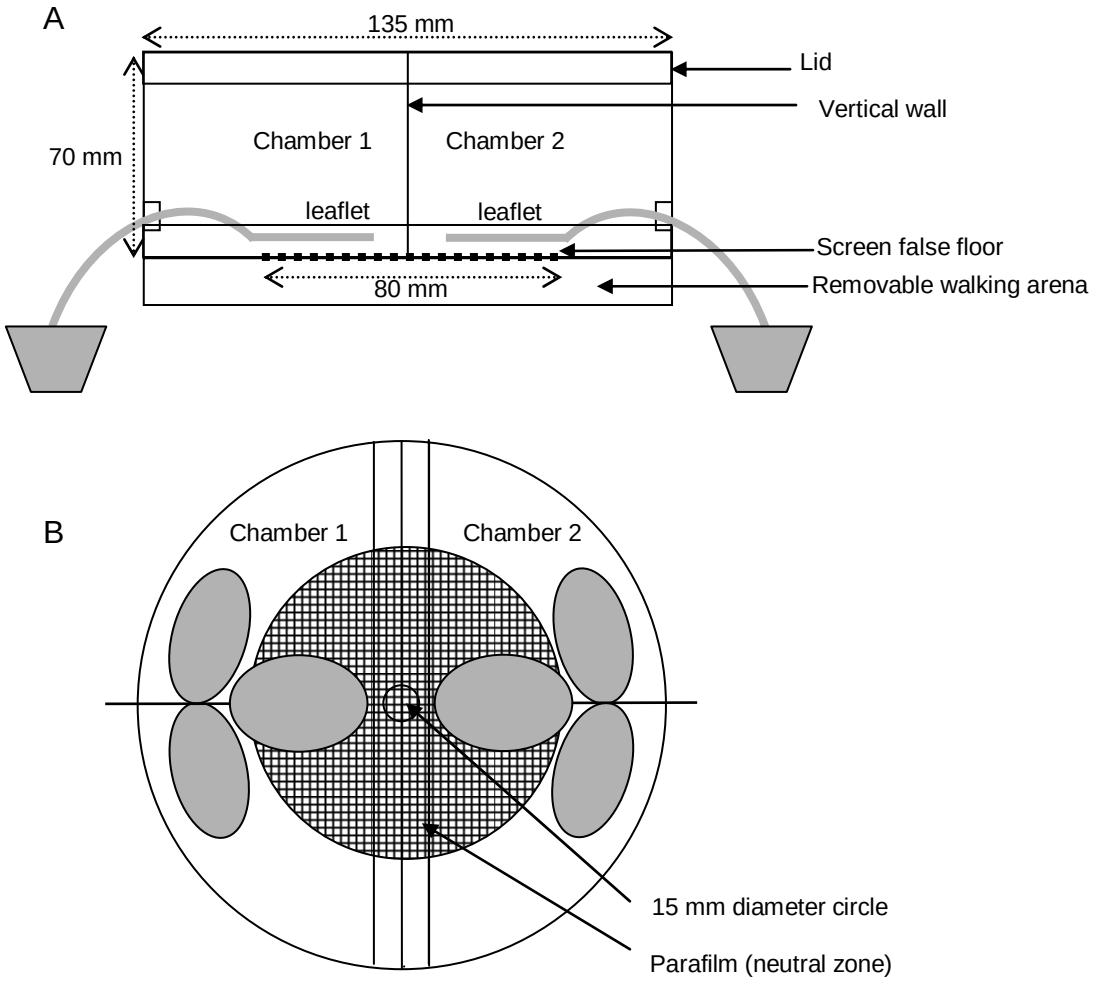

Figure 1. Static air two-chamber olfactometer (scheme not to scale). A, side view; B, top view. Forty aphids were released within the 15 mm diameter circle drawn on the centre of the removable walking arena (Petri dish beneath the screen false floor).The arena was used to close the olfactometer from below. The leaflets always remained attached to live plants (see Materials and methods for details).

This olfactometer was a modified version of the one-chamber olfactometer described by Eigenbrode et al. (2002). A plastic cylinder (diameter 135 mm) was divided into two chambers by a vertical plastic plate. The cylinder was closed at the bottom by a removable polystyrene Petri dish lid with a false floor. The false floor was made by opening a 80 mm diameter circle in the middle of the Petri

dish lid that was completely covered with polyethylene screen (200 µm mesh) (**Figure 1**); a line was drawn over the mesh indicating the position of the vertical wall in between the two chambers. To create a neutral zone between the chambers a 20 mm strip of Parafilm was put on the mesh over the line, just below the vertical wall between the two chambers. Two leaflets that were still attached to plants were placed above the false-floor Petri dish, one in each chamber (**Figure 1**). A strip of Parafilm was used to support the leaves in the correct position at a distance of ≈ 3 mm above the screen, to avoid leaflets from touching the screen, and then the top of the cylinder was closed with a Petri dish lid. The aphid walking arena was a second Petri dish in which a circle of 15 mm in diameter was drawn in the centre and where 40 apterous adult aphids were released at the start of each replicate. After releasing the aphids the Petri dish was immediately used to close the olfactometer from the bottom. To avoid visual cues, the trials were performed in a dark room. Aphids on the walking arena could move freely but could not touch the leaves so they would not be able to acquire any gustatory or contact cues.

The two-chamber olfactometer was used in three tests: 1) Non-infected mature leaflet vs. empty chamber, where one mature leaflet (5^{th} - 7^{th} leaf from the apex) of a non-infected control potato plant was enclosed in one chamber while the other chamber remained empty; 2) virus-infected apical leaflet vs. non-infected apical leaflet, leaflets of the 3^{rd} fully expanded leaves (from apex) were each enclosed in one of separated olfactometer chambers; 3) virus-infected mature leaflet vs. non-infected mature leaflet, leaflets of the 5^{th} – 7^{th} leaves (from apex) were each enclosed in an olfactometer chamber. Each test was repeated six times. The entire olfactometer was rotated 180 degrees after every recording and the plants were changed after two opposite recordings. In experiment 1) aphid positions were recorded only once, after 60 minutes.

In experiments 2) and 3) aphid positions were recorded at 10, 20, 30, 40, 50 and 60 min. During observation, the choice arena was illuminated from below by a red light for 30 seconds. Aphid contributed to scores if was present on the mesh directly below any leaflet part (except in the experiment comparing non-infected mature leaflet vs. empty chamber: any aphid present on the mesh of the empty chamber was counted as having chosen for the empty chamber). The total number of aphids showing a choice was counted and the mean number of aphids was calculated over time for each of the experiments.

EPG monitoring

The electrical penetration graph (EPG-DC system) technique was used to monitor plant penetration by young apterous adult aphids for 8 h (Alvarez et al., 2006) on PLRV-infected and non-infected plants at three different times: 1) on day 0, the same day of the inoculation with PLRV (8 days-old plants); 2) 27 days after PLRV inoculation (plant age, 35 days), when PLRV symptoms were not yet visibly expressed (called PLRV-27 and control-27 in **Table 1**); and 3) 65 days after PLRV inoculation (plant age, 73 days), when PLRV symptoms were evident on infected plants (called PLRV-65 and control-65 in **Table 1**).

Four plants of each treatment were placed in a Faraday cage and signals of two individual aphids per plant (8 aphids in total) were simultaneously monitored. Fifteen to twenty replicates (individual aphids) per treatment were obtained (n, **Table 1**). Aphids were placed on the abaxial side of the 3rd leaf from the apex, which was nearly fully expanded. EPGs were recorded at about 20 °C and constant light, immediately after aphid wiring.

EPG waveforms, waveform patterns and parameters

The recorded EPGs were analysed by distinguishing the following waveforms or waveform patterns: 1) Waveform C, stylet pathway phase; in fact waveform C includes 4 pooled pathway waveforms/activities that are not all well separated, i.e., waveform A, first stylet contact and epidermis penetration; waveform B, intercellular sheath salivation; waveform C, stylet movements (mainly); and waveform pd (potential drop), an intracellular stylet puncture. Waveform E, phloem phase, is separated into 2) waveform E1, sieve element salivation and 3) waveform E2, phloem sap ingestion with concurrent salivation; 4) waveform E1e, assumed to reflect extracellular watery salivation; 5) waveform F, derailed stylet mechanics (stylet penetration difficulties); and 6) waveform G, active drinking of water from xylem elements (Tjallingii, 1990).

Waveform features were characterised in a number of EPG parameters, divided here in four categories: (1) number of times waveforms occurred, (2), total (summed) duration of each waveform, (3) time to the first occurrence of waveforms, and (4) aphids with sustained phloem ingestion (sE2) on each plant treatment (**Table 1**).

Statistical analysis

The response of aphids to either of two odour sources at each time point (olfactometer assay) was scored by counting the numbers of aphids present directly underneath a leaflet or in the case of the empty chamber any aphid present on the mesh below the empty chamber. The binomial test was used to analyse the data after 60 minutes. Testing the null hypothesis of no preference (distribution is 50-50%) between the treatments (aphids not found below any leaflet were not taken into account in the statistical analysis).

Proportions of aphids preferring PLRV-infected leaves or control leaves (relative to the total number of aphids per treatment) over time were studied by regression analysis. Student's *t* analysis was used to test for linear relationship between % of aphids and time (regression coefficient) (SPSS 12.0.1 for Windows).

EPG parameters were calculated for individual aphids, then means and standard errors of the mean (SEM) over all replicates per treatment. Times to first E2 and to first sE2 since the first probe in the experiment were calculated only for those aphids that showed any E2 or sE2, respectively. The Mann-Whitney U-test was used to test for plant penetration differences between infected and control plants (SPSS 12.0.1 for Windows). Differences in number of aphids showing sustained phloem ingestion (sE2) in the 8 h EPG recordings were tested by Fisher's exact test (Preacher and Briggs, 2001). In order to assess the probing and feeding behaviour of *M. persicae* on Kardal leaves of the same developmental stage (3rd leaf from apex) but increasing plant age, we analyzed the EPG data of the three non-infected controls (control-0, -27, and -65) by Kruskall-Wallis followed by multiple comparisons with Bonferroni correction, hence the alpha value was lowered to account for the number of comparisons performed (Weisstein, 1999).

Results

Two-chamber olfactometer test

The results of the preference tests (**Figure 2**) show: (1) a preference of aphids for a non-infected mature leaflet to an empty chamber ($P < 0.001$); (2) no difference in attraction between apical infected and non-infected leaves observed after one hour ($P = 0.22$); (3) a preference of aphids for mature PLRV-infected leaves over non-infected mature leaves ($P < 0.001$).

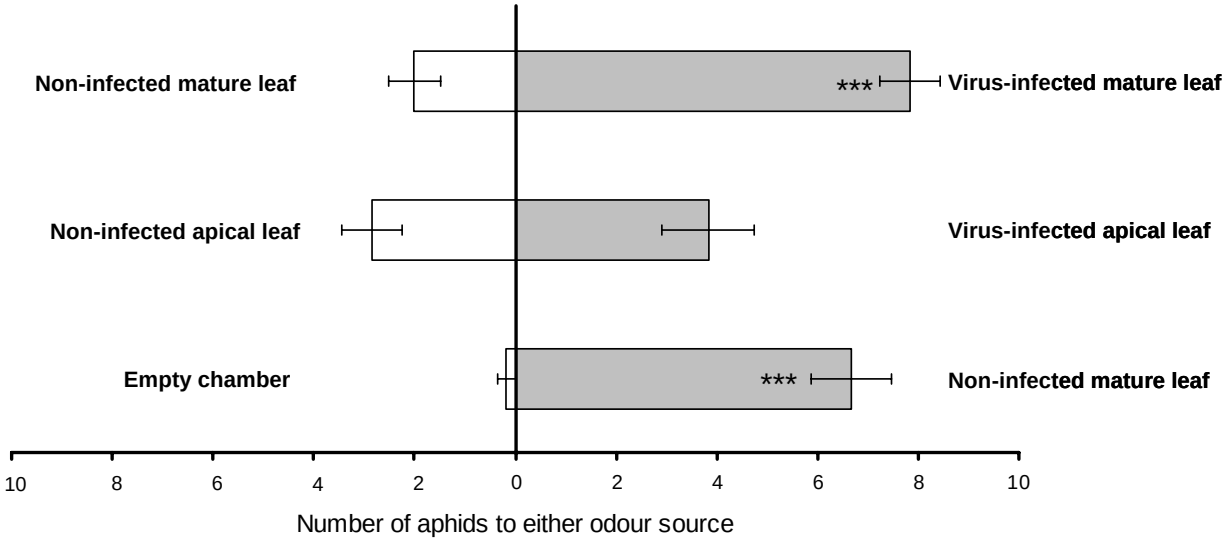

Figure 2. Choice test results of *Myzus persicae* to headspace volatiles in olfactometer assay: 1) non-infected mature leaflet vs. empty chamber (bottom bars), 2) virus-infected apical leaflet vs. non-infected apical leaflet (middle bars), and 3) virus-infected mature leaflet versus non-infected mature leaflet (upper bars) are compared. Only the number of aphids located directly below potato leaves was scored after 60 min. For each replicate test 40 aphids have been used. Error bars (SEM) are indicated next to each bar. *** = significant preferences within test ($P < 0.001$), binomial test.

There was a clear increase in the proportion of aphids preferring virus-infected mature leaves over time ($R = 0.968$, $t = 7.713$, $P = 0.002$) than the non-infected mature leaves ($R = 0.140$, $t = 0.808$, $P = 0.464$). On the contrary no such increase in proportion was found with virus-infected apical leaves ($R = 0.751$, $t = 2.276$, $P = 0.085$) or non-infected apical leaves ($R = 0.567$, $t = 2.287$, $P = 0.084$) (Figure 3).

EPG monitoring of plant penetration behaviour

Electrical penetration graph (EPG) parameters were divided into four categories show in **Table 1**. Twenty-seven days after infection of the plants with PLRV, the time to the 1st probe was the only parameter that differed significantly between aphids on infected plants not yet showing PLRV symptoms (PLRV-27) and aphids on non-infected control plants (Control-27) (Mann-Whitney U test: $P<0.05$). In contrast, we found several EPG parameters to differ at 65 days after infection between aphids on PLRV-infected plants with symptoms (PLRV-65) and non-infected control plants (control-65) (**Table 1**).

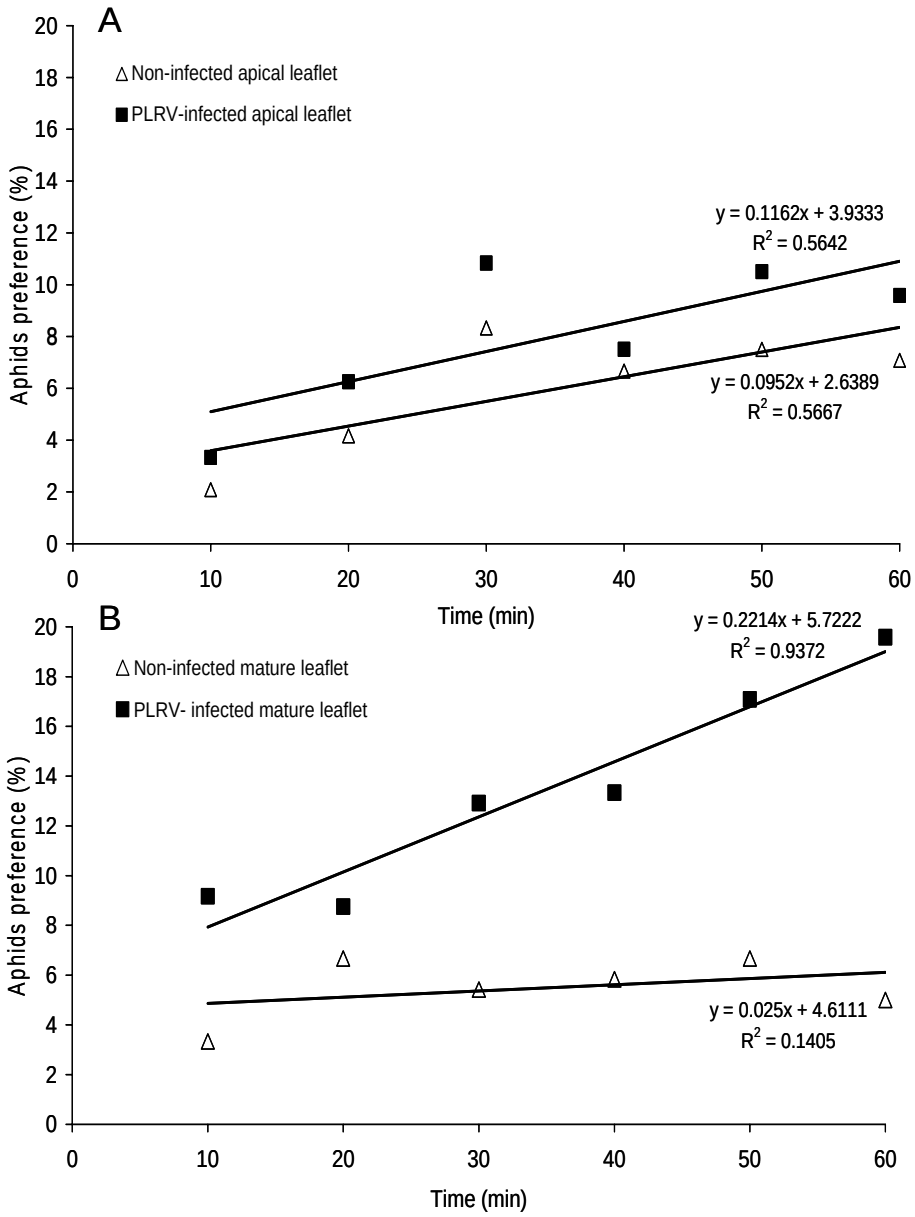

Figure 3. Choice test results of *Myzus persicae* to headspace volatiles from potato leaves of different age. Regression analysis was used to study the relationship between observation time and treatment of two separate experiments contrasting, A) PLRV-infected apical leaflets vs non-infected apical leaflets, and B) PLRV-infected mature vs non-infected mature leaflets. Numbers of aphids located directly below potato leaves were scored every 10 min for 1 h. For each replicate 40 aphids had been used. The percentages of aphids located either, below PLRV-infected leaflets or control leaflets for each treatment were calculated over the total number of aphids. Dots are means of 6 replicates. Regression lines are shown for each treatment. PLRV-apical leaflet, $t = 2.276$, $P = 0.085$, non-infected apical leaflet, $t = 2.287$, $P = 0.084$; PLRV-mature leaflet, $t = 7.713$, $P = 0.002$, non-infected mature leaflet, $t = 0.808$, $P = 0.464$.

EPG parameters showed fewer number of probes shorter than 3 minutes before the first phloem salivation activity E1, and lower number of F periods (waveform reflecting derailed stylet mechanics) in aphids on PLRV-65 plants than on control-65 plants; also aphids on PLRV-65 plants had shorter summed duration of F periods than on control-65 plants (Mann-Whitney U test: $P<0.05$)

(**Table 1**). But higher number of extra-cellular salivation (E1e) periods and sieve element salivation (E1) were shown on PLRV-65 plants than on control-65 plants (Mann-Whitney U test: $P<0.05$) (**Table 1**). Most of these E1e periods started as E1, i.e., the trans-membrane potentials of punctured sieve elements. Furthermore, aphids on control plants had decreased the total duration on E1 and the number of E1e with plant age (control-27 to control-65: total time on single E1, 37 ± 5 to 25 ± 8 min respectively; and number of E1e, 4 ± 0.5 to 1 ± 0.3 respectively; Mann-Whitney U test, $P<0.05$).

The start of F events within probes could give us information on the tissue depth at which the possibly causal factors/constraints are located. We ordered all the F periods starting times from the beginning of probes in two classes (irrespective aphid individuals) for each treatment, i.e. starting between 0 to 8 min, and later than 8 min (**Figure 4**).

The time to the first phloem activity (sieve element salivation E1) from the first probe was shorter on PLRV-65 than on control-65 plants (Mann-Whitney U test: $P<0.05$) (category 3, **Table 1**).

The number of aphids showing sustained phloem ingestion during the 8 h of recording period (n aphids sE2) on the PLRV-65 plants compared to non-infected control plants were not significantly different (number of aphids showing sE2: 6 out of 17 and 3 out of 15 aphids respectively; Fisher's exact test, $P = 0.287$).

We found no differences between EPG parameters on control-0 (8 days old plants) and control-27 (35 days old plants), but control-65 (73 days old plants) showed more waveform F (number of periods and total time), more total G (xylem drinking), and a longer time to the first phloem activity (in experiment) than control-0 leaves (Table 2; Mann-Whitney U test: $P<0.05$)

Table 1. EPG parameters (Mean ± SEM) for 8-hour monitoring of *Myzus persicae* on potato cultivar Kardal plants, 27 and 65 days after PLRV infection and on non-infected control plants of the same ages. The parameters are divided in 4 categories of data processing: number of waveform periods, total time, time to a certain event, and number of aphids with sustained phloem-sap ingestion.

Treatment[1]	Symptoms PLRV	Category 1: Number of waveform periods[3]										
		n[2]	Probes	Probes < 3min	C	E1e	single E1[4]	E1 frac.[4]	E2	sE2	F	G
Control-27	non-infected	20	59 ± 4	14 ± 2	70 ± 4	4 ± 0.5	9.6 ± 1.0	0.7 ± 0.2	1.1 ± 0.3	0.2 ± 0.1	1.0 ± 0.3	0.5 ± 0.1
PLRV-27	no symptoms	20	56 ± 3	13 ± 3	62 ± 5	5 ± 0.6	11.3 ± 1.3	0.3 ± 0.1	0.3 ± 0.1	0	1.4 ± 0.5	1.1 ± 0.2
Control-65	non-infected	15	42 ± 6	12 ± 2	52 ± 6	1 ± 0.3	6.7 ± 1.5	0.8± 0.2	1.3 ± 0.4	0.3 ± 0.2	2.7 ± 1.0	0.8 ± 0.2
PLRV-65	symptoms	17	41 ± 5	6 ± 1*	51 ± 5	3 ± 0.6*	9.4 ± 1.0*	1.5 ± 0.4	2.0 ± 0.6	0.6 ± 0.2	0.5 ± 0.2*	0.7 ± 0.2

		Category 2: Total time (minutes)								
		n	probing	C	E1e	single E1[4]	E1 frac.[4]	E2	F	G
Control-27	non-infected	20	297 ± 12	183 ± 12	2.5 ± 0.3	37 ± 5	12.9 ± 7.4	10 ± 8	35 ± 12	16 ± 5
PLRV-27	no symptoms	20	332 ± 11	203 ± 14	10.7 ± 4.8	39 ± 4	2.1 ± 1.3	1 ± 1	33 ± 11	26 ± 5
Control-65	non-infected	15	322 ± 20	174 ± 17	1.3 ± 0.7	25 ± 8	7.2 ± 2.7	28 ± 15	44 ± 13	45 ± 15
PLRV-65	symptoms	17	312 ± 21	186 ± 15	0.7± 0.2	26 ± 6	10.5 ± 4.2	62 ± 26	11 ± 5*	17 ± 7

		Category 3: Time (minutes) to event							Cat 4: sE2[6]	
		n	First probe from start of recording	First E1 in the exp. from first probe	First E1 from start of the probe	First E2 in exp. from first probe [5]		First sE2 in exp. from first probe [5]	n	%
Control-27	non-infected	20	2.9 ± 0.8	95 ± 19	9.0 ± 0.9	253 ± 51	(n = 9)	358 ± 23 (n = 3)	3	15
PLRV-27	no symptoms	20	0.9± 0.2*	104 ± 27	12.8 ± 1.2	397 ± 31	(n = 6)	- (n = 0)	0	0
Control-65	non-infected	15	5.0 ± 1.7	189 ± 35	13.1 ± 2.4	262 ± 56	(n = 8)	151 ± 109 (n = 3)	3	20
PLRV-65	symptoms	17	4.4 ± 1.7	61 ± 19*	10.0 ± 1.7	190 ± 32	(n = 11)	176 ± 57 (n = 6)	6	35

[1] Treatment: Control-27 and Control-65, non-infected plants 27and 65 days respectively after the exposure to non-viruliferous aphids; PLRV-27 and PLRV-65, infected plants 27 and 65 days, respectively after the exposure to viruliferous aphids; [2]n, number of replicates (aphids). [3]Category 1: probes < 3min: probes shorter than 3 minutes before first E1; C: pathway phase; E1e: salivation at extra cellular voltage level; E2: ingestion at intracellular voltage level; sE2: sustained ingestion at phloem level (E2 lasting > 10 min); F: derailed stylet mechanics; G: xylem ingestion; [4]salivation E1 periods split into single E1, i.e., without subsequent E2, and E1 fractions, i.e., embedded in periods with one or more subsequent E2 periods; E2 periods only occur in phloem phase together with E1.[5]First E2 in experiment from first probe and first sE2 in experiment from first probe were calculated only with the aphids (n) showing E2 and sE2 respectively (n is indicated next to each value between brackets). [6]Category 4: number and percentage of aphids with sustained E2. *difference statistically significant between control and PLRV infected plants of the same age, $P < 0.05$, Mann-Whitney U-test.

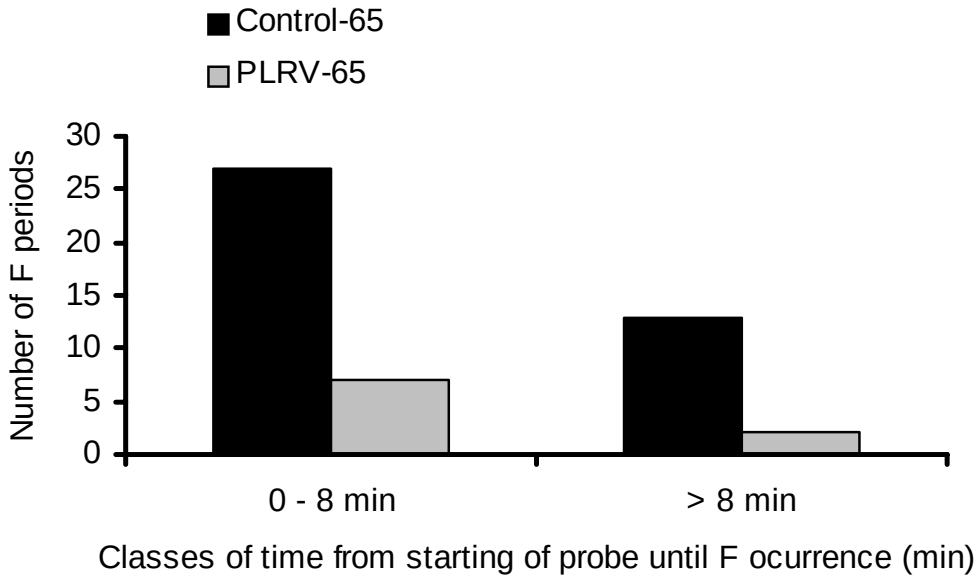

Figure 4. Number of derailed stylet mechanics (F waveform) per classes of time from the starting of the probe. The time until the first F on each probe across all aphids per treatment (control-65 and PLRV-65) were counted, then the F periods were sorted on two classes of time, 0 to 8 min, and > 8 min.

Table 2. EPG parameters (Mean ± SEM) differing with plant age for 8-hour monitoring of *Myzus persicae* on PLRV non-infected potato cv. Kardal plants

		Number of waveform periods	
Treatment[1]	n	E1e	F
Control-0	35	2 ± 0.4ab	0.3 ± 0.2a
Control-27	20	4 ± 0.5a	1.0 ± 0.3ab
Control-65	15	1 ± 0.3b	2.7 ± 1.0b
		Total time (minutes)	
	n	F	G
Control-0	35	6 ± 4a	9 ± 3a
Control-27	20	35 ± 12ab	16 ± 5ab
Control-65	15	44 ± 13b	45 ± 15b
		Time (minutes) to event	
	n	First E1 in the exp. From first probe	
Control-0	35	69 ± 14a	
Control-27	20	95 ± 19ab	
Control-65	15	189 ± 35b	

[1]Treatment, Control-0, -27, -65, non-infected plants, on day 0, 27 and 65 days respectively after the exposure to non-viruliferous aphids; the age of the plants on EPG recording were 8, 35, and 73 days old respectively; parameters description are the same of **Table 1**; numbers in a column followed by different letters are significantly different at $P < 0.002$ according to Kruskal-Wallis test followed by multiple comparisons with Bonferroni correction.

Discussion

Attraction of aphids by headspace volatiles

The resistance to aphids present in cultivar Kardal strongly depends on the developmental stage of the leaves (Alvarez et al., 2006). This cultivar has a high level of resistance to aphids in young apical leaves but older leaves become susceptible with maturity and senescence. Production of different headspace volatiles could be one of the factors contributing to the increased attraction of *M. persicae* to mature leaves of potato cultivar Kardal. The response of *M. persicae* to headspace volatiles from PLRV-infected and non-infected potato plants also appeared to depend on the age of the leaf. Mature leaves of virus-infected plants attracted more aphids than mature non-infected leaves. In contrast, virus-infection of apical leaves did not result in an increase in aphid attraction, suggesting that also the volatile induction is age dependant.

Plant penetration behaviour

In this study we used EPG parameters to evaluate whether the resistance to aphids in young leaves of potato cultivar Kardal can be suppressed by the presence of the virus. PLRV infection appeared to change feeding behaviour of aphids only after visual symptoms of PLRV disease had developed.

Electrical penetration graph data were processed into 23 quantified behavioural features, listed in **Table 1**; six of these parameters differ between treatments. Three parameters suggest enhanced plant penetration by the aphid stylets on PLRV-infected plants at the epidermal/mesophyll level, i.e., (1) the lower number of probes shorter than 3 minutes before first phloem activity (E1), (2) the lower number and (3) shorter total duration of derailed stylet mechanics (F) events.

Probes shorter than 3 min before the 1st E1 waveform suggest the presence of some resistance factors in these tissues that act before the stylets reach the phloem. The lower number of early withdrawals therefore suggests a breakdown of resistance components.

On virus infected plants (PLRV-65) waveform F occurred less often and total time was shorter than in control-65 (non-infected plants), mainly within the first 8 minutes of a probe (**Figure 4**). Stylets are assumed to penetrate about one cell layer per min. Thus the mechanical derailment of stylet movements (waveform F) seems to be caused mainly by some factors in epidermal/mesophyll tissue. In general, waveform F has been reported to occur in the mesophyll (Tjallingii, 1987).

Waveform F was also found more often and with longer duration in other *Solanum* spp plants resistant to *M. persicae* (Alvarez et al., 2006). The constraints to stylet movement diminished with the visual developments of PLRV infection symptoms. Leaf roll symptoms induced by PLRV are observed visually by upward curling of the pale yellowish leaf edges (Beemster and de Bokx, 1987), thickening of cell walls in primary phloem cells of stems and petioles, accumulated callose in sieve elements (Thomas, 1996) , and by phloem necrosis and excessive callose formation in the phloem (de Bokx, 1987). From these PLRV symptoms, especially those related to cell wall thickening or reinforcement an opposite effect on F occurrence would be expected. Therefore other, yet unknown factors seem responsible for diminished mechanical constraint of the stylet movements in PRLV-65 plants as the results suggest. On the other hand aphids on non-infected plants show an increase of F with plants age (**Table 2**). Because all EPG recordings used the youngest fully expanded leaves, namely leaves on the same developmental stage, regarding F waveform on cv. Kardal plant age seems to matter. These phenomena need further studies.

Aphids probing on plants with PLRV symptoms (PLRV-65) had a higher number of extracellular salivation periods (E1e) than on non-infected control plants of the same age. Waveform E1e is very similar to waveform E1, which is related to salivation at phloem sieve elements (Tjallingii and Hogen Esch, 1993). However, E1e occurs without initial potential drop indicative for the trans-membrane measured in the EPG when the stylet tips puncture a cell. Thus during E1e the stylet tip remains extracellular whereas the other waveform features shown suggest the watery salivation activity as in a sieve element. Waveform E1e is embedded in stylet pathway (waveform A, B and C) and mostly occurs in the mesophyll or non-phloem vascular tissue. In our study, however, E1e was mainly occurring after normal E1, i.e., during normal sieve element salivation the voltage level changed from intra- to extra-cellular which suggests that the membrane potential of the punctured sieve element collapsed, likely a symptom of cell death. E1 salivation always precedes E2, phloem sap ingestion and seems necessary to suppress primary wound reaction in sieve elements (Tjallingii, 2006; Will et al., 2006). Long periods of sieve element salivation (E1) are normally evoked in young leaves of Kardal (Tjallingii, 2006); on Kardal appears that E1 periods lead to the death of a sieve element after which the aphids continue with watery salivation. Hence, extended sieve element salivation and sieve element death can be considered as resistance factors at phloem level, which show a decrease with plant age (control-27 to control-65); on Kardal PLRV-65 leaves the number of E1e remained at the initial control-27 level (**Table 1**); our data do not provide enough information to understand the E1e phenomena and further studies will be needed.

The period before the first phloem activity in the experiment (time to 1st E1 in experiment from 1st probe, **Table 1**) is determined by epidermal, mesophyll, general vascular, and early phloem factors. The significantly shorter time to 1st E1 on PLRV-infected than on non-infected mature leaves suggests reduced resistance in these tissues. This parameter increases with plant age from control-0 to control-65, but on PLRV-65 leaves the time to first salivation at sieve-element remained at the initial control-0 level. As phloem salivation is essential for inoculation of persistently transmitted viruses like PLRV (Prado and Tjallingii, 1994), early E1 likely implies early virus inoculation after landing but in plants already infected (such as our PRLV-65) this has no advantage for the virus.

Early phloem sap ingestion (E2) on PLRV-infected plants, on the other hand, will enhance virus transmission since aphids will acquire the virus sooner. However, we found that PLRV-infected plants did not significantly affect the time to 1st E2. Other EPG parameters related to phloem sap ingestion were not different either, e.g., the number of E2, total time in E2, and total number of aphids with sE2. Thus, the phloem-sap uptake by aphids on PLRV-infected plants is not different from non-infected plants. Our EPG recording period of 8 h did not provide data with respect to long term sap ingestion, but Castle and Berger (1993) showed a better performance of *M. persicae* feeding on PLRV-infected potato plants. Whether this is due to a better phloem sap quality or to improved feeding behaviour remains unclear.

The presence of PLRV leads to important structural and metabolic changes in the host plant: Herbers et al., (1997) found that distorted plasmodesmata occur within the phloem tissue of infected plants, and there is an altered carbohydrate allocation causing impaired phloem sucrose loading, an accumulation of soluble sugars and starch, and a reduced photosynthetic capacity of the leaves. Phloem exudates of *Cucurbita pepo* infected by ZYMV show a changed amino acid composition (although total concentration of amino acids remains the same), which may change the performance of aphids (Blua et al., 1994). Changes in phloem sap composition may affect the dispersal of the vector as well and hence the epidemiology of viruses. Fiebig et al. (2004) found that BYDV-infection affect plants suitability for its aphid vector and promoted the production of aphid alatae; hence this might be the driving force for increased virus spread in the field. According to McElhany et al. (1995) the implications of vector preferences on patterns of disease spread are complex. The dynamics of vector-borne diseases depend on ecological factors. They are the complex result of changing frequency of diseased plants in the population, local spatial structure of the host, pathogen

and vector populations. Results of McElhany et al. (1995) show that for barley yellow dwarf virus, the vector remains infective for a long period after visiting a diseased host. Therefore, a vector preferring healthy hosts would spread the disease more than a vector preferring diseased hosts. Predictions of how a vector preference toward PLRV infected plants would affect the spread of the disease should be investigated under field conditions.

Conclusions

In our plant-virus-vector system, PLRV infection directly or indirectly affects the plant-aphid interaction since, 1) odour of infected plants attract significantly more vectors than non-infected plants, 2) virus infected plants enhance stylet penetration into the plant tissue as a result of reduced number of short probes, reduced constraints in terms of mechanical derailment, reduced time to the first phloem salivation. On the other hand, 3) phloem factors showed opposite effect as shown by increased E1e and E1 numbers and phloem feeding not distinctly improved. During the 8 h-monitoring the phloem-sap uptake was not affected by PLRV-infection of the plants. Therefore, better aphid performance on PLRV-infected potato plants reported by Castle and Berger (1993) might be related to enhanced phloem quality. It will be interesting to investigate whether PLRV infection results in increased nutritional quality of phloem sap on cv. Kardal.

The transmission efficiency of the persistent circulative PLRV is also expected to be affected on PLRV-infected plants because these plants attract more aphids than non-infected plants. It will be interesting to investigate the effects reported in this study on virus epidemiology.

CHAPTER 7

General discussion: New considerations on the interactions between *Solanum* species and aphids

Abstract

Aphids are phloem sap feeding insects that interact intensively with their host during colonisation even before feeding. To understand the full complexity of that relationship, integrated studies on the interacting organisms, i.e., plants and aphids, are required. Studies on plant performance and responses before and after insect attack (morphology, histology, physiology, development, functional genomics analysis, etc.) need to be combined with aphid behaviour and performance. The aim of this chapter is to integrate the data of this thesis on interactions and resistance mechanisms in *Solanum* species with respect to *Myzus persicae* (Sulzer) in the general perspective of the present knowledge on plant-aphid-virus interactions. Evidence provided in this thesis indicates that the aphid's endo-symbiotic bacteria *Buchnera* sp. should be taken into account in studies on plant-aphid interactions. In this Chapter, the interactions between *Myzus persicae* and *Buchnera* are compared in perspective with those between plants and pathogenic bacteria.

Contents

Figures

Introduction

More than 60 wild *Solanum* species have been reported to be equipped with effective resistance to *Myzus persicae* (Flanders et al., 1999; Flanders et al., 1992; Gibson & Pickett, 1983; Novy et al., 2002; Radcliffe & Lauer, 1968; Tingey & Sinden, 1982). These species represent an important potential source of resistance, which can be exploited to enhance resistance in cultivated potatoes to the main vector of devastating plants viruses, *Myzus persicae*. However, further exploration of the underlying biochemical, physiological, and molecular mechanisms of resistance is first needed. This thesis focuses on the mechanisms of the resistance to the aphid *M. persicae* in a number of *Solanum* species.

Where are the resistance factors to *M. persicae* located?

Aphids, as phloem feeders, insert their mouthparts into the plant (probing) to select a suitable host and to reach the phloem sieve elements. Therefore, knowledge on probing behaviour of aphids is crucial for a better understanding of host-plant resistance and might point at tools for aphids control.

In Chapter 2, twenty tuber-bearing *Solanum* genotypes were evaluated for susceptibility or resistance towards *M. persicae*. A combination of an aphid colony-development assay with the evaluation of probing activities with the electrical penetration graph (EPG) technique, showed to be effective in localising resistance factors in tissues of *Solanum* genotypes. Mechanisms of resistance to *M. persicae* were detected at three plant tissue levels.

Surface resistance

Surface resistance is the first line of defence against attack and is especially important to avoid virus infection. A long non-probing time before the first probe, as shown in EPGs, mainly reflects mechanical or olfactory factors acting on the leaf surface or at the cuticle-epidermis level. These factors may include repellent volatiles or colours, leaf toughness, wax structures, or the presence of trichomes (van Helden & Tjallingii, 1993). We found clear surface resistance in species with glandular trichomes, *S. berthaultii*, *S. polyadenium*, and *S. tarijense*, and we found evidence that the resistance is caused mainly by these morphological structures, that secrete chemicals affecting the performance of the aphids.

These glandular trichomes-containing species have been studied extensively because of their effective resistance to aphids (Gibson, 1971; 1974; 1976; Gibson & Turner, 1977; Tingley & Laubengayer, 1981). However, the glandular trichome-based resistance is genetically complex and it is strongly associated with poor agronomical characteristics (Bonierbale et al., 1994; Kalazich & Plaisted, 1991). Therefore, it has not yet been used successfully in breeding programs for aphid resistance.

Epidermis/mesophyll resistance

On *S. stoloniferum* Schlechtd *M. persicae* EPGs showed increased numbers of test probes before the first phloem activity. Furthermore, the number and total duration of the EPG waveforms indicating derailed stylet mechanics (waveform F), which only occurs in the mesophyll (Tjallingii, 1987), were notably longer in *S. stoloniferum.*

Mesophyll/phloem resistance

Sieve element salivation (waveform E1) is considered as the first established sieve element activity of the aphid, which always precedes phloem sap ingestion (E2). Both activities play a central role in the selection of host plants by aphids. Sustained phloem sap ingestion (sE2, i.e. E2 activity longer than 10 min) has been considered as committed phloem ingestion and an indicator of phloem acceptance (Tjallingii and Mayoral, 1992). We found genotypes showing a relatively long time before the first E1 but not to the first sE2 (i.e., *S. okadae*, *S. hondelmannii*, and *S. multiinterruptum*) most likely reflecting resistance factors at the mesophyll level. In contrast, we found other genotypes (i.e., *S. jamesii* and *S. tuberosum* cv. Kardal) with a relatively long time to first sE2 but not to first E1, which most likely indicates resistance factors at the phloem level.

The expression of resistance to *M. persicae* appeared to change with the age of the plants as well as with the age of plant parts. Some of the plants, resistant at the young stage, became susceptible at the flowering mature stage, either by changes in the whole plant or by reduced resistance in mature and senescent leaves. Overall, genotypes with some degree of resistance at the young and the flowering plant stage are: *S. jamesii*, *S. tuberosum* cv. Kardal, *S. capsicibaccatum*, *S. stoloniferum*, *S. berthaultii*, *S. polyadenium*, and *S. tarijense*.

The generalist herbivore *M. persicae* is the major virus-vector in potato crops. However, development of plants with a pre-phloem mechanism of resistance will only be advantageous to

avoid inoculation of persistently transmitted viruses, since this type of viruses is only effectively inoculated directly in the sieve elements. Among *Solanum* species, *S. stoloniferum* showed pre-phloem resistance to *M. persicae* that was not based on glandular trichome traits. Moreover, we found that young leaves are resistant to *M. persicae*, while senescent yellowing leaves of this species were more or less susceptible (Chapters 2 and 3).

***Solanum stoloniferum* resistance to aphids is species specific**

Although *S. stoloniferum* showed poor host features for *M. persicae*, we observed that it was accepted and colonised by *Macrosiphum euphorbiae*. In Chapter 3, *S. stoloniferum* was used as a model plant to unravel and characterise the different interactions of a single host plant with different aphid species. We studied aphid performance, settling behaviour, and probing of *M. persicae* and *M. euphorbiae* on *S. stoloniferum* and on *S. tuberosum* control plants, and we found that both plants could be a host plant for *M. euphorbiae* while confirming that *S. stoloniferum* is a poor host for *M. persicae*.

***Solanum stoloniferum* resistance: physical constraint or repellence and deterrence?**

The strong resistance found in *S. stoloniferum* against *M. persicae* seems to rely on constitutively expressed physical traits with some age effects. To find out whether *M. persicae* or *M. euphorbiae* infestation can induce an additional repellence or deterrence resistance in *S. stoloniferum*, we studied settling behaviour with free aphids. These tests were carried out on previously infested leaves (local responses), and on non-infested leaves of infested plant (systemic responses). There was no evidence for an induction of repellence or deterrence in plants that had been previously infested with *M. persicae* or *M. euphorbiae* compared to plants that had never been infested. (Chapters 3 and 4).

Solanum spp. responses to aphid infestation at molecular level

***Solanum stoloniferum* response to *M. persicae* and *M. euphorbiae* attack**

In Chapter 4 we selected *S. stoloniferum* and the two aphid species, *M. persicae* and *M. euphorbiae*, as a model to study plant responses in compatible and incompatible interactions. To characterize both plant-aphid interactions we analyzed the transcriptional responses in *S. stoloniferum* due to aphid colonisation at the local and systemic level.

About twice as many genes were differentially regulated (i.e. up or down) in response to attack by *M. euphorbiae* (compatible interaction) than by *M. persicae* (incompatible interaction). This stronger response to *M. euphorbiae* involves mainly up-regulation of genes in the functional categories pathogenesis related (PR), regulatory, and protein metabolism and down-regulation in the functional categories regulatory, general metabolism and photosynthesis related genes.

In addition, *M. euphorbiae* elicits genes related to regulated-proteolysis (proteasome system), plasmodesmata gateway, and extracellular transport. It is interesting to note that none of these genes are activated in the incompatible interaction with *M. persicae.*

We also found induction of genes related to cell differentiation and development. These are the same genes differentially expressed in growing tissues, which are characterized for a high sink strength (Herbers and Sonnewald, 1998). Perhaps these genes are related to a process of changing the physiological status of the tissue towards generating a local metabolic sink.

We hypothesize that especially the high number of regulatory genes that are up-regulated in local leaves in the compatible plant-aphid interaction is related to this change in the physiology of the plant, as a results from the aphid attack. Subsequently, the shift in plant condition contributes to the acceptance of *S. stoloniferum* as a host for *M. euphorbiae.* The capability of the aphids to manipulate the plant apparently seems to occur mainly in local leaves, i.e. at colonisation sites, more than in the remote systemic areas. De Vos et al. (2005) found evidence for induced expression of the SA-responsive PR-1 promoter in cells surrounding the feeding sites, however they did not find changes in the production of the signalling compound SA in Arabidopsis after *M. persicae* infestation compared to non-infested controls. Increases in other phytohormones such as jasmonic acid (JA) and ethylene (ET) were also not found. The authors explained the lack of evidence on the induction of signal transduction pathways as a consequence of the small damage that aphids do while feeding. Yet, De Vos et al. (2005) found that, strikingly, *M. persicae* in Arabidopsis induced the largest number of changes in gene expression, followed by pathogenic bacteria when compared with other plant-attackers. In agreement, our data also suggest that like pathogens, aphids in a plant-compatible interaction can manipulate the plant metabolism for their own benefit and might also suppress the plant's defence responses.

Most of the PR genes up-regulated upon the attack by either aphid species are SA-responsive, indicating that the SA signaling pathway was activated, as was demonstrated for several other plant-aphid interactions (Smith and Boyko, 2007). Genes related to the ethylene signal-transduction pathway were also differentially activated. In our system the ET signal-transduction pathway plays a larger role in compatible plant-aphid interactions than in incompatible interactions. *M. euphorbiae* directly activates genes responsible for ET biosynthesis and ET regulation. Also *M. persicae* colonisation of *A. thaliana* (compatible interaction) leads to up-regulation of ET-related transcripts (Moran et al., 2002). Regarding the jasmonic acid-responsive genes, we observed the activation of a transcript for jasmonic acid synthesis and two JA-responsive genes in the compatible interaction, but no activation of JA-responsive genes in the incompatible interaction.

***Solanum tuberosum* responses to *M. persicae* infestation depend on foliage maturity**

Leaves of different age stages offer different environments to aphids. We found that young leaves of cultivar Kardal are resistant to *M. persicae* whereas mature to senescent leaves are susceptible (Chapter 2). This leaf preference could reflect changes in tissue chemistry. In Chapter 5, we evaluated the plant response in young and old leaves to the *M. persicae* colonisation by studying gene expression and the aphid settling behaviour. In old (susceptible) leaves *M. persicae* attack elicits a substantially higher number of differentially regulated genes (up and down-regulated) than in young (resistant) leaves. *M. persicae* attack of young leaves of *S. tuberosum* cv Kardal results in a low number of down-regulated genes.

Comparative transcriptomics in *S. stoloniferum* and *S. tuberosum* after aphid attack

In order to find similarities in gene regulations in compatible and incompatible plant-aphid interactions we compared the transcriptomic results obtained with the two systems: (1) *S. stoloniferum* after the attack of *M. persicae* and *M. euphorbiae* (Chapter 4), and (2) *S. tuberosum* cv. Kardal after *M. persicae* attack at different maturity leaf stages (Chapter 5). In both systems, after the attack with either aphid we found the differential expression of mainly pathogenesis related, regulatory, and protein metabolism related genes. The gene-expression studies provide evidence that both plants, *S. tuberosum* and *S. stoloniferum*, respond to aphid attack by activating the salicylic acid (SA) and ethylene (ET) pathways. However, genes responsive to jasmonic acid were only found to be differentially regulated in the *S. stoloniferum-M. euphorbiae* and not in cv. Kardal- *M. persicae* interaction.

There is a group of 16 genes that are up-regulated in both systems used, i.e., *S. tuberosum* cv. Kardal after *M. persicae* infestation, and *S. stoloniferum* after *M. persicae* or *M. euphorbiae* infestation, and might characterize both aphid-*Solanum* spp interactions. Half of those genes are pathogenesis related. Like in both *S. stoloniferum*-aphid interactions, also in cv. Kardal up-regulated genes were found that could play a role in switching the tissue status from source to sink.

We did not find commonly down-regulated genes in both *M. persicae*-infested Kardal leaf stages and *M. persicae*- or *M. euphorbiae*-infested *S. stoloniferum.* Hence, down-regulated genes might be more host-species specific than are up-regulated genes. In *S. tuberosum* cv. Kardal plants infested with *M. persicae*, in young and old leaves, we find 28 down-regulated genes, among them were homologues to genes directly related with defence compound biosynthesis (e.g., two chalcone synthase, one chalcone isomerase and one proteinase inhibitor). After *M. persicae* attack Arabidopsis infested by *M. persicae* also represses chalcone synthase genes (Moran et al., 2002).

Tissue reactions of *S. stoloniferum* after *M. persicae* infestation

Chapter 4 describes that infestation of *S. stoloniferum* leaves with several confined *M. persicae* aphids leads to the development of obvious water-soaked pustules. Microscopic analysis of the pustules showed a burst of the tissue at the abaxial side of the leaf, which is probably the result of cell enlargement and cell division of vascular parenchyma cells and vacuolated bundle sheath cells surrounding the vascular bundle. Later the pustules collapse from the centre to the margin, the tissue becomes necrotic and finally holes are formed from the centre. In contrast, the infestation with *M. euphorbiae* did not induce any visible cellular changes.

Similar pustules were found also in mesophyll cells of two different host plants infected with the bacterial pathogen *Xanthomonas* spp., i.e., in pepper plants infected with *Xanthomonas campestris* pv. *vesicatoria*, causing bacterial spot disease in pepper and tomato; and in citrus plants infected with *Xanthomonas citri*, (Swarup et al., 1991; Leach and White, 1996). Furthermore, Marois et al. (2002) found also similar pustules in mesophyll cells of *Solanum tuberosum* (non-host of *X. campestris*) carrying a transient expression of the *X. campestris* effector gene construct. *Xanthomonas* spp are biotrophic pathogens that grow in the extracellular space in the mesophyll. These bacteria have a secretion system involved in host-pathogen interactions, i.e., in charge of delivering effector proteins directly inside the host cells. The effectors are involved in virulence by

targeting specific steps of the host cell metabolism to the benefit of the bacteria. (Bonas et al.,1989; 1991; for review see Talbot, 2004).

Additionally, in the transcriptomic response of *S. stoloniferum* and *S. tuberosum*, we observed the induction of an *auxin-induced SAUR* (*small auxin up RNA*)-*like protein* after *M. persicae* and *M. euphorbiae* attack (Chapters 4 and 5). Interesting, induction of *SAUR* transcripts has also been reported after inoculation of pepper and tomato plants by the virulent bacterial pathogen *X. campestris* pv. *vesicatoria* (Marois et al., 2002). Most likely, *SAURs* are involved in the disease response of the tissue of susceptible pepper and tomato, and the bacteria may possess effectors that steer host gene expression (Marois et al., 2002).

In general, in plant-pathogen interactions, pathogen effectors suppressing host basal defences play an important role (reviewed by Nomura et al., 2005). The observed pustule formation and the induction of an auxin-induced *SAUR* in *S. stoloniferum* strongly suggest that a similar situation exists in plant-aphid relationships. Although no elicitors have been isolated from aphid saliva so far, the parallel with the bacterial infections mentioned above is striking. Possibly, the watery saliva includes effector proteins of endo-bacterial origin that may play a role in plant-aphid interactions. As was also suggested by Zhu-Salzman et al. (2004) and Moran et al. (2002), I speculate that endosymbionts could be responsible for the pathogen-like responses in gene expression and pustule-like reactions at tissue level. Therefore, more research into the role of the aphid endosymbionts seems necessary.

Stylet penetration of *M. persicae* on *S. stoloniferum* and tissue reaction

Myzus persicae probing is constrained on *S. stoloniferum* as shown by aphids needing 3 hours or more of probing in mesophyll and vascular tissue before the first phloem activity and at least 5 h before the first period of sustained phloem feeding (Chapter 2). On susceptible *S. tuberosum* the aphid needs an average of 1 hour before the first phloem activity and an 3 to 4 h to start sustained phloem feeding, which, although quite a long period, can be considered as normal for host plant acceptance (Chapter 2; Tjallingii and Mayoral, 1992; Cole , 1997; Prado and Tjallingii, 1999).

Overall, most *M. persicae* aphids on *S. tuberosum* will feed on phloem sap after 3 h of probing but on *S. stoloniferum* they will continue pathway activities and puncturing different cell types.

Consequently, they will probably inject effectors present in the aphid saliva, into more cells and into the same cell multiple times in *S. stoloniferum* (Chapter 3).

Concerning probing differences between susceptible *S. tuberosum* and resistant *S. stoloniferum* we found that although the total number and total time of brief cell punctures (pd waveform) were similar, the average duration of the individual punctures lasted longer on *S. stoloniferum*, which indicates that in individual cells stylets spent more time, and more saliva might be injected (Chapter 3). The role of these brief cell punctures with watery salivation and sampling of cell contents remains unclear. Do aphids need to inject saliva in order to prepare a plant to become suitable for phloem feeding? Do aphids use cell sampling to establish plant suitability or are they getting cues to find phloem sieve elements? For our specific plant-aphid interaction we hypothesise that long pathway periods and more probes prior to sustained phloem ingestion on suitable host plants are a requirement for host acceptance and this time could be needed as 'waiting time' for changes in plants to increase the food quality or to reduce natural hurdles.

During the stylet pathway phase aphids salivate substantially, secreting intercellular (gelling) sheath material. Additionally, intracellular watery salivation is occurring regularly during brief cell punctures lasting about 5 seconds and clearly recognisable as waveform pd in the EPG (Tjallingii, 1988; Tjallingii and Hogen Esch, 1993). During these brief punctures, some watery saliva is injected into the cell and there is also ingestion of minute amounts of cell content (Martin et al., 1997). The cell punctures occur in all tissues and the cells show little damage and very few cells die (**Figure 1**) (Tjallingii and Hogen Esch, 1993). Saliva delivered inside the punctured cells could be the primary target to elicit plant responses (Tjallingii, 2006) but also, the salivary sheath material may be an apoplast signal that plants can respond to. Evidence has been found that even after reaching a sieve element, aphid stylets may continue exploring vascular and non-vascular tissues puncture cells. While in the mesophyll most, but not all, cells seemed punctured and sampled (Tjallingii and Hogen Esch, 1993), cells in vascular bundles showed multiple punctures, with a maximum number in phloem companion cells of more than 4 punctures per cell.

I think that the pustules developed on our incompatible plant-aphid interactions (resistant plants), could be a consequence of a physical constraint for the aphid to find the phloem. Aphids then punctured more cells and injected more saliva and with it, probably more effectors than in susceptible plants, ending in the hypertrophy in the mid vein.

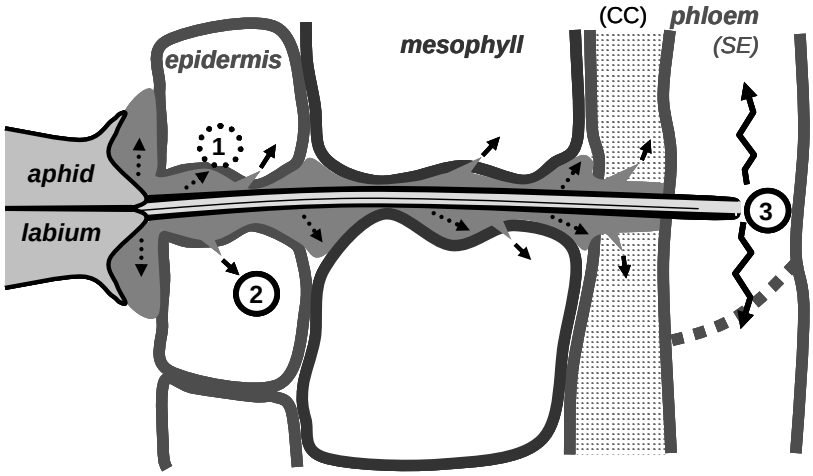

Figure 1. Aphid salivary secretion into the plant during stylet penetration. (1) gelling saliva (dotted arrows), forming the salivary sheath, (2) watery saliva (small solid arrows), intracellularly secreted during brief stylet punctures (pd waveform), (3) watery saliva (long undulated arrows), into phloem sieve elements (E1 waveform), preceding phloem feeding. Salivation (3) might be mainly responsible for the induced resistance that is systemically spread, whereas salivation (1) and (2) may have more local effects. CC, companion cell; SE, sieve element (Tjallingii, 2006).

Could apoplast-defence products affect aphids?

Aphids have developed a highly specialized plant penetration mechanism to take up nutrients from plants without killing them or consuming complete parts. This strategy shows similarity to the way biotrophic plant-pathogens utilize and interact with their host plants (for review see Talbot, 2004). Furthermore, aphid-infested plants show induction of the salicylic acid signal-transduction pathway which is related to plant reaction to pathogens. A differentially increased transcription of homologues to apoplastic PR enzymes (*PR1, PR2*), *β-1,3 glucanase, chitinase, cell death related protein, osmotin like protein*, supports the role of SA in the response of plants to aphids (Chapter 4 and 5; Moran and Thompson, 2001; Moran et al., 2002; van der Westhuizen et al., 1998a, 1998b; Zhu-Salzman et al., 2004).

The apoplast plays an important role in a plant's defence mechanism against pathogens (bacteria and fungi) since many defence-related products accumulate there (Bowles, 1990). But aphids feed on phloem sieve elements contents exclusively and only use the apoplast space as an intercellular route to the phloem (for review see Will and Van Bel, 2006). Aphids, by secreting gelling saliva on the pathway to the phloem, seem to be able to avoid apoplast-defence responses. The gelling saliva contains phospholipids, conjugated carbohydrates, and many proteins (phenoloxidases, peroxidases, pectinases, β-glycosidases) with enzyme activities that can polymerize both insect and plant derived

compounds (Cherqui and Tjallingii, 2000; Urbanska et al., 1998; Miles, 1999). Salivary sheath enzymes may suppress wound-triggered phenolic accumulation by the host plant by sequestering oxidized phenolics into the sheath (Miles and Oertli, 1993). Instantly the gelling saliva fills the stylet tunnels made in cell walls after withdrawal thus avoiding cell bleeding and enabling the plasmalemma to be repaired.

Watery saliva is also secreted by aphids during cellular punctures along the stylet pathway and during salivation (E1) and feeding (E2) in phloem sieve elements. Watery saliva may perform critical roles in feeding (i.e., lubrication of stylets, mainteinance of favorable redox conditions, detoxification of phenolics, prevention of sieve elements blockage by callose or polymerized P-proteins) (Miles, 1999).

The induction of pathogenesis-related plant defences by aphids, to which the aphid seems protected, may be important in preventing the opportunistic infection with a pathogenic bacteria or fungi.

Effect of Potato leafroll virus on *M. persicae*

Potato leafroll virus (PLRV; genus *Polerovirus*, family Luteoviridae) is a persistently transmitted circulative virus that depends on aphid vectors, mainly *M. persicae*. In Chapter 6 we investigated whether PLRV-infection in potato plants of cv. Kardal affected aphid behaviour.

Chapter 6 shows that *M. persicae* responses to headspace volatiles emitted from PLRV-infected and non-infected potato plants appeared to depend on the age of the leaf. For young apical leaves no difference in aphid attraction was found between PLRV-infected and non-infected leaves; hardly any aphids were attracted. In contrast, for mature leaves headspace volatiles from virus-infected leaves attracted more aphids than non-infected leaves. In addition, we studied the effects of PLRV infection on probing of *M. persicae* using the EPG technique. Several differences were observed between plant penetration in PLRV-infected and non-infected plants, but only after infected plants showed visual symptoms of PLRV infection. Virus-infected plants enhance stylet penetration into the plant tissue, have reduced constraints in terms of mechanical derailment, and aphid on such plants have reduced time to the first phloem salivation. On the other hand, phloem factors showed opposite effects as shown by increased salivation at the extracellular level (E1e) and during the 8 h-monitoring the phloem-sap uptake is not distinctly improved. Therefore, better aphid performance

on PLRV-infected potato plants reported by Castle & Berger (1993) might be related to enhanced phloem quality. It will be interesting to investigate whether PLRV infection results in increased nutritional quality of phloem sap in cv. Kardal.

Overall the transmission efficiency of the persistent circulative PLRV is also expected to be affected in PLRV-infected plants because these plants attract more aphids than non-infected plants. It will be interesting to investigate the effects reported in this study on virus epidemiology.

Myzus persicae and the endosymbiont *Buchnera*

While working on this thesis, some experimental evidence directed my attention to the possible role of the aphid endosymbiont *Buchnera* in plant-aphid interactions. What follows is a review-like-discussion on some known aspects of *Buchnera* and some hypothetical considerations.

The microbiology of aphids is unusual in having few or no micro-organisms detected in their gut lumen by microscopy as compared to most other insects that have a great diversity and abundance of microorganisms (Grenier et al., 1994; Harada et al., 1996; Wilkinson et al., 1997). *Myzus persicae* has a symbiosis with a γ-proteobacterium of the genus *Buchnera* (Munson et al., 1991), located in the aphid haemocoel inside huge specialized cells called bacteriocytes (Buchner, 1965). *Buchnera* represents 10% of the total volume of the insect (Baumann et al., 1994; Humphreys and Douglas, 1997; Wilkinson et al., 2001). *Buchnera* cells are absolutely dependent on their intracellular habitat in aphids and, as a result of an evolutionary reduction of genome size, they cannot be maintained long-term in axenic culture (Whitehead and Douglas, 1993). Equally, aphids benefit from their association with *Buchnera* because it provides the aphids with essential amino acids (Prosser and Douglas 1991; Douglas, 1996). Phylogenetic analysis indicates that the relationship between *Buchnera* and aphids was established 200-250 million years ago and led to co-speciation of both partners (Moran et al. 1993).

Was *Buchnera* originally a biotrophic bacterium?

A plant pathogenic bacterium has to face the plant defence response; the plant perception of pathogen associated molecular patterns (PAMPs), initiates PAMP-triggered immunity (PTI), which usually halts infection before the microbe gains a hold in the plant (Chisholm et al., 2006). From the bacterial side, a symbiotic relationship has the great advantage to avoid these PAMP-triggered plant

reactions. *Buchnera* is present far away from the plant defence response released in the apoplast. Aphids might not have to face apoplast defences because they feed on the phloem, which in spite of having the disadvantage of being poor in nutrients, has the advantage of having fewer toxicological hazards than other plant cells (Douglas, 2003b). Whether *Buchnera* was ancestrally a biotrophic bacteria yet remains an exciting, but still open, question.

Why should type III secretion system genes be conserved in Buchnera?

Although *Buchnera* cells are non-mobile, their surface is covered with flagellar basal bodies. The flagellar apparatus belongs to the family of type III secretion systems (T3SS) and therefore seems to be related to protein transport (Maezawa et al., 2006). Surprisingly, in *Buchnera* the genome is reduced, but transporter genes of the T3SS type are conserved (Shigenobu et al., 2000). The T3SS is like a molecular syringe used to inject effector proteins directly into the host cell (Jin et al., 2001; Li et al., 2002). T3SS is a key transmembrane system involved in host-pathogen interactions that mediates the secretion of proteins across bacterial membranes into the extra-cellular milieu and the translocation of effector proteins (termed effector proteins because of their role in inducing responses in host cells) directly into eukaryotic cells, thereby subverting the host's cellular processes (He et al., 2004). Plant pathogenic bacteria possess a large number of effectors (Chang et al., 2005) which presumably interfere in a collective manner with host cellular pathways. The highly dynamic feature of T3SS coding genes may contribute to pathogen fitness by allowing the pathogen to evade the host surveillance mechanism and support a role in the adaptation to different hosts (Guttman et al., 2002).

Another surprising discovery was the identification of T3SSs in plasmids and genomes of several nitrogen fixation *Rizhobium* strains (Freiberg et al., 1997, Kaneco et al., 2000; Göttfert et al., 2001; Meinhardt et al., 1993). The presence of T3SSs in *Rizhobium* was unexpected because T3SSs were originally thought to be restricted to pathogenic bacteria (Marie et al., 2001). The induction of nodule formation is co-regulated with the expression of T3SSs genes (Viprey et al.,1998) suggesting that type III secreted proteins, termed nodulation outer proteins (Nops), contribute to symbiosis (Bartsev et al., 2004).

The function of T3SSs in *Buchnera* yet remains to be uncovered.

What are the roles of symbiotic bacteria in plant attackers?

I found in the literature some important discoveries pointing at symbionts in unexpected roles with their symbionts-counterparts.

1) Role of symbiotic bacteria in a plant-pathogenic fungus interaction. *Rhizopus* (a plant-pathogenic fungus causing rice seedling blight) harbours intracellular symbiotic bacteria of the genus *Burkholderia.* It was found that the bacteria are in charge of producing the phytotoxin rhizoxin, a macrocyclic polyketide that is the causative agent of rice seedling blight (Partida-Martinez and Hertweck, 2005).

2) Role of symbiotic bacteria in a vector-virus interaction. Potato leafroll virus (PLRV; *Luteoviridae*), is transmitted by aphids in a persistent circulative manner. Thus, virions are ingested with phloem sap during aphid feeding, circulate in the digestive system, cross the epithelial cells of the gut, diffuse through the haemolymph and finally pass through the accessory salivary gland membranes to be transmitted to another plant via the saliva (Gildow, 1987). The haemolymph of an aphid acts as a reservoir in which acquired virus particles are retained in an infective form without replication for the life span of the aphid. This circulation is made possible by the chaperone homologue of GroEL in *E. coli*, from the aphid's endosymbiotic bacteria *Buchnera* sp. (Hogenhout et al., 2000). Chaperones are small, acidic and leucine-rich proteins that interact with effector proteins and stabilize these and protect them from degradation. *Buchnera* chaperone GroEl has been immuno-detected in the haemolymph of aphids and plays a crucial role in the persistence of luteoviruses in the aphid's body fluid (van den Heuvel et al., 1994; 1997). Many chaperones are present in the *Buchnera* genome (Shigenobu et al., 2000).

3) Role of symbiotic bacteria in a plant-aphid interaction. *Myzus persicae* is a generalist phloem feeder; hence it needs to deal with a large number of plant compounds. The same clone of *M. persicae* feeding on different species, *Brassica napus* L. (Brassicaceae), and *Solanum tuberosum* L. (Solanaceae), develops a different aphid protein profile according to the host plant; surprisingly a chaperone from *Buchnera* was over-expressed when *M. persicae* fed on *B. napus* (Francis et al., 2006). This supports the hypothesis that the endosymbionts have an important role in the plant-aphid relationships.

Taking into consideration the examples above, it will be exciting to address a potential active role of *Buchnera* on aphid-host plant interactions.

A model of co-operation between *M. persicae* and *Buchnera*

The intimate aphid-endosymbiont relationship may be a 'work division in equilibrium, a model of co-operation'. In this hypothetic co-operation model, *Buchnera* produces type III protein effectors and aphids inject them with the watery saliva into plant cells during their brief stylet punctures altering plant metabolism, and possibly suppressing defence responses, while *Buchnera* remains safe in the aphid shelter. Moreover, the symbionts supply the aphid with the essential amino acids that are scarce in the phloem sap and in return they receive non-essential amino acids from the aphid (Shigenobu et al., 2000). An additional feature in this co-operation model is that pathogenesis-related induced plant defences, for which the aphid seems protected (by secreting gelly saliva), would prevent the opportunistic infection of a bacterial or fungus disease (**Figure 2**).

Salivary secretions are mostly ingested during sap uptake presumably to keep the food canal unblocked by coagulated phloem proteins (Knoblauch and Van Bell, 1998; Tjallingii, 2006). Once in the phloem, the re-ingestion of saliva with the uptake of phloem sap might mitigate systemic unwanted plant defence responses by avoiding the drain of saliva to other plant parts.

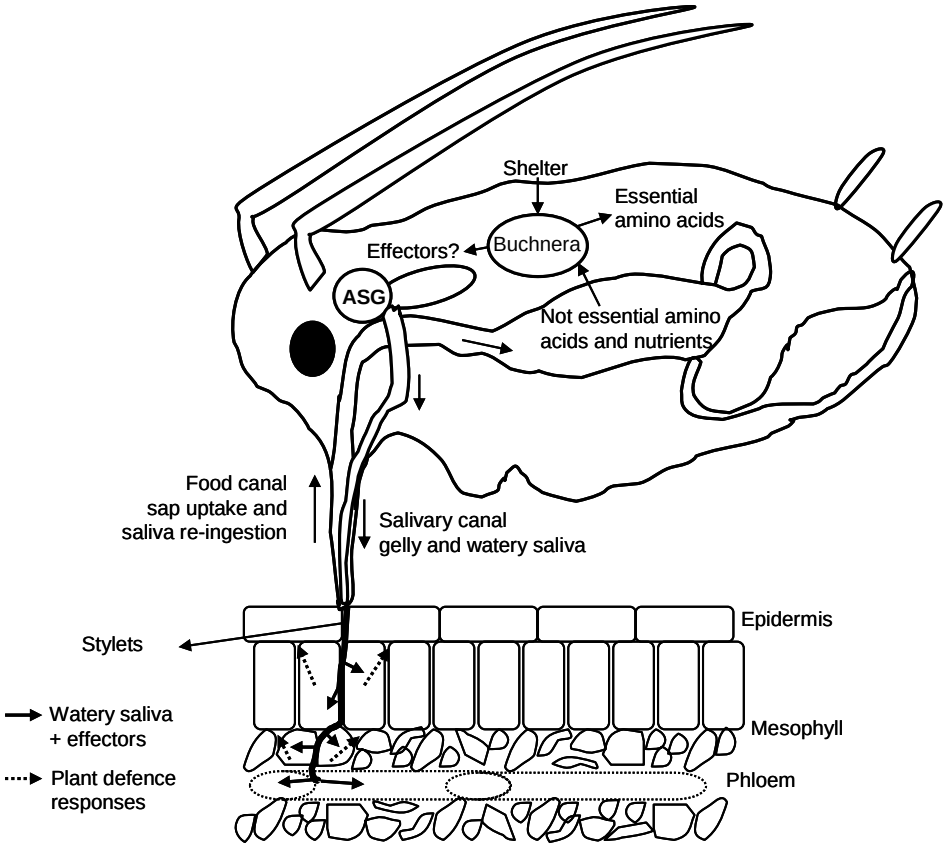

Figure 2. Aphid-*Buchnera* interaction. Co-operation model (see text for details). ASG, accessory salivary gland

Results supporting the model

Summarized below are some results analysed in perspective of the hypothetical model in plant-aphid-*Buchnera* interactions but this remains to be tested.

- Pustules development on resistant *S. stoloniferum* plants after infestation with *M. persicae* (Chapter 4). This could be a consequence of a physical constraint for the aphid to find the phloem. *M. persicae* cannot feed on *S. stoloniferum* therefore the aphid will continue puncturing cells and injecting more saliva and probably more effectors, resulting in pustule formation. It will be interesting to further investigate the mechanisms of pustule formation in *S. stoloniferum* in the context of the proposed model.

- An *auxin-induced SAUR* (*small auxin up RNA*)-*like protein* was induced in *S. stoloniferum* after *M. persicae* attack (Chapters 4 and 5). Interestingly, induction of *SAUR* transcripts had been reported after inoculation of pepper and tomato plants by virulent *Xanthomonas campestris* pv. *vesicatoria*, also causing hypertrophy and cell division (Marois et al., 2002). It would be interesting to search for genes homologous to pathogenic-bacteria effectors in the *Buchnera* genome.

- Surprisingly, the number of genes differentially regulated in *M. persicae*-infested Arabidopsis is similar to that in response to pathogenic bacterium (De Vos et al., 2005). The attack of other, more damaging insects such as caterpillars or thrips results in far fewer differentially regulated genes (De Vos et al., 2005). Furthermore the larger number of genes belongs to pathogenic-related genes, regulation and metabolism (Chapter 4). The plant response to aphid attack seems to be more related to the plant response to pathogenic bacteria than to other herbivorous insects.

Conclusions and research needs

Plant-aphid interactions are dynamic and complex. Depending on the host and aphid species, there is a potential for variation between host susceptibility on one hand, to host resistance on the other. The gene expression analysis of *Solanum* spp. attacked by aphids provided us with starting points for future research into plant-aphid interactions. We found a group of genes involved in defence responses that may affect aphid colonisation, but we also found genes related to plant metabolism and stress responses that can be beneficial to the aphid.

In a compatible interaction, aphids seem to be capable to benefit from host cell processes and to change the nutrient streams inside the plant to their advantage; they appear to be able to transform their feeding site into a sink, which perhaps is a major requirement for host acceptance.

We found general responses in *S. tuberosum* to *M. persicae*, and *S. stoloniferum* to the two aphids, *M. euphorbiae* and *M. persicae* but also responses unique to each of the plants. A compatible plant-aphid interaction resulted in a broader gene expression response, which in part represents the plant need to cope with the removal of phloem sap during aphid feeding. Among these responses we identified genes related to changes in sink-source relationship at the feeding site which may indicate a plant manipulation by the aphids; also genes related to signal-transduction pathways, regulation and signalling, protein metabolism, maintenance of cell homeostasis, transport, secondary metabolism, and structural features were found to be differentially regulated. On the contrary, in incompatible interactions the transcriptional response of the plant seems to be more restricted.

An intriguing question concerns the role of the high number of pathogenesis-related and SA-responsive genes activated upon aphid attack. Is this response elicited by the aphid itself or by compounds produced by the aphid endosymbiont? Until now, there is no evidence that bacteria-derived or aphid-borne effectors are secreted in aphid saliva. In order to improve the understanding of plant responses to aphid attack, it will be interesting to determine whether *Buchnera* effector proteins do occur in the aphid saliva. The identification of *Buchnera* effector genes homologous to *Xanthomonas* genes needs further research.

Prospects for breeding for aphid resistance in potato

Until now no effective resistance of potato plants to aphids has been achieved. The current perspectives to minimize the aphid-borne damage in potato crops are by combining plant resistances to aphids and viruses. The contribution of this thesis to understanding aphid-resistance in potato can be summarized as follows:

– Until now, in wild *Solanum* spp the best resistance observed against a broad range of aphids was associated with the presence of glandular trichomes (Chapter 2, Bonierbale et al., 1994; for a review see Yencho et al., 2000). QTL analysis of trichome-mediated insect resistance in potato has shown that the trait is complex, involving several chromosomal regions and that the

presence of glandular trichomes is associated whit a number of undesirable traits. Using molecular marker technologies it should be possible to separate the desired and undesired characteristics and produce a potato variety that is fully resistant to a range of insect pests.

- Several aphid-resistant *Solanum* species have been identified, some with resistance at the epidermis/mesophyll others at the phloem level. Combining different types of resistance might result in plants highly resistant to aphids. Again, molecular makers will be essential for introduction of genes involved into the cultivated potato

- It was shown that susceptibility towards aphids in *S. tuberosum* cv Kardal and S. *stoloniferum* was directly linked/correlated to the induction of senescence. For the moment it is unclear what is the role of aphids on the induction of senescence in potato, but genes involved in triggering might be candidates for improving aphid resistance. When senescence can be postponed, resistance might be increased.

- The transcriptomic analysis resulted in the identification of several genes that are up or down regulated upon aphid attack. Some of the genes identified might be possible targets for increasing aphid resistance, for instance by reducing the suitability of *S. tuberosum* as a host for *M. persicae*. Since in the compatible plant-aphid interactions, the down-regulation of some genes appears to be important, the effect that these genes have on aphid performance and feeding should be further investigated.

- It is important to notice that all results in this study of aphid-resistance in tuber-bearing *Solanum* genotypes were obtained under greenhouse and laboratory conditions. Such results are a basis for further unravelling the resistance mechanisms at the metabolic, molecular, and genetic levels. However, for breeding purpose field experiments will be necessary to evaluate resistance under crop conditions.

- Finally, efforts to find additional fully aphid resistant plants should continue.

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SUMMARY

Resistance mechanisms of *Solanum* species to *Myzus persicae*

The framework

The cultivated potato *Solanum tuberosum* L. is among the four most important crops worldwide, and it is generally susceptible to aphids. Aphids like *Myzus persicae* (Sulzer) constitute a threat to potato crops because their high efficiency to transmit viruses. In wild *Solanum* species more than 60 have been reported to have effective resistance to *Myzus persicae*. These species represent an important potential source of resistance, which can be exploited to enhance resistance in cultivated potatoes. However, further exploration of the underlying biochemical, physiological, and molecular mechanisms of resistance is first needed. Until now no *S. Tuberosum* cultivated plants with effective resistance to aphids has been achieved.

The aim

The aim of this thesis was to find and study in wild tuber-bearing *Solanum* species different mechanisms of resistance to *M. persicae* with the aim to combat virus problems, especially Potato leafroll Virus (PLRV).

For this, it is essential that we extend our knowledge on the plant-aphid interaction on susceptible and resistant host plants. To achieve this goal, I characterised phenotypic aspects of resistance and susceptibility in wild and cultivated potatoes and tried to link that to plant-aphid interactions with respect to aphid behaviour and performance on the one hand, and plant responses to aphid attack on the other.

The work

In Chapter 1 a comprehensive literature review on the current knowledge on potato-*M. persicae* interactions including PLRV was presented and research needs were identified.

In Chapter 2, twenty tuber-bearing *Solanum* genotypes were evaluated for susceptibility or resistance towards *M. persicae*. A combination of an assay of aphid colony-development with the evaluation of probing activities as measured with the electrical penetration graph (EPG) technique, showed to be effective in localising resistance factors in tissues of *Solanum* genotypes. Mechanisms of resistance to *M. persicae* were detected at three plant tissue levels.

Clear surface resistance was observed in species with glandular trichomes, i.e. *S. berthaultii, S. polyadenium,* and *S. tarijense*. However, the glandular trichome based resistance is genetically complex and it is strongly associated with poor agronomical traits. Therefore, it has not yet been used successfully in breeding programs. *Solanum stoloniferum* was found to have resistance to *M. persicae* at the epidermal/mesophyll level. *Solanum jamesii* and *S. tuberosum* cv. Kardal appear to have resistance factors at the phloem level, but this resistance can be overcome by the aphids. The expression of resistance to *M. persicae* changes with plant age and plant parts. Overall, genotypes with some degree of resistance at the young and the flowering plant stage are: *S. jamesii, S. tuberosum* cv. Kardal, *S. capsicibaccatum, S. stoloniferum, S. berthaultii, S. polyadenium,* and *S. tarijense*.

Plants with a pre-phloem mechanism of resistance will be advantageous to avoid inoculation of persistently transmitted viruses. Among *Solanum* species, *S. stoloniferum* showed pre-phloem resistance to *M. persicae* that was not based on glandular trichome traits (Chapters 2 and 3).

In Chapter 3, *S. stoloniferum* was used as a model plant to unravel and characterise the different interactions of a single plant with different aphid species. *S. stoloniferum* resistance was found to be aphid-species specific. Although *S. stoloniferum* showed poor host features for *M. persicae,* we observed that it was successfully colonised by *Macrosiphum euphorbiae*. Aphid performance, settling behaviour, and probing studies of *M. persicae* and *M. euphorbiae* on *S. stoloniferum* and on *S. tuberosum* showed that both plants can be a host plant for *M. euphorbiae* while these studies confirmed that *S. stoloniferum* is a poor host for *M. persicae*.

The strong resistance found in *S. stoloniferum* against *M. persicae* seems to rely on constitutively expressed physical traits with some age effects. To find out whether *M. persicae* or *M. euphorbiae* infestation can induce an additional repellence or deterrence component of resistance, settling

behaviour with free aphids was investigated. There was no evidence for an induction of repellence or deterrence, neither locally no systemically, when compared to plants that had not been infested (Chapters 3 and 4).

In Chapter 4 we selected *S. stoloniferum* and the two aphid species, *M. persicae* and *M. euphorbiae*, as a model to study transcriptional plant responses in compatible and incompatible interactions due to aphid colonisation on local and systemic level. About twice as many genes were differentially regulated in response to attack by *M. euphorbiae* (compatible interaction) than by *M. persicae* (incompatible interaction). This stronger response to *M. euphorbiae* involves mainly up-regulation of genes in the functional categories pathogenesis related (PR), regulatory, and protein metabolism and down-regulation in the functional categories regulatory, general metabolism and photosynthesis related genes. Induction of genes related to cell differentiation and development were also found. Possibly these genes are related to a process of changing the physiological status of the tissue from source to sink. In addition, *M. euphorbiae* elicits genes related to regulated-proteolysis (proteasome system), plasmodesmata gateway, and extracellular transport. We hypothesize that especially the high number of regulatory genes that are up-regulated in local leaves in the compatible plant-aphid interaction is related to the change in the physiology of the plant.

Infestation of *S. stoloniferum* leaves with *M. persicae* aphids leads to the development of water-soaked pustules. Microscopic analysis of the pustules showed a burst of the tissue, which is the result of cell enlargement and cell division of cells surrounding the vascular bundle. In contrast, the infestation with *M. euphorbiae* did not induce any visible cellular changes. Additionally, after *M. persicae* and *M. euphorbiae* attack, *S. stoloniferum* up-regulates an *auxin-induced SAUR* (*small auxin up RNA*)-like protein (Chapters 4 and 5). Induction of *SAUR* transcripts has also been reported after inoculation of pepper and tomato plants by the virulent bacterial pathogen *X. campestris* pv. *vesicatoria*. The formation of pustules and the induction of *auxin-induced SAUR* in *S. stoloniferum* suggest that a similar situation exists in this plant-aphid relationship too. Possibly, the aphid watery-saliva includes effector proteins of endo-bacterial origin that may play a role in plant-aphid interactions.

In Chapter 5, the response of *S. tuberosum* cv. Kardal in young and old leaves to the attack of *M. persicae* was evaluated by studying gene expression and the aphid settling behaviour. We found that young leaves of cv. Kardal are resistant to *M. persicae* whereas mature-senescent leaves are

susceptible (Chapter 2). This differential resistance in leaves could reflect changes in tissue chemistry. In old susceptible leaves *M. persicae* attack elicits higher number of differentially regulated genes than in young resistant leaves. The attack of *M. persicae* of young leaves results in a low number of down-regulated genes.

In order to find similarities in gene regulations in compatible and incompatible plant-aphid interactions the transcriptomic changes in two systems were compared: (1) *S. stoloniferum* after attack by *M. persicae* or *M. euphorbiae* (Chapter 4), and (2) attack of *M. persicae* on leaves of *S. tuberosum* cv. Kardal of different age (Chapter 5). In both systems, after the attack with either aphid differential expression of genes related to PR, regulatory, and protein metabolism were recorded. The gene-expression studies provide evidence that both plants, *S. tuberosum* and *S. stoloniferum*, respond to aphid attack by activating the salicylic acid (SA) and ethylene (ET) pathways. However, genes responsive to jasmonic acid were only found to be differentially regulated to some extent in the *S. stoloniferum*-*M. euphorbiae* interactions and not in the *S. tuberosum* cv. Kardal- *M. persicae* interaction.

There is a group of genes that was up-regulated in both systems used, i.e., *S. tuberosum* cv. Kardal after *M. persicae* infestation, and *S. stoloniferum* after *M. persicae* or *M. euphorbiae* infestation, and might characterize both aphid-*Solanum* spp. interactions. Half of those genes belong to the PR category. Like in both *S. stoloniferum*-aphid interactions, also in *S. tuberosum* cv. Kardal up-regulated genes were found that could play a role in switching the tissue status from source to sink. In contrast, commonly down-regulated genes were not found in *M. persicae*-infested Kardal (both leaf ages) or *M. persicae*- or *M. euphorbiae*-infested *S. stoloniferum.* Hence, down-regulated genes might be more host-species specific than up-regulated genes.

A compatible plant-aphid interaction resulted in a broader gene expression response, which may represent the plant's need to cope with the removal of phloem sap during aphid feeding. But the up-regulation of genes related to local source-sink relationship at the feeding site may indicate plant manipulation by the aphids. Also genes related to signal-transduction pathways, regulation and signalling, protein metabolism, maintenance of cell homeostasis, transport, secondary metabolism, and structural features were found to be differentially regulated. The plant's transcriptional changes in incompatible interactions remained more restricted.

In Chapter 6 we investigated whether PLRV-infection in cv. Kardal plants affected aphid behaviour. In a choice test *M. persicae* was attracted more by headspace volatiles emitted from PLRV-infected plants than that of non-infected leaves but only in mature leaves. For young apical leaves no difference in aphid attraction was found between PLRV-infected and non-infected leaves. In addition, we studied the effects of PLRV infection on probing of *M. persicae* using the EPG technique. Several differences were observed between plant penetration in PLRV-infected and non-infected plants, but only after infected plants showed visual symptoms of PLRV infection. Aphids on PLRV-infected plants have reduced time to the first phloem salivation which indicates that stylet penetration is enhanced. Overall the transmission efficiency of PLRV is also expected to be affected in PLRV-infected plants because these plants attract more aphids than non-infected plants.

In Chapter 7 the findings of this thesis are discussed in an integrated way. Some experimental evidence found in Chapters 4 and 5 direct the attention to the possible role of the aphid endosymbiont *Buchnera* in plant-aphid interactions. Therefore the possibility of an effect of the aphid endosymbiont is discussed and compared with those between plants and pathogenic bacteria. From this, a new model for plant-aphid-endosymbiont interaction is hypothesised.

The results obtained in this research project, on aphid performance, probing behaviour, colony development, settling behaviour, and gene expression contribute to the understanding of plant responses towards aphid attack and form the basis for further unravelling the resistance mechanisms at the metabolic, molecular, and genetic level. To minimize the aphid-borne damage in potato crops combining plant resistances to aphids and viruses seems a good perspective. These results will also be the starting point for future breeding for aphid resistance.

SAMENVATTING

Resistentie mechanismen in *Solanum* soorten tegen *Myzus persicae*

Het framework

De aardappel *Solanum tuberosum* L. is wereld wijd het op drie na belangrijkste voedselgewas. Het gewas is gevoelig voor bladluizen zoals *Myzus persicae* (Sulzer) en op dit moment zijn er nog geen rassen met een goede resistentie tegen die bladluizen. Bladluisresistentie is belangrijk omdat de bladluizen zeer efficiënt virussen overgedragen en deze virusziekten kunnen de oogst ernstig in gevaar brengen. Van meer dan 60 wilde aardappelsoorten is bekend dat ze meer of minder resistent zijn tegen *Myzus persicae*. Deze soorten vertegenwoordigen een belangrijke bron die benut kan worden om de resistentie in gecultiveerde soorten te brengen. Voordat hieraan begonnen wordt is eerst meer inzicht nodig in de onderliggende biochemische, fysiologische en moleculaire mechanismen van de resistentie.

Doelstelling

Het doel van het in dit proefschrift beschreven onderzoek was het vinden en bestuderen van wilde aardappelsoorten met verschillende resistentiemechanismen tegen *M. persicae* om op deze manier een belangrijk deel van de virusproblematiek aan te pakken, met name die van het aardappel bladrolvirus (PLRV),. Om dit te kunnen moeten we eerst meer kennis krijgen van de plant-insect interacties op gevoelige en resistente planten. Hiervoor heb ik verschillende phenotypische aspecten van resistentie en vatbaarheid onderzocht in gecultiveerde en wilde aardappelplanten. Ook heb ik geprobeerd deze phenotypische aspecten te koppelen aan het gedrag en de groei en reproductie (performance) van de bladluizen en de reactie van de plant op de aanval van bladluizen.

Het werk

In hoofdstuk 1 wordt een overzicht gegeven van de in de literatuur beschikbare kennis over de aardappel-*M. persicae* interactie, inclusief PLRV, en worden de onderzoeksbehoeftes in kaart gebracht.

Hoofdstuk 2 beschrijft de evaluatie van 20 knol-dragende *Solanum* genotypen met betrekking tot resistentie en vatbaarheid voor *M. persicae*. Hiervoor is een ontwikkelings-assay van bladluiskolonies gebruikt in combinatie met de evaluatie van de plant-penetratie en voedselopname (probing) activiteiten van de bladluis zoals die gemeten wordt met de electrisch penetration gram (EPG) techniek. Deze aanpak bleek geschikt te zijn voor het lokaliseren van de resistentiefactoren in verschillende plantenweefsels. Er werden resistentiemechanismen gevonden op drie verschillende niveaus.

Een duidelijke resistentie aan het bladoppervlak werd gevonden bij soorten die klierharen bezitten zoals *S. berthaultii, S. polyadenium*, en *S. tarijense*. Uit de literatuur is bekend dat deze op klierharen gebaseerde resistentie genetisch gezien complex is en ook dat die helaas geassocieerd is met minder gewenste productie-eigenschappen. Mede daardoor is men er nog niet in geslaagd om deze resistentie te benutten in veredelingsprogramma's. *Solanum stoloniferum* bleek resistentie tegen *M. persicae* te bevatten op het niveau van epidermis/mesophyl weefsel. *Solanum jamesii* en *S. tuberosum* cv. Kardal beschikken over resistentiefactoren op floëem-niveau, maar deze resistentie tegen *M. persicae* bleek te sterk te verminderen met de leeftijd van de bladeren. In zijn algemeenheid bleek een zekere mate van resistentie in de jonge en bloeiende delen van de plant, aanwezig te zijn in de soorten: *S. jamesii*, *S. tuberosum* cv. Kardal, *S. capsicibaccatum, S. stoloniferum, S. berthaultii, S. polyadenium,* en *S. tarijense*.
Planten met een resistentiemechanisme in de pre-floëem fase zijn te prefereren omdat hiermee de overdracht van persistente virussen zoals het PLRV wordt vermeden. Onder de onderzochte *Solanum* soorten, bezit *S. stoloniferum* een dergelijke pre-floëem resistentie tegen *M. persicae* die niet op klierharen is gebaseerd (hoofdstukken 2 en 3).

Hoofdstuk 3 beschrijft het gebruik van *S. stoloniferum* als modelplant om de reactie op twee verschillende bladluissoorten te karakteriseren. De in *S. stoloniferum* gevonden resistentie bleek specifiek te zijn voor *M. persicae. Macrosiphum euphorbiae* had geen problemen om deze plant te

koloniseren. Bladluis performance, 'settling-', penetratie–gedragstudies met *M. persicae* en *M. euphorbiae* op *S. stoloniferum* en op *S. tuberosum* lieten zien beide planten geschikt zijn als waardplant voor *M. euphorbiae* maar dat *S. stoloniferum* een slechte waardplant is voor *M. persicae*.

De sterke resistentie die jonge bladeren van *S. stoloniferum* bezitten tegen *M. persicae*, lijkt te berusten op een constitutief tot expressie komende fysieke barrière. Door gebruik te maken van een settling gedragstest is onderzocht of een aanval van *M. persicae* of *M. euphorbiae* additionele afstotende resistentie-componenten kon induceren in *S. stoloniferum*. Dit bleek niet het geval te zijn; niet in de bladeren waarop de bladluizen gezeten hebben (lokale) en ook niet in de overige (systemische) bladeren van de plant (hoofdstuk 3 en 4).

In hoofdstuk 4 wordt de reactie van *S. stoloniferum* op de twee bladluissoorten, *M. persicae* en *M. euphorbiae,* beschreven op het niveau van genexpressie. Hierbij kan de reactie op *M. euphorbiae* worden gezien als de compatible interactie en die op *M. persicae* als de incompatibele. Er is zowel aan locale bladeren, waarop de bladluizen zaten, als aan systemische bladeren gemeten. Uit de analyses bleek dat er ongeveer tweemaal zoveel genen differentieel werden gereguleerd als reactie op een *M. euphorbiae* aanval dan bij een *M. persicae* (incompatibele interactie) aanval. Deze sterkere reactie op een aanval van *M. euphorbiae* betrof vooral een verhoogde expressie van genen in de categorieën pathogenese gerelateerde genen (PR genen), regulerende genen, en genen betrokken bij eiwitmetabolisme. Daarnaast kwamen genen uit de functionele categorieën algemeen metabolisme en fotosynthese verminderd tot expressie in reactie op *M. euphorbiae*. Ook werd er een inductie gevonden van genen, betrokken bij celdifferentiatie en plantontwikkeling. Mogelijk zijn deze genen betrokken bij het veranderen van de fysiologische staat van het weefsel, waarbij lokaal een 'metabole sink' wordt gevormd als reactie op de bladluizen. Daar komt nog bij dat, *M. euphorbiae* ook genen activeert die betrokken zijn bij gereguleerde proteolyse (proteasome system), plasmodesmata vorming en extracellulair transport. Dit brengt mij tot de hypothese dat met name het grote aantal genen met een verhoogde expressie in de bladeren waarop de bladluizen zitten in de compatibele interactie is gerelateerd aan veranderingen in de fysiologie van de plant als gevolg van de bladluisaanval.

Infectie van *S. stoloniferum* bladeren met *M. persicae* bladluizen leidt tot de ontwikkeling van watergevulde puist-achtige structuren. Microscopische analyse van deze puistachtige structuren laat

veel kapot/gebarsten weefsel zien, wat het gevolg is van extra celdelingen en celvergroting van cellen rond de vaatbundel. Bij een infectie met *M. euphorbiae* zien we een dergelijke reactie niet. Interessant is in dit verband ook dat een aanval van *M. persicae* of *M. euphorbiae* in *S. stoloniferum* leidt tot de activatie van een gen dat codeert voor een *auxine-induceerd SAUR* (*small auxin up RNA*)-achtig eiwit (Hoofdstuk 4 en 5). Inductie van een *SAUR* transcript is in het verleden ook waargenomen na inoculatie van peper- of tomatenplanten met het virulente pathogeen *X. campestris* pv. *vesicatoria*. Ook dit ging gepaard met de vorming van watergevulde puistachtige structuren. De vorming van de puisten en de inductie van het *SAUR*-transcript in *S. stoloniferum* suggereren dat er parallellen zijn tussen de twee processen. Mogelijk bevat het waterige speeksel van de bladluis effector eiwitten die van de endo-symbionte bacteriën afkomstig zijn en die een rol spelen in de plant-bladluis interactie.

In hoofdstuk 5 wordt de reactie van *S. tuberosum* cv. Kardal op een aanval van *M. persicae* beschreven. Hiervoor is gebruik gemaakt van een settling gedragstest en van genexpressiestudies in jonge en oude bladeren. Ik heb hierbij gevonden dat jonge bladeren van cv. Kardal resistent zijn tegen *M. persicae* en oude vergeelde bladeren vatbaar. Dit verschil in resistentie zou een gevolg kunnen zijn van verschillen in chemische samenstelling van de bladeren. Een aanval van *M. persicae* op oude bladeren leidt tot differentiële regulatie van een groter aantal genen dan een aanval op jonge bladeren. Verder blijkt er in jong blad van cultivar Kardal slechts een klein aantal genen in expressieniveau verlaagd te worden na een aanval *M. persicae.*
Om overeenkomsten in genregulatie te vinden tussen de compatibele en incompatibele plant-bladluis interactie zijn de transcriptomics-resultaten van de twee systemen met elkaar vergeleken: (1) *S. stoloniferum* na een aanval van *M. persicae* of *M. euphorbiae* (Hoofdstuk 4) en (2) *S. tuberosum* cv. Kardal na een aanval van *M. persicae* op blad van verschillende leeftijd (Hoofdstuk 5). In beide systemen wordt na een aanval van elk van de bladluizen een differentiële expressie van PR en regulatorische genen, en van genen betrokken bij het eiwitmetabolisme waargenomen. De genexpressie studies leveren bewijs voor activatie van de signaal-transductie-route via salicylzuur en ethyleen, in zowel *S. tuberosum* als *S. stoloniferum* na een bladluisaanval. Genen die onderdeel uitmaken van de signaal-transductie-route via jasmonzuur werden slechts in beperkte mate differentieel gereguleerd in de *S. stoloniferum-M. euphorbiae* interactie en niet in de *S. tuberosum* cv. Kardal-*M. persicae* interactie.

Er is ook een groep genen die in beide gebruikte systemen, *S. tuberosum* cv. Kardal na *M. persicae* infectie, en *S. stoloniferum* na *M. persicae* of *M. euphorbiae* infectie in expressie werden verhoogd. Deze genen karakteriseren beide bladluis-*Solanum* spp. interacties. De helft daarvan behoort tot de PR-eiwitten. Net als in de interactie tussen beide bladluissoorten en *S. stoloniferum*, worden ook in de interacties met *S. tuberosum* cv. Kardal genen in expressie verhoogt die mogelijk een rol spelen in het veranderen van de status van het bladweefsel van source naar sink. Daar staat tegenover dat er geen genen waren die zowel in *M. persicae*-geïnfecteerde Kardal (oude of jonge bladeren) als in *M. persicae*- of *M. euphorbiae*-geinfecteerde *S. stoloniferum* planten in expressie verlaagd werden. Dit suggereert dat de verlaging van genexpressie meer soortspecifiek is dan een verhoging.

Een compatibele plant-insect interactie resulteerde in een bredere reponse voor wat betreft het aantal genen dat differentieel werd gereguleerd dan een niet-compatibele interactie. Dit zou een reactie van de plant kunnen zijn op de onttrekking van het floëemsap door de bladluis. Onder betrokken genen bevinden zich genen die gerelateerd kunnen worden aan het veranderen van de sink-source relatie op de plek waar de bladluis aan het voeden is, hetgeen suggereert dat de bladluis de genexpressie van de plant kan manipuleren. Onder de differentieel gereguleerde genen bevonden zich genen betrokken bij signaal transductie routes, regulering en signalering, eiwit metabolisme, celstofwisseling, transport en secundair metabolisme. In de incompatibele interactie lijkt de transcriptionele response van de plant beperkt te zijn.

In hoofdstuk 6 beschrijf ik het onderzoek naar het effect van een PLRV infectie van planten van het ras Kardal op het bladluisgedrag. De reactie van *M. persicae* op de geurstoffen van met PLRV geïnfecteerde en van niet-geïnfecteerde planten bleek af te hangen van de leeftijd van het blad. Voor wat betreft de jonge, apicale blad bleek er geen verschillen te zijn in het aantal bladluizen dat door PLRV geïnfecteerde en niet-geïnfecteerde bladeren werd aangetrokken. Voor oud blad bleek dit wel het geval; hier bleken virus-geïnfecteerde bladeren veel meer bladluizen aan te trekken dan het niet geïnfecteerde. Ook hebben we het penetratiegedrag van de bladluizen onderzocht met de EPG techniek. Daarbij zijn een aantal verschillen gevonden in plant-penetratie tussen geïnfecteerde en niet geïnfecteerde planten, waarbij de verschillen alleen meetbaar waren als planten zichtbare symptomen van de PLRV-infectie vertoonden. Bladluizen op PLRV-geïnfecteerde planten hebben minder tijd nodig om tot floëem activiteiten te komen, wat er op wijst dat stiletpenetratie gemakkelijker verloopt. Op basis van dit alles kunnen we verwachten dat verspreiding van het

PLRV virus beïnvloed zal worden omdat geïnfecteerde planten meer bladluizen aantrekken dan niet-geïnfecteerde.

In hoofdstuk 7 worden de resultaten van het beschreven onderzoek op een geïntegreerde manier bediscussieerd. Een aantal resultaten, beschreven in de hoofdstukken 4 en 5, wijzen op een mogelijke rol van de endosymbiont *Buchnera* in de plant-bladluis interactie. Daarom is de rol van *Buchnera* in plant-bladluis interacties bediscussieerd en vergeleken me de interactie tussen pathogene bacteriën en planten. Op basis hiervan is een nieuwe hypothese opgesteld voor de interactie plant-bladluis-endosymbiont.

De resultaten verkregen in dit onderzoeksproject m.b.t. bladluis performance, ‘probing’ gedrag, kolonie-ontwikkeling, ‘settling-gedrag’ en genexpressie dragen bij aan een beter begrip van de plantenreactie op de aanval van bladluizen en vormen de basis voor het ontrafelen van het resistentiemechanisme op metaboliet, moleculair en genetisch niveau. Perspectieven om schade in aardappel te minimaliseren zitten vooral in een combinatie van bladluis- en virusresistentie. De resultaten van dit onderzoek zijn ook het startpunt voor toekomstige veredeling op bladluisresistentie.

RESUMEN GENERAL

Mecanismos de Resistencia de *Solanum* al pulgón *Myzus persicae*

El marco de estudio

En el cultivo de papa (*Solanum tuberosum* L.), considerado entre los cuatro cultivos más importantes del mundo, los pulgones constituyen una verdadera amenaza por su capacidad de actuar como vectores de virus patógenos de plantas. Siendo el pulgón verde del duraznero *Myzus persicae* (Sulzer) uno de los vectores de virus más eficaces. Muchas especies silvestres de papa (*Solanum* sp) poseen resistencia a los pulgones, por lo tanto constituyen una fuente de resistencia que puede ser utilizada para la creación de nuevas variedades de papa. Sin embargo, se sabe muy poco sobre los mecanismos de resistencia a pulgones, por lo que es necesario profundizar en el estudio de los procesos que están relacionados con la resistencia, tanto a nivel fisiológico y bioquímico, como a nivel molecular.

El objetivo

El objetivo de esta tesis fue la búsqueda y estudio de mecanismos de resistencia a *M. persicae* entre genotipos de papa silvestres con el fin de utilizarlos en los programas de desarrollo de variedades resistentes para reducir la incidencia de virus en los cultivos de papa, especialmente el virus del Enrollado de la hoja de papa (Potato Leaf Roll Virus, o PLRV).

Para llevar a cabo este objetivo, es esencial ampliar nuestros conocimientos en la interacción planta-pulgón tanto en plantas resistentes como en plantas susceptibles. Para ello, se caracterizaron aspectos fenotípicos de resistencia y susceptibilidad en plantas de papa silvestres y cultivadas en función del comportamiento y de los parámetros de vida del pulgón, relacionándose finalmente con la respuesta molecular de las plantas infestadas por pulgones.

El trabajo

En el Capítulo 1 se presenta una completa revisión bibliográfica sobre los conocimientos actuales en el tema de la interacción papa-*Myzus persicae* incluyendo el PLRV para identificar los aspectos que requerían una mayor investigación .

En el Capítulo 2 se describe la evaluación y caracterización de la resistencia a *M. persicae* en 20 genotipos silvestres y cultivados de *Solanum*. Para ello, se combinaron estudios sobre parámetros de vida del insecto junto a la técnica de gráficos de penetración eléctrica (Electrical Penetration Graph o EPG) para el estudio del comportamiento alimenticio del pulgón. La técnica de EPG resultó ser muy eficaz para localizar factores de resistencia en los distintos tejidos de los genotipos de *Solanum*, llegándose a identificar mecanismos de resistencia a *M. persicae* en tres diferentes tejidos de la planta.

Se ha observado una clara resistencia a nivel superficial en las especies de papa que presentan tricomas glandulares (*S. berthaultii, S. polyadenium* y *S. tarijense*). Sin embargo, la base de la resistencia a tricomas glandulares es genéticamente compleja y está fuertemente asociada a caracteres negativos desde el punto de vista agronómico por lo que, hasta el momento, no han sido efectivamente utilizados en el mejoramiento de papa. *S. stoloniferum* posee factores pre-floemáticos (epidermis y mesófilo) de resistencia a *M. persicae* mientras que *S. jamesii* y el cultivar Kardal (*S. tuberosum*) parecen presentar factores de resistencia a nivel floemático. Se ha encontrado cierto grado de resistencia al pulgón tanto en plantas jóvenes como maduras en los siguientes genotipos: *S. jamesii, S. tuberosum* cv. Kardal, *S. capsicibaccatum, S. stoloniferum, S. berthaultii, S. polyadenium* y *S. tarijense.*

Los mecanismos de resistencia pre-floemáticos presentes en la planta serían más eficaces para prevenir la inoculación de virus circulativos transmitidos de manera persistente como es el caso del PLRV. Por lo tanto, la resistencia encontrada en *S. stoloniferum* (Capítulos 2 y 3) hace que sea la especie elegida para investigar en profundidad las interacciones planta- pulgón.

En el Capítulo 3, *S. stoloniferum* fue utilizada como planta modelo para estudiar la resistencia a *M. persicae*. Con el objetivo de caracterizar las interacciones entre planta-pulgón, se incluyó una segunda especie de pulgón (*Macrosiphum euphorbiae*) para la cual la planta resultó ser susceptible.

Se realizaron estudios de no preferencia evaluando el numero de pulgones asentados en cada genotipo, se evaluaron parámetros de vida y se analizó el comportamiento alimenticio del pulgón mediante la técnica de EPG. La resistencia de *S. stoloniferum* resultó ser de naturaleza constitutiva y depende de la edad de la planta, siendo además sólo efectiva frente a *M. persicae.*

Con el objetivo de determinar si la previa infestación con pulgones en *S. stoloniferum* inducía algún tipo de respuesta de repelencia, se realizaron experimentos de no preferencia con pulgones libres para evaluar el comportamiento de asentamiento del pulgón en la planta. No se detectó respuesta de repelencia previa infestación con *M. persicae* y *M. euphorbiae* a nivel local (hojas previamente infestadas) ni a nivel sistémico (hojas no previamente infestadas) (Capítulos 3 y 4).

El Capítulo 4 describe la respuesta de *S. stoloniferum* frente al ataque de *M. persicae* (interación incompatible) o de *M. euphorbiae* (interacción compatible) mediante el estudio de la expresión génica a nivel local y sistémico. La respuesta al ataque de *M. euphorbiae* en la planta infestada fue mayor (cambiando el nivel de expresión el doble de los genes) que la producida por el ataque de *M. persicae.* Los genes con mayores niveles de expresión pertenecían principalmente a las siguientes categorías funcionales: genes relacionados con patogenicidad (PR), genes reguladores y genes relacionados con el metabolismo de las proteínas. Además, algunos de los genes diferencialmente expresados se relacionaban con procesos de diferenciación y desarrollo celular. Los genes reprimidos pertenecían principalmente a genes reguladores, del metabolismo general y relacionados con la fotosíntesis.

Posiblemente estos genes con expresión diferenciada se relacionan con procesos de cambio de estado fisiológico de los tejidos para generar un sumidero metabólico. Notoriamente, la interacción con *M. euphorbiae* indujo mayor nivel de expresión de genes relacionados con proteólisis controlada, sistema de plasmodesmata (sistema de transferencia controlada de nutrientes) y transporte extracelular. Las interacciones con pulgones compatibles indujeron un aumento en la expresión de un gran número de genes reguladores a nivel local en las hojas infestadas. La respuesta a nivel sistémico fue muy débil.

La infestación de *S. stoloniferum* with *M. persicae* indujo la formación de pústulas sobre las venas en el envés de la hoja. El análisis microscópico reveló que las pústulas se formaban como consecuencia de la proliferación de células del haz vascular, ocasionando una ruptura del tejido

desde el centro de la pústula. Al contrario, la infestación con *M. euphorbiae* no provocó lesiones visibles en la hoja.

Además, el ataque de ambos pulgones indujo mayor expresión de un gen *SAUR* (*small auxin up-regulated RNA*) que es inducido por auxinas (Capítulos 4 y 5). La inducción de genes *SAUR*s también se observa en tomate y pimiento, infectados por la bacteria virulenta *Xanthomonas campestris* pv. *vesicatoria*. Tanto la formación de pústulas como el aumento de la expresión de genes *SAUR* indican que en la interacción entre *M. persicae* y *S. stoloniferum* existe cierta similitud con la infección bacteriana. Los pulgones inyectan saliva acuosa en las células vegetales y quizás en la saliva acuosa haya proteínas efectoras que cumplan un rol en la infestación.

En el Capítulo 5 se estudió la respuesta de *S. tuberosum* cultivar Kardal al ataque de *M. persicae* mediante el estudio de la expresión génica en hojas maduras y en hojas jóvenes. También se estudió el comportamiento de asentamiento del pulgón. Las hojas jóvenes de Kardal son resistentes a *M. persicae* mientras que las maduras y senescentes son susceptibles (Capítulo 2). Esta diferencia entre las hojas de distintas edades puede deberse a cambios bioquímicos que ocurren a nivel del tejido vegetal. En las hojas maduras (susceptibles), la infestación con *M. persicae* provocó una mayor respuesta a nivel de regulación génica que el ataque en hojas jóvenes. Esta diferencia fue especialmente mayor en relación a los genes reprimidos.

Con el fin de encontrar similitudes en la regulación génica de interacciones compatibles e incompatibles entre plantas y pulgones, se compararon los perfiles transcriptómicos de los sistemas estudiados en el Capítulo 4: (1) *S. stoloniferum* atacado por *M. persicae* o *M. euphorbiae*, y en el Capítulo 5: (2) hojas de distintas edades de *S. tuberosum* cv. Kardal atacadas por *M. persicae*. En ambos casos se observó expresión diferenciada de genes relacionados con patogenicidad, genes reguladores y genes relacionados con el metabolismo de las proteínas. Los perfiles transcriptómicos indicaron que el ataque de pulgones en ambos sistemas activó genes relacionados con la transducción de señales dependientes del ácido salicílico y del etileno, mientras que sólo se encontraron unos pocos genes regulados por el ácido jasmónico en el sistema de *S. stoloniferum*-*M. euphorbiae* y ningún gen en Kardal-*M. persicae.*

Genes que fueron inducidos en ambos sistemas caracterizarían la interacción de ambas especies de pulgones con las especies de *Solanum* en general. La mitad de ese grupo de genes está relacionado

con patogenicidad (genes PR). En ambos sistemas se encontró un grupo de genes relacionados con procesos de cambio de estado fisiológico, de fuente (source) a sumidero (sink). No se encontraron coincidencias entre los genes reprimidos en ambos sistemas, por lo que se concluye que los genes reprimidos son más específicos de cada interacción que los genes expresados.

La cantidad de genes con cambios en la expresión fue mayor en las interacciones compatibles entre las plantas y pulgones que en las interacciones incompatibles. Probablemente, parte de estos cambios estén relacionados con los mecanismos de la planta huésped de compensar por la pérdida de savia. Parte de los genes podrían también estar relacionados a una posible manipulación por parte del pulgón de la planta huésped para beneficio propio. Se observó diferencia en la expresión de genes relacionados con la transmisión de señales, regulación, metabolismo de las proteínas, mantenimiento de la homeostasis, transporte, metabolismo secundario y genes estructurales. En lo que respecta al número de genes, la respuesta de la planta al ataque de pulgones para los que resulta resistente (incompatible), fue muy limitada.

En el Capítulo 6 se investigó si las plantas de papa del cultivar Kardal infectadas por PLRV afectaban la atracción y el comportamiento alimenticio de los pulgones. Se observó que la respuesta de *M. persicae* a los volátiles emitidos por las hojas de las plantas infectadas dependía de la edad de la hoja. Se vio que los pulgones preferían las hojas maduras-infectadas sobre las maduras-no infectadas. Sin embargo, en hojas apicales no se detectó diferencia entre la atracción de los pulgones hacia hojas infectada o no infectadas. El efecto del PLRV sobre el comportamiento alimenticio se estudió por medio de la técnica de EPG. Se observó que el tiempo necesario para que el pulgón salivara en el floema era menor en plantas infectadas que en las plantas no infectadas, lo cual indica mejor penetración a través del tejido vegetal. Las diferencias fueron notorias sólo después de aparecer los síntomas en la planta de la infección de virus. Se supone que las plantas infectadas incidirían en la eficiencia de transmisión de PLRV debido al cambio que producen en el comportamiento del pulgón vector.

En el Capítulo 7 se integran y discuten los resultados obtenidos en esta tesis. Los resultados obtenidos en los Capítulos 4 y 5 llamaron la atención hacia un posible rol del endo-simbionte de *M. persicae Buchnera* sp en relación con la interacción con la planta huésped. Se hizo una comparación hipotética con otras bacterias patógenas de plantas. Partiendo de allí, se propuso un modelo de interacción planta-pulgón-endosimbionte.

Los resultados obtenidos en esta tesis en relación a la interacción entre *Solanum* y *Myzus persicae*, por un lado estudio sobre desarrollo de colonias, comportamiento de asentamiento, parámetros de vida, comportamiento alimenticio del pulgón, y por otro lado el estudio de la respuesta en expresión génica de la planta huésped, contribuyen ampliamente al entendimiento de la interacción planta-pulgón y constituyen una base para revelar y entender mecanismos de resistencia a *M. persicae* a nivel metabólico, molecular y genético.

La tendencia actual para reducir al mínimo el daño producido por los pulgones a los cultivos de papa es utilizar de manera combinada la resistencia a pulgones y a virus. En ese contexto, los resultados de esta tesis constituyen un aporte para el mejoramiento vegetal de la papa contra el ataque de los pulgones.

Appendix - Pictures

Figure 1. Aphids. A-B-C, *Myzus persicae* (Sulzer). D, *Macrosiphum euphorbiae* (Thomas) (Photos by E. Garzo)

Figure 2. *Solanum tuberosum* cultivar Kardal infested by Myzus persicae. Aphids (green spots) were only found on senescent leaves (Chapter 5, Figure 1, page 87)

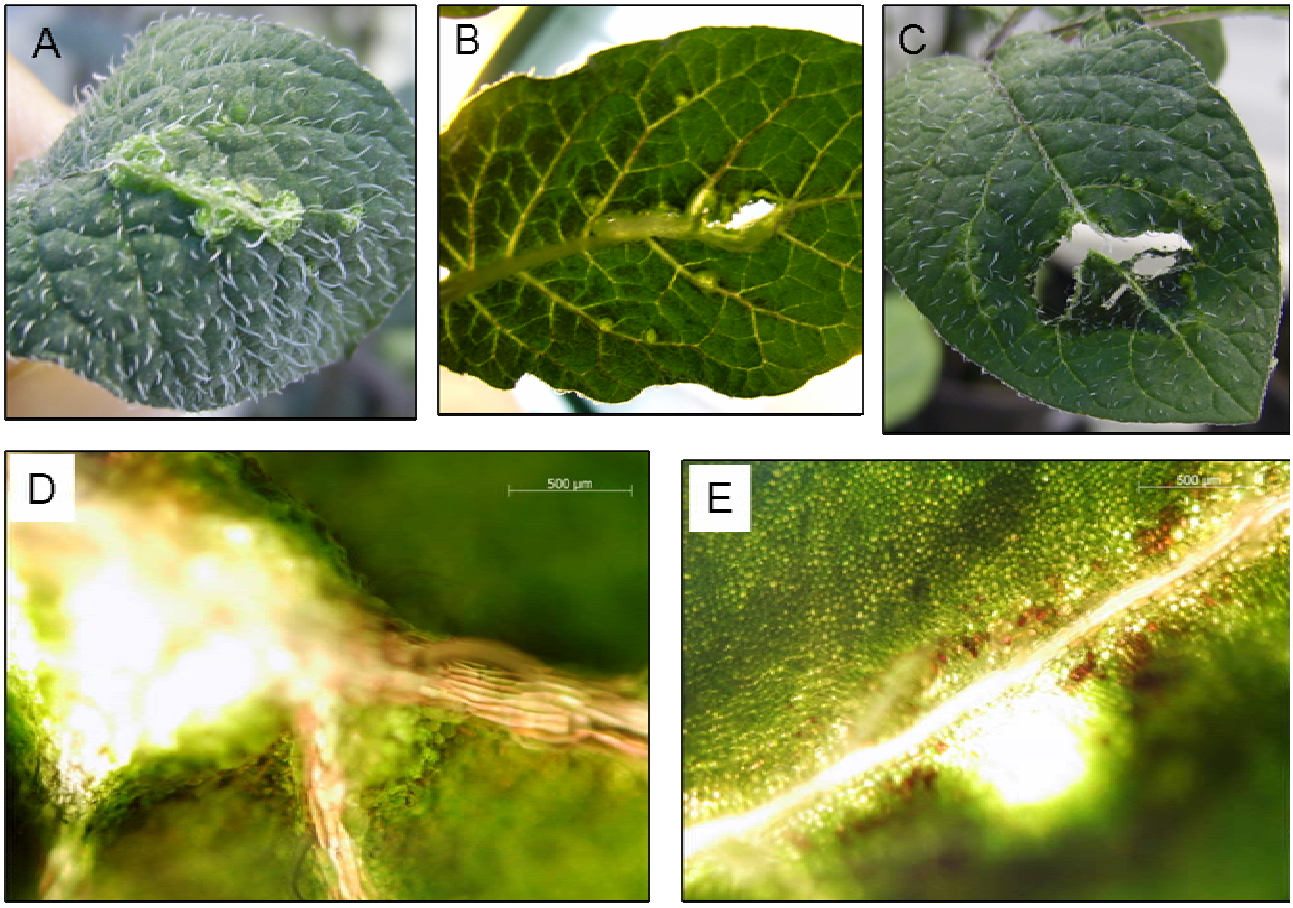

Figure 3. Pustules developed on the midrib and secondary veins on *S. stoloniferum* after infestation with *M. persicae* for 96 h. A, pustule. B-C, Pustules collapsed in the centre, and holes are formed. D, pustule from the abaxial side of the leaf. E, pustule from the adaxial side of the leaf, D-E, 5X enlargement (Chapter 4, A-B, Figure 4, page 64).

Appendix - Microscopic pictures

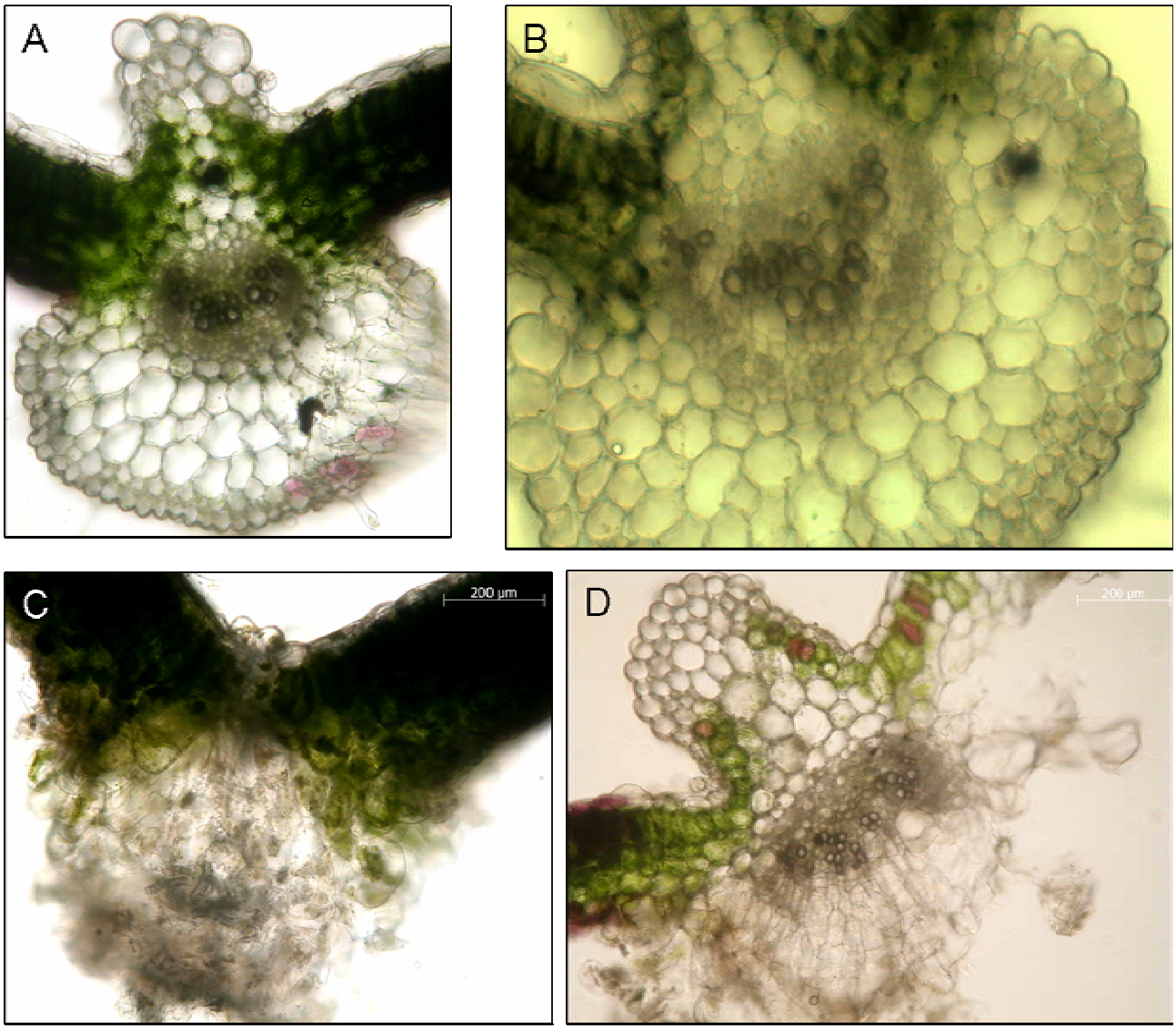

Figure 4. Sections of *S. stoloniferum* at the midrib leaf tissue. A-B, Non-infested control leaf tissue. C-D, leaf pustules after infestation with *M. persicae* for 96 h; C, burst of tissue to the abaxial side of the leaf causing epidermis rupture; D, the pustule is caused by hypertrophy (cell enlargement) in the tissue together with hyperplasia (cell division) of the vascular parenchyma cells and the vacuolated bundle sheath cells surrounding the vascular bundle at the abaxial side of the leaf. A, C, and D, 10X enlargement; B, 20X enlargement (Chapter 4, Figure 5, page 65).

CURRICULUM VITAE

Adriana Elisabet Alvarez was born on March 1st, 1964, in Morón, Buenos Aires, Argentina. In 1992 she graduated as Biologist from the National University of Salta (UNSa), Argentina. She is married since 1992 to Lucas Seghezzo and she has two children: Natalia (9) and Mateo (6). She was trained on cell biology and biochemistry techniques at the Biochemistry Research Institute 'Dr. F. Leloir' (Argentina, 1991), on genetic markers at the Laboratory of Genetics, National University of Buenos Aires, and INTA – Castelar (National Institute of Agricultural Technology) (Argentina, 1993), and on molecular markers at Plant Research International (PRI) (The Netherlands, 2003). Since 1992 she was appointed Assistant professor in Biochemistry at UNSa. From 1995 to 1997 she studied at Wageningen University, The Netherlands. In 1997 she obtained a M.Sc. degree in Biotechnology, specialization Plant and Microbial Production, diploma with distinction. In her M.Sc. thesis she studied the 'Use of microsatellite polymorphism for the evaluation of genetic diversity and phylogenetic reconstruction in the genus *Lycopersicon*'. This Ph.D. dissertation was done between 2003 and 2007 at the Laboratory of Entomology and PRI (Wageningen University). Back in Argentina, she will continue with her teaching and research activities at UNSa.

Publications

Alvarez AE, Garzo E, Verbeek M, Dicke M, Vosman B, Tjallingii WF. Infection of potato plants with potato leafroll virus changes attraction and feeding behaviour of *Myzus persicae*. Entomologia Experimentalis et Applicata (forthcoming)

Alvarez AE, Tjallingii WF, Garzo E, Vleeshouwers V, Dicke M, Vosman B. 2006. Location of resistance factors in the leaves of potato and wild tuber-bearing *Solanum* species to the aphid *Myzus persicae*. Entomologia Experimentalis et Applicata 121: 145-157

Alvarez AE, van de Wiel CCM, Smulders MJM, Vosman B. 2001. Use of microsatellites to evaluate genetic diversity and species relationships in the genus *Lycopersicon*. Theoretical and Applied Genetics 103:1283-1292

Brignoni M, Pignataro OP, Rodriguez ML, Alvarez A, Vega-Salas DE, Rodriguez-Boulan E, Salas PJI. 1995. Cyclic AMP modulates the rate of 'constitutive' exocytosis of apical membrane proteins in Madin-Darby canine kidney cells. Journal of Cell Science 108:1931-1943

To be submitted

Alvarez A, Alberti D'Amato A, Garzo E, Dicke M, Vosman B, Tjallingii WF. Aphid-plant interactions: probing and performance differences between *Myzus persicae* and *Macrosiphum euphorbiae* on two *Solanum* species

Alvarez A, Broglia V, Alberti D'Amato A, Wouters D, van der Vossen E, Tjallingii WF, Dicke M, Vosman B. *Solanum stoloniferum* responses in compatible and incompatible interactions with aphids

Alvarez A, Alberti D'Amato A, Tjallingii WF, Dicke M, Vosman B. Responses of *Solanum tuberosum* cv. Kardal to *Myzus persicae* infestation depend on foliage maturity

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PROPOSITIONS

Belonging to the thesis "Resistance mechanisms of *Solanum* species to *Myzus persicae*"

Adriana E. Alvarez

Wageningen, June 20, 2007

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1. *Solanum tuberosum* cultivar Kardal reacts upon the attack of *Myzus persicae* with the expression of many more genes in old susceptible leaves than in young apical resistant leaves, indicating that the plant changes its physiological status once the interaction has been established (this thesis).

2. The strong resistance found in the wild potato *Solanum stoloniferum* against *Myzus persicae* is constitutively expressed, is located at the epidermis/mesophyll level, and it decreases in senescent leaves (this thesis).

3. The ability of a plant to avoid senescence-like changes makes it more resistant to the attack of aphids.

4. It is difficult to find scientists with the same altruistic approach to science as Marie Curie, who wanted to "share the profits of [her] discoveries equally" with everybody (Marie Curie, 1867-1934).

5. Patenting genes can be seen as biopiracy and a new form of colonialism because the food crops grown today have been developed by indigenous farmers during centuries of innovation.

6. In Wageningen, only 11.6% of professors are women. You don't need the weather(wo)man to know which way the wind blows (Bob Dylan, 1965).

7. The True (science), the Good (moral), and the Beautiful (art), the three dimensions of knowledge, can pursue their own goals without violence and domination from the others (Wilber, 1998).

   Wilber, K. 1998. The marriage of sense and soul: Integrating Science and Religion. Random House, New York, USA.

8. The ultimate green/gene revolution would be the incorporation of chloroplasts into the human body.

PROPOSICIONES

Pertenecientes a la tesis "Mecanismos de resistencia de *Solanum* al pulgón *Myzus persicae*"

Adriana E. Alvarez

Wageningen, 20 de junio de 2007

1. *Solanum tuberosum* cultivar Kardal reacciona al ataque de *Myzus persicae* expresando muchos más genes en hojas viejas susceptibles que en hojas apicales jóvenes resistentes, lo cual indica que la planta cambia su estado fisiológico una vez que la interacción se ha establecido (esta tesis).

2. La fuerte resistencia encontrada en la papa silvestre *Solanum stoloniferum* contra *Myzus persicae* se expresa de manera constitutiva, se localiza a nivel de epidermis/mesófilo, y disminuye en hojas senescentes (esta tesis).

3. La capacidad de la planta para evitar cambios de tipo senescente la hacen más resistente al ataque de los áfidos.

4. Es difícil encontrar científicos con una visión altruista de la ciencia como la de Marie Curie, quien quería "compartir los beneficios de sus descubrimientos" con todo el mundo (Marie Curie, 1867-1934).

5. El patentamiento de genes puede ser visto como "biopiratería" y como una nueva forma de colonialismo porque los cultivos que se siembran hoy han sido desarrollados por los campesinos indígenas durante siglos de innovación.

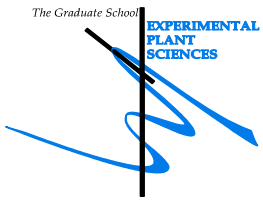
6. En Wageningen, sólo el 11.6% de los profesores (máximo rango en el escalafón docente) son mujeres. You don't need the weather(wo)man to know which way the wind blows [No hace falta el hombre (o la mujer) que anuncia el pronóstico del tiempo para saber en qué dirección sopla el viento] (Bob Dylan, 1965).

7. Lo Verdadero (ciencia), lo Bueno (moral), y lo Bello (arte), son las tres dimensiones del conocimiento; cada dimensión puede perseguir sus propios objetivos sin violencia ni dominación sobre las demás (Wilber, 1998).

   Wilber, K. 1998. The marriage of sense and soul: Integrating Science and Religion. Random House, New York, USA.

8. La mayor revolución verde/genética sería la incorporación de cloroplastos en el cuerpo humano.

Education Statement of the Graduate School
Experimental Plant Sciences



Issued to: **Adriana E. Alvarez**
Date: **20 June 2007**
Group: **Entomology and Biodiversity & Breeding,**
Wageningen University and Research Centre

1) Start-up phase	*date*
► **First presentation of your project**	
Oral Presentation at Laboratory of Entomology and Plant Research International: "Molecular mechanisms of Solanum resistance to Myzus persicae – Impact on PLRV transmission"	2004
► **Writing or rewriting a project proposal**	
Mol. characterisation of mechanisms of *Solanum* resistance to *Myzus persicae* – Impact on PLRV	Oct 2003
► **Writing a review or book chapter**	
► **MSc courses**	
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*7.5 credits**

2) Scientific Exposure	*date*
► **EPS PhD student days**	
EPS PhD student day, Vrije Universiteit Amsterdam	Jun 3, 2004
EPS PhD student day, Radboud University Nijmegen	Jun 2, 2005
EPS PhD student day, Wageningen University	Sep 19, 2006
► **EPS theme symposia**	
Theme 2 symposium 'Interactions between plants and biotic agents', Wageningen University	Dec 12, 2003
Theme 2 symposium 'Interactions between plants and biotic agents', Utrecht University	Sep 17, 2004
Theme 2 symposium 'Interactions between plants and biotic agents', Leiden University	Jun 23, 2005
► **NWO Lunteren days and other National Platforms**	
NWO-ALW Plant Sciences meeting, Lunteren	Apr 5-6, 2006
NWO-ALW Plant Sciences meeting, Lunteren	Apr 2-3, 2007
17e Meeting Netherlands Entomological Society, Ede-Wageningen	Dec 16, 2005
► **Seminars (series), workshops and symposia**	
Symposium on "System Biology" in Honor of Prof. dr. Pierre de Wit, Wageningen University	Nov 4, 2004
Symposia Current Themes in Ecology, 'Ecological and Evolutionary Genomics', WICC, Wageningen	Apr 29, 2005
Symposia Current Themes in Ecology, 'Darwinian Agriculture', WICC, Wageningen	Apr 13, 2007
Symposia YELREM, 'Biodiversity and Convergence of Sciences' de Bosbeek, Renkum	Jun 29, 2005
Symposia YELREM, 'Molecular Ecology and Insect Learning' de Bosbeek, Renkum	Jun 20, 2006
Workshop Plant-Insect Interactions: 'From Molecular Biology to Ecology', Wageningen University	Apr 25, 2006
Seminar series Entomology	2003-2007
Seminar series Plant Breeding and Biodiversity	2003-2007
► **Seminar plus**	
► **International symposia and congresses**	
1st Solanaceae Genome Workshop, WICC, Wageningen	Sep 19-21, 2004
1st Conference ALBAN-EU program, Universidad Politécnica de Valencia, España	May 13-14, 2005
IOBC Workshop: 'Breeding for inducible resistance against pests and diseases', Heraklion-Crete, Greece	Apr 27-29, 2006
Intl Joint Workshop on "PR Proteins" and "Induced Resistance Against Pathogens and Insects" Doorn, NL	May 10-14, 2007
► **Presentations**	
Poster, EPS PhD student day	Jun 3, 2004
Poster, 1st Solanaceae Genome Workshop, WICC, Wageningen/EPS Phd student day 2005	Sep 19-21, 2004/Jun 2, 2005
Poster, IOBC Workshop 2006/EPS PhD student day 2006	Apr 27-29, 2006/Sep 19, 2006
Oral, EPS theme 2 symposium	Sep 17, 2004
Oral, Workshop Plant-Insect Interactions	Apr 25, 2006
Oral, IOBC Workshop	Apr 27-29, 2006
Oral, NWO-ALW Plant Sciences meeting, Lunteren	Apr 2-3, 2007
Oral, Joint workshop PR Proteins and Induced Resistance	May 10-14, 2007
► **IAB interview**	Sep 18, 2006
► **Excursion**	
Subtotal Scientific Exposure	*16.7 credits**

3) In-Depth Studies	*date*
► **EPS courses or other PhD courses**	
Springschool Bioinformatics, De Wageningse Berg, Congress Centre, Wageningen	Mar 31-Apr 2, 2004
Workshop on Metabolomics, Laboratory of Plant Physiology, WU	May 2-4, 2005
► **Journal club**	2003-2007
Plant-Insect interactions, Laboratory of Entomology, WU PhD discussion group, Laboratory of Entomology, WU Theme group 'Abiotic and Biotic stress', Plant Research International Weekly meetings Plant Breeding Institute, Plant Research International	
► **Individual research training**	
Subtotal In-Depth Studies	*4.8 credits**

4) Personal development	*date*
► **Skill training courses**	
English Scientific Writing, CENTA, Wageningen University	Oct 13-Dec 1, 2005
► **Organisation of PhD students day, course or conference**	
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*1.5 credits**

TOTAL NUMBER OF CREDIT POINTS*	**30.5**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 credits

** A credit represents a normative study load of 28 hours of study*

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Cover design

Microphotography of a leaf cross section of *Solanum stoloniferum* stained with calcofluor white (cellulose staining). Drawings by Natalia Seghezzo Alvarez and Mateo Seghezzo Alvarez

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